**Identification of novel genes involved in high hydrostatic pressure resistance
of *Escherichia coli***

Elisa Gayán^a, Nele Rutten^a, Jan Van Impe^b, Chris W. Michiels^a, Abram Aertsen^{a*}

**^aLaboratory of Food Microbiology, Department of Microbial and Molecular Systems, KU
Leuven, 3000 Leuven, Belgium**

**^bChemical and Biochemical Process Technology and Control, Department of Chemical
Engineering, KU Leuven, 9000 Ghent, Belgium**

***Address correspondence to Abram Aertsen**

abram.aertsen@kuleuven.be

Faculty of Bioscience Engineering

Laboratory of Food Microbiology

Kasteelpark Arenberg 22

B-3001 Leuven

Belgium

Tel: +32 16 32 17 52

Abstract

High hydrostatic pressure (HHP) is an interesting hurdle in minimal food processing that aims to synergistically combine different stresses to improve food microbiological safety and stability without compromising quality. For a proper understanding and design of hurdle technology, the cellular impact of the applied stresses on foodborne pathogens should be well-established. To study the mechanism of HHP-mediated cell injury and death, we screened for loss-of-function mutations in *E. coli* MG1655 that affected HHP sensitivity. More specifically, *ca.* 6,000 random transposon insertion mutants were individually exposed to HHP, after which the phenotype of the most resistant or sensitive mutations was confirmed by *de novo* gene deletions in the parental strain. We found that disruption of *rbsK*, *rbsR*, *hdfR* and *crl* decreased HHP resistance, while disruption of *sucC* and *sucD* (encoding subunits of the succinyl-CoA synthetase) increased HHP resistance. More detailed study of the tricarboxylic acid cycle enzymes encoded by the *sdhCDAB-sucABCD* operon surprisingly showed that disruption of the *sucA* or *sucB* gene (encoding subunits of the 2-oxoglutarate dehydrogenase complex) notably decreased HHP survival. We also found that the increased HHP resistance of a Δ *sucC* and Δ *sucD* mutant was mediated by increased basal RpoS activity levels, although it did not correlate with their heat resistance. Our results reveal that compromising TCA cycle enzymes can profoundly affect HHP resistance in *E. coli*.

Key-words: *E. coli*; high hydrostatic pressure; heat; resistance; TCA cycle

1. Introduction

Consumers' tendency for healthy fresh-like foods requires the development of mild food preservation methods that minimally affect the sensorial and nutritional properties of the food product while at the same time ensuring food safety. To avoid intensive heat processing, a number of non-thermal food processing techniques capable of inactivating pathogenic and spoilage microorganisms have emerged, including high hydrostatic pressure (HHP), pulsed electric fields, ultrasound and UV-based irradiation (Barba et al., 2017). HHP processing is one of the most accepted technologies, being already used in the food industry to improve safety and extend shelf life of a variety of products (Huang et al., 2017). Unfortunately, full exploitation of HHP pasteurization is hampered in part by the considerable HHP resistance that some pathogenic bacteria tend to display naturally (Bruschi et al., 2017; Liu et al., 2015) or can readily acquire upon recurrent exposure to HHP processing (Vanlint et al. 2012). In addition, the high capital investment in HHP equipment and the need to combine pressure and thermal treatment to inactivate bacterial endospores limit the use of this technology in certain type of foods (Elamin et al., 2015; Khan et al., 2017). Rather than using intense HHP processing to ensure inactivation of resistant bacteria at the expense of food quality, HHP can be combined with other stresses in a hurdle-type approach. In fact, bacteria surviving HHP exposure tend to be sublethally injured (Sokołowska et al., 2014; Somolinos et al., 2008) and therefore sensitized to a number of subsequent stresses (Koseki et al., 2008; Somolinos et al., 2008). As such, combining mild HHP treatment with other preservation methods at moderate intensity could be a promising strategy to adequately control microorganisms while reducing the loss of food quality and processing costs (Khan et al., 2017; Oliveira et al., 2015). Indeed, the combination of HHP with mild heat, low pH, oxidative stress and certain natural antimicrobial compounds, such as reuterin, α , β -

65 unsaturated aldehydes or isothiocyanates, has been demonstrated to synergistically enhance
66 inactivation (Feyaerts et al., 2015; Gao et al., 2006; Montiel et al., 2015; Muñoz et al., 2006;
67 Wang et al., 2012). However, the combination of HHP with other compounds, such as carvacrol,
68 linalool or eugenol, only exerted an additive or even an antagonistic lethal effect (Feyaerts et al.,
69 2015).

70 Paramount to the proper design of effective hurdle approaches is a better understanding of the
71 cellular impact of available preservation stresses. With respect to HHP processing, current
72 knowledge on its effects on bacteria is mainly coined from the study of (i) HHP on biomolecules
73 (Gayán et al. 2017a; Rivalain et al., 2010), (ii) cellular responses to HHP stresses (Bowman et
74 al., 2008; Malone et al., 2006), and (iii) the genetic analysis of bacterial mutants that were
75 selected to become HHP resistant through directed evolution approaches (Gayán et al. 2017b;
76 Vanlint et al., 2013a, 2013b). As such, HHP was shown to hamper the functionality of several
77 cellular components including phospholipid bilayers, proteins, and ribosomes, which in turn
78 pleiotropically compromises cellular homeostasis (Gayán et al. 2017a; Rivalain et al., 2010). The
79 RpoS general stress response has proven to be important for HHP resistance in *E. coli*, and the
80 absence of this sigma factor renders *E. coli* hypersensitive to HHP (Charoenwong et al., 2011;
81 Gayán et al. 2017b). Furthermore, basal RpoS activity levels correlate well with HHP resistance
82 variability among Shiga-toxin producing *E. coli* isolates (Álvarez-Ordóñez et al., 2013; Robey et
83 al., 2001), while mutants with increased RpoS activity are rapidly selected for after iteratively
84 exposing *E. coli* O157:H7 to HHP stress (Vanlint et al., 2013a). However, other response
85 pathways were shown to be important as well, and *E. coli* MG1655 mutants lacking the RpoS
86 sigma factor can restore HHP resistance by acquiring mutations causing cAMP/CRP (cyclic
87 adenosine monophosphate/cAMP receptor protein) downregulation (Gayán et al. 2017b).

Furthermore, upregulation of the heat shock response has been reported in *E. coli* after HHP stress (Aertsen et al., 2004; Malone et al., 2006), and HHP-resistant mutants of *E. coli* and *L. monocytogenes* with increased basal levels of heat shock proteins have been isolated (Aertsen et al., 2004; Gayán et al., 2016a; Van Boeijen et al., 2010).

Nevertheless, our view on the cellular impact of HHP still remains fragmentary and needs to be further refined with alternative complementary experimental approaches. Whereas the above mentioned directed evolution approach specifically revealed genes and functions that are altered in mutants selected to acquire HHP resistance, we decided to embark in assessing the contribution of individual *E. coli* genes to HHP sensitivity or resistance. More specifically, *ca.* 6,000 single-gene disruption mutants of a random *E. coli* transposon insertion library were examined for their ability to survive a HHP shock.

2. Material and Methods

2.1. Bacterial strains, mutant construction and growth conditions

E. coli K-12 MG1655 (Blattner et al., 1997) and its derivatives described in Table 1 and 2 were used throughout this study. Transposon insertion mutagenesis was performed using the Tn10dCm transposon system and λ NK1324 as described by Kleckner *et al.* (1991). In-frame gene deletions were performed according to the method of Datsenko and Wanner (2000), using the MG1655 parental strain equipped with the plasmid pKD46 (encoding the λ red recombinase

genes behind the *araBAD* promoter) and an amplicon prepared on pKD13 (containing the kanamycin resistance cassette) using the primers listed in Baba *et al.* (2006). The kanamycin marker was flanked by FRT sites and was further excised by transiently equipping the strain with the plasmid pCP20 (expressing the Flp site-specific recombinase (Cherepanov and Wackernagel, 1995)) to obtain the desired deletion mutant. Deletion constructs were verified by PCR and sequencing (Macrogen; Amsterdam, The Netherlands) using the locus specific primers listed in Table S1. Where indicated, the strains were transformed with pFPV-P_{*bolA*}-*gfp* (encoding the *E. coli* MG1655 *bolA* promoter upstream of *gfp* (Gayán et al., 2016a)) or pFPV-P_{*dnaK*}-*gfp* (encoding the *E. coli* MG1655 *dnaK* promoter upstream of *gfp* (Aertsen et al., 2004)) by electroporation.

Strains were grown in Lysogeny Broth (LB) medium (Miller, 1992) and when necessary, a final concentration of 50 µg/ml of kanamycin (Km; Panreac-AppliChem, Darmstadt, Germany), 100 µg/ml of ampicillin (Ap; Thermo Fisher Scientific, Waltham, MA, USA) or 30 µg/ml of chloramphenicol (Cm; Sigma-Aldrich, St. Louis, MO, USA) was added to select for the presence of recombined amplicons (Datsenko and Wanner, 2000) or pKD46 and pCP20 vectors (Cherepanov and Wackernagel, 1995; Datsenko and Wanner, 2000) or Tn10dCm transposon insertions (Kleckner et al., 1991), respectively. For inactivation experiments, test tubes containing 4 ml of LB were inoculated with a single colony and then incubated aerobically with shaking (300 rpm) for 18 h at 37°C to obtain stationary phase cultures containing *ca.* 10⁹ Colony Forming Units per milliliter (CFU/ml). For pFPV-P_{*bolA*}-*gfp* and pFPV-P_{*dnaK*}-*gfp* reporter assays, cells were grown in LB supplemented with Ap to select for the presence of the plasmids (Aertsen et al., 2004; Gayán et al., 2016a) and for the β-galactosidase assay, a final concentration of 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG; Acros Organics, Morris Plains, NJ, USA) was added to induce the *lac* operon.

131

132 **2.2. HHP and heat treatment**

133 Cells from a stationary phase culture were harvested by centrifugation (4000×g, 5 min) and
134 resuspended in an equal volume of 10 mM potassium phosphate buffer of pH 7.0. For HHP
135 treatment, 200 µl of the cell suspension was heat-sealed in a sterile polyethylene bag after
136 exclusion of the air bubbles and subjected to 300 MPa or 400 MPa for 15 min in an 8-ml
137 pressure vessel (HPIU-10000, 95/1994; Resato, Roden, The Netherlands), held at 20°C with an
138 external water jacket connected to a cryostat. Both the slow pressure increase (100 MPa/min)
139 and the external water jacket attenuated adiabatic heating during pressure build-up. Finally,
140 decompression was almost instantaneous. For heat treatment, three sterile PCR tubes were
141 aseptically filled with a 65 µl portion of resuspended cells and subjected to 57.0°C for 15 min
142 using a PCR apparatus (T-personal 48; Biometra GmbH, Goettingen, Germany). After HHP or
143 heat treatment, samples were aseptically retrieved from the polyethylene bags or PCR tubes, and
144 survival was determined as described below.

145

146 **2.3. Determination of viability**

147 Samples were serially diluted in 10 mM potassium phosphate buffer, and subsequently a 5-µl
148 sample of each dilution was spotted onto LB agar in triplicate, as previously described
149 (Sieuwerds et al., 2008). After 24 h of incubation at 37°C, spots containing between 5–50
150 colonies were counted, so that the quantification limit was 1,000 CFU/ml. The logarithmic

reduction factor was calculated as $\log_{10} (N_0/N)$, in which N_0 and N represent the number of survivors in CFU/ml prior and after treatment, respectively.

2.4. Screening for transposon insertion mutants of *E. coli* MG1655 with altered HHP resistance

To screen for changes in HHP resistance, *ca.* 6,000 transposon insertion mutants of *E. coli* MG1655 were individually grown in LB in 24-well microtiter plates and individually exposed to a HHP shock of 300 MPa (15 min, 20°C). Mutants that presented at least a 2-log higher or lower HHP inactivation compared to the WT strain were kept for further study. To confirm that the transposon insertion was involved in the HHP resistance change of selected mutants, the locus with the transposon was transferred by P1-transduction (Sambrook and Russell, 2001) to the WT strain and the transductant was then subjected to 400 MPa (15 min, 20°C) in potassium phosphate buffer (10 mM, pH 7.0). The transposon location in the bacterial genome was mapped according to the method of Kwon and Rieke (2000). In brief, mutants' genomic DNA was digested with NlaIII (Thermo Fisher Scientific) and ligated to the Y-shaped linker. Subsequently, the transposon flanking region was amplified using the Y-linker primer (specific to Y-linker sequence) and a transposon specific primer (NK_Cm_DWN; Table S1) and sequenced. Sequences were compared against the *E. coli* MG1655 genome published at the GenBank database (Blattner et al., 1997) using the NCBI BLASTn (Nucleotide Basic Local Alignment Search Tool, blast.ncbi.nlm.nih.gov).

2.5. Measurement of RpoS, RpoH and cAMP/CRP activity reporter

The basal levels of RpoS sigma factor (directing expression of the general stress response) were quantified by the activity of the *bolA* promoter (P_{bolA}) using the pFPV- P_{bolA} -*gfp* construct (Gayán et al., 2016a), while RpoH levels (directing expression of the heat shock response) were measured by the *dnaK* promoter activity (P_{dnaK}) using the pFPV- P_{dnaK} -*gfp* vector (Aertsen et al., 2004). To determine the fluorescence derived from the reporter, 200 µl of the stationary phase cultures equipped with pFPV- P_{bolA} -*gfp* or pFPV- P_{dnaK} -*gfp* were transferred to microplate wells and placed in a Fluoroscan Ascent FL (Thermo 180 Labsystems, Brussels, Belgium). The basal GFP fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The obtained fluorescence values were subsequently divided by the optical density at 600 nm (OD₆₀₀) of the same sample to obtain the relative fluorescence units. Differences in RpoS and RpoH activity are expressed as fold change with respect to the parental strain.

The cAMP/CRP activity was indirectly tested by measuring β-galactosidase activity of permeabilized cells using ortho-nitrophenyl-β-galactoside (ONPG; Acros Organics) as a substrate in a Multiskan RC (Thermo Labsystems, Vantaa, Finland). Enzyme activity was expressed in Miller units (MU) (Miller, 1992).

2.5. Sensitivity to hydrogen peroxide

The susceptibility of *E. coli* to hydrogen peroxide was evaluated by the size of the growth inhibition zone. An aliquot of 100 µl of stationary phase cultures was plated into 15 ml of LB

soft agar. After drying, a volume of 5 μ l of a 30% (w/w) hydrogen peroxide solution (Acros Organics) was dropped on the center of the plate. The diameter of the inhibition zone was measured after overnight incubation at 37°C.

2.6. Statistical analysis

Statistical analyses (ANOVA, Bonferroni multiple comparison 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 ≤ 0.05 . All microbial inactivation, fluorescence and enzymatic activity data shown in figures correspond to averages and standard deviations calculated from three replicates performed in different working days.

3. Results and Discussion

3.1. Screening for mutants of *E. coli* MG1655 with HHP resistance change

To identify novel genes involved in stationary phase HHP resistance, *ca.* 6,000 random transposon insertion mutants of *E. coli* MG1655 were individually examined for their ability to survive a HHP shock. From this library, six and two mutants showed a confirmed ≥ 2.0 log cycle higher ($P \leq 0.05$) sensitivity and resistance to a 400 MPa (15 min, 20°C) shock than the wild-type (WT) strain, respectively (Table 2). The exact position of the transposon in each mutant

212 with the function of the gene disrupted is included in Table 2. The transposon insertion in the
213 two resistant mutants was located in the *sucD* gene and in the intergenic region between *sucB*
214 and *sucC*, whereas in the six sensitive mutants the transposon was mapped in either the *rbsK*,
215 *rbsR*, *hdfR* or *crl* gene (with *crl* and *hdfR* disruptions independently found twice).

216 In order to confirm the role of the identified genes in pressure resistance, each gene was deleted
217 *de novo* in the WT strain, and the corresponding mutants were exposed to a 400 MPa (15 min,
218 20°C) shock that caused *ca.* 3.2 log reductions of the WT strain (Fig. 1). As shown in Fig. 1,
219 deletion of the transcriptional regulator HdfR, which positively controls the glutamate-dependent
220 acid resistance response (Krin et al., 2010; Reynolds et al., 2017) and negatively controls the
221 FlhDC regulator for flagellum biogenesis (Ko and Park, 2000), caused the highest HHP
222 sensitivity reaching 5.9 log cycles of inactivation. Mutants $\Delta rbsR$, $\Delta rbsK$ and Δcrl showed *ca.* 2
223 log cycles lower ($P \leq 0.05$) HHP resistance than the WT strain. The *rbsR* and *rbsK* genes form
224 part of the *rbs* operon involved in ribose catabolism and transport, and encode the ribokinase and
225 the transcriptional repressor of the operon, respectively (Shimada et al., 2013). On the other
226 hand, Crl enhances RpoS activity by facilitating the assembly of σ^S to the RNA polymerase core
227 in detriment to σ^{70} (involved in the transcription of most genes during exponential phase) and
228 protecting σ^S from proteolysis, and it specifically promotes the activity of certain σ^S -dependent
229 promoters as well (Dudin et al., 2013; Robbe-Saule et al., 2007; Typas et al., 2007). Indeed,
230 disruption of *crl* was previously shown to decrease the resistance to thermal, oxidative and acidic
231 stress in *Salmonella enterica* subsp. *enterica* serovar Typhimurium (Robbe-Saule et al., 2008;
232 Robbe-Saule et al., 2007).

Regarding HHP-resistant mutants, the absence of either of the succinyl-CoA synthetase (SCS) subunits (encoded by *sucC* and *sucD* genes) significantly ($P \leq 0.05$) increased the HHP resistance of the WT strain by *ca.* 2 log cycles (Fig. 1). SCS catalyzes the reversible conversion of succinyl-CoA to succinate, accompanied by the generation of a nucleoside triphosphate molecule and coenzyme A (Park et al., 1997). The *sucC* and *sucD* genes form part of the *sdhCDAB-sucABCD* operon (Cunningham and Guest, 1998; Park et al., 1997), which contributes to two additional tricarboxylic acid (TCA) cycle enzymes: the succinate-quinone oxidoreductase complex (SQR; encoded by *sdhCDAB*) and the 2-oxoglutarate dehydrogenase complex (OGDH), with the last consisting of the oxoglutarate decarboxylase (E1; encoded by *sucA*), the dihydrolipoyltranssuccinylase (E2; encoded by *sucB*) and the lipoamide dehydrogenase (E3; encoded by *lpd*) subunit. OGDH converts 2-oxoglutarate to succinyl-CoA and CO₂ coupled to NADH formation (Park et al., 1997), while SQR oxidates succinate to fumarate with the reduction of ubiquinone to ubiquinol (Hagerhall, 1997).

Although some disruptions independently appeared twice in our screening (*cfr. hdfR* and *crl*), the screening of *ca.* 6,000 random transposon mutants was likely not sufficient to redundantly cover all non-essential genes of *E. coli*, since some gene disruptions previously known to significantly modulate HHP resistance (such as *rpoS*, *crp* or *cyaA* (Gayán et al., 2017b; Vanlint et al., 2013a, 2013b) did not appear in the screening.

Because of their resistance conferring ability, the impact of *sucC* and *sucD* disruptions were the subject of further scrutiny.

3.2. Deletion of *sucA* and *sucB* sensitizes *E. coli* to HHP treatment

Since the *sucCD* genes are part of a larger *sdhCDAB-sucABCD* operon, we decided to assess the impact of the upstream genes on *E. coli*'s HHP resistance. Fig. 1 correspondingly shows the inactivation (400 MPa, 15 min, 20°C) of MG1655 harboring an individual deletion of each gene within the operon. While the lack of any of the SQR subunits (*i.e.* SdhC/D/A/B) did not significantly ($P > 0.05$) affect HHP resistance, the deletion of *sucA* or *sucB* surprisingly sensitized ($P \leq 0.05$) the parental strain up to a 1,800-fold at 400 MPa. Please note that it was likely impossible to isolate the corresponding *sucA* nor *sucB* transposon insertion mutants in our screening because either *sucAB* or *sucCD* are essential for cell viability (Yu et al., 2006), while insertion of the transposon in the promoter or *sucAB* region would likely have (polarly) affected the expression of *sucABCD* as a whole.

Li *et al.* (2006a) described the different metabolic impact of Δ *sucA* and Δ *sucC* on *E. coli* BW25113. As such, the blockage of OGDH in the Δ *sucA* mutant decreased the carbon flow through isocitrate dehydrogenase (involved in the reversible reaction of isocitrate to 2-oxoglutarate producing CO₂ and NADPH) and activated the glyoxylate shunt to provide oxaloacetate, while the Δ *sucC* mutant refilled the TCA cycle intermediates without activating the glyoxylate cycle (Li et al., 2006a). Surprisingly, deletion of the *lpd* gene (encoding the lipoamide dehydrogenase subunit), which likewise compromises the OGDH complex and activates the glyoxylate shunt (Li et al., 2006b), did not significantly ($P > 0.05$) alter HHP resistance (Fig. 1). Moreover, upregulating the glyoxylate shunt by deleting the isocitrate dehydrogenase (*icd*) gene (Kabir and Shimizu, 2004) also failed ($P > 0.05$) to confer HHP sensitivity, suggesting that the shortcut of the TCA cycle itself cannot explain the increased HHP sensitivity of Δ *sucA* and Δ *sucB* mutants.

276

277 **3.3. HHP resistance of $\Delta sucA/B/C/D$ correlates with RpoS levels but not with RpoH or**
278 **cAMP/CRP activity**

279 In order to obtain more mechanistic insights into the opposing HHP tolerance encountered in
280 $\Delta sucAB$ and $\Delta sucCD$ mutants, we wondered to which extent their phenotype could stem from
281 underlying alterations in the global regulatory pathways (such as cAMP/CRP, RpoH or RpoS
282 regulation (Aertsen et al., 2004; Gayán et al., 2017b, 2016a; Vanlint et al., 2013a, 2013b) that
283 have previously been found to affect HHP resistance in *E. coli*.

284 More specifically, since attenuated cAMP/CRP regulation was shown to coincide with increased
285 HHP resistance (Gayán et al., 2017b; Vanlint et al. 2013b), the β -galactosidase activity in
286 $\Delta sucA/B/C/D$ mutants, and for comparison in a Δcrp and $\Delta cyaA$ mutant (lacking the cAMP
287 receptor protein and the cAMP synthase, respectively), was evaluated as a proxy of cAMP/CRP
288 activity (Fig. 2). Compared to the wild-type strain, the $\Delta sucC$ and $\Delta sucD$ mutants displayed
289 similar ($P > 0.05$) β -galactosidase levels, indicating an unaltered cAMP/CRP regulation that
290 therefore cannot explain their increased HHP resistance. The observation that $\Delta sucA$ and $\Delta sucB$
291 mutants actually displayed slightly attenuated ($P \leq 0.05$) levels of β -galactosidase activity
292 compared to the parental strain, seems likewise to rule out involvement of cAMP/CRP
293 upregulation in their HHP hypersensitive phenotype. Indeed, the lower β -galactosidase activity
294 tends to agree with the fact that accumulation of intracellular 2-oxoglutarate (as occurs in a
295 $\Delta sucA$ mutant (Li et al., 2006a)) decreases cAMP levels (Doucette et al., 2011).

296 Increased expression of heat shock proteins has previously also been shown to contribute to HHP
 297 resistance in *E. coli* (Aertsen et al., 2004; Gayán et al., 2016a). However, when examining
 298 expression of the *dnaK* promoter (*i.e.* P_{dnaK} , using the pPFV- P_{dnaK} -*gfp* reporter plasmid (Aertsen
 299 et al., 2004)) as a proxy of the heat shock response governed by the RpoH sigma factor, the
 300 $\Delta sucC$ and $\Delta sucD$ mutants showed lower ($P \leq 0.05$) P_{dnaK} activity than the WT strain, while no
 301 significant ($P > 0.05$) differences were observed among WT and its $\Delta sucA/B$ mutants (Fig. 3).
 302 This indicates that decreased or increased levels of the heat shock response are not the
 303 underlying cause of the HHP sensitive $\Delta sucA/B$ or the HHP resistant $\Delta sucC/D$ phenotypes,
 304 respectively.

305 Finally, expression of the RpoS-dependent *bolA* promoter (*i.e.* P_{bolA} , which can be fluorescently
 306 monitored using the pFPV- P_{bolA} -*gfp* reporter plasmid (Lange and Hengge-Aronis, 1991; Gayán et
 307 al., 2016a)) was quantified as a proxy of basal cellular RpoS activity (Fig. 4A). For proper
 308 comparison, P_{bolA} expression in a $\Delta rssB$ mutant with attenuated RpoS quenching (since RssB is
 309 the RpoS anti-sigma factor (Battesti et al., 2011; Gayán et al., 2017b)), a $\Delta rpoS$ mutant lacking
 310 the σ^S factor, and the Δcrl mutant (also originating from this screen) lacking the RpoS
 311 holoenzyme assembly factor (Typas et al., 2007) were included (Fig. 4A). This revealed that,
 312 compared to the WT parent, the $\Delta sucA$ mutant exhibited a decreased ($P \leq 0.05$) P_{bolA} expression
 313 similar to that of the Δcrl mutant, while the $\Delta sucB$ mutant displayed even lower ($P \leq 0.05$) levels
 314 than the $\Delta sucA$ and Δcrl mutants (Fig. 4A). However, $\Delta sucA/B$ mutants were shown to display a
 315 slower growth and a later entrance into stationary phase (Fig. S1), which could potentially
 316 explain their attenuated RpoS activity levels. Interestingly, the survival of the $\Delta sucA$ and $\Delta sucB$
 317 mutants to a 300 MPa (15 min, 20°C) shock was still significantly lower ($P \leq 0.05$) than that of

the $\Delta rpoS$ and Δcrl mutants (Fig. 4B), indicating that besides an attenuated RpoS activity additional factors might contribute to their HHP sensitivity.

Interestingly, the $\Delta sucC$ and $\Delta sucD$ mutants displayed no growth defects (Fig. S1) but a significantly ($P \leq 0.05$) increased P_{bolA} expression compared to the WT strain, with their P_{bolA} expression being equal ($P > 0.05$) to that of the $\Delta rssB$ strain (Fig. 4A). Importantly, this increase was shown to be truly RpoS dependent since additionally deleting the $rpoS$ gene in the $\Delta sucC$ and $\Delta sucD$ mutants (resulting in MG1655 $\Delta sucC \Delta rpoS$ and $\Delta sucD \Delta rpoS$, respectively) abolished P_{bolA} expression to the same extent ($P > 0.05$) as in a $\Delta rpoS$ mutant (Fig. 4A). Moreover, since lack of RpoS activity also reduced the HHP resistance of the $\Delta sucC \Delta rpoS$ and $\Delta sucD \Delta rpoS$ double mutants to the same level ($P > 0.05$) as a single $\Delta rpoS$ mutant (Fig. 4B), the increased basal RpoS activity seems to be causally involved in the HHP resistance of the $\Delta sucC$ and $\Delta sucD$ mutants.

3.4. Heat and hydrogen peroxide resistance of *sucA/B/C/D* mutants

Since our data suggested that the HHP resistance of $\Delta sucC/D$ mutants (and partially of $\Delta sucA/B$ mutants) was modulated by varying basal RpoS activity levels, we examined the resistance of these mutants to other stresses such as hydrogen peroxide and heat. Like the $\Delta rpoS$ strain, the $\Delta sucA$ and $\Delta sucB$ mutants showed larger ($P \leq 0.05$) zones of growth inhibition by hydrogen peroxide than the WT strain (Fig. 5A), indicating the higher susceptibility of these mutants to oxidative stress. In agreement, Ma *et al.* (2010) revealed the higher sensitivity of a BW25113 *sucB* deletion mutant to hydrogen peroxide and also to low pH, salicylate acid and various

antibiotics than its parent. In contrast, the deletion of neither the *sucC/D* or *rssB* genes reduced ($P > 0.05$) the growth inhibition zone of hydrogen peroxide, indicating that further upregulation of the RpoS response does not necessarily improve hydrogen peroxide protection in MG1655. However, the Δ *sucA/B/C/D* mutants showed a peculiar behavior against thermal stress (Fig. 5B). Contrary to their HHP resistance, the Δ *sucC* and Δ *sucD* mutants were on average 1.3 log cycles more sensitive ($P \leq 0.05$) to a heat shock (57°C, 15 min) than the parental strain, while the Δ *sucA* mutant was *ca.* 1.2 log cycles more resistant ($P \leq 0.05$) and the Δ *sucB* mutant equally resistant ($P > 0.05$) to the WT strain. Therefore, while disruption of the *sucA/B/C/D* genes seems to differentially modulate the general stress response, which correlates with HHP and oxidative stress resistance of Δ *sucA/B/C/D* mutants, the lack of these genes has a lower and different impact on heat survival suggesting that they might affect thermal tolerance by an additional RpoS-independent mechanism.

3.5. *sucC* and *sucD* genes are unaffected in spontaneous HHP-resistant mutants of *E. coli* MG1655

Finally, since we identified that loss-of-function mutations in *sucC* or *sucD* gene could cause significant HHP resistance in *E. coli* MG1655 and could hence support evolutionary routes towards acquisition of HHP resistance, the corresponding *sucABCD* loci of five HHP-resistant mutants of MG1655 previously obtained by directed evolution (*i.e.* LMM1010, LMM1020, LMM1030, DVL1 and DVL20 (Hauben et al, 1997; Vanlint et al., 2012, 2011)) were sequenced. In fact, we recently found that some of these mutants (*i.e.* LMM1010, LMM1020 and DVL1) incurred loss-of-function mutations in the *crp* or *cyaA* gene, which confers HHP resistance but

could still not completely explain their extreme piezoresistance (Gayán et al., 2017b). However, none of these mutants harboured any mutation in *sucABCD* genes, although the fact that the *sucABCD* operon as a whole cannot be compromised could limit the adaptive evolution through loss-of-function mutations in this locus.

4. Conclusions

In this study, the screening of a transposon insertion library of *E. coli* MG1655 for gene disruptions affecting HHP inactivation revealed a set of genes and operons (*hdfR*, *rbs* and *suc*) that were previously not recognized to be important for HHP survival. Upon further scrutiny of the *sucABCD* operon, we found that *sucA/B* or *sucC/D* deletions had opposing effects on HHP resistance. In fact, compromising SCS functionality by deleting *sucC/D* significantly improved HHP resistance through a mechanism relying on increased RpoS activity rather than an increased heat shock (RpoH) or decreased cAMP/CRP response. Although RpoS activity is known to be regulated at many levels, including alterations in carbon metabolism (Battesti et al., 2011, 2015), the input of the SCS complex and/or its metabolic network on quenching RpoS activity was previously unreported and deserves further attention. In contrast, compromising OGDH functionality by deleting *sucA/B* tremendously sensitized cells against HHP, and this phenotype could only partially be attributed to decreased RpoS activity levels.

The considerable impact of disruptions of the *hdfR* gene and within the *rbs* and *suc* operons also underscores a close link between cellular metabolism and HHP susceptibility. In fact, such a link

was also recently suggested by directed evolution of an MG1655 $\Delta rpoS$ strain towards HHP resistance, which selected for a number of HHP-resistant mutants harboring loss-of-function mutations in the *crp* gene (Gayán et al., 2017b). Interestingly, the observed lack of heat (cross-)resistance in the HHP resistant $\Delta cyaA$ or Δcrp mutants (Gayán et al., 2017b) agrees with the current observation that the HHP resistant $\Delta sucC/D$ mutants did not display an improved heat resistance. This clearly suggests that HHP and heat resistance mechanisms are not necessarily functionally equivalent. In fact, the cellular impact of HHP and heat stress is likely to be very different, as was also recently evidenced by the observation that heat injured *E. coli* cells proved to be heat sensitive but at the same time remarkably HHP resistant compared to non-injured control cells (Gayán et al., 2016b).

Such insights into differential bacterial stress response and adaptation mechanisms will become important for the proper design and prediction of successful hurdle technology approaches.

Acknowledgements

This work was supported by a postdoctoral fellowship (12P9818N; to E.G.) and a grant (1526416 N) from the Research Foundation - Flanders (FWO-Vlaanderen), and a postdoctoral fellowship (F + /13/040; to E.G.) and a grant (STRT1/10/036) from the KU Leuven Research Fund.

References

- Aertsen, A., Vanoirbeek, K., De Spiegeleer, P., Sermon, J., Hauben, K., Farewell, A., Nystrom, T., Michiels, C.W. 2004. Heat shock protein-mediated resistance to high hydrostatic pressure in *Escherichia coli*. Appl. Environ. Microbiol. 70, 2660-2666.
- Álvarez-Ordóñez, A., Alvseike, O., Omer, M.K., Heir, E., Axelsson, L., Holck, A., Prieto, M. 2013. Heterogeneity in resistance to food-related stresses and biofilm formation ability among verocytotoxigenic *Escherichia coli* strains. Int. J. Food Microbiol. 161, 220-230.
- Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., Mori, H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. 2, 2006.0008-2006.
- Barba, F.J., Ahrné, L., Xanthakis, E., Landerslev, M.G., Orlén, V. 2017. Innovative technologies for food preservation, in: Barba F.J., Sant'Ana, A.S., Orlén, V., Koubaa M. (Eds.), Innovative Technologies for Food Preservation: Inactivation of Spoilage and Pathogenic. Elsevier LTD, Academic Press, Oxford, 25-47.
- Battesti, A., Majdalani, N., Gottesman, S. 2015. Stress sigma factor RpoS degradation and translation are sensitive to the state of central metabolism. Proc. Natl. Acad. Sci. USA 112, 5159-64.
- Battesti, A., Majdalani, N., Gottesman, S. 2011. The RpoS-mediated general stress response in *Escherichia coli*. Annu. Rev. Microbiol. 65, 189-213.

421 Blattner, F.R., Plunkett, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J.,
 422 Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden,
 423 M.A., Rose, D.J., Mau, B., Shao, Y. 1997. The complete genome sequence of *Escherichia coli*
 424 K-12. *Science* 277, 1453-14562.

425 Bowman, J.P., Bittencourt, C.R., Ross, T. 2008. Differential gene expression of *Listeria*
 426 *monocytogenes* during high hydrostatic pressure processing. *Microbiol.-Sgm* 154, 462-475.

427 Bruschi, C., Komora, N., Castro, S.M., Saraiva, J., Ferreira, V.B., Teixeira, P. 2017. High
 428 hydrostatic pressure effects on *Listeria monocytogenes* and *L. innocua*: Evidence for variability
 429 in inactivation behaviour and in resistance to pediocin bacHA-6111-2. *Food Microbiol.* 64, 226-
 430 231.

431 Cunningham, L., Guest, J.R. 1998. Transcription and transcript processing in the *sdhCDAB-*
 432 *sucABCD* operon of *Escherichia coli*. *Microbiol-Uk* 144, 2113-2123.

433 Charoenwong, D., Andrews, S., Mackey, B. 2011. Role of *rpoS* in the development of cell
 434 envelope resilience and pressure resistance in stationary-phase *Escherichia coli*. *Appl. Environ.*
 435 *Microbiol.* 77, 5220-5229.

436 Cherepanov, P.P., Wackernagel, W. 1995. Gene disruption in *Escherichia coli*: TcR and KmR
 437 cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene*
 438 158, 9-14

439 Datsenko, K.A., Wanner, B.L. 2000. One-step inactivation of chromosomal genes in *Escherichia*
 440 *coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 97, 6640-6645.

441 Doucette, C.D., Schwab, D.J., Wingreen, N.S., Rabinowitz, J.D. 2011. α -ketoglutarate
 442 coordinates carbon and nitrogen utilization via Enzyme I inhibition. *Nat. Chem. Biol.* 7, 894-
 443 901.

444 Dudin, O., Lacour, S., Geiselman, J. 2013. Expression dynamics of RpoS/Crl-dependent genes
 445 in *Escherichia coli*. *Res. Microbiol.* 164, 838-847.

446 Elamin, W.M., Endan, J.B., Yosuf, Y.A., Shamsudin, R., Ahmedov, A. 2015. High pressure
 447 processing technology and equipment evolution: A review. *J. Eng. Sci. Tech. Rev.* 8, 75-83.

448 Feyaerts, J., Rogiers, G., Corthouts, J., Michiels, C.W. 2015. Thiol-reactive natural
 449 antimicrobials and high pressure treatment synergistically enhance bacterial inactivation. *Innov.*
 450 *Food Sci. Emerg. Technol.* 27, 26-34.

451 Gao, Y.L., Ju, X.R., Jiang, H.H. 2006. Statistical analysis of inactivation of *Listeria*
 452 *monocytogenes* subjected to high hydrostatic pressure and heat in milk buffer. *Appl. Microbiol.*
 453 *Biotechnol.* 70, 670-678.

454 Gayán, E., Govers, S.K., Aertsen, A. 2017a. Impact of high hydrostatic pressure on bacterial
 455 proteostasis. *Biophys. Chem.* 231, 3-9.

456 Gayán, E., Cambré, A., Michiels, C.W., Aertsen, A. 2017b. RpoS-independent evolution reveals
 457 the importance of attenuated cAMP/CRP regulation in high hydrostatic pressure resistance
 458 acquisition in *E. coli*. *Sci. Rep* 7, 8600.

459 Gayán, E., Cambré, A., Michiels, C.W., Aertsen, A. 2016a. Stress-induced evolution of heat
 460 resistance and resuscitation speed in *Escherichia coli* O157:H7 ATCC 43888. Appl. Environ.
 461 Microbiol. 82, 6656-6663.

462 Gayán, E., Govers, S.K., Michiels, C.W., Aertsen, A. 2016b. Severely heat injured survivors of
 463 *E. coli* O157:H7 ATCC 43888 display variable and heterogeneous stress resistance behavior.
 464 Front. Microbiol. 7, 1845.

465 Hagerhall, C. 1997. Succinate: quinone oxidoreductases – Variations on a conserved theme.
 466 Biochim. Biophys. Acta, Bioenergetics 1320, 107-141.

467 Hauben, K.J., Bartlett, D.H., Soontjens, C.C., Cornelis, K., Wuytack, E.Y., Michiels, C.W. 1997.
 468 *Escherichia coli* mutants resistant to inactivation by high hydrostatic pressure. Appl. Environ.
 469 Microbiol. 63, 945-950.

470 Huang, H.W., Wu, S.J., Lu, J.K., Shyu, Y.T., Wang, C.Y. 2017. Current status and future trends
 471 of high-pressure processing in food industry. Food Control 72, 1-8.

472 Kabir, M.M., Shimizu, K. 2004. Metabolic regulation analysis of *icd*-gene knockout *Escherichia*
 473 *coli* based on 2D electrophoresis with MALDI-TOF mass spectrometry and enzyme activity
 474 measurements. Appl. Microbiol. Biotechnol. 65, 84-96.

475 Khan, I., Tango, C.N., Miskeen, S., Lee, B.H., Oh, D.H. 2017. Hurdle technology: A novel
 476 approach for enhanced food quality and safety – A review. Food Control 73, 1426-1444.

477 Kleckner, N., Bender, J., Gottesman, S. 1991. Uses of transposons with emphasis on Tn10
 478 Methods Enzymol. 204, 139-80.

479 Ko, M., Park, C. 2000. H-NS-dependent regulation of flagellar synthesis is mediated by a LysR
 480 family protein. J. Bacteriol. 182, 4670-4672.

481 Koseki, S., Mizuno, Y., Yamamoto, K. 2008. Use of mild-heat treatment following high-pressure
 482 processing to prevent recovery of pressure-injured *Listeria monocytogenes* in milk. Food
 483 Microbiol. 25, 288-293.

484 Krin, E., Danchin, A., Soutourina, O. 2010. Decrypting the H-NS-dependent regulatory cascade
 485 of acid stress resistance in *Escherichia coli*. BMC Microbiol. 10, 273.

486 Kwon, Y.M., Ricke, S.C. 2000. Efficient amplification of multiple transposon-flanking
 487 sequences. J. Microbiol. Methods 41, 195-199.

488 Lange, R., Hengge-Aronis, R. 1991. Growth phase-regulated expression of *bolA* and morphology
 489 of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor sigma S. J.
 490 Bacteriol. 173, 4474-4481.

491 Li, M., Ho, P.Y., Yao, S., Shimizu, K. 2006a. Effect of *sucA* or *sucC* gene knockout on the
 492 metabolism in *Escherichia coli* based on gene expressions, enzyme activities, intracellular
 493 metabolite concentrations and metabolic fluxes by ¹³C-labeling experiments. Biochem. Eng. J.
 494 30, 286-296.

495 Li, M., Ho, P.Y., Yao, S., Shimizu, K. 2006b. Effect of *lpdA* gene knockout on the metabolism in
 496 *Escherichia coli* based on enzyme activities, intracellular metabolite concentrations and
 497 metabolic flux analysis by ¹³C-labeling experiments. J. Biotechnol. 122, 254-266.

498 Liu, Y., Gill, A., McMullen, L., Gaenzle, M. G. 2015. Variation in heat and pressure resistance
 499 of verotoxigenic and nontoxigenic *Escherichia coli*. J. Food Prot. 78, 111-120.

500 Ma, C., Sim, S., Shi, W., Du, L., Xing, D., Zhang, Y. 2010. Energy production genes *sucB* and
 501 *ubiF* are involved in persister survival and tolerance to multiple antibiotics and stresses in
 502 *Escherichia coli*. Fems Microbiol. Lett. 303, 33-40.

503 Malone, A.S., Chung, Y.K., Yousef, A.E. 2006. Genes of *Escherichia coli* O157:H7 that are
 504 involved in high-pressure resistance. Appl. Environ. Microbiol. 72, 2661-2671.

505 Miller, J.H. 1992. A short course in bacterial genetics: A laboratory manual and handbook for
 506 *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, New York.

507 Montiel, R., Martín-Cabrejas, I., Medina, M. 2015. Reuterin, lactoperoxidase, lactoferrin and
 508 high hydrostatic pressure on the inactivation of food-borne pathogens in cooked ham. Food
 509 Control 51, 122-128.

510 Muñoz, M., De Ancos, B., Sanchez-Moreno, C., Cano, M.P. 2006. Evaluation of chemical and
 511 physical (high-pressure and temperature) treatments to improve the safety of minimally
 512 processed mung bean sprouts during refrigerated storage. J Food Prot. 69, 2395-2402.

513 Oliveira, T.L.C., Ramos, A.L.S., Ramos, E.M., Piccoli, R.H., Cristianini, M. 2015. Natural
 514 antimicrobials as additional hurdles to preservation of foods by high pressure processing. Trends
 515 Food Sci. Technol. 45, 60-85.

516 Park, S.J., Chao, G., Gunsalus, R.P. 1997. Aerobic regulation of the *sucABCD* genes of
 517 *Escherichia coli*, which encode alpha-ketoglutarate dehydrogenase and succinyl coenzyme A

518 synthetase: Roles of ArcA, Fnr, and the upstream *sdhCDAB* promoter. J. Bacteriol. 179, 4138-
519 4142.

520 Reynolds, T.S., Courtney, C.M., Erickson, K.E., Wolfe, L.M., Chatterjee, A., Nagpal, P., Gill,
521 R.T. 2017. ROS mediated selection for increased NADPH availability in *Escherichia coli*.
522 Biotechnol. Bioeng. 114, 2685-2689.

523 Rivalain, N., Roquain, J., Demazeau, G. 2010. Development of high hydrostatic pressure in
524 biosciences: Pressure effect on biological structures and potential applications in
525 biotechnologies. Biotechnol. Adv. 28, 659-672.

526 Robbe-Saule, V., Carreira, I., Kolb, A., Norel, F. 2008. Effect of growth temperature on Crl-
527 dependent regulation of $\sigma(S)$ activity in *Salmonella enterica* serovar Typhimurium. J. Bacteriol.
528 190, 4453-4459.

529 Robbe-Saule, V., Lopes, M.D., Kolb, A., Norel, F. 2007. Physiological effects of Crl in
530 *Salmonella* are modulated by sigma(S) level and promoter specificity. J Bacteriol. 189, 2976-
531 2987.

532 Robey, M., Benito, A., Hutson, R.H., Pascual, C., Park, S.F., Mackey, B.M. 2001. Variation in
533 resistance to high hydrostatic pressure and rpoS heterogeneity in natural isolates of *Escherichia*
534 *coli* O157:H7. Appl. Environ. Microbiol. 67, 4901-4907.

535 Sambrook, J., Russell, D.W. 2001. Molecular cloning: A laboratory manual. Cold Spring Harbor
536 Laboratory Press, New York.

537 Shimada, T., Kori, A., Ishihama, A. 2013. Involvement of the ribose operon repressor RbsR in
538 regulation of purine nucleotide synthesis in *Escherichia coli*. Fems Microbiol. Lett. 344, 159-
539 165.

540 Sieuwerts, S., De Bok, F.A.M., Mols, E., De Vos, W.M., Van Hylckama Vlieg, J.E.T. 2008. A
541 simple and fast method for determining colony forming units. Lett. Appl. Microbiol. 47, 275-
542 278.

543 Sokołowska, B., Skapska, S., Niezgoda, J., Rutkowska, M., Dekowska, A., Rzoska, S.J. 2014.
544 Inactivation and sublethal injury of *Escherichia coli* and *Listeria innocua* by high hydrostatic
545 pressure in model suspensions and beetroot juice. High Pressure Res., 34, 147-155.

546 Somolinos, M., Garcia, D., Pagan, R., Mackey, B. 2008. Relationship between sublethal injury
547 and microbial inactivation by the combination of high hydrostatic pressure and citral or tert-butyl
548 hydroquinone. Appl. Environ. Microbiol. 74, 7570-7577.

549 Typas, A., Barembruch, C., Possling, A., Hengge, R. 2007. Stationary phase reorganisation of
550 the *Escherichia coli* transcription machinery by Crl protein, a fine-tuner of sigma(s) activity and
551 levels. EMBO J. 26, 1569-1578.

552 Van Boeijen, I.K.H., Chavarroche, A.A.E., Valderrama, W.B., Moezelaar, R., Zwietering, M.H.,
553 Abee, T. 2010. Population diversity of *Listeria monocytogenes* LO28: Phenotypic and genotypic
554 characterization of variants resistant to high hydrostatic pressure. Appl. Environ. Microbiol. 76,
555 2225-2233.

556 Vanlint, D., Rutten, N., Govers, S.K., Michiels, C.W., Aertsen, A. 2013a. Exposure to high
 557 hydrostatic pressure rapidly selects for increased RpoS activity and general stress-resistance in
 558 *Escherichia coli* O157:H7. Int. J. Food Microbiol. 163, 28-33.

559 Vanlint, D., Pype, B.J.Y., Rutten, N., Vanoirbeek, K.G.A., Michiels, C.W., Aertsen, A. 2013b.
 560 Loss of cAMP/CRP regulation confers extreme high hydrostatic pressure resistance in
 561 *Escherichia coli* O157:H7. Int. J. Food Microbiol. 166, 65-71.

562 Vanlint, D., Rutten, N., Michiels, C.W., Aertsen, A. 2012. Emergence and stability of high-
 563 pressure resistance in different food-borne pathogens. Appl. Environ. Microbiol. 78, 3234-3241.

564 Vanlint, D., Mitchell, R., Bailey, E., Meersman, F., McMillan, P.F., Michiels, C.W., Aertsen, A.
 565 2011. Rapid acquisition of gigapascal-high-pressure resistance by *Escherichia coli*. Mbio 2,
 566 e00130-10.

567 Wang, L., Pan, J., Xie, H.M., Yang, Y., Zhou, D.F., Zhu, Z.N. 2012. Pasteurization of fruit juices
 568 of different pH values by combined high hydrostatic pressure and carbon dioxide. J. Food Prot.
 569 75, 1873-1877.

570 Yu, B.J., Sung, B.H., Lee, J.Y., Son, S.H., Kim, M.S., Kim, S.C. 2006. *sucAB* and *sucCD* are
 571 mutually essential genes in *Escherichia coli*. Fems Microbiol. Lett. 254, 245-250.

572
 573
 574

575 **Table 1.** Bacterial strains and plasmids used in this study.

Bacteria and plasmids	Characteristics	Reference
<i>Bacteria</i>		
<i>Escherichia coli</i>		
MG1655 (WT)	Parental wild-type strain	Blattner et al. (1997)
MG1655 Δ <i>crl</i>	MG1655 carrying in frame deletion of <i>crl</i>	This study
MG1655 Δ <i>crp</i>	MG1655 carrying in frame deletion of <i>crp</i>	Gayán et al. (2017b)
MG1655 Δ <i>cyaA</i>	MG1655 carrying in frame deletion of <i>cyaA</i>	Gayán et al. (2017b)
MG1655 Δ <i>hdfR</i>	MG1655 carrying in frame deletion of <i>hdfR</i>	This study
MG1655 Δ <i>icd</i>	MG1655 carrying in frame deletion of <i>icd</i>	This study
MG1655 Δ <i>lpd</i>	MG1655 carrying in frame deletion of <i>lpd</i>	This study
MG1655 Δ <i>sdhA</i>	MG1655 carrying in frame deletion of <i>sdhA</i>	This study
MG1655 Δ <i>sdhB</i>	MG1655 carrying in frame deletion of <i>sdhB</i>	This study
MG1655 Δ <i>sdhC</i>	MG1655 carrying in frame deletion of <i>sdhC</i>	This study
MG1655 Δ <i>sdhD</i>	MG1655 carrying in frame deletion of <i>sdhD</i>	This study
MG1655 Δ <i>sucA</i>	MG1655 carrying in frame deletion of <i>sucA</i>	This study
MG1655 Δ <i>sucB</i>	MG1655 carrying in frame deletion of <i>sucB</i>	This study
MG1655 Δ <i>sucC</i>	MG1655 carrying in frame deletion of <i>sucC</i>	This study
MG1655 Δ <i>sucC</i> Δ <i>rpoS</i>	MG1655 carrying in frame deletion of <i>sucC</i> and <i>rpoS</i>	This study
MG1655 Δ <i>sucD</i>	MG1655 carrying in frame deletion of <i>sucD</i>	This study
MG1655 Δ <i>sucD</i> Δ <i>rpoS</i>	MG1655 carrying in frame deletion of <i>sucD</i> and <i>rpoS</i>	This study
MG1655 Δ <i>rbsK</i>	MG1655 carrying in frame deletion of <i>rbsK</i>	This study
MG1655 Δ <i>rbsR</i>	MG1655 carrying in frame deletion of <i>rbsR</i>	This study
MG1655 Δ <i>rpoS</i>	MG1655 carrying in frame deletion of <i>rpoS</i>	Gayán et al. (2017b)
MG1655 Δ <i>rssB</i>	MG1655 carrying in frame deletion of <i>rssB</i>	Gayán et al. (2017b)
LMM1010	HHP-resistant derivative of MG1655	Hauben et al. (1997)
LMM1020	HHP-resistant derivative of MG1655	Hauben et al. (1997)
LMM1030	HHP-resistant derivative of MG1655	Hauben et al. (1997)
DVL20	HHP-resistant derivative of MG1655	Vanlint et al. (2012)
DVL1	HHP-resistant derivative of MG1655	Vanlint et al. (2011)
<i>Plasmids</i>		
pKD46	Expression of γ , β and <i>exo</i> recombination genes of phage λ under the control of <i>araBAD</i> promoter, temperature-sensitive, Ap ^R .	Datsenko and Wanner (2000)
pKD13	Template plasmid containing <i>kan</i> gene flanked by FRT sites, Ap ^R and Km ^R .	Datsenko and Wanner, (2000)
pCP20	Expression of Flp recombinase, temperature-sensitive, Ap ^R and Cm ^R .	Cherepanov and Wackernagel (1995)
pFPV-P _{<i>bolA</i>} - <i>gfp</i>	<i>bolA</i> promoter of MG1655 upstream of <i>gfp</i>	Gayán et al. (2016a)
pFPV-P _{<i>dnaK</i>} - <i>gfp</i>	<i>dnaK</i> promoter of MG1655 upstream of <i>gfp</i>	Aertsen et al. (2004)

576

577 **Table 2.** Overview of transposon insertion mutants with altered resistance (≥ 2 log cycles in
 578 comparison to the WT strain) to a 400 MPa (15 min, 20°C) shock in potassium phosphate buffer
 579 (10 mM, pH 7.0), position of the transposon insertion site and corresponding function of the
 580 genes affected. Inactivation data correspond to the strains obtained after transduction of the
 581 transposon from mutants screened for HHP-resistance change to the WT background.

Mutant	Log reduction	Gene	Transposon position	Function
WT	3.59 (0.16) ^a			
95/1	$\geq 6.5^*$	<i>hdfR</i>	+371	DNA-binding transcriptional dual regulator HdfR (H-NS-dependent <i>flhDC</i> regulator). Repressor for <i>flhDC</i> operon (encoding the master regulator for flagellar biosynthesis and swarming migration) and activator of the <i>gltBDF</i> operon (encoding the glutamate synthase)
101/19	6.38 (0.11) ^b	<i>hdfR</i>	+356	
107/16	6.14 (0.45) ^b	<i>crl</i>	+222	σ^{38} (RpoS) RNA polymerase holoenzyme assembly factor Crl
189/16	6.27 (0.25) ^b	<i>crl</i>	+231	
76/37	5.78 (0.49) ^b	<i>rbsK</i>	+748	Ribokinase (involved in ribose catabolism)
31/5	6.04 (0.24) ^b	<i>rbsR</i>	+36	DNA-binding transcriptional dual regulator RbsR. Repressor for <i>rbs</i> operon (involved in ribose transport and catabolism)
126/27	1.93 (0.23) ^c	<i>sucD</i>	+540	Succinyl-CoA synthetase subunit α (TCA cycle)
2/39	1.71 (0.49) ^c	<i>sucC</i>	-102	Succinyl-CoA synthetase subunit β (TCA cycle)

The position of the transposon gives the nucleotide after which the transposon was inserted, starting from the first base of the start codon. In the case of *sucC*, the insertion was 102 bp upstream of the *sucC* open reading frame.

Letters indicate statistically significant differences ($P \leq 0.05$) among the inactivation of all the strains.

*Survival counts fell below de quantification limit (1,000 CFU/mL)

582

583

584

Figure legend

Figure 1. Logarithmic reduction factor of *E. coli* MG1655 WT and indicated mutants by a HHP treatment at 400 MPa (15 min, 20°C). The dotted line represents the quantification limit (1,000 CFU/ml). Letters indicate statistically significant differences ($P \leq 0.05$) among the inactivation of all strains.

Figure 2. β -Galactosidase activity of stationary phase cultures of *E. coli* MG1655 WT and its indicated mutants. Letters indicate statistically significant differences ($P \leq 0.05$) among the Miller Units (MU) calculated for each strain.

Figure 3. Fluorescence derived from pFPV- P_{dnaK} -*gfp* (encoding the *E. coli* MG1655 *dnaK* promoter upstream of *gfp*) in the indicated strains. Values are expressed as fold change with respect to the average value of the parental strain. Letters indicate statistically significant differences ($P \leq 0.05$) among the fluorescence values of all strains.

Figure 4. (A) Fluorescence derived from pFPV- P_{bolA} -*gfp* (encoding the *E. coli* MG1655 *bolA* promoter upstream of *gfp*) in the indicated strains. Values are expressed as fold change with respect to the average value of the parental strain. (B) Logarithmic reduction factor of *E. coli* MG1655 WT and indicated mutants by a HHP treatment at 300 MPa (15 min, 20°C). The dotted line represents the quantification limit (1,000 CFU/ml). Letters indicate statistically significant differences ($P \leq 0.05$) among the inactivation and fluorescence values of all strains.

Figure 5. Resistance of *E. coli* MG1655 WT and indicated mutants to (A) hydrogen peroxide and (B) heat. The resistance to hydrogen peroxide was measured by the inhibition growth diameter of a 5- μ l aliquot of the chemical (30% w/w) on stationary phase cells plated in 15 ml of

LB soft agar, while heat resistance was assayed by the logarithmic reduction factor by a heat treatment at 57.0°C for 15 min. The dotted line represents the quantification limit (1,000 CFU/ml). Letters indicate statistically significant differences ($P \leq 0.05$) among the inhibition growth diameter and inactivation of all the strains.

Figure 1

Figure 2

659 **Figure 3**

660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675

676 **Figure 4**

677

678

679 **Figure 5**

681