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

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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.* 6000 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*.

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

Paramount to the proper design of effective hurdle approaches is a better understanding of the cellular impact of available preservation

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stresses. With respect to HHP processing, current knowledge on its effects on bacteria is mainly coined from the study of (i) HHP on biomolecules (Gayán et al., 2017a; Rivalain et al., 2010), (ii) cellular responses to HHP stresses (Bowman et al., 2008; Malone et al., 2006), and (iii) the genetic analysis of bacterial mutants that were selected to become HHP resistant through directed evolution approaches (Gayán et al., 2017b; Vanlint et al., 2013a, 2013b). As such, HHP was shown to hamper the functionality of several cellular components including phospholipid bilayers, proteins, and ribosomes, which in turn pleiotropically compromises cellular homeostasis (Gayán et al., 2017a; Rivalain et al., 2010). The RpoS general stress response has proven to be important for HHP resistance in *E. coli*, and the absence of this sigma factor renders *E. coli* hypersensitive to HHP (Charoenwong et al., 2011; Gayán et al., 2017b). Furthermore, basal RpoS activity levels correlate well with HHP resistance variability among Shiga-toxin producing *E. coli* isolates (Álvarez-Ordóñez et al., 2013; Robey et al., 2001), while mutants with increased RpoS activity are rapidly selected for after iteratively exposing *E. coli* O157:H7 to HHP stress (Vanlint et al., 2013a). However, other response pathways were shown to be important as well, and *E. coli* MG1655 mutants lacking the RpoS sigma factor can restore HHP resistance by acquiring mutations causing cAMP/CRP (cyclic adenosine monophosphate/cAMP receptor protein) down-regulation (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.* 6000 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 Tables 1 and 2 were used throughout this study. Transposon insertion mutagenesis was performed using the Tn*10*dCm transposon system and λNK1324 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 (*C*m; 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 Tn*10*dCm 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.* 10^9 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.

2.2. HHP and heat treatment

Cells from a stationary phase culture were harvested by centrifugation (4000 × *g*, 5 min) and resuspended in an equal volume of 10 mM potassium phosphate buffer of pH 7.0. For HHP treatment, 200 μl of the cell suspension was heat-sealed in a sterile polyethylene bag after exclusion of the air bubbles and subjected to 300 MPa or 400 MPa for 15 min in 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. Both the slow pressure increase (100 MPa/min) and the external water jacket attenuated adiabatic heating during pressure build-up. Finally, decompression was almost instantaneous. For heat treatment, three sterile PCR tubes were aseptically filled with a 65 μl portion of resuspended cells and subjected to 57.0 °C for 15 min using a PCR apparatus (T-personal 48; Biometra GmbH, Goettingen, Germany). After HHP or heat treatment, samples were aseptically retrieved from the polyethylene bags or PCR tubes, and survival was determined as described below.

2.3. Determination of viability

Samples were serially diluted in 10 mM potassium phosphate buffer, and subsequently a 5-μl sample of each dilution was spotted onto LB agar in triplicate, as previously described (Sieuwerts et al., 2008). After 24 h of incubation at 37 °C, spots containing between 5 and 50 colonies were counted, so that the quantification limit was 1000 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.* 6000 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 Ricke (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

Table 1
Bacterial strains and plasmids used in this study.

Bacteria and plasmids	Characteristics	Reference
Bacteria		
Escherichia coli		
MG1655 (WT)	Parental wild-type strain	Blattner et al. (1997)
MG1655 Δ*crl*	MG1655 carrying in frame deletion of *crl*	This study
MG1655 Δ*crp*	MG1655 carrying in frame deletion of *crp*	Gayán et al. (2017b)
MG1655 Δ*cyaA*	MG1655 carrying in frame deletion of *cyaA*	Gayán et al. (2017b)
MG1655 Δ*hdfR*	MG1655 carrying in frame deletion of *hdfR*	This study
MG1655 Δ*icd*	MG1655 carrying in frame deletion of *icd*	This study
MG1655 Δ*lpd*	MG1655 carrying in frame deletion of *lpd*	This study
MG1655 Δ*sdhA*	MG1655 carrying in frame deletion of *sdhA*	This study
MG1655 Δ*sdhB*	MG1655 carrying in frame deletion of *sdhB*	This study
MG1655 Δ*sdhC*	MG1655 carrying in frame deletion of *sdhC*	This study
MG1655 Δ*sdhD*	MG1655 carrying in frame deletion of *sdhD*	This study
MG1655 Δ*sucA*	MG1655 carrying in frame deletion of *sucA*	This study
MG1655 Δ*sucB*	MG1655 carrying in frame deletion of *sucB*	This study
MG1655 Δ*sucC*	MG1655 carrying in frame deletion of *sucC*	This study
MG1655 Δ*sucC* Δ*rpoS*	MG1655 carrying in frame deletion of *sucC* and *rpoS*	This study
MG1655 Δ*sucD*	MG1655 carrying in frame deletion of *sucD*	This study
MG1655 Δ*sucD* Δ*rpoS*	MG1655 carrying in frame deletion of *sucD* and *rpoS*	This study
MG1655 Δ*rbsK*	MG1655 carrying in frame deletion of *rbsK*	This study
MG1655 Δ*rbsR*	MG1655 carrying in frame deletion of *rbsR*	This study
MG1655 Δ*rpoS*	MG1655 carrying in frame deletion of *rpoS*	Gayán et al. (2017b)
MG1655 Δ*rssB*	MG1655 carrying in frame deletion of *rssB*	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)
Plasmids		
pKD46	Expression of γ, β and *exo* recombination genes of phage λ under the control of *araBAD* promoter, temperature-sensitive, Ap^R.	Datsenko and Wanner (2000)
pKD13	Template plasmid containing *kan* 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_{bolA}-*gfp*	*bolA* promoter of MG1655 upstream of *gfp*	Gayán et al. (2016a)
pFPV-P_{dnaK}-*gfp*	*dnaK* promoter of MG1655 upstream of *gfp*	Aertsen et al. (2004)

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

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 (OD_{600}) of the same sample to obtain the relative fluorescence units. Differences in

Table 2
Overview of transposon insertion mutants with altered resistance (≥2 log cycles in comparison to the WT strain) to a 400 MPa (15 min, 20 °C) shock in potassium phosphate buffer (10 mM, pH 7.0), position of the transposon insertion site and corresponding function of the genes affected. Inactivation data correspond to the strains obtained after transduction of the 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	≥6.5*	*hdfR*	+371	DNA-binding transcriptional dual regulator HdfR (H-NS-dependent *flhDC* regulator). Repressor for *flhDC* operon (encoding the master regulator for flagellar biosynthesis and swarming migration) and activator of the *gltBDF* operon (encoding the glutamate synthase)
101/19	6.38 (0.11)[b]	*hdfR*	+356	
107/16	6.14 (0.45)[b]	*crl*	+222	σ^{38} (RpoS) RNA polymerase holoenzyme assembly factor Crl
189/16	6.27 (0.25)[b]	*crl*	+231	
76/37	5.78 (0.49)[b]	*rbsK*	+748	Ribokinase (involved in ribose catabolism)
31/5	6.04 (0.24)[b]	*rbsR*	+36	DNA-binding transcriptional dual regulator RbsR. Repressor for *rbs* operon (involved in ribose transport and catabolism)
126/27	1.93 (0.23)[c]	*sucD*	+540	Succinyl-CoA synthetase subunit α (TCA cycle)
2/39	1.71 (0.49)[c]	*sucC*	−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 (1000 CFU/ml).

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).

2.6. 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 µl 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.

2.7. 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 a change in HHP resistance

To identify novel genes involved in stationary phase HHP resistance, *ca*. 6000 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 with the function of the gene disrupted is included in Table 2. The transposon insertion in the two resistant mutants was located in the *sucD* gene and in the intergenic region between *sucB* and *sucC*, whereas in the six sensitive mutants the transposon was mapped in either the *rbsK*, *rbsR*, *hdfR* or *crl* gene (with *crl* and *hdfR* disruptions independently found twice).

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

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 CO_2 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*. 6000 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

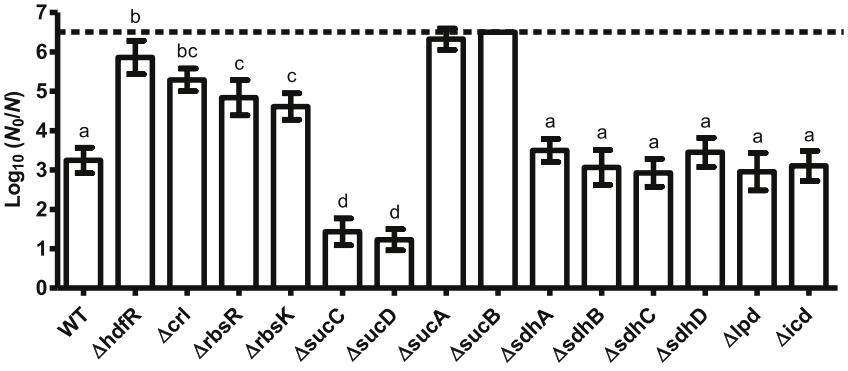

Fig. 1. Logarithmic reduction factor of *E. coli* MG1655 WT and indicated mutants by a HHP treatment at 400 MPa (15 min, 20 °C). The dotted line represents the quantification limit (1000 CFU/ml). Letters indicate statistically significant differences ($P \leq 0.05$) among the inactivation of all strains.

(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 1800-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-oxoglutare producing CO_2 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.

3.3. HHP resistance of ΔsucA/B/C/D correlates with RpoS activity but not with RpoH or cAMP/CRP activity

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

More specifically, since attenuated cAMP/CRP regulation was shown to coincide with increased HHP resistance (Gayán et al., 2017b; Vanlint et al., 2013b), the β-galactosidase activity in Δ*sucA/B/C/D* mutants, and for comparison in a Δ*crp* and Δ*cyaA* mutant (lacking the cAMP receptor protein and the cAMP synthase, respectively), was evaluated as a proxy of cAMP/CRP activity (Fig. 2). Compared to the wild-type strain, the Δ*sucC* and Δ*sucD* mutants displayed similar ($P > 0.05$) β-galactosidase levels, indicating an unaltered cAMP/CRP regulation that therefore cannot explain their increased HHP resistance. The observation that Δ*sucA* and Δ*sucB* mutants actually displayed slightly attenuated ($P \leq 0.05$) levels of β-galactosidase activity

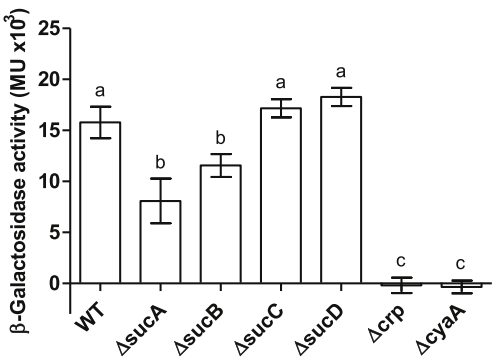

Fig. 2. β-Galactosidase activity of stationary phase cultures of *E. coli* MG1655 WT and its indicated mutants. Letters indicate statistically significant differences ($P \leq 0.05$) among the Miller Units (MU) calculated for each strain.

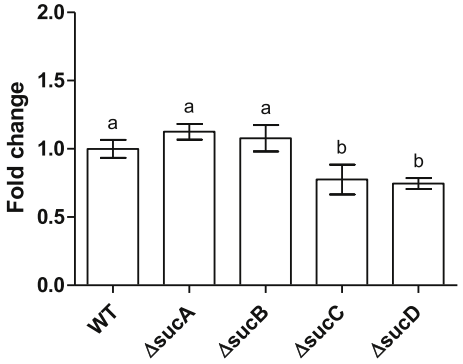

Fig. 3. Fluorescence derived from pFPV-P_{dnaK}-*gfp* (encoding the *E. coli* MG1655 *dnaK* promoter upstream of *gfp*) in the indicated strains. Values are expressed as fold change with respect to the average value of the parental strain. Letters indicate statistically significant differences ($P \leq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

compared to the parental strain, seems likewise to rule out involvement of cAMP/CRP upregulation in their HHP hypersensitive phenotype. Indeed, the lower β-galactosidase activity tends to agree with the fact that accumulation of intracellular 2-oxoglutarate (as occurs in a Δ*sucA* mutant (Li et al., 2006a)) decreases cAMP levels (Doucette et al., 2011).

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

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

Interestingly, the Δ*sucC* and Δ*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 Δ*rssB* strain (Fig. 4A). Importantly, this increase was shown to be truly RpoS dependent since additionally deleting the *rpoS* gene in the Δ*sucC* and Δ*sucD* mutants (resulting in MG1655 Δ*sucC* Δ*rpoS* and Δ*sucD* Δ*rpoS*, respectively) abolished P_{bolA} expression to the same extent ($P > 0.05$) as in a Δ*rpoS* mutant (Fig. 4A). Moreover, since lack of RpoS activity also reduced the HHP resistance of the Δ*sucC* Δ*rpoS* and Δ*sucD* Δ*rpoS* double mutants to the

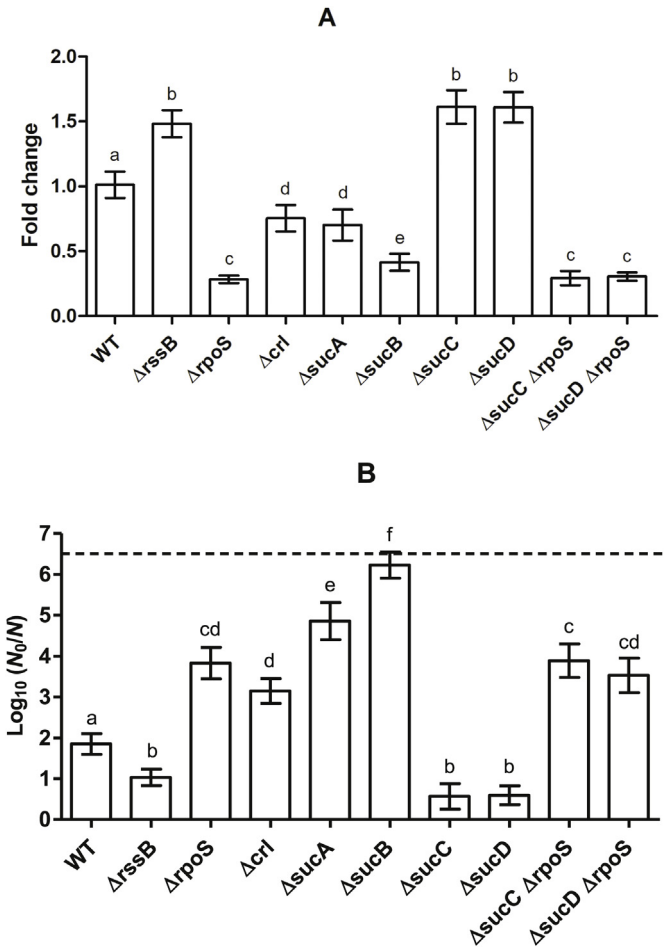

Fig. 4. (A) Fluorescence derived from pFPV-P_{bolA}-*gfp* (encoding the *E. coli* MG1655 *bolA* promoter upstream of *gfp*) in the indicated strains. Values are expressed as fold change with respect to the average value of the parental strain. (B) Logarithmic reduction factor of *E. coli* MG1655 WT and indicated mutants by a HHP treatment at 300 MPa (15 min, 20 °C). The dotted line represents the quantification limit (1000 CFU/ml). Letters indicate statistically significant differences ($P \leq 0.05$) among the inactivation and fluorescence values of all strains.

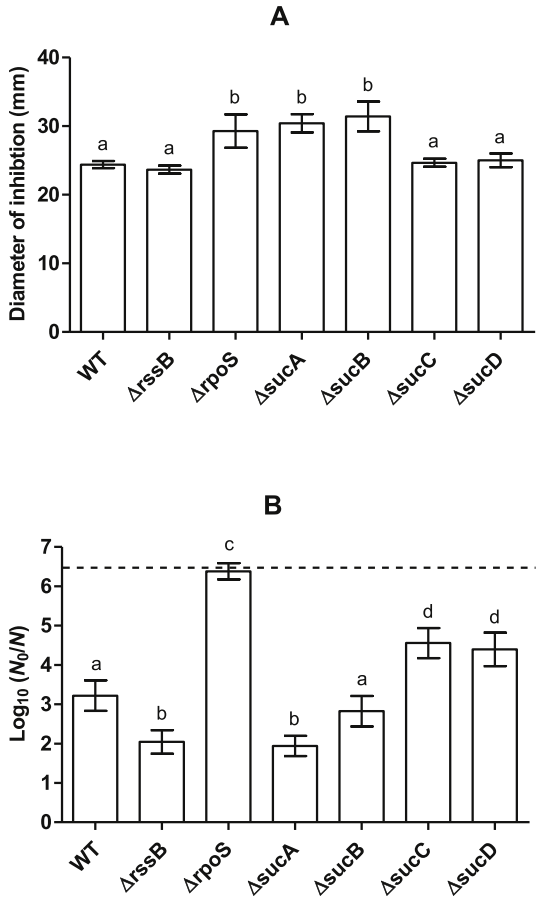

Fig. 5. Resistance of *E. coli* MG1655 WT and indicated mutants to (A) hydrogen peroxide and (B) heat. The resistance to hydrogen peroxide was measured by the inhibition growth diameter of a 5-µl aliquot of the chemical (30% w/w) on stationary phase cells plated in 15 ml of LB soft agar, while heat resistance 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 (1000 CFU/ml). Letters indicate statistically significant differences ($P \leq 0.05$) among the inhibition growth diameter and inactivation of all the strains.

same level ($P > 0.05$) as a single Δ*rpoS* mutant (Fig. 4B), the increased basal RpoS activity seems to be causally involved in the HHP resistance of the Δ*sucC* and Δ*sucD* mutants.

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

Since our data suggested that the HHP resistance of Δ*sucC/D* mutants (and partially of Δ*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 Δ*rpoS* strain, the Δ*sucA* and Δ*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 Δ*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 Δ*cyaA* or Δ*crp* mutants (Gayán et al., 2017b) agrees with the current observation that the HHP resistant Δ*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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.10.007.

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