Isolation and characterization of *Salmonella* Typhimurium SL1344 variants with increased resistance to different stressing agents and food processing technologies

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ABSTRACT

In this study, resistant variants of *Salmonella enterica* serovar Typhimurium SL1344 to different stressors were selected. In addition, a genetic and phenotypic study was performed to explore the mechanisms underlying the acquisition of resistance. We isolated 4 variants with increased stable resistance to acid, osmotic stress, high hydrostatic pressure (HHP) and Ultraviolet-C light (UV-C) after repeated rounds of exposure to these agents and outgrowth of survivors. A PEF-resistant variant (SL1344-RS), previously isolated by Sagarzazu et al. (2013), was also included in the analysis. The results indicated that the isolated variants showed resistance to at least one other agent. This increased resistance, in general terms, had a fitness cost in growth, and exerted a variable impact on virulence (mainly in cell adhesion capacity), increased antibiotic resistance but did not influence in biofilm formation capacity. Whole Genome Sequencing (WGS) analysis allowed us to identify the genetic changes responsible for these phenotypic differences, and revealed that in 3 out of the 5 variants (including SL1344-RS) a mutation was found in *hnr* gene, an anti-sigma factor that promotes RpoS proteolysis. Hence the expression of several *rpoS*-regulated genes was quantified and found higher in these variants. This increase in RpoS activity would explain the lower growth rates observed in these 3 variants, as it would lead to increased transcription of genes involved in growth arrest and resistance to various types of stress. However, further analysis of a set of 22 additional *Salmonella* strains obtained from different culture collections indicated that a direct relationship between RpoS activity and stress resistance might not exist within *Salmonella*.

1. Introduction

Salmonella are the second most frequent zoonotic agent in the European Union and the United States (EFSA, 2023; Marder et al., 2018). In Europe, about 65,208 confirmed cases of salmonellosis in humans were reported in 2022, with the cost of salmonellosis estimated at more than €3 billion annually (EFSA, 2023; Havelaar, 2011). The microorganisms of the genus *Salmonella* are a successful example of evolution and adaptation to different niches and hosts. The emergence of variants and the generation of population heterogeneity are factors that may contribute to the survival and adaptive capacity of *Salmonella*. In fact, these emerging resistant variants constitute an excellent model to deepen the understanding of mechanisms involved in cell survival and resistance to the different technologies and stressors. There are several

approaches to obtain such variants with stable tolerance, either after several cycles of treatment and growth of survivors (Karatzas et al., 2008; Sagarzazu et al., 2013), or after a single stressor treatment or food processing technology (Karatzas and Bennik, 2002; Metselaar et al., 2013).

The ability of *Salmonella* cells to resist and adapt to adverse conditions is one of the main characteristics that have made this microorganism such a relevant health hazard, but the impact of these resistance responses on other aspects of *Salmonella* physiology, such as virulence and growth fitness, is much less well known (Guillén et al., 2021). In Guillén et al. (2021), the impact of the resistance responses to stress conditions encountered in food and food processing environments of stable variants on different aspects of non-typhoidal Salmonellae physiology (with special emphasis on virulence and growth fitness), was

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reviewed. Nevertheless, further studies are needed to characterize the phenotype of these variants to understand the variability of pathogen populations and the impact of stress resistance on the overall phenotype, since for some food technologies or agents there is insufficient information. And also, the emergence of variants with increased resistance to food processing technologies may have important implications for the application of such technology in the food industry. Therefore, genetic and physiological studies to characterize emerging variants would greatly facilitate the design of efficient processes for industrial applications.

In previous work, a pulsed electric field (PEF)-resistant variant of *Salmonella* Typhimurium SL1344 (SL1344-RS) was obtained in our laboratory after repeated rounds of PEF treatment and outgrowth of survivors (Sagarzazu et al., 2013). This increased PEF resistance was accompanied by an increased resistance to some other agents, such as hydrogen peroxide, sodium chloride, ethanol and acid pH, and was linked to the entry into stationary growth phase of *Salmonella* cells (Guillén et al., 2023). Considering the relevance of this, in this study, other environmental stressors and food processing technologies, included in Guillén et al. (2020b), were explored for the isolation of a variant of *Salmonella enterica* serovar Typhimurium SL1344 with increased resistance after a selection procedure consisting of alternating rounds of exposure to the stressors or food technologies and outgrowth of the surviving population. In addition, a genetic and phenotypic study was performed to explore the mechanisms underlying the acquisition of resistance.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Salmonella enterica serovar Typhimurium SL1344 was used in this study as the model/parental strain. The strain was maintained frozen at $-80\ °C$ in a cryovial 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 wells microtiter plates and incubated at 37 °C under static conditions as described in Guillén et al. (2020b). For some experiments also an additional set of 22 *Salmonella* strains (listed in Supplementary Table S1) was used. The same preservation and growth conditions described for *Salmonella* Typhimurium SL1344 were used for the preservation and growth of the variants isolated in this study (see below) and for the set of 22 *Salmonella* strains.

2.2. Selection and isolation of resistant cells

Resistant variants were selected by subjecting *S.* Typhimurium SL1344 cells to successive rounds of agents or food technologies, followed by growth of the surviving cells in liquid medium. The isolation method was described by Sagarzazu et al. (2013) for the selection of *S.* Typhimurium SL1344 cells resistant to PEF. Briefly, one hundred microliters of the cell suspension exposed to various stresses were inoculated into 50 mL of TSB-YE and incubated at 37 °C for 24 h. Simultaneously, plate counts on Xylose Lysine Deoxycholate agar were performed after each treatment to follow the acquisition of tolerance to the different agents and verify that no contamination occurred during the screening process.

The conditions, and the description of the assay, for exposing the *Salmonella* cells to the different agents are shown in Table 1 and in section 2.3. Thus, *S.* Typhimurium SL1344 cells were exposed to successive cycles of stressors at an intensity leading to an inactivation of around 5 logarithmic cycles.

2.3. Environmental stress and food preservation technologies treatments

Environmental stress treatments and food preservation technologies are described in detail in Guillén et al. (2020b). The following sections

Table 1
Conditions used for the isolation of variants resistant to different environmental stresses and food preservation technologies. The initial concentration of the treatments was 10^9 CFU/mL. Treatment temperature was room temperature (25 °C) except for heat (58 °C) and NaCl (37 °C).

Environmental stresses and food technologies	Conditions	Treatment time
Acid	pH 2.5	180 min
Hydrogen peroxide	50 mM	80 min
Sodium chloride	30% (w/v) NaCl	24 h
Heat	58 °C	5 min
HHP	300 MPa	10 min
UV-C	0.09 mW/cm^2	150 s

contain a brief description of the treatments performed.

2.3.1. Acid, hydrogen peroxide, and sodium chloride resistance determinations

The treatment medium for acid-resistance determinations was citrate-phosphate McIlvaine buffer adjusted to pH 2.5. Hydrogen peroxide resistance was evaluated in 100 mM Tris–HCl buffer (pH 7.0) with hydrogen peroxide added at final concentrations of 30 mM (Sigma, St Louis, USA). Resistance to osmotic medium was evaluated in TSB-YE supplemented with 30% w/v of sodium chloride (VWR International; NaCl). In all cases, cells were added at an initial concentration of approximately 10^9 CFU/mL, for the selection of resistant variants, or 10^7 CFU/mL, for the determination of resistance to the treatment media at room temperature (25 °C), except for the NaCl determinations, which were carried out at 37 °C due to the low lethality of this agent. After the selected contact time, which ranged from 50 min to 32 h, depending on the agent, subsequent serial dilutions were prepared in buffered peptone water (Oxoid; BPW) and pour-plated for survival counts as described below.

2.3.2. Heat treatments

In the case of heat treatments, the experimental method used for the selection of resistant variants was different to that used for heat resistance determinations. Selection of variants was carried out in glass tubes, which contained TSB as a treatment medium, submerged and prewarmed at $58 \pm 0.2\ °C$ in a thermostated water bath, at initial concentrations of 10^9 CFU/mL.

Heat treatments were carried out in a specially designed resistometer (Condón et al., 1993). Once treatment temperature had attained stability ($58 \pm 0.1\ °C$), 0.1 mL of the microbial cell suspension was injected into the main chamber containing the treatment media, TSB, at initial concentrations of 10^7 CFU/mL. After inoculation, samples were collected at different heating times up to 20 min and immediately pour plated and incubated for survival counting.

2.3.3. 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). Cell suspensions were prepared at an initial concentration of 10^9 CFU/mL, for the selection of resistant variants, or 10^7 CFU/mL, for the determination of resistance, approximately, 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.

2.3.4. Pulsed electric field (PEF) treatments

The PEF equipment used in this investigation was supplied by ScandiNova (Modulator PG, ScandiNova, Uppsala, Sweden). The equipment and treatment chamber have been previously described by Saldaña et al. (2009). Prior to PEF treatments, 0.1 mL of the microbial cell suspension were dissolved in citrate-phosphate McIlvaine buffer (pH 7.0 and 1 mS/cm of conductivity) at an initial concentration of

approximately 10^7 CFU/mL. Samples were placed with a sterile syringe in the treatment chamber, which had a gap of 0.25 cm. Treatments were based on square pulses with a width of 3 µs and a frequency of 1 Hz. Electric field strength was set at 25 kV/cm, being the energy per pulse 1.88 kJ/kg. Treatments of up to 50 pulses (150 µs) were applied. Under these conditions, the final temperature of the treatment media was always below 35 °C.

2.3.5. Ultraviolet-C light (UV-C) treatments

UV-C treatments were carried out in a Petri dish under agitation (300 rpm), for the selection of resistant variants, or in a microtiter plates under static conditions, for the determination of resistance. Microtiter plates were coated with 1 layer of a microplate sealing film (BREATH-seal, Greiner bio-one, Frickenhausen, Germany) or the Petri dish lid and located 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, intensities of 0.47 or 0.09 ± 0.02 mW/cm^2 were attained. The treatment medium was citrate-phosphate McIlvaine buffer of pH 7.0, and the initial concentrations were of approximately 10^9 CFU/mL, for the selection of resistant variants, or 10^7 CFU/mL, for the determination of resistance. Treatment times of up to 180 s were applied and temperature never exceeded 30 °C.

2.3.6. Recovery after different treatments and survival counting

After treatments, samples were adequately diluted in Buffered Peptone Water (Oxoid; BPW) and plated in the recovery medium, TSA-YE. Plates were incubated for 24 h at 37 °C, after which the number of colony-forming units (CFU) per plate was counted.

2.3.7. Survival curves and fitting of data

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 acid, heat, HHP, and peroxide treatments; s for UV-C treatments and µs for PEF treatments). Since deviations from linearity were observed in survival curves to the majority of agents/technologies, 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.

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

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

In these equations, N_t represents the number of survivors ($\log_{10}$ CFU/ml), N_0 the initial count ($\log_{10}$ CFU/ml), and t the treatment time. This model describes the survival curves by means of three parameters: shoulder length (S_l (units of t)), defined as the time before exponential inactivation begins; inactivation rate (K_{max} (units of t^{-1})), defined as the slope of the exponential portion of the survival curve; and N_{res} which describes residual population density (tail; ($\log_{10}$ CFU/ml)). Therefore, the traditional decimal reduction time value (*D*-value) can be calculated from the K_{max} parameter using Equation (3).

$$D - \text{value} = \ln (10)/K_{max} \qquad \text{Eq. 3}$$

2.4. Growth fitness characterization assays

Growth fitness characterization assays were carried out in three different media: TSB-YE, Luria-Bertani (LB; Oxoid) broth supplemented with 100 µM 2-2'dipyridyl (DPY; Sigma), an iron chelator, and minimal medium, M9-broth, supplemented with 20 mM gluconate (GLU; VWR), as the principal carbon source in the intestine as described in 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). Growth curves were constructed by plotting the decimal logarithm of the number of cells against time. Model fitting was performed on each of the three replicates separately. The growth curves obtained were fitted with the Baranyi and Roberts model (Baranyi and Roberts, 2000):

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

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

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); μ' is the growth rate ($\log_{10}$/h); λ is the lag phase (h); and M and h_0 are curvature parameters, taking them as constant values, and with both 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 (ln/h) was calculated from the growth rate, in $\log_{10}$ scale, using Equation (6).

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

2.5. 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.

2.6. 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:

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

2.7. Antibiotic resistance assays

The minimum inhibitory concentration (MIC) of seven antibiotics representative of different classes (ampicillin, cephalexin, chloramphenicol, kanamycin, nalidixic acid, oxytetracycline, and sulfanilamide) 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.

2.8. Whole genome sequencing (WGS) of the Salmonella variants

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

The genomes of the resistant variants and of the parental strain were sequenced by the company STAB VIDA (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 the produced data was determined by Phred quality score at 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, a variant calling algorithm was applied to detect the variants that satisfy 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 a 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 (PRJNA1126312).

2.9. RNA extraction

RNA of the parental strain and each resistant variant was isolated by phenol-chloroform extraction with a subsequent cleanup procedure using the RNeasy Mini Kit (Qiagen) following the protocol described in Guillén et al. (2023). The samples, once purified, were treated with DNase to remove residual DNA using the Rapidout Removal Kit (Thermo Fisher Scientific, Massachusetts, USA), also following the manufacturer's instructions and extracted RNA samples were frozen at −80 °C until complementary DNA (cDNA) synthesis.

2.10. Quantitative Reverse Transcriptase PCR (qRT-PCR)

First, 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 qPCR assays were carried out.

Afterwards, expression of three RpoS-dependent genes (*katE*, *katN* and *otsB*) of the parental and the different strains/variants 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 2. 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).

2.11. Statistical analysis

All the determinations were carried out in triplicate on different working days. Standard deviations (SD) and statistical analyses (ANOVA, Tukey tests and Pearson's correlation coefficients; *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

3.1. Isolation of variants with increased resistance to environmental stresses and food processing technologies

S. enterica serovar Typhimurium SL1344 cells were exposed to different stresses and food processing technologies, including acid, oxidative and osmotic stress, heat, HHP and UV-C treatments, during successive treatments at lethal doses and subsequent growth of surviving cells in liquid medium. An acid resistant variant (SL-Acid), an osmotic resistant variant (SL-NaCl), a variant resistant to high hydrostatic pressure (SL-HHP) and a variant resistant to Ultraviolet-C light (SL-UV) were obtained. They were isolated after five, six, eight and eight cycles of acid, osmotic, HHP and UV-C stress, respectively. *Salmonella* cells were also exposed to lethal oxidative and heat treatments but no resistant variants could be isolated after 8 cycles.

Fig. 1 shows the survival curves of *S. enterica* serovar Typhimurium SL1344 (parental strain) and resistant variants to acid and osmotic stress, HHP and UV-C treatments. As can be observed in the curves, the isolated variants were more resistant than the parental, indeed differences quite small. Thus, the inactivation reached after 50 min at pH 2.5 was approximately 0.54 $\log_{10}$ cycles higher for the parental than for the SL-Acid variant (Fig. 1A). Similarly, differences in the $\log_{10}$ cycles of inactivated cells between the parental and the resistant variants of 0.84 (parental *vs* SL-NaCl; 1B), 0.82 (parental *vs* SL-HHP; 1C) and 1.15 (parental *vs* SL-UV; 1D) were observed after exposure to 30% (w/v) NaCl

Table 2
Primer sequences used in Real-Time PCR to quantify the expression of RpoS-dependent genes.

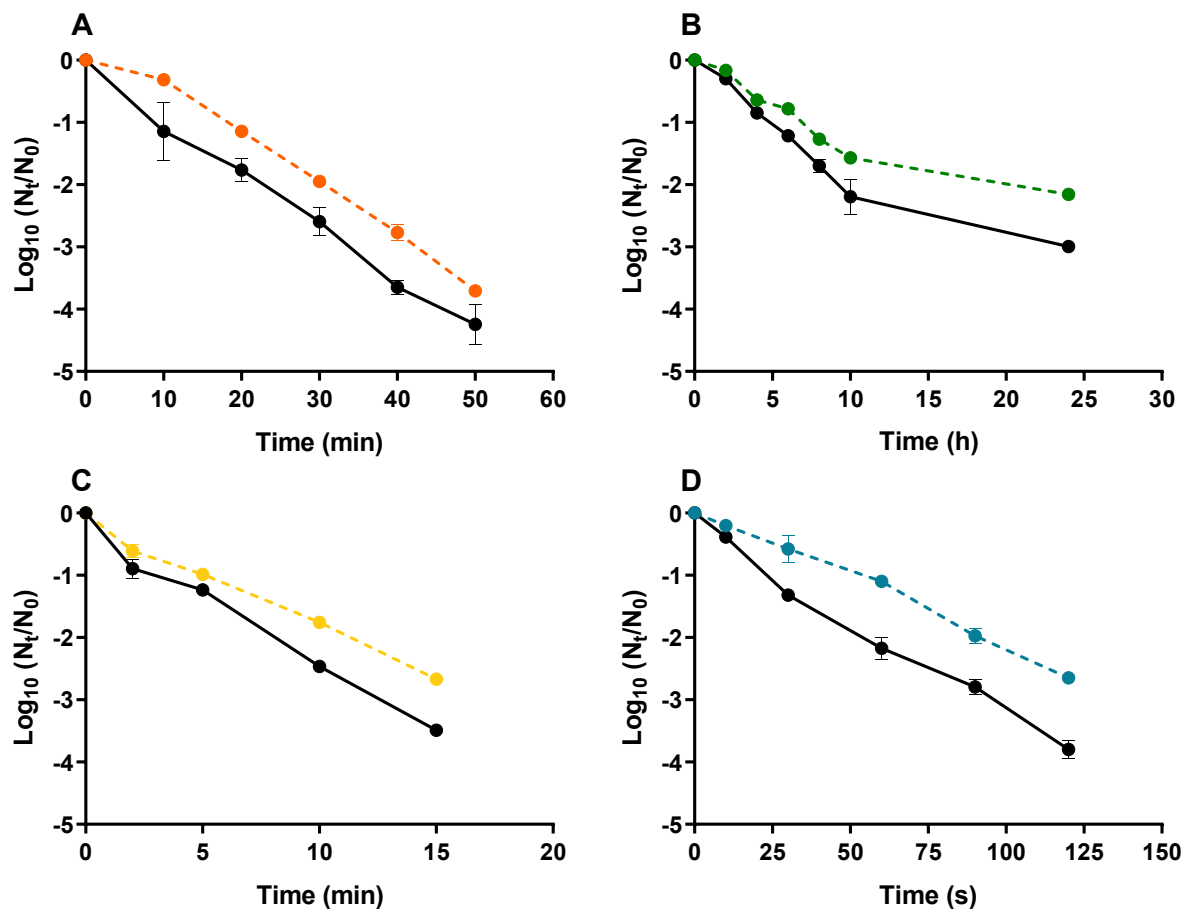| Gene target	Forward primer (5′ -to- 3′)	Reverse primer (5′ -to- 3′)
rpoZ	CGAAGAAGGTCTGATTAAC	GACGACCTTCAGCAATA
katE	GGCGTCTGTTCTCTTAT	CTGGAAGTTATGGTAGGG
katN	TGAGTCATCTGGAAATTAT	CGATAAAGTTCCGCTTC
otsB	TTAACCGTATCCCCCGAACTC	CCGCGAGACGGTCTAACAAC

Fig. 1. Survival curves of parental *S.* Typhimurium SL1344 (continuous line) and the different variants (discontinuous line) obtained resistant to A) acid pH (SL-Acid variant, pH 2.5), B) osmotic medium (SL-NaCl variant, TSB-YE + 30% NaCl + 37 °C), C) high hydrostatic pressure (SL-HHP variant, 300 MPa), and D) UV-C light (SL-UV variant, 0.09 mW/cm^2).

for 24 h, a 15 min treatment at 300 MPa and a UV-C treatment of 120 s at 0.09 mW/cm^2, respectively.

Since it has been reported that the acquisition of resistance to an environmental stress might lead to the development of cross-resistance responses and often results in a loss in the growth fitness or virulence of the cells (Guillén et al., 2022), in the following sections the resistance to other stressors, the growth capacity, the virulence capacity, the bio-film formation ability and the antibiotic resistance of these resistant variants isolated were determined.

3.2. Characterization of S. Typhimurium SL1344 variants to different environmental stresses and food processing technologies

First, the resistance to seven different preservation technologies and environmental stresses of the 4 isolated variants of *S.* Typhimurium SL1344 was determined and subsequently compared to that of the parental strain, *S.* Typhimurium SL1344. The resistance of the PEF-resistant variant (SL1344-RS) isolated by Sagarzazu et al. (2013) to these agents (Guillén et al., 2023) is also included in the figures for comparison purposes. The adequacy of the methodology used has already been discussed in Guillén et al. (2020b). Survival curves to 7 agents were obtained by plotting the logarithm of the survival fraction versus treatment time. The profiles of some of the curves showed deviations from linearity; for example, the survival curves to acid and hydrogen peroxide showed shoulders, whereas those to NaCl and PEF showed tails. Therefore, to describe them accurately, the nonlinear Geeraerd model (Geeraerd et al., 2000) was used 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, are included in Supplementary Table S2. The traditional decimal reduction time (*D*) value of each survival curve was calculated from its corresponding K_{max} (Eq. (3)). In order to establish meaningful comparisons, the *2D*-value parameter (time required to inactivate the first 2 $\log_{10}$ cycles) was used, as described in Guillén et al. (2020b). Since the *2D*-values obtained for each agent/technology cannot be directly compared due to the different time scale of the survival curves, they were normalized against the mean *2D*-value corresponding to parental strain, *S.* Typhimurium SL1344. This ratio (*2D*-value/*2D*-value parental strain) offers an indication of the relative acquisition of resistance of each variant to each one of the agents studied (Fig. 2).

As can be observed in Fig. 2 and in Supplementary Table S2, all the variants displayed, to some extent, cross-resistance responses. Excluding the preservation technology or environmental stresses of selection for each variant, the SL-Acid variant displayed increased resistance to 2 of the tested agents/technologies, the SL-NaCl variant to 3, the SL-HHP variant to 1, the UV-C variant selected variant to 3 and the PEF-selected variant to 5. Additionally, some sensitizations to other agents were also found in certain cases, such as towards PEF for the acid-selected variant and to HHP for SL-UV variant. It should also be noted that, in some cases, a selected variant acquired more resistance to a given agent than the variant selected with this particular agent (e.g. the resistance to PEF of the UV-C variant was higher than that of the PEF-selected one). In any case the observed differences in resistance among the variants are of low magnitude, with a change in the *2D*-value less than 2.8 fold in all cases. Furthermore, resistance data obtained for
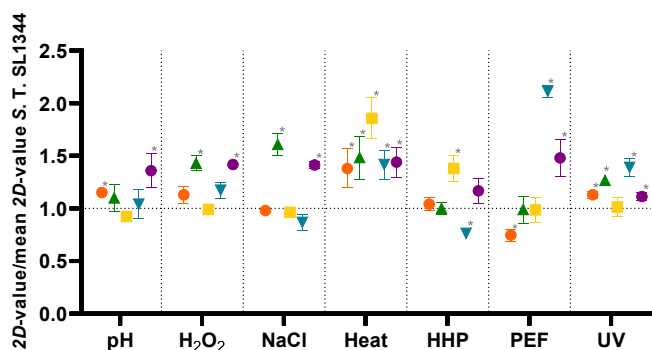
Fig. 2. Resistance to seven different preservation technologies and environmental stresses of the 5 variants of *S.* Typhimurium SL1344 isolated, SL-Acid (●), SL-NaCl (▲), SL-HHP (■), SL-UV (▼) and SL1344-RS (●). The parameter plotted is the mean *2D*-value for each strain divided by the mean *2D*-resistance value of the parental strain, *S.* Typhimurium SL1344. Error bars correspond to the standard deviation of the means and the asterisk (*) indicates statistically significant differences ($p < 0.05$) between the parental and the variant.

all the variants and agents studied (Table S2) were in the range of those previously described for 22 strains obtained from culture collections, following the same methodology (Guillén et al., 2020a, 2020b).

3.3. Characterization of the growth capacity of S. Typhimurium SL1344 variants in different growth media

Growth curves of the 4 resistant variants and the parental strain were obtained in three different media: in TSB-YE, a nutrient-rich medium, LB medium with iron limitation caused by the addition of DPY, and a minimal medium containing gluconate as the sole carbon source. The selection of this growth media has been previously discussed in Guillén et al. (2022). The μ_{max} (log$_{10}$/h) values calculated in the three growth media for each variant and the parental strain are shown in Fig. 3 (growth parameters and goodness of the fit parameters are included in Supplementary Table S3). As expected, growth rates were higher in TSB-YE (complex medium), and lower in M9-Gluconate (restrictive medium), for all the variants studied, including the parental strain.

As a general observation, the isolated variants showed an impaired growth compared to the parental strain, but this was influenced by the growth medium studied. In TSB-YE, the highest growth rate of the variants was that of SL1344-RS (0.914 ± 0.017), and the lowest that of SL-UV (0.873 ± 0.014). No statistically significant differences ($p > 0.05$) were observed among the resistant variants, but they were when compared to the parental strain, except for SL1344-RS. In LB-DPY, significant differences ($p < 0.05$) were only found between the parental
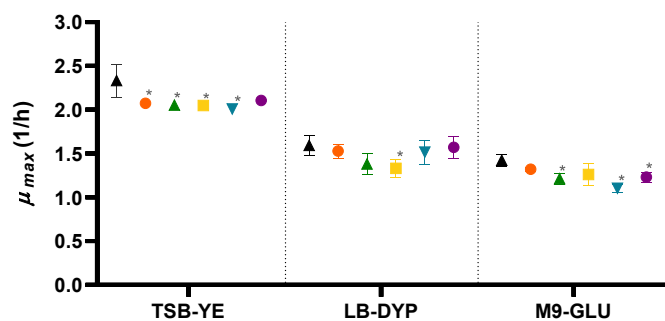

Fig. 3. Maximum specific growth rates (μ_{max} (ln/h)) of *S.* Typhimurium SL1344 (▲), SL-Acid (●), SL-NaCl (▲), SL-HHP (■), SL-UV (▼) and SL1344-RS (●) in TSB-YE, LB supplemented with 100 μM 2-2′dipyridyl (DPY) and M9-broth supplemented with 20 mM gluconate at 37 °C. Error bars correspond to the standard deviation of the means and the asterisk (*) indicates statistically significant differences ($p < 0.05$) between the parental and the variants.

strain and SL-HHP. Finally, in M9-Gluconate, the lowest growth rate was observed in SL-UV (0.479 ± 0.018) and significant differences ($p < 0.05$) were found between the parenteral strain and SL-NaCl, SL1344-RS and SL-UV. None of the variants displayed a statistically significant lag phase (h) (different from 0; $p > 0.05$) in any of the three media tested.

As reported for the stress resistance parameters, the growth parameters obtained for all the resistant variants (Table S3) were in the range of those obtained previously following the same methodology (Guillén et al., 2022).

3.4. Characterization of virulence capacity of S. Typhimurium SL1344 variants

The virulence capacity of the resistant variants was evaluated by calculating the percentage of cells capable to adhere to and invade Caco-2 cells (Fig. 4). The SL-NaCl, SL-HHP and SL1344-RS variants showed a superior adhesion capacity than the parenteral strain, 5.1, 4.0 and 5.2 vs 2.0%, respectively, while the SL-UV variant showed a lower adhesion capacity (0.8%) than the parental strain. Despite the variability observed in the adhesion, the invasion was not found to be significant. Except for the SL-NaCl variant, which had a higher invasion capacity than the parental strain, 0.3 vs 0.1%, the rest of the variants displayed a similar percentage of invasion and no differences were found with the parental strain ($p > 0.05$).

3.5. Static biofilm formation ability of S. Typhimurium SL1344 variants

The results of the static biofilm formation assay are represented in Fig. 5, with comparisons established through the calculation of Area Under the Curve (AUC) values. In this case, no statistically significant differences ($p > 0.05$) were identified between the biofilm formation abilities of the resistant variants and the parental strain. This implies that, despite the development of resistance, the capability to form biofilms remained unaltered.

3.6. Antibiotic resistance of S. Typhimurium SL1344 variants

Resistance to seven antibiotics was also determined for the resistant variants (Table 3). Overall, these variants showed an increased resistance to the antibiotics tested and none of them showed increased sensitivity to any antibiotic, as compared to the parental strain. Three strains showed and increased resistance to six out of seven of the antibiotics tested (SL-NaCl, SL-HHP and SL-RS) and two to five out of seven (SL-Acid and SL-UV). In the case of sulfonamides, no variant exhibited greater resistance than the parental strain. Nevertheless, the antibiotic resistance patterns varied depending on the strain. Thus, for instance, the SL-UV variant, displayed up to 8-fold resistance to kanamycin and oxytetracycline, and up to 32-fold resistance to cephalexin. Nonetheless, its resistance to nalidixic acid was not increased. By contrast, strains SL-NaCl and SL-HHP exhibited a lower increase in resistance to ampicillin, cephalexin and oxytetracycline than that of the SL-UV strain but displayed a two-fold increase in resistance to nalidixic acid as compared to the parental strain.

3.7. WGS of S. Typhimurium SL1344 variants

In addition to the phenotypical characterization of these variants a WGS analysis was performed to identify mutations associated with increased resistance.

As already pointed out in our earlier work (Guillén et al., 2023), upon comparing the strain we used as the parental one (*S.* Typhimurium SL1344) with the reference genome of this same strain (GenBank: Accession No. FQ312003.1) (Kröger et al., 2012), we found that our strain had suffered four mutations (4 single nucleotide variations, SNVs) (Table S4). Thus, here we will report and discuss the differences between our parental strain and the variants isolated from it, i.e., the mutations
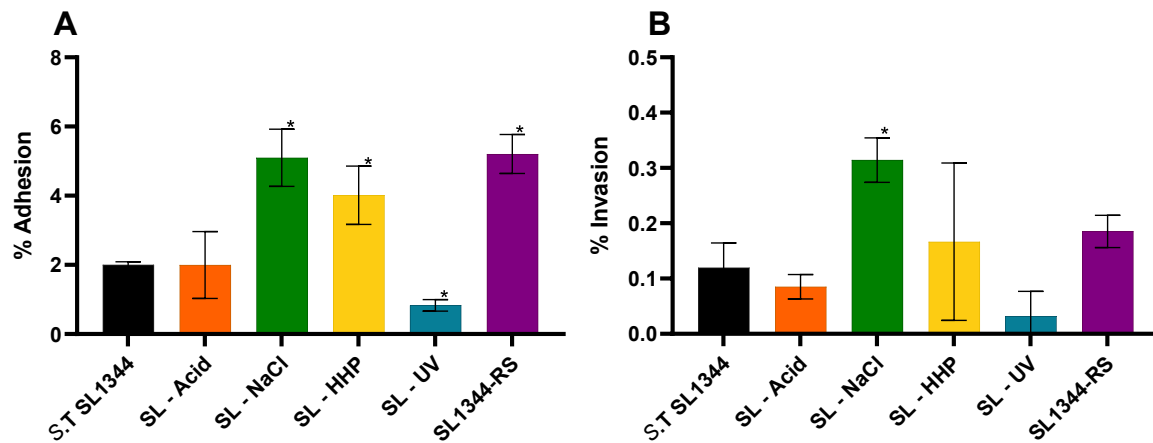
Fig. 4. Capacity of adhesion to (A) and invasion of (B) Caco-2 cells of the 5 resistant variants and the parental strain, *S.* Typhimurium SL1344. Error bars correspond to the standard deviation of the means and the asterisk (*) indicates statistically significant differences ($p < 0.05$) between the parental and the variants.
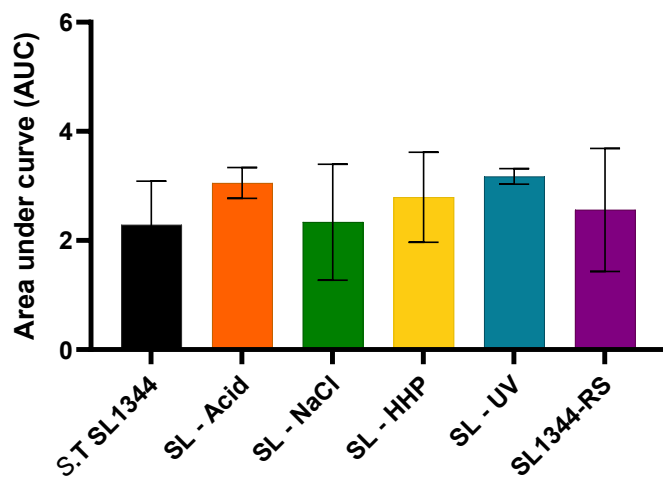

Fig. 5. Biofilm-forming ability of the 5 resistant variants and the parental strain, *S.* Typhimurium SL1344. Values correspond to the area under the curve and error bars correspond to the standard deviation.

Table 3
Minimum inhibitory concentrations (MIC) of the 5 resistant variants and the parental strain, *S.* Typhimurium SL1344. Units are in µg/ml.

	SL1344	SL-Acid	SL-NaCl	SL-HHP	SL-UV	SL1344-RS
Ampicillin	4	8	8	8	16	8
Cephalexin	8	16	16	16	256	32
Chloramphenicol	4	8	8	8	8	8
Kanamycin	8	32	64	64	64	64
Nalidixic acid	8	8	16	16	8	8
Oxytetracycline	1	2	4	2	8	4
Sulfonamide	4096	4096	4096	4096	4096	4096

fixed throughout the selection process applied. As can be observed in Table 4, sequencing of the genomes of these variants revealed that in SL-Acid, SL-NaCl and SL-HHP there was a single SNV in each one, as compared to the parental strain, whereas in SL-UV 2 SNVs were found.

The genes in which mutations were observed are also included in Table 4. As can be observed, the SL-Acid variant displayed a SNV in *yhfk* gene at position 959 bp, resulting in the substitution of a histidine (His) by a Leucine (Leu). In the SL-HHP variant, the *dnaJ* gene was mutated at position 826 bp, resulting in the substitution of a Leucine (Leu) by an Arginine (Arg). The SL-UV variant displayed 2 SNPs. One of them was found in the *caiD* gene at position 198 bp, replacing a Leucine (Leu) with a Phenylalanine (Phe). The other SNP was found in the *hnr* gene, being a missense mutation at position 493 bp, causing the substitution of a Glutamine (Gln) for a premature stop codon. A SNP was also found in this gene in the SL-NaCl variant, located 8 bp upstream from the AUG start codon, which corresponds to the Ribosome Binding Site (RBS) or Shine-Dalgarno sequence, and, therefore, this change might potentially lead to changes in the level of transcription of *hnr* mRNA. It should be noted that in the SL1344-RS variant, also a SNP in the *hnr* gene has been found (Guillén et al., 2023), in this case at position 902 bp, resulting in the substitution of a Leucine (Leu) by a Proline (Pro).

3.8. Quantification of RpoS activity in S. Typhimurium SL1344 variants

Results obtained indicated that the isolated variants were multi-resistant and in three of them (including the PEF resistant one) mutations in *hnr* were found, which regulates RpoS activity. Therefore, it was hypothesized that, especially in the case of variants SL-NaCl and SL-UV, their phenotypic characteristics might be linked to an increased RpoS activity, i.e., that the mutations found resulted in a decreased *hnr*-dependent RpoS proteolysis. Thus, qPCR was used to quantify the expression of several genes regulated by RpoS, which were used as reporters of its activity: *katE*, *katN* and *otsB*.

Table 4
Mutations identified in the resistant variants (obtained by cyclic exposure to different environmental stressors and food processing technologies) by whole genome sequencing (WGS). All detected mutations were single nucleotide variations (SNV).

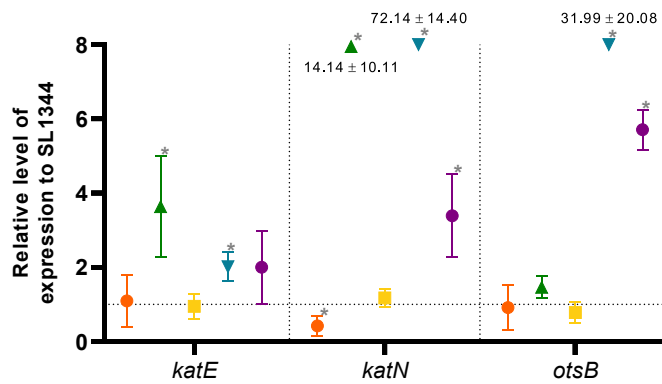| Variant	Region	Genes	Locus tag	Mutation type	Amino acid change	Description
SL-Acid	3638767	*yhfk*	SL1344_3434	c.959A > T	His320Leu	conserved membrane protein
SL-NaCl	1806813	*hnr*	SL1344_1684	c.-8G > A	Change in the Shine-Dalgarno sequence	hypothetical regulatory protein
SL-HHP	14430	*dnaJ*	SL1344_0013	c.836T > G	Leu279Arg	chaperone protein DnaJ
SL-UV	82316	*caiD*	SL1344_0071	c.198A > T	Leu66Phe	carnitine racemase
	1806313	*hnr*	SL1344_1684	c.493C > T	Gln165*	hypothetical regulatory protein

Fig. 6. Relative expression of the three genes studied ($2^{-\Delta\Delta Ct}$) in the resistant variants as compared to the parental strain. SL-Acid (●), SL-NaCl (▲), SL-HHP (■), SL-UV (▼) and SL1344-RS (●). Error bars correspond to the standard deviation of the means and the asterisk (*) indicates statistically significant differences ($p < 0.05$) between the parental and the variants.

As can be observed in Fig. 6, none of the three genes studied were overexpressed for the SL-Acid and SL-HHP variants, except for the *katN* gene in the SL-Acid variant, which showed a lower expression compared to the parental strain (what would indicate that their RpoS activity would be similar). In SL-NaCl and SL-UV, as it was observed in the SL1344-RS strain, the three genes were overexpressed, with the exception of the *otsB* gene in the SL-NaCl variant. Particularly notable was the case of the SL-UV variant, where overexpression was especially pronounced.

3.9. Relationship between RpoS and stress resistance in non-typhoidal Salmonellae

Various authors have suggested that the differences in stress resistance among *Salmonella* strains and serovars might be linked to differences in RpoS activity, and similar hypothesis have been offered for other microorganisms (and their correspondent alternative sigma factors) (Ait-Ouazzou et al., 2012; Cebrián et al., 2016; Hengge-Aronis, 1996). Therefore, the expression of *katE* and *otsB* (as reporters of RpoS activity) was determined in 22 additional *Salmonella* strains (Table S5) and subsequently compared with their previously published stress resistance parameters (*2D*-values to several agents), growth capacity (growth rates), virulence (percentage of adhesion and invasion), biofilm formation ability (AUC values) and antibiotic resistance (MIC values) (Guillén et al., 2020a, 2020b, 2022). The expression of *katE* and *otsB* of each strain was expressed as the C_t values normalized to the *rpoZ* level gene (ΔC_t). For this purpose, resistant variants were excluded and only the parental strain was included in the analysis. The iterative Grubbs' test was applied to identify potential outliers that could exert a disproportionate influence on further data analysis and lead to non-valid conclusions. Grubbs' test detected several outliers: the *2D*-value to heat and biofilm percentage of *S.* Senftenberg 775W, the invasion percentage of *S.* Enteritidis 7160 and the MIC values of *S.* Typhimurium 7162 for ampicillin, chloramphenicol, and oxytetracycline. These values, along with the MIC values for sulfonamides, were subsequently excluded from the analysis.

No significant correlation ($p > 0.05$) between stress resistance and expression of RpoS-dependent genes, *katE* and *otsB*, was observed for the 23 *Salmonella* strains studied SL1344 (Table S6). There was also no significant correlation between the expression levels of *katE* and *otsB* and growth fitness, biofilm formation ability, antibiotic resistance or virulence parameters. Therefore, for this dataset, no association with the level of RpoS-dependent genes expression was found.

4. Discussion

In this study we report the isolation and pheno- and genotypical characterization of four resistant variants derived from *S. enterica* serovar Typhimurium SL1344 after its exposure to several rounds of acid stress, osmotic stress, high hydrostatic pressure and UV-C light treatments. A PEF-resistant variant was previously isolated in our laboratory following the same protocol (Sagarzazu et al., 2013), and we also attempted to isolate stable variants resistant to heat and oxidative stress, but without success. This latter phenomenon might be due to several reasons, including that, maybe, insufficient selective pressure for their isolation was applied or an insufficient number of cycles of exposure and outgrowth. This technique has been used to isolate other *Salmonella* variants resistant to different stressors, after several cycles of treatment and growth of survivors. Examples of stable variants of *Salmonella* obtained after successive exposure to different selection agents are reviewed in Guillén et al. (2021). At this point it should be noted that the increase in resistance of the variants here isolated was relatively of small magnitude, if compared to other ones reported in the bibliography. There are also various possible explanations for this phenomenon. The fact that they were isolated after only few cycles of exposure and outgrowth would probably be one of the explanations, as hypothesized for explaining our unsuccessful isolation of variants after the application of heat and hydrogen peroxide treatments. Nevertheless, this approach has also the advantage that it reduces the probability of accumulating mutations not related to the phenotypic effect observed. Thus, three of the variants isolated only displayed a SNP and the other only two, facilitating the study of the mechanisms of acquisition of stress resistance (see below).

Our results indicate that all the variants isolated displayed resistance to at least, another agent that was not the selective one. However, whereas the SL-HHP variant only displayed an increased resistance to HHP and heat, some of the strains displayed an increased resistance to up to four out of the seven agents tested, such as SL-NaCl or SL-UV. It has been reported that pressure resistant mutants and/or acid resistant mutants of *L. monocytogenes*, *S. enterica* and *S. aureus* are frequently more tolerant to other stresses, particularly to heat treatments (Greenacre and Brocklehurst, 2006; Karatzas and Bennik, 2002; Karatzas et al., 2007). Similarly, the PEF resistant variant isolated by Sagarzazu and co-workers in 2013 also displayed an increased resistance to acid pH, hydrogen peroxide and ethanol. This latter strain also displayed an increased resistance to other agents such as antibiotics (Guillén et al., 2023). The occurrence of cross-tolerance between two stresses is *Salmonella* may be attributed to altered expression levels of proteins involved in the general stress response, such as RpoS, the master regulator of the general stress response (Hengge, 2011). As will be discussed later, this might be the case for the SL-NaCl and SL-UV variants. Besides, the occurrence of cross-tolerance may be attributed to similarities between the cellular targets affected by both agents, as might be the case for the SL-HHP variant, which displayed an increased resistance to heat.

It is also known that the development of stress resistance, including the resistance to certain antimicrobials can impose a fitness cost on bacteria, including *Salmonella* (Andersson and Hughes, 2010; Karatzas et al., 2008; Urdaneta et al., 2019; Zambrano et al., 1993). It is noteworthy that in *Escherichia coli* it has been demonstrated that rpoS activity would be a key factor determining the balance between stress resistance and growth fitness (Notley-McRobb et al., 2002). However, there is not always a direct relationship between resistance and fitness costs, and it is also possible that bacteria can reverse fitness costs by acquiring compensatory mutations (Andersson and Hughes, 2010; Guillén et al., 2021). Results here reported also indicate that stress resistance development can involve a fitness cost but also demonstrate that this might only be evident under some conditions (e.g. nutrient limitation), which is also in agreement with the results of Notley-McRobb and co-workers.

Finally, our results also indicate that stress resistance development

might also affect *Salmonella* virulence, as observed by Karatzas et al. (2008). Nevertheless, in our case resistant strains tended to display higher virulence that the parental one. In any case from our data it can also be concluded that the virulence capacity would be differently affected depending on the selecting agent/stressor. This is not surprising given the complexity of *Salmonella* virulence mechanisms and, particularly, their regulation (Erhardt and Dersch, 2015; Lou et al., 2019; Marcus et al., 2000).

On the other hand, the WGS analysis enabled us to identify the genetic changes responsible for these phenotypic changes. Regarding the SL-Acid variant, a SNP was found in the *yhfK* gene, which encodes a putative inner membrane protein. Although its functions are not fully elucidated, it is known that *yhfK* is required in moderate iron-restricted conditions for *S.* Typhimurium growth (Bjarnason et al., 2003; Karash et al., 2022) and that in *E. coli*, its expression is regulated by Crp, whose gene encodes cAMP receptor protein (CRP), which regulates the transcription of a magnitude of operons related to sugar transport and catabolic functions (Zheng et al., 2004). *Salmonella* acid tolerance mechanisms, reviewed in Álvarez-Ordoñez et al. (2011), include a number of transport systems and therefore, it is plausible that this protein might be involved in one of them. Results obtained also indicated that this mutation also led to an increase in heat resistance, which occurred when cells were recovered in non-selective (TSA-YE) and also in selective medium (XLD). Therefore, it can be speculated that this increase would not be related to an increased ability to repair sublethal damages caused by heat, but to direct heat protective changes, for instance, an increase in membrane stability. On the other hand, it should also be noted that results obtained in growth assays indicate that this mutation did not negatively affect *Salmonella*'s ability to grow in low iron media, what might had been expected, given phenotypical role that has been attributed to *yhfK* gene. Finally, it should also be noted that the resistance to acid medium of the SL-Acid variant, depended on whether growth occurred under agitation or in static culture conditions, with differences of up to 1.3-fold (data not shown), a phenomenon that was not observed for any other variant and or stress studied. This could be because there are transcriptional factors that are only activated under specific conditions, for example under agitation. Thus, for instance, Lim et al. (2012) noted that the *invF-2* promoter was not activated when cells were grown in static culture conditions. Further work will be required in order to fully elucidate all these questions regarding the mutation observed in the *yhfK* gene.

In the case of the SL-HHP variant, the mutation was found in the *DnaJ* gene. DnaJ, also known as Hsp40, is a co-chaperone that, together with DnaK, actively participates in the heat stress shock response by preventing the aggregation of stress-denatured proteins and by disaggregating proteins. It contributes to protein quality control by facilitating the folding of nascent proteins, polypeptides emerging through the Sec system, partially unfolded proteins, and protein aggregates (Kim et al., 2021; Rosenzweig et al., 2019). A similar hypothesis has been proposed for explaining the fact that its deletion also leads to a decreased baro-resistance in *E. coli* cells (Gänzle and Liu, 2015). This will mean that the allele found in this variant will probably be more efficient in preventing and/or repairing protein aggregation induced by these 2 agents, HHP and heat, which, in addition, have a lot of common cellular targets (Cebrián et al., 2016). Nevertheless, it should be noted that DnaJ also plays a relevant role in protecting *Salmonella* against oxidative and hyperosmotic stress. It catalyzes the formation and reduction of disulfide bonds (de Crouy-Chanel et al., 1995; Kim et al., 2021), and facilitates the interaction of oxidized DksA, an RNA polymerase-binding protein known to play a key role in the stringent response, with RNA polymerase (Cohen et al., 2022; Kim et al., 2018). However, in our case, no differences were found between the parental and the mutant strain. In any case, it should be noted that in this study we are also comparing 2 alleles (both of them functional), whereas in the aforementioned studies the comparison was established with a knock-out strain, in which the protein, and therefore, its activity, was

completely absent. DnaJ is also required for growth at high temperatures in *E. coli* (Sell et al., 1990) and for *Salmonella* invasion of epithelial cells (Takaya et al., 2004). However, in our case the DnaJ allele providing a higher stress resistance (SL-HHP variant) also led to a decreased growth rate and although it resulted in a higher adhesion, it did not significantly change the invasion of Caco-2 cells. Again, these discrepancies might be attributed to different factors that should be further investigated.

In any case, the most relevant finding of this study is that half of the strains isolated (SL-NaCl and SL-UV) displayed mutations in the *hnr* gene, the same gene presenting a mutation in the PEF resistant strain isolated in Sagarzazu et al. (2013). As pointed out above, *hnr* regulates RpoS proteolysis and, therefore, these mutations resulted in an altered RpoS activity in these strains. Thus, these strains presented an increase in RpoS activity, as demonstrated by the higher expression (as compared to the parental strain) shown by the SL-UV variant to all three RpoS activity reporters (*katE*, *katN* and *otsB*), SL-NaCl to two of the three reporters (*katE* and *katN*) and SL1344-RS to *katE* and *otsB*. Due to the different locations of these SNPs within the *hnr* gene they would be expected to have different impacts on Hnr function/activity. Thus, whereas the Hnr protein of the SL-UV mutant would be truncated due to the presence of a stop codon (the protein would lack 172 amino acids), the mutation in the Shine Dalgarno sequence of the NaCl strain would only result in a probably weaker promoter. However, further work would be required in order to verify this hypothesis.

It is generally acknowledged that alternative sigma factors are probably the most relevant strategy developed by bacteria when they face adverse conditions (Abee and Wouters, 1999) and the alternative sigma factor σ^S (also called σ^{38} or RpoS) of RNA polymerase (RNAP) is regarded as the master regulator of the general stress response in many Gram-negative bacteria, including *Salmonella* (Battesti et al., 2011; Hengge, 2009; Lago et al., 2017; Österberg et al., 2011). Thus, as it would be expected, the higher RpoS activity of these three strains led to the development of resistance to several agents in all of them. The differences observed in the pattern of multi-resistance might be probably due to the combination of several factors, including the level of RpoS activity in each strain, the relevance of RpoS in resistance to each particular agent and, in the case of the SL-UV strain, the presence of an additional mutation in *CaiD*. CaiD is involved in the pathway of carnitine metabolism, a molecule that has several functions in bacterial cells, such as providing osmotolerance, cryotolerace, bile tolerance and barotolerance but it also can serve as a nutrient or as an electron acceptor (Meadows and Wargo, 2015). CaiD was initially suggested to function as a racemase, as the *caiD* gene is required for racemase activity (Eichler et al., 1994), to convert D-carnitinyl-CoA to L-carnitinyl-CoA after being activated by CaiC (Bernal et al., 2008). Hence, this mutation, alone, might have caused a reduction in *S.* Typhimurium NaCl and HHP tolerance, and when combined with the overexpression of RpoS, might have led to the particular stress resistance pattern of this strain (the highest UV-C and PEF resistance but a decreased HHP and NaCl resistance). The precise role of RpoS and RpoS-dependent gene expression on *Salmonella* stress resistance, virulence, growth fitness and other phenotypic characteristics was recently reviewed by Guillén et al. (2021). However, its role in *Salmonella* resistance to some agents, such as PEF, UV-C and HHP, still remains to be fully elucidated. In this sense, results here obtained strongly suggest that RpoS would play a relevant role in *Salmonella* resistance to osmotic stress, UV-C and PEF, as it was previously suggested in Guillén et al. (2021). It is noteworthy that not only using these different agents led to mutations in a RpoS repressor, and therefore, led to the selection of variants with a higher RpoS activity, but also that positive correlation between PEF and UV-C resistance (Guillén et al., 2020b) and between PEF and NaCl resistance has been demonstrated among *Salmonella* strains (Guillén et al., 2022).

In addition, the increased RpoS activity would explain the lower growth rates observed for these three variants since it would lead to an increased transcription of genes involved in growth arrest and resistance

to a variety of stresses (Bearson et al., 1996), at least if we assume that, as in *E. coli* and *S.* Typhi, 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). However, it should also be noted that whereas stress-sensitive *rpoS* mutants (also called GASP phenotype: Growth Advantage in Stationary Phase) are surprisingly common among natural isolates of the closely related microorganisms *E. coli* and *S.* Typhi, they are not common in *S.* Typhimurium (Robbe-Saule et al., 2003), a phenomenon that has not yet been clarified.

Explaining the results obtained in the virulence assays for these three strains would be much more difficult since they displayed very different adhesion and invasion abilities. However, it does fit with the fact that the role of RpoS on *Salmonella* virulence seems to be very complex as reviewed in Guillén et al. (2021). Thus, RpoS seems to reduce the expression of some virulence factors while inducing other ones and, therefore, predicting what would be the virulence of a strain on the basis of its RpoS activity results extremely difficult.

As pointed out above, it has been suggested that the differences in stress resistance among *Salmonella* strains and serovars might be linked to differences in RpoS activity (Abdullah et al., 2018; Wang et al., 2021), and similar hypothesis have been made for other microorganisms (and their correspondent alternative sigma factors) (Lianou and Koutsoumanis, 2013; Robey et al., 2001). However, our results indicate that a direct relationship between RpoS activity and stress resistance would not exist within Salmonellae with the exceptions of PEF and, maybe, NaCl resistance. In any case, this conclusion should be taken with care given the relatively low number of strains tested (23). It should also be taken into account that, due to the complexity of *Salmonella* stress responses, the influence of RpoS activity might result masked, for instance, due to the occurrence of other mechanisms/phenomena more relevant for the resistance to a given particular stress. Hence, our results do not imply that RpoS activity plays no role in *Salmonella* resistance to agents other than PEF and NaCl, nor do they suggest that RpoS does not contribute to the observed differences in resistance among strains. Instead, our findings highlight NaCl and PEF as agents against which RpoS would play a significant role.

Finally, the fact that three out of five of the strains displayed mutations in *hnr* also suggest that this might constitute a conserved strategy within *Salmonella* in order to rapidly acquire stress resistance through the appearance of a subpopulation with increased stress resistance but also with reduced growth ability. Furthermore, it can also be hypothesized that the absence of GASPs in *S.* Typhimurium might be due to the fact that the generation of subpopulations with different RpoS activity in this microorganism would be achieved through changes in RpoS-regulated genes and not in *rpoS* itself. Further work is being carried out at the moment in order to validate these hypotheses as well as to study the reversibility of these genetic changes.

5. Conclusions

In this study, four resistant variants derived from *S. enterica* serovar Typhimurium SL1344 after its exposure to several rounds of acid stress, osmotic stress, high hydrostatic pressure and UV-C light treatments were isolated, and they showed cross-resistance to at least another stressor. This increased resistance, in general terms, had a fitness cost in growth, and had a variable impact on virulence (much greater and significant in adhesion than in invasion). Additionally, it led to increased antibiotic resistance but did not influence in the ability for biofilm formation. The increase in resistance could be due to an increase in RpoS activity, since the WGS analysis revealed that in 3 of the 5 variants (including SL1344-RS) a mutation was found in the *hnr* gene, an anti-sigma factor that promotes RpoS proteolysis, in addition to elevated *katE, katN* and *otsB* expression levels. However, extrapolating our results to a larger set of strains (22) indicates that a direct relationship between RpoS activity

and stress resistance within *Salmonella* cannot be established. In any case, this conclusion should be taken with caution, since most probably other cellular mechanisms also influence the resistance to the variety of stresses here studied.

CRediT authorship contribution statement

S. Guillén: Writing – original draft, Methodology, Investigation, Formal analysis. **L. Nadal:** Writing – review & editing, Methodology, Investigation. **N. Halaihel:** Writing – review & editing, Methodology, Formal analysis. **P. Mañas:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **G. Cebrián:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Guillermo Cebrián reports financial support was provided by Spain Ministry of Science and Innovation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2024.104714.

Data availability

Data will be made available on request.

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