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# Dynamics of high hydrostatic pressure resistance development in RpoS-deficient *Escherichia coli*

Elisa Gayán [a,1], Zhiying Wang [b], Maika Salvador [c], Michael G. Gänzle [b], Abram Aertsen [a,*]

[a] Department of Microbial and Molecular Systems, KU Leuven, Faculty of Bioscience Engineering, Kasteelpark Arenberg 20, 3001 Leuven, Belgium
[b] Department of Agricultural, Food and Nutritional Science, 4-10 Ag/For Centre, University of Alberta, Edmonton, AB T6G 2P5, Canada
[c] Department of Animal Production and Food Science, AgriFood Institute of Aragon (IA2), University of Zaragoza-CITA, Faculty of Veterinary, Miguel Servet 177, 50013 Zaragoza, Spain

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ABSTRACT

High hydrostatic pressure (HHP) treatment is one of the most widely accepted non-thermal food processing methods, but HHP-resistance development in pathogenic or spoilage bacteria might compromise the safety and stability of HHP-treated foods. Charting the possible routes and mechanisms of HHP resistance development in foodborne bacteria is therefore essential to anticipate or prevent the appearance of resistant variants. While upregulation of the RpoS-governed general stress response is a well-established route for increased HHP resistance in *Escherichia coli*, previous work revealed that mutations causing attenuated cAMP/CRP activity or aggregation-prone TnaA variants can evolve to overcome the HHP-hypersensitivity of an *E. coli* Δ*rpoS* mutant. In this study, further directed evolution and genetic analysis approaches allowed us to demonstrate that both kinds of mutants tend to co-emerge and compete with each other in *E. coli* Δ*rpoS* populations evolving towards HHP resistance, because of the higher HHP resistance of cAMP/CRP mutants and the faster growth rate of the TnaA mutants. Moreover, closer scrutiny of evolving populations revealed RpoS, cAMP/CRP and TnaA independent routes of HHP resistance development, based on downregulation of YegW or RppH activity.

## 1. Introduction

High hydrostatic pressure (HHP) treatment is one of the most widely used non-thermal processing methods to improve the microbial stability and safety of foods while retaining their sensorial and nutritional properties (Sehrawat, Kaur, Nema, Tewari, & Kumar, 2021). While some commercial HHP applications (ranging from 400 MPa to 600 MPa) aim for post-packaging decontamination, others aim for pasteurization of acidic products that sensitize pathogenic bacteria to HHP inactivation and inhibit outgrowth of potential survivors in the cold chain (Huang, Wu, Lu, Shyu, & Wang, 2017). However, the safety and stability of HHP-treated foods can be compromised because of the presence or emergence of HHP resistant bacteria. Indeed, while HHP resistant isolates are commonly encountered in food and other environments (González-Angulo et al., 2021; Liu, Gill, McMullen, & Gaenzle, 2015; Tamber, 2018; Wang et al., 2021), HHP resistant variants could also emerge in the food production line after recurrent HHP exposures (Vanlint, Rutten, Michiels, & Aertsen, 2012). In particular, strains of *Escherichia coli* including Shiga toxin-producing variants (STEC) have demonstrated the ability to readily acquire extreme HHP resistance after recurring exposure to HHP shocks (Vanlint et al., 2011; Vanlint et al., 2012). Furthermore, some HHP-resistant *E. coli* mutants also display resistance to fosfomycin (Gayán, Cambré, Michiels, & Aertsen, 2017), one of few antibiotics that are suitable for treatment of STEC infections (Kakoullis, Papachristodoulou, Chra, & Panos, 2019). Therefore, understanding the mechanisms of HHP resistance development in foodborne bacteria is essential to anticipate the emergence of HHP resistant variants and is critical to develop novel strategies for effective pathogen control, such as combining pressurization with other preservative hurdles.

Using directed evolution approaches towards increased HHP resistance development, we reported that selected mutants of *E. coli* O157:H7 displayed an upregulated RpoS-dependent general stress response (Vanlint, Rutten, Govers, Michiels, & Aertsen, 2013), which coincides with previous observations on the crucial role of this sigma factor for

* Corresponding author at: Faculty of Bioscience Engineering, Laboratory of Food Microbiology, Kasteelpark Arenberg 22, B-3001 Leuven, Belgium.
*E-mail address:* abram.aertsen@kuleuven.be (A. Aertsen).
[1] Present address: Department of Animal Production and Food Science, AgriFood Institute of Aragon (IA2), University of Zaragoza-CITA, Faculty of Veterinary, Miguel Servet 177, 50013 Zaragoza, Spain.

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pressure survival in stationary phase cells (Charoenwong, Andrews, & Mackey, 2011; Gayán, Cambré et al., 2017; Robey et al., 2001). Moreover, the large variability in HHP resistance among natural STEC isolates has previously been correlated with polymorphisms in their *rpoS* alleles and variations in cellular RpoS activity (Álvarez-Ordóñez et al., 2013; Robey et al., 2001). More recently, RpoS-independent evolutionary routes towards HHP-resistance were discovered as well. Indeed, loss-of-function mutations in *cyaA* (encoding the adenylate cyclase or cAMP synthetase) or *crp* (encoding the cAMP receptor protein), or gain-of-function mutations in *tnaA* (encoding the tryptophanase) were found to be acquired to overcome the HHP-hypersensitivity of an *E. coli* Δ*rpoS* mutant (Gayán, Cambré et al., 2017; Mortier et al., 2021; Vanlint, Pype et al., 2013). While it remains mechanistically unclear how attenuated cAMP/CRP activity exactly contributes to HHP resistance, specific mutations in *tnaA* were found to provoke aggregation of the TnaA protein and concomitant upregulation of heat shock proteins that, in agreement with earlier findings (Aertsen et al., 2004; Gayán, Van den Bergh, Michiels, Michiels, & Aertsen, 2020), exert a HHP protective effect (Mortier et al., 2021).

Since both cAMP/CRP- and TnaA-mediated evolutionary routes towards increased HHP resistance rapidly emerged in the absence of RpoS (Gayán, Cambré et al., 2017; Mortier et al., 2021), it can be assumed that the presence of this sigma factor might preclude the emergence of alternative and RpoS-independent HHP resistance mechanisms that could also be informative to understand HHP stress. Furthermore, many natural *E. coli* isolates as a result of ecological trade-offs actually harbour an attenuated or compromised *rpoS* allele (Chiang, Dong, Edge, & Schellhorn, 2011; Ferenci, 2005) and might therefore be inclined to follow such RpoS-independent evolutionary routes to acquire HHP resistance. For these reasons, the current study aimed to further scrutinize the dynamics of HHP resistance development in *E. coli* Δ*rpoS* populations.

## 2. Material and methods

### 2.1. Strain construction

The *E. coli* strains and plasmids used throughout this investigation are listed in Table S1, and the primers used for constructions are listed in Table S2. For strain constructions, LB broth and agar (Miller, 1992) were routinely used, and when necessary, a final concentration of 50 µg/ml of kanamycin (Panreac-AppliChem, Darmstadt, Germany), 20 µg/ml of tetracycline (Sigma-Aldrich, St. Louis, MO, USA) and/or 100 µg/ml of ampicillin (Thermo Fisher Scientific, Waltham, MA, USA) was added to select for the presence of plasmids or recombined amplicons.

In frame gene deletions were performed according to the recombineering method of Datsenko & Wanner (2000), using an *frt*-flanked *nptII* (encoding kanamycin resistance) PCR amplicon prepared on pKD13. Afterwards, the kanamycin resistance gene was excised by transiently equipping the strain with the plasmid pCP20 (expressing the Flp site-specific recombinase; Cherepanov & Wackernagel, 1995). On the other hand, mutations identified by whole genome sequencing in HHP-resistant mutants were identically reconstructed in the parental strain by the dual counter-selection system described by Li, Thomason, Sawitzke, Costantino, & Court (2013). Firstly, the region containing the target mutation in the parental strain, equipped with the plasmid pKD46, was replaced by a PCR amplicon containing the *tetA-sacB* -marker prepared on *E. coli* XTL298. In a second step, counter-selection was used to recombine the *tetA-sacB* cassette with a PCR amplicon obtained on the appropriate spontaneous mutant using primers flanking the desired mutation. For the point mutation located in the *rpoH* allele (*rpoH*$^{I54F}$), the *tetA-sacB* cassette was inserted downstream of the gene since it is essential for proper cell fitness (Goodall et al., 2018), and the clones lacking the cassette after the counterselection step with an amplicon harbouring the mutant allele were screened for the presence of the mutation by sequencing.

All genetic constructs were initially confirmed by PCR with primer pairs attaching outside of the engineered region (Table S2) and further verified by sequencing (Macrogen, the Netherlands).

### 2.2. HHP and heat treatment

For evaluating HHP and heat resistance, stationary phase cultures were obtained by inoculating test tubes containing 4 ml of Tryptone Soy Broth (TSB; Oxoid, Basingstoke, UK) with a single colony of the required strains, which were then incubated aerobically on an orbital shaker (200–300 rpm) for 18 h at 37 °C. Cells from stationary phase cultures, containing ca. $2 \times 10^9$ Colony Forming Units per milliliter (CFU/ml), were harvested by centrifugation (4000 × *g*, 5 min) and resuspended in an equal volume of 0.85 % KCl (Sigma-Aldrich).

To select for spontaneous HHP-resistant mutants, 200 µl of the suspension was heat sealed in a sterile polyethylene bag (Grade Packaging ltd., Coalville, UK) after exclusion of the air bubbles, and subjected to pressure (200–470 MPa) for 15 min using an 8-ml pressure vessel (HPIU-10000, 95/1994; Resato, Roden, The Netherlands), held at 20 °C with an external water jacket connected to a cryostat. To test HHP resistance of synthetic reconstructed mutants, 200 µl of the suspension was transferred to a polypropylene tube (Thermo Fisher Scientific), sealed on both sides after excluding air bubbles, and subjected to 400 MPa for 15 min using an 8-ml pressure vessel (high pressure multivessel apparatus U111, Agri-Food Discovery Place, Canada) immersed in a water bath at 20 °C. This treatment condition was chosen as it yields approximately 4 log cycles of inactivation of the parental strain. Pressure build-up was done at a rate of 100 MPa/min, and decompression was almost instantaneous.

In addition, the thermal resistance of spontaneous and reconstructed mutants was determined to evaluate if the acquisition of mutations increasing HHP resistance concomitantly provided cross-resistance to other food processing methods such as heat. For heat treatment, a sterile PCR tube was aseptically filled with 60 µl of cell suspension and subjected to 55 °C for 15 min using a PCR apparatus (T100; Biorad, Hercules, CA, USA). This treatment condition was chosen as it yields approximately 4 log cycles of inactivation of the parental strain.

After HHP or heat treatment, samples were aseptically retrieved from their container, and survival was determined as described below.

### 2.3. Selection of HHP-resistant mutants by directed evolution

To select for *E. coli* MG1655 Δ*rpoS* and Δ*rpoS* Δ*tnaA* mutants with increased pressure resistance, five independent cultures of each strain were reiteratively exposed to HHP shocks (15 min), progressively increasing pressure by 30 MPa each cycle (from 200 MPa to 470 MPa). After each HHP shock, an aliquot of the treated sample was inoculated 1/100 into fresh, prewarmed TSB and regrown for 23 h at 37 °C prior to the next round of pressurization. After ten cycles of selection, five surviving clones from each of the independently evolved cultures of the strain Δ*rpoS* Δ*tnaA* were purified and rechallenged to the last HHP treatment (470 MPa) in order to select a clone whose HHP resistance represented that of its corresponding evolved lineage.

### 2.4. Determination of viability and extent of sublethal injury

Samples of treated and control cultures were serially diluted in 0.85 % KCl, and a 5-µl volume of each dilution was spotted onto Tryptone Soy Agar (TSA; Oxoid) in triplicate, as previously described (Sieuwerts, de Bok, Mols, de Vos, & van Hylckama Vlieg, 2008). When indicated, cells were also recovered on Violet Red Bile Glucose Agar (VRBGA; Oxoid) as a selective medium to determine the extent of sublethal injury. After 24 h of incubation at 37 °C, spots containing between around 5 and 50 colonies were counted, so that the limit of quantification was 1,000 CFU/ml. The logarithmic reduction factor was calculated as log ($N_0/N$), in which $N_0$ and $N$ represent the number of survivors in CFU/ml prior

and after treatment, respectively. The number of sublethal injured cells was calculated by the difference between the counts on the non-selective (TSA) and the selective medium (VRBGA).

### *2.5. Evaluation of cAMP/CRP and tryptophanase activity*

The functionality of cAMP/CRP regulation in *E. coli* mutants during the directed evolution process was screened indirectly by their ability to ferment lactose on MacConkey agar No 3 (Lab M, Lancashire, UK). A sample of each stationary phase culture obtained after resuscitation from each HHP shock was diluted and plated on MacConkey agar to achieve approximately 500 CFU per plate. *E. coli* cells with fully functional cAMP/CRP activity and therefore lactose catabolism form pink colonies and a hazy precipitate of bile salts because of acidification (Gayán, Cambré et al., 2017; Shuman & Silhavy, 2003). Yellowish or pale-pinkish colonies not surrounded by a halo were considered as lactose-negative clones that incurred mutations downregulating cAMP/CRP activity. The lactose-negative phenotype of isolated clones was confirmed by striking the corresponding colonies on a fresh MacConkey plate. The percentage of lactose-negative mutants was calculated over the number of examined colonies on MacConkey agar (ca. 500 CFU), leading to a detection limit of ca. 0.2 %.

Tryptophanase activity was evaluated by the ability of indole production only in those clones that presented normal lactose fermentation on MacConkey agar, since dysfunctionality of cAMP/CRP complex downregulates the expression of *tnaA* (Isaacs, Chao, Yanofsky, & Saier, 1994; Li & Young, 2014). For indole test, 36 lactose-positive clones from each lineage were grown in 200 μl of TSB in microtiter plates overnight at 37 °C. Subsequently, a volume of 100 μl of Kovac's reagent (Sigma-Aldrich) was added to each well and visually examined for the presence of a pink layer on top of the culture after 2 min of incubation. Clones showing a colourless or low-intensity coloured layer compared to the parental strain were considered as lactose-positive/indole-negative mutants that harboured gain-of-function *tnaA* alleles. The percentage of lactose-positive/indole-negative mutants was calculated over 36 lactose-positive clones, leading to a detection limit of ca. 14 %.

When indicated, *crp*, *cyaA* and *tnaA* loci and their corresponding promoter regions were sequenced using the primers listed in Table S2.

### *2.6. Co-cultivation experiments*

The growth fitness of mutants and the parental strain was compared by confronting two strains in co-cultivation. One of them harboured a kanamycin resistance cassette disrupting the *rpoS* gene (*rpoS::nptII* or *rpoS::nptII* $tnaA^{\Delta 106}$) to be selectively counted against the markerless competitor strain (Δ*rpoS* Δ*crp*, Δ*rpoS* Δ*cyaA* or Δ*rpoS* $tnaA^{\Delta 106}$). Overnight stationary phase cultures of each couple of strains to be tested were diluted 1/100 in TSB, after which 500-μl portions of both suspensions were mixed. Subsequently, a 40-μl volume of this mixed suspension was inoculated in four tubes containing 4 ml of TSB, which were then incubated at 37 °C with shaking. Every 24 h, the obtained cultures were successively passaged by diluting them 1/10,000 in prewarmed growth medium until ca. 200 generations were achieved. Before each passage, a sample of each culture was appropriately diluted and spread-plated on TSA to achieve ca. 300 CFU per plate. After incubation, plates were counted to obtain the total cell count ($N_{\mathrm{TOT}}$), and subsequently replica-plated on TSA supplemented with kanamycin (50 μg/ml; $\mathrm{TSAKm}^{50}$). Counts on $\mathrm{TSAKm}^{50}$ yielded the number of CFU of strain 2 ($N_{\mathrm{st2}}$), and the number of CFU of strain 1 ($N_{\mathrm{st1}}$) was calculated as $N_{\mathrm{st1}} = N_{\mathrm{TOT}} - N_{\mathrm{st2}}$. The competition index was determined as $N_{\mathrm{st1}}/N_{\mathrm{st2}}$, and the detection limit was reached when $N_{\mathrm{st1}}/N_{\mathrm{st2}}$ fell below 0.003. Both TSA and $\mathrm{TSAKm}^{50}$ plates were incubated for 24 h at 37 °C. The presence of the *nptII* cassette in *rpoS::nptII* and *rpoS::nptII* $tnaA^{\Delta 106}$ strains did not entail any competitive fitness loss upon co-cultivation with their corresponding markerless homologues (i.e. the competition index remained ca. 1 over 200 generations; data not shown). Growth curves of individual strains were obtained by plating using 4 ml-TSB tubes inoculated to a starting concentration of ca. $10^4$ CFU/ml and incubated with shaking at 37 °C.

### *2.7. Growth ability at high osmotic pressure*

The functionality of RppH in *E. coli* strains was assessed by examining growth in the presence of an elevated NaCl concentration, since the lack of RppH activity has been related to osmotic stress sensitivity (Choi, Park, Kim, Seok, & Lee, 2017). Stationary phase cultures obtained in TSB were diluted 1/100 in 0.85 % KCl, and 5 μl of this suspension was spotted on TSA supplemented with 6 % of NaCl (Panreac-AppliChem). Plates were incubated at 37 °C for 48 h, and intensity of growth was scored relatively from ++++ to +.

### *2.8. Whole genome sequencing*

High-quality genomic DNA of MG1655 Δ*rpoS* Δ*tnaA* and its HHP-resistant derivatives were isolated from overnight LB cultures incubated aerobically at 37 °C, using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific). Paired-end libraries were prepared using Illumina Flex Library Prep kit (Illumina, USA) combined with Nextra DNA CD Index kit (Illumina, CA, USA) and sequenced by Illumina MiniSeq platform (Illumina). The whole genome sequencing runs yielded from 1,782,956 to 5,627,110 paired-end reads (150 bp), and the average coverage ± standard deviation ranged from 56.2 ± 12.0 to 147.5 ± 27.3. CLC Genomics Workbench version 7.5.1 (CLC Bio, Aarhus, Denmark) was used for analysis of the sequences, as previously described by Van den Bergh et al. (2016). Following quality assessment of the raw data, reads were trimmed using quality scores of the individual bases (quality limit = 0.01; maximum number of ambiguous bases = 2). Reads shorter than 15 bases were discarded from the set. Mapping of trimmed reads was performed by the CLC "Map Reads to Reference" algorithm (mismatch cost = 2; insertion cost = 3; deletion cost = 3; length fraction = 0.8; similarity fraction = 0.8) using as a reference the *E. coli* MG1655 genome (NC_000913.2) (Blattner et al., 1997). Mutations were detected using the CLC Fixed Ploidy Variant Detection tool (minimum coverage = 10; minimum frequency = 20 %; required significance = 1 %) and the InDels and Structural Variants tool (*P*-value threshold = 0.0001; maximum number of mismatches = 3; minimum number of reads = 2). Furthermore, the presence of large deletions or insertions was examined by inspecting coverage distribution and unmapped regions manually. Mutations found in the Δ*rpoS* Δ*tnaA* parent and all its mutants compared to the reference genome were ignored. Finally, the identified mutations were further confirmed with Sanger sequencing analysis, using the primers listed in Table S2.

### *2.9. Statistical analysis*

ANOVA and *t*-test were carried out using the software GraphPad PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA), and differences were regarded as significant when $P$ was $\leq 0.05$. All microbial inactivation outcomes shown in figures correspond to averages and standard deviations calculated from three biological replicates.

## 3. Results

### *3.1. Monitoring the emergence of cAMP/CRP- and TnaA-affected mutants in evolving lineages of MG1655 ΔrpoS towards increased HHP resistance*

Our previous research showed that five selected HHP-resistant mutants derived from MG1655 Δ*rpoS* by directed evolution harboured either mutations downregulating cAMP/CRP activity (by loss-of-function mutations in the *crp* gene) or mutations boosting the heat shock response (by gain-of-function mutations in the *tnaA* gene that

typically compromise tryptophanase activity) (Gayán, Cambré et al., 2017; Mortier et al., 2021). In this investigation, we therefore closely monitored the proportion of each type of mutant appearing in a new set of five independent lineages of MG1655 Δ*rpoS* evolving towards HHP resistance (Fig. 1A and Table 1). To this end, a number of clones from each lineage after each selection step were evaluated for their attenuated ability to ferment lactose (i.e. lactose-negative, as a proxy of downregulated cAMP/CRP activity; Gayán, Cambré et al., 2017) and, in case of lactose-positive clones, for their attenuated ability to produce indole (i.e. lactose-positive/indole-negative, as a proxy of gain-of-function *tnaA* alleles; Mortier et al., 2021).

As previously observed (Gayán, Cambré et al., 2017), all the Δ*rpoS* evolving lineages developed extensive HHP resistance after ten cycles of selection: the inactivation over all the lineages reached 2.4 log cycles on average at 470 MPa, while the survival of the Δ*rpoS* parent fell below the quantification limit (Fig. 1A). In all of the lineages, both lactose-negative and lactose-positive/indole-negative mutants began to appear between the fourth and sixth cycle of selection (i.e. after exposure to pressures between 290 MPa and 350 MPa; Table 1), although the prevalence of each phenotype changed over selective cycles and reached different end points. While in lineage 3 the prevalence of both types of mutants was virtually equal, lactose-negative mutants prevailed over lactose-positive/indole-negative mutants (lineages 1 and 5) or *vice versa* (lineages 2 and 4) in the other lineages. Remarkably, all the examined clones in all the lineages were either lactose-negative or lactose-positive/indole-negative, except for a small subpopulation with clearly fully functional cAMP/CRP and tryptophanase activity that co-existed with both type of mutants in lineage 4.

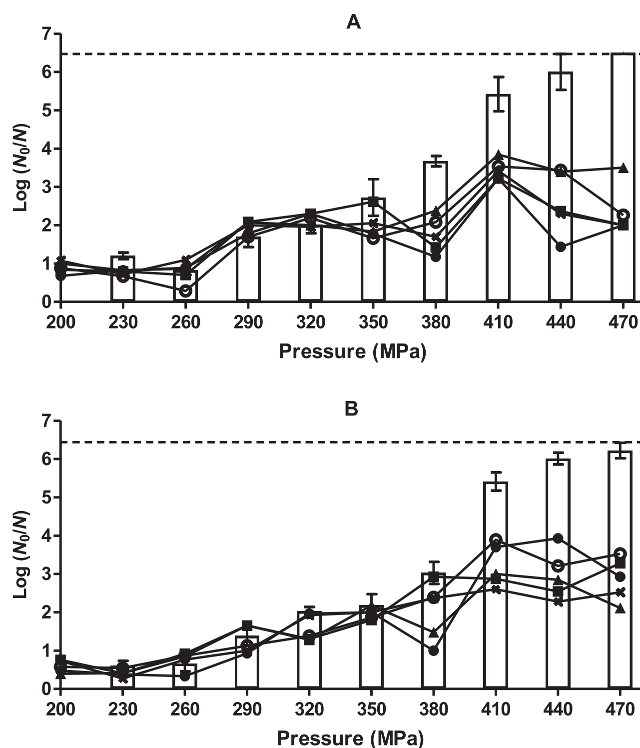


**Fig. 1.** Evolution of HHP resistance in five independent cultures of *E. coli* MG1655 (A) Δ*rpoS* and (B) Δ*rpoS* Δ*tnaA* iteratively exposed to progressively intensifying HHP shocks (30 MPa increments, 15 min) with intermittent resuscitation. The line graphs show the logarithmic reduction factor (log ($N_0$/$N$)) of the five evolving lineages (●, lineage 1; ■, lineage 2; ▼, lineage 3; ▲, lineage 4; ×, lineage 5) of each strain during the stepwise selection regime, while the bars represent the resistance of the unevolved MG1655 Δ*rpoS* or Δ*rpoS* Δ*tnaA* parental strain (i.e. without previous HHP exposure). Survivors were recovered on TSA. The dotted line represents the quantification limit (1,000 CFU/ml).

To confirm that our metabolic-phenotypic approach for detecting cAMP/CRP- or TnaA-affected mutants was accurate, we sequenced the *crp*, *cyaA* and *tnaA* loci in two lactose-negative and two lactose-positive/indole-negative clones isolated from each evolved lineage after ten cycles of selection. This confirmed that the lactose-negative isolates indeed harboured mutations in either *crp* or *cyaA* loci and carried an intact *tnaA* locus, while the lactose-positive/indole-negative isolates incurred mutations in the *tnaA* locus and carried intact *crp* or *cyaA* loci (data not shown). As such, all the analysed clones either incurred mutations in *crp*/*cyaA* or *tnaA*, but none of them had alterations in both. This is consistent with the fact that suppression of cAMP/CRP activity naturally inhibits *tnaA* expression (Isaacs et al., 1994; Li & Young, 2014), thereby offsetting any benefit of a *tnaA* gain-of-function mutation. In agreement, no additive HHP resistance effect of a *tnaA* gain-of-function allele could be observed in a *crp* or *cyaA* compromised background (Fig. S1).

### 3.2. *Differential fitness impact of crp/cyaA and tnaA mutations*

Because of their apparently consistent co-emergence and co-existence, we further characterized the fitness benefits of loss-of-function *crp*/*cyaA* or gain-of-function *tnaA* mutations in terms of HHP survival and growth. As such, we analysed the inactivation and the extent of sublethal injury of the Δ*rpoS* strain synthetically equipped with the three *tnaA* alleles formerly isolated in HHP-resistant mutants (Δ*rpoS* $tnaA^{Q240P}$, Δ*rpoS* $tnaA^{\Delta 106}$ and Δ*rpoS* $tnaA^{V224E}$; Mortier et al., 2021) and of the Δ*rpoS* strain lacking the *crp* or *cyaA* gene (Δ*rpoS* Δ*crp* and Δ*rpoS* Δ*cyaA*) at different HHP intensities (Fig. 2). In addition, the Δ*rpoS* Δ*tnaA* strain was included in the study as a control to confirm that the advantage of having spontaneous *tnaA* mutant alleles is not linked to the lack of tryptophanase activity. Indeed, the Δ*rpoS* Δ*tnaA* control strain displayed similar inactivation ($P > 0.05$) to the Δ*rpoS* strain when recovered on both the non-selective (TSA) and selective (VRBGA) media at all tested pressures. In contrast, Δ*rpoS* strains carrying $tnaA^{Q240P}$, $tnaA^{\Delta 106}$ or $tnaA^{V224E}$ increased ($P \leq 0.05$) their resistance over the parental Δ*rpoS* strain on the non-selective medium by 0.6, 1.8 and 2.0 log cycles on average at 290 MPa, 380 MPa and 470 MPa, respectively. Interestingly, their inactivation on the selective medium remained similar to that of the parental Δ*rpoS* strain ($P > 0.05$) at all pressures, suggesting that the three *tnaA* alleles likely improved HHP survival by favouring the recovery of sublethal injury.

At the lowest pressure (200 MPa), the Δ*rpoS* parent and Δ*rpoS* $tnaA^{Q240P}$/$tnaA^{\Delta 106}$/$tnaA^{V224E}$ variants showed the same resistance ($P > 0.05$) with the Δ*rpoS* Δ*crp*/Δ*cyaA* mutants on the non-selective medium; however, the number of sublethal injured cells in the later strains was ca. 5-fold lower ($P \leq 0.05$) compared to the other strains. At higher pressures, both lethal and sublethal injury of the Δ*rpoS* Δ*crp*/Δ*cyaA* mutants became significantly reduced compared to the different Δ*rpoS tnaA* mutants. More specifically, the inactivation of the Δ*rpoS* Δ*crp*/Δ*cyaA* mutants on the non-selective medium was 0.6 and 2.1 log cycles lower ($P \leq 0.05$) than the Δ*rpoS* $tnaA^{Q240P}$/$tnaA^{\Delta 106}$/$tnaA^{V224E}$ strains at 380 MPa and 470 MPa, respectively. Furthermore, the difference in counts of sublethally injured cells between Δ*rpoS* Δ*crp*/Δ*cyaA* and Δ*rpoS* $tnaA^{Q240P}$/$tnaA^{\Delta 106}$/$tnaA^{V224E}$ mutants reached up to 25-fold at 380 MPa, indicating attenuation of cAMP/CRP activity is better at preventing lethal and sublethal HHP injury compared to TnaA variants.

Additionally, we compared the growth fitness of cAMP/CRP- and TnaA-affected mutants to the parental Δ*rpoS* strain, in order to evaluate whether the enrichment steps within the directed evolution approach could favour or discriminate any type of mutants. This initially revealed that growth curves of axenically grown cultures yielded no obvious fitness cost in the Δ*rpoS* Δ*crp*, Δ*rpoS* Δ*cyaA* and Δ*rpoS* $tnaA^{\Delta 106}$ strains compared to their Δ*rpoS* parent (Fig. S2). However, when comparing relative growth fitness of these parent and mutant strains in pairwise competitive growth assays, significant differences were detected (Fig. 3). While the Δ*rpoS* $tnaA^{\Delta 106}$ mutant barely lost competitive fitness upon co-cultivation with the parental strain (i.e. the competition index

**Table 1**
Rate of emergence of lactose-negative (Lac-) and lactose-positive/indole-negative (Lac+/Ind-) mutants during the evolution of five independent lineages of MG1655 Δ*rpoS* towards increased HHP resistance.

| | | Selective cycle[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 (200 MPa) | 2 (230 MPa) | 3 (260 MPa) | 4 (290 MPa) | 5 (320 MPa) | 6 (350 MPa) | 7 (380 MPa) | 8 (410 MPa) | 9 (440 MPa) | 10 (470 MPa) |
| Lin 1 | Lac- | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | 3.2 | 74.9 | 81.6 | 91.4 | 100.0 | 100.0 |
| | Lac+/Ind- | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | 5.2 | 5.4 | 2.1 | ND[d] | ND[d] |
| Lin 2 | Lac- | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | 6.4 | 18.1 | 18.9 | 21.9 | 7.5 | 10.8 |
| | Lac+/Ind- | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | 27.3 | 82.0 | 81.1 | 78.1 | 92.5 | 89.2 |
| Lin 3 | Lac- | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | 7.4 | 55.3 | 73.0 | 74.8 | 56.8 | 46.3 |
| | Lac+/Ind- | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | 27.9 | 22.5 | 17.8 | 3.8 | 53.7 |
| Lin 4 | Lac- | ND[b] | ND[b] | ND[b] | ND[b] | 0.3 | 3.1 | 58.9 | 46.5 | 60.5 | 19.0 | 26.8 |
| | Lac+/Ind- | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | 8.0 | 6.9 | 49.0 | 34.5 | 77.7 | 57.9 |
| Lin 5 | Lac- | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | 32.6 | 82.9 | 89.6 | 90.2 | 100.0 | 100.0 |
| | Lac+/Ind- | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | ND[c] | 10.7 | 9.6 | 5.5 | ND[d] | ND[d] |

ND: Not Detected.
[a] Phenotypes were evaluated in stationary phase cultures obtained after resuscitation from the indicated HHP treatment.
[b] The detection limit for lactose-negative mutants was 0.2%.
[c] The detection limit for lactose-positive/indole-negative mutants was 13.9%.
[d] The screening for indole-negative mutants could not be performed.

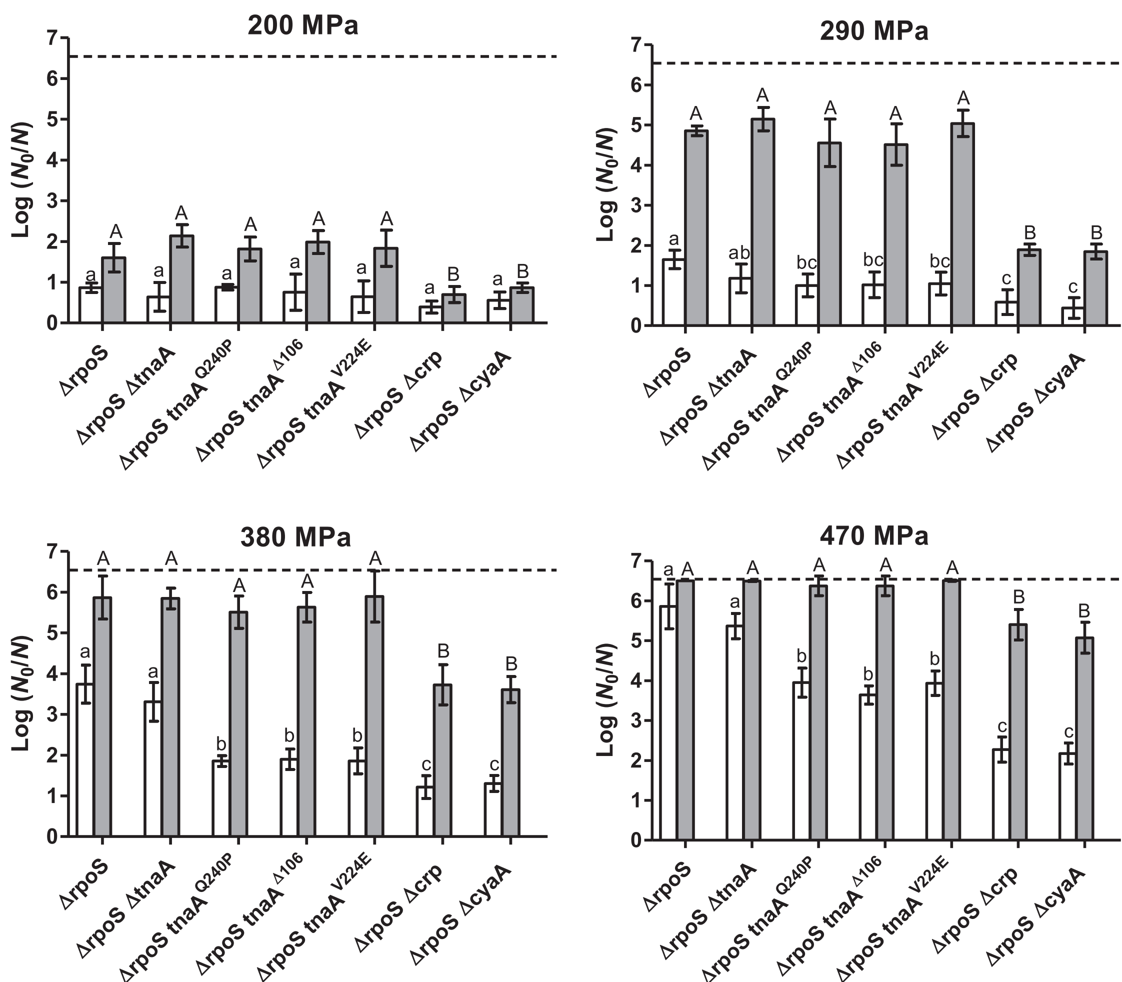


**Fig. 2.** Reduction of cell counts of *E. coli* MG1655 Δ*rpoS* and indicated derivatives by HHP treatments at different pressures (200 MPa, 290 MPa, 380 MPa and 470 MPa; 15 min). Survivors were recovered on TSA (white bars) and VRBGA (gray bars). The dotted line represents the limit of quantification (1,000 CFU/ml). Different lowercase and capital letters indicate statistically significant ($P \leq 0.05$) differences among the inactivation of all the strains obtained on TSA or VRBGA, respectively, at the same pressure.

decreased from 1.00 to 0.26 on average after ca. 200 generations), the initial 1/1 ratio between the Δ*rpoS* Δ*crp*/Δ*cyaA* and Δ*rpoS* strains diminished to $< 10^{-2}$ after ca. 80–100 generations (Fig. 3). Similarly, counts of Δ*rpoS* Δ*crp*/Δ*cyaA* strains rapidly vanished upon co-cultivation with the Δ*rpoS tnaA*$^{\Delta 106}$ variant.

As such, the co-existence of both cAMP/CRP- and TnaA-affected

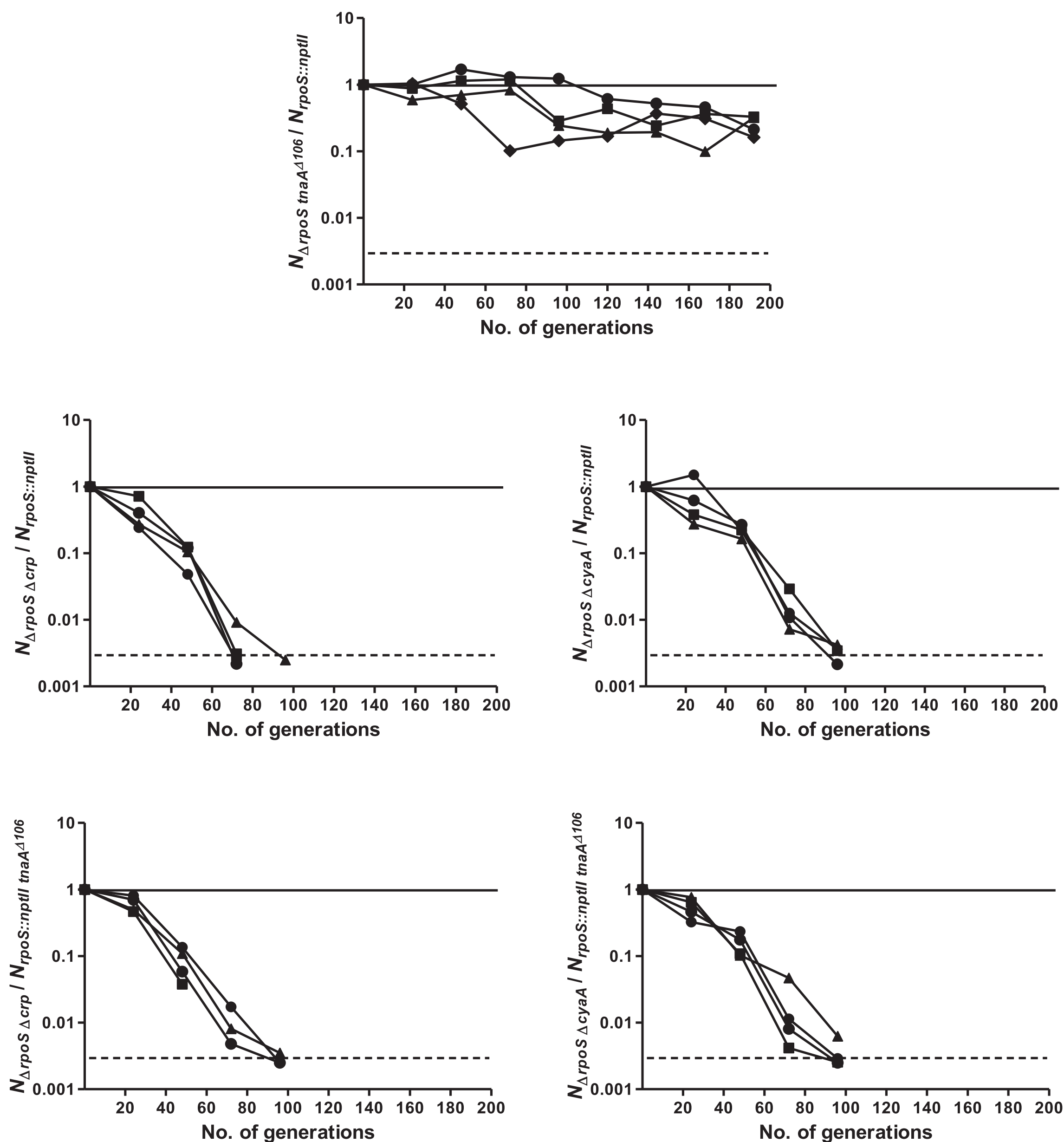


**Fig. 3.** Competition index over generation number during co-cultivation of four independent lineages of the indicated couple of strains of *E. coli*. The relative abundance of the corresponding strains is expressed by the ratio of CFU of strain 1 ($N_{st1}$; markerless strain) over the CFU of strain 2 ($N_{st2}$; harbouring the *nptII* cassette). The detection limit is indicated by a dotted line and was reached when $N_{st1}/N_{st2}$ fell below 0.003.

mutants in HHP-cycled Δ*rpoS* populations seems to be sustained by the improved HHP resistance of the former and the improved competitive growth fitness of the latter.

**Table 2**
Rate of emergence of lactose-negative (Lac-) mutants during the evolution of five independent lineages of MG1655 Δ*rpoS* Δ*tnaA* towards increased HHP resistance.

| | | Selective cycle[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 (200 MPa) | 2 (230 MPa) | 3 (260 MPa) | 4 (290 MPa) | 5 (320 MPa) | 6 (350 MPa) | 7 (380 MPa) | 8 (410 MPa) | 9 (440 MPa) | 10 (470 MPa) |
| **Lin 1** | Lac- | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | 2.1 | 10.7 | 70.3 | 91.9 | 93.5 |
| **Lin 2** | Lac- | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | 6.5 | 41.0 | 32.6 | 86.9 | 97.2 |
| **Lin 3** | Lac- | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | 4.6 | 10.5 | 1.0 | 2.2 | 51.9 |
| **Lin 4** | Lac- | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | 2.1 | 17.5 | 35.4 | 83.6 | 14.9 |
| **Lin 5** | Lac- | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | ND[b] | 2.2 | 30.9 | 9.6 | 3.7 |

ND: Not Detected.
[a] Phenotypes were evaluated in stationary phase cultures obtained after resuscitation from the indicated HHP treatment.
[b] The detection limit for lactose-negative mutants was 0.2%.

### 3.3. Evolution of ΔrpoS ΔtnaA strain towards increased HHP resistance independently of cAMP/CRP downregulation

Given the observation that some HHP-resistant clones with apparently normal lactose fermentation and indole production could arise in the Δ*rpoS* evolved populations (cfr. lineage 4, Table 1), we wondered whether alternative evolutionary routes towards HHP resistance could be discovered independent of RpoS, cAMP/CRP or TnaA. Therefore, five independent lineages of an MG1655 Δ*rpoS* Δ*tnaA* mutant (unable to acquire mutations that upregulate RpoS activity or change TnaA folding) were subjected to directed evolution towards HHP resistance, while focusing on the emergence of lactose-positive (i.e. unaffected in cAMP/CRP activity) HHP resistant clones (Fig. 1B and Table 2). This revealed that HHP resistance emerged in each of the five independent Δ*rpoS* Δ*tnaA* lineages (on average 3.4 log cycles compared to the parental strain at 470 MPa), and that lactose-positive clones could indeed still be observed in each of the lineages. In fact, while lactose-negative (i.e. likely cAMP/CRP affected) mutants dominated (>90 %) in lineages 1 and 2, a large fraction of the clones screened from lineages 3 (48 %), 4 (85 %) and 5 (96 %) retained an apparently normal lactose metabolism.

Subsequently, we characterized the resistance of one lactose-positive clone isolated from each independent lineage (designated as MT1 to MT5), after confirming by sequencing that their *crp* and *cyaA* loci were unaltered. Each of the five independent clones displayed lower inactivation ($P \leq 0.05$) than their MG1655 Δ*rpoS* Δ*tnaA* parent at 400 MPa (Fig. 4A). More specifically, compared to the parent strain, MT1, MT2 and MT5 increased survival by ca. 2.5 log cycles, while MT4 and MT3 increased survival by 2.1 and 1.7 log cycles, respectively. As such, the viability of MT1, MT2 and MT5 after 400 MPa exposure equalled ($P >$ 0.05) to that of the Δ*rpoS* Δ*crp/cyaA* mutants (data not shown) and even to that of wild-type (i.e. RpoS proficient) MG1655 (Fig. 4A).

Interestingly, most of the HHP-resistant mutants also developed cross-resistance to heat (55 °C), except for MT2, which became significantly ($P \leq 0.05$) more sensitive compared to its Δ*rpoS* Δ*tnaA* parent (Fig. 4B). MT1 and MT4 were the most heat-resistant mutants reaching 1.8-log cycle lower inactivation ($P \leq 0.05$) than their parent.

### 3.4. Genomic analysis of the evolved ΔrpoS ΔtnaA mutants

The whole genome of the five independently evolved lactose-positive HHP-resistant Δ*rpoS* Δ*tnaA* mutants was sequenced and compared with that of the parental strain in order to identify mutations potentially linked to their increased HHP and heat (cross-)resistance. As observed in Table 3, most of the mutants (except MT4) incurred more than one mutation and became affected in many different genes, although two genes (*yegW* and *rppH*) were recurringly hit by mutations in different strains. The three most HHP-resistant mutants (i.e. MT1, MT2 and MT5) harboured a mutation in the open reading frame (ORF) of the *yegW* gene, encoding the putative DNA-binding transcriptional regulator YegW with yet unknown function. More specifically, MT1 incurred a ca. 2.5-kb insertion probably corresponding to a IS2 element, MT2 a single nucleotide polymorphism (SNP) leading to an amino acid (AA) substitution (L53V, denoted as the $yegW^{L53V}$ allele) in its predicted HTH motif domain (https://www.uniprot.org/uniprot/P0ACM5), and MT5 a 1-bp frameshifting deletion (denoted as the $yegW^{162fs}$ allele) theoretically leading to a truncated YegW with partially abnormal AA sequence.

On the other hand, both MT1 and MT4 incurred a mutation in the *rppH* ORF, encoding the RNA pyrophosphohydrolase that initiates 5′-end-dependent mRNA degradation (Luciano, Vasilyev, Richards, Serganov, & Belasco, 2018). MT1 incurred a 1-bp deletion that resulted in a truncation and a non-sense sequence from AA 25 to 57 (denoted as the $rppH^{25fs}$ allele) thus disrupting its nudix motif (Vasilyev & Serganov, 2015). MT4, in turn, incurred a SNP giving an AA substitution (L101H, denoted as the $rppH^{L101H}$ allele) in the nudix hydrolase domain, this being the single mutation found across its genome.

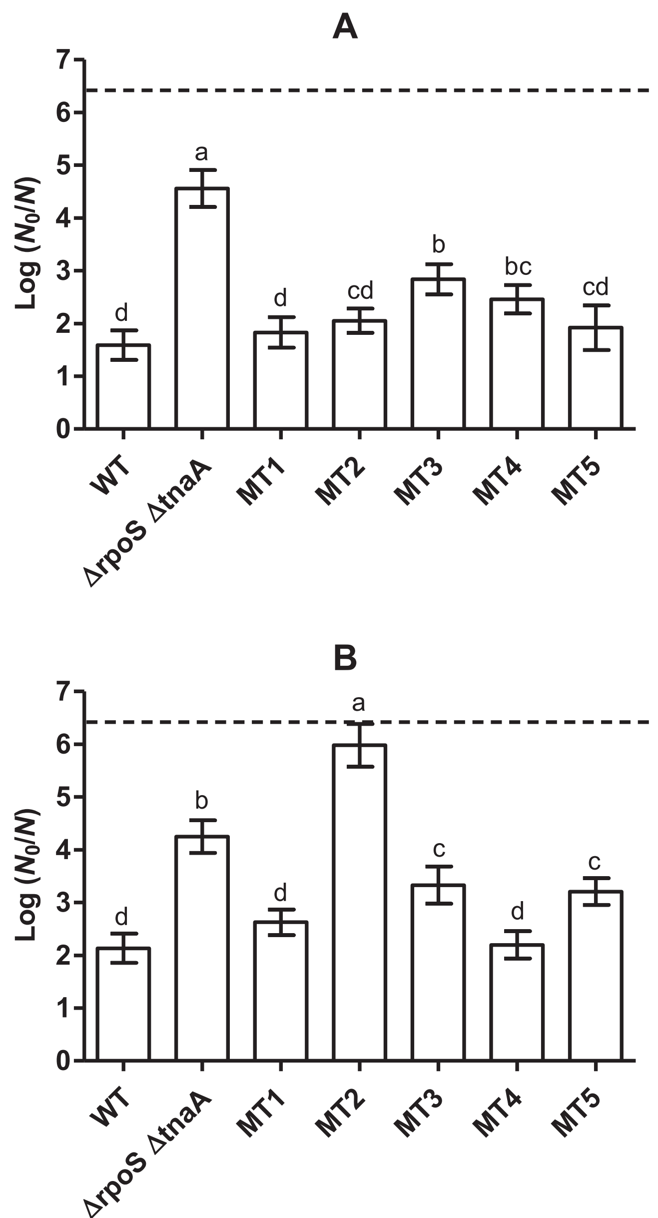


**Fig. 4.** Reduction of cell counts of *E. coli* MG1655 WT, Δ*rpoS* Δ*tnaA* and spontaneous HHP-resistant Δ*rpoS* Δ*tnaA* mutants (from MT1 to MT5) by (A) HHP (400 MPa, 15 min) and (B) heat (55 °C, 15 min) treatment. Survivors were recovered on TSA. The dotted line represents the limit of quantification (1,000 CFU/ml). Different letters indicate statistically significant ($P \leq 0.05$) differences among the inactivation of all the strains within each panel.

Due to the importance of the heat shock response for HHP survival (Gaenzle & Liu, 2015; Gayán, Govers, & Aertsen, 2017), it is important to highlight that MT5 incurred a missense mutation in its *rpoH* gene, encoding the RpoH ($\sigma^{32}$) sigma factor that governs the expression of the heat shock response (Horikoshi, Yura, Tsuchimoto, Fukumori, & Kanemori, 2004; Obrist & Narberhaus, 2005), giving rise to an AA substitution (I54F, denoted as the $rpoH^{I54F}$ allele) in the *N*-terminal half of the 2.1 region. This exact mutation was formerly found in *E. coli* mutants selected for enhanced RpoH stability leading to increased basal expression of heat shock genes (Obrist & Narberhaus, 2005).

### 3.5. Role of yegW and rppH mutations in increased HHP and heat (cross-) resistance of ΔrpoS ΔtnaA mutants

Subsequently, we aimed to investigate the role of the genes that were

**Table 3**
Mutations identified in *E. coli* Δ*rpoS* Δ*tnaA* mutants.

| Mutant | Gene region | Nucleotide mutation | Protein mutation | Function |
|---|---|---|---|---|
| **MT1** | ORF of *rppH* | 1-bp deletion (G) at position 73 | Truncated protein with a non-sense sequence from, and including, AA 25 to 57[b] (normal length: 176 AA) | RNA pyrophosphohydrolase |
| | ORF of *yegW* | ca. 2.5-kb insertion at position[a] 525 | Truncated protein with a non-sense sequence from, and including, AA 175 to 201[b] (normal length: 248 AA) | Putative DNA-binding transcriptional regulator YegW |
| | ORF of *mtn* | T636G | – | 5′-methylthioadenosine/ S-adenosylhomocysteine nucleosidase |
| | ORF of *iclR* | T378C | – | DNA-binding transcriptional repressor IclR |
| **MT2** | ORF of *allE* | T659G | I220S | (S)-ureidoglycine aminohydrolase |
| | ORF of *yegT* | C158T | S53L | Putative nucleoside transporter YegT |
| | ORF of *yegW* | T157G | L53V | Putative DNA-binding transcriptional regulator YegW |
| | ORF of *rodZ* | 1-pb deletion (G) at position 442 | Truncated protein with a non-sense sequence from, and including, AA 147 to 153[b] (normal length: 337 AA) | Transmembrane component of cytoskeleton |
| **MT3** | ORF of *ynfM* | T431C | V144A | Putative transporter YnfM |
| | ORF of *hldE* | G772T | D258Y | Bifunctional heptose 7-phosphate kinase/heptose 1-phosphate adenyltransferase |
| **MT4** | ORF of *rppH* | T302A | L101H | RNA pyrophosphohydrolase |
| **MT5** | ORF of *rpoH* | A160T | I54F | RNA polymerase sigma factor H ($\sigma^{32}$) |
| | ORF of *yegW* | 8-bp insertion at position[a] 574 | Truncated protein with a non-sense sequence from, and including, AA 162 to 211[b] (normal length: 248 AA) | Putative DNA-binding transcriptional regulator YegW |

ORF: open reading frame; AA: amino acid.
[a] The position gives the nucleotide after which the insertion took place.
[b] Total length of the truncated protein.

most frequently mutated in the selected HHP- and heat-(cross-)resistant Δ*rpoS* Δ*tnaA* mutants (i.e. *yegW* and *rppH* genes) by reconstructing some of their alleles (i.e. $yegW^{L53V}$ and $yegW^{162fs}$, and $rppH^{25fs}$ and $rppH^{L101H}$) in the parental strain. In order to approach the functional impact of the spontaneous mutations, the HHP and heat resistance of the reconstructed mutants was compared to that of the parent, and of the strains completely lacking either the *yegW* or *rppH* gene (Fig. 5).

The spontaneous $yegW^{L53V}$ and $yegW^{162fs}$ alleles and the Δ*yegW* deletion increased ($P \le 0.05$) HHP resistance of the parental strain by 1.7 log reductions on average and equalled ($P > 0.05$) to that of the corresponding spontaneous mutants. Conversely, the three *yegW* mutants decreased ($P \le 0.05$) heat resistance of the Δ*rpoS* Δ*tnaA* strain by more than ca. 1.5 log cycles. While MT2 showed equal ($P > 0.05$) heat sensitivity as the Δ*rpoS* Δ*tnaA* $yegW^{L53V}$ strain, the resistance of MT5 was much higher ($P \le 0.05$) than that of the Δ*rpoS* Δ*tnaA* $yegW^{162fs}$ strain. Similarly, MT1 showed higher ($P \le 0.05$) heat survival than the parental strain despite harbouring a truncated mutation in its *yegW* allele. These results suggest that heat sensitisation provided by loss-of-function *yegW* mutations could be rescued by the $rppH^{25fs}$ allele in MT1 (see below) and by the gain-of-function mutation in the *rpoH* gene in MT5, which was proven to remarkably increase heat resistance of the parental strain on its own (Fig. S3).

Regarding *rppH* mutations, the HHP resistance of the Δ*rpoS* Δ*tnaA* $rppH^{L101H}$ strain was 1.0-log cycle higher ($P \le 0.05$) than that of the parental Δ*rpoS* Δ*tnaA* strain and equal ($P > 0.05$) to that of MT4, while the inactivation of the Δ*rpoS* Δ*tnaA* $rppH^{25fs}$ and Δ*rpoS* Δ*tnaA* Δ*rppH* strains was similar ($P > 0.05$) to that of the parental strain. Thus, the increased HHP resistance developed by MT1 likely stemmed from the disruption of *yegW* by a 2.5-kb insertion and not from its $rppH^{25fs}$ allele. By contrast, both spontaneous *rppH* alleles, and the synthetic *rppH* deletion to a lower extent, increased ($P \le 0.05$) the heat survival of the

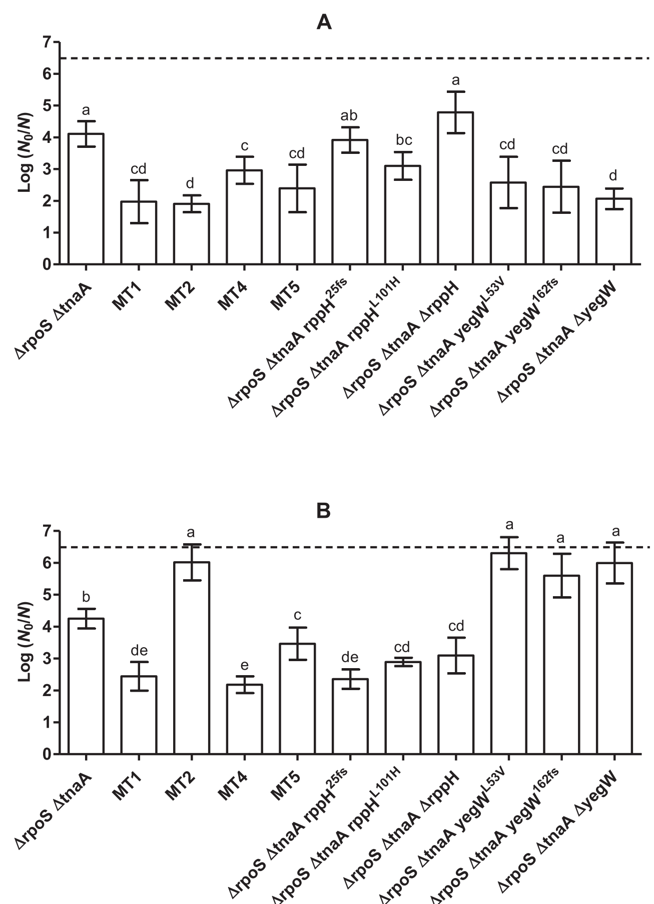


**Fig. 5.** Reduction of cell counts of *E. coli* MG1655 Δ*rpoS* Δ*tnaA* and its indicated spontaneous and reconstructed mutants by (A) HHP (400 MPa, 15 min) and (B) heat (55 °C, 15 min) treatment. Survivors were recovered on TSA. The dotted line represents the limit of quantification (1,000 CFU/ml). Different letters indicate statistically significant ($P \le 0.05$) differences among the inactivation of all the strains within each panel.

parental strain by ca. 1.6 log cycles, thus mimicking ($P > 0.05$) the heat resistance of MT1 and MT4.

Because of the different outcomes observed in the HHP resistance of Δ*rpoS* Δ*tnaA* *rppH*$^{25fs}$ and Δ*rpoS* Δ*tnaA* *rppH*$^{L101H}$ strains, we explored the functionality of spontaneous *rppH* alleles by the growth ability at a high sodium chloride concentration, since deprivation of RppH activity has been related to osmotic stress sensitivity (Choi et al., 2017). Both strains, together with the spontaneous mutants where the alleles originated from, showed impaired growth compared to the parental strain (Table 4). The growth of MT4 and Δ*rpoS* Δ*tnaA* *rppH*$^{L101H}$ strains was less restricted than that of MT1 and Δ*rpoS* Δ*tnaA* *rppH*$^{25fs}$, followed by the Δ*rpoS* Δ*tnaA* Δ*rppH* strain. These results point out that the *rppH*$^{25fs}$ allele, giving rise to a truncated protein with a nonsense sequence coinciding with its entire nudix motif, debilitated RppH activity more than the *rppH*$^{L101H}$ allele, incurring an AA substitution in its nudix hydrolase domain. Therefore, the weak attenuation of RppH activity or a change in its RNA specificity derived from the *rppH*$^{L101H}$ allele, but not its stronger or complete inhibition observed in *rppH*$^{25fs}$ and Δ*rppH* alleles, might be required for HHP resistance development, while any small to large depletion of RppH activity (stemming from *rppH*$^{L101H}$, *rppH*$^{25fs}$ and Δ*rppH* alleles) can contribute to increased heat resistance.

## 4. Discussion

Mutations upregulating RpoS activity reflect a relevant evolutionary strategy in *E. coli* in both natural and laboratory settings to acquire increased stress resistance, with such mutations often found among the many genes modulating the extensive regulatory network of the general stress response (Battesti, Majdalani, & Gottesman, 2011; Donovan, Norton, Bower, & Mulvey, 2013; Gayán, Cambré, Michiels, & Aertsen, 2016; Gayán et al., 2020; Tenaillon et al., 2012; Vanlint, Rutten et al., 2013). This straightforward mutational upregulation of RpoS activity, however, obstructs the evolutionary emergence of informative RpoS-independent genetic routes towards resistance development that might expedite our understanding of cryptic pleiotropic stresses such as HHP stress. Moreover, since many *E. coli* isolates are naturally deficient in RpoS activity (Álvarez-Ordóñez et al., 2013; Bhagwat et al., 2006; Chiang et al., 2011; Ferenci, 2005; Robey et al., 2001), their HHP resistance development might more likely proceed via RpoS-independent ways.

In the (synthetically engineered) absence of RpoS, novel pathways towards increased HHP resistance in *E. coli* were revealed, such as acquiring loss-of-function *cyaA/crp* mutations or gain-of-function *tnaA* mutations (Gayán, Cambré et al., 2017; Mortier et al., 2021). In this context, our current study revealed that these two RpoS-independent resistance mechanisms to a large extent co-emerge and co-exist in MG1655 Δ*rpoS* populations evolving towards HHP resistance. In addition, these two mechanisms are not complementary, since deprivation of cAMP/CRP activity abolishes expression of gain-of-function TnaA alleles (Isaacs et al., 1994; Li & Young, 2014) and therefore their potential advantages. Comparing benefits of carrying loss-of-function *crp/cyaA* mutations with gain-of-function *tnaA* alleles, it was demonstrated that the former provided better survival and lower extent of sublethal injury than mutants harbouring *tnaA* mutations, especially at the higher pressures tested. In contrast, *crp/cyaA* mutants suffered from attenuated growth fitness when competing with their parent, as previously observed in the strain ATCC 43888 (Vanlint, Pype et al., 2013), and TnaA variants. Therefore, due to the outstanding HHP resistance of cAMP/CRP-affected mutants (despite being counterselected during the growth steps in between the subsequent HHP exposures) and the higher competitive growth fitness of *tnaA* variants (despite being more HHP sensitive than cAMP/CRP-affected mutants), both type of mutants managed to co-emerge and prevail in most evolved populations. While often RpoS activity is considered to play an important role in modulating a strain's balance between growth fitness and stress resistance (Ferenci, 2005), our data indicate that *E. coli* can navigate this balance even in the complete absence of RpoS.

Interestingly, we could also select for the emergence of HHP resistance via evolutionary routes that are independent of RpoS, cAMP/CRP or TnaA. Genome analysis indicated that such routes were (at least in part) supported by mutations in the *rppH* or *yegW* genes that have so far not been connected with HHP resistance. Abolishing YegW activity, a putative DNA-binding transcriptional regulator, could be causally linked to the increased HHP resistance of the spontaneous Δ*rpoS* Δ*tnaA* mutants incurring deleterious mutations in the encoding gene (i.e. MT1, MT2 and MT5), while providing them heat sensitivity. Further characterization of the currently cryptic YegW regulator is required to further unravel its impact on HHP survival.

The RNA pyrophosphohydrolase encoded by the *rppH* gene is a nudix hydrolase that converts the 5′-terminal triphosphate or diphosphate of mRNA to a monophosphate for initiating 5′-end-dependent degradation by RNase E (Luciano et al., 2018). The loss of RppH activity in *E. coli* affects several critical cellular processes including virulence and stress adaptation responses (Badger, Wass, & Kim, 2000; Deana, Celesnik, & Belasco, 2008; Lee, Kim, Park, Kim, & Seok, 2014). In fact, *rppH* deletion mutants have demonstrated reduced invasiveness, higher sensitivity to a variety of antibiotics and chemicals (including rifampicin, colistin, sodium chloride and ethanol), but also faster growth at a cold temperature (Badger et al., 2000; Bessman et al., 2001; Choi et al., 2017; Lee et al., 2014). In this study, we reported that weak attenuation of RppH activity as derived from the *rppH*$^{L101H}$ allele but not its stronger or complete inhibition as derived from the *rppH*$^{25fs}$ and Δ*rppH* alleles is required for HHP resistance development, while any degree of depletion stemming from *rppH*$^{L101H}$, *rppH*$^{25fs}$ and Δ*rppH* alleles is sufficient to provide heat resistance. Downregulating RppH affects hundreds of different transcripts (Deana et al., 2008; Luciano et al., 2012), so further research is required to mechanistically understand its impact on HHP and heat survival.

In conclusion, our work demonstrates that preventing the preferential evolutionary routes for HHP resistance development in *E. coli* readily enables the emergence of alternative mechanisms for increased HHP survival. While upregulation of RpoS activity is a relevant evolutionary strategy, downregulation of cAMP/CRP activity and acquisition of gain-of-function TnaA variants appear to become the predominant routes in the absence of the general stress sigma factor. Furthermore, we also revealed yet alternative evolutionary routes towards HHP resistance (independent of RpoS, cAMP/CRP or TnaA), based on downregulation of RppH and YegW activity. Since the species of *E. coli* includes important foodborne pathovars that often display attenuated RpoS activity (Álvarez-Ordóñez et al., 2013; Bhagwat et al., 2006; The, Thanh, Holt, Thomson, & Baker, 2016; Robey et al., 2001), its genetic resourcefulness with regard to HHP resistance development might attenuate the safety of HHP treated foods.

**Table 4**
Growth ability of *E. coli* MG1655 Δ*rpoS* Δ*tnaA* and its indicated mutants on TSB supplemented with 6 % sodium chloride. The growth intensity of 5 µl of stationary phase cultures diluted 1/100 was relatively scored from ++++ to + with respect to the Δ*rpoS* Δ*tnaA* and Δ*rpoS* Δ*tnaA* Δ*rppH* strain, respectively.

| Strain | Growth intensity |
|---|---|
| Δ*rpoS* Δ*tnaA* | ++++ |
| Δ*rpoS* Δ*tnaA* Δ*rppH* | + |
| MT1 | ++ |
| Δ*rpoS* Δ*tnaA* *rppH*$^{25fs}$ | ++ |
| MT4 | +++ |
| Δ*rpoS* Δ*tnaA* *rppH*$^{L101H}$ | +++ |

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.112280.

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