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Loss of cAMP/CRP regulation confers extreme high hydrostatic pressure resistance in *Escherichia coli* O157:H7

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ABSTRACT

Application of high hydrostatic pressure (HHP) constitutes a valuable non-thermal pasteurization process in modern food conservation. Triggered by our interest in the rapid adaptive evolution towards HHP resistance in the food-borne pathogen *E. coli* O157:H7 (strain ATCC 43888) that was demonstrated earlier, we used genetic screening to identify specific loci in which a loss-of-function mutation would be sufficient to markedly increase HHP survival. As such, individual loss of RssB (anti RpoS-factor), CRP (catabolite response protein) and CyaA (adenylate cyclase) were each found to confer significant HHP resistance in the 300 MPa range (i.e. >1,000-fold), and this phenotype invariably coincided with increased resistance against heat as well. In contrast to loss of RssB, however, loss of CRP or CyaA also conferred significantly increased resistance to 600 MPa (i.e. >10,000-fold), suggesting cAMP/CRP homeostasis to affect extreme HHP resistance independently of increased RpoS activity. Surprisingly, none of the rapidly emerging HHP-resistant mutants of ATCC 43888 that were isolated previously did incur any mutations in *rssB*, *crp* or *cyaA*, indicating that a number of other loci can guide the rapid emergence of HHP resistance in *E. coli* O157:H7 as well. The inability of spontaneous *rssB*, *crp* or *cyaA* mutants to emerge during selective enrichment under HHP selection likely stems from their decreased competitive fitness during growth. Overall, this study is the first to shed light on the possible genetic strategies supporting the acquisition of HHP resistance in *E. coli* O157:H7.

1. Introduction

High hydrostatic pressure (HHP) processing is increasingly being implemented in modern food industry as a non-thermal pasteurization technique, and can inactivate microbial contaminants while minimally affecting the sensorial properties of the food (Aertsen et al., 2009; Considine et al., 2008). As a novel processing technique entering the food production chain, however, the impact of HHP exposure on the conduct and evolution of food-borne pathogens and spoilage microorganisms needs to be thoroughly investigated so that the resulting insights can be used either to increase the lethality of HHP processing or to prevent the possible occurrence of unwanted side-effects.

In this context, it has already been established that HHP can facilitate the uptake of lysozyme in Gram-negative bacteria (Masschalck et al., 2001; Nakimbugwe et al., 2006), and can sensitize bacteria against subsequent challenge with acid (García-Graells et al., 1999; Pagán et al., 2001; Van Opstal et al., 2006) or oxidative stress (Aertsen et al., 2005; Malone et al., 2008). On the other hand, bacterial resistance against HHP was shown to be induced transiently by a prior heat shock (Aertsen et al., 2004), or to be acquired permanently by mutants that emerge during repeated exposure to HHP (Hauben et al., 1997; Karatzas et al., 2003; Vanlint et al., 2011, 2012). Notably, this latter ability to genetically acquire HHP resistance has been shown to be particularly prominent in strains of *Escherichia coli*, where sequential exposures to gradually increasing pressures can reproducibly select for resistance in the gigapascal range without obviously compromising cellular fitness (Hauben et al., 1997; Vanlint et al., 2011, 2012).

While many of the genomic changes and molecular mechanisms underlying HHP resistance development in *E. coli* remain to be elucidated, it has already clearly been established that loss or attenuation of RpoS activity significantly increases the cell's susceptibility to HHP inactivation (Charoenwong et al., 2011; Robey et al., 2001). RpoS is the stationary phase sigma factor (σ^S) that tends to redirect the RNA polymerase to promoters of genes involved in general stress resistance of the cell (Battesti et al., 2011), and reduced RpoS activity therefore increases the overall frailty of the cell. In addition to the complex and elaborate cellular regulation of RpoS activity, sequence polymorphisms in the *rpoS* locus have been shown to be very common in natural *E. coli* strains (Chiang et al., 2011; Dong et al., 2009; Spira and Ferenci, 2008). Since RpoS competes with the housekeeping sigma factor (i.e. RpoD or σ^{70}) for the limited amount of RNA polymerase in the cell, the latter tuning of RpoS activity reflects a strain's potential to balance the allocation of its resources between *s*elf-*p*reservation on the one hand *a*nd *n*utritional *c*ompetence on the other (i.e. the SPANC balance) (Ferenci, 2005; Nyström, 2004).

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We recently focused on the course of HHP resistance development in *E. coli* O157:H7 (strain ATCC 43888) as a representative of the notorious and low infectious dose EHEC strains that are known for their ability to cause hemorrhagic colitis and hemolytic uremic syndrome in humans (O'Loughlin and Robins-Browne, 2001). Interestingly, the attenuated RpoS activity and concomitant HHP hypersensitivity naturally displayed by this strain could rapidly and reproducibly be alleviated within merely 35 generations under a HHP selection regimen (Vanlint et al., 2013). More specifically, 5 cycles of progressively intensifying HHP treatments (from 200 to 300 MPa) were sufficient for mutants to emerge that became resistant to 300 MPa and displayed a number of other phenotypes reminiscent of increased RpoS activity as well (Vanlint et al., 2013).

In this study we aimed to elucidate the adaptive evolutionary paths that could allow such a rapid emergence of HHP resistance in *E. coli* O157:H7, and for the first time identify abolition of cAMP/CRP homeostasis as an effective strategy towards the acquisition of extreme HHP resistance.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli O157:H7 strain ATCC 43888 (lacking the genes for Shiga-like toxin I and II; obtained from the American Type Culture Collection) and its derivatives used throughout this study were listed in Table 1. For challenge studies (see below), stationary phase cultures of these strains were obtained by aerobic growth with shaking (200 rpm) for 18 h in Tryptone Soy Broth (TSB) (Oxoid, Basingstoke, United Kingdom) at 37 °C. When appropriate, a final concentration of the following antibiotics (Applichem, Darmstadt, Germany) was used in the growth medium: (i) 100 μg/ml ampicillin (Amp^{100}) to select for the presence of pSB367 (containing the *spvR* gene and the *spvA* promoter transcriptionally fused to the *luxCDABE* genes; i.e. P_{spvA}-*lux* reporter) (Robey et al., 2001; Swift and Stewart, 1994), (ii) 30 μg/ml of chloramphenicol (Cm^{30}) to select for the presence of the pACYC184-based (Rose, 1988) complementation plasmids (encoding the ATCC 43888 *rssB*, *crp* or *cyaA* locus), and/or 50 μg/ml of kanamycin (Km^{50}) to select for the presence of the mini-Tn5Km2 transposon (see below).

Table 1
Bacterial strains and plasmids used in this study.

Bacteria and plasmids	Characteristics	Source or reference
Bacteria		
Escherichia coli ATCC 43888		
wild-type	Parental strain, O157:H7 serotype	ATCC[a]
rssB (MT1)	HHP-resistant derivative of ATCC 43888; *rssB*::mini-Tn5Km2, Km^R	This study
crp (MT2)	HHP-resistant derivative of ATCC 43888; *crp*::mini-Tn5Km2, Km^R	This study
cyaA (MT3)	HHP-resistant derivative of ATCC 43888; *cyaA*::mini-Tn5Km2, Km^R	This study
5-cycle mutant	Evolved HHP-resistant mutant of ATCC43888	Vanlint et al., 2013
Escherichia coli S17-1λ*pir*	Tp^R, Sm^R, *recA*, *thi*, *pro*, *hsdR*$^-$, M^+, RP4:2-Tc:Mu:KmTn7, λ*pir*	Herrero et al., 1990
Plasmids		
pUTmini-Tn5Km2	Delivery plasmid for mini-Tn5Km2, Ap^R and Km^R	de Lorenzo et al., 1990; Hensel et al., 1995
pACYC184	Cloning vector, Tc^R and Cm^R	Rose, 1988
pACYC184-*rssB*	*rssB* locus of ATCC 43888 cloned in pACYC184, Cm^R	This study
pACYC184-*crp*	*crp* locus of ATCC 43888 cloned in pACYC184, Cm^R	This study
pACYC184-*cyaA*	*cyaA* locus of ATCC 43888 cloned in pACYC184, Cm^R	This study
pSB367	*spvR* gene and *spvA* promoter transcriptionally fused to the *luxCDABE* genes; i.e. P*spvA-luxCDABE* reporter	Swift and Stewart, 1994; Robey et al., 2001

Ap^R: ampicillin; Km^R: kanamycin; Tc^R: tetracycline; Cm^R: chloramphenicol; Km^R: kanamycin; Sm^R: spectinomycin; Tp^R: trimethoprim.
[a] ATCC: American Type Culture Collection, Manassas, Virginia.

2.2. Challenge with high hydrostatic pressure (HHP) or heat

Cells from a stationary phase culture were harvested by centrifugation (4000 ×*g*, 5 min) and resuspended in an equal volume of 0.85% KCl. For treatment with HHP, a 200 μl portion of resuspended cells was heat sealed in a sterile polyethylene bag after exclusion of the air bubbles, and subjected to the appropriate pressure (300 or 600 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. Please note that both the slow pressure-increase (100 MPa/min) and the external water jacket attenuate adiabatic heating during pressure build-up, and conservative estimates indicate only a transient increase in sample temperature of less than 13 °C at 600 MPa. Finally, at the end of the holding time, decompression was almost instantaneous. For treatment with heat, on the other hand, a 75 μl portion of resuspended cells was transferred aseptically into a sterile PCR tube and subjected to 56 °C for 15 min using a PCR apparatus (Westburg, Leusden, The Netherlands).

After HHP or heat treatment, samples were aseptically retrieved from the polyethylene bags or PCR tubes, respectively, after which they were serially diluted in 0.85% KCl with 0.1% peptone, and subsequently spread-plated on Tryptone Soy Agar (TSA). After overnight incubation at 37 °C, colonies on the plates were counted and the logarithmic reduction factor was calculated as $\log(N_0/N)$, in which N_0 and N represent the number of viable cells in colony forming units (CFU) per ml prior to and after treatment, respectively. Please note that the detection limit was 10 CFU/ml for spread-plated samples.

2.3. Measurement of reporter gene activity

Strains of *E. coli* ATCC 43888 were first transformed with pSB367 (encoding P_{spvA}-*lux*; as a bioluminescent indicator of RpoS activity) by electroporation, after which cultures of the corresponding transformants were grown to stationary phase in TSB Amp^{100} at 37 °C. Subsequently, 200 μl samples were transferred to microplate wells and placed in a Fluoroscan Ascent FL (Thermo Labsystems, Brussels, Belgium) for the determination of bioluminescence. The obtained bioluminescence values were subsequently divided by the OD_{600} of the same sample to obtain the relative bioluminescence units. Differences in bioluminescence are expressed as fold change, standardized with respect to the average value of the parental strain.

2.4. Construction and screening of a random transposon knock-out library in *E. coli* O157:H7 ATCC 43888 for loss-of-function mutations leading to HHP resistance

A random mini-Tn5Km2 transposon (de Lorenzo et al., 1990) knock-out library of the pressure-sensitive *E. coli* ATCC 43888 strain was constructed by conjugation. Briefly, *E. coli* S17-1 λ-pir carrying a pUTmini-Tn5Km2 plasmid (donor) (Hensel et al., 1995) was mated with *E. coli* ATCC 43888 (acceptor), and from this conjugation ATCC 43888 exconjugants were selected on M9 minimal medium (Miller, 1992) containing kanamycin (Km^{50}). The corresponding clones were collected, until a pool of ca. 20,000 independent and random insertion mutants was obtained. From this 20,000 clone pool, 120 independent sub-pools of 100 clones each were derived via cell sorting (Cytopeia Influx System Flow Cytometer/cell sorter apparatus; Becton Dickinson, Erembodegem, Belgium). More specifically, single cell sorting was used to randomly combine 100 individual cells of the original library