Improving E.coli performance under stress by rewiring its global regulator Camp Receptor Protein (CRP)

Souvik Basak

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

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BY REWIRING ITS GLOBAL REGULATOR cAMP RECEPTOR

PROTEIN (CRP)

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SOUVIK BASAK

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SOUVIK BASAK

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Executive Summary

Strain engineering tools are usually adopted to improve microorganism tolerance towards stressful environments in bioindustries. However, classical strain engineering techniques of using UV/chemical mutagens are often both labor- and time- intensive. In recent years, transcriptional engineering approach has started to attract attention in strain engineering. The better efficiency of this method has made it more preferable over the classical methods. Here in this thesis, I would adopt error-prone PCR technique to engineer global transcription factor cAMP receptor protein (CRP), which can regulate more than 400 genes in *Escherichia coli*, to enhance its tolerance against organic solvents, oxidative stress, and low pH.

E. coli DH5 α - Δ *crp* strain was transformed with the plasmid-containing *crp* mutants, which were generated from error-prone PCR, followed by a selection under various stresses. All selected mutants exhibited much better tolerance than the wild type (WT) against respective stress. For example, the best toluene tolerant mutant showed growth even in 0.23% (v/v) toluene 0.51 h⁻¹ whereas the growth of WT was completely inhibited. The best oxidative stress mutant demonstrated viability in 12 mM H₂O₂, while WT growth was halted at 6 mM H₂O₂. The best mutant identified under various stresses were then subjected to various characterizations, including cross-tolerance check, DNA microarray analysis, quantitative real time PCR and enzyme assay. For instance, toluene-tolerant mutant was also able to have improved growth against *n*-hexane, cyclohexane and *p*-xylene while oxidative-stress-tolerant mutant also exhibits thermotolerance. DNA microarray analysis and qRT-PCR results have demonstrated that the modifications to CRP would not only bring differential expression in CRP-regulated genes but also those non-CRP-regulated genes. In conclusion, random mutagenesis of CRP can provide an efficient alternative for *E. coli* strain engineering.

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Chapter 1: Introduction

1. Introduction

Applications of *E. coli* to biocatalytic reactions are often limited by its tolerance to the surrounding stressful conditions that are often encountered inside bioreactors. Strain engineering of *E. coli* through the implementation of genotype-phenotype relationship has been a popular approach over the last few decades, to enhance cell phenotypes against such stresses. Classical strain engineering of *E. coli* has been achieved *via* two major strategies: i) the ‘rational’ method involves either exogenous gene incorporation or innate gene knock-out; ii) the second method is ‘random’ method based on the isolation of a good stress-tolerant trait from a pool of population constructed by randomly incorporating mutations in either a specific target gene or entire genome, using UV or chemical mutagens. However, rational approach guided strain engineering requires illustrative exploration of the metabolic space. Additionally, knocking out or over-expression of genes may lead to heavy metabolic burden over cells. The isolation of mutant through random approach may be time and labor-intensive due to the search for a good functional mutant from a wide range of randomly created variants.

As for random approach, the induction of mutations is often achieved by functionally improved strain evolution under sublethal dose of stress condition—mutagenic treatment such as UV, chemicals, or with genomic amplification *via* DNA polymerase enzymes without proof reading activity. The directed evolution tool subsequently converged with classical random approach by direct manipulation of a specific gene of interest that has eventually led to transcriptional engineering over the last few years. The manipulation of transcription factor reframes the entire genetic hierarchy of microbes thereby potentiating the probability of strain engineering.

Among many stress conditions encountered during *E. coli* involved biotransformation, some major stresses are organic solvent stresses, oxidative stress and stresses due to low pH condition. The goal of this thesis is to apply transcriptional engineering approach to enhance *E. coli* performance under these stresses. The target transcription factor is cyclic AMP Receptor Protein (CRP), the global transcription factor that regulates more than 400 genes in *E. coli*. CRP is reported as the widest controlling global transcription factor in *E. coli* but has remained unexplored for strain engineering.

This report is organized in 6 chapters. Chapter 2 is the background study and literature review covering strain engineering; its application against certain stress tolerances; an overview of global transcription regulators and CRP; studies about organic solvent tolerance, oxidative stress and low pH tolerance of *E. coli* and the objectives of this thesis. Chapter 3 is the detailed study about finding *E. coli* mutants against organic solvent stress through CRP engineering whereas chapter 4 and chapter 5 are on the improvement of *E. coli* tolerances against oxidative stress and low pH respectively. Chapter 6 is about the conclusion and possible future work that can be investigated to provide further insights into this study.

Chapter 2: Background

2. Background

2.1. Strain engineering

In strain engineering, bacterial genotype is often manipulated to bring in changes in their phenotypes. Engineering a particular microorganism can be accomplished by classical techniques such as ‘rational’ and ‘random’ methodologies. The rational engineering method requires intense knowledge about the working set of genes, their intracellular orchestration and functional characteristics of individual gene within the subset. Acquisition of randomly created variants has been achieved commonly by using mutagens such as UV, nitrosoguanidine (NTG), alkylating acridine mustard, alkyl methane sulphonate etc [1]. Directed evolution methods such as error-prone PCR [2] and DNA shuffling [3] have also been implemented as popular tools of random mutation by uncontrolled incorporation of mismatched nucleotides during amplification of the target nucleotide.

2.1.1. Rational approach

As mentioned earlier, the rational approach is based on preplanned modification of metabolic pathways thus requires sufficient knowledge about the genetic repertoire to be manipulated [4, 5]. The rational approach is adopted to enhance cell performance by switching on or off specific pathways. For example, the cloning of α -glucomethylloside-producing gene helped reduce acetate accumulation in bioreactors and subsequently enhanced the production of recombinant protein [6]. Metabolic pathway modification for biofuel production such as ethanol [7] and butanol [8] has been a popular approach over the last few decades. Rational engineering of microbes has also been implemented in other bioengineering sectors such as biosynthesis of aromatic amino acids from glucose or production of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP, a potential intermediate of aromatic biosynthesis pathway in *E. coli*) by over-expressing DAHP

synthetase [9]. Furthermore, usage of aspartate type of amino acids in food or nutritional supplement has been a popular strategy over the last few years [10]. Isoleucine, which is utilized as a nutritional supplement thereby, is also synthesized rationally in *Clostridium lactofermentum* with a 15 g/l titer [11]. In addition, to address the waste management and biodisposal problems in polyester industries, biodegradable polyesters are being manufactured (24 g/l) via modulating *E. coli* metabolic pathways [12]. Not only in bioprocesses, but application of metabolic engineering has also been extended to improve endogenous transcription profile in *E. coli*. As for example, introduction of exogenous *Vitreoscilla* haemoglobin has increased the transcriptional activity of *E. coli* oxygen regulated promoters thus improving the cell growth during cultivation [13].

2.1.2. Random approach

The random approach of strain engineering involves mutation in the genome or within a specific gene sequence to construct diverse variants enabling isolation of good functional trait/s from a plethora of possible mutants [14]. The point mutations are often induced randomly by various techniques such as spontaneous adaptation under stressful environment, treatment with physical or chemical mutagens. Over the past few years, a new method of random engineering has evolved and termed as combinatorial engineering which works on accumulation of beneficial point mutations created in form of a pool of variants.

2.1.2.1. Spontaneous adaptation

This method is based on allowing microorganism to create mutation by itself via adjusting to outside environment that has a mild dosage of stress over it. For example, the expression of heat shock protein hsp 22.4 from *Chaetomium globosum* in *Saccharomyces cerevisiae* (*S. cerevisiae*) enhanced the organism's thermotolerance after 4 hr adaptation to 51°C [15]. Again, exposure to repeated freeze thaw cycle is reported to increase the stress tolerance in

S.cerevisiae [16]. *Pichia stipitis* increased ethanol productivity and titer after sufficient incubation to partially neutralized or alkalinized hardwood hydrolysate [17]

2.1.2.2. UV and chemical mutagens

Short wavelength (180-380 nm) thereby high penetration power of UV makes it a potential tool for physical mutagenesis. DNA damage by intercalation of double strands through thymine-thymine dimer formation is the base mode of action of UV ray [18]. The point mutations over the DNA strands are generated during strand regeneration by error-prone repair method [19]. Chemical mutagens such as methyl methane sulphonate, ethyl methane sulphonate and nitrosoguanidine derivative N-methyl-N'-nitro-N-nitrosoguanidine (NTG) [20] also mutagenize cellular DNA *via* similar mechanism of DNA alkylation—damage is followed by random repair of affected strands. The potential problem of such mutagenesis is that, the mutation is highly non-specific and depends on the UV or chemical dose employed. Another problem is that it's time consuming and resource-oriented [21].

UV or chemical mutagenesis requires a large population of cells to confer genetic diversity to microorganism, the population could be around 10^8 - 10^9 cells per library [1, 22]. Moreover, NTG creates mutation by killing 40-60% population of cells [22], thus diminishing the chance of identifying good mutants [23]. Because of the non-specific lethal effect of mutagens over the entire genome, challenges remain in engineering complex phenotypes whose phenotypic output is the result of a cluster of interrelated gene functions [24]. In order to overcome the deleterious effect of random classical engineering, combinatorial engineering was designed to focus on a particular gene for first diversification, then the selection of good mutants with desired phenotype.

2.1.3. Combinatorial engineering

Since classical strain improvement often precludes simultaneous perturbations in functionally related network of genes and also generates deleterious or silent mutations due to uncontrolled approach [25], combinatorial strain engineering method has evolved as an alternative for strain engineering [26, 27]. Through different combinatorial approaches, starting from random over-expression and gene knockouts, a wide set of genetic cascades are unlocked through transposon mutagenesis, synthetic promoter libraries [28], ribosome engineering [29, 30], genome shuffling [31-33], exploiting artificial transcription factors [34] to DNA binding motifs and global transcription machinery engineering (gTME) [35].

2.1.3.1. Promoter Engineering

This approach basically involves creation of diverse promoters from the native cell genome either by shuffling the pool of the same or by random incorporation of nucleotides within the promoter itself. The chief objective of the promoter engineering is to outlay an optimal promoter assembly (or a single engineered promoter) in order to acquiesce maximum gene expression induced thereof [36, 37]. Applying the first methodology, Lu and Jeffries, 2007 organized a set of *E. coli* native promoters involved in xylose fermentation and innate respiratory pathway [28]. The authors reported that multiple-gene-promoter shuffling eventually elicited the optimal combination of promoters for ethanol production inside *E. coli*.

Jensen and Hammer undertook the second methodology for modification of the spacer sequence between -10 and -35 regions of the promoter and subsequently succeeded to achieve 400 fold activity upregulation of certain enzymes in *Lactococcus lactis* [37]. Promoter engineering, subsequently, has been also implemented in bioprocesses. It has been

published that error-prone PCR mediated randomization and selection of promoters resulted in optimum expression of *dxs* gene together with highest lycopene titer in *E. coli* [35].

2.1.3.2. Post transcriptional control

Together with the combinatorial strategy to regulate gene expression by promoter engineering, investigations have also been done on post transcriptional control of gene expression. Incorporation of tunable intergenic regions (TIGR) such as RNase cleavage sites or ribosome binding site (RBS) sequestering sequences has been reported to differentially influence gene transcription and translation [38]. This strategy has been successfully used to optimize flux in *E. coli* mevalonate pathway [35, 38].

2.1.3.3. Gene target specific combinatorial search

Although knockout and overexpression strategies require prior knowledge of the gene role in the metabolic landscape, combinatorial approaches have been developed for effective and unbiased manipulation of cell genotype. For example, an antisense DNA based random cDNA library has been used under GAL1 promoter for selective inhibition of gene expression [39]. Combinatorial gene knockout approach has been implemented in order to bring in the best phenotypic output in microbe. It has been demonstrated that combinatorial library construction and subsequent screening of 800,000 knockout clones led to selection of the best clone producing lycopene in *E. coli* [40]. In addition, combinatorial libraries have been used to selectively overexpress certain useful genes. As for example, sialylation of human erythropoietin has been improved in Chinese hamster ovary cells by overexpression of combinatorially engineered genes into it [41]. Ano et al 2009 used this technology to delete URA3 gene of sake yeast followed by combinatorial manipulation of the yeast genome. The produced *sah1-1/sah1Δ::URA3* strain yielded higher amounts of S-adenosylmethionine compared to wild type [42].

2.1.3.4. Genome shuffling

Genome shuffling after inducing several random point mutations is another strategy of combinatorial engineering [43]. The mutation points in parent genomes are generated by error-prone PCR, and the mutated genomes are crossed by DNA shuffling to yield chimeras. The technology has been applied successfully in *Lactobacillus rhamnosus* to improve its glucose tolerance with increased production of lactic acid [43]. Genome shuffling has also been successfully implemented to elevate acetic acid tolerance in *Candida krusei* [44]. Tolerance to organic solvents has been engineered too by genome shuffling. For example, ethanol tolerance has been efficiently improved in industrial yeast strain with increment in ethanol productivity [45].

Combinatorial approaches though rely on biological diversification to generate a broad range of genetic variants, often fail to perturbate a cascade of genetic network owing to single gene based sequential search optimization [35, 46]. Indeed, a spectrum of phenotypic characters can be attributed by single gene controlled functional organization and randomly incorporated chromosomal mutation often remains unsuccessful to re-orchestrate the diverse genomic space. Moreover, erratic or gene specific mutations often lead to local deconvolution of phenotypic space hence keeps short in reaching a global phenotypic maximum. Since transcription factor regulates important intracellular phenomenon such as the transcription and translation of a large span of genes, manipulation of transcription factor promises potential of unlocking wide functional genomic space as compared to other combinatorial methods [46].

2.1.3.5. Transcription engineering

Transcription engineering basically involves rewiring gene-expressional landscape by manipulating cellular transcription and translational machinery [47]. Since transcription

factors can regulate a plethora of gene expression, small modifications to these transcription factors may result in broad changes in cell genotype, its metabolic pathways and the components belonging to it [48]. Transcription engineering can be accomplished either by directly manipulating global transcription factors or by introducing artificial transcription factor to change the transcription profile of a subset of genes [46]. In addition, transcription engineering also can be achieved through selective inhibition of gene transcripts by silencing respective mRNAs [49, 50]. Individual strategies of transcription engineering have been described hereunder.

2.1.3.5.1. Modulating global transcription factors

In this methodology, transcription factors especially global regulators are targeted. For example, modifications to *E. coli* sigma factor (σ^{70} , which is a part of binding unit of RNA polymerase) has been undertaken to increase *E. coli* ethanol tolerance together with metabolite overproduction [46]. *SPT15*, a TATA binding protein can alter the binding pattern RNA polymerase [51] Hence manipulation of this regulator has been successfully implemented to improve yeast ethanol tolerance [52]. *rpoA*, a sigma factor encoded in *E. coli*, has also been engineered to enhance its butanol tolerance as well as tyrosine production [53].

In recent studies, scientists have suggested the role of global regulators in controlling biofilm dispersal. It has been reported that engineering global transcription factors such as Hha, H-NS, c-di-GMP binding protein could significantly potentiate biofilm dispersal [54-57]. Pursuing transcription engineering, Chen et al. cloned exogenous global regulator IrrE from *Deinococcus radiodurans* into *E. coli* and showed that manipulating IrrE could improve cell phenotype against ethanol, butanol and acetate [58]. This strategy has also been named as global transcription machinery engineering (gTME) [46].

2.1.3.5.2. Engineering artificial transcription factors

Artificial transcription factor with zinc fingers is another target of transcription engineering. Zinc fingers are binding parts of some transcription regulators and associated factors. Hence fabrication in zinc fingers could reframe the binding domain of the concerned transcription factor, resulting in the alteration of DNA activation [59, 60]. Strain engineering can be achieved by the introduction of artificial zinc finger combined with suitable activators or repressors. This strategy has been successfully implemented in elevating the stress tolerance of *S. cerevisiae* against heat, osmotic pressure and antifungal drug ketoconazole [61] .

2.1.3.5.3. Ribosome engineering

Apart from mutations in the transcription factor itself, sh-RNA and miRNA based gene silencing and subsequently inhibiting gene transcription has also been investigated as a tool of transcription engineering. [62]. Besides gene based silencing, enzyme based repression of transcription factor has emerged as a tool of transcription engineering. For example, engineered ribozymes are often targeted to deregulate the mRNA transcript of several transcription factors either to nullify the translation of detrimental proteins or to study the functional character of specific transcriptional regulator [49, 50]. In recent years, extensive randomization has been performed to generate a library of specific binding ribozymes which has been subsequently utilized at transcription level to investigate the differentiation of a set of neuronal cells [63].

2.1.4. Error-prone PCR

Directed evolution methods, including error-prone PCR and DNA shuffling, are random mutagenesis techniques utilized extensively to introduce mutations into target genes. Error-prone PCR is the modification of standard PCR leading to DNA amplification with one or

several nucleotide misincorporation/s. The modification often involves high percentage of MgCl_2 [64]. Sometimes MnCl_2 addition [65] or artificially created nucleotide imbalance in the reaction system [66] can also induce mutation during error-prone PCR. *Taq* DNA polymerases are the most commonly employed enzymes in error-prone PCR attributed to their low fidelity and high error rate [67]. dITP has also been used in some error-prone PCR protocol to create mutated DNA [68].

Error-prone PCR is an established tool in directed evolution to engineer both proteins and strains. In protein engineering, this tool is often applied to increase both enzyme activity and stability together with the specificity of proteins. For example, *Penicillium janczewskii* *zalesk* α -galactosidase expression and activity in *Pichia pastoris* has been improved by error-prone PCR [69]. Again, activity enhancement of an endochitinase has been achieved through error-prone PCR mediated directed evolution exploiting cDNA of *Trichoderma viride* as template [70]. Protein specificity is also greatly improved by exploitation of this technique. The lectin binding specificity for 6-sulpho-galactose, which is a facile determinant of glioblastoma, has been augmented through error-prone PCR [71]. This directed evolution technique has also been implemented to increase protein stabilities such as thermostability of *Orpinomyces* sp. xylanase [72] and acid stability of *Bacillus licheniformis* α -amylase [73].

In recent years, this technique has been adopted for mechanistic studies to deconvolute either protein's mode of action or its active domain. As instance, random mutagenesis by Craig et al. has revealed that β -galactosidase activity variations in *E. coli* do not depend on sequence specific errors in the catalytic sites of the enzyme [74]. Again, error-prone PCR mediated random mutagenesis has also uncovered the functional domain of the bacterial transcription factor TraJ [75].

In a last few years, error-prone PCR has been undertaken to convert protein engineering into strain engineering. For example, *E. coli* transcription factors have been engineered by this technique to improve both bacterial tolerance as well as secondary metabolite production as reviewed in earlier sections. Recently, our group has also isolated 1-butanol and osmotolerant *E. coli* mutants through error-prone PCR mediated library creation and selection [76, 77].

2.2. Global transcription factors

Gottesman in 1984 defined global regulator as the regulon regulating more than one subject operon and the operons should represent genes for multiple metabolic pathways [78]. The global transcription factors can sense changes of specific metabolites and exert their action by either activation or repression of their target genes. For instance, CRP would sense changes in intracellular concentration of cAMP through formation of the cAMP-CRP complex that would affect target genes. A global transcription factor is able to regulate genes of different functional groups, as presented in Fig. 2.1 below [79].

Fig. 2.1: Number of global environmental conditions that can be regulated by each global transcription factor in *E. coli*. (Reprinted with permission from Ref. [80], Copyright 2003, Elsevier)

According to a recent report published on Ecocyc database, there are 175 transcription factors in *E. coli* [63]. One hundred and one of them are involved in either the catabolism of carbohydrates and amino acids or intracellular transport. Seven of them are global transcription regulators namely ArcA, CRP, FIS, FNR, HNS, IHF, Lrp, rest are local regulators [81].

As mentioned earlier, CRP is a very important global transcription factors as it is able to regulate more than 400 genes [82]. CRP is mainly responsible for sensing the energy level of *E. coli* and the catabolism of carbon sources besides glucose [83, 84]. The presence of glucose would inhibit the catabolism of alternative carbon sources in *E. coli*, presumably due to lowered concentration of cAMP that is required to induce CRP. In addition, CRP, acting as a dual regulator, is also essential in the regulation of genes involved in energy production, amino acid metabolism, nucleotide metabolism and ion transport systems. Furthermore, CRP

can exert indirect control on other genes through regulating other transcription factors such as Fis, MelR, RpoH and PdhR [84].

Based on sequence homology, it was found that the consensus sequence for FNR is actually similar to that of CRP, and thus FNR is considered a homologue of CRP [85]. Previous studies on FNR had also revealed that FNR and CRP activate the initiation of transcription in a similar manner. However, although FNR shows some resemblance to CRP, FNR is more important in the regulation of genes during anaerobic growth [84]. This is due to the involvement of FNR in sensing the redox potential balance between oxygen and alternative electron acceptors such as nitrate and fumarate. Like FNR, ArcA is also responsible for energy production *via* regulation of the respiratory modes. Both ArcA and NarL are important co-regulators of FNR in the regulation of energy production related genes during anaerobic growth [83]. Among the other global regulators, Lrp contributes in *E. coli* nitrogen assimilation while IHF and FIS regulate DNA topology during binding to regulatory elements [65].

It has been reported earlier that CRP is the most wide controlling global transcriptional regulator in *E. coli* and can harmonize other global regulators (Fig. 2.2) [86]. Matinez-Antonio et al 2008 reported that CRP can regulate 413 genes i.e. almost 10% of the whole *E. coli* genome [87]. Except RpoD, the housekeeping sigma factor of *E. coli*, no other global regulator could regulate more genes than CRP. The authors also suggested that CRP is the master regulator of the cell as it can sense the energy status of the cell thus activating relevant transcriptional machinery to regulate the intracellular energy. Furthermore, CRP can co-act with other global and local regulators such as FNR, FIS, MelR, CytR, MetY, AnsB [80] to exert regulation on specific promoters. Thus CRP has more perturbation in the functional genomic space hence sharing a broader transcriptional network (TRN). As mentioned earlier [86], CRP shares the topmost layer of *E. coli* TRN and orchestrate other regulators directly

or indirectly to programme the whole cell transcriptome. Thus if CRP is modified or mutated, there is higher chance to re-cascade the cell transcriptional profile by re-defining its interaction with specific promoters, other transcription factors and its binding proteins. Also, CRP being an intracellular energy sensor, it holds plausible rationality to exploit CRP for combating cell stress, as cellular metabolism is often hampered under stressful condition to re-switch energy production cycles. Hence considering extent of regulation of CRP compared to other global and local transcription factors, we aim to manipulate *crp* of *E. coli* DH5 α to enhance cell performance under various stresses.

Fig. 2.2: Transcriptional hierarchy in *E. coli* including the global regulators. (Reprinted with permission from Ref [86], Copyright 2004, Oxford University Press)

2.2.1. Global Transcription Factor CRP

2.2.1.1. cAMP

cAMP (Fig. 2.3) or 3'-5'-cyclic adenosine monophosphate is a second messenger involved in many biological processes. cAMP is involved in signal transduction of many cellular responses *via* the activation of protein kinases. cAMP is synthesized from ATP (adenosine triphosphate) by the enzyme adenylyl cyclase.

A

B

Fig. 2.3: Molecular structure of Cyclic AMP; A. Two dimensional structure B. Three dimensional structure (http://en.wikipedia.org/wiki/Cyclic_adenosine_monophosphate)

2.2.1.2. CRP structure

CRP is a global regulator in *E. coli* and activates more than 100 promoters (Fig. 2.4). The CRP molecule is activated by the allosteric binding of cAMP to the specific sites and thus enables the RNA polymerase holozyeme to bind with certain promoters[88]. CRP was the first transcription factor to be isolated [89], its structure was subsequently analyzed, and the transcriptional activation was subjected to extensive biophysical, biochemical and genetic investigations.

Fig. 2.4: 3-D structure of Catabolite Activator Protein (PDB, 1G6N)

CRP is a homodimeric protein, with each monomer comprising of two domains—the cAMP binding N-terminal domain contains 1-137 residues whereas the C-terminal domain contains residues from 138-209 [90]. The gap between these two domains is known as the hinge region (L134-D138). The β -stranded N-terminal domain (NTD) is known for cAMP binding and CRP dimerization. The C-terminal domain is the DNA binding domain. CRP can recognize specific DNA binding site *via* its C-terminal F-helix (α F) that takes the shape of helix-turn-helix together with the neighbouring E-helix (α E). These two α -helices are thought to provide the major interaction sites of CRP with the DNA [91]. There is a cleft between the two domains inside CRP [90]. The cleft is present inside the CRP molecule in the closed form.

2.2.1.3. RNA polymerase

The RNA polymerase (RNAP) has a molecular mass 450 kDa and composed of subunits $\alpha_2\beta\beta'\sigma$. The α subunit is responsible for the recognition of the upstream promoter (UP) element, response for various activators, repressors, elongation factors etc [88]. The α -subunit comprises of two folded domains, α -NTD and α -CTD. The α -NTD governs the dimerization

of α domain and determines its interaction with the remainder of the RNA polymerase. The α -CTD of the RNAP determines the interaction of the UP element of DNA with the RNAP *via* specific and non-specific interactions [92]. There is a linker region between the two domains inside CRP about 13 amino acid long. This region is flexible and unstructured. The relative distance between α -CTD and α -NTD can vary leading to flexible binding of α -CTD to different transcription complexes.

The β (151 kDa) and β' (155 kDa) subunits are responsible for the catalytic activity of RNAP and are capable of interacting with other subset of activators, repressors, elongation factors and termination factors [88, 93].

The σ subunit is actually σ^{70} subunit responsible for the recognition of consensus sequences at -35 element and -10 element of a promoter, binding to that region and thus responding to activators [94].

2.2.1.4. CRP-dependent promoters

CRP-dependent promoters could be classified as Class I, Class II and Class III CRP dependent promoters [95].

- i. The Class I CRP-dependent promoter requires only CRP for transcription activation and only a single DNA binding site at the upstream of the DNA site for RNAP [96] as depicted in Fig. 2.5 .

Fig. 2.5: CRP and RNAP binding with Class I type CRP dependent

promoters. (Reprinted with permission from Ref. [88], Copyright: 1999, Elsevier)

- ii. The class II CRP-dependent promoter also requires only one CRP site for transcription initiation with a lone DNA binding site overlapping the binding region for RNA polymerase, altering the promoter at -35 element [96] as depicted in Fig. 2.6.

Fig. 2.6: CRP and RNAP binding with Class II type CRP dependent

promoters. (Reprinted with permission from Ref. [88], Copyright 1999, Elsevier)

- iii. The class III CRP-dependent promoter depends on multiple activator molecules for full transcription activation [97]. The multiple activator molecules include one

or more CRP molecules and some other regulon specific activator molecule as depicted in Fig. 2.7.

Fig. 2.7: CRP and RNAP binding with Class III type CRP dependent promoters. (Reprinted with permission from Ref. [88], Copyright 1999, Elsevier)

2.2.1.5. cAMP-CRP Binding Mechanism

Two regions of CRP are reported to be most important for binding with cAMP. The amino acids G71 and E72 of CRP form the bonds with the sugar portion of cAMP whereas T127 and S128 binds with the adenine base of the cAMP [90]. The cAMP-CRP binding occurs *via* two types of conformations *syn*-conformation and *anti*-conformation [90]. Among these two conformations only the *anti*-conformation is responsible for the global allosteric changes in CRP binding. Another model of explaining cAMP-CRP binding stated that this binding complex had two types of conformations, active and inactive [91]. The active conformation is responsible for the cAMP binding, higher affinity for DNA and susceptible for disulphide cross linking and proteolytic hydrolysis by enzymes.

2.2.1.6. DNA Binding Mechanism

X-ray crystallographic data of CRP-(cAMP)₂-DNA complex has been resolved at 3.0 Å resolution where CRP-(cAMP)₂ complex has been bound to a 30 bp fragment. Within the 30 bp fragment, the actual target DNA recognition and binding site for cAMP-CRP complex consists of 22 bp [98]. The 22 bp recognition site contains the consensus sequence 5' aaaTGTGAtctagaTCACAtt 3' bearing an inverted repeat of 11 bp [98, 99]. Though the major interaction occurs *via* C-terminal domain, the αF helix in the N-terminal domain is parallel to the base pairs and penetrates through the major groove of the DNA. The majority of the interactions between the protein and the DNA occur *via* weaker interactions of the “Phosphate backbone” (K26, K166, R169, Q170, S179, T 182, H199) [100]. Some of these interactions do not form by the amino acid bonding; rather they occur by hydrogen bonding between the two amino acids (169 NH, 170 NH, and 179 NH) [100]. Only three amino acids make specific contacts with the base pairs; they are R180, E181, and R185. The CRP after binding bends the DNA at an angle 80° reframing DNA structure wrapped over and out of CRP [88]. DNA downstream position 6 and 7 makes an angle of 40° between themselves where a slight bend is cited by the authors between position -1 and 1.

2.2.1.7. Mechanism of transcription initiation by cAMP-CRP complex

RNA polymerase mediated bacterial transcription initiates through recognition of the consensus sequences at -10 and -35 positions of the DNA promoter [101, 102]. The optimum promoter configuration involves 17 bp spacing between the two loci, although 20 bp spacing might retain some partial activity of RNA polymerase [91]. First, apo-CRP (CRP without any activator) is inactive and attains active conformation only upon two cAMP molecules binding with it [90]. Ample amount of research has been carried out on the conformation of cAMP binding with CRP. In outcome, it has been proposed that optimum CRP activation is achieved by anti-conformational combination of cAMP with CRP [100, 103]. The anti-combined CRP undergoes allosteric modulation and in turn, attaches with the effector DNA through the 22

bp recognition sequence as stated earlier [99]. The cAMP-CRP-DNA complex acquires a specific bending topography rendering RNA polymerase access its binding site and thus initiate transcription [100].

With all the strategies of strain engineering, we tried to improve *E. coli* strain tolerance against certain stresses such as organic solvent, oxidative stress and low pH. Since these stresses are often encountered in bioreactors during biocatalysis, we reviewed some previous background approaches to endow microbes with tolerances against these stresses.

2.3. Organic solvent tolerance

One of the common stresses that are often encountered during microbial biocatalysis in bioreactors is organic solvent stress. It disrupts cell membrane by dissolving the low polarity membrane bound lipids, leading to change of fatty acid isomerization [104, 105], or causing ion leakage [106, 107]. This phenomenon ultimately ceases the cell growth which is aggravated at higher temperature and also is influenced by feedstock generated inhibitors [108]. The phenomenon has been a potentially challenging problem in industrial microbiology.

The first strain isolated from a culture containing organic solvents was *Pseudomonas putida* strain IH-2000 which grew in the presence of 50% v/v toluene [109]. Hereafter six toluene resistant mutants of *Pseudomonas putida* KT2442TOL were generated by transposon mutagenesis which employed mutation of the membrane efflux pump transporting the organic solvent out of the cell [110].

Engineering bacterial strain against organic solvent was evaluated as mathematical correlation between the physicochemical properties of solvents and the lipophilicity of bacterial membrane. This has revealed that bacteria can grow in the presence of an organic

solvent whose log P_{ow} (octanol-water partition coefficient) is equal or greater than a certain value called index value of the solvent [111].

Study of organic solvent tolerance has been performed on many microorganisms like *Pseudomonas putida*, *Pseudomonas fluorescens*, *Achromobacter delicatulus*, *Alcaligenes faecalis*, *Agrobacterium tumefaciens*, *Bacillus subtilis* [112]. Among these, almost all the microorganisms can tolerate higher alkanes like octane, nonane, decane, dodecane etc. with ether compounds such as n-Hexyl ether because these organic solvents have high octanol-water partition coefficients greater than the index value of any of the microbes stated above. But organic solvents like n-Hexane, cyclohexane, propyl benzene, ethylbenzene, benzene, toluene, *p*-xylene are selectively tolerated by the microbes. It has been proposed that the *E. coli* strains having low surface hydrophobicity have more organic solvent tolerance [111, 112].

Engineering strain like yeast in the TATA binding protein coded by *SPT15* increases the yeast tolerance against high glucose and ethanol concentrations [51]. This technique has also been applied for engineering ethanol tolerant *E. coli* and [52] butanol tolerant *C. acetobutylicum* [113]. Gene knockout also has been implemented to engineer an ethanol tolerant *S. cerevisiae* strain [114].

2.3.1. Industrial feasibility

E. coli has been a popular choice of microbe in industrial biocatalysis. But the application of *E. coli* as industrial biocatalyst is limited due to its poor tolerance to various organic solvents such as toluene. The enhancement of *E. coli* tolerance against toluene has several advantages as following:

- Microbes can be used to catalyze the conversion of several non-polar compounds such as steroids, cholesterol that are poorly soluble in water and provide higher biocatalytic rates only when solubilized in non-aqueous phase [115]. *Pseudomonas putida* has been used in biocatalysis as organic solvent tolerant microbe but the low protein fraction and thus low reaction rate limits the use of this microbe in practice [116]. Toluene is a highly non-polar solvent capable of dissolving various hydrophobic substrates. Hence *E. coli* strains having tolerance against toluene can be efficiently used in this biphasic biocatalytic process.
- Non-aqueous enzymology has been popular in industrial biocatalysis because of added advantages of using solvent tolerant enzymes in catalytic conversion of hydrophobic substrates. The enzyme has an altered stereo and regio- specificity in presence of the organic solvent [117] and thus can be used to produce optically active intermediates and various food products [118]. By construction of toluene tolerant strain of *E. coli*, suitable toluene tolerant enzyme can be isolated from the microbial cell that can be further used in this bioconversion reaction.
- Another advantage of toluene tolerant *E. coli* strain can be to detoxify the fermentation medium by extracting toxic hydrocarbon metabolites from the intracellular fluid of the microbe. For example, epoxide and diepoxide containing metabolites can be effectively extracted from the bacterial cell by using a highly apolar solvent such as toluene ($\log P_{ow}$ 2.6) [115]. Hence performing the biocatalysis in presence of toluene with the mutant *E. coli* strain can be an effective tool for this kind of biocatalytic process.
- Lastly, mutant *E. coli* strain can be used for bioremediation in different contaminated sites containing toluene.

2.3.2. Methods to improve toluene tolerance of *E. coli*

The toluene resistance study of *E. coli* started from 1965 when Jackson and DeMoss studied the effect of toluene and found that toluene addition in the range from 1.5 to 10 µl/ml of culture can alter the growth pattern of *E. coli* quite significantly [119]. Toluene treatment incurred loss of cellular protein and 25% and 85% of cellular RNA which was not dependent on the toluene concentration only, but also on incubation temperature of the cells.

Since toluene can damage cell wall, researchers have been using toluene to make holes in cell wall to transport biomolecules inside cells [120-123]. Toluene is toxic for most of the bacteria except *Pseudomonas putida* that can tolerate toluene and grow in toluene liquid culture. Toluene has a log P_{ow} 2.6, below the index value of *E. coli* 3.7, and *E. coli* cannot survive in an organic solvent having its partition coefficient lower than its index value [105].

To counter the binding and subsequent disruption by organic solvents to the *E. coli* cell membrane, investigation has begun for the enhancement of bacterial tolerance against such solvent stress, including increase in chain length of the fatty acids, increase in proportion of the saturated fatty acids, increase in the amount of trans-unsaturated fatty acids, deletion of the OprF porin channel, modification of the lipopolysaccharides, and efflux system for the hydrophobic compounds were employed to remodel the strain against such solvent stress. Studying effect of toluene, *p*-xylene, cyclohexane, cyclooctane and *n*-nonane on different *E. coli* mutants, it was found that among all the solvents, toluene binds cell wall most abundantly (0.053-0.16 micromoles of solvent per milligram of cellular protein) [111]. So it seems that toluene damages and disrupts the cell wall more than any other organic solvent undertaken for the study.

Generation of *E. coli* mutants has been undertaken by strain engineering but found survival of none of them against toluene in solid culture selection method. NTG induced mutation has been tried in *E. coli* JA 300 cells in this objective which led to the evolution of one mutant

that survived 10% v/v xylene but died in toluene [124]. Only solvents like *n*-hexane or *p*-xylene whose logP_{ow} value is greater than the Index value of the organism allowed the growth of the cells. Some *E. coli* strains like OST 3101 and OST 3121 could survive in the solvents like cyclohexane, *n*-pentane and *p*-xylene but could not grow in toluene at all. This indicated that toluene is very toxic for the *E. coli* microorganism [124].

Study about the genome responsible for the organic solvent tolerance in *E. coli* was subsequently started. *soxRS* genes in the *E. coli* genome was found to increase the bacterial tolerance against organic solvent like cyclohexane. But this resistance of *E. coli* limited by the degree of expression of the gene inside the cell. When the gene was cloned in a high copy vector, the bacteria exhibited solvent tolerance. But when the bacteria harbored the gene in a low copy plasmid, there was no organic solvent tolerance shown by it [125].

Mutation in *ahpC* gene which encodes alkyl hydroperoxidase enzyme in *E. coli* was another combinatorial method utilized to increase organic solvent tolerance of *E. coli*. This enzyme converts organic solvent like tetralin to tetralin hydroperoxide and transforms many other organic solvents to their corresponding hydroperoxide metabolites. Screening of the *ahpC*F mutant library by solid medium overlay method exhibited that there was no growth of control as well as mutated *E. coli* against toluene [126].

E. coli JA300 cells were investigated by inducing mutation with organic solvent overlaid solid LBGMg-agar medium method which successfully tolerated cyclohexane and *n*-pentane. The mutants also can be adopted to tolerate *p*-xylene in the solid agar medium and if treated suitably with NTG chemical, can grow in the medium containing 10% *p*-xylene upto 10⁷ cells/ml. However, the mutant did not show any growth in presence of toluene in solid culture overlay method implying the mutation was not enough to induce tolerance against toluene in *E. coli* JA300 cells. Six genes known as organic solvent tolerant genes (*ost*) are involved in

the organic solvent tolerance of *E. coli* named *acrA* and *B*, *tolC*, *marA*, *soxS* and *robA*. Among these, the *marA*, *soxS* and *robA* genes only induce the organic solvent tolerance if transformed into the cell with high copy vector and thus activating the AcrAB-TolC efflux pump [112].

TolC protein in the outer membrane of *E. coli* has been estimated to be most important for tolerance against solvent like cyclohexane and that, in turn, is positively regulated by three other genes named *marA*, *rob* and *soxS* [127].

Expression of genes like *man X* and *man Y* facilitates adherence of the hydrocarbon to the bacterial cell wall. By expression of these genes, *E. coli* can bind upto 10% toluene to its cell wall. The phenomenon exhibits that bacterial tolerance to the hydrocarbons increases due to the expression of *man X* and *man Y* gene [128].

Strain engineering involving recombinant DNA technology to increase the organic solvent tolerance in *E. coli* has also been performed. It involved cloning of an organic solvent tolerant gene *ostA* from an *n*-hexane resistant strain of *E. coli* JA300 and recombinant plasmids to transform into the non-resistant strain OST4251. The results exhibited that the cells engineered like this, showed more organic solvent tolerance by dense growth of the cells at the site of inoculation [129]. This type of strain engineering of bacteria is dependent on both the promoter activity as well as the copy number of the plasmid used [130].

Recombinant DNA method has been adopted for the production of the *cis*-glycol by cloning toluene dioxygenase gene from *Pseudomonas putida* into *E. coli*. Efficiency of engineered *E. coli* strain towards biocatalysis did not depend only on the manipulation in the gene level, but also on the strain itself. Supporting to this, the metabolite formation increased from 0.25 g/l to 0.41 g/l by switching the strain from JM105 to TG2 [116]. Following this, effect of membrane lipoprotein (Braun's lipoprotein) started drawing attention as a second tool of

strain engineering. As an outcome, the activity of the enzyme Toluene dioxygenase (TDO) inside the *E. coli* cell was also investigated against the control of the membrane lipoprotein Braun's lipoprotein (Fig. 2.8) [131].

Fig. 2.8: Chemical pathway of toluene metabolism by Toluene dioxygenase [131]

Comparative study was carried on the biocatalytic rate between the control harboring the cloned plasmid and a mutant one where Braun's lipoprotein has been mutated by transposon mutagenesis (Tn10 insertion) [131]. The mutant exhibited improved (1.5-3.9 fold) enhanced reaction rate for the toluene metabolism compared to control. Membrane disruption by EDTA was compared with the mutation in Braun's lipoprotein. Furthermore, mutation in Braun's lipoprotein is found to be superior to EDTA treatment (greater than 2 fold) in enhancing the biocatalytic reaction concluding mutation is a key step in strain engineering process. Kinetically, EDTA concentration more than 0.5 mM caused a decrease in the TDO activities.

2.4. Oxidative stress

Oxidative stress is the effect produced by accumulation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) [132]. Generally, these species are produced as the byproducts of intracellular aerobic respiration [133, 134]. The common ROS responsible for cell damage are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\cdot$) (ref) [135]. The ROS initiate cell necrosis by destroying cell membrane, damaging

biological macromolecules such as lipids, proteins and DNA [132]. The *in vivo* produced hydrogen peroxide, in turn, reacts with intracellular transition metals thus yields hydroxyl radicals [136].

2.4.1. Application of oxidative stress tolerant mutants in bioreactors

Oxidative stress is a common burden over the cells when subjected to cultivation under a multitude of conditions. Especially, submerged culture of microbes often requires intense aeration inside fermentor, that in turn, results in oxidative stress [137]. Earlier, Gregory and Fridovich have reported that growth of *Streptococcus faecalis* grown under 20 atm of O₂ and *Escherichia coli* grown under 5 atm of O₂ could induce superoxide dismutase (SOD) 16 times and 25 times more than that of the endogeneous level of the enzyme respectively [138]. This indicates that molecular oxygen itself can induce oxidative stress in microbes. Further, it has been acknowledged that enrichment of 50% oxygen (v/v) in culture condition of *E. coli* could upregulate SOD level which is often encountered during shifting of *E. coli* culture from shake flask to bioreactors [139]. It has also been shown that exposure to oxygen could lead to activation of cellular antioxidant defense machinery [140] thus signifying the role of superincumbent oxygen induced oxidative stress. Recombinant protein production in *E. coli* is also limited by the oxidative stress, induced by partial pressure of oxygen inside the bioreactor [141].

Production of biofuel in reactors could also be retarded due to oxidative stress of the microbes often originating from the solvent toxicity itself. Earlier publication suggests that 6.4 g/L of *n*-butanol or 8g/L of isobutanol could attenuate *E. coli* cell growth due to solvent lethality, oxidative stress being one of its principle mediators [142].

2.4.2. General oxidative stress response of *E. coli*

In order to address this genre of problems, improved *E. coli* phenotype against oxidative stress is under search through decades. The prototype response of *E. coli* against oxidative stress is the induction of antioxidant enzymes involved in ROS scavenging and DNA repair [143], which is *via* global transcriptional activation of redox-sensing regulators SoxR and OxyR [144]. Actually, *E. coli* has two endogeneous defense mechanisms to combat oxidative stress: those that ameliorate the superincumbent stress by direct protection against it and those that repair the cellular DNA affected by the stress. The former involves a series of antioxidant proteins or enzymes [145]. The latter involves intracellular enzymes, known as recombinases that reconstruct cellular DNA by recombination.

The antioxidant enzymes, in general, are controlled by two major regulons, OxyR [135, 144, 146] and SoxRS [133, 144, 147]. Usually, OxyR regulon encounters the stress induced by hydrogen peroxide together with cumene hydroperoxide and ethanol [148, 149]. Chief antioxidant enzymes contributing in cellular defense under OxyR control are catalases, peroxidases and glutathione reductase [144]. The SoxRS regulon primarily regulates the response due to superoxide anion which is one of the free radicals responsible for cellular oxidative damage [150]. The recombinase enzymes responsible for DNA repair, is controlled by the cellular SOS response [151, 152].

In addition, oxidative stress in *E. coli* also induces chaperone such as Hsp33 to protect the plentiful of cellular proteins from stress generated shock [153]. Furthermore, heat shock proteins such as DnaK, GroEL, GroES also contribute in shielding the biologically important proteins from oxidative shock to refold in their proper conformation [153, 154].

Earlier publications suggest that other stress response mechanism inside microbial cell may overlap with the defense mechanism against oxidative stress [155-157]. Those stress response mechanisms include thermal stress, osmotic stress, starvation, acid tolerance and antibiotic

resistance. The overlap of these mechanisms suggests that the stress responsive proteins inside cells may contribute in multiple defense machineries for stress tolerances.

Three principal regulators inside *E. coli* control the oxidative stress namely RpoS, SoxRS and OxyR. There are two kinds of catalases inside *E. coli* cells HP-I (encoded by *katG*) and HP-II (encoded by *katE*), among which HP-II catalase is regulated by RpoS [158, 159]. DNA repair and protection is also controlled by RpoS. In fact, RpoS and OxyR share a significant interlinkage because σ^{70} and σ^{38} guided regulation of them share a common origination from RNA polymerase [160, 161].

2.4.3. SoxRS regulon

The SoxRS regulon in *E. coli* orchestrate the expression of *sodA* gene in which encodes the enzyme superoxide dismutase. Superoxide dismutase (SOD) catalyzes the dismutation reaction to convert intracellular superoxide (O_2^-) to hydrogen peroxide and water:

$2 O_2^- + 2 H^+ \rightarrow H_2O_2 + 2 H_2O$ [162]. Three kinds of SOD are present in *E. coli*, manganese containing superoxide dismutase (MnSOD) [163], iron containing superoxide dismutase (FeSOD) [164] and copper-zinc containing superoxide dismutase (CuZnSOD) [165]. SODs are endogeneously produced within bacterial cell as a first line defense mechanism against oxidative stress. CuZnSOD is abundant in the periplasmic area of the cell whereas MnSOD and FeSOD exist in the cytosol and are often working coherently. MnSOD contributes in dismutation of lethal oxygen radical produced in stress-induced cells such as after heat shock and often combines with iron metabolism for complete effect [136]. MnSODs remains active even after treatment with H_2O_2 whereas other SODs get inactivated [166]. SODs also enable cell to survive in the stationary phase as well as under thermal stress [167]. In anaerobic growth condition also, toxic oxygen radical may damage the bacterial cell where FeSOD plays a protective role against the ROS [158].

The *soxRS* operon comprises of two genes *soxR* and *soxS* [168] interplaying together for the target regulation. The proteins SoxR and SoxS regulate gene transcription by binding with DNA. Usually SoxR activates SoxS in the process, which takes part in transcriptional regulation of the associated gene. Ten genes have been reported so far to be transcriptionally regulated by SoxRS [144].

Superoxide dismutase protects bacterial cell against oxidative stress that can originate from any other stress such as acid shock, organic solvent, thermal stress or antibiotics. The protection increases in the stationary phase due to elevated level of the enzyme resulting in from the dual regulation of SoxRS and RpoS [160, 169].

2.4.4. OxyR regulon

OxyR regulon of *E. coli* mainly confers resistance to the cell against oxidative stress due to hydrogen peroxide. Hydrogen peroxide induces about thirty proteins [132, 135] which include enzymes such as catalase (from *katG* and *katE*), glutathione reductase (from *gor*) and alkyl hydroperoxide reductase (*ahpCF*) [146, 170]. OxyR regulates resistance against hydrogen peroxide through HPI type of catalase which is encoded from *katG*. The response mechanism is complex and often, occurs *via* encoding HP II catalase translated from *katE*. Interestingly, *katE* is not governed by OxyR but in turn, is regulated by RpoS [159].

2.4.5. Strategies undertaken for tolerance improvement

Traditional approaches have been adopted to construct mutant *E. coli* strain *via* spontaneous adaptation [171] and cloning of exogenous antioxidant gene [172, 173]. 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 [174]. Previous publications have also illustrated that starvation induces oxidative stress resistance in *E. coli* [175]. However, global

transcriptomic approach to reveal maximum peroxide tolerance is lacking. In this study, we have engineered CRP to reprogramme the transcriptional network thereby modifying the cell oxidative stress tolerance.

2.5. Enhanced cell growth at low pH

One of the major obstacles in the industrial application of *E. coli* K-12 is its survival in the low pH condition. The acid tolerance of *E. coli* determines productivity of organic acids in industrial scale such as lactic acid and succinic acid. The survival of *E. coli* in low pH is reported to depend upon externally added components such as glucose whereas the acid tolerance response (ATR) is found to be independent of transcriptional regulators such as IHF, H-NS, Lrp etc. as discussed in details below.

2.5.1. Industrial feasibility of acid tolerance of *E. coli*

Acid tolerance of *E. coli* has been a marked importance in industrial biocatalysis. Acid mediated fermentation has been employed in the bioreactor to produce lactic acid by recombination of the enzyme lactate dehydrogenase (LDH) [176]. The problem in this method is the downfall of yield of lactic acid with the increase of acidity in the medium. The productivity of lactic acid is 50-75 g/l under neutral pH (pH=7.0) but falls down to 10-20 g/l when the media turns acidic due to the production of acid [177]. Choice of host strain is also a variable parameter for the productivity of lactic acid in which *E. coli* K-12 serves in the lower range. Industrial productions of succinic acid [178] and 3-Hydroxy propionic acid [179] by *E. coli* biocatalysis are also limited due to the appreciable decrease in the yield when the pH of the medium gets lowered by continuous production of the acids. Hence an acid tolerant strain of *E. coli* with higher titer value for the products is still under search.

2.5.2. Strain engineering of *E. coli* against low pH

As stated earlier, shortcomings of using *E. coli* strain in industrial biocatalysis led to intensive search for the improved strain against low pH. Various strain enhancement methodologies have been adopted to maneuver the cell tolerance against acid stress and hence isolate better stress performing mutants. Majority of the strategies investigated thereof, exploited classical strain engineering approaches.

2.5.2.1. Rational approach

Rational approach involving alteration of the metabolic pathways by cloning exogenous gene has been a popular strategy to increase *E. coli* acid tolerance. For example, heterologous expressions of *orfB* and *orfC* (encoding a small heat shock protein Lo18) genes from acidophilic bacterium *Oenococcus oeni*, elevated tolerance response of *E. coli* at low pH [180]. Again, exogenous cloning of *dnaK* (encoding heat shock protein DnaK) from *E. coli* into *Lactococcus lactis* improved strain performance against acidic environment and thus increased the lactic acid titre up to 2 g/L from less than 0.3 g/L [181].

2.5.2.2. Random approach

Random approach has been undertaken to enhance cell stress performance by mutating associated genes or genetic pathways without having specific knowledge about it. In general, spontaneous adaption of the cells under specific stress has been undertaken as the most popular approach to augment cell tolerance.

2.5.2.2.1. Spontaneous adaptation

Strain engineering of acid tolerance have also been reported by acid or glucose adaptation method [182, 183]. It has been revealed that glucose itself can trigger that acid tolerance in *E. coli*, the dose ranging from 0.01% to 1% of the total medium [183]. A work on *E. coli* K-12 revealed that sufficient adaptation to HCl medium at pH 3.0 can retain its survival close to

100% [184]. But the method is sensitive to the presence of other antibiotics like tetracycline and cations like potassium or calcium. Tetracycline can inhibit the acid tolerance of *E. coli* K-12 while cations like potassium or calcium can restore the tolerance. Another limitation of this method is that, due to high osmotic pressure at higher percentage of glucose, the biomass production is on the lower side [183].

Activation of *E. coli* acid resistance machinery has also been achieved by pre-adaptation to heterogeneous stressors such as heat shock or starvation or exposure to medium filtrates from acid habituated cells. Wang and Doyle have illustrated that heat shock of *E. coli* O157:H7 at 48°C for 10 min improved cell survival 10-100 times better in minimal glucose medium (pH 2.5) at 37 °C [185]. They suggested the improved tolerance due to two newly synthesized outer membrane proteins of 22 and 15 kDa. Similar improvement of stress tolerance was observed in cold shock-treated *E. coli* cells (2 h at 10 °C) in tryptic soy broth (TSB) at pH 4.0 [186]. Arnold and Casper also acknowledged that starvation or stationary phase adapted *E. coli* O157:H7 cells exhibited acid tolerance almost twice higher compared to mid log phase grown cells [187]. Interestingly in another study, it has been shown that exposure of medium filtrates from acid habituated cells to mid log phase grown cells at pH 7.0, improved ATR of the latter [188].

Interestingly, it has been revealed that type of acidulant could influence the survival and gene expression of *Escherichia coli*. Challenging three *E. coli* O157:H7 strains with citric, malic and acetic acids produced differential survival patterns for the respective strains [189]. Recently, a global transcriptomic analysis of *E. coli* K-12 over acid challenge demonstrated that acetic, lactic and hydrochloric acid (HCl) could trigger in individualistic gene expression profile besides a common transcriptional response [190].

The analysis of acid tolerance of *E. coli* by this method revealed that external addition of cAMP reduced the acid tolerance of *E. coli* thereby reducing the percentage survival of it [183]. Regulatory factors like IHF, H-NS, Fur, RelA, Lrp, CysB, NhaA have been found inactive in modulation of the acid tolerance response of *E. coli* though study of influence of CRP over acid tolerance was lacking in this paper.

2.5.2.2.2. Increasing acid tolerance through mutagenesis

It has been reported that using NTG mutagenesis has increased acetic acid tolerance of *Zymomonas mobilis* together with increased productivity of ethanol [191]. Physical and chemical mutagenesis has also been applied to improve strain acid tolerance in order to increase inorganic acid productivity and titer. For example, *Megasphaera elsdenii*, the main lactate utilizing bacteria in the ruminant animals, has been subjected to UV and NTG mutagenesis to increase acid tolerance as well as propionic acid productivity [192]. The mutants thus obtained, were treated as starting population for genome shuffling to finally achieve propionic acid titre 45~50 g/L from 20 g/L.

2.5.2.3. Combinatorial approach

Combinatorial approach have also been undertaken to construct better acid tolerant microbial variants. For example, acid tolerance in the fermentation medium has been a problem especially against microbes like *E. coli*. Genome shuffling of ethanologenic *Candida krusei* also performed to yield better acetic acid tolerant strain [44]. Furthermore, directed evolution of Homoserine o-succinyl transferase (a thermostable MetA protein) evolved potentially improved thermo tolerant as well as acid tolerant *E. coli* strains. But the limitation of this method was that biomass production was too slow to be feasible for industrial fermentation[193].

2.5.2.4. Mechanism studies of cell acid tolerance

Earlier studies on activation of *E. coli* acid tolerance response (ATR) by adaptation revealed three acid resistance (AR) mechanisms. AR1 involves adaptation of the cells to slightly acidic media (pH 5.5) without external glucose or amino acids. Aerobically grown *Escherichia coli* cells under this condition could withstand pH as low as 2.5 [194]. Further investigations proved that this system might be regulated by either RpoS or CRP [195, 196] thus known as oxidative or glucose repressed system. AR2 involves cell growth in complex acidic media supplemented with external glucose or glutamate which in turn, encode glutamate decarboxylase (GadAB) together with glutamate : GABA antiporter [197]. Successful phenotypes, able to withstand pH as low as 2.0, have been scored through this strategy. AR3 requires arginine replacing glutamate in AR2 under analogous circumstances to elevate cell tolerance by encoding arginine decarboxylase as well as arginine : arginine antiporter [194, 196, 197].

Genomic level investigation was initiated to search for the gene responsible for *E. coli* acid tolerance. It had already been reported that glutamate decarboxylase (GadAB) mediated acid resistance system plays a central role in proton consumption during decarboxylation reaction thus increasing cell pH [194, 198]. Also, glutamate : GABA antiporter system helps proton extrusion from the cell thus maintaining intracellular H^+ balance during acid stress [176, 197]. Further studies revealed that membrane lipoprotein composition bears significant correlation with cell acid tolerance [199]. For example, PhoE porin in the outer membrane could allow proton influx thereby increasing cell acid sensitivity [200]. By constructing PhoE mutant or including polyphosphate to block the channel, improvement of cell phenotype towards acid stress has been achieved [200]. Furthermore, K^+ / H^+ or Na^+ / H^+ membrane transporters' roles towards cell proton balance have been published [201]. The authors proposed that manipulation in these transporter proteins may result in modification of cell

acid tolerance. Even, the fatty acid composition in membrane phospholipid could also play a crucial role in acid tolerance [202]. Proton-translocating F_1F_0 -ATPase is observed to contribute significantly in cell pH maintenance by extruding H^+ ion to the extracellular medium [203]. Incorporation of cyclopropane fatty acids in membrane phospholipid could enhance the resistance against proton influx, hence improving the acid resistance [202].

The *fur* (ferric uptake regulator) gene is found to regulate the acid survival of *E. coli* because the mutation in Fur protein can impair the proton tolerance of it [204]. In a separate study, insertion of a new gene in *E. coli* colanic acid producing genes *wcaD* and *wcaE*, is reported to reduce the acid tolerance of it [205].

A global genetic analysis work over the genome of *E. coli* revealed that expression of a number of genes is related quantitatively with the acid tolerance response of *E. coli* [206]. Expressional downregulation was observed for 60 genes while 26 genes revealed upregulation more than 2 fold; among which five genes have been reported to be member of the RpoS regulon. Furthermore, the authors also reported that disruption of the *rpoS* gene led to the impairment of cell acid tolerance [206]. This work has been in compliance with the earlier findings acknowledging the contribution of RpoS towards cell acid tolerance [207-209]. A correlation between mRNA level of glutamate decarboxylase genes (*gad AB*) with acid tolerance of *E. coli* has been investigated and it was revealed that the level of the transcript increases with the acid stress depending on the superincumbent temperature [210].

2.6. Objectives

In this thesis, I intend to employ transcription engineering tools to enhance *E. coli* performance under various stresses. The target transcription factor is the global regulator of *E. coli*, cAMP receptor protein (CRP). Error-prone PCR technique would be adopted to introduce mutations to *crp* and the random mutagenesis libraries would be subjected to

variant selection under different stresses. My target individual *E. coli* phenotypes are as follows: i) organic solvent tolerance, such as toluene tolerance ii) oxidative stress tolerance iii) low pH tolerance. The aim of this work is to select the best *E. coli* mutant strain under the above mentioned stresses, and characterize the mutants with DNA microarray, quantitative real-time PCR and enzyme assays to reveal the mechanism underneath their phenotype improvement.

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Chapter 3: “Error-prone PCR of Global Transcription Factor Cyclic AMP Receptor Protein for Enhanced Organic Solvent (Toluene) Tolerance”

(This whole chapter has been a direct extract from the reference: **Basak, S.**; Song, H.; Jiang, R. Error-prone PCR of global transcription factor cyclic cAMP receptor protein of *E. coli* for enhanced organic solvent (toluene) tolerance. *Process Biochemistry* 2012, 47, 2152-2158)

3.1. Introduction

Development of strain engineering strategies has always aroused much interest in both industry and academic for improving microbial performance under stressful conditions. Generally, these strain engineering methodologies can be categorized into two categories: “rational” approach harnessing metabolic engineering tools to manipulate pre-explored genetic pathways [1], and “random” approach dealing with non-specific genotypic changes either caused by mutagens such as UV and NTG (nitrosoguanidine) [2] or exogenous addition of adaptive stressors [3]. The strain engineering approaches have been commercially implemented for the production of a broad spectrum of compounds such as enzymes and amino acids [4-7], antibiotics [8], food additives and biofuels [9], or for the enhancement of substrate uptake [10]. Metabolic engineering approach is usually limited by lack of detailed information on metabolic landscape as well as the complex genotype-phenotype relationship [11]. On the other hand, UV/chemical mutagen treatment is both time and labor intensive, and often requires a breadth of 10^8 - 10^{10} cells for strain selection [12, 13].

In recent years, transcriptional engineering has emerged as a new approach for strain improvement. Since cellular functions are often elicited through a diverse perturbations of interrelated genomic space regulated by transcription factors [14], they have become good targets for strain engineering. So far, this manipulation has been done with transcription factors such as SPT15 [15], sigma factor [14], artificial zinc finger containing transcription factor [16], Hha and H-NS [17, 18], and IrrE [19] to improve cell performance under stress and alter biofilm formation. Here, we will target cAMP receptor protein (CRP), the global transcriptional regulator in *E. coli*, to improve cell organic solvent tolerance. CRP can regulate over four hundred genes in *E. coli*, including organic solvent tolerance related genes such as *marA*, *glpC* and *manXYZ*, via dictating the promoter specificity of RNA polymerase [20-25]. Previously, we have successfully engineered CRP to improve cell osmotolerance and

butanol tolerance [26, 27]. Now we want to explore the possibility of engineering CRP to improve cell organic solvent tolerance. Toluene has been chosen as our model organic solvent in this study. Organic solvent is often applied in biphasic biocatalysis to perform the bioconversion of lipophilic substrate. It is used either to increase the product yield or to extract any hydrophobic toxic by-product detrimental to cells [28]. In addition, effluents such as benzene, toluene, ethylbenzene and xylene are often present at contaminated sites during bioremediation [29]. As an empirical rule, microbe can only tolerate and survive in the organic solvent whose Log P_{ow} value is greater than the index value of the microorganism [30-32]. *E. coli*, a popular microbial host elsewhere, is constrained to use in biphasic biocatalysis due to its high index value (3.7), indicating low tolerance to a wide range of organic solvents [30]. NTG treatment or pre-adaptation of cells to organic solvents has evolved mutant *E. coli* strain against *p*-xylene and *n*-hexane [31], but not toluene. Although recombinant DNA technology involving isogenic or exogenous organic solvent tolerant gene enabled the use of *E. coli* in the biphasic media [33-35], this method was limited by the choice of vector or exogeneous donor. Furthermore, toluene (Log P_{ow} =2.6) being extremely toxic to *E. coli*, evolution of a specific toluene resistant *E. coli* strain is still a difficult and complicated task.

In this study, we applied error-prone PCR to introduce mutations to *crp* and selected three variants (M1 ~ M3) with better tolerance towards toluene [36]. The performance of the best mutant M2 was further confirmed with other organic solvents such as *n*-hexane, cyclohexane, and *p*-xylene. A set of genes associated with cell organic solvent stress response were chosen for quantitative real-time reverse transcription PCR analysis. The cell surface characterization was performed by evaluating surface hydrophobicity while cell morphologies were examined by scanning electron microscopy.

3.2. Materials and Methods

3.2.1. Materials

E. coli DH5 α was obtained from Invitrogen (San Diego, USA) and Δcrp strain was constructed via a method described previously [26]. *E. coli* Δcrp strain was routinely cultured in LB medium (Bacto Tryptone 10g/l, Yeast extract 5g/l, NaCl 10g/l) supplemented with 0.2% (w/v) glucose and 10 mM MgSO₄ · 7H₂O (LBGMg). Gene morph[®] Random Mutagenesis kit was purchased from Stratagene (La Jolla, USA). All restriction enzymes were from Fermentas (Burlington, Canada). T4 DNA ligase was obtained from New England Biolabs (Ipswich, MA, USA). QIAquick gel extraction kit and QIAprep spin miniprep kit were procured from Qiagen. Toluene was purchased from Sigma-Aldrich (St. Louis, MO, USA). Low copy number plasmid pKSCP that contains the native *crp* operon was obtained from our previous work [26]. The Δcrp strain harbouring plasmid pKSCP is denoted as wild-type (WT) in this work.

3.2.2. Construction of variant library

crp was subjected to error-prone PCR using Genemorph II random Mutagenesis kit (Stratagene) with primers *crp_sense* (5'-gagaggatccataacagaggataaccgcgcatg-3') and *crp_anti* (5'-agatggtaccaaacaataatggcgcgctaccaggtaacgcgcga-3'). The PCR product was recovered from 1.2% low melting agarose gel using QIAquick Gel Extraction Kit (Qiagen), digested by restriction enzymes *Bam* HI and *Kpn* I, and inserted into the digested pKSCP backbone with T4 DNA ligase. The ligation mixture was transformed into Δcrp competent cells by electroporation.

3.2.3. Mutant selection

One percent (v/v) of the overnight cell culture was subcultured into 10 ml LBGMg-kan medium containing 0.25% (v/v) toluene. The selection was continued for the next two rounds under the same selection pressure while toluene concentration was increased to 0.28% for another two repeats. Colonies were grown on LB-kan agar plates at 37 °C overnight. Individual colonies were picked up and mutations were verified by DNA sequencing. The “selected” plasmids containing mutated *crp* were re-transformed into fresh Δcrp background to eliminate background interference.

3.2.4. Cell growth in toluene

To evaluate the growth profile of the selected mutants under stress, cells were cultivated in LBGMg-kan medium fed with 0.2% (v/v) or 0.23% toluene. All cells were grown in screw capped glass tubes to prevent evaporation of organic solvent and the overnight cell culture was inoculated into fresh LBGMg-kan media to an OD₆₀₀ value between 0.05 and 0.06. Cells were then cultured under various toluene concentrations and their growth was monitored by measuring their absorbance at 600 nm at periodic time intervals.

3.2.5. Quantitative real-time reverse transcription PCR (qRT-PCR)

Both WT and mutant were grown with or without 0.1 % (v/v) toluene (Fig. 4). Cells were harvested for RNA isolation using RNeasy Mini Kit (Qiagen, Germany) according to manufacturer’s protocols. The genomic DNA from both samples was removed by RNase free DNase-I (Qiagen, Germany). The purity of the isolated RNA was confirmed by gel electrophoresis and the concentration of the extracted RNA was estimated by spectrophotometric measurements using Eppendorf Biophotometer (Eppendorf, Germany).

The qRT-PCR analysis was carried out by a two-step reaction. First, the isolated RNA samples having OD_{260/280} values in between 1.9 to 2.1 were taken for cDNA synthesis. The cDNA synthesis from the respective RNA sample was carried out using iScript cDNA Synthesis Kit (Bio-Rad, CA) with 500 ng of cellular RNA as template. Secondly, the PCR reaction was carried out using pooled cDNA in the Bio-Rad iQ5 real-time PCR detection system with iQ SYBR Green Supermix (Bio-Rad, CA). Each reaction contained 2 µl of 1:20 diluted cDNA as template and the working concentration of each primer pair was 1 µM. The primer sequences are summarized in Table 3.2. The match of PCR amplification efficiencies of the primers was confirmed by plotting standard curves against that of housekeeping gene. qRT-PCR was carried out with the following program: 3 min at 95 °C, 40 cycles at 95 °C for 15 s, followed by 60 °C for 30 s. The threshold cycle (C_t) values were determined from the Bio-Rad iQ5 Optical System Software. The absence of genomic DNA was determined by subjecting same fold diluted primer pair as controls under the same qRT-PCR temperature program. To evaluate the fold change in gene expression level, 2^{-ΔΔC_t} method was followed. Bacterial 16S rRNA gene (*rrsG*) was taken as the housekeeping gene in this study. Normalization of each target gene was performed with the housekeeping gene to yield differential threshold crossings (ΔC_t gene). Average ΔC_t of each WT gene was used as platform ΔC_t to calculate ΔΔC_t for the respective gene in WT as well as mutant. The relative quantification of the fold change of a specific gene was ascertained based on the average gene expression ratio. Gene expression comparison was performed with both wild type and mutant under the same conditions.

3.2.6. Mutant tolerance to other organic solvents

Both WT and mutant were subjected to stresses exerted by other organic solvents namely *n*-hexane, cyclohexane, and *p*-xylene. Both were inoculated in 10 ml LB-kan medium and

grown to the stationary phase. One percent of the stationary phase culture was used to inoculate 10-ml LBGMg-kan medium containing different organic solvents (3% (v/v) *n*-hexane, 1% (v/v) cyclohexane, 0.23% (v/v) *p*-xylene). All were incubated at 37°C, 200 rpm and the growth of cells was monitored spectrophotometrically at 600 nm by sampling the culture periodically.

3.2.7. Cross tolerance to oxidative and low pH stress

In order to testify mutant applicability to other heterogeneous stresses, both mutant and WT were subjected to oxidative and low pH stresses. For oxidative stress, 1% (v/v) overnight seed of both the strains were inoculated in LB-kan (25 µg/ ml) medium fed with 6 mM hydrogen peroxide (H₂O₂). Starting from initial OD₆₀₀ ~ 0.05, the culture was subjected to continue at 37 °C and 200 rpm and cell growth was monitored spectrophotometrically at 600 nm.

For verification of mutant low pH tolerance, 1% of both the overnight grown cells were subjected to phosphate citrate buffered [37] LB medium at pH 4.50 (37 °C, 200 rpm) and cell accumulation was measured at 600 nm at periodic intervals.

3.2.8. Microbial adhesion to hydrocarbon (MATH) test

To characterize cell surface hydrophobicity, MATH test was performed for both wild type as well as the mutant. A previously published protocol for MATH test was followed [38]. Both the mutant and wild type cells were cultured up to stationary phase in LB-kan medium. The overnight seed cells were then diluted (1:100) in fresh LB-kan medium with 0.10% or without toluene and cultured at 37°C, 200 rpm for 8 h. Cells were collected by centrifugation at 4°C, 5000 x g for 10 min, washed thrice with ice cold 0.1M Potassium phosphate buffer (PPB) and resuspended in the same buffer at an optical density 0.6 (OD₆₀₀). 0.9 ml of both

MT and mutant cell suspension cultured in the presence or absence of toluene were taken in 2-ml eppendorf tubes and added with 200 μ l of toluene. The closed biphasic systems were vortexed vigorously for 2 min followed by standing at ambient temperature for 15 min for phase separation. The aqueous phase was carefully taken off and transferred into a cuvette for measuring cell density at 600 nm. The MATH value was calculated as $MATH = [(A_0 - A_t)/A_0] \times 100$ (A_0 = Initial absorbance of the cell suspension; A_t = Final absorbance of the cell suspension). The cell surface is considered as strongly hydrophobic if the MATH value is above 50%, the cell surface is moderately hydrophobic if the MATH value is within 20%-50% while a MATH value below 20% indicates the cell surface being hydrophilic [39].

3.2.9. Cell morphology studies by FESEM (Field Emission Scanning Electron Microscopy)

The stationary phase cultures of both WT and mutant were used as the inoculums, which was diluted 1:100 into different fresh LBGMg-kan medium. The incubation was at 37°C, 200 rpm with 0.10% or without toluene. Cells were harvested by centrifugation at 5000 x g for 10 min after 8 h culture, washed thrice with 0.9% NaCl and fixed with 2.0% glutaraldehyde at 4°C overnight. Cells were then collected by centrifugation, washed thrice with DI water, and resuspended in DI water. Approximately 3-4 μ l of this suspension was coated over prewashed glass slides. After drying at room temperature, cells were dehydrated by ethanol at increasing concentrations of 10%, 30%, 50%, 70%, 90% and 100% (v/v). The slides were mounted on FESEM stubs followed by coating of the cell surface with platinum sputter for 90 s. Images were captured after magnification with a JSM-6701F field emission scanning electron microscope (JEOL, Japan).

3.3. Results

3.3.1. Mutant isolation and growth profile

In order to isolate CRP mutant strains with better toluene tolerance, error-prone PCR was performed to construct *crp* mutant libraries. The libraries were then subjected to enrichment selection with high toluene pressure. After two rounds of error-prone PCR, approximately 10^6 clones with modified *crp* were subjected to growth selection under toluene stress. After four rounds of subculture, the surviving colonies were picked randomly from LB plates, and the location and identity of their mutations were ascertained by DNA sequencing. Three CRP variants were identified (M1 ~ M3) and their relevant plasmids were re-transformed into fresh Δcrp background to eliminate false positives. The amino acid mutations of these CRP variants are displayed in Table 3.1.

Table 3.1: Amino acid substitutions in M1 ~ M3

Mutant	Amino acid substitution
M1	T127N
M2	F136I
M3	T127N, V176A

Since the mutants were isolated based on their toluene tolerance, we confirmed the growth of all mutants, with WT as control, against 0.2 % and 0.23 % toluene, respectively (Fig. 3.1). Both WT and the mutants exhibited similar growth pattern in the absence of toluene in LBGMg medium and the growth rate of both WT and mutants was found to be around 0.9 h^{-1} (Fig. 3.1A). At high toluene stress, all mutants exhibited better growth compared to WT. When toluene concentration was at 0.2% (Fig. 3.1B), all three variants shared similar growth rate at around 0.68 h^{-1} , with WT exhibiting null growth. In order to differentiate the performance of the three mutants, the toluene concentration in the culture medium was

enhanced to 0.23% (Fig. 3.1C). M2 showed better toluene tolerance than the other two mutants at this stress with the growth rate of 0.51 h^{-1} while that of M1 and M3 was around 0.38 h^{-1} . WT revealed null growth at this toluene concentration. Based on these results, M2 was chosen for further studies hereon.

Fig. 3.1: Cell growth profile with different concentrations of toluene in LBGMg-kan medium at 37°C, 200 rpm. A: 0%; B: 0.2% (v/v); C: 0.23% (v/v). Each data point is the mean of three independent observations. The OD of cell growth is measured at 600 nm.

3.3.2. qRT-PCR

We have chosen fifteen genes for qRT-PCR mediated expressional analysis, with eight associated with *E. coli* organic solvent tolerance (*tolC*, *acrA*, *acrB*, *marA*, *glpC*, *manX*, *manY* and *manZ*) [40-43]. The other seven selected genes included *soxRS*, which was reported to

be related with *E. coli* cyclohexane tolerance [44], *crp*, *cya*, and three SoxRS-regulated respiratory pathway genes *zwf*, *fldA* and *fumC*.

Table 3.2: Primers for qRT-PCR

Primer	Gene	Sequence (5'→3')
acrA_F	<i>acrA</i>	GGAAGGCGCATTGGTACAGAAC
acrA_R	<i>acrA</i>	GGGAACCTTAATGCCGTCCTGG
acrB_F	<i>acrB</i>	GGTATGTTCGTGCTGCTGATGG
acrB_R	<i>acrB</i>	GTAGGCAGTTTGCTCGCCAGTT
tolC_F	<i>tolC</i>	TCAACTCTAACGCGACCAGTGC
tolC_R	<i>tolC</i>	GCGGTGTTGAGGATCAAGGTTT
marA_F	<i>marA</i>	CAATACATCCGCAGCCGTAAGA
marA_R	<i>marA</i>	GTATTTATGCGGCGGAACATCA
glpC_F	<i>glpC</i>	TTAACGGTGCTGGATTCCCAGT
glpC_F	<i>glpC</i>	TTACAGGTTTCGCAGTCGGTGA
manX_F	<i>manX</i>	GGGCCAAACGACTACATGGTTATT
manX_R	<i>manX</i>	ACCTGGGTGAGCAGTGTCTTACG
manY_F	<i>manY</i>	CAGAGCATTGGTGCAGGTATCG
manY_R	<i>manY</i>	GAAACGTGGATCCAGGAAATCG
manZ_F	<i>manZ</i>	ACTGACCAGACGGGCAAAGAAC
manZ_R	<i>manZ</i>	TAACCAGCGATACCGATGACGA
rrsG_F	<i>rrsG</i>	TCAAGGGCACAACCTCCAAGTC
rrsG_R	<i>rrsG</i>	GGTGTAGCGGTGAAATGCGTAG

cya_F	<i>cya</i>	AGATTGATCAGGTGCGTGAGGC
cya_R	<i>cya</i>	AAATCTGCGGGTTTACCAGCGT
crp_F	<i>crp</i>	TCAGAGAAAGTGGGCAACCTGG
crp_R	<i>crp</i>	GATGGTTTTACCGTGTGCGGAG
fldA_F	<i>fldA</i>	CAGCTTGGTAAAGACGTTGCCG
fldA_R	<i>fldA</i>	ACAACCAAACAGCGCAACCAGT
fumC_F	<i>fumC</i>	AACGTCTTCCGTCCAATGGTGA
fumC_R	<i>fumC</i>	CACCAGCATCAGCGATTTCATTG
soxR_F	<i>soxR</i>	CATTAAAGCGCTGCTAACCCCC
soxR_R	<i>soxR</i>	TGCCGCTGTTACGGATACTGGT
soxS_F	<i>soxS</i>	GCATCAGACGCTTGGCGATTAC
soxS_R	<i>soxS</i>	GACATAACCCAGGTCCATTGCG

In the absence of toluene, three genes (*manXZ*, *crp*) showed differential expression in M2 (> 2-fold) as compared to WT with *p*-value less than 0.05 (Table 3.3A). To be more specific, *manX* and *manZ* displayed downregulation, 4.79-fold and 3.69-fold respectively, whereas *crp* demonstrated 9.64-fold upregulation in M2. In the presence of toluene stress, the expression level of six genes in M2, namely *acrAB*, *glpC*, *manXY* and *crp*, was elevated for more than two-fold ($p < 0.05$) (Table 3.3B). In particular, *glpC* and *crp* exhibited 19.18- and 9.48-fold increase in M2 with respect to WT. *acrA*, *acrB*, *manX*, and *manY*, were also found to have 2.77-, 5.17-, 2.61- and 3.13-fold elevated expression in M2, respectively. On the other hand, *soxS*, part of SoxRS regulon, exhibited 3.2-fold downregulation. *marA* and *zwf*, revealed less than two-fold downregulation (1.77- and 1.9- fold respectively) compared to WT.

Table 3.3A: qRT-PCR analysis of certain genes without toluene treatment (Each data point is the mean of three independent observations)

Gene	Fold Change		<i>p</i> - value
	WT	M2	
<i>tolC</i>	1.001 ± 0.055	1.260 ± 0.267	0.232
<i>acrA</i>	1.002 ± 0.080	0.723 ± 0.211	0.099
<i>acrB</i> *	1.010 ± 0.178	0.624 ± 0.055	0.023
<i>marA</i>	1.003 ± 0.088	0.916 ± 0.035	0.192
<i>glpC</i> *	1.001 ± 0.064	0.569 ± 0.158	0.012
<i>manX</i> ***	1.001 ± 0.148	0.209 ± 0.027	4.3 x 10 ⁻⁵
<i>manY</i>	1.012 ± 0.198	1.217 ± 0.265	0.344
<i>manZ</i> **	1.012 ± 0.198	0.274 ± 0.062	0.003
<i>cya</i>	1.000 ± 0.053	0.960 ± 0.254	0.803
<i>crp</i> **	1.015 ± 0.207	9.789 ± 1.494	0.009

<i>zwf</i>	1.053 ± 0.387	0.555 ± 0.055	0.153
<i>fldA</i>	1.014 ± 0.208	0.767 ± 0.075	0.167
<i>fumC</i>	1.016 ± 0.355	1.500 ± 0.458	0.114
<i>soxR</i>	1.081 ± 0.458	1.616 ± 0.553	0.266
<i>soxS</i>	1.009 ± 0.173	0.994 ± 0.164	0.914

* - $p < 0.05$ ** - $p < 0.01$ *** - $p < 0.001$

Table 3.3B: qRT-PCR analysis of certain genes with toluene treatment (Each data point is the mean of three independent observations)

Gene	Fold Change		<i>p</i> - value
	WT	M2	
<i>tolC</i>	1.017 ± 0.217	0.924 ± 0.201	0.618
<i>acrA</i> ^{**}	1.014 ± 0.199	2.804 ± 0.489	0.004
<i>acrB</i> ^{***}	1.014 ± 0.213	5.241 ± 0.428	1.06 x 10 ⁻⁴
<i>marA</i> ^{**}	1.004 ± 0.108	0.567 ± 0.041	0.003
<i>glpC</i> ^{**}	1.044 ± 0.361	20.03 ± 1.80	0.002
<i>manX</i> ^{***}	1.001 ± 0.066	2.609 ± 0.225	2.89 x 10 ⁻⁴
<i>manY</i> ^{**}	1.029 ± 0.314	3.222 ± 0.420	0.002
<i>manZ</i> ^{**}	1.000 ± 0.023	1.469 ± 0.102	0.001
<i>cya</i>	1.004 ± 0.110	0.974 ± 0.296	0.878
<i>crp</i> ^{***}	1.033 ± 0.338	9.789 ± 1.494	1.1 x 10 ⁻⁴

<i>zwf</i> [*]	1.062 ± 0.416	0.555 ± 0.055	0.048
<i>fldA</i>	1.011 ± 0.180	1.489 ± 0.498	0.193
<i>fumC</i>	1.012 ± 0.193	1.216 ± 0.264	0.340
<i>soxR</i>	1.062 ± 0.428	0.727 ± 0.058	0.307
<i>soxS</i> ^{**}	1.010 ± 0.170	0.316 ± 0.053	0.003

* - $p < 0.05$ ** - $p < 0.01$ *** - $p < 0.001$

3.3.3. Cross tolerance to other organic solvents

Earlier studies acknowledged that toluene had a lower Log P_{ow} value (2.6) than many other organic solvents such as *n*-hexane (Log P_{ow} 3.9), cyclohexane (Log P_{ow} 3.4), and *p*-xylene (Log P_{ow} 3.1) [32]. Since lower Log P_{ow} value suggests higher toxicity of the organic solvent [30, 32, 45], improvement of toluene tolerance in M2 encouraged us to test its tolerance against other organic solvents as well. In order to test M2 tolerance against *n*-hexane, both M2 and WT were cultured in LBG medium instead of LBGMg medium. In 3% *n*-hexane (Fig. 3.2A), the absorbance of the cell culture at 600 nm rose up to 2.5 after 60-h culture, whereas WT only attained its stationary phase at an OD₆₀₀ value of 0.2. As for the other organic solvents, 1% cyclohexane (Fig. 3.2B) and 0.23% *p*-xylene (Fig. 3.2C), M2 also exhibited excellent growth whereas WT showed almost no growth till last observation. These findings suggest that the toluene-tolerant M2 may also have tolerance towards other organic solvents.

Fig. 3.2: Growth profiles of both WT and M2 in various organic solvents at 37°C, 200 rpm. A: 3% (v/v) *n*-hexane, B: 1% (v/v) cyclohexane, C: 0.23% (v/v) *p*-xylene. Each data point is the average of three biological replicates. The OD of cell growth is measured at 600 nm.

3.3.4 Cross tolerance to oxidative stress and low pH

Apart from showing cross tolerance to other kind of organic solvents, the mutant also revealed resistance against stressors like oxidative stress and low pH. When subjected to 6 mM H₂O₂, M2 revealed continuous cell accumulation and reached stationary phase OD₆₀₀ ~ 2.9 while WT growth was completely inhibited. Similarly at pH 4.50, M2 reached OD ~ 0.6 at 12 h whereas WT reached OD ~ 0.5 at the same time (Fig. 3.3). Thus M2 exhibited

significant tolerance improvement against H_2O_2 whereas it showed some, if not very high, elevated growth performance against pH as low as 4.50.

Fig 3.3: Cross tolerance of M2 against heterogeneous stressors. A: 6 mM H_2O_2 ; B: pH 4.50. For H_2O_2 , cells were challenged in LB-kan medium containing 6mM stressor and for pH 4.50, cells were allowed to grow in modified LB/kan-phosphate-citrate buffered medium. The cells were incubated at 37°C, 200 rpm. The OD of cell growth is measured at 600 nm.

3.3.5. MATH test

The MATH test was conducted to characterize cell surface hydrophobicity of both WT and M2 strains. Allowance of growth of both WT and M2 in the absence of toluene yielded MATH scores at 61.2% and 33.2%, respectively, suggesting the cell surface to be strongly and moderately hydrophobic for the respective organisms (Fig. 3.4). In contrast, when grown in the presence of 0.10% toluene, the MATH values changed to 45.8 and 26.2% for WT and M2, respectively, and both could be considered as moderately hydrophobic. The MATH value of M2 was always much lower than that of WT, with or without toluene. The cell surface adjustment of the organisms was likely due to the interaction with surrounding hydrophobic environment.

Fig. 3.4: MATH values for WT and M2 grown in the absence or presence of 0.1% (v/v) toluene. Each result is the mean of three biological replicates.

3.3.6. Cell morphology studies by FESEM (Field Emission Scanning Electron Microscopy)

In order to characterize cell morphology alterations, we visualized both M2 and WT cells in the presence or absence of toluene *via* FESEM. Without toluene, the cellular size of M2 and WT were noted to be $2.12 \pm 0.35 \mu\text{m}$ and $2.87 \pm 0.42 \mu\text{m}$ in length (based on ~ 50 cells), respectively. With toluene, the cell length of M2 and WT were measured as $2.09 \pm 0.36 \mu\text{m}$ and $2.10 \pm 0.46 \mu\text{m}$, respectively (Fig. 3.5). It could be deduced that, there was no significant change in both cellular size, with or without toluene. Both cell surfaces were notably smooth

without much roughness or indentations. The rod shape of both cells also remained unchanged, with no cell elongation or filament formation after toluene treatment.

Fig. 3.5: Field Emission Scanning Electron Micrographs (FESEM) WT and M2 after 8-h culture in LBGMg-kan medium A: WT, no toluene, B: WT, 0.1% (v/v) toluene, C: M2, no toluene, D: M2, 0.1% (v/v) toluene

3.4. Discussion

In this study, we aimed to improve *E. coli* organic solvent tolerance via engineering its global transcription factor CRP. The model organic solvent was chosen to be toluene. In pursuit, the random mutagenesis libraries were first constructed *via* error-prone PCR method

and the libraries were then challenged with toluene stress. Three mutants M1~M3 were identified with enhanced performance in toluene. The best mutant M2 also showed good tolerance to other organic solvents such as *n*-hexane, cyclohexane, and *p*-xylene. Together with our previous results on engineering CRP to improve cell osmotolerance, we believe that direct manipulation of CRP probably could be developed as a reliable strategy to remodel cell genetic framework and obtain certain desired cell phenotypes.

We have studied the possible roles of the amino acid substitution of M2. CRP is a homodimeric protein and each subunit consists of three domains: the N-terminal domain (residue 1-134) is responsible for the allosteric activation of CRP through cAMP binding; the C-terminal domain (residue 140-209) recognizes and binds with the DNA; and the short intermediate region (residue 134-139) which connects the N- and C-terminal domains, is the hinge region [46]. The hinge region is responsible for regulating the interdomain network thus determining the conformation of CRP [47]. The best mutant M2 only has one amino acid substitution F136I. The F136 position belongs to the hinge region and is situated near the cAMP binding pocket. This is possibly involved in the formation of the β -hairpin flap that enables sufficient interactions between N- and C-terminal domains [46]. Mutation F136I in M2 may alter this β -hairpin flap and hence modify CRP conformation. It's interesting that one single amino acid substitution in CRP could already lead to enhanced cell performance. CRP is a global transcription factor that can regulate hundreds of genes through a cascade of complex genetic networks [48-52]. Hence point mutations over CRP might result in the change of transcription profile of relevant genes. Here in this study, we have carried out qRT-PCR analysis with eight genes that are related with *E. coli* organic solvent tolerance to prove gene expression changes upon CRP mutation.

Among the eight organic solvent tolerance associated genes, only *marA*, *glpC* and *manXYZ* are directly or indirectly regulated by CRP according to Regulon DB [53]. The *acrAB-tolC*

system is a major efflux pump that can extrude intracellular solvent from cells and thus enhance *E. coli* organic solvent tolerance [42]. Though *acrAB* prefer to release antibiotics and amphiphilic charged compounds, they also play essential roles in maintaining cell resistance against hydrophobic organic solvents [54]. As for *glpC*, which encodes glycerol-3-phosphate dehydrogenase—a member of anaerobic respiratory chain C [55], was upregulated in organic solvent-tolerant *E. coli* strain through lowering intracellular solvent level inside the cell [40]. *manXYZ*, encoding a mannose PTS permease, a sugar transporter of the phosphotransferase system, also showed elevated expression, and cells would exhibit modifications to their surface properties and thus lower their adherence to hydrocarbons upon *manXYZ* upregulation [41]. Our qRT-PCR results concurred with these previous findings on the upregulation of *acrAB*, *glpC*, *manXY* in M2 when facing toluene challenge. However, *tolC* did not show any significant expression level change, which might be due to the passive diffusion of toluene *via tolC*-independent process [42]. Although high expression of stress responsive proteins such as MarA could enhance cell organic solvent tolerance [56], *marA* demonstrated downregulation in M2 with toluene present. The reason is still unknown and requires further investigation.

Fig. 3.6: Mutant growth profile in 0.1 % (v/v) toluene. The growth was monitored in LBGMg-kan medium at 37°C, 200 rpm. The OD of cell growth is measured at 600 nm.

We have also evaluated the transcriptional scores of both *crp* and *cya* (encoding adenylate cyclase for cAMP generation) in WT and M2. The unaltered expression profile of *cya* under both normal and stressful conditions with concomitant significant *crp* upregulation in M2 may suggest that the elevated expression of *crp* is independent of *cya* in M2. In addition, we deconvoluted the expressional profile of certain SoxRS-regulated respiratory pathway genes. *zwf*, which encodes glucose-6-phosphate dehydrogenase, a well characterized enzyme involved in pentose phosphate pathway, superpathway of glycolysis and Entner-Doudoroff pathway, was downregulated in M2 in the presence of toluene (1.91 fold), which suggested that modifications to CRP might also alter the expression of genes that are not regulated by

CRP. However, the other respiratory pathway enzymes such as *fldA* (encoding flavodoxin 1 involved in both aerobic and anaerobic growth conditions of *E. coli*), *fumC* (encoding fumarase C, a member of the TCA cycle and highly active under aerobic conditions of *E. coli* growth) did not display differential expression in both strains irrespective of the presence of toluene.

Besides CRP, other global regulators such as RpoS may also respond to organic solvent stress. A major solvent tolerant gene cluster *acrAB* is regulated by RobA, which is regulated by RpoS [57]. Furthermore, RpoS has been found to be upregulated in isobutanol tolerant strains of *E. coli* [58].

Mutants with improved phenotype towards one stress may also have improved performance under other stressful conditions, which is due to the overlap of stress response mechanism in microbes. Thus, we evaluated mutant performance against other kinds of stresses such as oxidative and acid stress. In both the cases, M2 showed improved growth performance under the same condition implying co-activation of multistress response pathways within the same strain. The M2 tolerance against oxidative stress is in compliance with the previous finding that alkylhydroperoxide reductase, an enzyme regulating cell oxidative stress resistance, was found to increase organic solvent tolerance in *E. coli* [61]. However, the reason for M2 low pH tolerance is still unknown and needs further investigation. Not only oxidative and acid tolerance, earlier publications suggest that cell solvent tolerance can co-exist with diverse set stress tolerances. For example, AcrAB is responsible for releasing antibiotics as stated earlier, hence the organic solvent mutant might also have antibiotic tolerance as suggested by other authors [59]. Moreover, expression of exogenous prefoldin, a thermoresistant protein, also demonstrated improved *E. coli* organic solvent tolerance [60].

We have demonstrated that M2 had much better growth than WT when cultured against various organic solvents. When cultured in LBGMg medium, *n*-hexane (Log P_{ow} =3.9) did not inhibit cell growth because it has a higher Log P_{ow} value than that of *E. coli* (Log P_{ow} = 3.7). It has been reported that $MgSO_4$ reduces the charge-charge repulsion between cell membranes and thus confers stabilization [30], which promotes cell growth in LBGMg medium. This prompted us to evaluate *E. coli* cell tolerance against *n*-hexane in modified LB (LBG) medium without $MgSO_4$. Persuade of cell growth in this medium against *n*-hexane eventually discriminated the tolerance profile between WT and M2. Both cyclohexane (Log P_{ow} =3.4) and *p*-xylene (Log P_{ow} =3.1) can inhibit *E. coli* growth significantly even in LBGMg medium.

MATH test is performed to characterize cell surface hydrophobicity in correlation with our organic solvent tolerance study [41]. Organic solvent propagates through dissolution of the lipoidal cell membrane together with in-cell permeation to disrupt the intracellular organelles [28]. M2 has always demonstrated a lesser MATH value than that of the WT with or without toluene stress. These findings suggest lesser hydrophobic cell surface of M2 than WT, which probably leads to lesser organic solvent penetration through cell membrane [62]. The lesser adherence of M2 to the hydrocarbons also supports previous reports that better tolerant microbes bind less abundantly to the hydrophobic solvents [40, 45]. .

Cell morphologies becomes more susceptible to change during their adjustment to stressful environment [63]. Nevertheless, in this study, the cell length of both WT and M2 remained approximately the same in the presence or absence of toluene stress. Common stress responsive morphologies such as cell membrane roughness [64] or cell elongation [65] were not clearly observed in either WT or M2.

Researchers have made efforts over the last few decades to isolate organic solvent tolerant mutants, but neither NTG mutagenesis nor the introduction of exogenous alkyl hydroperoxide

reductase gene (*ahpC*) has generated satisfactory mutants [61, 66-69]. Only metabolic engineering approach by cloning exogenous toluene dioxygenase gene from *Pseudomonas putida* has been applied successfully to construct a toluene-tolerant *E. coli* strain [33, 34, 70], viable against 0.18%-0.25% (v/v) toluene [70]. Similar strategies such as cloning of isogenic *ostA* have also been undertaken to enhance *E. coli* *n*-hexane tolerance [35]. These methods were either based on the choice of high copy plasmid and promoters for over-expression [44] or relied on intensive search of relevant genes from foreign microbes [33, 34]. In this study, through CRP engineering strategy, we could isolate CRP mutants that could well tolerate toluene concentration up to 0.23% (v/v) without introducing any foreign genes. The mutant performance is also comparable to the previously published toluene-tolerant strain obtained *via* metabolic engineering approach. Moreover, the traditional methods of isolating organic solvent tolerant mutants also exhibited limitations in terms of frequency of obtaining variants. For example, only one cyclohexane-tolerant variant was selected from a pool of 10^6 *E. coli* cells by spontaneous adaptation, whereas one *p*-xylene tolerant strain was constructed through classical NTG treatment from a population of 10^{19} cells [31]. In comparison, three toluene-tolerant variants were easily selected within a few days from a variant library size around 10^5 ~ 10^6 . Moreover, researchers also found that engineering exogenous regulator IrrE could greatly enhance *E. coli* tolerance towards butanol and ethanol [19]. In a nutshell, this transcription engineering methodology may be considered as an alternative strain engineering strategy to construct microbes with improved phenotypes worthy of application in bioprocesses.

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Chapter 4: “Enhancing *E. coli* tolerance towards oxidative stress via engineering its global regulator cAMP receptor protein (CRP)”

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4.1. Introduction

Using UV/chemical mutagens or rewiring metabolic pathways through gene addition/knockout have been traditional approaches for strain improvement [1]. Classical strain engineering strategies are often time-consuming and labor-intensive [2]. 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 [3]. Moreover, a functional cluster of genes orchestrate phenotypic modulation only when perturbed altogether [4] and that 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 the genetic control circuit by the modification of the entire genomic hierarchy inside microorganisms [5]. Global regulators are able to organize a large repertoire of genetic switches [6]. These regulators can also impart pleiotropic phenotype changes through the regulation of operons belonging to various functional groups [7]. Transcriptional engineering has been evolved as a potential tool for strain engineering over the last few years to alter strain stress tolerance [8-10], biofuel production [11-13], and biofilm formation [14, 15].

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 [16]. Among them, CRP can regulate more than 400 genes [17] and harmonize certain genetic circuits by directly or indirectly regulating other transcriptional regulators [18], which makes it a potential target for altering cellular phenotypes. Previously, we have shown that engineering CRP can improve *E. coli* osmotolerance [19] and 1-butanol tolerance [20]. Here, we aim to explore the possibility of rewiring CRP against oxidative damage often encountered inside bioreactors

under stressful conditions [21, 22]. *E. coli* DH5 α was used as host strain for its suitability in plasmid stability and in bioprocess usage [23-25].

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 [26]. The prototype response of *E. coli* against oxidative stress is the induction of antioxidant enzymes involved in ROS scavenging and DNA repair [27], which is *via* global transcriptional activation of redox-sensing regulators SoxR and OxyR [28]. In addition, oxidative stress in *E. coli* also induces chaperone such as Hsp33 to protect plenty of cellular proteins from stress generated shock [29]. Traditional approaches have been adopted to construct mutant *E. coli* strain *via* spontaneous adaptation [30] and cloning of exogenous antioxidant genes [31, 32]. 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 [33], which encouraged us to manipulate relevant *E. coli* response through CRP. Here, we have constructed a CRP library through error-prone PCR [34, 35] 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.

4.2. Materials and Methods

4.2.1. Materials

E. coli DH5 α was procured from Invitrogen (San Diego, USA) and *E. coli* Δ crp strain was obtained according to a previous published protocol [19]. Luria Bertani (LB) or Lysogeny

broth (Bacto tryptone (Oxoid) 10g/l, yeast extract (Merck) 5g/l, sodium chloride (Merck) 10g/l) was routinely used for bacterial culture since it has been a popular medium choice for *E. coli* growth under oxidative stress [31, 32, 36, 37]. SOC medium (yeast extract 5g/l, tryptone 20g/l, NaCl 10 mM, KCl 2.5 mM, MgCl₂ 10 mM, MgSO₄ 10 mM, glucose 20 mM) was used for cultivation of transformed cells. 30% (w/w) hydrogen peroxide (H₂O₂) and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) 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.

4.2.2. Cloning and library construction

The native *crp* was amplified *via* error-prone PCR with the following primers: *crp_sense* (5' -gagaggatccataacagaggataaccgcgcatg-3') and *crp_anti* (5' -agatggtaccaaacaataatggcgcgctaccaggtaacgcgccca-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 Δcrp competent cells and cultured at 37°C, 200 rpm.

4.2.3. Mutant selection

The mutant library was cultured in SOC medium at 37°C and 200 rpm for 4 h after electroporation and then subjected to enrichment selection. In order to select mutants against oxidative stress, H₂O₂ was used as stressor and LB medium was fed with increasing concentration of H₂O₂. The selection was carried out in 1.5 mM H₂O₂ for three repeats and challenged with 2.0 mM H₂O₂ 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 Δ *crp* backgrounds in order to nullify plasmid or genome borne false positives. The pKSCP plasmid containing native *crp* operon was also transformed into Δ *crp* background and is designated as wild type (WT) in this study.

4.2.4. 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 OD₆₀₀ value of 0.05. Each clone was cultivated at 37°C, 200 rpm in 0-12 mM H₂O₂, 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.

In order to evaluate oxidative stress tolerance of mid log phase cells, 12 mM H₂O₂ was introduced into the culture after cell had grown to mid-log phase (OD₆₀₀ 0.65) and cell growth was monitored.

4.2.5. 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.

4.2.6. Mutant thermotolerance

Stationary phase culture of the mutant and WT was used to inoculate fresh LB-kan media up to an OD₆₀₀ value between 0.05 and 0.06. With the same starting OD₆₀₀, both were allowed to reach stationary phase at 48 °C. Cell density was tracked by sampling from the cultures and measuring the OD₆₀₀ values at periodic time intervals.

4.2.7. Measurement of intracellular reactive oxygen species (ROS) level

The intracellular peroxide level was measured by using ROS sensitive probe 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) as described previously [38]. In brief, both the mutant and WT were grown to OD₆₀₀ 0.6 with or without 4mM H₂O₂. Cells were harvested by centrifugation, washed with 10 mM, pH7.0 potassium phosphate buffer (PPB), and resuspended in the same buffer. Cells were incubated with 10 μM H₂DCFDA (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. An 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 (Sigma-Aldrich, USA) using an Eppendorf biophotometer (Hamburg, Germany).

4.2.8. DNA microarray

Cells were grown with or without 4 mM H₂O₂ to OD₆₀₀ 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 × 15K slides were used and Cy3/Cy5 hybridized slides were scanned under Agilent High Resolution Scanner. Data organization and analysis

was performed by Agilent Genespring GX software. The expression ratio and *p*-value was calculated based on two biological replicates of each strain under all conditions.

4.2.9. Quantitative real time reverse transcription PCR (qRT-PCR)

cDNA was synthesized from cellular RNA extracted from both OM3 and WT grown in the presence or absence of H₂O₂. It was performed with iScript cDNA synthesis kit (Bio-Rad, CA, US) using 500 ng cellular RNA as template. Ten genes were chosen for qRT-PCR analysis to validate the microarray results. The primers used are listed in Table 4.4. qRT-PCR was carried out using Bio-Rad iQ5 thermocycler with the following programme: 3 min at 95 °C, 40 cycles at 95 °C for 15 s, followed by 60 °C for 30 s. 2^{-ΔΔCt} method was used for gene expression analysis. The observations were based on two biological replicates.

4.2.10. Enzyme activity assay

For all enzyme activity assay, fresh cells were cultivated up to OD₆₀₀~0.6, harvested by centrifugation at 1900 × g for 15 min, lysed by ultrasonication and cell lysate was subjected to assay under specific conditions. Cellular protein concentration was determined by Bradford assay.

i) Superoxide Dismutase (SOD)

SOD activity assay was performed by pyrogallol autoxidation method as described before [39]. Briefly, pH 8.2, 50 mM Tris-Cl buffer with 1 mM EDTA was used as reaction medium. Cell lysate containing 40~60 μg cellular proteins was added to 0.2 mM pyrogallol (dissolved in pH 6.5, 50 mM PPB) to initiate the reaction, and the absorbance decrease of pyrogallol was monitored at 420 nm. The percentage inhibition of pyrogallol autoxidation was calculated by the following formula:

$$\% \text{ inhibition of pyrogallol autoxidation} = [1 - (\Delta A / \Delta A_{\max})] \times 100, \text{ where}$$

ΔA = Absorbance change due to pyrogallol autoxidation in the sample reaction system

$\Delta A_{\max}$ = Absorbance change due to pyrogallol autoxidation in the control (without cell lysate)

One unit of SOD activity was defined as the amount required for inhibiting pyrogallol autoxidation by 50% per min.

ii) Catalase assay

Catalase assay was carried out according to a previous protocol with modifications [32]. About 40~60 μg cellular protein and 10 mM H_2O_2 was added to pH 7.2, 50 mM PPB reaction medium. The absorbance drop of H_2O_2 at 240 nm ($\epsilon_{240} = 43.6 \text{ mM}^{-1} \text{ cm}^{-1}$) was recorded at 25°C [40]. One unit of catalase activity was defined as the μmoles of H_2O_2 decomposed per min.

iii) Alkyl hydroperoxide reductase (AhpCF) assay

As per the method published earlier [41], the reaction system comprised of 1mM cumene hydroperoxide, 240 μM NADH, 40~60 μg cellular protein dissolved in pH 7.2, 50 mM PPB containing 0.5 mM EDTA. The reaction was monitored at 340 nm (NADH, ϵ : $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) [42]. One unit of alkyl hydroperoxide reductase activity was defined as the μmoles of NADH consumed per min.

iv) Glutamate decarboxylase (GAD) assay

The assay was followed as described before [43]. Briefly, the analysis was based on the pH indicator assay where the color of Bromocresol green changes within pH 3.8-5.4. The reaction system was comprised of 10 mM monosodium salt of L-glutamic acid (MSG), 10 mM pyridoxal 5'-phosphate, 70 μM Bromocresol green as pH indicator, 60 μg of cell protein

and 20 mM acetate buffer (pH 4.8) as medium. The reaction was carried out at 40°C and the absorbance change was monitored at 620 nm. One unit of glutamate decarboxylase activity was defined as μ moles of MSG consumed per min.

4.2.11. Field Emission Scanning Electron Microscopy (FESEM)

Cells were first grown in the absence or presence of 4 mM H₂O₂ and cell pellets were harvested by centrifugation, washed with ice-cold 0.9% NaCl, and thereafter fixed with 2.0% glutaraldehyde overnight. The fixed cells were harvested and washed with DI water to remove all residual glutaraldehyde and NaCl. Cells were then resuspended in DI water, and 2-3 μ l cell suspension was used to coat a glass slide. The cells were dehydrated with increasing concentrations (10%-100%) of ethanol and dried overnight at 37°C. Cell surface was coated with platinum sputter for 75 s and observed with a JEOL JSM 6701 Microscope (JEOL, Japan).

4.3. Results

4.3.1. Random 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 $\sim 10^5$ clones containing *crp* were obtained after two rounds of error-prone PCR. With enrichment selection of 1.5 mM $\sim$ 2.0 mM H₂O₂, three mutants (OM1~OM3) that exhibited better tolerance towards stress were selected from the library. The mutation rate was approximately 1-3 amino acid substitutions over the CRP open reading frame and the mutations on these three variants are listed in Table 4.1.

Table 4.1: Amino acid substitutions in OM1~OM3

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

4.3.2. Mutant growth in H₂O₂

Mutant growth was evaluated by subjecting mutants as well as WT in 0 mM to 12 mM H₂O₂ (Fig. 4.1). In the absence of H₂O₂, all three mutants exhibited similar growth profiles as WT, with the growth rate around 0.31~0.36 h⁻¹ (Fig. 4.1A). With 8 mM H₂O₂ present (Fig. 4.1B), all mutants behaved similarly to each other with a growth rate of 0.45 h⁻¹, whereas WT exhibited null growth under the same condition. When the pressure was further increased to 12 mM H₂O₂ (Fig. 4.1C), the growths of OM1 and OM2 were hindered completely within the time frame of observation, while OM3 achieved stationary phase OD₆₀₀ of 2.7 with a growth rate of 0.6 h⁻¹.

Fig. 4.1: Cell growth in the absence or presence of H₂O₂ (A) 0 mM H₂O₂, (B) 8 mM H₂O₂, (C) 12 mM H₂O₂. Cells were cultured in LB-kanamycin medium at 37°C, 200 rpm. Each data point is the mean of three replicates. The OD is measured at 600 nm.

Since the aforementioned growth performances were evaluated by seeding the overnight grown stationary phase cells, we also investigated the oxidative stress tolerance of mid log phase cells. For this purpose, we introduced 12 mM H₂O₂ 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 (Fig. 4.2). The inhibition was more prominent in the WT as its OD only reached 1.03. Because OM3 displayed the best viability at high H₂O₂ concentration, it was chosen for subsequent investigation.

Fig. 4.2: Cell growth profile after the introduction of 12mM H₂O₂ during mid log phase (OD₆₀₀ 0.65). Each data point is the mean of three replicates. The OD is measured at 600 nm.

4.3.3. Mutant thermotolerance and its tolerance to cumene hydroperoxide

Since inorganic hydroperoxide H₂O₂ 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 OD₆₀₀ ~2.8 (Fig. 4.3A). Moreover, earlier publications on the interrelationship between oxidative stress and thermotolerance encouraged us to evaluate the thermotolerance of OM3 [44]. As shown in Fig. 4.3B, OM3 demonstrated better growth (0.52 h⁻¹) than WT (0.38 h⁻¹) at 48°C.

Fig. 4.3: 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. The OD is measured at 600 nm.

4.3.4. 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 Appendix Table S1 and Table S2. 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 [28, 45]. 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 H₂O₂ treatment (Table 4.2A). 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 H₂O₂ 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 4.2B). 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.

Table 4.2A: DNA microarray data of certain genes in OM3 after H₂O₂ treatment ($p < 0.05$, Log₂ Fold Change > 2.0)

Regulator	b number	Gene	Log ₂ Fold Change
CRP	b4036	<i>lamB</i>	-4.780
	b4034	<i>malE</i> *	-4.257
	b0598	<i>cstA</i> *	-5.093
RpoS	b3517	<i>gadA</i> *	7.766
	b1493	<i>gadB</i>	7.096
	b1492	<i>gadC</i>	7.024
	b1732	<i>katE</i> *	3.801
	b1482	<i>osmC</i>	2.755
	b4376	<i>osmY</i>	3.062
	b1896	<i>otsA</i> *	2.996
OxyR	b1684	<i>sufA</i>	3.336
	b1683	<i>sufB</i>	3.483
	b1681	<i>sufD</i>	3.140
	b1680	<i>sufE</i>	2.551
	b1679	<i>sufS</i>	2.771

* - Analyzed by qRT-PCR (Table 4.3)

Table 4.2B: DNA microarray data of certain endogenous genes in OM3 ($p < 0.05$, Log₂ Fold Change > 2.0)

Regulator	b number	Gene	Log ₂ Fold Change
CRP	b4036	<i>lamB</i>	-8.997
	b4034	<i>malE</i> *	-8.930
	b4035	<i>malK</i>	-8.502
RpoS	b3517	<i>gadA</i> *	4.517
	b1493	<i>gadB</i>	4.571
	b1732	<i>katE</i> *	2.701
	b1896	<i>otsA</i> *	2.581

* - Analyzed by qRT-PCR (Table 4.3)

qRT-PCR was carried out on ten selected genes to validate the microarray results [28, 46, 47]. Without H₂O₂, the expression of *katE*, *gadA*, *crp*, *cya* and *otsA* were all up-regulated in OM3 as compared to WT, whereas *sodA*, *cstA*, *ahpF* and *malE* demonstrated down-regulation, which agreed with the microarray data (Table 4.3). 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 4.3).

Table 4.3: DNA microarray and qRT-PCR data comparison of ten selected genes in OM3

Gene	Log ₂ Fold Change in DNA microarray		Log ₂ Fold Change in qRT-PCR	
	Without Stress	Under Stress	Without Stress	Under Stress
<i>sodA</i>	-0.115	0.719	-0.176	0.185
<i>katE</i>	2.701	3.801	3.132	3.048
<i>ahpC</i>	-0.518	0.667	-0.302	-0.031
<i>ahpF</i>	-1.051	-0.374	-0.893	-0.483
<i>gadA</i>	4.517	7.766	5.023	7.646
<i>cstA</i>	-3.757	-5.117	-1.903	-5.441
<i>otsA</i>	2.580	2.996	3.199	2.906
<i>malE</i>	-8.930	-4.257	-9.587	-4.192
<i>crp</i>	4.189	3.890	3.362	2.634
<i>cya</i>	2.240	1.263	0.215	0.755

4.3.5. Intracellular reactive oxygen species (ROS) level

Oxidative stress in extracellular medium may alter the intracellular peroxide and other ROS level [48] and thus determine cell viability [27]. The normalized fluorescence intensity suggested that the ROS level in OM3 was always lower than that of WT irrespective of growth with or without H₂O₂ (Fig. 4.4). In the absence of oxidative stress, OM3 possessed 2.5 times lower intracellular ROS compared to WT. Incubation with 4 mM H₂O₂ elevated the free radical level in both strains, diminishing the difference to around 1.4 times.

Fig. 4.4: Intracellular ROS level in OM3 and WT with cells treated with or without 4 mM H₂O₂. Mid exponential phase grown cells (OD₆₀₀ 0.6) were incubated with 10 μ M H₂DCFDA (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.

4.4. Discussion

In this study, we have successfully enhanced *E. coli* oxidative stress tolerance *via* engineering its global regulator CRP. H₂O₂ was preferred as the stress-inducing agent in this study because it is a highly oxidizing agent and on dissolution, it forms reactive oxygen species to simulate *in vivo* oxidative stress condition. A pool of variants ($\sim 10^5$) was created by error-prone PCR. The library was then screened with H₂O₂ and three mutants (OM1~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 (Fig. 4.5) [49]. R82 sets a pivotal role in cAMP binding due to the electrostatic interaction between the guanidium group of R82 and the electronegative oxygen of the phosphate group of cAMP [50]. V139 is in the hinge region and participates in the interdomain interaction between N- and C-terminals of CRP [51]. We assume that the amino acid substitutions in OM3 might have altered CRP conformation and cAMP binding, which probably led to the altered transcription profile in *E. coli* as proven by the microarray data.

Fig. 4.5: Amino acid mutations in OM3 (pdb: 1G6N). The main carbonyl of F69 interacts with the amine group of R123. The guanidinium 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.

Since our target regulator CRP is a global regulator of *E. coli*, 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 [52]. The repression of these genes in OM3 supported previous reports on the overlap between oxidative stress and acid tolerance response [47]. *mal* operon is regulated by CRP directly [53], 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* [54], among which *gad* superfamily, namely *gadABC*, displayed maximum up-regulation under stress by microarray (~7-fold). Glutamate decarboxylase (encoded by *gad*) can convert intracellular glutamate to γ -amino butyric acid and is also associated with acid tolerance response of *E. coli* [46]. 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 (Fig. 4.6A). The direct reason of GAD activation due to oxidative stress is still unknown. However, it has been acknowledged that *gad* expression may result from extracellular acetate accumulation [55] where acetate is a by-product of *E. coli* metabolism[56-58]. Hence, we believe that stress induced increased metabolism may have led to elevated acetate formation which in turn, enhances *gad* transcriptional output. Engineered CRP in OM3 might have improved this transcriptional machinery through activating other positive regulator such as RpoS.

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 H₂O₂. 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 (Fig. 4.6B).

Table 4.4: qRT-PCR primers used in this study^a

Primer	Sequence
<i>crp_F</i>	TCAGAGAAAGTGGGCAACCTGG
<i>crp_R</i>	GATGGTTTTACCGTGTGCGGAG
<i>cya_F</i>	AGATTGATCAGGTGCGTGAGGC
<i>cya_R</i>	AAATCTGCGGGTTTACCAGCGT

<i>sodA_F</i>	GCTATCGAACGTGACTTCGGCT
<i>sodA_R</i>	TCAGCGGAGAATCCTGGTTAGC
<i>katE_F</i>	GCCAAACTGCTCTACTCCCGAA
<i>katE_R</i>	CTTCACCCTGGTCAGCGATCTT
<i>gadA_F</i>	GAAGAATATCCGCAATCCGCAG
<i>gadA_R</i>	GAGCATACAGGCCTCGGAAGAA
<i>cstA_F</i>	TTCCATGCGCTGATCTCTTCTG
<i>cstA_R</i>	TGTTTCATGGCAAAATACACGCC
<i>otsA_F</i>	TGCCGACATATGACACCTTGCT
<i>otsA_R</i>	TCAATGCCGATCGGGTAGACTT
<i>malE_F</i>	CGAAACAGCGATGACCATCAAC
<i>malE_R</i>	CCGCTTCCAGACCTTCATCAGT
<i>ahpC_F</i>	GCAGGGTATCATCCAGGCAATC
<i>ahpC_R</i>	GAGACGGAGCCAGAGTTGCTTC
<i>ahpF_F</i>	GATCCCAGCAGCAGTTGAAGGT
<i>ahpF_R</i>	ACGCCTTTGGTGCGATACTGAT
<i>rrsG_F</i>	TCAAGGGCACAACCTCCAAGTC
<i>rrsG_R</i>	GGTGTAGCGGTGAAATGCGTAG

^a - Primers are designed by Primer3 software (www.simgene.com/Primer3)

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]. Moreover, *katE*, *osmCY* being regulated by RpoS [55], transcriptional up-shift of these genes in OM3 again supported the idea of stress induced activation of the trio: engineered CRP-RpoS-stress response genes.

Other major regulons associated with *E. coli* oxidative stress are SoxRS and OxyR, with the latter being suggested as a more specific regulator of H₂O₂ responsive pathways [28]. 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 H₂O₂. Although *suf* operon is regulated by OxyR, it is interesting to find the upregulation of the former despite later's downregulation. Actually, *suf* operon is regulated by several regulators such as OxyR, IHF [61], Fur (ferric uptake regulator) and IscR (Iron-sulphur cluster regulator). Among these, Fur represses the *suf* expression whereas the rest regulators activate it. In our study, Fur and IscR exhibited little down and upregulation (fold change < 1.0) respectively, whereas IhfA showed notable upregulation (1.389 fold). We suggest that all these factors, coherently contributed to the activation of *suf* operon in OM3 under oxidative stress. 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 (Fig. 4.6C). Since AhpC is only active with AhpF present [62-64], our findings probably have suggested that *ahpCF* are not major players in oxidative stress defense of OM3. In this context, the microarray finding of stress specific *ahpC* upregulation in OM3 seems to be an artifact. 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 (Fig. 4.6D), indicating that SOD, similar to alkyl hydroperoxide reductase, did not play an important role in the antioxidant machinery of OM3. Since SoxRS and OxyR are two independent regulators working *via* different mechanisms to relieve oxidative stress, the results imply that OxyR mediated antioxidant defense mechanism is more prevalent in OM3 than that of SoxRS.

Fig. 4.6. Enzyme activity assay. (A) glutamate decarboxylase (GAD) (B) catalase (C) alkyl hydroperoxide reductase (AhpCF) (D) superoxide dismutase (SOD). Each data was the mean of three independent observations.

OM3 also exhibited better thermotolerance than WT when exposed to 48°C, which was consistent with the earlier finding that there was an overlap between heat shock and oxidative stress defense mechanism *via* heat shock protease HtrA [65] and heat shock proteins IbpA/B [66]. 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 and down-regulation of *sohA* (putative protease of HtrA [67]). The findings also comply with the previous report that manipulating *cya* gene (encoding adenylate cyclase responsible for CRP

activation) could trigger in *E. coli* thermotolerance without inducing heat shock proteins [68]. 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 [69]. 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 Fig. 3, baseline concentration of endogenous 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. 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 [70]. 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.

Exertion of stress often induces modification to cellular morphology [71]. Interestingly, OM3 underwent no significant change in cell length in the presence or absence of H₂O₂ as shown by the micrographs (Fig. 4.7). However, the exterior examination of cells revealed that OM3 cell surface had undergone uneven modifications in either conditions exposing coarser topology in presence of H₂O₂. We consider that these modifications might be a morphological response towards oxidative stress.

Fig. 4.7. FESEM micrographs of WT and OM3. (A) WT, 0 mM (B) OM3, 0 mM (C) WT, 4 mM H₂O₂ (D) OM3, 4 mM H₂O₂

In this work, OM3 could survive and reach stationary phase of OD₆₀₀~3.0 in 12 mM H₂O₂ whereas the maximal survival limit of WT was 4 mM H₂O₂ (data not shown). The only report so far to acquire non-pathogenic *E. coli* tolerance over 12 mM H₂O₂ was after adapting cells to glucose starved condition [72]. Metabolic engineering approaches of introducing heterogeneous genes such as *grx* (glutaredoxin) [73], *oscyp2*(rice cyclophilin) [74], and *prrA* (a pleiotropic protein promoting DNA repair in radiation-induced damage) [75] could only improve *E. coli* cell tolerance against 5 mM H₂O₂, while incorporating *Brgr* (encoding glutathione reductase from *Brassica rapa*) [31] helped improve *E. coli* tolerance against 1.5

mM H₂O₂. Classical strain engineering approaches using ethyl methane sulphonate (EMS) or UV did not result in significant improvement of *E. coli* tolerance towards oxidative stress [76, 77]. Besides H₂O₂, earlier research has improved *E. coli* tolerance towards 0.1-0.4 mM cumene hydroperoxide *via* spontaneous adaptation [78] or chemical mutagen (diethyl sulfate) treatment [79]. Previous studies suggested that a population size $\sim 10^9$ cells was required to isolate bacterial mutants with tolerance towards cumene hydroperoxide [79]. By comparison, we were able to isolate three oxidative stress tolerant mutants from a library size of $\sim 10^5$. Also, it took only 6 ~7 days to isolate potential mutants through our methodology compared to weeks or months by other strain engineering methods. Without pretreatment, the best mutant OM3 exhibited efficient growth against 0.3 mM cumene hydroperoxide, which was comparable to other publications [78]. Hence, together with our previous works, we believe that this isogenic transcriptional engineering approach could provide a promising alternative for *E. coli* strain engineering.

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Chapter 5: Error-prone PCR of cyclic AMP
receptor protein (CRP) for improvement of cell
low pH tolerance

5.1. Introduction

Bioprocesses through large scale fermentation have become popular in last few decades. One of the shortcomings of using microbes during fermentation is the stringent reaction conditions that often, remain adverse for microbial survival inside bioreactor. Moreover, biocatalytic reactions often require multiple extreme conditions which native microbial hosts rarely satisfy. In order to address such problems, strain engineering has emerged as a popular strategy. Microbial strains have been traditionally manipulated basically *via* two approaches. The first is rational approach, which aims to engineer microbial metabolism either by cloning of exogenous gene(s) or knocking out of specific gene(s) [1]. With the metabolic landscape of a microbe being difficult to deconvolute, challenges remain in rewiring metabolic network to bring in satisfactory phenotypic outputs [2]. Furthermore, metabolic engineering often requires proper choice of vector to score phenotype with adequate metabolic fitness. Depending on single gene based sequential search optimization, it is difficult to orchestrate a broader subset of genetic space and hence the method often falls back to reach the desired optimum [3, 4]. The second approach, namely random approach, is strategized to manipulate bacterial genome by introducing mutations randomly either by spontaneous adaptation or by using physical (UV) and chemical (nitrosoguanidine, ethyl methane sulphonate) mutagens [5]. Although the method has been successful in retrieving phenotypes in certain cases such as enhancing *E. coli* organic solvent tolerance [6] or enhancing microbial productivity of lipase [7]; this method needs huge population of cells as well as serial rounds of mutagenesis for isolation of a single desired variant [6].

Transcription engineering, a new approach from combinatorial engineering, has started to attract attention in strain engineering over the last few years [8-15]. Since transcription engineering undertakes manipulation on transcriptomic level, chances are more likely to reprogramme cell behaviour through remodelling the genetic network [16]. Global regulators

regulate the expression of a cascade of genes from various metabolic pathways [17]. Thus, modifications to these global regulators could reframe a broader subset of genetic space inside host cell. Our lab has successfully rewired global regulator cAMP receptor protein (CRP) to enhance *E. coli* tolerance towards 1-butanol, NaCl and organic solvents [18-20]. The objective of this study is to explore the potential of engineering CRP, the widest controlling *E. coli* global regulator, to harness acid tolerant mutant cells.

Acquisition of low pH or acid tolerant mutant is of industrial importance since certain biocatalytic operations suffered limitations due to cell viability decline at low pH especially during the production of lactic acid, succinic acid and 3-hydroxy propionic acid [21-23]. Measures have been taken to elevate cell acid tolerance such as pre-adaptation to acid [24] or inducer of acid tolerance response such as glucose [25], cross adaptation to other stresses such as heat shock [26] and cold shock [27]. One of the shortcomings of these methods is that, the acidulant type may influence cell acid response [28, 29]. The presence of inducer glucose may also exert elevated osmotic pressure over cells hence lowering biomass production [25]. Chemical mutagenesis with nitrosoguanidine (NTG) has also been applied to improve cell low pH tolerance [30] together with combinatorial approach such as genome shuffling [31]. Nonetheless, the adopted methods failed to engineer *E. coli* strain capable of withstanding pH below 4.4.

CRP is known to have negative regulatory effect on acid-tolerance-associated genes *gadAB* [32] and *fur* [33, 34], which suggests that CRP is a potential target for engineering *E. coli* strain under low pH conditions. Here, I intend to perform error-prone PCR to create random mutagenesis CRP libraries followed by enrichment selection. The “winners” would be verified by their growth performance in low pH systems. Due to the overlap between acid and thermal stress response, the thermotolerance of the best mutant would also be checked.

5.2. Materials and Methods

5.2.1. Materials

E. coli DH5 α was obtained from Invitrogen (San Diego, USA) and Δcrp strain was constructed *via* a method described previously [19]. Modified LB medium (Bacto Tryptone 10g/l, yeast extract 5g/l, NaCl 5g/l) was used as culture medium. All restriction enzymes were procured from Fermentas (Burlington, Canada). All the components of LB were procured from Merck (E-Merck, Germany). T4 DNA ligase from New England Biolabs (Ipswich, MA, USA) was used for ligation. Citric acid and dipotassium hydrogen phosphate (K₂HPO₄) were from Amresco (Solon, USA) and E-Merck (Germany) respectively. Low copy number plasmid pKSCP containing native *crp* operon was obtained from our previous work [19]. The Δcrp strain harbouring plasmid pKSCP is denoted as wild-type (WT).

5.2.2. Construction of variant library

Error-prone PCR of *crp* was performed using Genemorph II random Mutagenesis kit (Stratagene, La Zolla, USA)) with primers 5'-gagaggatccataacagaggataaccgcgcatg-3' and 5'-agatggtaccaaacaaaatggcgcgctaccaggtaacgcgcca-3'. The error-prone PCR was performed with 30 ng of pKSCP (containing native *crp* operon) plasmid obtained from our previous studies as template [18-20], 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 PCR product was purified from 1.2% low melting agarose gel using QIAquick Gel Extraction Kit (Qiagen, Germany). Restriction enzymes *Bam* HI and *Kpn* I were used to clone mutated *crp* into the digested pKSCP vector. The ligation mixture was transformed into *E. coli* Δcrp competent cells by electroporation.

5.2.3. Mutant Selection

The mutants were selected based on acid shock method [35, 36]. The random mutagenesis library was cultured overnight at 37°C, 200 rpm in 10 ml modified LB medium to stationary phase. One percent of the overnight culture was inoculated into fresh 10 ml medium and incubated at 37°C, 200 rpm. After the culture had grown up to mid log phase ($OD_{600} \sim 0.6$), 110 μ l of concentrated HCl was added into the medium to bring to pH 3.0. The cells were then incubated for 2 h, harvested, washed with fresh LB thrice, and spread onto LB-kanamycin agar plates incubated at 37°C for 18 h .

5.2.4. Plasmid isolation and sequencing

Total 29 individual colonies were selected and their plasmids were isolated by QIAprep. The mutations were verified by DNA sequencing. The mutant *crp* genes were then digested, cloned into fresh pKSCP plasmids, back-transformed into fresh Δcrp background to eliminate background interference.

5.2.5. Mutant growth

Individual fresh clones (both mutants and WT) were cultured overnight in LB medium. One percent (v/v) of the overnight culture was inoculated into phosphate-citrate buffered [37] LB medium containing 25 μ g/ml kanamycin with an OD_{600} of 0.05~0.06. The cells were subjected to various pH conditions ranging from pH 7.5 to 4.34. Samples were withdrawn periodically and cell growth was evaluated at 600 nm.

5.2.6. Thermotolerance

Both mutant and WT were cultured overnight up to stationary phase. One percent of the overnight culture was inoculated into LB-kan medium to an OD_{600} around 0.5. The cells were

then allowed to grow at 48°C, 200 rpm. Samples were withdrawn at periodic intervals and cell growth was monitored spectrophotometrically at 600 nm.

5.2.7. Glutamate decarboxylase assay

Evaluation of the Glutamate decarboxylase (GAD) enzyme activity has been performed as published earlier [38, 39]. Briefly, Bromocresol green (BCG) is a pH indicator dye that can change color within pH 3.8-5.4. At a temperature of 40°C, decarboxylation of monosodium salt of L-glutamic acid (MSG) is catalyzed by GAD resulting in pH drift towards alkaline range. This pH up-shift causes a color change of BCG from yellow to blue that can be monitored at 620 nm. In this investigation, the reaction system was comprised of 10 mM MSG, 10 mM pyridoxal 5'-phosphate, 70 µM BCG as pH indicator, 60 µg of cell protein and 20 mM acetate buffer (pH 4.8) as medium. One unit of glutamate decarboxylase activity was defined as µmoles of MSG consumed per min.

5.2.8. Reporter gene assay for CRP-Promoter interaction

As reported earlier [39], three classes of CRP - dependent promoters have been cloned on plasmid pPR9TT plasmid [40] to construct recombinant plasmids pPRO1, pPRO2, and pPRO3 respectively. Each promoter bearing plasmid, together with either control or mutant plasmid, was co-introduced in CRP knockout strain of DH5α. The resulting colonies were cultured up to mid log phase of growth (OD₆₀₀~ 0.6 – 0.8). 350 µl of cell suspension from each dual plasmid harbouring strain was used for the assay. The assay was continued with yeast β-galactosidase assay kit (Thermo Scientific, USA) according to manufacturer's instructions. The end point was determined by the formation of yellow color of *o*-nitrophenol formed from *o*-nitrophenyl-β-D-galactopyranoside (ONPG) due to the β-galactosidase activity. The quantization of CRP-promoter binding was scaled on the degree of β-

galactosidase activity that exhibited differential color changes for different promoters measured at 420 nm.

5.3. Results

5.3.1. Mutant selection

After electroporation and cultivation in 1 ml SOC medium, the library size was approximately $\sim 10^4$. Following the acid shock and plating of the colonies, we selected 29 survived colonies. Plasmid isolation, retransformation into fresh host cell followed by preliminary screening by growth performances at pH 4.50, scored two clones significantly better than the WT. Sequencing of the respective *crp* genes showed that two mutants had been isolated through our library creation and selection (Table 5.1). The mutant AcM1 revealed two amino acid substitutions F69C and V139M whereas mutant AcM2 contained only one point mutation V86L.

Table 5.1: Amino acid substitutions in mutants obtained

Mutant	Amino acid substitution	
AcM1	F69C	V139M
AcM2	V86L	

5.3.2. Mutant growth profile

Mutant growth was analyzed by cultivating cells at low pHs with normal medium (pH 7.5) as control. In normal LB medium (pH 7.5) (Fig. 5.1A), mutants and WT shared similar growth pattern, with mutants had slightly higher growth rate (0.79 h^{-1}) than WT (0.74 h^{-1}). At pH 4.50 (Fig. 5.1B) and 4.34 (Fig. 5.1C), all mutants exhibited better growth than WT. At pH 4.50, AcM1 had the best growth rate of 0.141 h^{-1} while that of WT was 0.129 h^{-1} . Mutants

stationary phase OD were also found higher compared to WT. AcM1 attained best stationary phase OD₆₀₀ at 1.76 while AcM2 and WT grew up to OD 1.42 and 1.16 respectively.

Improved phenotype of the mutants was also confirmed by the evaluation of the cell growth profile at pH 4.34. In this pH too, all mutants' growth performances were superior compared to WT. For example, both AcM1 and AcM2 showed similar growth rates of 0.1171 h⁻¹ and 0.1186 h⁻¹ respectively while WT growth rate was 0.0918 h⁻¹. AcM1, which had demonstrated the best growth performance, was considered as the best mutant and chosen as the model organism for subsequent studies.

Fig. 5.1: Cell growth profile at various pH. A: normal LB at pH 7.5, B: pH = 4.50; C: pH = 4.34. Overnight grown cells were freshly inoculated into modified LB-kan medium buffered with phosphate-citrate buffer at desired pH. Cells were cultivated in 10 ml final medium at 37°C, 200 rpm. The growth was monitored spectrophotometrically at 600 nm.

5.3.3. Mutant thermotolerance

In order to evaluate mutant tolerance to multiple stresses, we subjected both mutant and WT to high temperature. As shown in Fig. 5.2, AcM1 displayed much better growth than WT at 48°C—AcM1 reached stationary phase OD₆₀₀ at 0.121, while the growth of WT was completely inhibited at this temperature (OD ~ 0.05).

Fig. 5.2: Cell thermotolerance of AcM1 and WT. The OD is measured at 600 nm.

5.3.4. Glutamate decarboxylase assay

Glutamate decarboxylase (GAD) activity evaluation was performed both in AcM1 and WT. It showed almost similar activity in either strain in absence of stress; although under stress, the enzyme revealed more than two fold activity enhancement in the mutant (Fig. 5.3). Specifically, without stress, GAD activity in AcM1 and WT was noted be around 150.40

U/mg and 138.56 U/mg respectively. However, upon stress exertion, the induction of GAD activity was more pronounced in AcM1 (1625.92 U/mg) compared to WT (727.437 U/mg).

Fig. 5.3: Glutamate decarboxylase activity of AcM1 and WT cell lysates both at normal (6.85) and acidic pH (4.50). The assay was performed by L-MSG decarboxylation method and end point was determined by yellow to blue conversion of Bromocresol green at 620 nm.

5.3.5. Reporter gene assay for CRP-Promoter interaction

The assay was performed by β -galactosidase activity evaluation resulted from downstream coding of the reporter gene by CRP- promoter interaction. The findings revealed that WT and mutant CRP bind differentially with Class-I and Class-III promoters, although the effect is less pronounced in case of Class-II promoter (Fig. 5.4). For example, for Class-I promoter, the mutant and WT β -galactosidase activities were recorded as 51.16 and 242.92 $\text{ml}^{-1} \text{min}^{-1}$

respectively. Likewise, when CRP was allowed to bind with Class-III promoter, β -galactosidase activity was noted as 43.70 $\text{ml}^{-1} \text{min}^{-1}$ for mutant and 150.93 $\text{ml}^{-1} \text{min}^{-1}$ for WT. However for Class-II promoter, the activity induction by both mutant and WT CRP was found similar (30.66 and 35.30 $\text{ml}^{-1} \text{min}^{-1}$).

Fig. 5.4: Reporter gene assay for CRP-Promoter interaction in both AcM1 and WT. This was evaluated by activity estimation of β -galactosidase, which had been expressed due to binding of both mutant and WT CRP with respective promoters.

5.4. Discussion

In this study, we have successfully improved *E. coli* acid tolerance *via* engineering its global regulator CRP. Phosphate-citrate buffer at different pH values was used to induce low pH stress over the cells. Two acid tolerant mutants (AcM1~AcM2) were selected from a

library of 10^4 cells based on acid shock method. The best mutant AcM1 also revealed resistance against elevated temperature.

We found that simple modifications to global regulator CRP could result in enhanced strain tolerance towards oxidative stress. As for the best mutant AcM1, it obtained two mutations *via* error-prone PCR (F69C, V139M). F69 contributes in CRP conformation, which is reoriented upon cAMP binding with the interaction between F69 and R123 [41]. V139 is in the hinge region and participates in the interdomain interaction between N- and C-terminals of CRP [42]. We assume that the amino acid substitutions in AcM1 might have altered CRP conformation and cAMP binding, which probably led to the altered transcription profile in *E. coli*.

CRP is a global transcription regulator that initiates transcriptional activation by binding with specific promoters [43-45]. Since modifications to CRP molecule had reprogrammed transcription profile of a plethora of genes, we tried to investigate CRP-DNA binding pattern in both the strains. The CRP-promoter binding assay indicated that mutation in CRP has altered the binding affinity especially with Class-I and Class-III promoters. We assume that change in promoter binding has resulted in differential transcriptional activation which, in course, has rewired the cell transcriptome.

The mutants showed better cell density than WT in all the low pH cultivation systems. In this study, we used modified LB as the media by adding NaCl (5g/L). Earlier studies reported that incorporation of more than 150 mM sodium chloride increases acid sensitivity of the cells [46]. Since normal LB contains 10g/l NaCl i.e. ~172 mM NaCl, we reduced NaCl to half of its original concentration (5g/l or 86 mM NaCl).

All the mutants exhibited improved growth performance at both pH 4.50 and 4.34 compared to WT. It has been published earlier that *E. coli* could not grow below pH 4.4 [47]

either in the complex or in the defined minimal medium. But the mutants obtained in our study could withstand pH as low as 4.34. Moreover, the acid adapted cells exhibited about 10% growth rate at pH 4.5 compared to control [47] while our mutants elicited an 18% growth rate compared to control (grown at normal LB). Hence our mutant performances are quite comparable to the results published so far.

CRP is reported to have important role in cell acid tolerance. Reportedly, it has four acid tolerance (AR) mechanisms. First one, the mechanism is independent on the amino acid synthesis and relies heavily on catabolite repression [48]. Activation of the system is triggered when *E. coli* is grown in complex media such as LB in presence or absence of glucose. The authors reported the chief regulators of this acid resistance mechanism are CRP and sigma factor (σ^s). Glucose induces acid tolerance in logarithmic phase cells of *E. coli* which is reverted by addition of cAMP in the medium [25]. This experimental outcome first suggested that CRP activation represses the acid tolerance mechanism of *E. coli* whereas glucose induced CRP repression actually increases the cell acid tolerance. In addition, CRP being a negative regulator of σ^s in *E. coli* [49], it can be inferred that glucose-repression mediated σ^s activation can be related with improved cell acid tolerance. Our reporter gene assay results also suggest that mutant CRP has weaker promoter binding than that of the WT. This implies partial deactivation of CRP which probably has promoted σ^s activation inside mutant cell. However, total deletion of CRP gene can have a significant deleterious effect on the cell phenotype [48] probably due to damage of several other transcriptional networks.

CRP also contributes to cell acid tolerance by regulating acid resistance system 2 (AR2). This system requires activation of glutamate decarboxylase enzymes (GAD ABC). The GAD system encompasses two putative enzymes GAD AB for glutamate conversion to γ -amino butyric acid where GAD C encodes a putative glutamate: GABA antiporter [50]. Researchers have acknowledged that GAD is regulated directly by σ^s which as described earlier, is

negatively regulated by CRP [48]. In fact, CRP has a weak promoter binding site located between -52 bp to -73 bp from GAD transcriptional start site [51] which may interfere with the σ^S mediated regulation of GAD. In order to investigate CRP mediated GAD regulation and in our mutant acid tolerance, we evaluated GAD activity in both our mutant as well as WT. Interestingly, under acidic environment, we found about 2-fold activity up-regulation of GAD in the mutant with respect to the WT. This finding suggests that, CRP engineering has de-repressed the GAD activity which concurs with the previous finding of partial CRP deactivation and subsequent σ^S upregulation.

Another important question is that did AR1 contribute to mutant acid tolerance just because of CRP mediated de-repression of σ^S or there was any other mechanism behind? Castanie-Cornet et al 1999 reported that excess glutamate addition into the media could eventually restore the acid resistance of a CRP-knockout mutant [48]. They proposed that CRP might contribute to intracellular glutamate synthesis by some metabolic pathway. Hence it is plausible to believe that certain point mutations in our CRP may have activated this pathway thereby inducing the RpoS mediated AR1 system.

Both the mutants and the WT were subjected to thermotolerance investigation where mutants exhibited better thermotolerance by achieving more cell density in the same culture condition. The better tolerance to elevated temperature suggested the overlap between acid tolerance and thermotolerance which has been also reported by other authors [26, 29, 52]. In a recent work, Lee et al. 2008 also reported that engineered CRP encoded artificial transcription factor could lead to increased thermotolerance of *E.coli* [53]. A set of genes such as *ompW*, *cpxP*, *yliH* and regulons like MarABR were differentially expressed indicating that CRP engineering can perturb certain genes to improve cell thermotolerance. We also believe that CRP modification has reprogrammed similar kind of thermosensor genes to elevate cell thermotolerance.

Previous strain engineering approaches on acid tolerance reported improvement of strain performance by strategies such as pre-adaptation [28, 54], activation of acid tolerance system by glucose or arginine [25, 55], tolerance induction by introducing medium filtrate to exponential phase of cells [56] or cross adaptation of cells to other kinds of stresses [57]. But the limitations of these methods are these methods are either time consuming depending on heavy population of cells or rely on exogenous addition of components. In addition, strategies have been furnished to harness acid tolerant *E. coli* cells by recombinant introduction of foreign genes such as *dnaK* [58] or *orfB*, *orfC* [21]. But our method of random mutagenesis had scored successful phenotypes within a few days. Furthermore, CRP being a global regulator, engineering CRP could harness tolerant cells against various other stresses such as high temperatures. Hence CRP engineering could be an alternative approach in engineering microbes against low pH.

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Chapter 6: Summary and Conclusion

In order to surpass time and labour intensive metabolic engineering approach strategies together with the classical mutagenesis approach that requires multiple rounds of mutagenesis as well as very broad library width, transcription engineering has started captivating interest for last few decades. Among various kinds of transcription engineering, global transcription machinery engineering (gTME) has emerged as a potential tool for its viability to perturb larger sets of functional genomic space thus rewiring the transcription network inside cell. Cyclic AMP receptor protein (CRP) is widest controlling global transcription regulator controlling more than 400 genes in *E. coli*. Thus in this study we have undertaken CRP engineering to reprogramme cell transcriptional network with subsequent selection of desired trait. To isolate desired traits we have chosen phenotypes that are industrially important and tolerant against stresses like organic solvent, oxidative stress and low pH stress.

For the organic solvent stress tolerant phenotype we isolated variants that showed enhance growth performance against 0.23% (v/v) toluene. The F136I CRP variant showed best strain improvement and was chosen as model organism for the subsequent studies. It also showed tolerance against 0.23% (v/v) *p*-xylene, 3% (v/v) *n*-hexane as well as 1% (v/v) cyclohexane. It showed improved growth against 6 mM H₂O₂ and pH 4.50 as well to demonstrate mutant's multistress resistant potential. We performed MATH test to characterize cell surface hydrophobicity of both our mutant and the WT which revealed that our mutant has always low cell surface hydrophobicity compared to WT. qRT-PCR analysis demonstrated that certain previously reported stress tolerant genes such as *acrAB*, *manXYZ*, *glpC* also showed upregulation in our mutant than the WT. These genes basically relating with membrane bound efflux pumps as well as sugar transport, it seems that solvent extrusion together with energy production are important underlying mechanisms behind the engineered cell tolerance. In contrast, genes such as *marA* and *zwf* exhibited downregulation whose reason was unclear and need further investigation. Metabolic genes such as *fumC* and *fladA* did not

reveal much difference in their transcript levels in between mutant and WT signifying undisturbed central metabolism in cells both in presence and absence of stress. Although mutant strain showed significant difference in cell surface hydrophobicity as well as gene expression profile, interestingly it did not reveal morphological change when studied under Field Emission Scanning Electron Microscopy (FESEM).

We also engineered CRP to improve cell oxidative stress. Error prone PCR was the random mutagenesis technique to introduce point mutations in CRP. The best mutant (OM3) isolated contained three amino acid substitutions such as F69C, R82C and V139M. OM3 exhibited growth against H₂O₂ as high as 12 mM. Furthermore, it also demonstrated growth against other stressor such as cumene hydroperoxide (0.3 mM) and temperature as high as 48°C. DNA microarray analysis and qRT-PCR revealed that RpoS regulated genes such as *gadABC*, *katE* showed intensive upregulation whereas genes such as *ahpCF* (OxyR regulated), *lamB*, *malE*, *malK* (CRP regulated) showed downregulation. The results indicated that RpoS contributed significantly to augment mutant cell tolerance. The observations also suggested that *gadAB* mediated acetate detoxification together with *katE* (encoding catalase HP-II) mediated free radical scavenging were two important machineries for improved cell oxidative stress tolerance. qRT-PCR and enzymatic assays also confirmed the findings. Interestingly, this phenotype revealed stress induced morphological roughness revealed in both mutant and WT.

After successful construction of the *E. coli* phenotype against organic solvent and oxidative stress, we tried to construct low pH tolerant variants through gTME. Variant CRP library construction and subsequent phenotype selection at pH 3.0 were persuaded to isolate strains AcM1 and AcM2. Both AcM1 and AcM2 CRP were sequenced to identify the point mutations as F69C V139M and V86L on respective CRP reading frames. The low pH tolerance of the mutants was confirmed at pH 4.50 and 4.34 where both the mutants

demonstrated improved growth performance than the WT. The variants, furthermore, showed better growth profile at 48°C as well compared to the WT. Since glutamate decarboxylase (GAD) is one of the chief enzymes contributing to cell acid tolerance, we tried to characterize our cell acid defense system by evaluating this enzyme activity inside our best mutant (AcM1). Although both the cells exhibited similar GAD activity without stress, AcM1 GAD activity increased about two fold compared to WT at pH 4.50. Because modifications to CRP has resulted in change of enzyme activity as well as acid tolerance, we further tried to reveal the role of the CRP mutations in changing the transcriptional activation of the corresponding genes. We studied mutant as well as WT CRP binding profiles on all three CRP dependent promoters. Interestingly, mutant CRP displayed differential binding affinity to Class-I and Class-III promoters than the WT suggesting CRP engineering could really alter the DNA activation and subsequent transcription profile of the regulated genes.

Altogether, we have proved that engineering global transcription factor such as CRP could bring in desired traits by rewiring cell transcriptome. The method needs only a few days to be accomplished compared to other strain engineering methods that require weeks to months to yield a single phenotype. Also, the strategy required lower library size ($10^4 \sim 10^6$) of cells compared to traditional mutagenesis methods. For example, from earlier citations, isolation of one cyclohexane tolerant strain through spontaneous adaptation required 10^6 cells while isolation of one *p*-xylene tolerant strain by NTG mutagenesis required a library population of 10^{19} . Moreover, the mutant performances from our methods are also comparable to that of other traditional methods. For instance, in case of oxidative stress tolerant trait construction, our best mutant could tolerate 12 mM H₂O₂ whereas recombinant DNA introduction could only elevate *E. coli* cell tolerance upto 5 mM H₂O₂. We have also proved that our mutants could tolerate multigenic stresses such as withstanding different organic solvent stresses altogether by a single engineered mutant or both oxidative and thermotolerance by a specific

CRP variant strain. In fact, low pH tolerant variant also revealed thermotolerance that eventually simulate construction of multistress resistant strain through CRP engineering. Thus, we propose that engineering global transcription factor such as CRP could be a promising and potential strategy for strain engineering. The synopses of the methodologies and outcome of our studies have been summarized in Table 7.1.

Table 7.1. Table of summary of the mutants, relevant phenotype and genotype characterizations

Sl No.	Stress encountered	Mutants isolated	Amino acid substitutions	Best Mutant	Cross tolerance	Other comments
1	Organic solvent (Toluene)	M1 M2 M3	T127N F136I T127N V176A	M2	3% (v/v) <i>n</i> -hexane, (v/v) cyclohexane, 0.23% (v/v) <i>p</i> -xylene	M2 cell surface hydrophobicity is lowered than WT; stress genes such as <i>acrAB</i> , <i>glpC</i> , <i>manXYZ</i> are upregulated in M2; no morphological change was observed with or without stress
2	Oxidative stress (H ₂ O ₂)	OM1 OM2 OM3	T127N D138V T146I F69C R82C V139M	OM3	Organic hydroperoxide (0.3 mM cumene hydroperoxide); Thermotolerance (48°C)	ROS level is lower in OM3 than WT; stress tolerant genes such as <i>gadAB</i> , <i>katE</i> , <i>osmCY</i> , <i>sufABDES</i> have been upregulated, Glutamate decarboxylase (encoded by <i>gadAB</i>) and Catalase (encoded by <i>katE</i>) activities have been higher in OM3 as well. Improved OM3 thermotolerance might be correlated with

						upregulation of heat shock proteins such as HtrC and HscA. Stress induced cell surface roughness was also observed.
3	Low pH	AcM1	F69C		Thermotolerance	Glutamate decarboxylase activity is elevated in AcM1 (~2.0 fold than WT), differential promoter binding between AcM1 and WT CRP was also revealed
	(Citric acid as acidulant)		V139M	AcM1	(48°C)	
		AcM2	V86L			

Chapter 7: Future Work

7.1. Future work as a direct extension of present study

1. In this study, we have undertaken error-prone PCR to introduce random point mutations in global regulator CRP followed by construction of *E. coli* variant library; and finally selecting better functional mutants through enrichment selection methodology. Since the mutation points have been attributed randomly, we want to perform saturation mutagenesis in order to deconvolute the best amino acid substitution/s at the relevant points. The best mutant is to be chosen as template for saturation mutagenesis. We assume that, the location of point mutations as well as resultant amino acid combinations will yield the super-mutant with greatest performance of all the variants.

2. With the best low pH tolerant mutant, numerous characterization studies are on the scheme. Since our best mutant obtained by error-prone PCR has shown cross tolerance towards other stressor such as high temperature, the newly scored mutant will also be subjected to heterogeneous stressors such as temperature and oxidative stress. We expect that, this study will suggest more scientific insights on the stress response overlaps between low pH and other stresses.

3. As investigated earlier, we also want to perform qRT-PCR of relevant genes to have a mechanistic insight of the cell stress response mechanism. Genes such as *gadABC*, *dps*, *wcaD*, *wcaE*, *fur*, have been established to contribute in cell acid tolerance. Hence, it is our aim to check these genes expression profiles through qRT-PCR. This study will provide significant ideation of the role of CRP engineering in genomic reprogramming against acid stress; thus will reinforce the concept of transcriptome-genome relationship to improve cell phenotype. Furthermore, since *gad* encodes enzyme glutamate decarboxylase crucial for imparting cell acid tolerance (reviewed in the background section), we also intend to check this gene role in translational level by performing the enzymatic assay.

4. If possible, we also want to check cell morphological characteristics of both mutant and WT by scanning electron microscopy as performed previously by FESEM.

7.2. Other possible studies

1. We have performed qRT-PCR on certain genes in organic solvent tolerant microbes. Although this investigation has unfolded some of the genomic profiles in both mutant as well as the WT, did not reveal the whole transcriptomic profile inside cell. Hence, performing whole transcriptomic analysis by DNA microarray or whole proteomic analysis by iTRAQ (isobaric tag for relative and absolute quantification) could provide a more complete exploration of intracellular genomic/ transcriptomic/ proteomic profiles.

2. Earlier oxidative stress tolerance studies demonstrated that oxidative stress tolerant mutants are characterized by various intracellular phenomena such as lesser protein carbonylation, lesser lipid peroxidation and other antioxidant enzyme such as glutathione reductase (GSH) activity. These kinds of studies could also be exploited in case of our mutants that would provide more validation of our investigation.

Appendix

TABLE S1: Endogenous (untreated) genes in OM3 with expression ratio ≥ 2 and a p -value threshold < 0.05

b number	GeneSymbol	Function	p -value	Log ₂ Fold-Change [#]
b4036	<i>lamB</i>	phage lambda receptor protein; maltose high-affinity receptor	0.039	-8.997
b4240	<i>treB</i>	PTS system enzyme II, trehalose specific	0.042	-9.071
b1966	<i>tnaA</i>	tryptophanase	0.049	-9.058
b4034	<i>malE</i>	periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis	0.043	-8.930
b4239	<i>treC</i>	trehalase 6-P hydrolase	0.021	-8.536
b4035	<i>malK</i>	ATP-binding component of transport system for maltose	0.039	-8.502
b3115	<i>tdcD</i>	putative kinase	0.047	-8.075
b3118	<i>tdcA</i>	transcriptional activator of <i>tdc</i> operon	0.039	-8.020
b2149	<i>mgIA</i>	ATP-binding component of methyl-galactoside transport and galactose taxis	0.044	-7.653
b4033	<i>malF</i>	part of maltose permease, periplasmic	0.044	-7.592
b2150	<i>mgIB</i>	galactose-binding transport protein; receptor for galactose taxis	0.037	-7.230
b3126	<i>garL</i>	orf, hypothetical protein	0.043	-6.819
b4037	<i>malM</i>	periplasmic protein of mal regulon	0.040	-6.619
b3709	<i>tnaB</i>	low affinity tryptophan permease	0.043	-6.596
b3113	<i>tdcF</i>	orf, hypothetical protein	0.032	-6.399
b3112		putative L-serine dehydratase	0.039	-6.340
b2148	<i>mgIC</i>	methyl-galactoside transport and galactose	0.037	-6.168

		taxis		
b1498	<i>ydeN</i>	putative sulfatase	0.043	-6.125
b4032	<i>malG</i>	part of maltose permease, inner membrane	0.021	-5.942
b3128	<i>garD</i>	putative hydrolase	0.039	-5.720
b4119	<i>mela</i>	alpha-galactosidase	0.039	-5.703
b3111		putative L-serine dehydratase	0.036	-5.678
b1073	<i>flgB</i>	flagellar biosynthesis, cell- proximal portion of basal- body rod	0.021	-5.635
b3125	<i>garR</i>	putative dehydrogenase	0.039	-5.590
b1074	<i>flgC</i>	flagellar biosynthesis, cell- proximal portion of basal- body rod	0.039	-5.370
b3225	<i>nana</i>	N-acetylneuraminate lyase	0.042	-5.240
b1075	<i>flgD</i>	flagellar biosynthesis, initiation of hook assembly	0.037	-5.225
b4189	<i>yjfO</i>	orf, hypothetical protein	0.042	-5.047
b2704	<i>srlB</i>	PTS system, glucitol/sorbitol-specific enzyme IIA component	0.047	-5.031
b1076	<i>flgE</i>	flagellar biosynthesis, hook protein	0.037	-4.963
b2000	<i>flu</i>	outer membrane fluffing protein, similar to adhesin	0.021	-4.879
b3124	<i>garK</i>	orf, hypothetical protein	0.044	-4.836
b3926	<i>glpK</i>	glycerol kinase	0.041	-4.733
b3927	<i>glpF</i>	facilitated diffusion of glycerol	0.044	-4.717
b4189	<i>yjfO</i>	orf, hypothetical protein	0.040	-4.660
b1922	<i>fliA</i>	flagellar biosynthesis; alternative sigma factor 28; regulation of flagellar operons	0.027	-4.655
b3528	<i>dctA</i>	uptake of C4-dicarboxylic acids	0.039	-4.617
b2789	<i>gudP</i>	putative transport protein	0.039	-4.572
b1493	<i>gadB</i>	glutamate decarboxylase isozyme	0.039	4.571
b3566	<i>xylF</i>	xylose binding protein transport system	0.047	-4.564
b3517	<i>gadA</i>	glutamate decarboxylase isozyme	0.044	4.517
b4188	<i>yjfN</i>	orf, hypothetical protein	0.039	-4.445

b3666	<i>uhpT</i>	hexose phosphate transport protein	0.042	-4.398
b0929	<i>ompF</i>	outer membrane protein 1a	0.037	-4.312
b1963	<i>yedR</i>	orf, hypothetical protein	0.039	4.255
b1945	<i>fliM</i>	flagellar biosynthesis, responsible for motor activation, rotation and direction	0.021	-4.246
b1077	<i>flgF</i>	flagellar biosynthesis, cell-proximal portion of basal-body rod	0.027	-4.060
b4299	<i>yjhI</i>	putative regulator	0.039	-4.031
b2001	<i>yeeR</i>	orf, hypothetical protein	0.040	-4.010
b4085	<i>alsE</i>	putative epimerase	0.040	-3.961
b3133	<i>agaV</i>	PTS system, cytoplasmic, N-acetylgalactosamine-specific IIB component 2	0.021	-3.961
b0485	<i>ybaS</i>	putative glutaminase	0.039	3.942
b1944	<i>fliL</i>	flagellar biosynthesis	0.039	-3.935
b1946	<i>fliN</i>	flagellar biosynthesis, responsible for motor switch and activation, conferring rotation and ascertaining its direction	0.042	-3.913
b2801	<i>fucP</i>	fucose permease	0.043	-3.870
b2788	<i>gudX</i>	putative glucarate dehydratase	0.037	-3.807
b1497	<i>ydeM</i>	putative enzyme	0.039	-3.790
b1005		orf, hypothetical protein	0.039	3.773
b4055	<i>aphA</i>	diadenosine tetraphosphatase	0.039	-3.767
b4084	<i>alsK</i>	putative NAGC-like transcriptional regulator	0.039	-3.762
b1078	<i>flgG</i>	participates in distal cell flagellar biosynthesis, encodes basal-body rod protein	0.027	-3.710
b4307	<i>yjhQ</i>	orf, hypothetical protein	0.043	-3.689
b1079	<i>flgH</i>	flagellar biosynthesis, basal-body outer-membrane	0.037	-3.659
b1938	<i>fliF</i>	flagellar biosynthesis; basal-body MS	0.039	-3.655
b1901	<i>araF</i>	L-arabinose-binding periplasmic protein	0.039	-3.634
b3946	<i>fsaB</i>	putative transaldolase	0.040	-3.628
b4120	<i>melB</i>	melibiose permease II	0.039	-3.605

b4086	<i>alsC</i>	putative transport system permease protein	0.038	-3.548
b3020	<i>ygiS</i>	putative transport periplasmic protein	0.042	-3.457
b3571	<i>malS</i>	alpha-amylase	0.021	-3.449
		partial putative periplasmic transport protein Z2474	0.039	-3.443
b2799	<i>fucO</i>	L-1,2-propanediol oxidoreductase	0.046	-3.442
b0651	<i>rihA</i>	putative tRNA synthetase	0.042	-3.381
b2219	<i>atoS</i>	sensor protein AtoS for response regulator AtoC	0.039	-3.324
b3092	<i>uxaC</i>	uronate isomerase	0.043	-3.309
b1080	<i>flgI</i>	homolog of Salmonella P-ring of flagella basal body	0.040	-3.308
b4322	<i>uxuA</i>	mannonate hydrolase	0.036	-3.275
b1776	<i>ydjL</i>	putative oxidoreductase	0.039	-3.266
b3417	<i>malP</i>	maltodextrin phosphorylase	0.037	-3.261
b2800	<i>fucA</i>	L-fuculose-1-phosphate aldolase	0.045	-3.256
b3223	<i>nanE</i>	putative enzyme	0.044	-3.183
b1940	<i>fliH</i>	flagellar biosynthesis; possibly related to export of flagellar proteins	0.045	-3.156
b4321	<i>gntP</i>	gluconate transport system permease 3	0.027	-3.150
b2924	<i>mscS</i>	putative transport protein	0.047	3.127
b3418	<i>malT</i>	positive regulator of mal regulon	0.039	-3.102
b1521	<i>uxaB</i>	altronate oxidoreductase	0.044	-3.100
b1947	<i>fliO</i>	flagellar biosynthesis	0.043	-2.933
b2869	<i>ygeV</i>	putative transcriptional regulator	0.037	-2.877
b3091	<i>uxaA</i>	altronate hydrolase	0.042	-2.849
b2220	<i>atoC</i>	response regulator of ato, ornithine decarboxylase antizyme	0.037	-2.820
b3949	<i>frwC</i>	PTS system, fructose-like enzyme II component	0.047	-2.807
b1921	<i>fliZ</i>	orf, hypothetical protein	0.037	-2.788
b1976	<i>mtfA</i>	orf, hypothetical protein	0.040	-2.784
b3087	<i>ygjR</i>	orf, hypothetical protein	0.039	-2.766
b4352	<i>yjiA</i>	orf, hypothetical protein	0.044	-2.745
b2272	<i>yfbM</i>	orf, hypothetical protein	0.021	-2.742
b3135	<i>agaA</i>	putative N-acetylgalactosamine-6-phosphate deacetylase	0.041	-2.740

b1897	<i>otsB</i>	trehalose-6-phosphate phosphatase, biosynthetic	0.037	2.717
b1732	<i>katE</i>	catalase; hydroperoxidase HPH	0.027	2.701
b0871	<i>poxB</i>	pyruvate oxidase	0.021	2.696
b2238	<i>yfaH</i>	orf, hypothetical protein	0.021	-2.686
b1795	<i>yeaQ</i>	orf, hypothetical protein	0.037	2.653
b4216	<i>ytfJ</i>	orf, hypothetical protein	0.041	-2.639
b2464	<i>talA</i>	transaldolase A	0.047	2.627
b4568	<i>ytjA</i>	predicted protein	0.039	2.594
b1311	<i>ycjO</i>	putative binding-protein dependent transport protein	0.037	-2.592
b1896	<i>otsA</i>	trehalose-6-phosphate synthase	0.037	2.581
b1900	<i>araG</i>	ATP-binding component of high-affinity L-arabinose transport system	0.037	-2.580
b3748	<i>rbsD</i>	D-ribose high-affinity transport system; membrane-associated protein	0.039	-2.525
b4003	<i>zraS</i>	sensor kinase for HydG, hydrogenase 3 activity	0.043	-2.518
b3516	<i>gadX</i>	putative ARAC-type regulatory protein	0.046	2.483
b0897	<i>ycaC</i>	orf, hypothetical protein	0.044	2.482
	<i>ybdD</i>	conserved protein b4512	0.043	-2.431
b4089	<i>rpiR</i>	transcriptional repressor of rpiB expression	0.043	-2.401
b1171	<i>ymgD</i>	orf, hypothetical protein	0.048	2.392
b1518	<i>lsrG</i>	orf, hypothetical protein	0.048	-2.380
b3263	<i>yhdU</i>	orf, hypothetical protein	0.040	-2.379
b4193	<i>ulaA</i>	orf, hypothetical protein	0.039	-2.354
b1308	<i>pspE</i>	phage shock protein	0.036	-2.352
b3371	<i>frlB</i>	putative transport protein	0.037	-2.344
b4353	<i>yjiX</i>	orf, hypothetical protein	0.039	-2.330
b1072	<i>flgA</i>	flagellar biosynthesis; assembly of basal-body periplasmic P ring	0.037	-2.271
b2465	<i>tktB</i>	transketolase 2 isozyme	0.042	2.259
b3426	<i>glpD</i>	sn-glycerol-3-phosphate dehydrogenase	0.050	-2.237
b1384	<i>feaR</i>	regulatory protein for 2- phenylethylamine catabolism	0.039	-2.230
b1081	<i>flgJ</i>	flagellar biosynthesis	0.039	-2.220

b0044	<i>fixX</i>	putative ferredoxin	0.046	-2.218
b2341	<i>fadJ</i>	putative enzyme	0.039	-2.188
b3515	<i>gadW</i>	putative ARAC-type regulatory protein	0.042	2.170
b2366	<i>dsdA</i>	D-serine dehydratase	0.039	-2.110
b3357	<i>crp</i>	cyclic AMP receptor protein	0.027	2.067
b3544	<i>dppA</i>	dipeptide transport protein	0.024	-2.065
b3880	<i>yihS</i>	orf, hypothetical protein	0.044	-2.031
b4090	<i>rpiB</i>	ribose 5-phosphate isomerase B	0.048	-2.022
b3239	<i>yhcO</i>	orf, hypothetical protein	0.047	2.022
b0453	<i>ybaY</i>	glycoprotein/polysaccharide metabolism	0.039	2.021
b0061	<i>araD</i>	L-ribulose-5-phosphate 4- epimerase	0.039	-2.012

- Logarithmic (base 2) value of expression ratio of genes in OM3 compared to WT without H₂O₂ treatment

TABLE S2: Genes in OM3 with expression ratio ≥ 2 and a p -value threshold < 0.05 after H_2O_2 treatment

b number	GeneSymbol	Function	p -value	Log ₂ Fold-Change [*]
b3517	<i>gadA</i>	glutamate decarboxylase isozyme	0.024	7.766
b1493	<i>gadB</i>	glutamate decarboxylase isozyme	0.033	7.097
b1492	<i>gadC</i>	acid sensitivity protein, putative transporter	0.031	7.025
b2850	<i>ygeF</i>	orf, hypothetical protein	0.024	6.323
b1005		orf, hypothetical protein	0.024	6.199
b3117	<i>tdcB</i>	threonine dehydratase, catabolic	0.046	-6.162
b3566	<i>xylF</i>	xylose binding protein transport system	0.024	-6.078
b3491	<i>yhiM</i>	orf, hypothetical protein	0.028	5.820
b1259	<i>yciG</i>	orf, hypothetical protein	0.031	5.757
b3115	<i>tdcD</i>	putative kinase	0.029	-5.722
b3116	<i>tdcC</i>	anaerobically inducible L-threonine, L-serine permease	0.025	-5.632
b3708	<i>tnaA</i>	tryptophanase	0.024	-5.501
b3118	<i>tdcA</i>	transcriptional activator of <i>tdc</i> operon	0.028	-5.493
b0929	<i>ompF</i>	outer membrane protein 1a	0.024	-5.363
b4307	<i>yjhQ</i>	orf, hypothetical protein	0.025	-5.133
b0598	<i>cstA</i>	carbon starvation protein	0.027	-5.093
b4240	<i>treB</i>	PTS system enzyme II, trehalose specific	0.025	-5.075
b4518	<i>ymdF</i>	conserved protein	0.025	5.003
b3511	<i>hdeD</i>	orf, hypothetical protein	0.024	4.967
b2844	<i>yqeF</i>	putative acyltransferase	0.028	-4.791
b4036	<i>lamB</i>	phage lambda receptor protein; maltose high-affinity receptor	0.024	-4.780
b3081	<i>fadH</i>	putative NADPH dehydrogenase	0.025	-4.761
b2342	<i>fadI</i>	putative acyltransferase	0.036	-4.695
b1074	<i>flgC</i>	flagellar biosynthesis, cell-proximal portion of basal-body rod	0.028	-4.692

b2150	<i>mglB</i>	galactose-binding transport protein; receptor for galactose taxis	0.025	-4.616
b4566	<i>yjhX</i>	conserved protein	0.033	-4.615
b1073	<i>flgB</i>	flagellar biosynthesis, cell-proximal portion of basal-body rod	0.031	-4.592
b0978	<i>appC</i>	probable third cytochrome oxidase, subunit I	0.024	4.592
b2801	<i>fucP</i>	fucose permease	0.028	-4.532
b3567	<i>xylG</i>	putative ATP-binding protein of xylose transport system	0.028	-4.484
b3509	<i>hdeB</i>	orf, hypothetical protein	0.024	4.458
b1422	<i>ycdI</i>	putative transcriptional regulator LYSR-type	0.040	-4.457
b4055	<i>aphA</i>	diadenosine tetraphosphatase	0.039	-4.340
b2957	<i>ansB</i>	periplasmic L-asparaginase II	0.024	-4.338
b3846	<i>fadB</i>	4-enzyme protein: 3-hydroxyacyl- CoA dehydrogenase; 3- hydroxybutyryl-CoA epimerase; delta	0.032	-4.294
b1075	<i>flgD</i>	flagellar biosynthesis, initiation of hook assembly	0.024	-4.266
b4034	<i>malE</i>	periplasmic maltose-binding protein; substrate recognition for transport and chemotaxis	0.032	-4.257
b0753	<i>ybgS</i>	putative homeobox protein	0.024	4.238
b2537	<i>hcaR</i>	transcriptional activator of <i>hca</i> cluster	0.046	-4.234
b0722	<i>sdhD</i>	succinate dehydrogenase, hydrophobic subunit	0.024	-4.214
b4187	<i>aidB</i>	putative acyl coenzyme A dehydrogenase	0.028	4.197
b1256	<i>ompW</i>	putative outer membrane protein	0.024	-4.196
b2802	<i>fucI</i>	L-fucose isomerase	0.032	-4.177
b3364	<i>tsgA</i>	putative transport	0.025	4.166
b1497	<i>ydeM</i>	putative enzyme	0.027	-4.162
b4069	<i>acs</i>	acetyl-CoA synthetase	0.024	-4.143
b0721	<i>sdhC</i>	succinate dehydrogenase, cytochrome	0.022	-4.139
b1536	<i>ydeI</i>	orf, hypothetical protein	0.028	4.125
b0622	<i>pagP</i>	orf, hypothetical protein	0.029	4.109

b1137	<i>ymfD</i>	orf, hypothetical protein	0.033	-4.105
b1963	<i>yedR</i>	orf, hypothetical protein	0.029	4.098
b3528	<i>dctA</i>	uptake of C4-dicarboxylic acids	0.027	-4.086
b0979	<i>appB</i>	probable third cytochrome oxidase, subunit II	0.024	4.077
b2135	<i>yohC</i>	orf, hypothetical protein	0.024	4.046
b3510	<i>hdeA</i>	orf, hypothetical protein	0.024	4.020
b4189	<i>yjfO</i>	orf, hypothetical protein	0.040	-3.998
b4068	<i>yjcH</i>	orf, hypothetical protein	0.024	-3.963
b0972	<i>hyaA</i>	hydrogenase-1 small subunit	0.033	3.961
b2924	<i>mscS</i>	putative transport protein	0.037	3.897
b2241	<i>glpA</i>	<i>sn</i> -glycerol-3-phosphate dehydrogenase	0.033	-3.864
b0806	<i>ybiM</i>	orf, hypothetical protein	0.028	3.846
b3512	<i>gadE</i>	orf, hypothetical protein	0.031	3.821
b1138	<i>ymfE</i>	orf, hypothetical protein	0.030	-3.811
b1732	<i>katE</i>	catalase; hydroperoxidase HP II	0.035	3.801
b2980	<i>glcC</i>	transcriptional activator for <i>glc</i> operon	0.045	-3.772
b3749	<i>rbsA</i>	ATP-binding component of D-ribose high-affinity transport system	0.031	-3.768
b4030	<i>psiE</i>	orf, hypothetical protein	0.026	3.767
b4299	<i>yjhI</i>	putative regulator	0.032	-3.718
b0485	<i>ybaS</i>	putative glutaminase	0.031	3.707
b3092	<i>uxaC</i>	uronate isomerase	0.034	-3.690
b2172	<i>yeiQ</i>	putative oxidoreductase	0.024	-3.681
b2149	<i>mgIA</i>	ATP-binding component of methyl-galactoside transport and galactose taxis	0.040	-3.660
b0806	<i>ybiM</i>	orf, hypothetical protein	0.046	3.635
b2165	<i>yeiN</i>	orf, hypothetical protein	0.024	-3.632
b1612	<i>fumA</i>	fumarase A = fumarate hydratase Class I; aerobic isozyme	0.028	-3.622
b0486	<i>ybaT</i>	putative amino acid/amine transport protein	0.036	3.620
b3927	<i>glpF</i>	facilitated diffusion of glycerol	0.028	-3.619
b2464	<i>talA</i>	transaldolase A	0.026	3.608
b4216	<i>ytfJ</i>	orf, hypothetical protein	0.024	-3.599
b1685	<i>ydiH</i>	orf, hypothetical protein	0.024	3.599
b2151	<i>galS</i>	<i>mgI</i> repressor, galactose operon inducer	0.031	-3.572

b2240	<i>glpT</i>	<i>sn</i> -glycerol-3-phosphate permease	0.028	-3.561
b2206	<i>napA</i>	probable nitrate reductase 3	0.040	-3.558
b0871	<i>poxB</i>	pyruvate oxidase	0.024	3.536
b4239	<i>treC</i>	trehalase 6-P hydrolase	0.030	-3.516
b3006	<i>exbB</i>	uptake of enterochelin; tonB-dependent uptake of <i>B. colicins</i>	0.024	3.515
b1076	<i>flgE</i>	flagellar biosynthesis, hook protein	0.027	-3.513
b1015	<i>putP</i>	major sodium/proline symporter	0.024	-3.504
b1683	<i>sufB</i>	orf, hypothetical protein	0.044	3.483
b1528	<i>ydeA</i>	putative resistance / regulatory protein	0.043	3.477
b4189	<i>yjfO</i>	orf, hypothetical protein	0.041	-3.474
b1967	<i>hchA</i>	orf, hypothetical protein	0.024	3.469
b1415	<i>aldA</i>	aldehyde dehydrogenase, NAD-linked	0.033	-3.467
b3241	<i>aaeA</i>	putative membrane protein	0.044	3.454
b3513	<i>mdtE</i>	putative membrane protein	0.040	3.442
b1922	<i>fliA</i>	flagellar biosynthesis; alternative sigma factor 28; regulation of flagellar operons	0.026	-3.439
b2766	<i>ygcN</i>	orf, hypothetical protein	0.044	-3.431
b3091	<i>uxaA</i>	altronate hydrolase	0.024	-3.426
b2876	<i>yqeC</i>	orf, hypothetical protein	0.039	-3.423
b3506	<i>slp</i>	outer membrane protein induced after carbon starvation	0.033	3.423
b2166	<i>yeiC</i>	putative kinase	0.026	-3.405
b3753	<i>rbsR</i>	regulator for rbs operon	0.044	-3.398
b4067	<i>actP</i>	putative transport protein	0.030	-3.396
b3555	<i>yiaG</i>	orf, hypothetical protein	0.038	3.386
b3113	<i>tdcF</i>	orf, hypothetical protein	0.024	-3.382
b2997	<i>hybO</i>	putative hydrogenase subunit	0.024	-3.374
b2239	<i>glpQ</i>	glycerophosphodiester phosphodiesterase, periplasmic	0.044	-3.372
b4367	<i>fhuF</i>	orf, hypothetical protein	0.024	3.371

b1737	<i>chbC</i>	PEP-dependent phosphotransferase enzyme II for cellobiose, arbutin, and salicin	0.024	-3.370
b0848	<i>ybjM</i>	orf, hypothetical protein	0.028	3.364
b4199	<i>yjfY</i>	orf, hypothetical protein	0.025	3.358
b1478	<i>adhP</i>	alcohol dehydrogenase	0.024	3.355
b3926	<i>glpK</i>	glycerol kinase	0.038	-3.343
b3132	<i>kbaZ</i>	putative tagatose 6-phosphate kinase 2	0.028	-3.337
b1684	<i>sufA</i>	orf, hypothetical protein	0.024	3.336
b2767	<i>ygcO</i>	orf, hypothetical protein	0.036	-3.335
b3752	<i>rbsK</i>	ribokinase	0.024	-3.314
b3750	<i>rbsC</i>	D-ribose high-affinity transport system	0.028	-3.276
b2869	<i>ygeV</i>	putative transcriptional regulator	0.033	-3.273
b4188	<i>yjfN</i>	orf, hypothetical protein	0.035	-3.265
b2996	<i>hybA</i>	hydrogenase-2 small subunit	0.024	-3.245
b0342	<i>lacA</i>	thiogalactoside acetyltransferase	0.024	-3.244
b1930	<i>yedF</i>	orf, hypothetical protein	0.024	-3.235
b1901	<i>araF</i>	L-arabinose-binding periplasmic protein	0.050	-3.228
b4298	<i>yjhH</i>	putative lyase/synthase	0.024	-3.210
b4342	<i>yjiT</i>	orf, hypothetical protein	0.024	-3.170
b1467	<i>narY</i>	cryptic nitrate reductase 2, beta subunit	0.024	3.169
b1681	<i>sufD</i>	orf, hypothetical protein	0.043	3.140
b0681	<i>ybfM</i>	orf, hypothetical protein	0.031	-3.130
b3005	<i>exbD</i>	uptake of enterochelin; tonB-dependent uptake of <i>B. colicins</i>	0.028	3.110
b1309	<i>ycjM</i>	putative polysaccharide hydrolase	0.041	-3.099
b4060	<i>yjcB</i>	orf, hypothetical protein	0.031	3.077
b2220	<i>atoC</i>	response regulator of ato, ornithine decarboxylase antizyme	0.025	-3.072
b0897	<i>ycaC</i>	orf, hypothetical protein	0.034	3.071
b1466	<i>narW</i>	cryptic nitrate reductase 2, delta subunit, assembly function	0.024	3.070
b0594	<i>entE</i>	2,3-dihydroxybenzoate-AMP ligase	0.026	3.067

b4376	<i>osmY</i>	hyperosmotically inducible periplasmic protein	0.024	3.062
b1258	<i>yciF</i>	putative structural proteins	0.033	3.058
b1537	<i>ydeJ</i>	orf, hypothetical protein	0.037	3.056
b0030	<i>rihC</i>	orf, hypothetical protein	0.024	-3.054
b1661	<i>cfa</i>	cyclopropane fatty acyl phospholipid synthase	0.024	3.046
b2243	<i>glpC</i>	<i>sn</i> -glycerol-3-phosphate dehydrogenase	0.025	-3.040
b4323	<i>uxuB</i>	D-mannonate oxidoreductase	0.034	-3.037
b1945	<i>fliM</i>	flagellar biosynthesis, responsible for motor activation, rotation and direction	0.033	-3.036
b1795	<i>yeaQ</i>	orf, hypothetical protein	0.047	3.023
b1897	<i>otsB</i>	trehalose-6-phosphate phosphatase, biosynthetic	0.035	3.014
b3873	<i>yihM</i>	orf, hypothetical protein	0.032	-3.001
b0980	<i>appA</i>	phosphoanhydride phosphorylase; pH 2.5 acid phosphatase; periplasmic	0.025	3.000
b1896	<i>otsA</i>	trehalose-6-phosphate synthase	0.012	2.996
b3073	<i>ygiG</i>	probable ornithine aminotransferase	0.024	2.969
b3748	<i>rbsD</i>	D-ribose high-affinity transport system; membrane-associated protein	0.025	-2.961
b2462	<i>eutS</i>	orf, hypothetical protein	0.039	-2.959
b3112		putative L-serine dehydratase	0.024	-2.955
b0723	<i>sdhA</i>	succinate dehydrogenase, flavoprotein subunit	0.028	-2.929
b2310	<i>argT</i>	lysine-, arginine-, ornithine-binding periplasmic protein	0.035	-2.901
b4227	<i>ytfQ</i>	putative LACI-type transcriptional regulator	0.024	-2.889
b0974	<i>hyaC</i>	probable Ni/Fe-hydrogenase 1 b-type cytochrome subunit	0.026	2.889
b4346	<i>mcrB</i>	component of McrBC 5-methylcytosine restriction system	0.035	-2.883
b2460	<i>eutQ</i>	orf, hypothetical protein	0.031	-2.881

b1252	<i>tonB</i>	energy transducer; uptake of iron, cyanocobalimin; sensitivity to phages, colicins	0.043	2.877
b1465	<i>narV</i>	cryptic nitrate reductase 2, gamma subunit	0.024	2.874
b3240	<i>aaeB</i>	orf, hypothetical protein	0.022	2.856
b4293	<i>fecI</i>	probable RNA polymerase sigma factor	0.037	2.851
b3153	<i>yhbO</i>	orf, hypothetical protein	0.024	2.840
b2426	<i>ucpA</i>	putative oxidoreductase	0.033	-2.835
b0899	<i>ycaM</i>	putative transport	0.024	-2.834
b3133	<i>agaV</i>	PTS system, cytoplasmic, N-acetylgalactosamine-specific IIB component 2	0.024	-2.823
b4511	<i>ybdZ</i>	conserved protein	0.042	2.814
b1778	<i>msrB</i>	orf, hypothetical protein	0.038	-2.812
b2080	<i>yegP</i>	orf, hypothetical protein	0.022	2.798
b0596	<i>entA</i>	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, enterochelin biosynthesis	0.024	2.796
b4107	<i>yjdN</i>	orf, hypothetical protein	0.033	2.792
b1892	<i>flhD</i>	regulator of flagellar biosynthesis, acting on class 2 operons; transcriptional initiation factor	0.037	-2.790
b2204	<i>napH</i>	ferredoxin-type protein: electron transfer	0.036	-2.780
b1195	<i>ymgE</i>	orf, hypothetical protein	0.024	2.778
b4269	<i>yjgB</i>	putative oxidoreductase	0.024	2.775
b2148	<i>mglC</i>	methyl-galactoside transport and galactose taxis	0.024	-2.772
b1680	<i>sufS</i>	orf, hypothetical protein	0.024	2.771
b2344	<i>fadL</i>	transport of long-chain fatty acids; sensitivity to phage T2	0.031	-2.763
b0790	<i>ybhP</i>	orf, hypothetical protein	0.025	2.763
b1482	<i>osmC</i>	osmotically inducible protein	0.038	2.755
b0802	<i>ybiJ</i>	orf, hypothetical protein	0.037	2.746
b1891	<i>flhC</i>	regulator of flagellar biosynthesis acting on class 2 operons; transcription initiation factor	0.032	-2.743
b0789	<i>ybhO</i>	putative synthetase	0.044	2.742
	Z0666	orf Unknown function Z0666	0.035	-2.736

b1241	<i>adhE</i>	CoA-linked acetaldehyde dehydrogenase and iron-dependent alcohol dehydrogenase; pyruvate-formate-lyase deactivase	0.024	2.735
b1905	<i>ftnA</i>	cytoplasmic ferritin	0.040	-2.728
b2799	<i>fucO</i>	L-1,2-propanediol oxidoreductase	0.029	-2.723
b0720	<i>gltA</i>	citrate synthase	0.034	-2.697
b3365	<i>nirB</i>	nitrite reductase	0.030	2.688
b4321	<i>gntP</i>	gluconate transport system permease 3	0.040	-2.681
b3925	<i>glpX</i>	unknown function in glycerol metabolism	0.026	-2.675
b1329	<i>mppA</i>	putative transport periplasmic protein	0.026	-2.633
b0343	<i>lacY</i>	galactoside permease	0.024	-2.630
b0651	<i>rihA</i>	putative tRNA synthetase	0.028	-2.622
b1000	<i>cbpA</i>	curved DNA-binding protein; functions closely related to DnaJ	0.032	2.610
b0384	<i>psiF</i>	induced by phosphate starvation	0.035	2.609
b4122	<i>fumB</i>	fumarase B= fumarate hydratase Class I; anaerobic isozyme	0.028	-2.601
b3507	<i>dctR</i>	orf, hypothetical protein	0.041	2.585
b0999	<i>cbpM</i>	orf, hypothetical protein	0.024	2.583
b3077	<i>ebgC</i>	evolved beta-D-galactosidase, beta subunit; cryptic gene	0.041	-2.581
b1218	<i>chaC</i>	cation transport regulator	0.042	2.577
b2672	<i>ygaM</i>	orf, hypothetical protein	0.033	2.556
b1679	<i>sufE</i>	orf, hypothetical protein	0.032	2.551
b2347	<i>yfdC</i>	putative transport	0.033	2.540
b3909	<i>kdgT</i>	2-keto-3-deoxy-D-gluconate transport system	0.026	-2.529
b0585	<i>fes</i>	enterochelin esterase	0.024	2.515
b3134	<i>agaW</i>	PTS system N-acetylgalactosamine-specific IIC component 2	0.024	-2.505
b1751	<i>ydjY</i>	orf, hypothetical protein	0.043	-2.502
b3946	<i>fsaB</i>	putative transaldolase	0.024	-2.494
b0975	<i>hyaD</i>	processing of HyaA and HyaB proteins	0.024	2.493
b1036	<i>ycdZ</i>	orf, hypothetical protein	0.025	2.480
b1770	<i>ydjF</i>	putative DEOR-type transcriptional regulator	0.033	-2.480

b1927	<i>amyA</i>	cytoplasmic alpha-amylase	0.026	2.479
b3366	<i>nirD</i>	nitrite reductase	0.024	2.478
b2687	<i>luxS</i>	orf, hypothetical protein	0.037	2.467
b2665	<i>ygaU</i>	orf, hypothetical protein	0.028	2.455
b2943	<i>galP</i>	galactose-proton symport of transport system	0.025	2.451
b1838	<i>pphA</i>	protein phosphatase 1 changes phosphoproteins, indicates protein misfolding	0.032	2.447
b4045	<i>yjbJ</i>	orf, hypothetical protein	0.049	2.446
b4292	<i>fecR</i>	regulator for fec operon, periplasmic	0.030	2.435
b2302	<i>yfcG</i>	putative S-transferase	0.024	2.429
b4133	<i>cadC</i>	transcriptional activator of cad operon	0.038	2.425
b2870	<i>ygeW</i>	putative carbamoyl transferase	0.024	-2.425
b3546	<i>eptB</i>	orf, hypothetical protein	0.029	-2.416
b2203	<i>napB</i>	cytochrome c-type protein	0.029	-2.412
b1189	<i>dadA</i>	D-amino acid dehydrogenase subunit	0.028	-2.408
b2244	<i>yfaD</i>	orf, hypothetical protein	0.028	-2.408
b1946	<i>fliN</i>	flagellar biosynthesis, responsible for motor switch and activation, conferring rotation and ascertaining its direction	0.027	-2.393
b1320	<i>ycjW</i>	putative LACI-type transcriptional regulator	0.045	-2.387
b1805	<i>fadD</i>	acyl-CoA synthetase, long-chain-fatty-acid--CoA ligase	0.040	-2.377
b1217	<i>chaB</i>	cation transport regulator	0.041	2.377
b1678	<i>ynhG</i>	orf, hypothetical protein	0.028	2.368
b2258	<i>arnF</i>	putative transport/receptor protein	0.040	-2.362
b3452	<i>ugpA</i>	sn-glycerol 3-phosphate transport system, integral membrane protein	0.035	-2.356
b3568	<i>xylH</i>	putative xylose transport, membrane component	0.026	-2.356
b1950	<i>fliR</i>	flagellar biosynthesis	0.024	-2.352
b3519	<i>treF</i>	cytoplasmic trehalase	0.033	2.347
b3361	<i>fic</i>	stationary phase encoded gene interfering in cell division, regulated by rpoS	0.028	2.342

b4228	<i>ytfR</i>	putative ATP-binding component of a transport system	0.034	-2.341
b2800	<i>fucA</i>	L-fuculose-1-phosphate aldolase	0.027	-2.340
b2211	<i>yojI</i>	putative ATP-binding component of a transport system	0.038	2.319
b3922	<i>yiiS</i>	orf, hypothetical protein	0.024	2.317
b2463	<i>maeB</i>	putative multimodular enzyme	0.024	-2.312
b0724	<i>sdhB</i>	succinate dehydrogenase, iron sulfur protein	0.025	-2.307
b3418	<i>malT</i>	positive regulator of mal regulon	0.038	-2.300
b1190	<i>dadX</i>	alanine racemase 2, catabolic	0.034	-2.296
b0809	<i>glnQ</i>	ATP-binding component of glutamine high-affinity transport system	0.026	-2.295
b3362	<i>yhfG</i>	orf, hypothetical protein	0.037	2.294
b4021	<i>pepE</i>	peptidase E, a dipeptidase where amino-terminal residue is aspartate	0.024	-2.293
b1938	<i>fliF</i>	flagellar biosynthesis; basal-body MS	0.031	-2.287
b2871	<i>ygeX</i>	putative dehydratase	0.038	-2.284
b3403	<i>pck</i>	phosphoenolpyruvate carboxykinase	0.033	-2.279
b0976	<i>hyaE</i>	processing of HyaA and HyaB proteins	0.035	2.277
b2981	<i>yghO</i>	orf, hypothetical protein	0.031	-2.264
b2219	<i>atoS</i>	sensor protein AtoS for response regulator AtoC	0.026	-2.260
b4297	<i>yjhG</i>	putative dehydratase	0.042	-2.258
b3603	<i>lldP</i>	L-lactate permease	0.028	-2.250
b2242	<i>glpB</i>	sn-glycerol-3-phosphate dehydrogenase	0.024	-2.244
b4139	<i>aspA</i>	aspartate ammonia-lyase	0.024	-2.236
b4003	<i>zraS</i>	sensor kinase for HydG, hydrogenase 3 activity	0.041	-2.235
b0150	<i>fhuA</i>	outer membrane protein sensing ferrichrome, colicin M, and phages T1, T5, and phi80	0.026	2.234
b0220	<i>ivy</i>	orf, hypothetical protein	0.041	2.232
b1777	<i>yeaC</i>	orf, hypothetical protein	0.025	-2.229
b0380	<i>yaiZ</i>	orf, hypothetical protein	0.028	-2.226
b0592	<i>fepB</i>	ferric enterobactin	0.033	2.223

b2341	<i>fadJ</i>	putative enzyme	0.038	-2.221
b2888	<i>ygfU</i>	putative permease	0.033	-2.215
b3514	<i>mdtF</i>	putative transport system permease protein	0.033	2.204
b1646	<i>sodC</i>	superoxide dismutase precursor	0.031	2.195
b3023	<i>ygiV</i>	orf, hypothetical protein	0.024	2.194
b0810	<i>glnP</i>	glutamine high-affinity transport system; membrane component	0.033	-2.193
b3043	<i>ygiL</i>	putative fimbrial-like protein	0.046	-2.178
b2289	<i>lrhA</i>	NADH dehydrogenase transcriptional regulator, LysR family	0.032	-2.172
b4118	<i>melR</i>	regulator of melibiose operon	0.038	-2.171
b4229		putative ATP-binding component of a transport system	0.033	-2.166
b3020	<i>ygiS</i>	putative transport periplasmic protein	0.031	-2.165
b2878	<i>ygfK</i>	putative oxidoreductase, Fe-S subunit	0.041	-2.165
b0595	<i>entB</i>	2,3-dihydro-2,3-dihydroxybenzoate synthetase, isochroismatase	0.046	2.163
b4322	<i>uxuA</i>	mannonate hydrolase	0.036	-2.163
b0506	<i>allR</i>	putative regulator	0.046	2.161
b1953	<i>yodD</i>	orf, hypothetical protein	0.030	2.159
b2994	<i>hybC</i>	probable large subunit, hydrogenase-2	0.046	-2.155
b2309	<i>hisJ</i>	histidine-binding periplasmic protein of high-affinity histidine transport system	0.037	-2.152
b1611	<i>fumC</i>	fumarase C= fumarate hydratase Class II; isozyme	0.025	-2.147
b0564	<i>appY</i>	regulatory protein affecting appA and other genes	0.024	2.142
b4351	<i>mrr</i>	restriction of methylated adenine	0.035	-2.126
b1077	<i>flgF</i>	flagellar biosynthesis, cell-proximal portion of basal-body rod	0.024	-2.114
b2013	<i>yeeE</i>	putative transport system permease protein	0.048	-2.114

b1735	<i>chbR</i>	negative transcriptional regulator of <i>cel</i> operon	0.028	-2.113
b0118	<i>acnB</i>	aconitate hydratase B	0.033	-2.104
b2552	<i>hmp</i>	dihydropteridine reductase, ferrisiderophore reductase activity	0.035	-2.092
b3964	<i>yijD</i>	orf, hypothetical protein	0.041	-2.077
b3905	<i>rhaS</i>	positive regulator for <i>rhaBAD</i> operon	0.024	-2.077
b1750	<i>ydjX</i>	orf, hypothetical protein	0.028	-2.075
b0151	<i>fhuC</i>	ATP-binding component of hydroxymate-dependent iron transport	0.033	2.072
b3544	<i>dppA</i>	dipeptide transport protein	0.047	-2.070
b0977	<i>hyaF</i>	nickel incorporation into hydrogenase-1 proteins	0.038	2.069
b3917	<i>sbp</i>	periplasmic sulfate-binding protein	0.035	-2.063
b4037	<i>malM</i>	periplasmic protein of mal regulon	0.026	-2.061
b0624	<i>crcB</i>	orf, hypothetical protein	0.027	2.060
b3070	<i>yqjH</i>	orf, hypothetical protein	0.032	2.059
b0608	<i>ybdR</i>	putative oxidoreductase	0.037	2.059
b1101	<i>ptsG</i>	PTS system, glucose-specific IIBC component	0.024	2.056
b1504	<i>ydeS</i>	putative fimbrial-like protein	0.027	-2.055
b3004		orf, hypothetical protein	0.034	2.054
b2415	<i>ptsH</i>	PTS system protein HPr	0.028	2.045
b1078	<i>flgG</i>	participates in distal cell flagellar biosynthesis, encodes basal-body rod protein	0.035	-2.039
b2518	<i>ndk</i>	nucleoside diphosphate kinase	0.036	-2.029
b1676	<i>pykF</i>	pyruvate kinase I	0.028	2.026
b2879	<i>ssnA</i>	putative proteoglycan	0.026	-2.020
b3923	<i>uspD</i>	putative regulator	0.025	2.019
b1776	<i>ydjL</i>	putative oxidoreductase	0.033	-2.005
b2308	<i>hisQ</i>	histidine transport system permease protein	0.024	-2.000

* - Logarithmic (base 2) value of expression ratio of genes in OM3 compared to WT after treatment with 4 mM H₂O₂

List of publications

1. Full articles

1. **Basak S**, Song H, Jiang R. Error-prone PCR of global transcription factor cyclic cAMP receptor protein of *E. coli* for enhanced organic solvent (toluene) tolerance. *Process Biochemistry* 2012; 47: 2152-2158
2. **Basak S**, Jiang R. Enhancing *E. coli* tolerance towards oxidative stress *via* engineering its global regulator cAMP receptor protein (CRP) *PloS ONE* 2012; 7(12): e51179
3. **Basak S**, Song H, Jiang R. Enhancing *E. coli* tolerance towards low pH stress *via* engineering its global regulator cAMP receptor protein (CRP) *Manuscript communicated*

2. Conference publications

1. **Basak S**, Jiang R. Directed evolution of cAMP receptor protein for improved cell toluene tolerance. *Biopharma Asia Convention 2012*
2. Chong H, **Basak S**, Jiang R. Engineering global regulator cAMP receptor protein for improved cell performance under stress. *ASM 2012*
3. Zhang H, Chong H, **Basak S**, Huang L, Jiang R. Engineering global regulator cAMP receptor protein (CRP) of *E.coli* to improve strain performance under stress. *AIChE 2012*
4. Zhang H, Chong H, **Basak S**, Huang L, Jiang R. Engineering global transcription factor cAMP receptor protein to improve strain performance under stress. *IBS 2012*
5. Zhang H, Chong H, **Basak S**, Huang L, Jiang R. Engineering global transcription factor cAMP receptor protein (CRP) for improved cell performance under various stresses (1-butanol, ethanol, oxidative stress, organic solvent and osmotolerance. *FEMS 2013*