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Enhancing *E. coli* Tolerance towards Oxidative Stress via Engineering Its Global Regulator cAMP Receptor Protein (CRP)

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Abstract

Oxidative damage to microbial hosts often occurs under stressful conditions during bioprocessing. Classical strain engineering approaches are usually both time-consuming and labor intensive. Here, we aim to improve *E. coli* performance under oxidative stress *via* engineering its global regulator cAMP receptor protein (CRP), which can directly or indirectly regulate redox-sensing regulators SoxR and OxyR, and other ~400 genes in *E. coli*. Error-prone PCR technique was employed to introduce modifications to CRP, and three mutants (OM1~OM3) were identified with improved tolerance *via* H_2O_2 enrichment selection. The best mutant OM3 could grow in 12 mM H_2O_2 with the growth rate of 0.6 h^{-1}, whereas the growth of wild type was completely inhibited at this H_2O_2 concentration. OM3 also elicited enhanced thermotolerance at 48°C as well as resistance against cumene hydroperoxide. The investigation about intracellular reactive oxygen species (ROS), which determines cell viability, indicated that the accumulation of ROS in OM3 was always lower than in WT with or without H_2O_2 treatment. Genome-wide DNA microarray analysis has shown not only CRP-regulated genes have demonstrated great transcriptional level changes (up to 8.9-fold), but also RpoS- and OxyR-regulated genes (up to 7.7-fold). qRT-PCR data and enzyme activity assay suggested that catalase (*katE*) could be a major antioxidant enzyme in OM3 instead of alkyl hydroperoxide reductase or superoxide dismutase. To our knowledge, this is the first work on improving *E. coli* oxidative stress resistance by reframing its transcription machinery through its native global regulator. The positive outcome of this approach may suggest that engineering CRP can be successfully implemented as an efficient strain engineering alternative for *E. coli*.

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Introduction

Strain engineering approaches have been widely implemented for the production of a broad range of compounds [1,2,3,4,5,6,7]. Using UV/chemical mutagens or rewiring metabolic pathways through gene addition/knockout have been traditional approaches for strain improvement [8]. Classical strain engineering strategies are often time-consuming and labor-intensive [9]. The manipulation of metabolic pathways, however, needs comprehensive knowledge of the complex metabolic network together with the fitness of the manipulation in the phenotypic context [10]. Moreover, a functional cluster of genes orchestrate phenotypic modulation only when perturbed altogether [11], which is difficult to be achieved by metabolic approach.

The strategy of reprogramming a network of genes for phenotype enhancement has led to transcriptional engineering that enables reframing genetic control circuits by modification to entire genomic hierarchy inside microorganisms [12]. Global regulators are able to organize a large repertoire of genetic switches [13]. These regulators can also impart pleiotropic phenotype changes through the regulation of operons belonging to various functional groups [14]. Transcriptional engineering has been evolved as a potential tool for strain engineering over the last few years to alter strain stress tolerance [15,16,17], biofuel production [18,19,20], and biofilm formation [21,22].

In this work, we focus on engineering global regulator cAMP receptor protein (CRP) of *E. coli* to improve its performance under stress. Seven global regulators (ArcA, CRP, FIS, FNR, IHF, LRP and H-NS) in *E. coli* can regulate about half of the total genes [23]. Among them, CRP can regulate more than 400 genes and harmonize certain genetic circuits by directly or indirectly regulating other transcriptional regulators [24], which makes it a potential target for altering cellular phenotypes. Previously, we have shown that engineering CRP can improve *E. coli* osmotolerance [25], 1-butanol tolerance [26] and organic solvent tolerance [27]. Here, we aim to explore the possibility of rewiring CRP against oxidative damage often encountered inside bioreactors under stressful conditions [28]. *E. coli* DH5α was used as host strain for its suitability in plasmid stability and in bioprocess usage [29,30].

Oxidative modification of biological macromolecules and intracellular components by reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$), can lead to cell damage [31]. The prototype response of *E. coli* against oxidative stress is the

induction of antioxidant enzymes involved in ROS scavenging and DNA repair [32], which is *via* global transcriptional activation of redox-sensing regulators SoxR and OxyR [33]. In addition, oxidative stress in *E. coli* also induces chaperone such as Hsp33 to protect plenty of cellular proteins from stress generated shock [34]. Traditional approaches have been adopted to construct mutant *E. coli* strain *via* spontaneous adaptation [35] and cloning of exogenous antioxidant genes [36]. Earlier reports suggested that OxyR and RpoS, two major regulators of oxidative stress response in *E. coli*, were either directly or indirectly regulated by CRP [37]. The activation of RpoS (σ^s) is related with the down regulation of cAMP-CRP complex [37]. The complex was also suggested to stimulate the cleavage of LexA repressor, potentiate ROS generated SOS control and thus transcribe mutagenically important relevant genes upon cell damages [38]. Studies have also revealed that *cya* and *crp* deletion in *E. coli* may increase cellular H_2O_2 sensitivity [39]. These findings encouraged us to manipulate relevant *E. coli* response through CRP. Here, we have constructed a CRP library through error-prone PCR [40] and isolated three improved mutants (OM1~OM3) against oxidative stress *via* enrichment selection (H_2O_2). The stress response of the best mutant OM3 and wild type was further analyzed by DNA microarray and validated with quantitative real time reverse transcription PCR (qRT-PCR). Cell lysate of OM3 and WT were tested for antioxidant enzyme activities, namely catalase, alkyl hydroperoxide reductase, and superoxide dismutase.

Materials and Methods

Materials

E. coli DH5α was procured from Invitrogen (San Diego, USA) and *E. coli Δcrp* strain was obtained according to a previous published protocol [25]. Luria Bertinii (LB) medium (Bacto tryptone (Oxoid) 10 g/l, Yeast extract (Merck) 5 g/l, Sodium Chloride (Merck) 10 g/l) was routinely used for bacterial culture since it has been a popular medium choice for *E. coli* growth under oxidative stress [36,41]. SOC medium (Yeast extract 5 g/l, Tryptone 20 g/l, NaCl 10 mM, KCl 2.5 mM, $MgCl_2$ 10 mM, $MgSO_4$ 10 mM, Glucose 20 mM) was used for cultivation of transformed cells. 30% (w/w) hydrogen peroxide (H_2O_2) and 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Sigma-Aldrich (St. Louis, MO, US). Restriction enzymes from Fermentas (Burlington, US) and T4 DNA ligase from New England Biolabs (Ipswich, MA, US) were used for cloning and library construction. DNA fragments were purified by QIAquick gel extraction kit (Qiagen, Germany) whenever necessary and plasmid isolation was performed by QIAprep spin miniprep kit from the same manufacturer.

Cloning and library construction

The native *crp* was amplified *via* error-prone PCR with the following primers: *crp*_sense (5′-gagaggatccataacagaggataaccgcg-catg-3′) and *crp*_anti (5′-agatggtaccaaacaaaatggcgcgctaccagg-taacgcgcca-3′) using Genemorph® random mutagenesis kit from Stratagene (La Zolla, US). The error-prone PCR was performed with 30 ng of pKSCP (containing native *crp* operon) plasmid obtained from our previous studies as template [69,70], using the following program: 3 min at 95°C, 30 cycles of 45 s at 95°C, 45 s at 62°C followed by 1 min at 72°C, and 10 min at 72°C. The amplified PCR products were purified from 1.2% low-melting agarose gel, double digested with restriction enzymes *Bam* HI and *Kpn* I, and cloned into plasmid pKSCP. The resulting recombinant plasmid was transformed into Δ*crp* competent cells and cultured at 37°C, 200 rpm.

Mutant selection

The mutant library was cultured in SOC medium at 37°C and 200 rpm for 4 h after electroporation and thence subjected to enrichment selection. In order to select mutants against oxidative stress, H_2O_2 was used as stressor and LB medium was fed with increasing concentration of H_2O_2. The selection was carried out in 1.5 mM H_2O_2 for three repeats and challenged with 2.0 mM H_2O_2 during the fourth round. The 'winners' were cultured on LB-kanamycin (LB-kan) plates overnight at 37°C. Individual clones were selected randomly from the plates and sequenced to identify amino acid mutations in CRP. The mutated *crp* was re-cloned into fresh pKSCP plasmid and back-transformed to fresh *Δcrp* backgrounds in order to nullify plasmid or genome borne false positives. The pKSCP plasmid containing native *crp* operon was also transformed into *Δcrp* background and is designated as wild type (WT) in this study.

Mutant growth under stress

The freshly transformed colonies were cultured overnight in LB-kan medium and the overnight inoculums were used to seed cells in fresh LB-kan medium to an OD_{600} value of 0.05. Each clone was cultivated at 37°C, 200 rpm in 0–12 mM H_2O_2, 50-ml screw capped centrifuge tube shielded from light. Samples were withdrawn at periodic intervals and cell growth was monitored by measuring the optical density at 600 nm.

Instead of adding H_2O_2 at the very beginning, 12 mM H_2O_2 was also introduced into the culture after cells reaching mid-log phase (OD_{600} 0.65) and their growth was monitored.

Tolerance to cumene hydroperoxide

One percent overnight culture was seeded into fresh 10-ml LB-kan medium containing 0.3 mM cumene hydroperoxide. Cell growth was monitored spectrophotometrically at 600 nm.

Mutant thermotolerance

Stationary phase culture of the mutant and WT was used to inoculate fresh LB-kan media up to an OD_{600} value between 0.05 and 0.06. With the same starting OD_{600}, both were allowed to reach stationary phase at 48°C. Cell density was tracked by sampling from the cultures and measuring the OD_{600} values at periodic time intervals.

Measurement of intracellular reactive oxygen species (ROS) level

The intracellular peroxide level was measured by using ROS sensitive probe 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) as described previously [42]. In brief, both the mutant and WT were grown to OD_{600} 0.6 with or without 4 mM H_2O_2. Cells were harvested by centrifugation, washed with 10 mM, pH 7.0 potassium phosphate buffer (PPB), and resuspended in the same buffer. Cells were incubated with 10 μm H_2DCFDA (dissolved in dimethyl sulfoxide) at 30°C, 200 rpm in darkness for 30 min, harvested, washed again with PPB, and lysed by sonication in darkness. 100-μl cell lysate was pipetted into a 96-well black microplate. Cell fluorescence was measured by a BioTek microplate reader (Winooski, VT, US) with an excitation wavelength at 485 nm and emission at 528 nm. The fluorescence intensity was normalized against total protein concentration measured by Bradford reagent using an Eppendorf biophotometer (Hamburg, Germany).

DNA microarray

Cells were grown with or without 4 mM H_2O_2 to OD_{600} around 0.6~1 and harvested by centrifugation. RNA was extracted using Qiagen RNeasy kit (Germany) according to manufacturer's instructions. Microarray assay was performed at Genomax Technologies (Singapore). Agilent SurePrint *E. coli* 8×15 K slides were used and Cy3/Cy5 hybridized slides were scanned under Agilent High Resolution Scanner (C-model). Data organization and analysis was performed by Agilent Genespring GX software. Extraction of raw signal data was achieved from TIFF image with Agilent Feature Extraction Software (V10.7.1.1). The expression ratio and *p*-value was calculated based on two biological replicates of each strain under all conditions. A log base 2 transformation was used followed by percentile shift to 75th percentile of each sample (per chip normalization). The normalization was performed by shifting baseline to median of all samples (per gene normalization). *p*-value was calculated using unpaired Student T-Test –with a Benjamini-Horchberg False Discovery Rate (FDR) correction.

The rest of "Materials and Methods" is provided in Materials and Methods S1.

Results

Radom mutagenesis library construction and mutant selection

In order to select *E. coli* mutants with elevated tolerance towards oxidative stress, error-prone PCR was performed to introduce mutations to CRP and construct random mutagenesis libraries. Approximately ~10^5 clones containing *crp* were obtained after two rounds of error-prone PCR. With the enrichment selection of 1.5 mM~2.0 mM H_2O_2, three mutants (OM1~OM3) that exhibited better tolerance towards stress were selected from the library. The mutation rate was around 1–3 amino acid substitutions over the CRP open reading frame and the amino acid mutations of OM1~OM3 are listed in Table 1.

Mutant growth in H_2O_2

Mutant growth was evaluated by subjecting mutants as well as WT in 0 mM to 12 mM H_2O_2 (Figure 1). The stability of hydrogen peroxide was confirmed by its absorbance at 240 nm ($\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$) during the culturing period (Figure S1). In the absence of H_2O_2, all three mutants exhibited similar growth profiles as WT, with the growth rate around 0.31~0.36 h^{-1} (Figure 1A). With 8 mM H_2O_2 present (Figure 1B), all mutants behaved similarly to each other with the growth rate around 0.45 h^{-1}, whereas WT exhibited null growth. The cell growth of both *E. coli Δcrp* strain and *Δcrp* strain harboring blank plasmid was also completely inhibited under the same condition (Figure S2). When the pressure was further hiked to 12 mM H_2O_2 (Figure 1C), the growth of OM1 and OM2 were hindered completely within the time frame of observation, while OM3 achieved stationary phase OD_{600} of 2.7 with the growth rate of 0.6 h^{-1}.

Table 1. Amino acid substitutions in OM1~OM3.

Mutant	Amino acid substitution		
OM1	T127N		
OM2	D138V	T146I	
OM3	F69C	R82C	V139M

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We have also introduced 12 mM H_2O_2 into the culture after cells reached mid-log phase in LB-kan medium. OM3 demonstrated the highest stationary phase OD value at 1.84, whereas OM1 and OM2 could only reach ~1.5 (Figure 2). The inhibition was more prominent in WT as its OD only reached 1.03. Because OM3 displayed the best viability at high H_2O_2 concentration, it was chosen for subsequent investigation.

Mutant thermotolerance and its tolerance to cumene hydroperoxide

Since inorganic hydroperoxide H_2O_2 was used as oxidative stressor for mutant selection, we further characterized OM3 tolerance against organic hydroperoxide, cumene hydroperoxide. WT growth was completely inhibited in 0.3 mM cumene hydroperoxide while OM3 reached stationary phase at OD_{600} ~2.8 (Figure 3A). Moreover, earlier publications on the interrelationship between oxidative stress and thermotolerance encouraged us to evaluate the thermotolerance of OM3 [43]. As shown in Figure 3B, OM3 demonstrated better growth (0.52 h^{-1}) than WT (0.38 h^{-1}) at 48°C.

DNA microarray analysis and quantitative real time reverse transcription PCR

DNA microarray analysis of OM3 and WT revealed that OM3 had different transcription profile from WT in the presence or absence of oxidative stress, as shown in Table S1 and Table S2 (Gene Expression Omnibus (GEO): GPL13359). In response to oxidative stress, 202 genes in OM3 displayed over twofold up-regulation, while 266 genes showed down-regulation, with the *p*-value threshold less than 0.05. Previous investigation has shown that general stress sigma factor σ^s (or RpoS), OxyR and SoxRS regulons play essential roles in regulating *E. coli* oxidative stress response [33,44]. Here, we found that CRP-regulated genes also went through great expression level changes under oxidative stress—*lamB* (encoding outer membrane protein facilitating diffusion of maltose and other maltodextrins), *malE* (encoding component of maltose ABC transporter), and *cstA* (encoding a carbon starvation protein) were all down-regulated by more than 4.2-fold with H_2O_2 treatment (Table 2). Among RpoS-regulated genes, *gadA* (glutamate decarboxylase subunit A) had the maximum fold up-regulation in OM3 (7.76-fold), followed by its family members *gadB* (7.1-fold) and *gadC* (7.02-fold). In addition, increased induction of antioxidant gene *katE* (catalase HP-II, 3.8-fold) was observed. Genes associated with both osmotic as well as oxidative stress tolerance such as *osmC* and *osmY* demonstrated 2.75- and 3.06-fold up-regulation respectively in OM3 compared to WT. OxyR-regulated genes such as *sufABDES* (2.55~3.48 fold up-regulation) showed enhanced expression level as compared to WT. Without H_2O_2 treatment, all of these OxyR-regulated genes revealed less than 2.0-fold change with respect to WT. By contrast, the RpoS-regulated genes exhibited expressional increment, including *gadAB* (4.5-fold), *katE* (2.7-fold), *otsA* (trehalose-6-phosphatase synthase) (2.6-fold) with the threshold $p<0.05$ (Table 3). With or without stress, none of the SoxRS-regulated redox-sensing genes exhibited more than twofold changes as compared to WT. Interestingly, genes regulated by CRP also underwent copious down-regulation (>8.5 fold) in OM3, including *lamB*, *malE*, *malK* (ATP binding component of maltose ABC transporter), which are mainly associated with membrane formation and intracellular transport.

qRT-PCR was carried out on ten selected genes to validate the microarray results (Table S3) [33,45,46]. Without H_2O_2, the expression of *katE*, *gadA*, *crp*, *cya* and *otsA* were all up-regulated in

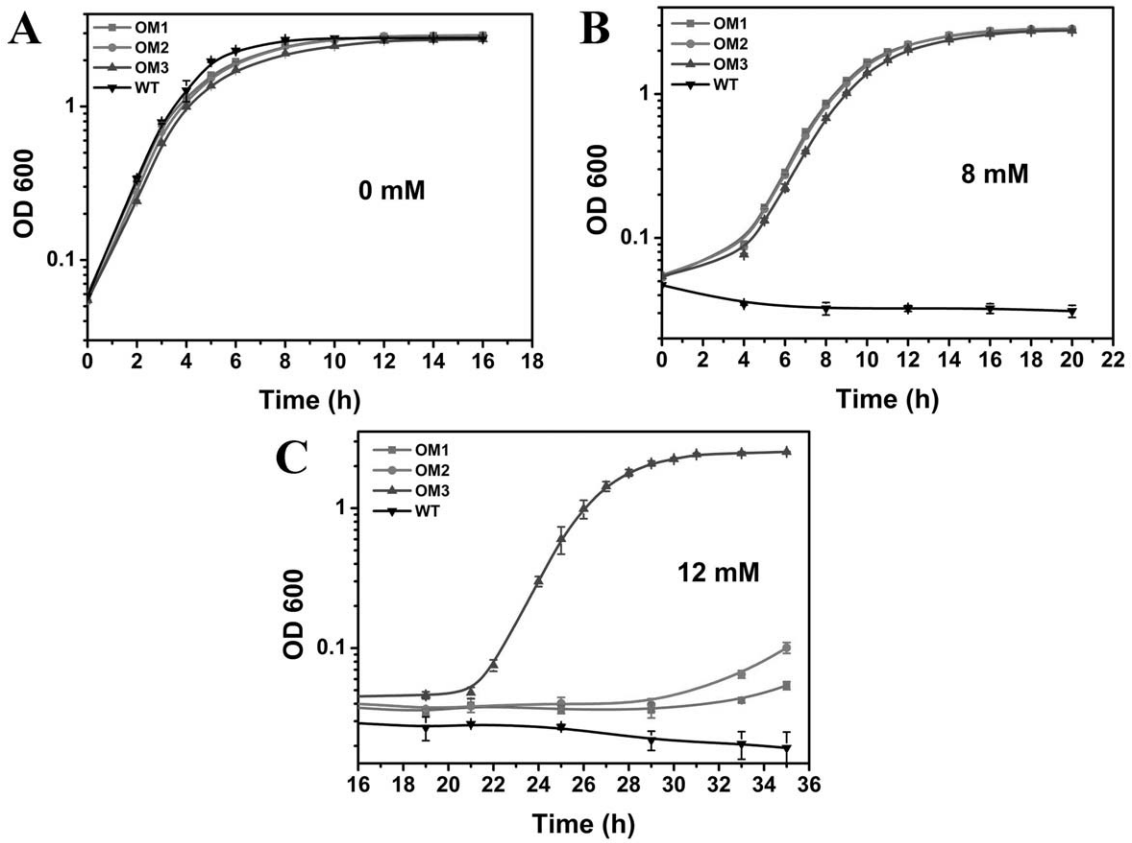

Figure 1. Cell growth in the absence or presence of H_2O_2 (A) 0 mM H_2O_2, (B) 8 mM H_2O_2, (C) 12 mM H_2O_2, Cells were cultured in LB-kanamycin medium at 37°C, 200 rpm. Each data point is the mean of three replicates.
doi:10.1371/journal.pone.0051179.g001

OM3 as compared to WT, whereas *sodA*, *cstA*, *ahpF* and *malE* demonstrated down-regulation, which agreed with the microarray data (Table S4). Under oxidative stress, antioxidant gene expression such as *sodA*, *katE*, *gadA* and *otsA* were elevated in OM3, while *cstA*, *ahpCF*, and *malE* were down-regulated, which also confirmed the microarray results. The only discrepancy we found was that *ahpC* (alkyl hydroperoxide reductase) revealed small activation through microarray under stress but qRT-PCR showed slight down-regulation (Table S4).

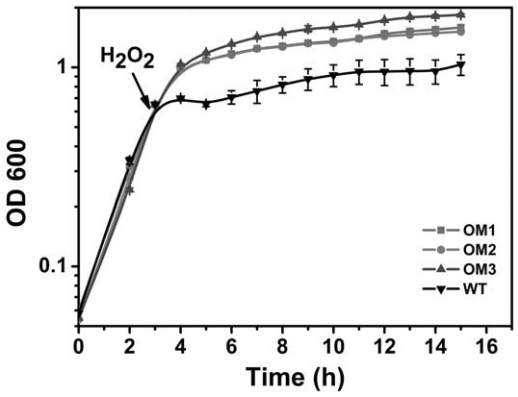

Figure 2. Cell growth profile after the introduction of 12 mM H_2O_2 during mid log phase (OD_{600} 0.65). Each data point is the mean of three replicates.
doi:10.1371/journal.pone.0051179.g002

Intracellular reactive oxygen species (ROS) level

Oxidative stress in extracellular medium may alter the intracellular peroxide and other ROS level [47] and thus determine cell viability [32]. The normalized fluorescence intensity suggested that the ROS level in OM3 was always lower than that of WT irrespective of growth with or without H_2O_2 (Figure 4). In the absence of oxidative stress, OM3 possessed 2.5 times lower intracellular ROS compared to WT. Incubation with 4 mM H_2O_2 elevated the free radical level in both strains, diminishing the difference to around 1.4 times.

Discussion

In this study, we have successfully enhanced *E. coli* oxidative stress tolerance *via* engineering its global regulator CRP. H_2O_2 was preferred as the stress-inducing agent in this study and a pool of variants ($\sim 10^5$) was created by error-prone PCR. The library was then screened with H_2O_2 and three mutants (OM1$\sim$OM3) with enhanced oxidative stress tolerance were selected. The best mutant OM3 also revealed resistance against cumene hydroperoxide and exhibited thermotolerance.

We found that simple modifications to global regulator CRP could result in enhanced strain tolerance towards oxidative stress. As for the best mutant OM3, it obtained three mutations *via* error-prone PCR (F69C, R82C and V139M). F69 is important in conferring CRP conformation, which is reoriented upon cAMP binding with the interaction between F69 and R123 (Figure 5) [48]. R82 sets a pivotal role in cAMP binding due to the electrostatic interaction between the guanidium group of R82 and

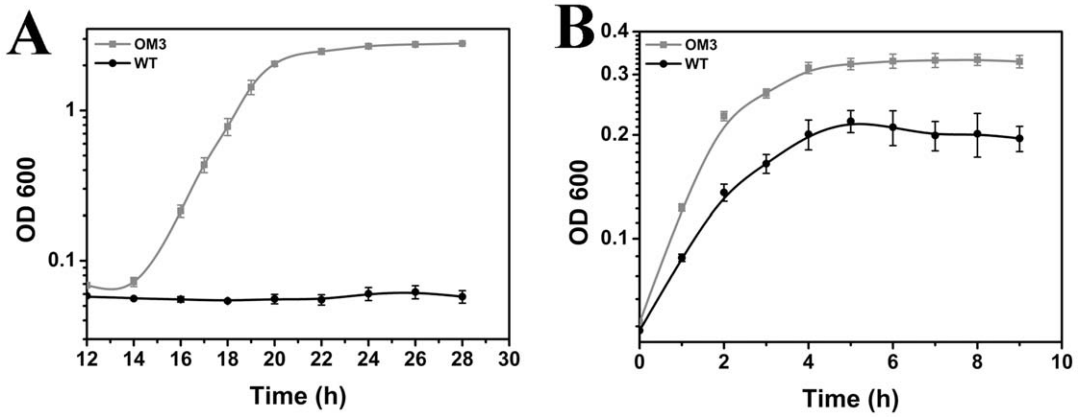

Figure 3. OM3 and WT growth in cumene hydroperoxide or at high temperature (A) 0.3 mM cumene hydroperoxide, (B) 48°C. Cells were grown in LB-kanamycin at 37°C, 200 rpm under above stressors. Each data point is the mean of three replicates.
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the electronegative oxygen of the phosphate group of cAMP [49]. V139 is in the hinge region and participates in the interdomain interaction between N- and C-terminals of CRP [50]. The secondary structure of OM3 CRP and native CRP didn't show any significant difference (Figure S3), but the DNA binding properties of the native CRP and OM3 CRP are very different with Class I, Class II and Class III CRP-dependent promoters as tested by reporter gene assay (Figure S4).

Since our target regulator CRP is a global regulator of *E. coli* and can regulate hundreds of genes [51,52,53,54,55], genome-wide microarray analysis of OM3 and WT in the presence or absence of H_2O_2 was performed to reveal the transcription profile change upon modifications to CRP. We found that CRP-regulated genes such as *lamB* and *malEK* showed differential expression in OM3 under either condition. *mal* operon, transcribing genes such as *lamB*, *malE* and *malK* in *E. coli*, is associated with membrane formation and intracellular transport [56]. The repression of these genes in OM3 supported previous reports on the overlap between oxidative stress and acid tolerance response [46]. *mal* operon is regulated by CRP directly [57], implying that genes outside the regulation of the three principle regulators could also play important roles for oxidative stress management in *E. coli*.

Previous publications have suggested that RpoS can regulate the expression of *gadABC*, *katE* and *osmCY* [58], among which *gad* superfamily, namely *gadABC*, displayed maximum up-regulation under stress by microarray (~7-fold). Glutamate decarboxylase (*gad*) can convert intracellular glutamate to γ-amino butyric acid and is also associated with acid tolerance response of *E. coli* [45]. qRT-PCR result confirmed its upregulation under stress and further enzymatic assay revealed that its activity was 2.9-fold higher in OM3 than in WT under stress (Figure S5A). Strong induction of *katE* (3.8-fold) might lead to a higher amount of catalase in OM3, and thus contributed crucially in the degradation of intracellular H_2O_2. qRT-PCR result concurred with microarray data and enzymatic assay proved about 4-fold increased catalase activity in the cell lysate of OM3 as compared to WT (Figure S5B). These findings implied that *katE* could contribute significantly towards OM3 cell protection from oxidative damage. The elevation of *osmCY*, induced upon hyperosmotic stress in OM3 under stress reinforced the paradigm overlap between osmotic stress and oxidative stress [59].

Other major regulons associated with *E. coli* oxidative stress are

Table 2. DNA microarray data of certain genes in OM3 after H_2O_2 treatment ($p<0.05$, Log_2 Fold Change>2.0).

Regulator	b number	Gene	Log_2 Fold Change
CRP	b4036	*lamB*	−4.780
	b4034	*malE**	−4.257
	b0598	*cstA**	−5.093
RpoS	b3517	*gadA**	7.766
	b1493	*gadB*	7.096
	b1492	*gadC*	7.024
	b1732	*katE**	3.801
	b1482	*osmC*	2.755
	b4376	*osmY*	3.062
	b1896	*otsA**	2.996
OxyR	b1684	*sufA*	3.336
	b1683	*sufB*	3.483
	b1681	*sufD*	3.140
	b1680	*sufE*	2.551
	b1679	*sufS*	2.771

*- Analyzed by qRT-PCR (Table S4).
doi:10.1371/journal.pone.0051179.t002

Table 3. DNA microarray data of certain endogenous genes in OM3 ($p<0.05$, Log_2 Fold Change>2.0).

Regulator	b number	Gene	Log_2Fold Change
CRP	b4036	*lamB*	−8.997
	b4034	*malE**	−8.930
	b4035	*malK*	−8.502
RpoS	b3517	*gadA**	4.517
	b1493	*gadB*	4.571
	b1732	*katE**	2.701
	b1896	*otsA**	2.581

*- Analyzed by qRT-PCR (Table S4).
doi:10.1371/journal.pone.0051179.t003

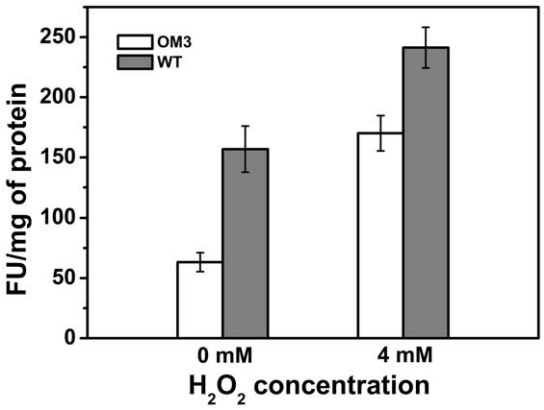

Figure 4. Intracellular ROS level in OM3 and WT with cells treated with or without 4 mM H_2O_2. Mid exponential phase grown cells (OD_{600} 0.6) were incubated with 10 μm H_2DCFDA (dissolved in dimethyl sulfoxide) at 30°C, 200 rpm. The oxidized fluorophore was quantified using excitation wavelength 485 nm and emission wavelength 528 nm. Each data point is the mean of five independent observations.
doi:10.1371/journal.pone.0051179.g004

SoxRS and OxyR, with the latter being suggested as a more specific regulator of H_2O_2 responsive pathways [33]. OxyR regulates *suf* operon (*sufABDES*), which is involved in the formation and repair of Fe-S cluster and encodes components of an ATP binding cassette transporter [60]. It was demonstrated by microarray that the expression of *sufABDES* was elevated by more than twofold in OM3 than in WT when treated with H_2O_2. Under the same condition, a very minor down-regulation was noted in OxyR-regulated *ahpC* (−0.031-fold) and *ahpF* (−0.483-fold) *via* qRT-PCR. The enzyme assay had also confirmed slightly lower alkyl hydroperoxide reductase activity in the cell lysate of OM3 (Figure S5C). Since AhpC is only active with AhpF present [61,62,63,64,65,66,67], our findings probably have suggested that *ahpCF* are not major players in oxidative stress defense of OM3 [68]. The SoxRS-regulated genes such as *sodA* (manganese-containing superoxide dismutase, SOD) failed to exceed two-fold transcriptional level change under either stressful or normal condition, which was confirmed by qRT-PCR. In addition, little difference was observed in SOD activities between OM3 and WT (Figure S5D), indicating that SOD, similar to alkyl hydroperoxide reductase, did not play an important role in the antioxidant machinery of OM3.

OM3 also exhibited better thermotolerance than WT when exposed to 48°C, which was in cope with the earlier finding that there was an overlap between heat shock and oxidative stress defense mechanism *via* heat shock protease HtrA [69] and heat shock proteins IbpA/B [70]. However, despite the repression of HtrA and IbpA/B or even the chaperones (DnaKJ, GroEL and GroES), the thermotolerance of OM3 was elevated. This phenotypic improvement might be due to the up-regulation of heat shock proteins HtrC and HscA, down-regulation of *sohA* (putative protease of HtrA [71]). The performance of OM3 at 48°C was comparable to *E. coli* MG1655 thermotolerant mutant isolated *via* spontaneous adaptation after two years and 620 generations [72]. In comparison, engineering CRP could greatly shorten the mutant selection period from years to days.

Toxicity of hydrogen peroxide and other oxidative stress is often mediated through generation of intracellular ROS, hence we have investigated relative ROS concentrations in both OM3 and WT. As portrayed in Figure 4, the baseline concentration of

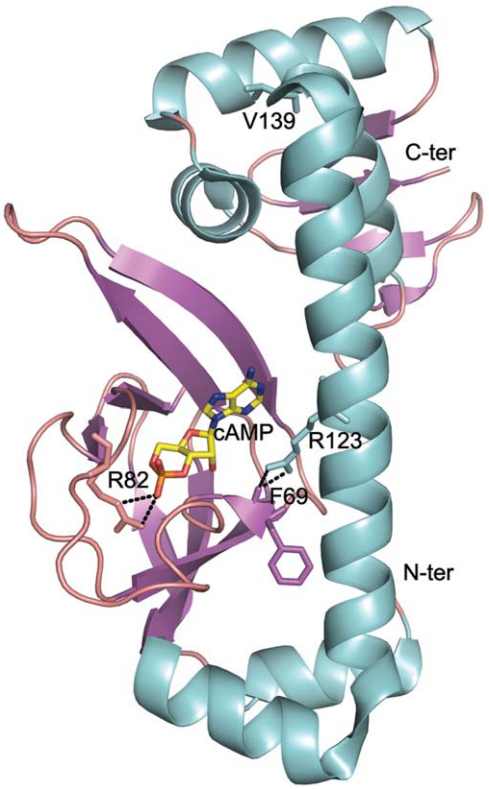

Figure 5. Amino acid mutations in OM3. The main carbonyl of F69 interacts with the amine group of R123. The guanidium group of R82 has the electrostatic interaction with the phosphate group of cAMP. V139 is in the hinge region that participates in the inter-domain interaction between N-terminal cAMP binding domain and the C-terminal DNA binding domain. The structural stereoview was prepared by PyMOL using native CRP structure as template (PDB: 1G6N).
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endogeneous ROS was 2.5 times lower in OM3 compared to WT in the absence of stress, indicating free radical scavenging system was more active in the mutant than WT. Seaver and Imlay *et al.* reported that Ahp^- (Alkyl hydroperoxidase) and Kat^- (Catalase) mutants of *E. coli* had a H_2O_2 production rate of 14 μM/s [73] whereas that of the wild type was only 1–2 μM/s [74] or 3 μM/s [73]. These findings indicated that the net H_2O_2 production of *E. coli* is a direct outcome of intracellular free radical scavenging mechanism. Exertion of stress led to more intracellular ROS accumulation in both OM3 and WT, which was probably due to the increased mass transfer of peroxide into the cells [75]. Since the antioxidant machinery of OM3 might be more active than that of WT, as shown by the elevated expression and activity of catalase, the ROS level in OM3 was lower than that of WT under stress. CRP mediated cellular metabolism could also play an important role in regulating oxidative stress. For instance, ferric uptake regulator protein (Fur) is associated with cell iron metabolism [76,77]. It helps in protecting intracellular Fe^{2+} ion, which binds with cellular O_2^- radical and depletes intracellular iron pool [78]. Since the cAMP-CRP complex is correlated with Fur activation, it is indirectly related with cellular ROS level [79]. Moreover, thiamine metabolism has been found activated with concomitant up-regulation of CRP-regulated genes in ROS affected cells [80].

Exertion of stress often induces modification to cellular morphology [81]. Interestingly, OM3 underwent no significant change in cell length in the presence or absence of H_2O_2 as shown

by the micrographs (Figure S6), but the exterior examination of cell revealed that OM3 cell surface has gone rough in either conditions and become even rougher in H_2O_2, which might be a morphological response towards oxidative stress.

In this work, OM3 could survive and reach stationary phase of OD_{600}~3.0 in 12 mM H_2O_2 whereas the maximal survival limit of WT was 4 mM H_2O_2 (data not shown). The only report so far to acquire non-pathogenic *E. coli* tolerance over 12 mM H_2O_2 was after adapting cells to glucose starved condition [82]. Metabolic engineering approaches of introducing heterogeneous genes such as *grx* (glutaredoxin) [83], *oscyp2*(rice cyclophilin) [84], and *pprA* (a pleiotropic protein promoting DNA repair in radiation-induced damage) [85] could only help *E. coli* improvise cell tolerance against 5 mM H_2O_2, while cloning *Brgr* (glutathione reductase from *Brassica rapa*) [41] helped improve *E. coli* tolerance against 1.5 mM H_2O_2. Classical strain engineering approaches using ethyl methane sulphonate (EMS) or UV did not result in significant improvement of *E. coli* tolerance towards oxidative stress [86,87]. Besides H_2O_2, earlier research has improved *E. coli* tolerance towards 0.1–0.4 mM cumene hydroperoxide *via* spontaneous adaptation [88] or chemical mutagen (diethyl sulfate) treatment [89]. Previous studies suggested that a population size $\sim10^9$ cells was required to isolate bacterial mutants with tolerance towards cumene hydroperoxide [89].By comparison, we were able to isolate three oxidative stress tolerant mutants from a library size of $\sim10^5$. Without pretreatment, the best mutant OM3 exhibited efficient growth against 0.3 mM cumene hydroperoxide, which was comparable to other publications [88]. Hence, together with our previous works, we believe that this isogenic transcriptional engineering approach could provide a promising alternative for *E. coli* strain engineering.

Supporting Information

Figure S1 H_2O_2 absorbance at 240 nm during the culturing period. The experiment was carried out in light shielded environment at 37°C, 200 rpm and H_2O_2 concentration was measured spectrophotometrically at 240 nm using molar extinction coefficient of 43.6 M^{-1} cm^{-1}.
(TIF)

Figure S2 Cell growth in 6 mM and 8 mM H_2O_2. Growth was evaluated in LB-kanamycin (25 μg/ml) medium in light shielded environment. Each data point is the average of two biological replicates.
(TIF)

Figure S3 CD spectra of WT and OM3 CRP. Spectra were obtained in a CHIRASCAN spectropolarimeter with pH 7.2 50 mM PPB buffer as blank. The two spectra were analogous with peaks obtained at 195 and 223 nm. Subsequent deconvolution with K2D2 software revealed that the β-strand percentages in both WT and mutant CRP were close to each other, i.e. 19.53 and 18.65% respectively. Small variation was observed in the relative quantity of α-helix, the percentage being 40.97 in WT and 45.69 in OM3.
(TIF)

Figure S4 DNA binding assay quantified through β-galactosidase activity. The WT and OM3 CRP-pKSCP vectors were co-introduced with distinct pPRO plasmids (pPRO1, pPRO2, and pPRO3) harboring Class I, Class II and Class III CRP-dependent promoters into Δ*crp* strain and the resulting DNA binding was quantified *via* β-galactosidase activity.
(TIF)

Figure S5 Enzyme activity assay. (A) glutamate decarboxylase (GAD) (B) catalase (C) alkyl hydroperoxide reductase (AhpCF) (D) superoxide dismutate (SOD). Each data was the mean of three independent observations.
(TIF)

Figure S6 FESEM micrographs of WT and OM3. (A) WT, 0 mM (B) OM3, 0 mM (C) WT, 4 mM H_2O_2 (D) OM3, 4 mM H_2O_2.
(TIF)

Materials and Methods S1 Supporting information on materials and methods.
(DOC)

Table S1 Endogenous (untreated) genes in OM3 with expression ratio ≥2 and a *p*-value threshold <0.05.
(DOC)

Table S2 Genes in OM3 with expression ratio ≥2 and a *p*-value threshold <0.05 after H_2O_2 treatment.
(DOC)

Table S3 qRT-PCR primers used in this study.
(DOC)

Table S4 DNA microarray and qRT-PCR data comparison of ten selected genes in OM3.
(DOC)

Author Contributions

Conceived and designed the experiments: SB RJ. Performed the experiments: SB. Analyzed the data: SB RJ. Contributed reagents/materials/analysis tools: RJ. Wrote the paper: SB RJ.

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