Engineering cAMP Receptor Protein (CRP) for improved cell phenotypes during fermentation

Chong, Hui Qing

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**Engineering cAMP Receptor Protein (CRP) for
Improved Cell Phenotypes during Fermentation**

Hui Qing Chong

**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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**Engineering cAMP Receptor Protein (CRP) for Improved
Cell Phenotypes during Fermentation**

Hui Qing Chong

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EXECUTIVE SUMMARY

Microorganisms are widely used in industries for various industrial applications, such as the production of commodity chemicals, biofuels, and recombinant proteins. However, a major limitation in using these microorganisms is their low tolerance towards various stresses during fermentation. Classical strain engineering approaches (UV/chemical mutagenesis, adaptive evolution and metabolic engineering) have been utilized to improve strain phenotypes valuable in industry, but are usually resource-intensive and only marginally efficient. Transcriptional engineering approach has gained much attention in strain engineering in recent years, which can greatly shorten variant selection period from months to just several days. In this work, error-prone PCR technique was adopted to engineer global transcription factor cAMP receptor protein (CRP) in *Escherichia coli*, to generate genetic and thus phenotypic diversity and obtain mutants with desired phenotypes, including ethanol and acetate tolerance.

Random mutagenesis libraries of CRP mutants were created and transformed into *E. coli* Δcrp microbial host. Cells were cultured under different stress, namely ethanol or acetate, and “winners” were selected based on growth. The best mutant under each stress condition was further characterized according to its cross-tolerance, changes in transcript profile and cell morphology. For instance, three *E. coli* mutants (E1-E3) with elevated ethanol tolerance were selected, and the best ethanol-tolerant strain E2 had single amino acid substitution M59T, which was then integrated into the genome to create iE2. When exposed to 150 g/L ethanol, the survival rate of iE2 after 15 min was

about 12 %, while that of parent strain was < 0.01 %. Real-time PCR analysis on CRP-regulated genes revealed that several genes involved in enterobactin biosynthesis (*entH*, *entD*) and iron ion transport (*fecA*, *fecB*), and general stress response (*osmY*, *rpoS*) were differentially expressed in iE2. Four mutants (A1-A4) with improved acetate tolerance were also identified, with A2 (D138Y) having the highest growth rate. Real-time PCR analysis using OpenArray® revealed that CRP-regulated genes, especially those involved in the uptake and utilisation of sugars, amino sugars and amino acids, were differentially expressed between A2 and WT.

Chapter 1: INTRODUCTION

Since the development of recombinant DNA technology, microbes have been used in industrial production of a variety of products, ranging from commodity chemicals, biofuels, small-molecule drugs, bioplastics, therapeutics proteins to industrial enzymes. Therefore, microorganisms that have short doubling time, able to reach high cell density, and easily manipulated genetically will be highly desired. However, the growth of microbial hosts and thus product yield are largely affected by the toxicity of the chemicals produced, as well as various stresses encountered during fermentation. As a result, engineering strains with robust cell growth and improved production under stress conditions become increasingly important, particularly when the number of chemicals that are synthesized by microorganisms are expected to rise in the near future.

Complex phenotypes such as cellular tolerance to stresses are difficult to engineer, since they do not depend on simple genotype-phenotype relationship, requiring coordinated response on both transcriptomic and proteomic level. Multiple modifications in genetic elements and metabolic pathways are needed before attaining the phenotype of interest. Therefore, genetic diversity is first generated among host cells, leading to phenotypic diversity that is useful for the selection of the desired trait in strain engineering.

Classical strain engineering approaches can be classified as either “random” or “rational”. The “random” approaches depend on the use of mutagens such as ultra-violet (UV) , N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS) to introduce random mutations into microorganisms,

or to culture microbial hosts under selection stress such that beneficial spontaneous mutations can be developed . The major drawback of these “random” approaches is that they are heavily dependable on recursive mutagenesis and selection procedure, making selection process both time-consuming and labour intensive, and therefore marginally effective. The “rational” approach to improve cell phenotype is to apply metabolic engineering tools, which are mainly based on gene overexpression or deletion. The lack of metabolic information for many host microorganisms restricts metabolic engineering to well-characterized microorganisms such as yeasts, *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*). Even so, it is still difficult to engineer complex phenotypes in simple microorganism like *E. coli*.

An alternative approach known as combinatorial strain engineering has been developed in recent years to resolve the limitations of classical strain engineering. It was suggested that random mutagenesis on transcription and translation machinery can create greater phenotypic diversity than the conventional strain engineering approaches. Among different combinatorial strain engineering methods, transcriptional engineering is of considerable interest. Transcription factors are usually mutated in transcriptional engineering in order to obtain global perturbations to the transcription network, thereby achieving phenotypic diversity at the transcription level. Transcription factors such as SPT15, artificial zinc-finger containing transcription factors, sigma factors, H-NS, Hha and IrrE have been engineered previously to alter a range of phenotypes in various microorganisms.

E. coli has been extensively used in industrial production due to: (i) rapid growth rate and high yield production; (ii) well-characterized genetics; (iii) low

cost of culture media; and (iv) availability of comprehensive tools for genetic manipulations. There are seven major transcription factors in *E. coli*, with cAMP receptor protein (CRP) being the most important and well-characterized. CRP can regulate more than 400 genes in *E. coli*. Hence, in this study, we will attempt to improve cell phenotypes through engineering of CRP in model microorganism *E. coli*.

The specific aims of this study are described as follows:

1. To enhance the ethanol tolerance of *E. coli* by engineering CRP and selecting mutants with better growth in high concentration of ethanol. The *crp* mutation(s) from the best mutant would be integrated into chromosome. Characterization of the ethanol tolerant mutant would be based on measurement of the growth rate at various ethanol concentrations, cell viability at lethally high ethanol concentration, as well as the ability to resist other alcohols. Transcript analysis of CRP-regulated genes is also performed using RT-PCR. SEM micrographs are used to observe any differences in cell morphologies between mutant and wild-type.
2. To engineer cAMP receptor protein (CRP) of *Escherichia coli* through error-prone PCR (ep-PCR) and select CRP mutants with improved acetate tolerance. The selected mutants will be characterized based on their growth rate in various concentrations of sodium acetate, measurement of extracellular acetate concentration, changes in the transcript profile of CRP-regulated genes using quantitative real-time

reverse transcription PCR (RT-PCR) analysis and differences in cell morphology using Scanning Electron Microscope (SEM).

This dissertation is categorized into six chapters. Chapter 1 presents the introduction and the specific aims of this study, while literature review is provided in Chapter 2. Chapter 3 and 4 describe the results and discussion of enhancing ethanol tolerance and acetate tolerance of *E. coli* respectively. Lastly, the conclusions and recommendation of future works are presented in Chapter 5.

Chapter 2: BACKGROUND

This chapter focuses on the literature review on the use of the microorganism *Escherichia coli* in industrial applications, as well as the various strain engineering approaches, including both conventional and combinatorial approaches. In addition, the physiological effects on *Escherichia coli* due to commonly encountered stresses during fermentation are also discussed at the end of this chapter.

2.1. Selection of Host Microorganism

Many microorganisms have been used in industrial production of a variety of products, ranging from commodity chemicals, biofuels, small-molecule drugs, bioplastics, therapeutics proteins to industrial enzymes. For example, *Saccharomyces cerevisiae* (*S. cerevisiae*) has traditionally been used for the production of bioethanol (Girio et al. 2010; Matsushika et al. 2009), while *Lactobacillus* is used for lactic acid production (Patnaik et al. 2002), and *Streptomyces* strains for antibiotics production (Santos and Stephanopoulos 2008).

Escherichia coli (*E. coli*) is also a popular and well-characterized workhorse of modern industrial biotechnology (Vickers et al. 2012), and has been used for the production of commodity chemicals, biofuels and recombinant proteins (Olano et al. 2008; Sabra et al. 2010). Currently, about 30% of FDA-approved recombinant therapeutic proteins are being produced in *E. coli* (Chou 2007; Huang et al. 2012). There is also a high level of interest in biofuel production, especially for bioethanol, through microbial fermentation of renewable biomass due to the increase in crude oil prices and in energy demand (Antoni et al. 2007;

Lee et al. 2011a; Mills et al. 2009). Although *S. cerevisiae* is the most commonly used microbial host in traditional production of bioethanol, its inability to ferment pentose sugars (D-xylose and L-arabinose) restricts its usage in lignocellulosic fermentation. In contrast, *E. coli*'s natural ability to ferment a variety of sugars, including pentose sugars, makes it an attractive biocatalyst for bioethanol production.

The popularity of *E. coli* in industrial application, as well as in academic research, can be attributed to the following reasons: (i) it is able to grow rapidly and reach high cell density; (ii) it is a simple microorganism with its genetics, metabolic pathways and fermentation properties being well-characterized; (iii) the cost of the culture media for *E. coli* fermentation is low; and (iv) there is a wide range of tools available for genetic manipulations in *E. coli*, which allows us to have better control over *E. coli* than other industrial microorganisms (Blattner et al. 1997; Eiteman and Altman 2006; Makino et al. 2011; Vickers et al. 2012). Owing to the advantages of using *E. coli* as a microbial host in industrial fermentation, we will be using *E. coli* as our model microorganism in this study.

2.2. Strain Engineering

The production of recombinant proteins and biofuels in industrial fermentation often makes use of high-level gene expression to increase production yield. However, this results in microorganisms becoming more stress-sensitive due to metabolic burden (Chou 2007). Furthermore, toxic compounds or environmental stresses can be present during fermentation that inhibit fermentation performance (Mills et al. 2009). Therefore, strain

engineering methods, which mimic the natural selection process, have been applied in the early days of biotechnology to develop strains with the following traits: (i) better cell growth, (ii) higher productivity, (iii) better tolerance to product toxicity and (iv) lower or no byproduct formation (Lee et al. 2011a; Olano et al. 2008). The ultimate aim of strain engineering is to produce strains that are more stress tolerant to replace the native strains in industrial fermentation.

Traditional strain engineering approaches consisting of evolutionary engineering, which includes both adaptive evolution and physical/chemical mutagenesis, and metabolic engineering have long been utilized for strain improvement (Olano et al. 2008). Engineering of complex phenotypes depends on the optimal coordination of multiple genes, and therefore requires detailed understanding of microorganism's metabolism, thereby limiting the application of metabolic engineering in strain engineering (Stephanopoulos 2002; Woodruff and Gill 2011). Moreover, the time- and labor-intensive nature of evolutionary engineering is considered to be only marginally efficient for strain improvement (Warnecke et al. 2010). In order to overcome the shortcomings of traditional strain engineering approaches, various strain engineering methods such as promoter engineering (Alper et al. 2005), ribosome engineering (Shima et al. 1996), genome shuffling (Patnaik et al. 2002), scalar analysis of library enrichments (SCALEs) (Lynch et al. 2007), trackable multiplex recombineering (TRMR) (Warner et al. 2010), multiplex automated genome engineering (MAGE) (Wang et al. 2009) and transcriptional engineering (Alper et al. 2006; Chen et al. 2011; Park et al. 2003) have been developed in the past decade. These methodologies not only provide a more efficient selection procedure for

strains with desired traits, but also enhance our understandings on genes that confer the trait improvement (Lee et al. 2011a; Woodruff and Gill 2011).

2.2.1. Classical Strain Engineering

The improvement methods in classical strain engineering include evolutionary engineering and metabolic engineering. Evolutionary engineering, which consists of adaptive evolution and physical or chemical mutagenesis, is considered as “random” approach since random mutations are introduced into the microorganism to create improved strains. In contrast, metabolic engineering is thought to be a “rational” approach as this method depends on carefully chosen metabolic pathways to modify to bring out the desired phenotype (Olano et al. 2008).

2.2.1.1. Adaptive Evolution

In the adaptive evolution method, microorganisms are cultivated either in batch repeatedly or in chemostat for a long time under selective pressure, so that the microorganisms can accumulate spontaneous mutations. Those variants with beneficial mutations would enable them to grow better than the original cells, and eventually dominate the culture (Cakar et al. 2012). Adaptive evolution can be applied to improve strains for industrially important traits without prior knowledge about the microorganism’s metabolic pathways (Asakura et al. 2011). However, the drawbacks of using adaptive evolution for strain engineering include time-consuming selection process, difficulty in mapping the locations of the mutations in the genome and linking the mutations to the adaptive traits (Asakura et al. 2011; Lee et al. 2011a). For instance, it took three months to obtain an ethanol tolerant strain with an increase of IC50

(concentration of ethanol inhibiting 50% of cell growth) from 20 g/L to 30 g/L ethanol (Mills et al. 2009). Nevertheless, adaptive evolution has been successful in the isolation of strains that display a variety of phenotypes, such as better stress resistance (Lee et al. 2011a; Steiner and Sauer 2003; Yomano et al. 1998), higher production of chemicals or recombinant proteins, improved plasmid stability, and ability to utilize alternative carbon source (Cakar et al. 2012).

With the recent development in high-throughput technologies, multiple samples may be processed simultaneously to reduce costs and to increase the precision of adaptive evolution process. This will also shorten the time required to obtain variants with phenotypes of interest (Cakar et al. 2012).

2.2.1.2. UV/Chemical Mutagenesis

Classical random mutagenesis makes use of mutagens such as ultra-violet (UV) (Reed et al. 1987; Sridhar et al. 2002), N-methyl N'-nitro-N-nitrosoguanidine (NTG) (Yu et al. 2008b; Zhou et al. 2010) and ethyl methanesulfonate (EMS) (Klein-Marcuschamer and Stephanopoulos 2008; Taylor et al. 2006) to introduce random mutations into the genome of microorganisms, followed by the direct selection in medium providing selection pressure. UV/chemical mutagenesis requires recursive mutagenesis and selection before the isolation of mutants with valuable phenotype (Cakar et al. 2012; Sandoval et al. 2011). The schematic diagram presenting how the repeated cycles of mutagenesis and selection works to obtain variant(s) with improved traits is shown in Figure 1 below.

Figure 1: Schematic diagram showing strain engineering process with mutagens. Recursive mutagenesis and selection are performed to select mutants with desired phenotypes for industrial applications. The process is repeated until mutants with satisfactory phenotypic improvement are selected.

Similar to adaptive evolution, this process is time-consuming and also labor-intensive, as many rounds of mutagenesis and selection are required prior to obtaining mutants with improved traits. The mutations are also difficult to be mapped in genome, and are not transferable to another microorganism for the same phenotypic change. Furthermore, the mutations present are often neutral or deleterious, resulting in majority of the mutants in the selection culture to be crippled, and in turn cause this method to be only marginally efficient in obtaining mutants with good phenotypic improvement (Cakar et al. 2012; Sandoval et al. 2011). Despite some of the drawbacks of using UV/chemical mutagenesis, this method is capable of producing mutant strains with properties

of industrial importance, such as increasing the total inhibition concentration of vanillin from 3 to 4 g/L through using NTG mutagenesis (Mills et al. 2009).

2.2.1.3. Metabolic Engineering

Metabolic engineering is based on the rational design of metabolic pathways in microorganisms to bring about the change in biochemical networks to achieve a desired phenotype (Cakar et al. 2012). Metabolic engineering has been used in strain engineering for more than two decades (Lynch and Gill 2012), and is heavily dependent on DNA recombinant technology whereby endogenous genes are either overexpressed or deleted, and heterologous genes are introduced into the host microorganisms (Yadav et al. 2011). For example, a 25-fold increment in protein production in a batch reactor was achieved through knocking out *ptsHI* in *E. coli* (Huang et al. 2012).

Although there are successful cases of strain engineering attained by application of metabolic engineering, this method is not without limitations. The major drawback of metabolic engineering is the requirement of extensive and comprehensive information regarding the metabolism of the host microorganism to design the system rationally (Asakura et al. 2011; Cakar et al. 2012; Patnaik 2008). The complexity of physiological responses of microorganisms further limits the use of metabolic engineering in strain engineering (Cakar et al. 2012). It is not easy to alter the phenotype of even simple microorganism like *E. coli* (Patnaik 2008). In addition, the lack of vectors or cloning methods for many industrial strains also causes difficulties in genetic manipulations (Cakar et al. 2012). The limitations of metabolic engineering are partially solved to date due to advancement in many genetic

tools and methods, such as genomics, proteomics, transcriptomics and metabolomics, that provides more information and details for the rational design of metabolic pathways to engineer different microorganisms (Lynch and Gill 2012; Olano et al. 2008).

2.2.2. Combinatorial Engineering

In order to overcome the limitations of classical strain engineering approaches, researchers have proposed combining strategies in evolutionary engineering and metabolic engineering, giving rise to a novel approach known as combinatorial engineering. Information from systems biology and tools from synthetic biology are utilized in combinatorial engineering to speed up the strain improvement process (Lynch and Gill 2012). Systems biology can provide information of potential target genes to engineering, while synthetic biology can provide tools to extensively modify existing metabolic pathways, or even to create non-natural metabolic pathway within the microbial host. Combinatorial engineering focuses on the creation of genetic diversity, which in turn leads to phenotypic diversity, followed by the screening and selection of strains with phenotype of interest (Santos and Stephanopoulos 2008). When coupled with information from systems biology, different methods of combinatorial engineering can regulate gene expression level to improve and optimize strain performance (Lee et al. 2012). Since high throughput assays are required for the screening and selection of variants in combinatorial engineering, the engineered traits are restricted to improving yield and titers of products with colorimetric properties and enhancing tolerance (Yadav et al. 2011).

2.2.2.1. Promoter Engineering

The main objective in promoter engineering is to fine-tune the expression of genes, with two methods being developed to achieve this aim. In the first method, the spacer sequences in the -10 and -35 regions are modified without altering the consensus regions. The second method allows the introduction of point mutations into well-characterized native promoters through error-prone polymerase chain reaction (ep-PCR). Promoter engineering was first proposed to create promoter libraries that enable constitutive gene expression, and had been successful in optimizing gene expression in various microorganisms such as *E. coli*, *Lactobacillus lactis*, *Lactobacillus plantanum*, and *S. cerevisiae*. In particular, promoter engineering was used to optimize the expression level of phosphoenolpyruvate and deoxyxylulose phosphate synthase in order to increase lycopene production (Alper et al. 2005; Lee et al. 2011a). However, promoter engineering can be time-consuming if the gene expression of more than one gene is to be optimized, since promoter engineering is only applicable to a single gene or an operon at a time. In cases whereby the gene expression of several genes in a metabolic pathway is to be fine-tuned, multiple rounds of cell modifications would be needed (Santos and Stephanopoulos 2008).

2.2.2.2. Ribosome Engineering

Ribosomal engineering was initially developed as a rational approach to increase antibiotics production, such as streptomycin, gentamicin, kanamycin and chloramphenicol, in various *Streptomyces* strains. These antibiotics are known to impact on the ribosomes, so random mutagenesis is performed on *rpsL* to create mutant ribosomes that are antibiotics resistant. Ribosome engineering also enables modifications to gene expression at the translational level, and has been applied in the enhancement of the tolerance to aromatic

compounds, such as 4-hydroxybenzoate, toluene and *m*-xylene in *Pseudomonas putida* (Olano et al. 2008). Since the mutations are directly introduced into the genome, deleterious mutations present in the mutant ribosomes may inhibit cell growth, thereby restricts its application in strain engineering.

2.2.2.3. Genome Shuffling

Genome shuffling is employed to combine beneficial mutations in an additive or synergistic manner from parental strains already with subtle advantage in the phenotype of interest (Santos and Stephanopoulos 2008). The combinatorial libraries can be created by recursive protoplast fusion of the parental strains to generate genetic diversity (Gong et al. 2009; Patnaik et al. 2002). Genome shuffling was first successfully applied to improve the acid tolerance of *Lactobacillus* to produce three times more lactic acid than wild-type strain at pH 4.0 (Patnaik et al. 2002), and also used to increase hydroxycitric acid production in *Streptomyces* sp. U121 (Olano et al. 2008). The tylosin production in *Streptomyces fradiae* was elevated by 6- to 8-fold in just two rounds of genome shuffling, whereas classical strain engineering approach required 20 rounds of mutagenesis and selection before obtaining mutant with similar improvement (Zhang et al. 2002), thereby proving that genome shuffling can be an efficient and important tool for strain improvement.

2.2.2.4. Scalar Analysis of Library Enrichment (SCALEs)

SCALEs depends on the selection of mixtures of plasmid libraries containing different sizes of inserts, ranging from single gene to an operon, followed by microarray and multi-scale analysis. The schematic diagram depicting the process of SCALEs is illustrated in Figure 2. This method works to identify

specific gene(s) within the genomic library that confer a change to the desired phenotype based on the unique signal intensity patterns produced. SCALEs also helps to assess the effect of increasing the expression level of the gene or operon on the trait of interest (Lynch et al. 2007). To date, SCALEs has been utilized to enhance the tolerance to 3-hydroxypropionic acid (3-HP) (Warnecke et al. 2010), and acetate in *E. coli* (Sandoval et al. 2011), as well as other chemicals such as 1-naphthol, antibiotics and antimetabolites (Mills et al. 2009).

Figure 2: Overview of SCALEs. The fragments of genomic DNA with different sizes were ligated into vectors and transformed into cell line. The pools of transformants are subjected to selective pressure whereby the fitter strains will survive. These

strains are analysed by microarray and non-linear multiscale decomposition to identify the trait-conferring genotype (Lynch et al. 2007).

2.2.2.5. Trackable Multiplex Recombineering (TRMR)

In TRMR, the promoters in the *E. coli*'s genome are replaced with synthetic DNA cassettes coupled to molecular barcodes to increase or decrease the expression of target genes. The molecular barcode attached to the DNA cassette helps in the identification of gene(s) that confer the change in phenotype through measurement of the barcode frequency, which can be correlated to the allele frequency, as shown in Figure 3. This technique has been proven in *E. coli* to improve the tolerance to a variety of growth inhibitors such as β -glucoside, D-fucose, valine, and methylglyoxal (Warner et al. 2010), as well as acetate, furfural, ethanol, butanol and isopentanol (Lynch and Gill 2012).

Figure 3: The TRMR method. Allele libraries were generated by replacing the promoter region in genomic DNA with synthetic DNA cassettes attached with

molecular barcode. The synthetic DNA cassettes can either increase or decrease the expression of target gene. Gene variants with phenotype of interest after screening or selection can be identified through their molecular barcode (Warnecke et al. 2010).

TRMR can play an important role in strain engineering due to its ability to engineer thousands of genes simultaneously. TRMR is also able to determine the genes that affect the growth of microorganisms under selection pressure within a week, providing valuable metabolic information of the microorganism that is useful for strain improvement by metabolic engineering, thereby speeding the process of strain evolution (Lee et al. 2011a; Lee et al. 2012).

2.2.2.6. Multiplex Automated Genome Engineering (MAGE)

MAGE makes use of single-stranded DNA oligos (ssDNA oligos) that can simultaneously target different locations of the genome to generate genetic diversity in the microorganism, as illustrated in Figure 4. Mutations introduced into the chromosome can include mismatch, insertions or deletions. There are two strategies employed in MAGE: (i) ribosome binding site optimization, and (ii) gene knockout. In the first strategy, the ssDNA oligos will target the ribosome binding sites (RBS) in the genome to bring about changes in expression of target genes. In contrast, the second strategy allows genes to be inactivated after being targeted by the ssDNA oligos (Lynch and Gill 2012; Wang et al. 2009). Using MAGE to simultaneously control the expression of 24 genes in *E. coli*, this approach has been successful in raising the lycopene production by 5-fold in just three days (Lee et al. 2012).

Figure 4: The MAGE method. Single-stranded DNA oligos (ssDNA oligos) target either ribosome binding site (RBS) or gene on genome to create genetic diversity among host microorganism for strain selection (Wang et al. 2009). Mutations introduced into the chromosome can include mismatch, insertions or deletions.

2.2.2.7. Transcriptional Engineering

Transcriptional engineering, which includes random mutagenesis on artificial, exogenous or native transcription factors, is considered to be both “random” and “rational” – “random” since the mutations made to the target protein are non-directed, and “rational” since the key regulatory proteins important for the target trait are carefully chosen (Klein-Marcuschamer et al. 2009). However, extensive information on the choice of target regulator is usually not required. Moreover, the selection procedure for mutant with improved trait in transcriptional engineering is shorter than that of classical strain engineering (Santos and Stephanopoulos 2008), and the genetic modifications are also easily mapped (Klein-Marcuschamer et al. 2009), thereby resolving some of the drawbacks faced by classical strain engineering

approaches (Warner et al. 2009). Transcriptional engineering has already been proven to be an efficient and valuable alternative strain engineering method to improve multigenic phenotypes such as tolerance to growth inhibitors (Kuhn et al. 2010).

2.2.2.7.1. Artificial Transcription Factors

Zinc-finger domains have highly specific binding to DNA through recognition of 3-base pair (bp) sequences, and can be found in many of the transcriptional regulatory proteins. When more than three zinc-finger domains are linked together, they form zinc-finger proteins. A three-finger protein would be able to recognize 9-bp DNA sequence, while a four-finger protein can identify DNA sequence of 12-bp. Each zinc-finger protein is fused to a transcription activation or repression domain to form an artificial transcription factor that is capable of modifying gene expression. Randomized libraries of artificial transcription factors can be created through randomizing the sequence of zinc-finger proteins such that they have different DNA-binding specificities. The libraries were screened to select for strains with improved phenotype (Park et al. 2003).

This method has been used to improve various phenotypes such as tolerance to osmotic shock, heat shock, and cold shock in both eukaryotic and prokaryotic systems (Park et al. 2005a; Park et al. 2003), as well as improving recombinant protein production in yeast (Park et al. 2005b). Zinc-finger proteins have also been fused to cAMP receptor protein (CRP), which acts as an effector domain for the artificial transcription factor, for greater fold activation or repression of target genes (Lee et al. 2008). More recently, libraries of zinc-finger-containing

artificial transcription factors have been used to improve *E. coli*'s tolerance to 1-butanol up to 1.5 % (v/v), which is higher than the complete inhibitory concentration of 1 % (v/v) (Lee et al. 2011b).

2.2.2.7.2. Exogenous Transcription Factor

Besides the creation of artificial transcription factor, exogenous transcription factor can also be mutagenized for trait improvement in microorganisms. For instance, global regulator IrrE from radiation-resistant bacterium *Deinococcus radiodurans* is engineered and overexpressed in *E. coli* to enhance alcohol, acetate, osmotic, and furfural tolerance, as well as ethanol production (Chen et al. 2011; Ma et al. 2011; Wang et al. 2012). Through this approach, the growth of *E. coli* is increased by 2- to 50-fold in the presence of various concentrations of ethanol, butanol or acetate, as well as elevating ethanol production of *E. coli* to about 40 g/L by enhancing *E. coli*'s ability to tolerate 25 % glucose.

2.2.2.7.3. Native Transcription Factors

Native transcription factors such as TATA-binding protein (SPT15) (Alper et al. 2006), sigma factors (*rpoD* and *rpoS*) (Alper and Stephanopoulos 2007; Klein-Marcuschamer and Stephanopoulos 2008; Yu et al. 2008a), alpha subunit of RNA polymerase (*rpoA*) (Klein-Marcuschamer et al. 2009), histone-like nucleoid-associated protein (H-NS) (Hong et al. 2010b), and high haemolysin activity (Hha) (Hong et al. 2010a) have already been engineered in various microorganisms (*S. cerevisiae*, *L. plantarum* or *E. coli*) for a range of phenotypes. Engineering of SPT15 produced ethanol-tolerant mutants with 70% rise in volumetric productivity of ethanol from glucose in yeast (Alper et al. 2006). Other phenotypic improvements, including more efficient production of

lycopene, L-tyrosine and hyaluronic acid, and better tolerance to other chemicals such as ethanol, butanol, sodium dodecyl sulphate (SDS), acetate, hexane, lactic acid and *p*-hydroxybenzoic acid, have been realized through engineering *rpoD*, *rpoS* and *rpoA* in *E. coli* and *L. plantanum* (Alper and Stephanopoulos 2007; Klein-Marcuschamer et al. 2009; Klein-Marcuschamer and Stephanopoulos 2008). This implies that engineering native transcription factors is able to elicit a variety of phenotypes suitable for different industrial processes. Random mutagenesis on H-NS and Hha was also performed to control biofilm formation or dispersal in *E. coli* (Hong et al. 2010a; Hong et al. 2010b).

2.3. cAMP Receptor Protein (CRP) in *E. coli*

There are seven major global transcription factors in *E. coli*, which are cAMP receptor protein (CRP), regulator of fumarate and nitrate reduction (FNR), integration host factor (IHF), factor for inversion stimulation (FIS), aerobic respiration regulatory protein (ArcA), histone-like nucleoid-associated protein (H-NS) and leucine-responsive regulatory protein (Lrp), and together they account for direct regulation of more than 51% of the genes in *E. coli* (Martínez-Antonio 2011; Martinez-Antonio and Collado-Vides 2003). Among these seven global transcription factors, CRP is known as the mastery regulatory protein essential for regulation of more than 400 genes, where most of these genes are involved in carbon metabolism and transport based on intracellular cAMP level (Keseler et al. 2011; Khankal et al. 2009; Martínez-Antonio 2011). Furthermore, CRP is reported to top the hierarchy of the regulatory network in *E. coli* (Ma et al. 2004) due to its ability to regulate the second most number of genes after *rpoD* (Martinez-Antonio et al. 2008). All

these information suggest that CRP is a potential target for transcriptional engineering.

2.3.1. CRP Structure

CRP, also known as catabolite activator protein (CAP), is a 47kDa dimer composing of two identical subunits in *E. coli*. Each subunit consists of 209 amino acids, and is able to bind to cAMP, thus there are three independent forms of CRP known, which are apo-CRP (inactive conformation of CRP, see Figure 5A), CRP:(cAMP)₂ complex (active conformation of CRP, see Figure 5B) and CRP:(cAMP)₄ complex (Baker et al. 2001; Harman 2001; Hudson et al. 2009). In the absence of cAMP, apo-CRP only displays weak and non-specific binding to DNA sequence (Gorecki et al. 2009). However, at micromolar concentrations of cAMP, cAMP binds to *anti*-sites of CRP to produce an allosteric conformational change in CRP such that the stability of CRP:(cAMP)₂ complex increases. This conformational change results in a sequence-specific binding to DNA with high affinity, and also promotes interactions between CRP-cAMP complex and RNA polymerase (Harman 2001), thus CRP-(cAMP)₂-DNA complex is considered the active form of all CRP conformers (Gorecki et al. 2009). At higher concentrations of cAMP, the CRP:(cAMP)₄ complex is formed due to additional binding of cAMP at *syn*-sites. The CRP:(cAMP)₄ complex would lose its transcriptional regulatory property of CRP due to severe reduction in the DNA binding affinity (Tutar 2008b; Won et al. 2009).

Figure 5: (A) Structure of apo-CRP. Image is obtained from the RCSB PDB (www.pdb.org) of PDB ID 2WC2 (Popovych et al. 2009). (B) Structure of cAMP-CRP. Image is obtained from the RCSB PDB (www.pdb.org) of PDB ID 2GZW (Kumarevel et al.).

Each CRP subunit is made up of two domains: the larger N-terminal domain (residue 1-133) and the smaller C-terminal domain (residue 139-209). These two domains are connected by a flexible short peptide known as the hinge region (residue 134-138) (Popovych et al. 2009; Tutar 2008b; Yu and Lee 2004).

The main function of the N-terminal domain is to bind to cAMP, therefore extensive β -sheet structures are present at this domain to form the cAMP binding pocket. Three α -helices (A, B and C) are also located in the N-terminal domain (Baker et al. 2001; Harman 2001; Popovych et al. 2009) with the C-helix acting as the hydrophobic core for the dimerization of the two CRP subunits (Sharma et al. 2009). The cAMP binding pocket constitutes of seventeen amino acids, among which seven are hydrophilic and the rest are hydrophobic. Nine of the hydrophobic amino acids (I30, V49, L61, L64, I70, G71, L73, A84 and V86) are important for interactions with the ribose ring and

the distal face of the adenine ring in cAMP. The seven hydrophilic residues are S62, E72, S83, R123, T127, S128 and L124. Five intra-subunit residues (G71, E72, R82, S83 and T127) are directly involved in the cAMP binding, while R123 forms a salt bridge with E72 to play a role in cAMP binding (Harman 2001). S128 from the opposite subunit is found to interact with the cAMP molecule by forming the hydrogen bond (Gorecki et al. 2009; Tutar 2008b).

The C-terminal domain, consisting of another three α -helices (D, E and F), is primarily involved in recognition and specific binding of DNA sequences through the helix-turn-helix motif. This helix-turn-helix motif is formed when F-helix interacts with the neighbouring E-helix to create the DNA binding surface (Baker et al. 2001; Popovych et al. 2009). In the inactive form of CRP, the F-helix is hidden within the core of apo-CRP structure, which accounts for its low affinity towards DNA (Sharma et al. 2009).

The hinge region connecting the N-terminal and C-terminal domains is important in transmission of signal between these two domains. Binding of cAMP to the N-terminal domain results in changes to interdomain interactions that leads to relocation of the hinge region, which in turn enables the F-helix to be exposed for binding to specific DNA sequences (Gorecki et al. 2009; Tutar 2008a).

There are three activating regions present in CRP that are important for interactions with RNA polymerase and activation of promoters, which are activating region 1 (AR1), activating region 2 (AR2) and activating region 3 (AR3). AR1, constituting of residue 156-164, form a surface loop that activates both Class I and Class II promoters (Tagami and Aiba 1998; Won et al. 2009).

The C-terminal domain of the alpha-subunit of the RNA polymerase also interacts with AR1 (Tutar 2008b). An additional contact between AR2 and N-terminal domain of the alpha-subunit of the RNA polymerase is also essential for activation of Class II promoters (Tagami and Aiba 1998). The main amino acid residues involved in AR2 are H19, H21, E96 and K101. AR3, which composed of K52, D53, E54, E55 and E58, is also found to be crucial for interactions with Class II promoters (Won et al. 2009). The binding of CRP to DNA is also known to induce a sharp bend that may be important for activation of promoters (Tagami and Aiba 1998; Won et al. 2009).

2.3.2. Allosteric Activation of CRP

The binding of cAMP to apo-CRP will result in allosteric activation of CRP to its active form. This allosteric signal in turn leads to reorientation of both the CRP subunits, as well as the domains within each subunit. The ultimate outcome of the reorientation is to allow protrusion of F-helix in order for site-specific DNA recognition and binding of CRP to occur (Harman 2001). The mechanism of CRP activation depends on the differences in lengths of the C- and D-helices upon cAMP binding. In the inactive form of CRP, the C-helix spans from residues 110-130, while the D-helix contains amino acids from position 135-152. C-helix in the active conformation of CRP consists of residues 110-136, whereas that of D-helix is from 139-152. Therefore, when cAMP binds to CRP, the D-helix becomes 4 residues shorter, while C-helix extends by 6 residues. The elongation of C-helix then causes the relocation of the hinge region and the destabilization of the D-helix, leading the F-helix to adopt an orientation that is vital for DNA recognition and binding (Popovych et al. 2009; Sharma et al. 2009). An additional low-affinity cAMP binding site

exists between the hinge region and the helix-turn-helix region due to relocation of the hinge region, enabling binding of another cAMP molecule in the *syn* conformation (Gorecki et al. 2009). However, this leads to the loss of transcriptional regulatory property as the DNA binding site on CRP's C-terminal domain is no longer accessible (Tutar 2008b).

2.3.3. Transcriptional Regulation of CRP

The CRP of *E. coli* regulates the transcription of more than 400 genes (Keseler et al. 2011), and is capable of acting as either an activator or a repressor, especially for genes involved in carbon metabolism (Martinez-Antonio and Collado-Vides 2003). Since its isolation in the 1970s, CRP is one of the most extensively studied transcription factor. It is believed that CRP's ability to regulate vast number of genes belonging to various functional classes would enable it to mediate response for a wide spectrum of environmental conditions in *E. coli* (Hudson et al. 2009).

Activated CRP is able to recognize and bind to a 22-base pairs sequence with the consensus sequence 5'-AAATGTGAN₆TCACATTT-3', which is located within or near its targeted promoters for regulation of transcription (Harman 2001; Hudson et al. 2009; Tutar 2008a). The CRP binding sites can exist at different locations relative to the transcription start site for different types of promoters, which can be broadly classified into two categories: Class I and Class II. Class I promoters contain CRP binding site that is usually centered at or near position -61.5. CRP binding site that is displaced 10.5 bp upstream from -61.5 can also be found in Class I promoters. In contrast, CRP binding site overlaps the -41.5 region in Class II promoters. Numerous studies had also

indicated that the interaction between CRP and RNA polymerase was crucial in activating CRP-dependent promoters (Harman 2001; Hudson et al. 2009; Tagami and Aiba 1998).

2.4. Error-prone polymerase chain reaction (ep-PCR)

Directed evolution techniques, including error-prone PCR, aim to mimic the process of natural selection in the laboratory. These techniques had been successfully applied in improving the catalytic properties of enzymes (Leemhuis et al. 2009; Stemmer 1994) and had been adopted in strain engineering approach such as transcriptional engineering and promoter engineering. Error-prone PCR is usually used to introduce random point mutations into gene of interest (Harayama 1998). This method uses either unbalanced concentrations of nucleotides (Wang et al. 2006) or low fidelity DNA polymerase such as MutazymeII (Leemhuis et al. 2009) to promote incorporation of mismatched bases, thereby enhancing the mutation rate. The mutation rate had to be controlled ideally between 1.5 to 5 base substitutions per targeted gene, since high mutation rate will result in many deleterious mutations, whereas low mutation rate will cause wild-type sequence to predominate in the library (Harayama 1998). In this study, I will be employing ep-PCR to introduce random mutations into *crp* gene to construct mutant libraries.

2.5. Types of Stresses Present during Fermentation

There are many kinds of stresses that microbial cells may encounter during fermentation, such as changes in temperatures and pH, toxicity from fermentation products and byproducts, as well as osmotic stress from carbon

source (Santos and Stephanopoulos 2008; Warner et al. 2009). The presence of stresses would often limit cell growth, and hinder production yield. As mentioned previously, *E. coli* is a potential biocatalyst for bioethanol production from lignocellulosic fermentation (Antoni et al. 2007; Girio et al. 2010), and a popular host microorganism for the production of recombinant therapeutic protein (Chou 2007; Huang et al. 2012). However, during bioethanol production, growth of *E. coli* is impeded mainly due to its low tolerance towards ethanol and lignocellulose-derived growth inhibitors (Girio et al. 2010). Moreover, acetate, one of the lignocellulose-derived growth inhibitors and a major fermentation byproduct, can hamper both cell growth and protein synthesis in *E. coli* (Chou 2007), leading to lower yield of recombinant products. As a result, it is vital for us to engineer *E. coli* to enhance its tolerance towards ethanol and acetate, so that *E. coli* can be applicable for industrial fermentation.

2.5.1. Ethanol Stress

Ethanol is known to induce toxicity on *E. coli* cells through interrupting cell membrane structure and integrity, and perturbing the activity of both membrane-bounded and solution enzymes leading to proton flux imbalance. These physiological changes will eventually cause cell death (Dombek and Ingram 1984; Goodarzi et al. 2010; Ingram 1976).

Although *E. coli* has weak tolerance towards ethanol, it is still armed with several protective mechanisms against ethanol stress. Sudden exposure of ethanol to growing *E. coli* culture causes growth arrest of about 30 min, after which it is followed by growth restoration with a lower growth rate. The lag

period is thought to allow time for *E. coli* to adapt to the ethanol stress by changing the length of fatty acid from 16 carbons to 18 carbons (Ingram 1976; Liu and Qureshi 2009). Other adaptive changes include inhibition of phosphatidylethanolamine synthesis and saturated fatty acid synthesis (Buttke and Ingram 1980). These ethanol-induced responses in turn result in increasing the proportion of acidic phospholipids in the membrane, while decreasing the total phospholipid content. Increasing the proportion of acidic phospholipids in the membrane seems to be vital for ethanol adaptation, since mutants that are unable to synthesize acidic phospholipids are also ethanol-sensitive (Dombek and Ingram 1984).

Transcriptome analysis on ethanol-tolerant strains also reveals that biosynthesis of osmoprotectants, including glycine and betaine, as well as certain amino acids can aid in ethanol tolerance (Gonzalez et al. 2003; Horinouchi et al. 2010). Higher expression level of genes *gcv* (for glycine metabolism), *betIBA* (for betaine synthesis from choline), and *betT* (for uptake of choline) is observed in ethanol-tolerant mutant, and supplement of glycine or betaine in the culture media indeed improve *E. coli*'s growth in ethanol. Ethanol, similar to sugars, would compete with water to form hydrogen bonds with biological molecules, thereby destabilizes many proteins and macromolecules. Presence of glycine or betaine probably helps to stabilize enzymes and biological structures within the cell to counteract the harmful effect of ethanol (Gonzalez et al. 2003). The biosynthesis of amino acids tryptophan and histidine is also up-regulated in the ethanol-tolerant strain, and supplements of these two amino acids also improve the growth of *E. coli* in the presence of ethanol. The genes encoding iron ion transport are also up-regulated in the

ethanol-tolerant strain, although addition of iron ion to medium does not shows any growth enhancement under ethanol stress (Horinouchi et al. 2010).

Many of the genes encoding TCA cycle enzymes are shown to increase in expression in ethanol-tolerant strains, including *sucABCD*, *sdhCDAB*, and *pflB* (Gonzalez et al. 2003; Goodarzi et al. 2010; Horinouchi et al. 2010). Majority of these genes are regulated by CRP (Keseler et al. 2011). In addition, some genes, such as *entA* and *fecA* that are involved in the iron ion transport, are also CRP-regulated.

2.5.2. Acetate Stress

The presence of acetate in culture media reduces the maximum cell density and biomass, as well as the yield of recombinant proteins (Aristidou et al. 1994; Luli and Strohl 1990). Cell growth can be retarded even in the presence of 0.5 g/L of acetate in minimal medium (Mills et al. 2009), while concentration of acetate as low as 15 to 25 mM can decrease production of recombinant proteins (Aristidou et al. 1994). Acetate toxicity is found to affect recombinant *E. coli* cells more than the wild-type cells, and is also influenced by the nutritional status of the culture medium, as acetate imposed more severe growth inhibition in a minimal medium than in a complex medium (Kleman and Strohl 1994; Koh et al. 1992; Lawford and Rousseau 1992).

There are two ways which acetate can induce inhibitory effects on *E. coli* cells. The first way is the uncoupling effect, whereby the protonated form of acetic acid penetrates the cell membrane and then dissociates within the cytoplasm to decreases the intracellular pH (Luli and Strohl 1990). Energy needs to be spent to counteract the proton influx, leading to reduced energy

available for cell growth (Lawford and Rousseau 1992). Furthermore, acetate interferes with homeostatic mechanisms that maintain a constant intracellular pH, and weakens the proton-motive force essential for oxidative phosphorylation and other endergonic membrane processes. The drop in intracellular pH can also impact cell growth by affecting the integrity of the purine bases, and denatures essential enzymes in the cell (De Mey et al. 2007; Warnecke and Gill 2005). It was reported that 50% inhibition was observed when the intracellular pH decreased to 6.85 (Lawford and Rousseau 1992; O'Beirne and Hamer 2000; Russell 1992). The second way whereby acetate can inhibit cell growth is through accumulation of anion within the cytoplasm (Russell 1992). The accumulation of anion can result in an increase in cell turgor pressure in cases whereby intracellular pH remained unchanged. In order to maintain a constant osmolarity in the cell, most of the glutamate is displaced from the cell, thus lowering the cell growth potential (O'Beirne and Hamer 2000; Warnecke and Gill 2005). Even after the removal of acetate from the culture medium, growth is only restored if the cells are able to synthesize glutamate (Roe et al. 1998; Roe et al. 2002).

Acetate is produced whenever an imbalance between glucose metabolism and biosynthesis, excess NADH, or repression of TCA cycle enzymes occurs (Luli and Strohl 1990). When the capacity of oxidative metabolism is exceeded, the metabolic processes are reorganized to meet the anabolic requirements. Therefore, *E. coli* synthesizes acetate from acetyl-CoA in order to satisfy the energy requirement (Castano-Cerezo et al. 2009; Han et al. 1992).

There are two major pathways that generate acetate in *E. coli* — the *ackA-pta* pathway and the *poxB* pathway. The two enzymes present in the *ackA-pta*

pathway are phosphotransacetylase and acetate kinase. Phosphotransacetylase (PTA) is required for the reversible conversion of acetyl-CoA and inorganic phosphate to acetyl phosphate and CoA, whereas acetate kinase (ACKA) catalyses the reaction between acetyl phosphate and ADP to acetate and ATP. The *ackA-pta* pathway is essential for maintaining the carbon influx within the cell during the exponential phase under both aerobic and anaerobic growth. The main enzyme for the *poxB* pathway is pyruvate oxidase. Pyruvate oxidase (POXB), which is more active during the late exponential and stationary phase, is important for catalysing the conversion of pyruvate to acetate (De Mey et al. 2007; Dittrich et al. 2005). Figure 6 below summarises the reactions in the *ackA-pta* and *poxB* pathways.

Figure 6: Central metabolic pathways producing acetate (Dittrich et al. 2005). POXB – pyruvate oxidase; PTA – phosphotransacetylase; ACKA - acetate kinase.

E. coli will undergo “acetate switch”, the instance whereby the rate of acetate formation is equivalent to the rate of acetate utilization, when its acetogenic carbon source is almost depleted. An example of acetogenic carbon

source is glucose (Veit et al. 2007; Wolfe 2005). After the “acetate switch”, *E. coli* starts consuming acetate as a carbon source. During the assimilation of acetate, acetate is transported into the cell and converted to acetyl-CoA via the acetyl-CoA synthetase (*acs*). The gene expression for *acs*, which is jointly regulated by several regulators such as CRP, increases in the presence of acetate (Polen et al. 2003). Acetyl-CoA is then metabolized in the tricarboxylic acid (TCA) cycle and the glyoxylate bypass pathway. *E. coli* strain B is found to produce less acetate than *E. coli* strain K since the glyoxylate bypass genes are being constitutively expressed in *E. coli* strain B (Polen et al. 2003; Veit et al. 2007).

In addition to the regulation of acetyl-CoA synthetase (*acs*), CRP is also reported to be involved in the regulation of many genes that show differential expression after exposure to acetate (De Mey et al. 2007; Oh et al. 2002; Peng and Shimizu 2003; Phue and Shiloach 2002; Polen et al. 2003; Veit et al. 2007), especially for genes encoding for enzymes in the carbon metabolism. These genes are regulated by CRP, and able to influence either acetate formation or assimilation, such as those involved in carbon metabolism (*gltA*, *mdh*, *sucCDAB*), sugar transport (*ptsG*), amino acid synthesis (*gadA*) and osmoregulation (*ompF*).

2.6. Conclusions

E. coli is a commonly used host microorganism for industrial fermentation, such as recombinant protein production and bioethanol production. However, *E. coli* has low tolerance towards inhibitory byproducts such as acetate and toxic products like ethanol, restricting its use as an industrial microorganism. Various

strain engineering approaches, either classical or combinatorial, have aimed to improve *E. coli*'s tolerance for industrial applications, and the mechanisms involved in conferring tolerance improvements have been partially analysed. In this thesis, I propose targeting a potential native global regulator, CRP, for transcriptional engineering to enhance *E. coli*'s resistance towards different stress conditions that may be encountered in fermentation, including ethanol toxicity and acetate inhibition. Enhancing *E. coli*'s tolerance towards these stresses would help broaden its applications in industrial fermentation.

Chapter 3: Improving ethanol tolerance of *Escherichia coli* by rewiring its global regulator cAMP receptor protein (CRP)

3.1. Introduction

The use of bioethanol as alternative fuel has drawn greater attention than ever due to recent energy crisis and environmental concerns (Hahn-Hagerdal et al. 2006). Production of ethanol from microbial fermentation is of practical value in replacing the utilization of fossil fuels. Different microorganisms, including yeast (Ho et al. 1999), *Zymomonas mobilis* (Ohta et al. 1991; Zhang et al. 1995) and *E. coli* (Ingram et al. 1987; Tao et al. 2001) have been engineered for selective production of ethanol in the past. Nevertheless, the yields and titers from microbial fermentation is usually held back by the accumulation of toxic end-product ethanol (Fischer et al. 2008; Wackett 2008). As such, it is essential to obtain an ethanol-tolerant microbial host for large-scale bioethanol production.

In general, there are two conventional approaches to improve strain performance under ethanol stress: i) “random approach” of mutagenesis with UV/chemical mutagens (Sridhar et al. 2002) and adaptive evolution (Goodarzi et al. 2010; Horinouchi et al. 2010; Yomano et al. 1998); ii) “rational approach” of using metabolic engineering tools (Chen et al. 2010; Kajiwara et al. 1996; Luo et al. 2009). However, the random introduction of mutations into microbial genetic materials followed by serial rounds of selection is usually time-consuming and laborious. As for “rational approach”, the lack of detailed metabolism knowledge for many microorganisms often limits its use (Patnaik 2008).

A new approach, transcriptional engineering, has gained attention in strain engineering in recent years. It has been reported before that cell performance can be altered by introducing modification to transcription factor Spt15 (Alper et al. 2006), sigma factor (Klein-Marcuschamer et al. 2009), and zinc-finger containing artificial transcription factor (Park et al. 2003), H-NS and Hha (Hong et al. 2010a; Hong et al. 2010b), as well as IrrE (Chen et al. 2011; Wang et al. 2012). In particular, sigma factor 70 from *E. coli* (Alper and Stephanopoulos 2007) and IrrE from *Deinococcus radiodurans* have been engineered to improve ethanol tolerance of *E. coli* DH5 α . Our lab has successfully improved the osmotolerance and 1-butanol tolerance of *E. coli* DH5 α through engineering its global regulator cAMP receptor protein (CRP) in the past (Zhang et al. 2012a; Zhang et al. 2012b). In this work, we would like to improve the ethanol tolerance of *E. coli* BW25113 by engineering CRP. *E. coli* BW25113 is a well-characterized strain that has been used for gene deletion or chromosomal integration (Baba et al. 2006). Both *E. coli* BW25113 and its isogenic mutants have also been engineered for production of chemicals such as alcohol (Shen and Liao 2008), hydrogen (Maeda et al. 2007) and D-lactate (Zhu and Shimizu 2004). CRP is a well-known *trans*-acting transcription factor that regulates the expression of more than 400 genes in *E. coli* (Keseler et al. 2011), and participates in various regulatory networks and different metabolic processes (Ma et al. 2004). It was also demonstrated previously that the deletion of *crp* could result in enhanced *E. coli* organic solvent tolerance (Okochi et al. 2008). In view of these discoveries, we speculated that the ethanol tolerance of *E. coli* could also be enhanced by rewiring the transcription profile of *E. coli* through CRP.

Here, we harnessed error-prone PCR technique to introduce mutations to CRP, and the random mutagenesis libraries were subjected to selection under ethanol stress. Three error-prone PCR variants (E1-E3) with enhanced ethanol resistance were identified. The amino acid substitutions in the best ethanol-tolerant mutant E2 was then integrated into the genome of *E. coli* JW5702 Δkan to create strain iE2, which was further investigated with respect to its survival and tolerance to other alcohol. Moreover, changes in the transcript profile of over four hundred CRP-regulated genes in iE2 were examined by quantitative real-time reverse transcription PCR (RT-PCR) using OpenArray® real-time PCR instrument.

3.2. Materials and methods

3.2.1. Materials

E. coli JW5702 (BW25113 Δcrp) from Keio collection (Baba et al. 2006) was purchased from *E. coli* Genetic Stock Center, and used as the host strain for gene manipulation as well as phenotype selection. The kanamycin resistance gene in JW5702 was removed using FLP helper plasmid following protocol to create JW5702 Δkan (Cherepanov and Wackernagel 1995; Datsenko and Wanner 2000). *E. coli* cells were grown in Luria–Bertani (LB) medium (w/v): 0.5 % yeast extract (Merck, Damstadt, Germany), 1 % tryptone (Oxoid, Hampshire, UK), and 1 % NaCl (Merck, Damstadt, Germany). Restriction enzymes and *pfu* DNA polymerase were purchased from Fermentas (Burlington, Canada), and T4 DNA ligase was from New England Biolabs (Ipswich, MA, USA). Gel purification and plasmid extraction were performed and QIAprep spin miniprep kit (QIAGEN, Germany), respectively. Plasmids pKSC and pKSCP were from our previous work (Zhang et al. 2012a; Zhang et al. 2012b).

3.2.2. Random Mutagenesis

crp was subjected to error-prone PCR using Genemorph II Random Mutagenesis kit (Stratagene) according to the manufacturer's instructions. 30 ng of DNA template was used. Primers A and B (see Table 1) were used to amplify full-length chimeric *crp* genes. The ep-PCR program was set as follows: 2 min at 95 °C, 30 cycles of 95 °C for 1 min, 62 °C for 1 min, followed by 72 °C for 1 min, and finally 10 min at 72 °C. The error-prone PCR products were recovered from 1.0 % agarose gel, digested with restriction enzymes *Bam* HI and *Kpn* I, and inserted into plasmid pKSC that was treated with the same enzymes. The recombinant plasmids constructed were transformed into *E. coli* JW5702 Δkan by electroporation using Eppendorf Multiporator (Hamburg, Germany).

Table 1: Primers used in this study with restriction site underlined.

Prime r	Sequence
A	5'-GAGAG <u>GATCC</u> CATAACAGAGGATAACCGCGCATG-3'
B	5'- AGAT <u>GGTACCA</u> AAACAAAATGGCGCGCTACCAGGTAACGCGCCA-3'
C	5'-GGAAAACATATGATTCCGGGGATCCGTCGACC-3'
D	5'- CGGTATCATATGTTTTCCTGACAGAGTACGCGTACTAACCAAATC G-3'
E	5'-GAATTC <u>GAGCTCG</u> TGTAGGCTGGAGCTGCTTCG-3'
F	5'-GGAAAACATATGATTCCGGGGATCCGTCGACC-3'
G	5'-ATCCGAATTCTGGAAGGAAAGAAAATCGAGTAACTCTGCT-3'
H	5'-CTACAC <u>GAGCTCT</u> TGACGCAGTGGAGTAGCAAAAATG-3'
I	5'-TACCCTCGAGCGATGTGGCGCAGACTGATTTATC-3'
J	5'-CCTAGGTTAATTAAGACTTAGTGACTCGCGCAGCGGTTGTTC-3'

3.2.3. Phenotype Selection

The transformants were cultivated in 50-ml LB medium containing 40 g/L ethanol at 37 °C, 200 rpm for 12-16 h, which was repeated for two subcultures. After each round of selection, cells were plated onto LB agar plates. Individual colony was randomly chosen for DNA sequencing. To exclude the possibility of false positive and accumulation of chromosomal mutation, the mutated *crp* genes were digested and re-ligated to plasmids pKSC and the recombinant plasmids were re-transformed into *E. coli* JW5702 Δkan .

3.2.4. Cell Growth Profile

For each mutant strain, overnight seed was inoculated into 10-ml fresh LB broth containing different ethanol concentrations (0 g/l, 55 g/l and 62 g/l) with similar initial OD600 at ~ 0.06. Cells were cultured at 37 °C with a constant agitation of 200 rpm. Their growth was recorded spectrophotometrically by measuring the absorbance at 600 nm. The *E. coli* JW5702 Δkan harboring pKSCP plasmid was used as control, and denoted as wild-type (WT) in this study.

3.2.5. Chromosomal integration

The *crp* operon from CRP mutant was integrated into the chromosome of *E. coli* JW5702 Δkan using the λ -Red recombination technique (Datsenko and Wanner 2000). The *crp* operon from CRP mutant was first amplified using primers C and D (Table 1), while the kanamycin marker (Kan) was amplified from pKD13 using primers E and F. The upstream homology region (H1) was amplified from *E. coli* BW25113 using primer pair G and H, while downstream homology region (H2) was amplified with primers I and J. All these PCR fragments were ligated into pETDuet-1 (Novagen, Madison, WI) after being

digested with appropriate restriction enzymes to produce recombinant plasmid *crp*-HKpETduet. pETDuet-1 was chosen due to availability of multiple restriction sites. The DNA cassette containing H1-Kan-*crp* operon-H2 was obtained after digesting *crp*-HKpetDuet with *Nde* I and *Pac* I. This DNA cassette was then electroporated into *E. coli* JW5702 Δkan for chromosomal integration, and the site specific localization into original *crp* operon chromosomal site was verified through PCR. DNA sequencing was performed to confirm the mutation in the integrated *crp* operon was exactly the same as in the original mutant.

3.2.6. Survival upon ethanol exposure

The survival of CRP variant was measured by exposing it to a high ethanol concentration. Both WT and the variant were first cultured in LB medium until their OD600 reached 0.6, followed by the exposure to 150 g/l of ethanol. Aliquots of 10- μ l cell culture were taken at an interval of 5 min for a total period of 60 min, and serial dilutions were plated onto LB agar plates containing 30 μ g/ml kanamycin. The number of colonies was counted after overnight incubation at 37 °C. “Survival” is defined as the ratio of CFU after exposure to ethanol to the CFU when ethanol is absent.

3.2.7. RNA isolation

One percent (v/v) overnight cell culture was inoculated into LB medium with or without 60 g/l ethanol. Cells were cultured for 2.5 h in the absence of ethanol, or for 8 h in 60 g/l ethanol. The isolation of total RNA was performed using PureLink RNA mini kit (Ambion, Carlsbad, CA, USA), with PureLink DNase (Invitrogen, Carlsbad, CA, USA) treatment according to the manufacturer’s instructions. The quality and integrity of the isolated RNA were determined

through spectrophotometer and agarose gel electrophoresis. About 800 ng total RNA was converted into cDNA by reverse transcription in 20- μ l reaction mixture using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with random primer mix provided following the recommended protocol.

3.2.8. Quantitative real-time reverse transcription PCR (RT-PCR) using OpenArray

RT-PCR using OpenArray Real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) was performed in duplicates. OpenArray is a high throughput real-time PCR platform, which allows gene expression of 224 genes to be assessed on a single plate. The Lightcycler® FastStart DNA Master SYBR Green I (Roche, Germany) was used in reformatted OpenArray® real-time PCR plates (Foster City, CA, USA). 33 nl reaction mixture was loaded into each through-hole on the OpenArray® real-time PCR plates with the OpenArray® AccuFill™ system (Applied Biosystems, Foster City, CA, USA). The real-time PCR conditions were carried as follows: 1 cycle at 90 °C for 10 min, 36 cycles at 48 °C for 18 s, 54 °C for 1 min, 77 °C for 22 s, 72 °C for 70 s, and 95 °C for 20 s, followed by 1 cycle at 70 °C for 5 min. The bacterial 16S rRNA (*rrsG*) was used as the internal standard and the sequence of the primers are given in Table 4 (see supplementary information). The values of cycle threshold (C_t) were provided by the OpenArray® Real-time qPCR Analysis Software Version 1.0.4, and $2^{-\Delta\Delta C_t}$ method of relative quantification was utilized to compute the relative expression level. The *p*-value for each gene was calculated by student's *t*-test using IBM SPSS Statistics Software Version 19.

3.2.9. Cross resistance to other alcohol

Cell culture was prepared by diluting overnight seed into fresh LB medium containing different alcohol. Cell growth was recorded by OD600 readings with incubation at 37 °C, 200 rpm. The alcohol concentrations used are presented in volume ratio as follows: 3.1 % 1-propanol, 1.3 % 1-butanol, and 0.45 % 1-pentanol.

3.3. Results

3.3.1. Isolation of ethanol-tolerant CRP mutants

Error-prone PCR was carried out to introduce 2 - 4 nucleotide substitutions per *crp* by varying the amount of DNA template, corresponding to an average of 1 - 3 amino acid mutations per mutant. Recombinant plasmids containing mutated *crp* inserts were transformed into competent *E. coli* JW5702 Δkan and the total library size was greater than 10^6 . The mutagenesis libraries were then enriched through repeated subcultures containing 40-45 g/l ethanol to identify “winners” with enhanced ethanol tolerance. The mutated *crp* inserts of these “winners” were digested, re-ligated to plasmid pKSCP (Figure 7), and the resulting recombinant plasmids were re-transformed into fresh *E. coli* JW5702 Δkan to eliminate the possibility of false positives or chromosomal mutations. Three ethanol-tolerant mutants (E1-E3) with improved growth under ethanol stress were finally selected and their amino acid substitutions are summarized in Table 2.

Figure 7: Vector map of plasmid pKSCP containing native *crp* operon. *crp* gene insert are ligated into the *Bam* HI and *Kpn* I restriction sites.

Table 2: Amino acid substitutions of the CRP mutants: E1 – E3.

Mutant	Amino acid substitutions
E1	H31D D53N G177A
E2	M59T
E3	V47E Q80L

3.3.2. Growth profile of CRP mutants

E. coli JW5702 Δkan harboring plasmid pKSCP, which contains the native *crp* operon, was used as control and designated as wild type (WT) in this study. The ethanol tolerance of E1-E3 was investigated at high ethanol concentration (62 g/l) by comparing their growth performance to that of WT. When cultured without ethanol, all strains presented similar cell growth rate at 0.47-0.48 h⁻¹

(Figure 8A). With increasing ethanol concentration in the culture medium, E1-E3 demonstrated better growth than that of WT with E2 displaying the best ethanol tolerance. When ethanol concentration reached 62 g/l (Figure 8B), the growth rate of E2 was 0.08 h^{-1} , as compared to WT's 0.06 h^{-1} . It also illustrated a longer exponential growth phase to reach a higher OD600 value (0.19) than WT (0.10).

Figure 8: Growth profiles of E1-E3 and WT in LB medium containing different concentrations of ethanol at 37 °C. **(A)** no ethanol, **(B)** 62 g/L ethanol. Values shown represent average of triplicate experiments.

3.3.3. Chromosomal integration

Since E2 demonstrated the best ethanol resistance among all mutants, its *crp* operon was integrated into the chromosome of *E. coli* JW5702 Δkan to create strain iE2. Chromosomal integration was performed to avoid disadvantages of using plasmid-based system, such as genetic instability due to segregation or horizontal gene transfer, metabolic burden resulting from plasmid maintenance in cells, and need for supplementing antibiotics (Westfall and Gardner 2011; Yadav et al. 2011). DNA sequencing results confirmed that iE2 had the same amino acid substitution as E2 in its *crp* operon. The growth of iE2 under ethanol stress was investigated with its parent strain BW25113 as control. In the absence of ethanol, iE2 shared similar growth pattern with BW25113, with a growth rate of 0.43 h^{-1} compared to BW25113's 0.5 h^{-1} (Figure 9). When both strains were facing high ethanol challenge (62 g/l), iE2 (0.07 h^{-1}) not only grew faster than BW25113 (0.055 h^{-1}) but also was able to reach a higher OD600 value (0.13) than BW25113 (0.09). As such, iE2 was used for further investigation.

Figure 9: Growth of iE2 and parent strain BW25113 in LB medium containing different concentrations of ethanol at 37 °C. **(A)** no ethanol, **(B)** 62 g/L ethanol. Values shown represent average of triplicate experiments.

3.3.4. Survival test

To further demonstrate iE2's improvement on ethanol tolerance, both iE2 and BW25113 were exposed to 150 g/l ethanol and their survival was measured (Figure 10). iE2 exhibited significantly better survival than BW25113 over the 1-h period examined. For instance, after 15-min exposure to 150 g/l ethanol, iE2 had over 10 % survival whereas BW25113 only demonstrated less than 0.01 % survival. Even after 1-h exposure, iE2 still demonstrated more than 1000-fold higher survival than BW25113.

Figure 10: Survival comparison between iE2 and parent strain BW25113 when exposed to 150 g/L ethanol. Cells were grown to mid-exponential phase ($OD_{600} \sim 0.6$) in LB medium before being exposed to 150 g/L ethanol for a period of 1 h.

Figure 11: Tolerance of iE2 towards other alcohol as compared to BW25113. Cells were cultivated at 37 °C in LB medium containing different alcohols: **(A)** 3.1 % (v/v) 1-propanol, **(B)** 1.3 % (v/v) 1-butanol, **(C)** 0.45 % (v/v) 1-pentanol.

3.3.5. Resistance towards other alcohols

The tolerance ability of iE2 towards other alcohols, namely 1-propanol, 1-butanol, and 1-pentanol, was also studied with BW25113 as the control. As shown in Figure 11, iE2 showed much better growth than BW25113 and was able to achieve a higher OD600 value at stationary phase with all alcohols tested. The presence of 3.1 % (v/v) 1-propanol resulted in the growth rate of 0.129 h^{-1} for iE2, which was higher than that of BW25113 at 0.087 h^{-1} . When iE2 was grown in 0.45% (v/v) 1-pentanol, its growth rate at 0.062 h^{-1} was still higher than BW25113 at 0.047 h^{-1} . Although iE2 and BW25113 shared similar growth rate (0.068 h^{-1}) when cultivated in 1.3 % (v/v) 1-butanol, iE2 was able to reach higher OD600 (Figure 11B).

3.3.6. RT-PCR analysis using OpenArray®

In order to investigate on the transcript profile changes of CRP-regulated genes in iE2, we have applied RT-PCR using OpenArray® to analyze 444 CRP-regulated genes listed in Ecocyc (Keseler et al. 2011). It was revealed that 203 genes were differentially expressed in iE2 by more than 2-fold (p -value threshold < 0.05) in the absence of ethanol, which consisted of 48 up-regulated genes and 155 down-regulated genes (see SI Table 5). Genes that were involved in acid stress (*gadA*, *gadB*, *gadC*) were highly up-regulated by more than 20-fold, whereas many of the genes encoding transporters, such as *tnaB*, *glpT*, *srlA* and *mglB*, were down-regulated by more than 50-fold. Several genes involved in central intermediary metabolism (*acnA*, *sdhD*, *sucA*) were up-regulated in iE2 by 2-4 folds (Table 3). In contrast, the presence of ethanol stress resulted in the differential expression of 92 genes in iE2 as compared to BW25113, including 71 genes with increased gene expression and 21 genes with decreased

expression (see SI Table 6). Many of the up-regulated genes and down-regulated genes encode for membrane proteins (*yjcH*, *nmpC*, and *osmY*) or transporters (*mdtE*, *fecD*, *ptsG*, *srlA*, and *srlB*) as illustrated in Table 3.

We have also investigated the transcript profile of E2 with WT as control. It was found that a total of 169 genes (34 up-regulated and 135 down-regulated) were differentially expressed in E2 in the absence of ethanol stress (see SI Table 7), while 159 genes, including 28 up-regulated and 131 down-regulated, were expressed differentially in E2 under ethanol stress (see SI Table 8).

By comparing the list of differentially expressed genes in iE2 and E2, it was discovered that 14 genes were up-regulated and 93 genes were down-regulated in both iE2 and E2 in the absence of ethanol (see SI Table 9). On the other hand, when challenged by ethanol, only 6 up-regulated genes and 12 down-regulated genes were common in both iE2 and E2 (see SI Table 10). Majority of the common down-regulated genes in iE2 and E2 were transport-related genes. In particular, genes encoding for glucitol/sorbitol sugar transporter for the phosphotransferase system (PTS), such as *srlA*, *srlB*, *srlD* and *srlE*, were highly down-regulated in both iE2 (16- to 40- fold) and E2 (7- to 14-fold).

3.4. Discussion

The toxicity of ethanol towards microbial host is one of the great challenges during bioethanol production. Hence in the present study, we used transcriptional engineering tools to enhance *E. coli* ethanol tolerance by targeting its global regulator CRP. Random mutations were introduced to CRP via error-prone PCR and three variants E1-E3 were identified with enhanced ethanol tolerance. The *crp* operon from the best error-prone PCR mutant E2

was then integrated into *E. coli* genome to create mutant strain iE2, which also demonstrated better ethanol tolerance than its parent strain BW25113 under ethanol stress.

Table 3: Summary of differentially expressed genes in iE2 or E2.

Function	Gene	Fold-change* (iE2/BW25113)		Fold-change* (E2/WT)	
		-Ethanol	+Ethanol	-Ethanol	+Ethanol
Transport	<i>srlA</i>	0.017	0.028	0.52	0.072
	<i>srlB</i>	0.041	0.025	0.38	0.088
	<i>mdtE</i>	17.2	5.5	2.8	0.26
	<i>tnaB</i>	0.012	4.3	0.039	0.35
	<i>glpT</i>	0.014	-	0.22	0.35
	<i>mglB</i>	0.026	2.5	0.030	0.60
	<i>ptsG</i>	0.72	0.23	3.3	0.20
Membrane protein	<i>yjcH</i>	0.59	8.8	0.13	-
	<i>nmpC</i>	0.065	3.3	-	0.38
Enterobactin biosynthesis	<i>entA</i>	0.063	-	0.10	7.5
	<i>entB</i>	0.11	1.4	2.05	2.1
	<i>entE</i>	0.1	0.61	0.50	3.8
	<i>entD</i>	0.078	2.4	-	2.1
Iron transport	<i>fecA</i>	0.59	1.7	2.5	0.31
	<i>fecB</i>	0.73	-	1.7	0.75
	<i>fecC</i>	0.66	3.9	1.8	-
	<i>fecD</i>	0.75	4.4	-	-
	<i>fecE</i>	-	2.9	1.4	-
Stress response	<i>gadA</i>	20.3	-	2.7	-
	<i>gadB</i>	50.5	-	7.2	-
	<i>gadC</i>	44.8	-	6.5	-

	<i>rpoS</i>	2.1	1.3	0.67	-
	<i>osmY</i>	18.7	2.2	1.5	-
	<i>marA</i>	1.3	3.1	0.48	2.7
	<i>bhsA</i>	3.5	2.5	0.41	4.1
TCA cycle	<i>acnA</i>	3.2	0.66	1.2	1.3
	<i>sdhD</i>	2.3	-	0.37	0.74
	<i>sucA</i>	2.0	-	1.6	-
Amino acid metabolism	<i>ilvN</i>	0.11	1.7	0.3	0.13
Energy metabolism	<i>fixA</i>	-	-	-	27.4
	<i>tdcE</i>	0.17	11.2	0.25	0.33
Carbon compound catabolism	<i>galE</i>	0.10	22.3	0.001	265
	<i>gatD</i>	0.15	-	0.3	0.11

*Fold-change values with $p > 0.05$ would not be shown in the table.

We have studied the amino acid mutations in E1-E3. CRP is composed of an N-terminal cAMP binding domain (residues 1–134) and a C-terminal DNA recognition and binding domain (residues 140 – 209) that are connected by a hinge region (residues 135-139) (Passner et al. 2000; Passner and Steitz 1997). The binding of cAMP activates CRP and the resulting CRP-cAMP complex induces sequence-specific DNA recognition within or near the promoters of the genes that are under its control (Toyama et al. 1991). Six amino acid mutations, including H31D (β -strand 2), V47E (β -strand 4), D53N (AR3), M59T (β -strand 5), Q80L (β -strand 7) and G177A (E-helix), were adopted by E1-E3. β -strand 4 and 5 are known to be involved in the interdomain contacts in CRP-cAMP complex, while β -strand 7 plays a role in sensing the cAMP-binding signal (Won et al. 2008). D53 resides in the activating region 3 (AR3), which is

essential in regulating the promoter activity in CRP-dependent Class II promoters (Harman 2001; Rhodius and Busby 2000). In addition, it was reported that mutations to D53 could result in CRP activation even in the absence of cAMP (Won et al. 2008). E-helix, where G177 is located, is responsible for the recognition and binding to specific DNA sequence after activation of CRP (Harman 2001; Popovych et al. 2009).

We have previously isolated CRP mutants with improved 1-butanol tolerance in *E. coli* DH5 α , which displayed better growth in 1.2 % (v/v) 1-butanol in LBGMg medium (Zhang et al. 2012b). When comparing the amino acid substitutions in E1-E3 with the 1-butanol tolerant CRP mutants, we found that there is no repeated mutation among the variants. The best 1-butanol-tolerant mutant, MT5, was found to grow twice faster than its control to reach a higher OD600. From this study, although iE2 can also tolerate 1.3 % (v/v) 1-butanol with a higher OD600 attained (Figure 11B) than its control, iE2 is dissimilar to MT5 as iE2 shared similar growth rate with its control. This implies that the choice of selection pressure essential in bringing out the desired cell phenotype. However, the difference in 1-butanol tolerance of iE2 and MT5 is incomparable, since both are engineered in different *E. coli* strains with different culture medium.

Ethanol tolerance can be attained through genetic modifications to alter structural components or to reprogram metabolic processes and stress responses (Goodarzi et al. 2010). Contrary to previous findings whereby elevated gene expression of tricarboxylic acid (TCA) cycle genes was found in ethanol-tolerant strain (Gonzalez et al. 2003; Goodarzi et al. 2010; Horinouchi et al. 2010), the fold-change for majority of the TCA cycle genes in iE2 or E2 was

either less than 2-fold or statistically insignificant ($p > 0.05$) when cultivated with or without ethanol. iE2 also demonstrated an up-regulation in genes involved in general stress response (*rpoS* and *osmY*) and drug resistance (*marA*), which is in agreement with previous report on transcriptome analysis of ethanol tolerant strain LY01 obtained via spontaneous adaptation (Gonzalez et al. 2003). Genes involved in iron ion transport (*fecA*, *fecB*, *fecC*, *fecD* and *fecE*) and enterobactin biosynthesis pathway (*entA*, *entB*, *entC*, *entD*, *entE* and *entH*) were also up-regulated in ethanol tolerant strain (Gonzalez et al. 2003; Goodarzi et al. 2010; Horinouchi et al. 2010). Likewise, we have found the expression level of *fecC*, *fecD* and *fecE* and *entD* were higher in iE2 under ethanol stress (2.4- to 4.4-fold). Besides *entD*, other enterobactin biosynthesis genes such as *entA*, *entB* and *entE* were also up-regulated in plasmid-based mutant strain E2.

When comparing genes that are both up-regulated in iE2 and E2, two genes, *bhsA* and *marA*, are of considerable interest. It was previously reported that *bhsA*, encoding for putative outer membrane protein, could be induced by several stress conditions and its deletion would result in acid, heat, hydrogen peroxide or cadmium sensitivity (Zhang et al. 2007), whereas elevated gene expression of *marA*, encoding multiple antibiotics resistance protein, was observed in ethanol tolerant strain LY01. Despite *marA* being up-regulated in both iE2 and E2 (SI Table S7), as well as in LY01 under ethanol stress, overexpression of *marA* was not found to improve ethanol tolerance in *E. coli* (Gonzalez et al. 2003).

We have made attempts to overexpress ten genes in *E. coli* BW25113, including genes encoding transporters (*mdtE*, *srlA*, and *srlB*), membrane protein (*yjcH*), and metabolic enzymes (*entA*, *tdcE*, *galE*, *gatD*, *ilvN*, *fixA*), but neither

improvement nor decline in ethanol tolerance were found after overexpression of these genes. Since ethanol tolerance is elicited through the involvement of multiple pathways in *E. coli*, it was previously suggested that single genetic modification was not sufficient to lead to substantial increase in ethanol tolerance (Gonzalez et al. 2003; Goodarzi et al. 2010).

Conventional strain engineering tools have been adopted on the selection and engineering of *E. coli* to enhance microbial ethanol tolerance in the past. For example, the ethanol tolerance of *E. coli* was increased by spontaneous adaptation on medium supplemented with ethanol (Chen et al. 2010; Horinouchi et al. 2010; Yomano et al. 1998) or metabolic engineering (Luo et al. 2009). However, these approaches are often resource-intensive, for instance, culturing *E. coli* for 1000 hours to obtain ethanol-tolerant strain in adaptive evolution (Horinouchi et al. 2010), or require specific genetic and metabolic information. As for using UV/chemical mutagens to improve cellular functions, mutations are often difficult to map in the genome, which makes the improvement mechanism inaccessible for new strain engineering. By contrast, random mutagenesis of *crp*, followed by a selection in 1-2 subcultures with increasing ethanol concentrations could already generate three mutants that could perform better in high concentration ethanol as shown in this study. The information obtained from the changes of gene expression level in iE2 and E2 may also be helpful in enhancing strain performance under other stresses due to the overlap of different strain phenotypes. Thus, we believe that transcriptional engineering can offer more edges over conventional strain engineering in the following aspects: i) transcriptional engineering can cover a vast gene space and it is easy to conduct; ii) little knowledge of genotype-phenotype

relationship and metabolic pathways is required; iii) compared to classical strain engineering methods of using mutagens or spontaneous adaptation, the selection period can be significantly shortened from months to days. In conclusion, transcriptional engineering offers advantage of perturbation on gene transcription level, resulting in large diverse phenotypic libraries and making the isolation of variants with markedly improved phenotypes more efficient (Alper et al. 2006).

3.5. Acknowledgement

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3.6. Supplementary Information

3.6.1. Cell morphology studies using Scanning Electron Microscopy (SEM)

Morphological changes of iE2 and parent strain BW25113 after their exposure to ethanol were examined by scanning electron microscopy (SEM). Cells were first cultured overnight and then inoculated (1 % (v/v)) into LB medium with or without 60 g/L ethanol. The culture was incubated for 8 h and collected by centrifugation at 4 °C, 4000 rpm for 5 min. Cell pellets were washed thrice with 0.9 % NaCl solution and resuspended in the same solution. 20 µl of cell suspension was fixed by immersion in 2 % glutaraldehyde for 3 h at 4 °C. Cells were then dehydrated by series of progressive ethanol at 10, 30, 50, 70, 90, and 100 % for 15 min and dried at room temperature. Samples were then mounted on SEM stubs and coated with platinum for 70 s using gold

sputter. Cell morphology was observed in a JSM-6700F field emission scanning electron microscope (JEOL, Japan).

Figure 12: Cell morphology of iE2 and BW25113 in the absence/presence of ethanol using scanning electron microscope. The cells were grown in the absence and presence of 60 g/L ethanol at 37 °C for 8 h. **(A)** BW25113, no ethanol. **(B)** BW25113, 60 g/L ethanol. **(C)** iE2, no ethanol. **(D)** iE2, 60 g/L ethanol.

The measurement of cell size was based on more than 70 cells. The average size of iE2 and BW25113 grown without ethanol was $1.66 \pm 0.30 \times 0.80 \pm 0.05 \mu\text{m}$ and $2.12 \pm 0.37 \times 0.91 \pm 0.08 \mu\text{m}$ respectively, i.e. BW25113 was longer than iE2. Exposure to 60 g/L ethanol led to cell elongation in both iE2 and BW25113. The cellular size of iE2 increased to $3.71 \pm 0.62 \mu\text{m} \times 1.11 \pm 0.12 \mu\text{m}$, whereas the size of BW25113 increased to $3.57 \pm 0.86 \times 1.05 \pm 0.11 \mu\text{m}$. By comparing Figure 12B and Figure 12D, it was also evident that the surface of

BW25113 was rougher and more wrinkled than iE2 when cultivated under ethanol stress.

Table 4: Primers used in OpenArray® real-time PCR.

Gene	Forward sequence (5'→3')	Reverse sequence (5'→3')
<i>aaeA</i>	GGTTGCGCTGGTGAAACAGAA	CCCTGCGGCAACACTATCAAC
<i>aaeB</i>	GCTGACGCTGATTACCCAATCC	ATAACTCTCCGCAGGCGTTTGA
<i>aaeR</i>	TAAACCGTAGCACACGCAGCAT	TGAAGAACAGCCAATGCGTAGC
<i>aceA</i>	GGCGAGGGTATGAAGCACTACG	ATTCTTCAGTGGAGCCGGTCAG
<i>aceB</i>	GCTGGAAGCCAATAACGGTCAC	GCATACCTTCTTCGGTGCGTTC
<i>aceE</i>	CGGCAAGATCATCAACGAAGT	TTCACGAACGTACGCACCATCT
<i>aceF</i>	CCGATATCACCGAGTTGGAAGC	CGCCACACCGATGTTGATGTAT
<i>aceK</i>	GGAAGTGAATTTCCGCGACATC	AGTAATCAGCGCGGAACAGGTC
<i>ackA</i>	TACGCCCTGCCTTACAACCTGT	CGGATAGCAGAAACGGAACCAC
<i>acnA</i>	CAAAGGCGATCCGGTTTATCTG	CTCCTGCCAACCGTAGGTATCG
<i>acnB</i>	GTGGATGATCGCGGAAGGTTAC	GGAGCACACAGGATTGGCTCTT
<i>acs</i>	GCGGCGCTGACCTTTAAATATG	CCAGTTGGGTACGCCTTCAAAC
<i>actP</i>	CTATATGTCCGCCGCCCTCATTC	GTAAGAGGCCACATCGGCAAAG
<i>aegA</i>	CTTGCTCACTTTCGGCATTCTT	GAAGACCGCGTCGTATTGTTCC
<i>aer</i>	GACGATATCGCCGACAGTACCC	GCTGGCTAAATGACGCACTTCC
<i>agaA</i>	TGGAAGTATAGCCGATGGTCA	CACCGTGCATCTGCACTTCTTC
<i>agaV</i>	ATGGTACTGGCAGAAGGGATCG	TTGTTGTTTGCCATTGGCGTAG
<i>agaW</i>	TACGCTGGAAGTGGTGTGGATG	TCTGTACTGCGACAGCGAAAGG
<i>agp</i>	CTCAGCAAACCTCCAGCTTACCGA	TCTACCAGCGAGTTGCCGACTT
<i>aldA</i>	CAATTTGGTAACCCCGCTGAAC	ATCGACATTTCTTGGCGAACAT
<i>aldB</i>	GTTTACCGGCTCAACGGAAGTG	AGGCAAACAGTGCAAAGCCTTC
<i>ampD</i>	TGTTGTTAGAACAGGGGTGGCTG	AATAATGCGTCGATCCACGGA
<i>ampE</i>	GATGCCGTGCTTCATGTACTGG	CACCTTATCGACATGCGGTTCA
<i>ansB</i>	AATATCGGCTCCCAGGACATGA	GTTTGTTCGATTTTACCGTCAG
<i>araA</i>	TGTCGCCAGCTTGATTGATCTC	GTGCATGGCTGAAGACGGTATG
<i>araB</i>	CGATTGCAATTGGCCTCGATT	TGTAGTCACGCGGATGATGACG
<i>araC</i>	GGGATACTCGTTTAAACGCCCATC	TAGTGATGAATCTCTCCTGGCGG
<i>araD</i>	CAAACGCCAGGTATTAGAAGCCA	CCATATCGTCAGCGGTCATGAC
<i>araE</i>	GTTGATGGTCACTCGGCATC	ACGCAATACTTCTTCCGCCTCA
<i>araF</i>	TACCGCCAACGAACTGGATACC	CGGATGTTGAACCAGCATTGAG
<i>araG</i>	ATATGCAGCAGGTTGACCACGA	AACCGCCAGACTTATTGGCGTA
<i>araH</i>	CATTGGCAACGATGCAGATTGT	ATAATCAGACACGCGACGGTGA
<i>araJ</i>	CAGGTGTGTTTGCCTGGTTCAG	CGCAGTGGTGAATAACGTCCTG
<i>argG</i>	GATTGCCTGCGGTTTCGACTAC	ATCCCAGAATTTACGCCCATA
<i>aroA</i>	CCACCGTGTTGATTGTTGTTGC	TGTGGTTCGGCGGTTTGTAGTC
<i>ascB</i>	AACCAGTCTGAAGGTGCGTTCC	TGCCATCAGGGCGATATCTTCT
<i>ascF</i>	ACCGTTTCACAAGCCTTTCAGG	CATTGCCAGCAGTTTGACCATC
<i>aspA</i>	TGGCCAATATCGGTCTGGAAGT	CACGCAGTTGGTTAATCGCATC
<i>bglB</i>	ATCCGGTTGGATTACGGGTTCT	TCGGCAATCGCTTCATTTACCT
<i>bglF</i>	TTAACAGTGTGGCAGGCCTTGA	CACCTTGTTCGGTCGTCCACTG
<i>bglG</i>	TTGCGATTAAACGCTTTCAGCA	TATTTCCGCTCATTTGGGCACT
<i>bhsA</i>	CGAAGTTCAGTCAACGCCAGAA	GCTGTTCCATGGAGGGTATTCTG
<i>caiA</i>	CGAGGCTATTGGTCGTTTCCAG	TGCATTGCGCTATCCACAACCT
<i>caiB</i>	CCGATGAAGTACTGTGGCAGCA	GCTGGTCAACATCACCGTTCTG
<i>caiC</i>	ACAGATTTCAGCAGGAAGATGCCA	AGAATTTCCGCCGTATCGTCAG
<i>caiD</i>	CATTACATCTGACCCGCAATGG	CTCTCCGGCACCGGTAATAATG

<i>caiE</i>	AGCGGGAGCCAATATTCAGGAT	AATGCTCTCTTCGCCAATGACC
<i>caiF</i>	AGCAAGGCGGTAAATACCCTGA	CTTCTGCTCGCGGTAAAGTTCC
<i>caiT</i>	TGGGTTGGGTGTTTCATTGTCAG	CACAGTACGACCACGGGAGATG
<i>cdd</i>	GTGAGCGGAACCTGGTATTTTCG	AACTGACGGCAGTGACCACAAG
<i>chbA</i>	ATCAGTCACGGATGGCATTGAA	TAATCAGTTCACGCGCAAGCAT
<i>chbB</i>	GTTCTGCGGGCATGTCTACCTC	ATCTGCGGCCCTAATAACACGA
<i>chbC</i>	AAGCAGCCACACGTTAATGCAA	TACGTTGCCGCAATACCTTTC
<i>chbF</i>	GCACGAGCGCAGGTAGTACAGA	CCCATGATGCGGGATATTAACG
<i>chbG</i>	GCACTACCCAGGGATTTCAGCAG	TCCGTTAAGCGTGGGAAACAGT
<i>chbR</i>	GCCTTATTGCTTTGTCTGCTTCG	AGCCACTGCGGTACATCATCAA
<i>chpA</i>	AGGTAGCGAGCAAGCTGGACAT	TCAGCTAACGCTACGCCATCAC
<i>cirA</i>	TTTCTTGTGCATGGCCTGTGTT	TGTACGCCAGGCACTTCTTTGA
<i>cpdB</i>	GCCAGTTGACCTTCCGTAATGC	GACTGTGGTTTCGTGCTGTTGG
<i>crp</i>	CATTCTGATGCGTTTGTCTGCAC	CCGACAATCTGACCAATTTCCTG
<i>crr</i>	AATATCGAAGACGTGCCGGATG	TGTCGATACCGAAGTGGACGAA
<i>csgD</i>	TCCATGGAGGATCAAGAACGTG	GTTATTAGACGCGCCGATACGC
<i>csgE</i>	GAATCTTCACGCCGTTGAGGTAG	AGTGATCCAGCTTCCCCATCG
<i>csgF</i>	TTGGTATTGAAACACCCTCAGCG	CGATCTGTACGTTCAACTGCAA
<i>csgG</i>	ACACGCAATACCAGCTCGATCA	CCGACATCAGGCACAGCATAAC
<i>csiD</i>	CGTCTGCTGGAACCTCACCTTCA	GCAACGTCTTCAACAGCAATGG
<i>csiE</i>	TTGAAAGTGAATACGGCGTCCA	TTCGCGTAACTGCTGCTCAATC
<i>cstA</i>	CAGGGAAATACCTCGTCTGGACA	CACATAGTCCAGCCCGTCGTTAT
<i>cyaA</i>	GTGCAGGTTGAAACCAATCACG	TGACGTCACGTACCAGCTCCTC
<i>cyoA</i>	TGGAAGTACCGTGCGAGCAATA	CGATGGTAATGGGCTTCTCGTC
<i>cyoB</i>	ACTTGATGCAGTCCCGTTCCAT	AATCAGTACTGCACCGCTGGCT
<i>cyoC</i>	TGGCAACTGATACTTTGACGCAC	CACCAGAACGGCATAGGTAGCA
<i>cyoD</i>	CGTTCTGGATGGTGATGACAGG	GCGATGATTAGCACGGTGAAGA
<i>cyoE</i>	TGCTGGCTATCTTCAGCCTGTG	CGTAACCGCCAAGAGAGAGCAT
<i>cysG</i>	ATGCAGGCATGTAAACCCTCGT	CGCATCGACCACGTTACAGAAG
<i>cytR</i>	GCATGTTGTTGCTGGGTTCAAG	CAATCCGTTTATGCCCTTGCTC
<i>dadA</i>	GTTCTTACTCGACGGCGATGCT	CAACACGAATGCGGTTATCGAA
<i>dadX</i>	GCTTACGGGCATGGTATTGAGC	GTTGCTGTGTACGCAGGTGGTC
<i>dctA</i>	CGACGGCTTCGTTAAGCTCATT	AATCAGCGCGATGGTACTGACA
<i>dcuA</i>	GGTCTGTTTCATTCTGCCGACCT	ATGAAGCTACCCAGCACGAAGC
<i>dcuB</i>	ATCGCTGTAGTCGCCCTTCTTG	CGATCATACCGGAACGGAAGAC
<i>dcuR</i>	CTGCATTACGGTGTCTGTTGATT	AGGATCCTGTTTCGTTGGAGCTG
<i>deoA</i>	CCGACCGACTTCGTTGAGAACT	CGACGCTGTAATCGATGGTGTC
<i>deoB</i>	TTCCGGTACGGTCATTCTGGAT	GATATTGTAGCCGCCGTTGGTC
<i>deoC</i>	GACGAGAAAGTGATCGCCCTGT	GCGATGTCGATGTCTGCTGTTAC
<i>deoD</i>	ATCCCGTCCTGCTCCATCTACA	AGTCAGCGATAGCGGCAAAGTC
<i>dgsA</i>	ACCGGCGTTCCGGTTTATATTC	TGTGTGGCCTATTTCCACGAGA
<i>dksA</i>	AAGTCGATCGCACCGTTACACAT	GCCGAAATCTTCGTCTTCCACTT
<i>dsdA</i>	TCTGGGGTTATCAATCGGCATT	GACTGCGCTGCTTTACGTCTT
<i>dsdX</i>	TTAGGCGCAACGTTATTCACCA	ATCGTCCCCATGCTCATATGCT
<i>dusB</i>	CCTCTTGCAGTACCCGATGTC	TCAGAGCCTGAATGCCACAGTC
<i>ebgA</i>	ATGAGTGGCAAACCGACATCCT	CAGCACTTCACCATCGCTCTGT
<i>ebgC</i>	ATGCGCTATTTACCGGGCATC	GATTTGCCCTTCGTGGACCTC
<i>entA</i>	CGCGTATTGGCATGAGTGCTTA	ATACGCTGTTCTTCGGCGTCAT
<i>entB</i>	CTGCCGGAGTCTCACGATATTCC	GCAATATTTCGCGATCACCTGC

<i>entC</i>	CCGCCATTGATAGTGGCGTATT	TCTGACGCCAGCAGACGATTAC
<i>entD</i>	CACTACGCACAACCTGCAACACG	GCCAATCGGTTGACGAGATACC
<i>entE</i>	GCTGTGAGTTGCAGCAGGTGTT	TTGTAATAGCCGCGGAAGGTGT
<i>entH</i>	TGATCTGGAAACGCCATTTAACG	CCATGTAGTAAACCGAACGGCTG
<i>envZ</i>	GTTAAGCAACTGGCGGATGACC	CAATGATGGCGTTGCACTCTTC
<i>epd</i>	TACGGAATTGCCGTTGGTCTCT	CTAACGTCGTGTCGAGCATTTCG
<i>exuT</i>	TCAGATCCTGCGTAACCGTCAG	ACCGAGGATAACAACCGAGGTCA
<i>fadD</i>	TGACCGGACAGTATCTGCTGGA	CCGGTTGACCTGGTGGTACTTC
<i>fadH</i>	CGGCATTGCACCAGATTTAACA	ACCAGATGCGGTTGGTAGCTGT
<i>fadL</i>	CCGATGATGCAGGTAACGTCAG	ATCGGTGCAACAAAGTGCATGT
<i>fbaA</i>	ACTGTACACCCAGCCGGAAGAC	TTCCTGAGAATCACGCAGGATG
<i>feaR</i>	GGCGCTGGCGATATTACGTTAC	ACAGGCGATGACTGAGTTGCAC
<i>fecA</i>	GCCAGCTATGCGTATGTGAACG	GTTATCCGCAAACCTGGCTGGAC
<i>fecB</i>	CCCACTATCGCGAAGAGAGCAT	TAAGCGGCTGATGGTGGAAGAT
<i>fecC</i>	TTCCTGCTCGGCATTAACAGC	GTATGACGAAATCCGCCTCCTG
<i>fecD</i>	TGCTGAAGATGCTGGCAAAGAC	TGGCAATCTTCACAAAGCTCCA
<i>fecE</i>	GGTCGTAATCCCTGGCTGTCAC	GTATTCTGGGCCAGGACCATTG
<i>fepA</i>	AACCTGACCGGTAACCTCCACCA	GGCACCCAGGAAGTATCACCAC
<i>fis</i>	ACGCGTAAATTCTGACGTACTGACC	GGCTGTTCTACTTCAGCCAGTACCA
<i>fiu</i>	GCGTGGTGCCGATACCTCTAAC	CGGCTGCTTGCTGATCATATTG
<i>fixA</i>	ACAGCCTTAAGTGTGGGCGGTA	GGGCATAAAGGTCGGAAGAACC
<i>fixB</i>	AACAGCTTTGCAAGGCGATAGG	ATTGATGGCGAAAATGGTTTGC
<i>fixC</i>	ACTGGCGGAATATCGTCAGCAT	GGTCACTCCTTTCATGCCATCC
<i>flhC</i>	ATGCTGCCATTCTCAACCGACT	ATGCCAGCAGTGGTCTCTTCTTC
<i>flhD</i>	AGCGTCCGCTATGTTTCGTCTC	TGATGCCGGTATGAATTTGCTG
<i>focA</i>	TGTTGATTGTTGTTGCTAAGGCG	TTTGTGGTCGGCGGTTTGTAG
<i>focB</i>	CCCGTCTTCACAGGCATTAACC	GGCAATACCTGCCAGATTACCG
<i>fucA</i>	TTGGAACACGCGAACTTTCTGA	TAAAGTTGCGCCAGCACTTCAA
<i>fucI</i>	GATGCCGATGACACATCGATTTC	CATATCCACCGCCTGGACTTTC
<i>fucK</i>	GCCCAGGTTGATACTTCGCTGT	CAACATTTGCCAGGGTGTTC
<i>fucO</i>	GGGCGTGAAAGTGGAAGGTATG	CCACCGGTACAAACATCATCCA
<i>fucP</i>	TTGCAGCCGGATTAGGTTGTCT	GAACGTCTTGCGATTGATGTGG
<i>fucR</i>	CGATAGTCGACCTGCTGCTGAA	ATGTGAAAGGGATCGCCACTGT
<i>fucU</i>	CCAGGCGATTATCCCGTTATTTG	AAACGATTGATGCGGATGATGTC
<i>fumA</i>	CGATGAAAGAGATCCTCGCACA	ACCCGCGTAGTAAATCGGATGA
<i>fumB</i>	CTGCCGGTTATCCATCAGGTTC	GGTAGAAGCCGCCGTGTTTATG
<i>fur</i>	AAGAAAGCTGGCCTGAAAGTAACG	CAGCGTCGTCAAACCTGGTTCAG
<i>gabD</i>	GCGCAACTGGTTCAATTTGATG	CTGATGACCAGGAATGGTGTCTG
<i>gabP</i>	TATTACGCGCCTGCGAAGGTAT	AAAGCCGATGACTAGCCAGGTG
<i>gabT</i>	TCGCCATTGAGCTGTTTGAAGA	TCAATGGTGAGCGGTACAAGGA
<i>gadA</i>	CTCTCTGAACGTCTGCGTCTGC	CTGGGCAATACCCTGCAGTTTC
<i>gadB</i>	GCTTATCTGGCGGATGAAATCG	GCAGACGCAGACGTTTCAGAGAG
<i>gadC</i>	ACTGCTGTTGCTGGGTGTTCTG	CGTAATCACCAGCTGCGAAATG
<i>gadE</i>	TGGTAAACACTTGCCCCATAAGAA	CAGGGTGACGATGTCGCTCATA
<i>gadX</i>	ATGCGAACACGAGTTTGTACGG	GTTGCAAAGCACGTTGCATTCT
<i>galE</i>	GCTGGACGTGGTTAATGCCTTC	ATATCCCTGTGGATGGCGTGAC
<i>galK</i>	GTCGGAACCGTATTGCAGCAG	GCAATCGATCAGCAAGGCATG
<i>galM</i>	ATGCCAACCGTATCGCCAATAG	CGTCGGTCAGACGATATTGCAC
<i>galP</i>	CCGGGATGAACGTCATCATGTA	CCCAGCGTTAGCGTTGGTTTAC

<i>galS</i>	ATAACAGCACGCTGGTCAGTGC	AACGCATCAGAAACGTCCATCA
<i>galT</i>	CAGAGCTCAGCGTTGCAGCAT	GCTATTTGCCCAAATCTGACCGT
<i>gapA</i>	ATGAAAGGCGTTCTGGGCTACA	GGAGTAACCGGTTTTCTGTTGTCG
<i>gatA</i>	GGCGATTTCATGCTAAGTCGTCA	TCGACGATATCGGGCTGTTGTA
<i>gatB</i>	AACGCAAGATTATTGTCGCTTGC	TCCACTTTGGCAGTGGTGCATA
<i>gatC</i>	CGCTAAGCAGGCTCGTAGTCGT	TGATTACCCGGCACACAAACAG
<i>gatD</i>	TTGAGCCGATTACCGTTGGTCT	CTAATGTCGATCGCCGTCACAC
<i>gatY</i>	GTGCAAGTCAATGAAGCCGATG	AGTTCACCCACTGGCGAATGTT
<i>gatZ</i>	ATATGTTTCGTGCCGGCTTCAGT	CGGTGCCAATGACATAGCTCAG
<i>gcd</i>	GGCAATTAACAATACAGGCTCGC	AGCATCACAAGGCCAGCGATA
<i>gdhA</i>	CTCGTGTGATCACTGCGTCAGA	GGCTGTTGGCCTTCGAGATAGA
<i>glcC</i>	TCGCCATTTGTCAGGCTTCTC	GCAACACCGCGTTGTAGATCC
<i>glgA</i>	GTTTCAGAGATGTTCCCGCTGCT	GACGGGATACTACCTGCGCATC
<i>glgC</i>	ACTCTGGTGCAGCACATTCAGC	TAACGGCGGATAATGTCGAGGT
<i>glgP</i>	AATCGGCAGCGGTGTATTCAGT	CCCATATTGGCAATGTTTCAGCA
<i>glnA</i>	AGATGGGTCTGGTGGTTGAAGC	GGAGCCGTTATCACCGAACATC
<i>glnG</i>	TAGTTCCATCCGTTGGGTGCTT	TGGCGCTGTTTAATCTGCTTGA
<i>glnL</i>	ATAACCAGCGCCGCTTAAGTCA	GGTCCGCCTGTTTCGATAATCAC
<i>glpA</i>	CACCGTTGATCCATTTTCGTCTG	GTTTCGCCGGTGAGATGGTTAC
<i>glpB</i>	CCAGTTACAGCGCCAGTTTGTG	CGTTACGTTCCGCTACCAGTCC
<i>glpC</i>	TTAACGGTGCTGGATTCCAGT	TTACAGGTTTCGCAGTCGGTGA
<i>glpD</i>	AGCGGAGCTGAAATACCTGGTG	CAGCGATAACCTCTGCTGCGTA
<i>glpE</i>	AGAGTTTCGCAATGGGACATGC	CGTAGCCCTGTTGCAGCAGATA
<i>glpF</i>	GGCTCCCTTGCTGATTGGTCTA	CGGCACCAGGAAGTAAGGAATG
<i>glpG</i>	ACCCACGCGTTAATGCACTTCT	TTGCTGCACATAGCCGCTTAAC
<i>glpK</i>	ATACCCTGGACTGGGACGACAA	TTCACGCACAACCTGACCAACA
<i>glpQ</i>	GACTGGAATGAAACGCAGCAGA	GCTGCGATGTCTCCTCAATCAA
<i>glpR</i>	GCGCATTTGTCACCAACAATCTC	GTCGCTATCGATGCCGCTTATC
<i>glpT</i>	ACACTGGTGACCATCGCGACTA	CAGGTAACCAAACAGCCCGGTA
<i>glpX</i>	AGGCGATAAAGGCTGCTTCCTC	GTGGCGTGGTTTAGCCAGAATC
<i>gltA</i>	GATCAGCTGGCGACCGATTCT	ACATGACTGCCATTGGATGCG
<i>gltB</i>	CACCAGCGCTTCTCCACTAACA	AGTCAGAGCCGGTTTCGTTGAC
<i>gltD</i>	AGCCGCTGGGTATTGAAGTGAA	TCCATGTTGTGTGGACGGAAAC
<i>gltF</i>	CGCTGCATCCATTGGTATTGAC	AGCAACTTGTGAGCGAGCAGAA
<i>gntK</i>	CTGAATGACGACGATCGCAAAC	CTTTCAGGCGGCTTTCAATCAC
<i>gntP</i>	CAGGTCTGATCCTGCCGAAGTT	TTGGCGATGGTGGTAGAGATCA
<i>gntT</i>	TGCTGATTGCGATGTTACCTT	ATGTTGGTTTTCGTGCATCATGG
<i>gntU</i>	TTCGATTGTTGTCAGCCACGTT	CAACAGCTGGAACGCAATCATC
<i>gntX</i>	ATGCCACTGGCGTTAGGTCATT	ATAGTCGGCAACCGTGACCAGT
<i>grpE</i>	AAATTTCGCGCTGGAGAAATTCA	CGCCAAACTTACGCACAACATC
<i>guaA</i>	AGAAGTGCCTAAAGCGGACCTG	CCAGTGTGCGGTCATAAAGTCG
<i>guaB</i>	GAAGGCGGTATCGGCTTTATCC	AACCGTTACGCTCGGTGAGTTC
<i>gutM</i>	GCATCGTCGACACCTTGTTTATGA	CGTTTCAGTTTAAGCGCCAATGAT
<i>gutQ</i>	GCGCTCGCTTGCTGAATAAAGT	CGTAAATCGCCGTCGGTAAAGA
<i>gyrA</i>	AGATGTCCGAGATGGCCTGAAG	ATGCGGACGATCGTGTATAGA
<i>hlyE</i>	CAGGAGTATTACAGGCAGCCTC	GGGCGGATGCTTTCTTCTCAT
<i>hofB</i>	GAATATTCCACAGCTCACTGCCC	GCGTCCAGCAGGTGATCTCAA
<i>hofC</i>	ATGGCGAGTAAGCAACTCTGGC	CAGAATTGATGGCGATTGCTT
<i>hofM</i>	GCAGCGTCATCACATTATGTTGG	CACGTTATAGGCGGGACTGAGTG

<i>hofN</i>	TTTCTGCGTTTCTGGTTGCTGA	TATCTGTAAGCTGCGGGCGAGT
<i>hofO</i>	TGGTAGACACCGCTCCCTTCA	CTCTGCCAGCCGTGTAAATGC
<i>hofP</i>	CGAGCGCATCATCGGTGTAAT	TGCGGCTATCCATTGCTTCAT
<i>hpt</i>	ATATGGTGCTGGTGGGTCTGCT	CGTCCTTGCCACGGATATCTTC
<i>hupB</i>	TGGCCGTGCGTTAGATGCTAT	CTGCACGGAAGCTCGGTACTTT
<i>hyfA</i>	GGTGAAATGCGACCTGTGTGAT	TTGCTCCTGAGTGAGGGAAAGG
<i>hyfB</i>	GATGATCGTCGCCATGCTACTG	ATGAAACGTGCCGGGTACAAGT
<i>hyfC</i>	CTTGTCGTTACGCTGCTCAAGG	CGGTTAACCAGAAGACCCATGC
<i>hyfD</i>	TCATGGCACTGGTCACCATTCT	GAACGCGTGGTTGACGATGTAG
<i>hyfE</i>	TTATTCCGTCCGGCAATGATG	CAGGATATTGCGCTGGCTGAC
<i>hyfF</i>	AAGCAATCGTCCTTGCTTGACC	AGTTCAGCAATACGGCGGAGAG
<i>hyfG</i>	GATGACTGGCCGGAAGATATGC	CATCCACAAACAAGCGGAAGTG
<i>hyfH</i>	TGACTATCCAGACCGACGACCA	CGCGGGTATAGAGATCGGCTTT
<i>hyfI</i>	ATCACAGCGTATCGCCATTGAG	TACGCGAAAGAGTTGCTGAACG
<i>hyfJ</i>	ATCACGTTGGCGTGATTGATTG	GTGTGGCTGGCGTCAATAACAA
<i>hyfR</i>	GAAATTGGCGATCTGCCGTTAG	AAAGAGATCGCTGCGAAACTGG
<i>idnD</i>	AACGACGACATGGATCACTGGA	ATCATTGGGAATTCTGCCATCG
<i>idnK</i>	AGCCCCCATGTTCAATTCCTCT	CGACATTGTTCCGGTGACATTGG
<i>idnO</i>	AAGCACTGGTTGAGGACGAAGC	TAACATGCCGCCATCAACAAAC
<i>idnR</i>	ATTACGATCGCGATTCCGAAGA	CAGCCGTTCTCCCTGTACATCC
<i>idnT</i>	GCCATCGCGACTATCTTTGAGG	TGTTCCAGAAGGAGGGCATCTC
<i>ilvB</i>	GGAAGTGGACACCTACGGCATC	AAACTGCCGTTTGACATCCTT
<i>ilvN</i>	CGTAATTCTGGAGCTACCGTTC	CTGGTCGTCATTGACCAGTAGCC
<i>infB</i>	GCGGTTAAAGGCTCTCAGGTCA	TCATTACCGCTCTTCCAGCTC
<i>kbaZ</i>	CGGCGATTGAAGAAGAACTGGT	CTGGCTGTCCGGCCAGTAATAG
<i>lacA</i>	CCGATATGTGCGAAGGCTTACC	GCGGCCTATATGGATGTTGGAA
<i>lacI</i>	CTGTGGAAGCTGCCTGCACTAA	GCGCCGAGACAGAACTTAATGG
<i>lacY</i>	TTAGTGATGTTTGCGCCGTTCT	GAACATGATGCCGACAATCGAG
<i>lacZ</i>	ACCATGATTACGATTCACTGGC	ATTCAGGCTGCGCAACTGTTG
<i>lamB</i>	CTCCACCATCTGGGCAGGTAAG	TTGCTGGCGAAAGAGGAAGAAC
<i>lhgO</i>	AAACGGCATTTCGCTACGACAA	GAGCCCGGTGATATTCGGTTC
<i>lpd</i>	GGTGTTCACGAAGGTCACGTTG	AAGCGATAGCACGACCAGAAGC
<i>lsrA</i>	GAGAACGTTTACTGCGCGGTCT	TTAATATTCAGCGCCCGACGAT
<i>lsrB</i>	TCGCCTTACTTAGCGCTCTTGG	CATCAACGCCAGCTCTTTACC
<i>lsrC</i>	GTTTATAGCAGCGCGCAAATCC	AAAGTCGCGACACAAGCAACAG
<i>lsrD</i>	TGGTGGTTCCGGTTCCATTATC	CGCCAGCCACTCTTTAATTTGC
<i>lsrF</i>	CAAAGATATGGTGCGCGATCAG	GATAGCCTGCCAGCACATTTCC
<i>lsrG</i>	TGCGCTTCGATGTCTTACAGGA	CGGCATCAAACCATTGAACAGA
<i>lsrK</i>	TTCGGGCTGTGATATTCGACCT	CGAAACGGCAGCGATATACTCC
<i>lsrR</i>	CAGGACGATGCGACAATCATTC	GCGCGCTTAAAGGTAAGCCAAT
<i>lyxK</i>	GGGGATCGCTGAAATCAATCAC	CCGCATTGAGGGTAAATTCGTC
<i>malE</i>	CGTACGTTACAACGGCAAGCTG	GCGCTCTTACCTTTCGCTTTCA
<i>malF</i>	GCACAAGGGGAATACCTGTTTCG	TGCAGACCAGAGGGAAGAGGAC
<i>malG</i>	AGTACCGATTCTGGCGGTGGTA	GGCAAAGTCACCCACAGGTAG
<i>malI</i>	GAATCAGGGTGTCGATGGTGTG	GATGCTCCGTCAACAACCTGTGC
<i>malK</i>	GCGTATCGAAATCTCCCGTCTG	AACGGTCTGCCGGATAGTGGTA
<i>malM</i>	GAACATGACCCCATCAGCCTTC	AGTTGGGTCTGCTGCTGGAGAT
<i>malS</i>	ACGGCGTGATGCAAAGTGACTA	CATGCGACGAGAGGTAGCTCAA
<i>malT</i>	CTTAATACGCTGCCGGAAGTGG	CAGTACCTGCCATTACCGTTGC

<i>malX</i>	GTCGGCCTGGTGATTCCATTAG	CCACCAGAATGTGATGCAGACC
<i>malY</i>	GGTGGTGTATGGCCCTTCTGTC	ACCAACCATCAGCCTGCTTCTC
<i>manX</i>	AAGGCGTGAAAGCACTGAAAGC	CGGGAGACATTGGTTTCTTTGG
<i>manY</i>	CAGAGCATTGGTGCAGGTATCG	GCATTGCTTGCAGGAACAGAGA
<i>manZ</i>	ACTGACCAGACGGGCAAAGAAC	ACGAAGAAGCCAACGATGATCC
<i>marA</i>	GGGTTACTCCAAATGGCACCTG	GGTTCGGGTCAGAGTTTGTGTC
<i>marR</i>	CTGGATATTACCGCGGCACAGT	TTCCACCCAGCCTTTACAGACC
<i>mdh</i>	CAACACCTTTGTTGCGGAAC TG	GTTCTGGATGCGTTTGGTCAGA
<i>mdtE</i>	TTCAGGGCAGTACGCCAGTACA	CAGCAAGTCACCATTTGGGTTG
<i>mdtF</i>	GTGCTGGCGATAGGGTTACTGG	GCGGAAAGAACAACGGCAATAC
<i>melA</i>	ACCCAATGGCGATGAATACCTG	TATGGTTGATACCTGCGCAACG
<i>melB</i>	CGTCTGCTGAAGGAAGCCATCT	CAAATCCGCATCACCGATAACA
<i>melR</i>	TGCCGATGCATCTGTTTCTCTC	CAATTTTCATCAATGGCGAGCTG
<i>metK</i>	AGCGCTATCGGCAAACAGTCTC	TCAGCCTGACGCTGTACCAGAC
<i>mglA</i>	GTTAATTGCCGAAC TGGCGAAG	ACGCAAGACGCAGAATTTCGTT
<i>mglB</i>	GCGCTGAAAGCACACAACAAGT	ACCTTTACCATCGGCCAGGTTT
<i>mglC</i>	GGCCTGAACCTGCTGATGATCT	GCTGAACGATACACCGCCTACC
<i>mhpA</i>	CCGTATTGATCGCGTACTGCTG	CGTCACGGACAGGTCAATCATC
<i>mhpB</i>	CAATAAACCGCTGCTGTTCTTG	ACTTCTCAGCGGCGCTAATCAC
<i>mhpC</i>	GCCTGGAAGCTAATCCGAAACAG	CACAGTCGCGGAAGATATGCAG
<i>mhpD</i>	AGCATACTCTTGAGCAACTGGCG	TTGCACTTTCGGATGTGT CAGG
<i>mhpE</i>	CGCCATTCGTCATCAGTATTCG	CCGCTTCAATCCATTCAAGGTC
<i>mhpF</i>	AGCGTAAAGTCGCCATTATCGGT	GCATGTTTCATCAGTCCGATCACC
<i>mhpR</i>	CACCGTTTCAGCCGCTTATCCT	CGCGGTAGTTCTGTCCGTAACC
<i>modA</i>	AGAGAAAGGCGTG GATGTGGTTT	TGTCTGACGCGTAGCTGTATCGA
<i>modB</i>	GGTTACTGGTGCGTTGCACGT	CCATCAGCGGAAACGACATGAC
<i>modC</i>	CTTATCTGCAACGGCTGACACG	CCACGGATTTCATCACGCTACTG
<i>mpl</i>	CGAATGGCGTCACGGTATATGA	GAAGACTTCATCGGCACGACCT
<i>mtlA</i>	TCGGGCCGATGATCACTTATCT	GCGGACCTGCAATCATAGAACC
<i>mtlD</i>	CGGTCATCAGACCATTTCGTGAC	GGCCTACGCGCTCTACATCATC
<i>mtlR</i>	CGAGGCGGTAAATCTTCTGGTG	TCAGCAGTTCCGCATCTTCGTA
<i>murP</i>	GGTACAGGATGCCGCAGAAATC	ATTAACGTCGCTATCCCCAGCA
<i>murQ</i>	CACCGCTGAGTTTGCCATTACA	CATCAGGTTGCCGAACACTTTG
<i>nadC</i>	CTCGCCGCTATAACCCTGACAC	ACACCTCTTCAACCCAGCGTTT
<i>nagA</i>	ATGTATGCATTAACCCAGGGCC	CAGCCGTTTAACTGCACATCGAT
<i>nagB</i>	TCGCCATATCGTCAATCGTATCA	TTCGTCCATGTTGAAGGTGACAA
<i>nagC</i>	ACCAGGCGGACAAGCTCAGAT	CGTTCGATAAGCTGACGCGTAA
<i>nagD</i>	ATGTAATTTGCGATATCGACGGC	ACACGCTGTCAGGTACATCGACA
<i>nagE</i>	GCTGGTGATGGGCGTTATCTTC	GTAAGGCGCAGACGGGTGATAC
<i>nanA</i>	CCGTCACGCCGTTCTACTATCC	ATCGCCAGAGGTCTGTTTCAGC
<i>nanC</i>	GCATGGCAAACGACGCTATACA	TCTGCCGTTGTAAACACCCTGA
<i>nanE</i>	AGCGTGCCGATTATTGGAATTG	GATGGTGAATACGTGCCAGCAG
<i>nanK</i>	AGAAGCCTTGCGTGATGCCTTAT	CGGCAAATTGGTAAGTTGTTCCA
<i>nanM</i>	AAGCTTGCTCCCGTCTCATCAC	CCATTTCCC GTTATGCCAAAGA
<i>nanT</i>	TACCTACAACGTTGGCGCATTG	CGTCATGAGTACGCAACGCTTC
<i>narQ</i>	GGATACCAACCTGCCGAATGAG	CGCCTGCGAACACTGATACAAC
<i>nfuA</i>	CGTATTTCCGATGCTGCACAAG	CTCATCAACATACGCGGTCAGC
<i>nirB</i>	GGCAAAGGCTACGGTTGTGAAG	GATCACCGAGTAGGTGCCGTCT
<i>nirC</i>	CGGTAATTTGCTCGATCCATCC	ACCAGGTTACCCAGCCAGGTTT

<i>nirD</i>	ACATCGACCCGTTCTTCGAGTC	CACTCGCGCTTCGTAATGTTTG
<i>nlpE</i>	CTCGGTGCTCGTGAAGAACCTT	AGGGTCATCGGCGTCATAGGTA
<i>nrdA</i>	TCAGTCGATCTCTGCCAACACC	CACCAGATCGTCTTGTGCGTCT
<i>nrdB</i>	GATCCTGAGATGGCGGAAATTG	TGCCTGCATACGGATATTGGTG
<i>nupC</i>	GCACTGTTTGCCACCGTTACTG	CAGATCCATCATCGCAACGAAC
<i>nupG</i>	TCGGCTTTATCATGGCAATGTG	AGGGTTGTCCAGCTCTGATTCTG
<i>nusA</i>	TTGAAGCCGCACGTTATGAAGA	CGGTGATGATTTACCTTCGTG
<i>ompA</i>	ACTCGTCTGGGTGGCATGGTAT	GTCACCGATGTTGTTGGTCCAC
<i>ompF</i>	CGCGGGTCTTAAATACGCTGAC	AGTTCAGGCCATCAACCAGACC
<i>ompR</i>	CGCCTGCTGACTCGTGAATCT	TAGTCGTCAGCGCCAATCTCC
<i>osmY</i>	AGAAGGCTCGGTGAAGGGCTAC	TTGTGCCTGAGAATCGACGGTA
<i>oxyR</i>	TATGCTGCACCAGACCTTTCCA	TAGCCAGCAACATTGGCTCATC
<i>paaA</i>	AACGCTTTGAGCAACGGATAGC	CTGCACTTTGGCCAACAGAATG
<i>paaB</i>	GGCAAACAGGGCTTATCACACC	TTGCGAGGCAACAATTTCACTC
<i>paaC</i>	TCAGGCACGCAACTTCTTATCG	GAAATACTGGCGTGCAATGGTG
<i>paaD</i>	GATCCCGGTGCTGACCATTACT	GAGTTGCAGCACAACTGAACG
<i>paaE</i>	GCAATTGCAGCAGGATCAGGTA	TGTCTTTCAGGTCTGCCAGTGC
<i>paaF</i>	ACCAGCATTTTCGGTCTGTGTGA	ATTGACGGCTGCGATAAGTGGT
<i>paaG</i>	TAATACCCGATTGCGGTGGAAC	CTGCCAGCGTTTCATCATCAAC
<i>paaH</i>	TTTGCTGTACCTGTTTCGGTGT	AACTGTCGCTTACCGCTTCCAG
<i>paal</i>	GCCCATGCAATGTATGAGAACG	CTATTGCAGGCGTAGGCAAAGG
<i>paaJ</i>	GCTGCGTTGATTATTGCCAGTG	CGCTTCGTTCAATTCAATCACG
<i>paaK</i>	CTGAAATGGACGCTCAAACACG	GGATAGTTGTCGCGCAGATCCT
<i>paaZ</i>	AGGAGGCGGTGAAGAATTAGGC	CTGTCGCCTGGTTGTAGCTCCT
<i>pdhR</i>	ATTCAACGTCTCGAAGCGAAGG	AGTTCACGGATGCGTTCCTTGT
<i>pflB</i>	ACGACATCTCTGGTCCGGCTAC	GCGCTTCTTGTTTCGGTGATCTT
<i>pgk</i>	CCTGGATATCGGTGATGCTTCC	TCGCTGTCTGCGATAGCGTTAG
<i>pncB</i>	ATGCAGCAAGCCGTGTTTCATC	CGTTGGACACGGTGACTTGTTT
<i>pnp</i>	GGCCGCGTCTACACTGGTAAAG	CACCGGTACTTCCTGACCCATC
<i>poxB</i>	CAGCAAGGTGGATATGGCACTG	TATTGCGGGTGAATGGCTTTCT
<i>ppdD</i>	ATGGTGGTTATTGGCATCATTGC	CACCGTCGCAGGTATCTAATCCA
<i>ppiA</i>	CGCCACCAGCCAGTTCTTTATC	CACATTCTGGTACGGACCAACG
<i>preA</i>	ATGTGTGCCAAAGCCTACGACA	CCAACGGATGTTTCAGCAATCTG
<i>preT</i>	GTCACGACGCTCCCTGTAGTCA	TAATTTCTCCGTCGGGCACACT
<i>proP</i>	ACCGTCCTACGACACGATTGGT	AACCCGGCAATAGAACCGAAGT
<i>prpB</i>	CATTGCAGATTGTTGGCACCAT	GTCGGTCAGCACATCATCAAGG
<i>prpC</i>	ACCGCGGCTACGATATTCTTGA	GTAAGGCTTCCAGCACGGTACG
<i>prpD</i>	CTTAACGGCGGCAGATTACGAG	GACCAATGGGGTACTCCACCAC
<i>prpE</i>	CAATGTTGCGTTCATTGGGTGT	ATCAGCACCGGTTTAGCGTCAT
<i>prpR</i>	ACTGCGCAATATGATGGAACGA	CCTAAATAAATCGCCGCTGCTG
<i>psiE</i>	CCGTACTIONAATCTTGGCCTGCT	CACAATCAGCGCGATAAATTCTG
<i>pta</i>	TGCTGAACGTGGTATCGCAACT	GGTTTCGGTCATGCCTTTGTTC
<i>ptsG</i>	TACTGGCGTTGTGCTGTCCTTC	CCAGATGTGGTGCAGACCAAAC
<i>ptsH</i>	GCCCTGCTGCCCAGTTTGTGA	TTCAACCGCTTTCTGCTCGTC
<i>ptsI</i>	AAATTTCTGCCGACCAGGTTGA	CCTGCTCCAGCTCCTCATCTTC
<i>putP</i>	TCACCATTTATTGCGCTTCGG	AGGCTGGCCTGTACAGTGTCACT
<i>rbfA</i>	GGTTAAAGCGGGCATCAAAGC	CCGGGTAAACACGACGTTCTTC
<i>rbsA</i>	GGCATGAAGTCGTTACCCGTTT	ACTCACAGCCTGCTGTTTCATCG
<i>rbsB</i>	TGCCAAAGTTATCGAGCTGCAA	GGATGAGCGGTCAACAGGTTCT

<i>rhsC</i>	GGCTGGTTTGGTATTGGTCGTC	TGATACCAGAAAGACGCGTTGC
<i>rhsD</i>	ATCGCGGAAGAGATCAAACACC	ACATTCTCCGCTGCGAATTACC
<i>rhsK</i>	TATGGGCTAGCGTGAATGGTGA	AGGTTGTGCGCCTTTACGTGTT
<i>rhsR</i>	CGCGTAGCCTCAAACCTCAATCA	AGATTGCGATTCAATCCGCTGTT
<i>relA</i>	GAAACCTGGGCGTATTGTCTGC	ACCGACTTACCGACGCTCTCAC
<i>rhaA</i>	GGTGCATATCGGCCTTGACTTC	ACGGCAACGATTTCTGCTCTTC
<i>rhaB</i>	GCCATTCGCCTTGGATTAAACA	GTTTGCCGAGTTGTTGTTGTGC
<i>rhaD</i>	CCGATATCGCACCATATCACGA	GATGCCTAAGTTAGCCGCAGGA
<i>rhaR</i>	ACCACACTGGCGCTTAGGTAGC	TGGTGTAACGATGGCGATTACAG
<i>rhaS</i>	GGATGGGCAGTATCCGTCTCAC	CCAGGTTCTCCTGCAAACCTGCT
<i>rhaT</i>	CCATCAGCGCCCTGTTACTACC	CGTCATCAGCGTACCGACAATC
<i>rimP</i>	CCACCAGGTAAGTGCTGTGCTG	GTACCGCCATACGGAGAACCAG
<i>rof</i>	TCTTGAGCTGAAAGATGGCGAAA	GGATTGCTTACCACCACCGTA
<i>rpoH</i>	CCGTTCACTGGATCAAAGCAGA	CTTTGCTGGTTACGCCCAGTTC
<i>rpoS</i>	GTTGATGACGTCAGCCGTATGC	CTCGAACAGCCATTTGACGATG
<i>rpsO</i>	GGTAGCACTGCTGACTGCACAGA	TCGATGAGCTGGGTGTAACGTG
<i>rrsG</i>	GGTGTAGCGGTGAAATGCGTAG	TCAAGGGCACAACCTCCAAGTC
<i>sbmC</i>	CGGTAGCTCGTGTAGTCGGTGA	GCTGCACCGCAACATACATTTT
<i>sdhA</i>	GTGGTGGGTTGTACCGCACTGT	TCCATATCCTGCACCGGTACG
<i>sdhB</i>	ATGCTGCTGGATGCGCTTATC	AATCTTCTTGCCCGGCTGGTT
<i>sdhC</i>	GCGATAGCGTCCATTCTCCATC	GTGATACGCCAGAGCGGTAAGG
<i>sdhD</i>	TAAGCAACGCCTCCGCATTAG	GGCGAAGAAACCGATCCAGAC
<i>serA</i>	ATTGGTACGCAATTGGGCATTC	TTTGGTGGACGGATTCTCTGGT
<i>serC</i>	CATTCTTTCCCGTCCGATTGAC	GAGCCGTTATCGTTGAGGATGG
<i>sfsA</i>	CGCTTTGGGCTAACAGGTTGAC	TCCCTGTTCTGTTCTCCGCTAAC
<i>sgbE</i>	CTTCGAAGAACGTGGCAGGAGT	CTGGCGCGAGAATAGACCCATA
<i>sgbH</i>	GTTCCGTAACTGGACGCTGGAC	CCGCCAGTAATGGAAAGCTCAA
<i>sgbU</i>	AAACTGGGCATTGACCGTATCG	TCGGTCCACATCTCAATCAGGA
<i>sodA</i>	GAAAGCGGCTATCGAACGTGAC	TCAGCGGAGAATCCTGGTTAGC
<i>sodB</i>	ACTTTGGTTCTGGCTGGACCTG	CAGATAGCCAGGACGTGCATTG
<i>sohB</i>	AGGTTGTGCTCCGTCTGGAAG	GCCACACAGGCCATCATGTAAC
<i>speC</i>	CGCCGATATGTGTAACGCTGAC	TAACAGCGCATTTCGTACCACT
<i>srlA</i>	CCGCTGTTGATTAGCCTGCTG	GGCTTAGGGTCATCGGATTGC
<i>srlB</i>	CAGTGACCAGATGCTCATCACCTT	CAGTTCGCGAAGGTTGTCTTCC
<i>srlD</i>	CAAAGTGGGCAGCAAACACAAC	TGTGGCAACAGTGACTGGAACA
<i>srlE</i>	CTGGCGCAGTACATTGTGGAAG	CCACCTTCGCCAGTAAACCATC
<i>srlR</i>	CTTTGACACCACAGGCACAACC	CTAACGGCAGCTTCTGCAATCA
<i>sucA</i>	CGATTGGTGCCGAGTATATGCA	GCGCCGAGGTAACGTTCAAGA
<i>sucB</i>	TCTACGTGAAAGCGGTGGTTGA	TTCTTCTCGATGTCTGCCATGC
<i>sucC</i>	CCGGCTAACTTCCTTGACGTTG	GTTCCGGCGTTGTTACCTTCCAG
<i>sucD</i>	CGGATTACGGTTTCGGTCAGTC	CCACAACCTGGCTTGGTAACGTG
<i>tam</i>	GTCGATATCTGGCGAACCACCT	TCTGTCCGTTCTCTTGCAGTGG
<i>tdcA</i>	GTTCCGAATCCATTACCCGTGA	GAACGAAGACAGTTGCGCTTCA
<i>tdcB</i>	GCGAAATTGTGCAAATGGAAGG	CAATTAACCGCCACCACCAAT
<i>tdcC</i>	CATCATCGCACTCGTGGCTATC	GTAGGCAACAACCCAGGTGGAG
<i>tdcD</i>	TATTCACCGGCGGAATAGGAGA	CGGAATAACGGCACAAATGACA
<i>tdcE</i>	TCTAACGTGGTGTACGGCCAGA	ATCCCATCTTTGGCGTAGGTGA
<i>tdcF</i>	GCGTTGATTTAGGCAGCATGG	GATGATATCGCCACGCTCAG
<i>tdcG</i>	TGACGGTATTGAACGTGGGATG	GCGCGTACATGTTGATCCAGTC

<i>tnaA</i>	TGCTGTGCATGAAAGACGACAG	CGATAAGCCAGCCAGTCGAGAT
<i>tnaB</i>	CATTGTTCTCGGGCTGAAGAT	GGAATATTGCCGTGGAAACCAA
<i>treB</i>	GTTCTGGTCTGGCAGGATTGCT	CAGGCGGTATTTCCGCTGATAG
<i>treC</i>	AATGTCCTCCACCAGCCTTGAG	TTGTGCATTCTTGTGGCCAGT
<i>trg</i>	GGCGATCTGACGATGAATGATG	CTTGTTCTTCGGTGCGAGATGA
<i>truB</i>	GTTATTTACCTGCGCCGTCTGG	CGGAAGATTCACCACCGGATAG
<i>trxA</i>	TCTGGATGAAATCGCTGACGAA	CCAGGTTAGCGTCGAGGAACTC
<i>tsx</i>	TGACCTTAGCTTCGGTCCGTTC	CGCATAGACGTTTCATGGACAGG
<i>ubiG</i>	TTCAGGTGGATTACGTGCAGGA	ACTTGCCGTTGCGGTAAAGTGT
<i>udp</i>	GAATGTACGACTGCGCTGGTTG	ATCGCCTGCCACTCTTCCATAG
<i>ugpA</i>	CGGTGAGTTTCTTCTGCTGGT	GAAGCCAGATCCAGTCCGGTAA
<i>ugpB</i>	GTTCAATAAGCCGGAGCAGGTG	ATTTGGCGTACTCGCGAATGTT
<i>ugpC</i>	TGAGCCGCTCTCTAACCTCGAT	ACCGCCGTTTCATCACCATTACT
<i>ugpE</i>	GATCCTCTTCCCGCTGTACGTG	TGTTAAGCAACATCCGCCAGAA
<i>ugpQ</i>	GCTGGGCTGCGTCTCTATTTCAT	GACCAATCACGTCAATCGCATC
<i>uhpT</i>	AACATCGCGCAGAACGATATGA	AGCATGAACGGCAGGAATTGTT
<i>uidA</i>	TATTGCCAACGAACCGGATACC	ATAACGGTTCAGGCACAGCACA
<i>uidB</i>	GCCGCTTCATTGACCTTTGTCT	ACATTCTCACGCGTCGATTTGA
<i>uidC</i>	GTTATTTGCGCCATGCAATGAA	TCCGCACATCTCGTTTACTGGA
<i>ulaA</i>	CCGTTGTTGCTGGGTATTGTGA	CGCCGTTAATGCCGTAGACTTC
<i>ulaB</i>	ACGGACAAGGCAGTTCCATGAT	GCAATGTGCGTAGAAGCGATGA
<i>ulaC</i>	CGCGTTACTACCAGGCGATTCT	GATGAGGATATCCACCGGATCG
<i>ulaD</i>	CGTCTTTCCGATATGGGCTTCA	CGATGGAACGTTTGAAGTACG
<i>ulaE</i>	CATGTGCCTTTCTGCTCATCGT	GCGTTTCGTTATTGGCTTCCTG
<i>ulaF</i>	TGCGACGCATCTCGAACTCTAC	TCAGTTCATACTCGCCCTGCAC
<i>uxaA</i>	GATAACGTCGCGGTCGCTTTAG	ATCCGCCAATGCATAACCAATC
<i>uxaB</i>	TGTTCTACGCTGGTGGACCGTA	CAGCACGTTGAGCGGATATTTG
<i>uxaC</i>	TCTGTGTACCGGTGATGCGTCT	AGTTATCCTGCGCCAGCAGTTC
<i>uxuA</i>	TCGTAGAAAGCGTGCCAATTCA	ATTCGAGGTCAGTACGGGTCCA
<i>uxuB</i>	TCCGTCAGTGGGTGATTGAAGA	TAAGCCGGGTTAGTCACGGTGT
<i>uxuR</i>	TTGCAACTGGAAGAGCGTGAAC	GTCATCCAGATGGCTGTGCAAC
<i>xseA</i>	GGCTGCTGCAACAGAAGTACGA	AGATGATCACCGGCAGAGAAGG
<i>xylA</i>	ACAATCCCGACGAACTGGTGTT	TGCGACATCTGCTTTACGCTTC
<i>xylB</i>	GCAGGCTTGCGACTTATCTCGT	TCAACCATTCCCACACCAACTG
<i>xylF</i>	GCCGGTAGATAACAACGCCAAG	GGCATCGTTTGAGGCAACTACA
<i>xylG</i>	ATATTACCCTCGCCGCACTCAA	TGCGCGGGTTAAGTAACAGACA
<i>xylH</i>	GGGTTTACAGTCTCCGGCCTCT	GCCATAAACATTCCGCCGAGTA
<i>xylR</i>	ACCCACCCGTTTCATTACATTGC	AAATGCATATTCGCGCTCAGTG
<i>yaeJ</i>	ATTCCCGATGGTGAGCTTGAGA	CTTCGCGGTTTCAGTTCCTGACT
<i>yaeQ</i>	TAAAGCGACGGTTAATGTGGCC	AACCACGGGTAAATTGCAGACG
<i>ybiS</i>	GCCTGCGTGTAAGTCATGGTTG	CGGTGGTAGACAGCGGGTTATG
<i>ybiT</i>	GGCGAAATGGACGGTACTCTG	GGTGTCGATGTCGAGGTTGTTG
<i>yefQ</i>	CACGCTATACGCGGAATTTACCA	TAAAGCAGTTGGCGATGGCAG
<i>ychH</i>	GGGTCTGGTGGTAATGGTGGTC	GTAACGGTCGCACACCTGTTCA
<i>yeiP</i>	TCCGTACCGGGCTGAAAGTAGA	GCCCTCTGGCATAAACAGCAAC
<i>yfiD</i>	TGCAGTAAGCAAACCTGGGTGACA	TAACCGGATACACGGATGGTCAG
<i>yhcH</i>	CATTAGCTGCCAGACCGCAAG	CTGCCATGCCAAACAGAATCC
<i>yhfA</i>	CCACGCCTGTTTACGCACATTA	GCGGCAACCACTTCATACGAG
<i>yiaJ</i>	TAACTTCTCCAGCCGCGAAGAC	TGACTTCACGTAGTCCGGGTGA

<i>yiaK</i>	CGATGGTGGCTTTGATGATGAG	CGTATTCGTCGCTGTTGTCCTG
<i>yiaL</i>	GTAAACCGTCCGGAAGTGCATC	CGTAGCTGCCCCGGTATCATTTC
<i>yiaM</i>	ATAACGCCCACGTTCAAGTCAC	CGGTGAATAATCGCTCCAGTCC
<i>yiaN</i>	GCGCTGTTTCTTCCAGTCATCA	CGAGGCCACCAGAAACATAACC
<i>yiaO</i>	GCGGGTAACTATCAGCGCAAAC	CTCCCTGCGGCACATCTTTAAC
<i>yjcH</i>	GCGCATTTCAAGGAGTTAGTCG	CAATTGGAATACCGCGTGTGAC
<i>ynfK</i>	CCGAAGGGCTGCGTAATAAAGA	GGCCGTTTGAAATGAGGGTGTA
<i>zraR</i>	CTGAATGTGGTGGCGATTGAAG	TATTTCCCGGCCAGTCGTAATG
<i>zraS</i>	TTAAACCGCGTGGTAAAGCGAGT	GTTGGCGGTAAAGCGTAACTGG

Table 5: Genes with >2-fold change in their expression level in iE2 as compared to BW25113 in the absence of ethanol stress, using a *p*-value threshold less than 0.05.

b-number	Gene	Function^a	Fold-change^b	<i>p</i>-value
b1493	<i>gadB</i>	glutamate decarboxylase B	50.525	6.81E-04
b1492	<i>gadC</i>	GadC GABA APC transporter	44.773	6.35E-05
b3517	<i>gadA</i>	glutamate decarboxylase A	20.320	3.63E-06
b4376	<i>osmY</i>	periplasmic protein	18.726	1.38E-07
b3512	<i>gadE</i>	GadE DNA-binding transcriptional activator	18.309	4.45E-07
b3513	<i>mdtE</i>	MdtEF-TolC multidrug efflux transport system - membrane fusion protein	17.156	2.92E-07
b3514	<i>mdtF</i>	MdtEF-TolC multidrug efflux transport system - permease subunit	7.786	1.64E-07
b1182	<i>hlyE</i>	hemolysin E	7.288	9.26E-03
b3516	<i>gadX</i>	GadX DNA-binding transcriptional dual regulator	7.209	6.57E-07
b4030	<i>psiE</i>	predicted phosphate starvation-inducible protein	5.844	2.79E-08
b4111	<i>proP</i>	ProP osmosensory MFS transporter	5.748	4.11E-07
b3453	<i>ugpB</i>	glycerol-3-phosphate / glycerol-2-phosphate ABC transporter - putative periplasmic binding protein	5.276	3.22E-06
b2508	<i>guaB</i>	IMP dehydrogenase	5.066	2.93E-04
b2661	<i>gabD</i>	succinate semialdehyde dehydrogenase, NADP+-dependent	5.064	6.84E-07
b2659	<i>csiD</i>	predicted protein	5.040	1.11E-02
b1519	<i>tam</i>	trans-aconitate methyltransferase	4.769	4.71E-05
b0343	<i>lacY</i>	LacY lactose MFS transporter	4.412	3.48E-02
b3449	<i>ugpQ</i>	glycerophosphodiester phosphodiesterase, cytosolic	4.266	1.70E-08
b4014	<i>aceB</i>	malate synthase A	4.008	9.09E-07
b3450	<i>ugpC</i>	glycerol-3-phosphate / glycerol-2-phosphate ABC transporter - putative ATP binding subunit	3.555	8.84E-04
b2507	<i>guaA</i>	GMP synthetase	3.551	3.63E-05
b0124	<i>gcd</i>	glucose dehydrogenase	3.541	4.13E-03
b1112	<i>bhsA</i>	protein involved in stress resistance and biofilm formation	3.488	2.76E-05
b1276	<i>acnA</i>	aconitate hydratase 1	3.177	2.47E-09
b1038	<i>csgF</i>	curli assembly component	3.163	3.26E-03
b0342	<i>lacA</i>	galactoside O-acetyltransferase	3.037	2.86E-02
b3172	<i>argG</i>	argininosuccinate synthase	2.811	2.74E-08
b2234	<i>nrdA</i>	ribonucleoside diphosphate reductase 1, α subunit dimer	2.653	1.94E-02
b3748	<i>rbsD</i>	ribose pyranase	2.614	1.05E-05

b3437	<i>gntK</i>	D-gluconate kinase, thermostable	2.598	9.57E-07
b0346	<i>mhpR</i>	MhpR transcriptional activator	2.522	1.82E-07
b1272	<i>sohB</i>	predicted inner membrane peptidase	2.513	2.78E-07
b4016	<i>aceK</i>	isocitrate dehydrogenase phosphatase / isocitrate dehydrogenase kinase	2.499	2.63E-05
b3749	<i>rbsA</i>	ribose ABC transporter - putative ATP binding subunit	2.477	8.66E-07
b2235	<i>nrdB</i>	ribonucleoside diphosphate reductase 1, β subunit dimer	2.474	4.38E-05
b4015	<i>aceA</i>	isocitrate lyase	2.455	6.59E-07
b2660	<i>lhgO</i>	L-2-hydroxyglutarate oxidase	2.444	2.52E-03
b2487	<i>hyfG</i>	hydrogenase 4, large subunit	2.381	5.99E-04
b0722	<i>sdhD</i>	succinate dehydrogenase membrane protein	2.268	3.92E-04
b0907	<i>serC</i>	phosphohydroxythreonine aminotransferase / 3-phosphoserine aminotransferase	2.231	4.39E-05
b0432	<i>cyoA</i>	cytochrome bo terminal oxidase subunit II	2.187	7.83E-06
b3451	<i>ugpE</i>	glycerol-3-phosphate / glycerol-2-phosphate ABC transporter - putative membrane subunit	2.133	6.01E-06
b0430	<i>cyoC</i>	cytochrome bo terminal oxidase subunit III	2.118	2.42E-07
b0819	<i>ybiS</i>	L,D-transpeptidase YbiS	2.116	8.18E-05
b2417	<i>crr</i>	glucose-specific enzyme IIA component of PTS	2.063	2.02E-03
b2965	<i>speC</i>	ornithine decarboxylase, biosynthetic	2.058	2.84E-05
b2741	<i>rpoS</i>	RNA polymerase, sigma S (sigma 38) factor	2.050	1.10E-05
b0726	<i>sucA</i>	2-oxoglutarate decarboxylase, thiamin-requiring	2.011	4.83E-05
b4067	<i>actP</i>	acetate / glycolate transporter	0.498	2.43E-02
b2492	<i>focB</i>	FocB formate FNT transporter	0.490	3.96E-04
b4197	<i>ulaE</i>	L-xylulose 5-phosphate 3-epimerase	0.484	1.04E-02
b2805	<i>fucR</i>	FucR transcriptional activator	0.482	1.42E-07
b4195	<i>ulaC</i>	L-ascorbate-specific enzyme IIA component of PTS	0.482	1.90E-03
b1620	<i>malI</i>	MalI DNA-binding transcriptional repressor	0.467	2.36E-07
b3414	<i>nfuA</i>	iron-sulfur cluster scaffold protein	0.450	6.07E-08
b0678	<i>nagB</i>	glucosamine-6-phosphate deaminase	0.442	3.74E-06
b3905	<i>rhaS</i>	RhaS transcriptional activator	0.437	1.60E-03
b4194	<i>ulaB</i>	L-ascorbate-specific enzyme IIB component of PTS	0.436	1.15E-03
b1819	<i>manZ</i>	mannose PTS permease - ManZ subunit	0.430	7.05E-07
b0347	<i>mhpA</i>	3-(3-hydroxyphenyl)propanoate hydroxylase	0.421	1.51E-02
b4138	<i>dcuA</i>	DcuA dicarboxylate Dcu transporter	0.415	1.50E-04
b0113	<i>pdhR</i>	PdhR DNA-binding transcriptional dual regulator	0.414	1.89E-06
b4196	<i>ulaD</i>	3-keto-L-gulonate 6-phosphate decarboxylase	0.404	1.79E-03
b2468	<i>aegA</i>	putative oxidoreductase, Fe-S subunit	0.400	2.02E-04
b3418	<i>malT</i>	MalT transcriptional activator	0.399	3.53E-07
b4213	<i>cpdB</i>	2',3'-cyclic nucleotide 2'-phosphodiesterase / 3'-	0.398	8.66E-08

		nucleotidase		
b0903	<i>pflB</i>	pyruvate formate-lyase (inactive)	0.396	4.66E-06
b3236	<i>mdh</i>	malate dehydrogenase	0.390	7.65E-05
b1621	<i>malX</i>	fused maltose and glucose-specific PTS enzymes: IIB component, IIC component	0.386	1.69E-03
b2799	<i>fucO</i>	L-1,2-propanediol oxidoreductase	0.384	4.27E-05
b2708	<i>gutQ</i>	D-arabinose 5-phosphate isomerase	0.384	3.29E-05
b1594	<i>dgsA</i>	DgsA DNA-binding transcriptional repressor	0.370	1.61E-03
b2428	<i>murQ</i>	N-acetylmuramic acid 6-phosphate etherase	0.370	3.73E-09
b1389	<i>paaB</i>	ring 1,2-phenylacetyl-CoA epoxidase subunit	0.369	3.13E-04
b3869	<i>glnL</i>	NtrB sensory histidine kinase	0.366	1.68E-07
b3723	<i>bglG</i>	BglG transcriptional antiterminator	0.361	8.59E-05
b1205	<i>ychH</i>	stress-induced protein	0.360	1.79E-05
b3934	<i>cytR</i>	CytR DNA-binding transcriptional repressor	0.359	1.51E-10
b2147	<i>preA</i>	NADH-dependent dihydropyrimidine dehydrogenase subunit	0.352	2.51E-06
b3368	<i>cysG</i>	uroporphyrin III C-methyltransferase [multifunctional]	0.351	1.71E-07
b3868	<i>glnG</i>	NtrC transcriptional dual regulator	0.350	9.74E-04
b0757	<i>galK</i>	galactokinase	0.343	1.52E-05
b1415	<i>aldA</i>	aldehyde dehydrogenase A, NAD-linked	0.339	1.90E-04
b4003	<i>zraS</i>	ZraS sensory histidine kinase	0.335	6.98E-06
b3566	<i>xylF</i>	xylose ABC transporter - periplasmic binding protein	0.330	3.23E-02
b1002	<i>agp</i>	3-phytase / glucose-1-phosphatase	0.323	2.66E-09
b2344	<i>fadL</i>	long-chain fatty acid outer membrane transporter; sensitivity to phage T2	0.320	8.21E-09
b2801	<i>fucP</i>	FucP fucose MFS transporter	0.313	4.44E-03
b2146	<i>preT</i>	NADH-dependent dihydropyrimidine dehydrogenase subunit	0.310	3.13E-07
b3925	<i>glpX</i>	fructose 1,6-bisphosphatase II	0.309	3.05E-03
b2964	<i>nupG</i>	NupG nucleoside MFS transporter	0.305	9.96E-10
b4268	<i>idnK</i>	D-gluconate kinase, thermosensitive	0.298	1.37E-03
b4460	<i>araH</i>	arabinose ABC transporter - membrane subunit	0.285	8.03E-07
b4471	<i>tdcG</i>	L-serine deaminase III	0.284	2.87E-03
b3135	<i>agaA</i>	predicted truncated N-acetylgalactosamine-6-phosphate deacetylase	0.283	7.24E-06
b3091	<i>uxaA</i>	D-altronate dehydratase	0.281	7.50E-08
b3113	<i>tdcF</i>	predicted L-PSP (mRNA) endoribonuclease	0.275	6.85E-05
b2803	<i>fucK</i>	L-fuculokinase	0.274	2.02E-07
b3579	<i>yiaO</i>	L-dehydroascorbate transporter, periplasmic binding protein	0.271	1.75E-03
b1392	<i>paaE</i>	ring 1,2-phenylacetyl-CoA epoxidase, reductase subunit	0.271	1.73E-05
b2614	<i>grpE</i>	phage lambda replication; host DNA synthesis;	0.271	3.37E-06

		heat shock protein; protein repair		
b3221	<i>yhcH</i>	conserved protein	0.268	3.54E-10
b4266	<i>idnO</i>	5-keto-D-gluconate 5-reductase	0.267	3.94E-05
b3092	<i>uxaC</i>	D-glucuronate isomerase / D-galacturonate isomerase	0.262	1.50E-05
b1391	<i>paaD</i>	phenylacetate degradation protein	0.254	1.10E-04
b3565	<i>xylA</i>	xylose isomerase	0.253	8.61E-06
b1521	<i>uxaB</i>	altronate oxidoreductase	0.249	8.87E-06
b0679	<i>nagE</i>	N-acetylglucosamine PTS permease	0.243	2.40E-07
b4323	<i>uxuB</i>	D-mannonate oxidoreductase	0.239	2.21E-05
b4069	<i>acs</i>	acetyl-CoA synthetase (AMP-forming)	0.236	6.09E-06
b2365	<i>dsdX</i>	DsdX Gnt transporter	0.234	6.24E-08
b1593	<i>ynfK</i>	predicted dethiobiotin synthetase	0.231	1.67E-12
b1818	<i>manY</i>	mannose PTS permease - ManY subunit	0.228	5.05E-11
b1817	<i>manX</i>	mannose PTS permease - ManX subunit	0.220	6.82E-11
b1514	<i>lsrC</i>	AI-2 ABC transporter - membrane subunit	0.219	4.76E-05
b3134	<i>agaW</i>	PTS system N-acetylgalactosamine-specific IIC component 2	0.218	2.63E-03
b3133	<i>agaV</i>	PTS system, cytoplasmic, N-acetylgalactosamine-specific IIB component 2 (EIIB-AGA)	0.215	1.30E-05
b3870	<i>glnA</i>	glutamine synthetase	0.213	2.96E-12
b3578	<i>yiaN</i>	L-dehydroascorbate transporter	0.204	5.71E-03
b3575	<i>yiaK</i>	2,3-diketo-L-gulonate reductase	0.202	9.19E-03
b2707	<i>srlR</i>	GutR DNA-binding transcriptional repressor	0.202	2.49E-06
b2841	<i>araE</i>	AraE arabinose MFS transporter	0.198	2.04E-05
b4322	<i>uxuA</i>	D-mannonate dehydratase	0.196	4.27E-11
b2095	<i>gatZ</i>	D-tagatose 1,6-bisphosphate aldolase 2, subunit	0.194	7.45E-05
b0411	<i>tsx</i>	nucleoside channel; receptor of phage T6 and colicin K	0.184	5.66E-12
b2802	<i>fucI</i>	L-fucose isomerase	0.180	2.25E-05
b3132	<i>kbaZ</i>	tagatose 6-phosphate aldolase 1, kbaZ subunit	0.171	1.14E-04
b3114	<i>tdcE</i>	2-ketobutyrate formate-lyase/pyruvate formate-lyase 4, inactive	0.167	1.44E-05
b4311	<i>nanC</i>	N-acetylneuraminic acid outer membrane channel	0.166	8.06E-05
b2092	<i>gatC</i>	galactitol-specific enzyme IIC component of PTS	0.164	1.26E-09
b3528	<i>dctA</i>	DctA dicarboxylate DAACS transporter	0.162	2.08E-05
b0598	<i>cstA</i>	peptide transporter induced by carbon starvation	0.161	1.20E-03
b2096	<i>gatY</i>	D-tagatose 1,6-bisphosphate aldolase 2, catalytic subunit	0.159	1.28E-04
b1617	<i>uidA</i>	β -D-glucuronidase	0.155	2.91E-07
b2094	<i>gatA</i>	galactitol-specific enzyme IIA component of PTS	0.151	6.45E-09

b0597	<i>entH</i>	proofreading thioesterase in enterobactin biosynthesis	0.149	5.05E-05
b2091	<i>gatD</i>	galactitol-1-phosphate dehydrogenase	0.147	3.16E-08
b4118	<i>melR</i>	MelR DNA-binding transcriptional dual regulator	0.144	1.66E-05
b2366	<i>dsdA</i>	D-serine deaminase	0.138	7.60E-11
b0584	<i>fepA</i>	FepA, outer membrane receptor for ferric enterobactin (enterochelin) and colicins B and D	0.136	1.39E-03
b2093	<i>gatB</i>	galactitol-specific enzyme IIB component of PTS	0.136	2.96E-14
b2579	<i>yfiD</i>	stress-induced alternate pyruvate formate-lyase subunit	0.136	1.81E-07
b0904	<i>focA</i>	FocA formate transporter	0.129	4.25E-07
b2151	<i>galS</i>	GalS DNA-binding transcriptional dual regulator	0.127	7.05E-05
b3927	<i>glpF</i>	GlpF glycerol MIP channel	0.123	8.58E-07
b0595	<i>entB</i>	apo-EntB multimer	0.115	2.43E-05
b3576	<i>yiaL</i>	conserved protein	0.115	5.25E-04
b3670	<i>ilvN</i>	acetolactate synthase I, small subunit	0.114	1.43E-06
b2243	<i>glpC</i>	glycerol-3-phosphate dehydrogenase (anaerobic), small subunit	0.112	7.69E-09
b0908	<i>aroA</i>	3-phosphoshikimate-1-carboxyvinyltransferase	0.109	3.90E-06
b4321	<i>gntP</i>	GntP Gluconate Gnt transporter	0.105	6.02E-07
b3077	<i>ebgC</i>	evolved β -D-galactosidase, β subunit	0.103	4.43E-06
b3222	<i>nanK</i>	N-acetylmannosamine kinase	0.102	9.61E-06
b2957	<i>ansB</i>	asparaginase II	0.101	2.15E-17
b0759	<i>galE</i>	UDP-glucose 4-epimerase	0.099	4.53E-05
b3581	<i>sgbH</i>	3-keto-L-gulonate 6-phosphate decarboxylase	0.099	6.96E-03
b3926	<i>glpK</i>	glycerol kinase	0.098	1.03E-04
b0594	<i>entE</i>	2,3-dihydroxybenzoate-holo-EntB ligase [multifunctional]	0.096	2.09E-05
b4267	<i>idnD</i>	L-idonate 5-dehydrogenase	0.093	4.17E-07
b3671	<i>ilvB</i>	acetolactate synthase I, large subunit	0.079	3.72E-06
b0593	<i>entC</i>	isochorismate synthase 1	0.078	1.55E-04
b0583	<i>entD</i>	phosphopantetheinyl transferase	0.078	4.37E-06
b3115	<i>tdcD</i>	propionate kinase	0.077	7.24E-04
b2706	<i>gutM</i>	GutM DNA-binding transcriptional activator	0.076	1.72E-05
b4032	<i>malG</i>	maltose ABC transporter - membrane subunit	0.076	8.94E-07
b3116	<i>tdcC</i>	TdcC threonine STP transporter	0.074	1.08E-07
b3076	<i>ebgA</i>	evolved β -D-galactosidase, α subunit	0.071	4.93E-08
b0929	<i>ompF</i>	outer membrane porin F	0.071	6.00E-10
b4239	<i>treC</i>	trehalose-6-phosphate hydrolase	0.069	2.93E-05
b0553	<i>nmpC</i>	outer membrane porin protein; locus of qsr prophage	0.065	6.29E-07
b0596	<i>entA</i>	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	0.063	2.54E-05

b2705	<i>srlD</i>	sorbitol-6-phosphate dehydrogenase	0.060	4.49E-06
b1900	<i>araG</i>	arabinose ABC transporter - ATP binding subunit	0.056	1.62E-02
b4240	<i>treB</i>	fused trehalose(maltose)-specific PTS enzyme: IIB component/IIC component	0.054	1.54E-04
b4033	<i>malF</i>	maltose ABC transporter - membrane subunit	0.052	2.27E-13
b0805	<i>fiu</i>	putative outer membrane receptor for iron transport	0.052	3.62E-06
b2155	<i>cirA</i>	outer membrane receptor involved in uptake of ferric dihydroxybenzoylserine	0.051	3.68E-08
b3224	<i>nanT</i>	NanT sialic acid MFS transporter	0.050	1.53E-04
b1901	<i>araF</i>	arabinose ABC transporter - periplasmic binding protein	0.049	5.32E-07
b3367	<i>nirC</i>	NirC nitrite FNT transporter	0.048	1.54E-05
b4037	<i>malM</i>	maltose regulon periplasmic protein	0.042	5.35E-07
b2704	<i>srlB</i>	glucitol/sorbitol-specific enzyme IIA component of PTS	0.041	2.10E-07
b4139	<i>aspA</i>	aspartate ammonia-lyase	0.037	3.49E-06
b2148	<i>mglC</i>	galactose ABC transporter - membrane subunit	0.036	6.34E-07
b4034	<i>malE</i>	maltose ABC transporter - periplasmic binding protein	0.034	1.85E-05
b2242	<i>glpB</i>	glycerol-3-phosphate dehydrogenase (anaerobic), membrane anchor subunit	0.034	5.07E-06
b2149	<i>mglA</i>	galactose ABC transporter - ATP binding subunit	0.032	2.51E-06
b3365	<i>nirB</i>	dimer of large subunit of nitrite reductase	0.030	1.31E-10
b3118	<i>tdcA</i>	TdcA DNA-binding transcriptional activator	0.030	1.13E-04
b3366	<i>nirD</i>	nitrite reductase, small subunit	0.028	5.57E-07
b2703	<i>srlE</i>	glucitol/sorbitol-specific enzyme IIB component of PTS	0.027	2.23E-06
b4036	<i>lamB</i>	phage lambda receptor protein; maltose high-affinity receptor	0.026	3.90E-06
b2150	<i>mglB</i>	galactose ABC transporter - periplasmic binding protein	0.026	1.09E-04
b2239	<i>glpQ</i>	glycerophosphoryl diester phosphodiesterase, periplasmic	0.024	3.60E-07
b2241	<i>glpA</i>	glycerol-3-phosphate dehydrogenase (anaerobic), large subunit	0.023	1.81E-07
b4035	<i>malK</i>	maltose ABC transporter - ATP binding subunit	0.022	4.09E-07
b3225	<i>nanA</i>	N-acetylneuraminate lyase	0.020	1.36E-06
b2702	<i>srlA</i>	glucitol/sorbitol-specific enzyme IIC component of PTS	0.017	1.08E-03
b3223	<i>nanE</i>	predicted N-acetylmannosamine-6-phosphate epimerase	0.014	5.95E-03
b2240	<i>glpT</i>	GlpT glycerol-3-P MFS transporter	0.014	1.43E-06
b3709	<i>tnaB</i>	TnaB tryptophan ArAAP transporter	0.012	5.74E-07
b3117	<i>tdcB</i>	catabolic threonine dehydratase	0.008	5.33E-07

b3708	<i>tnaA</i>	L-cysteine desulphydrase / tryptophanase	0.004	1.66E-07
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^aFrom the EcoCyc database (<http://ecocyc.org>)

^bFold-change in gene expression between iE2 and BW25113 (average of triplicate experiments)

Table 6: Genes with >2-fold change in their expression level in iE2 as compared to BW25113 in the presence of ethanol stress, using a *p*-value threshold less than 0.05.

b-number	Gene	Function^a	Fold-change^b	<i>p</i>-value
b0759	<i>galE</i>	UDP-glucose 4-epimerase	22.301	1.38E-03
b3114	<i>tdcE</i>	2-ketobutyrate formate-lyase/pyruvate formate-lyase 4, inactive	11.194	1.89E-06
b4068	<i>yjcH</i>	conserved inner membrane protein	8.782	1.29E-03
b3452	<i>ugpA</i>	glycerol-3-phosphate / glycerol-2-phosphate ABC transporter - putative membrane subunit	8.772	1.65E-04
b3581	<i>sgbH</i>	3-keto-L-gulonate 6-phosphate decarboxylase	8.016	1.67E-02
b3223	<i>nanE</i>	predicted N-acetylmannosamine-6-phosphate epimerase	7.062	6.27E-04
b3750	<i>rbsC</i>	ribose ABC transporter - membrane subunit	6.573	1.35E-03
b0036	<i>caiD</i>	crotonobetainyl-CoA hydratase	6.372	2.71E-05
b3513	<i>mdtE</i>	MdtEF-TolC multidrug efflux transport system - membrane fusion protein	5.532	1.67E-04
b3748	<i>rbsD</i>	ribose pyranase	4.834	3.77E-04
b3392	<i>hofP</i>	protein involved in utilization of DNA as a carbon source	4.648	8.20E-05
b4288	<i>fecD</i>	ferric dicitrate ABC transporter - membrane subunit	4.400	1.05E-03
b3709	<i>tnaB</i>	TnaB tryptophan ArAAP transporter	4.369	9.99E-03
b3907	<i>rhaT</i>	rhamnose RhaT transporter	4.340	1.53E-04
b2489	<i>hyfI</i>	hydrogenase 4, small subunit	4.251	1.80E-02
b4289	<i>fecC</i>	ferric dicitrate ABC transporter - membrane subunit	3.930	7.26E-03
b3393	<i>hofO</i>	protein involved in utilization of DNA as a carbon source	3.772	1.09E-03
b3366	<i>nirD</i>	nitrite reductase, small subunit	3.762	4.96E-02
b1900	<i>araG</i>	arabinose ABC transporter - ATP binding subunit	3.693	4.32E-03
b0431	<i>cyoB</i>	cytochrome bo terminal oxidase subunit I	3.661	1.85E-03
b3224	<i>nanT</i>	NanT sialic acid MFS transporter	3.637	2.73E-03
b1394	<i>paaG</i>	predicted ring 1,2-epoxyphenylacetyl-CoA isomerase (oxepin-CoA forming)	3.622	4.39E-02
b0035	<i>caiE</i>	predicted acyl transferase	3.578	3.08E-02
b3588	<i>aldB</i>	acetaldehyde dehydrogenase	3.454	6.76E-04
b3365	<i>nirB</i>	dimer of large subunit of nitrite reductase	3.370	1.57E-05
b0553	<i>nmpC</i>	outer membrane porin protein; locus of qsr prophage	3.344	1.84E-04
b3116	<i>tdcC</i>	TdcC threonine STP transporter	3.338	2.82E-04
b2148	<i>mgIC</i>	galactose ABC transporter - membrane subunit	3.316	2.38E-03

b3580	<i>lyxK</i>	L-xylulose kinase	3.237	4.64E-02
b3221	<i>yhcH</i>	conserved protein	3.175	2.76E-04
b1531	<i>marA</i>	MarA DNA-binding transcriptional dual regulator	3.072	8.82E-08
b4287	<i>fecE</i>	ferric dicitrate ABC transporter - ATP binding subunit	2.947	6.08E-04
b1389	<i>paaB</i>	ring 1,2-phenylacetyl-CoA epoxidase subunit	2.930	1.76E-02
b3566	<i>xylF</i>	xylose ABC transporter - periplasmic binding protein	2.884	7.67E-03
b3115	<i>tdcD</i>	propionate kinase	2.861	1.25E-04
b1615	<i>uidC</i>	membrane-associated protein	2.857	1.92E-02
b2492	<i>focB</i>	FocB formate FNT transporter	2.827	2.05E-02
b2535	<i>csiE</i>	stationary phase inducible protein	2.797	2.20E-05
b0034	<i>caiF</i>	CaiF transcriptional activator	2.784	7.45E-03
b3394	<i>hofN</i>	protein involved in utilization of DNA as a carbon source	2.743	2.00E-04
b3437	<i>gntK</i>	D-gluconate kinase, thermostable	2.737	3.47E-05
b0723	<i>sdhA</i>	succinate dehydrogenase flavoprotein	2.728	1.41E-02
b3721	<i>bglB</i>	6-phospho- β -glucosidase B	2.710	6.48E-03
b2147	<i>preA</i>	NADH-dependent dihydropyrimidine dehydrogenase subunit	2.663	3.33E-02
b0598	<i>cstA</i>	peptide transporter induced by carbon starvation	2.646	3.71E-02
b4268	<i>idnK</i>	D-gluconate kinase, thermosensitive	2.642	3.17E-03
b4197	<i>ulaE</i>	L-xylulose 5-phosphate 3-epimerase	2.576	1.34E-02
b0429	<i>cyoD</i>	cytochrome bo terminal oxidase subunit IV	2.561	7.16E-04
b3117	<i>tdcB</i>	catabolic threonine dehydratase	2.544	2.48E-02
b4265	<i>idnT</i>	L-idonate / 5-ketogluconate / gluconate transporter	2.537	2.24E-05
b2150	<i>mglB</i>	galactose ABC transporter - periplasmic binding protein	2.535	3.96E-02
b1112	<i>bhsA</i>	protein involved in stress resistance and biofilm formation	2.528	3.19E-07
b1517	<i>lsrF</i>	predicted class I aldolase	2.507	2.66E-02
b3904	<i>rhaB</i>	L-rhamnulose kinase	2.411	4.78E-02
b2487	<i>hyfG</i>	hydrogenase 4, large subunit	2.403	1.61E-04
b2803	<i>fucK</i>	L-fuculokinase	2.399	6.65E-04
b0583	<i>entD</i>	phosphopantetheinyl transferase	2.387	5.74E-03
b4471	<i>tdcG</i>	L-serine deaminase III	2.381	5.72E-03
b3222	<i>nanK</i>	N-acetylmannosamine kinase	2.376	3.22E-04
b2243	<i>glpC</i>	glycerol-3-phosphate dehydrogenase (anaerobic), small subunit	2.359	3.99E-03
b0346	<i>mhpR</i>	MhpR transcriptional activator	2.314	4.98E-03
b1518	<i>lsrG</i>	Autoinducer 2-degrading protein	2.272	7.91E-03
b4193	<i>ulaA</i>	L-ascorbate-specific enzyme IIC component of PTS	2.248	1.59E-03

b1040	<i>csgD</i>	CsgD DNA-binding transcriptional dual regulator	2.234	4.00E-02
b4376	<i>osmY</i>	periplasmic protein	2.179	9.92E-03
b3751	<i>rbsB</i>	ribose ABC transporter - putative periplasmic binding protein	2.150	1.27E-02
b3578	<i>yiaN</i>	L-dehydroascorbate transporter	2.139	1.86E-03
b1519	<i>tam</i>	trans-aconitate methyltransferase	2.118	1.24E-03
b2469	<i>narQ</i>	NarQ sensory histidine kinase	2.090	6.82E-04
b2393	<i>nupC</i>	NupC nucleoside NUP transporter	2.083	1.09E-03
b3582	<i>sgbU</i>	predicted L-xylulose 5-phosphate 3-epimerase	2.073	2.90E-02
b3260	<i>dusB</i>	tRNA dihydrouridine synthase	0.494	1.02E-06
b1819	<i>manZ</i>	mannose PTS permease - ManZ subunit	0.477	9.41E-06
b2092	<i>gatC</i>	galactitol-specific enzyme IIC component of PTS	0.459	9.40E-03
b4266	<i>idnO</i>	5-keto-D-gluconate 5-reductase	0.447	4.88E-03
b2913	<i>serA</i>	α -ketoglutarate reductase / D-3-phosphoglycerate dehydrogenase	0.444	2.69E-05
b0350	<i>mhpD</i>	2-oxopent-4-enoate hydratase	0.426	1.54E-04
b4460	<i>araH</i>	arabinose ABC transporter - membrane subunit	0.415	6.37E-03
b0428	<i>cyoE</i>	heme O synthase	0.407	1.36E-03
b3165	<i>rpsO</i>	30S ribosomal subunit protein S15	0.391	4.81E-07
b4118	<i>melR</i>	MelR DNA-binding transcriptional dual regulator	0.389	2.44E-05
b2663	<i>gabP</i>	GabP APC transporter	0.373	4.16E-02
b3367	<i>nirC</i>	NirC nitrite FNT transporter	0.373	5.00E-03
b0675	<i>nagD</i>	ribonucleotide monophosphatase	0.355	1.13E-04
b3213	<i>gltD</i>	glutamate synthase, small subunit	0.328	3.12E-05
b4194	<i>ulaB</i>	L-ascorbate-specific enzyme IIB component of PTS	0.281	6.21E-04
b0593	<i>entC</i>	isochorismate synthase 1	0.258	5.81E-04
b1101	<i>ptsG</i>	fused glucose-specific PTS enzymes: IIB component/IIC component	0.233	1.11E-05
b2703	<i>srlE</i>	glucitol/sorbitol-specific enzyme IIB component of PTS	0.062	1.51E-04
b2702	<i>srlA</i>	glucitol/sorbitol-specific enzyme IIC component of PTS	0.028	2.39E-08
b2705	<i>srlD</i>	sorbitol-6-phosphate dehydrogenase	0.026	1.64E-05
b2704	<i>srlB</i>	glucitol/sorbitol-specific enzyme IIA component of PTS	0.025	4.46E-05

^aFrom the EcoCyc database (<http://ecocyc.org>)

^bFold-change in gene expression between iE2 and BW25113 (average of triplicate experiments)

Table 7: Genes with >2-fold change in their expression level in E2 as compared to WT in the absence of ethanol stress, using a *p*-value threshold less than 0.05.

b-number	Gene	Function^a	Fold-change^b	<i>p</i>-value
b1493	<i>gadB</i>	glutamate decarboxylase B	7.246	2.34E-08
b2393	<i>nupC</i>	NupC nucleoside NUP transporter	6.749	4.27E-07
b1492	<i>gadC</i>	GadC GABA APC transporter	6.587	4.07E-08
b2508	<i>guaB</i>	IMP dehydrogenase	4.841	1.26E-06
b2507	<i>guaA</i>	GMP synthetase	4.564	2.82E-06
b0431	<i>cyoB</i>	cytochrome bo terminal oxidase subunit I	3.623	2.62E-07
b3512	<i>gadE</i>	GadE DNA-binding transcriptional activator	3.616	1.07E-04
b1101	<i>ptsG</i>	fused glucose-specific PTS enzymes: IIB component/IIC component	3.291	1.68E-06
b0428	<i>cyoE</i>	heme O synthase	3.117	2.25E-06
b0037	<i>caiC</i>	carnitine-CoA ligase	2.920	2.91E-02
b0597	<i>entH</i>	proofreading thioesterase in enterobactin biosynthesis	2.897	4.61E-06
b3513	<i>mdtE</i>	MdtEF-TolC multidrug efflux transport system - membrane fusion protein	2.827	1.53E-04
b1272	<i>sohB</i>	predicted inner membrane peptidase	2.822	1.48E-07
b0430	<i>cyoC</i>	cytochrome bo terminal oxidase subunit III	2.764	9.71E-08
b3517	<i>gadA</i>	glutamate decarboxylase A	2.690	8.59E-07
b2235	<i>nrdB</i>	ribonucleoside diphosphate reductase 1, β subunit dimer	2.578	4.80E-05
b0429	<i>cyoD</i>	cytochrome bo terminal oxidase subunit IV	2.537	2.82E-06
b4291	<i>fecA</i>	outer membrane receptor; citrate-dependent iron transport, outer membrane receptor	2.487	1.05E-03
b1038	<i>csgF</i>	curli assembly component	2.473	2.49E-02
b2171	<i>yeiP</i>	predicted dehydrogenase, NAD-dependent	2.449	6.40E-06
b0343	<i>lacY</i>	LacY lactose MFS transporter	2.441	3.52E-02
b1395	<i>paaH</i>	3-hydroxyadipyl-CoA dehydrogenase (NAD ⁺)	2.419	6.77E-04
b2942	<i>metK</i>	methionine adenosyltransferase	2.244	5.04E-05
b3806	<i>cyaA</i>	adenylate cyclase	2.212	1.89E-06
b3261	<i>fis</i>	Fis DNA-binding transcriptional dual regulator	2.162	1.53E-05
b0907	<i>serC</i>	phosphohydroxythreonine aminotransferase / 3-phosphoserine aminotransferase	2.129	2.06E-07
b2488	<i>hyfH</i>	hydrogenase 4, component H	2.115	3.73E-05
b3260	<i>dusB</i>	tRNA dihydrouridine synthase	2.093	1.44E-04
b3516	<i>gadX</i>	GadX DNA-binding transcriptional dual regulator	2.078	9.67E-05
b0595	<i>entB</i>	apo-EntB multimer	2.059	8.35E-04
b2344	<i>fadL</i>	long-chain fatty acid outer membrane transporter; sensitivity to phage T2	2.048	9.40E-09
b0411	<i>tsx</i>	nucleoside channel; receptor of phage T6 and	2.047	1.90E-08

		colicin K		
b0125	<i>hpt</i>	hypoxanthine phosphoribosyltransferase	2.017	7.39E-10
b0106	<i>hofC</i>	protein transport protein HofC	2.003	3.66E-03
b2492	<i>focB</i>	FocB formate FNT transporter	0.499	1.84E-04
b2705	<i>srlD</i>	sorbitol-6-phosphate dehydrogenase	0.496	7.17E-04
b0679	<i>nagE</i>	N-acetylglucosamine PTS permease	0.489	6.39E-07
b3961	<i>oxyR</i>	OxyR DNA-binding transcriptional dual regulator	0.488	7.90E-04
b0036	<i>caiD</i>	crotonobetainyl-CoA hydratase	0.483	1.01E-02
b1531	<i>marA</i>	MarA DNA-binding transcriptional dual regulator	0.481	5.91E-09
b3751	<i>rbsB</i>	ribose ABC transporter - putative periplasmic binding protein	0.477	6.64E-07
b3905	<i>rhaS</i>	RhaS transcriptional activator	0.475	2.97E-02
b2804	<i>fucU</i>	L-fucose mutarotase	0.474	1.98E-03
b2957	<i>ansB</i>	asparaginase II	0.473	1.00E-05
b3453	<i>ugpB</i>	glycerol-3-phosphate / glycerol-2-phosphate ABC transporter - putative periplasmic binding protein	0.468	4.79E-02
b3414	<i>nfuA</i>	iron-sulfur cluster scaffold protein	0.467	3.58E-08
b3430	<i>glgC</i>	glucose-1-phosphate adenylyltransferase	0.466	1.18E-07
b3425	<i>glpE</i>	thiosulfate sulfurtransferase	0.465	1.85E-04
b2093	<i>gatB</i>	galactitol-specific enzyme IIB component of PTS	0.457	9.78E-06
b4194	<i>ulaB</i>	L-ascorbate-specific enzyme IIB component of PTS	0.456	9.52E-05
b1594	<i>dgsA</i>	DgsA DNA-binding transcriptional repressor	0.454	4.27E-06
b0721	<i>sdhC</i>	succinate dehydrogenase membrane protein	0.449	1.22E-08
b2659	<i>csiD</i>	predicted protein	0.444	7.58E-03
b4197	<i>ulaE</i>	L-xylulose 5-phosphate 3-epimerase	0.442	4.01E-02
b4213	<i>cpdB</i>	2',3'-cyclic nucleotide 2'-phosphodiesterase / 3'-nucleotidase	0.435	4.99E-06
b2801	<i>fucP</i>	FucP fucose MFS transporter	0.433	1.97E-02
b3582	<i>sgbU</i>	predicted L-xylulose 5-phosphate 3-epimerase	0.429	4.09E-02
b1620	<i>mall</i>	Mall DNA-binding transcriptional repressor	0.428	6.69E-09
b2092	<i>gatC</i>	galactitol-specific enzyme IIC component of PTS	0.424	3.08E-11
b3750	<i>rbsC</i>	ribose ABC transporter - membrane subunit	0.424	6.98E-03
b3868	<i>glnG</i>	NtrC transcriptional dual regulator	0.422	2.06E-03
b4003	<i>zraS</i>	ZraS sensory histidine kinase	0.418	5.60E-03
b0764	<i>modB</i>	molybdate ABC transporter - membrane subunit	0.414	2.12E-06
b1112	<i>bhsA</i>	protein involved in stress resistance and biofilm formation	0.413	2.04E-04
b3461	<i>rpoH</i>	RNA polymerase, sigma 32 (sigma H) factor	0.404	5.73E-06
b3236	<i>mdh</i>	malate dehydrogenase	0.396	3.18E-06
b4239	<i>treC</i>	trehalose-6-phosphate hydrolase	0.395	5.89E-08
b4004	<i>zraR</i>	ZraR transcriptional activator	0.395	7.73E-03

b3426	<i>glpD</i>	glycerol 3-phosphate dehydrogenase, aerobic	0.392	5.37E-04
b3077	<i>ebgC</i>	evolved β -D-galactosidase, β subunit	0.390	1.16E-02
b2803	<i>fucK</i>	L-fuculokinase	0.383	1.54E-05
b2704	<i>srlB</i>	glucitol/sorbitol-specific enzyme IIA component of PTS	0.381	1.76E-04
b2805	<i>fucR</i>	FucR transcriptional activator	0.380	1.32E-07
b2660	<i>lhgO</i>	L-2-hydroxyglutarate oxidase	0.380	1.92E-02
b3091	<i>uxaA</i>	D-altronate dehydratase	0.379	1.09E-07
b3748	<i>rbsD</i>	ribose pyranase	0.378	1.44E-06
b3588	<i>aldB</i>	acetaldehyde dehydrogenase	0.376	3.92E-07
b0722	<i>sdhD</i>	succinate dehydrogenase membrane protein	0.371	1.11E-11
b1817	<i>manX</i>	mannose PTS permease - ManX subunit	0.369	2.26E-11
b3241	<i>aaeA</i>	AaeAB Hydroxylated, Aromatic Carboxylic Acid Efflux Transport System Protein A	0.365	2.31E-02
b3134	<i>agaW</i>	PTS system N-acetylgalactosamine-specific IIC component 2	0.360	1.22E-02
b3093	<i>exuT</i>	ExuT hexuronate MFS transporter	0.359	2.11E-04
b4265	<i>idnT</i>	L-idonate / 5-ketogluconate / gluconate transporter	0.352	1.47E-03
b2365	<i>dsdX</i>	DsdX Gnt transporter	0.348	8.01E-11
b3092	<i>uxaC</i>	D-glucuronate isomerase / D-galacturonate isomerase	0.347	4.43E-11
b3415	<i>gntT</i>	GntT Gluconate Gnt transporter	0.344	1.18E-03
b1615	<i>uidC</i>	membrane-associated protein	0.339	1.84E-03
b3576	<i>yiaL</i>	conserved protein	0.339	8.29E-03
b1002	<i>agp</i>	3-phytase / glucose-1-phosphatase	0.327	2.36E-08
b2800	<i>fucA</i>	L-fuculose-phosphate aldolase	0.325	2.19E-03
b4193	<i>ulaA</i>	L-ascorbate-specific enzyme IIC component of PTS	0.323	1.49E-02
b0061	<i>araD</i>	L-ribulose 5-phosphate 4-epimerase	0.322	1.15E-02
b2715	<i>ascF</i>	β -glucoside PTS permease	0.319	3.76E-02
b4119	<i>melA</i>	α -galactosidase	0.315	3.87E-03
b0720	<i>glhA</i>	citrate synthase	0.310	8.40E-07
b4037	<i>malM</i>	maltose regulon periplasmic protein	0.308	3.06E-06
b4118	<i>melR</i>	MelR DNA-binding transcriptional dual regulator	0.308	8.53E-04
b4033	<i>malF</i>	maltose ABC transporter - membrane subunit	0.306	2.02E-08
b2091	<i>gatD</i>	galactitol-1-phosphate dehydrogenase	0.302	2.78E-12
b3670	<i>ilvN</i>	acetolactate synthase I, small subunit	0.301	1.26E-07
b4034	<i>malE</i>	maltose ABC transporter - periplasmic binding protein	0.284	2.48E-09
b3113	<i>tdcF</i>	predicted L-PSP (mRNA) endoribonuclease	0.283	5.40E-08
b4032	<i>malG</i>	maltose ABC transporter - membrane subunit	0.283	1.25E-06
b3583	<i>sgbE</i>	L-ribulose-5-phosphate 4-epimerase	0.282	1.47E-02
b3081	<i>fadH</i>	2,4-dienoyl-CoA reductase	0.277	1.99E-03

b2802	<i>fucI</i>	L-fucose isomerase	0.270	5.28E-06
b4196	<i>ulaD</i>	3-keto-L-gulonate 6-phosphate decarboxylase	0.269	1.95E-02
b4321	<i>gntP</i>	GntP Gluconate Gnt transporter	0.267	2.05E-04
b4266	<i>idnO</i>	5-keto-D-gluconate 5-reductase	0.267	1.82E-02
b4035	<i>malK</i>	maltose ABC transporter - ATP binding subunit	0.266	1.78E-04
b4036	<i>lamB</i>	phage lambda receptor protein; maltose high-affinity receptor	0.264	3.76E-05
b0064	<i>araC</i>	AraC DNA-binding transcriptional dual regulator	0.262	3.49E-10
b3133	<i>agaV</i>	PTS system, cytoplasmic, N-acetylgalactosamine-specific IIB component 2 (EIIB-AGA)	0.258	4.05E-04
b3565	<i>xylA</i>	xylose isomerase	0.249	2.09E-04
b3114	<i>tdcE</i>	2-ketobutyrate formate-lyase/pyruvate formate-lyase 4, inactive	0.249	5.44E-07
b4240	<i>treB</i>	fused trehalose(maltose)-specific PTS enzyme: IIB component/IIC component	0.248	5.87E-10
b1205	<i>ychH</i>	stress-induced protein	0.246	3.67E-09
b2841	<i>araE</i>	AraE arabinose MFS transporter	0.245	3.74E-08
b1190	<i>dadX</i>	alanine racemase 2, PLP-binding	0.244	3.37E-06
b2799	<i>fucO</i>	L-1,2-propanediol oxidoreductase	0.244	8.12E-05
b3926	<i>glpK</i>	glycerol kinase	0.227	1.15E-06
b2239	<i>glpQ</i>	glycerophosphoryl diester phosphodiesterase, periplasmic	0.227	3.85E-08
b4268	<i>idnK</i>	D-gluconate kinase, thermosensitive	0.221	7.33E-04
b3221	<i>yhch</i>	conserved protein	0.220	2.50E-08
b2240	<i>glpT</i>	GlpT glycerol-3-P MFS transporter	0.220	2.00E-09
b0113	<i>pdhR</i>	PdhR DNA-binding transcriptional dual regulator	0.217	8.59E-13
b3566	<i>xylF</i>	xylose ABC transporter - periplasmic binding protein	0.217	1.34E-04
b3671	<i>ilvB</i>	acetolactate synthase I, large subunit	0.212	3.22E-07
b4460	<i>araH</i>	arabinose ABC transporter - membrane subunit	0.211	1.16E-11
b3076	<i>ebgA</i>	evolved β -D-galactosidase, α subunit	0.210	2.16E-04
b2614	<i>grpE</i>	phage lambda replication; host DNA synthesis; heat shock protein; protein repair	0.201	5.67E-06
b2148	<i>mglC</i>	galactose ABC transporter - membrane subunit	0.198	3.68E-07
b3575	<i>yiaK</i>	2,3-diketo-L-gulonate reductase	0.197	5.06E-03
b4310	<i>nanM</i>	N-acetylneuraminic mutarotase	0.193	2.17E-06
b3581	<i>sgbH</i>	3-keto-L-gulonate 6-phosphate decarboxylase	0.186	1.24E-02
b4322	<i>uxuA</i>	D-mannonate dehydratase	0.186	9.73E-12
b3528	<i>dctA</i>	DctA dicarboxylate DAACS transporter	0.179	4.19E-07
b4067	<i>actP</i>	acetate / glycolate transporter	0.177	1.50E-04
b4267	<i>idnD</i>	L-idonate 5-dehydrogenase	0.167	1.88E-02
b1415	<i>aldA</i>	aldehyde dehydrogenase A, NAD-linked	0.159	1.83E-08
b4311	<i>nanC</i>	N-acetylneuraminic acid outer membrane channel	0.153	2.37E-07

b1189	<i>dadA</i>	D-amino acid dehydrogenase	0.146	3.49E-09
b0346	<i>mhpR</i>	MhpR transcriptional activator	0.139	9.43E-06
b1901	<i>araF</i>	arabinose ABC transporter - periplasmic binding protein	0.136	1.11E-06
b0723	<i>sdhA</i>	succinate dehydrogenase flavoprotein	0.135	3.54E-09
b4068	<i>yjcH</i>	conserved inner membrane protein	0.133	8.15E-05
b3225	<i>nanA</i>	N-acetylneuraminate lyase	0.132	4.87E-05
b1900	<i>araG</i>	arabinose ABC transporter - ATP binding subunit	0.131	6.91E-06
b2151	<i>galS</i>	GalS DNA-binding transcriptional dual regulator	0.124	1.09E-04
b2146	<i>preT</i>	NADH-dependent dihydropyrimidine dehydrogenase subunit	0.123	2.36E-09
b3118	<i>tdcA</i>	TdcA DNA-binding transcriptional activator	0.122	5.38E-07
b3927	<i>glpF</i>	GlpF glycerol MIP channel	0.119	3.15E-05
b3222	<i>nanK</i>	N-acetylmannosamine kinase	0.109	1.56E-06
b2147	<i>preA</i>	NADH-dependent dihydropyrimidine dehydrogenase subunit	0.099	2.51E-05
b0596	<i>entA</i>	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	0.096	6.00E-03
b2149	<i>mglA</i>	galactose ABC transporter - ATP binding subunit	0.090	3.49E-08
b4323	<i>uxuB</i>	D-mannonate oxidoreductase	0.082	6.90E-06
b3224	<i>nanT</i>	NanT sialic acid MFS transporter	0.078	4.66E-08
b2980	<i>glcC</i>	GlcC transcriptional dual regulator	0.075	6.88E-04
b0598	<i>cstA</i>	peptide transporter induced by carbon starvation	0.063	8.00E-05
b3116	<i>tdcC</i>	TdcC threonine STP transporter	0.051	5.47E-06
b3117	<i>tdcB</i>	catabolic threonine dehydratase	0.040	4.58E-04
b3709	<i>tnaB</i>	TnaB tryptophan ArAAP transporter	0.039	4.95E-07
b3223	<i>nanE</i>	predicted N-acetylmannosamine-6-phosphate epimerase	0.035	1.33E-02
b2150	<i>mglB</i>	galactose ABC transporter - periplasmic binding protein	0.030	1.22E-06
b3115	<i>tdcD</i>	propionate kinase	0.026	1.44E-07
b3708	<i>tnaA</i>	L-cysteine desulfhydrase / tryptophanase	0.019	1.78E-06
b4069	<i>acs</i>	acetyl-CoA synthetase (AMP-forming)	0.014	4.57E-07
b0759	<i>galE</i>	UDP-glucose 4-epimerase	0.001	1.14E-04

^aFrom the EcoCyc database (<http://ecocyc.org>)

^bFold change in gene expression between E2 and WT (average of duplicate experiments)

Table 8: Genes with >2-fold change in their expression level in E2 as compared to WT in the presence of ethanol stress, using a *p*-value threshold less than 0.05.

b-number	Gene	Function^a	Fold-change^b	<i>p</i>-value
b0759	<i>galE</i>	UDP-glucose 4-epimerase	265.076	2.66E-02
b0041	<i>fixA</i>	probable flavoprotein subunit required for anaerobic carnitine metabolism	27.385	3.35E-03
b0040	<i>caiT</i>	L-carnitine/ γ -butyrobetaine antiport	8.127	2.36E-02
b0596	<i>entA</i>	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	7.540	7.89E-05
b0334	<i>prpD</i>	2-methylcitrate dehydratase	6.639	1.79E-02
b1112	<i>bhsA</i>	protein involved in stress resistance and biofilm formation	4.061	2.38E-05
b0594	<i>entE</i>	2,3-dihydroxybenzoate-holo-EntB ligase [multifunctional]	3.766	1.65E-05
b1779	<i>gapA</i>	glyceraldehyde 3-phosphate dehydrogenase-A complex	3.355	9.08E-13
b0764	<i>modB</i>	molybdate ABC transporter - membrane subunit	3.217	1.35E-03
b0429	<i>cyoD</i>	cytochrome bo terminal oxidase subunit IV	3.050	2.61E-06
b0428	<i>cyoE</i>	heme O synthase	2.780	1.91E-02
b0432	<i>cyoA</i>	cytochrome bo terminal oxidase subunit II	2.757	4.46E-05
b1531	<i>marA</i>	MarA DNA-binding transcriptional dual regulator	2.700	8.06E-04
b0431	<i>cyoB</i>	cytochrome bo terminal oxidase subunit I	2.641	4.38E-02
b0124	<i>gcd</i>	glucose dehydrogenase	2.543	3.24E-03
b0125	<i>hpt</i>	hypoxanthine phosphoribosyltransferase	2.537	9.19E-05
b0038	<i>caiB</i>	γ -butyrobetainyl-CoA:carnitine CoA transferase	2.430	2.48E-02
b0118	<i>acnB</i>	bifunctional aconitate hydratase 2 and 2-methylisocitrate dehydratase	2.393	2.54E-06
b4015	<i>aceA</i>	isocitrate lyase	2.376	3.33E-04
b0344	<i>lacZ</i>	β -galactosidase	2.337	1.92E-03
b2171	<i>yeiP</i>	predicted dehydrogenase, NAD-dependent	2.310	4.94E-04
b0116	<i>lpd</i>	lipoamide dehydrogenase	2.213	7.25E-04
b2925	<i>fbaA</i>	fructose bisphosphate aldolase class II	2.180	5.79E-04
b3868	<i>sodA</i>	superoxide dismutase (Mn)	2.144	1.51E-03
b0595	<i>entB</i>	apo-EntB multimer	2.090	1.64E-03
b0583	<i>entD</i>	phosphopantetheinyl transferase	2.089	6.92E-03
b0430	<i>cyoC</i>	cytochrome bo terminal oxidase subunit III	2.039	5.79E-07
b4016	<i>aceK</i>	isocitrate dehydrogenase phosphatase / isocitrate dehydrogenase kinase	2.007	9.27E-04
b2151	<i>galS</i>	GalS DNA-binding transcriptional dual	0.499	3.08E-02

		regulator		
b3425	<i>glpE</i>	thiosulfate sulfurtransferase	0.491	1.52E-05
b4034	<i>malE</i>	maltose ABC transporter - periplasmic binding protein	0.491	2.20E-02
b1512	<i>lsrR</i>	LsrR DNA-binding transcriptional repressor	0.491	4.03E-03
b4382	<i>deoA</i>	thymidine phosphorylase	0.489	7.51E-04
b2803	<i>fucK</i>	L-fuculokinase	0.486	3.51E-02
b0106	<i>hofC</i>	protein transport protein HofC	0.485	1.30E-03
b1521	<i>uxaB</i>	altronate oxidoreductase	0.482	1.28E-03
b4268	<i>idnK</i>	D-gluconate kinase, thermosensitive	0.479	3.38E-03
b3749	<i>rbsA</i>	ribose ABC transporter - putative ATP binding subunit	0.478	1.22E-03
b3751	<i>rbsB</i>	ribose ABC transporter - putative periplasmic binding protein	0.476	5.78E-03
b3367	<i>nirC</i>	NirC nitrite FNT transporter	0.476	2.61E-02
b2964	<i>nupG</i>	NupG nucleoside MFS transporter	0.476	1.07E-02
b3423	<i>glpR</i>	GlpR DNA-binding transcriptional repressor	0.475	7.46E-05
b1616	<i>uidB</i>	UidB glucuronides GPH transporter	0.474	3.61E-03
b3564	<i>xylB</i>	xylulokinase	0.474	1.53E-02
b4321	<i>gntP</i>	GntP Gluconate Gnt transporter	0.471	3.31E-04
b1594	<i>dgsA</i>	DgsA DNA-binding transcriptional repressor	0.470	1.38E-04
b4123	<i>dcuB</i>	DcuB dicarboxylate Dcu transporter	0.463	4.73E-02
b2715	<i>ascF</i>	β -glucoside PTS permease	0.460	2.50E-03
b2957	<i>ansB</i>	asparaginase II	0.459	1.88E-02
b4239	<i>treC</i>	trehalose-6-phosphate hydrolase	0.455	1.33E-02
b3424	<i>glpG</i>	intramembrane serine protease GlpG	0.454	3.26E-04
b4194	<i>ulaB</i>	L-ascorbate-specific enzyme IIB component of PTS	0.453	3.20E-03
b4139	<i>aspA</i>	aspartate ammonia-lyase	0.450	4.53E-04
b4289	<i>fecC</i>	ferric dicitrate ABC transporter - membrane subunit	0.450	2.51E-02
b0346	<i>mhpR</i>	MhpR transcriptional activator	0.448	3.89E-04
b0908	<i>aroA</i>	3-phosphoshikimate-1-carboxyvinyltransferase	0.441	5.35E-03
b2149	<i>mgla</i>	galactose ABC transporter - ATP binding subunit	0.427	9.55E-03
b1389	<i>paaB</i>	ring 1,2-phenylacetyl-CoA epoxidase subunit	0.427	2.14E-02
b3748	<i>rbsD</i>	ribose pyranase	0.426	1.66E-03
b3575	<i>yiaK</i>	2,3-diketo-L-gulonate reductase	0.424	2.27E-02
b3214	<i>gltF</i>	periplasmic protein	0.419	1.42E-02
b3666	<i>uhpT</i>	UhpT-hexose phosphate MFS transporter	0.418	1.16E-02
b1901	<i>araF</i>	arabinose ABC transporter - periplasmic binding protein	0.417	2.75E-04
b1615	<i>uidC</i>	membrane-associated protein	0.417	1.28E-02
b3905	<i>rhaS</i>	RhaS transcriptional activator	0.416	1.50E-03

b3240	<i>aaeB</i>	AaeAB Hydroxylated, Aromatic Carboxylic Acid Efflux Transport System Protein B	0.415	1.91E-04
b2841	<i>araE</i>	AraE arabinose MFS transporter	0.414	1.19E-04
b3926	<i>glpK</i>	glycerol kinase	0.413	5.48E-05
b3132	<i>kbaZ</i>	tagatose 6-phosphate aldolase 1, kbaZ subunit	0.412	1.99E-03
b4067	<i>actP</i>	acetate / glycolate transporter	0.406	2.08E-02
b0108	<i>ppdD</i>	prepilin peptidase dependent protein	0.406	3.23E-02
b3428	<i>glgP</i>	glycogen phosphorylase	0.405	8.53E-04
b3072	<i>aer</i>	aerotaxis sensor receptor, flavoprotein	0.404	5.23E-06
b0034	<i>caiF</i>	CaiF transcriptional activator	0.402	3.09E-02
b2707	<i>srlR</i>	GutR DNA-binding transcriptional repressor	0.400	2.14E-02
b2943	<i>galP</i>	GalP - galactose MFS transporter	0.400	2.12E-02
b2799	<i>fucO</i>	L-1,2-propanediol oxidoreductase	0.399	7.56E-06
b0553	<i>nmpC</i>	outer membrane porin protein; locus of qsr prophage	0.386	2.06E-03
b4122	<i>fumB</i>	fumarase B	0.386	1.26E-03
b4213	<i>cpdB</i>	2',3'-cyclic nucleotide 2'-phosphodiesterase / 3'-nucleotidase	0.379	4.82E-04
b4118	<i>melR</i>	MelR DNA-binding transcriptional dual regulator	0.379	1.66E-06
b2143	<i>cdd</i>	cytidine deaminase	0.379	3.78E-06
b3366	<i>nirD</i>	nitrite reductase, small subunit	0.378	2.80E-03
b3116	<i>tdcC</i>	TdcC threonine STP transporter	0.376	1.71E-03
b3934	<i>cytR</i>	CytR DNA-binding transcriptional repressor	0.376	6.14E-04
b3413	<i>gntX</i>	protein involved in utilization of DNA as a carbon source	0.363	3.41E-03
b3723	<i>bglG</i>	BglG transcriptional antiterminator	0.361	2.77E-03
b3709	<i>tnaB</i>	TnaB tryptophan ArAAP transporter	0.354	1.78E-02
b3925	<i>glpX</i>	fructose 1,6-bisphosphatase II	0.353	6.75E-05
b2240	<i>glpT</i>	GlpT glycerol-3-P MFS transporter	0.351	3.87E-04
b2706	<i>gutM</i>	GutM DNA-binding transcriptional activator	0.350	1.11E-02
b1892	<i>flhD</i>	DNA-binding transcriptional dual regulator with FlhC	0.345	3.13E-05
b4003	<i>zraS</i>	ZraS sensory histidine kinase	0.333	2.05E-03
b3514	<i>mdtF</i>	MdtEF-TolC multidrug efflux transport system - permease subunit	0.330	6.08E-04
b2468	<i>aegA</i>	putative oxidoreductase, Fe-S subunit	0.327	1.72E-05
b1002	<i>agp</i>	3-phytase / glucose-1-phosphatase	0.327	7.89E-05
b2241	<i>glpA</i>	glycerol-3-phosphate dehydrogenase (anaerobic), large subunit	0.327	1.75E-05
b3114	<i>tdcE</i>	2-ketobutyrate formate-lyase/pyruvate formate-lyase 4, inactive	0.325	1.77E-02
b3394	<i>hofN</i>	protein involved in utilization of DNA as a carbon source	0.321	1.71E-02
b0113	<i>pdhR</i>	PdhR DNA-binding transcriptional dual regulator	0.317	3.44E-11

b3415	<i>gntT</i>	GntT Gluconate Gnt transporter	0.316	9.34E-05
b4291	<i>fecA</i>	outer membrane receptor; citrate-dependent iron transport, outer membrane receptor	0.314	6.17E-03
b1516	<i>lsrB</i>	AI-2 ABC transporter - periplasmic binding protein	0.306	1.34E-02
b2488	<i>hyfH</i>	hydrogenase 4, component H	0.303	4.14E-03
b4267	<i>idnD</i>	L-idonate 5-dehydrogenase	0.300	7.75E-03
b1734	<i>chbF</i>	diacetylchitobiose-6-phosphate hydrolase	0.299	1.05E-02
b4265	<i>idnT</i>	L-idonate / 5-ketogluconate / gluconate transporter	0.291	5.43E-04
b3117	<i>tdcB</i>	catabolic threonine dehydratase	0.290	5.72E-03
b3093	<i>exuT</i>	ExuT hexuronate MFS transporter	0.287	2.96E-04
b3224	<i>nanT</i>	NanT sialic acid MFS transporter	0.283	1.25E-04
b4196	<i>ulaD</i>	3-keto-L-gulonate 6-phosphate decarboxylase	0.281	9.08E-05
b2663	<i>gabP</i>	GabP APC transporter	0.281	5.40E-06
b4266	<i>idnO</i>	5-keto-D-gluconate 5-reductase	0.280	5.20E-05
b3452	<i>ugpA</i>	glycerol-3-phosphate / glycerol-2-phosphate ABC transporter - putative membrane subunit	0.276	1.83E-02
b0333	<i>prpC</i>	methylcitrate synthase	0.272	4.32E-02
b3927	<i>glpF</i>	GlpF glycerol MIP channel	0.271	1.57E-05
b3708	<i>tnaA</i>	L-cysteine desulfhydrase / tryptophanase	0.268	2.54E-02
b3566	<i>xylF</i>	xylose ABC transporter - periplasmic binding protein	0.266	1.81E-02
b3513	<i>mdtE</i>	MdtEF-TolC multidrug efflux transport system - membrane fusion protein	0.263	5.28E-03
b3571	<i>malS</i>	α -amylase	0.262	2.83E-04
b3426	<i>glpD</i>	glycerol 3-phosphate dehydrogenase, aerobic	0.261	5.22E-06
b0107	<i>hofB</i>	protein involved in plasmid replication	0.260	2.80E-03
b4195	<i>ulaC</i>	L-ascorbate-specific enzyme IIA component of PTS	0.258	1.00E-03
b3393	<i>hofO</i>	protein involved in utilization of DNA as a carbon source	0.257	2.62E-02
b2095	<i>gatZ</i>	D-tagatose 1,6-bisphosphate aldolase 2, subunit	0.256	9.16E-07
b3752	<i>rbsK</i>	ribokinase	0.255	8.74E-03
b1819	<i>manZ</i>	mannose PTS permease - ManZ subunit	0.251	7.18E-06
b3134	<i>agaW</i>	PTS system N-acetylgalactosamine-specific IIC component 2	0.250	1.81E-02
b3599	<i>mtlA</i>	mannitol PTS permease	0.249	8.93E-06
b3750	<i>rbsC</i>	ribose ABC transporter - membrane subunit	0.249	4.41E-04
b4036	<i>lamB</i>	phage lambda receptor protein; maltose high-affinity receptor	0.246	1.69E-03
b4035	<i>malK</i>	maltose ABC transporter - ATP binding subunit	0.245	3.84E-04
b1415	<i>aldA</i>	aldehyde dehydrogenase A, NAD-linked	0.239	1.48E-03
b3578	<i>yiaN</i>	L-dehydroascorbate transporter	0.217	5.11E-05

b4471	<i>tdcG</i>	L-serine deaminase III	0.215	3.08E-03
b1387	<i>paaZ</i>	oxepin-CoA hydrolase/3-oxo-5,6-dehydrosuberyl-CoA semialdehyde dehydrogenase	0.212	7.08E-05
b3576	<i>yiaL</i>	conserved protein	0.202	3.15E-03
b1101	<i>ptsG</i>	fused glucose-specific PTS enzymes: IIB component/IIC component	0.202	4.96E-08
b3588	<i>aldB</i>	acetaldehyde dehydrogenase	0.201	6.40E-06
b2092	<i>gatC</i>	galactitol-specific enzyme IIC component of PTS	0.197	4.93E-06
b2800	<i>fucA</i>	L-fucose-phosphate aldolase	0.187	4.69E-04
b1038	<i>csgF</i>	curli assembly component	0.187	4.14E-04
b4032	<i>malG</i>	maltose ABC transporter - membrane subunit	0.186	5.47E-04
b1818	<i>manY</i>	mannose PTS permease - ManY subunit	0.185	2.19E-10
b2096	<i>gatY</i>	D-tagatose 1,6-bisphosphate aldolase 2, catalytic subunit	0.169	5.07E-09
b4310	<i>nanM</i>	N-acetylneuraminate mutarotase	0.169	2.35E-04
b3225	<i>nanA</i>	N-acetylneuraminate lyase	0.166	7.68E-04
b2093	<i>gatB</i>	galactitol-specific enzyme IIB component of PTS	0.157	8.18E-06
b3395	<i>hofM</i>	protein involved in utilization of DNA as a carbon source	0.157	1.05E-04
b1517	<i>lsrF</i>	predicted class I aldolase	0.154	1.15E-02
b1817	<i>manX</i>	mannose PTS permease - ManX subunit	0.151	1.36E-10
b3722	<i>bglF</i>	β -glucoside PTS permease	0.150	5.65E-04
b2705	<i>srlD</i>	sorbitol-6-phosphate dehydrogenase	0.136	6.52E-06
b2703	<i>srlE</i>	glucitol/sorbitol-specific enzyme IIB component of PTS	0.132	5.34E-06
b3670	<i>ilvN</i>	acetolactate synthase I, small subunit	0.127	2.34E-03
b2094	<i>gatA</i>	galactitol-specific enzyme IIA component of PTS	0.119	4.79E-09
b2091	<i>gatD</i>	galactitol-1-phosphate dehydrogenase	0.106	9.33E-09
b2704	<i>srlB</i>	glucitol/sorbitol-specific enzyme IIA component of PTS	0.088	5.25E-05
b2702	<i>srlA</i>	glucitol/sorbitol-specific enzyme IIC component of PTS	0.072	1.08E-08

^aFrom the EcoCyc database (<http://ecocyc.org>)

^bFold-change in gene expression between E2 and WT (average of duplicate experiments)

Table 9: Genes that are up-regulated OR down-regulated in both iE2 and E2 as compared to their control in the absence of ethanol stress.

b-number	Gene	Function^a	Fold-change (E2/WT)	Fold-change (iE2/BW25113)
b1493	<i>gadB</i>	glutamate decarboxylase B	7.246	50.525
b1492	<i>gadC</i>	GadC GABA APC transporter	6.587	44.773
b3517	<i>gadA</i>	glutamate decarboxylase A	2.690	20.320
b3512	<i>gadE</i>	GadE DNA-binding transcriptional activator	3.616	18.309
b3513	<i>mdtE</i>	MdtEF-TolC multidrug efflux transport system - membrane fusion protein	2.827	17.156
b3516	<i>gadX</i>	GadX DNA-binding transcriptional dual regulator	2.078	7.209
b2508	<i>guaB</i>	IMP dehydrogenase	4.841	5.066
b0343	<i>lacY</i>	LacY lactose MFS transporter	2.441	4.412
b2507	<i>guaA</i>	GMP synthetase	4.564	3.551
b1038	<i>csgF</i>	curli assembly component	2.473	3.163
b1272	<i>sohB</i>	predicted inner membrane peptidase	2.822	2.513
b2235	<i>nrdB</i>	ribonucleoside diphosphate reductase 1, β subunit dimer	2.578	2.474
b0907	<i>serC</i>	phosphohydroxythreonine aminotransferase / 3-phosphoserine aminotransferase	2.129	2.231
b0430	<i>cyoC</i>	cytochrome bo terminal oxidase subunit III	2.764	2.118
b4067	<i>actP</i>	acetate / glycolate transporter	0.177	0.498
b2492	<i>focB</i>	FocB formate FNT transporter	0.499	0.490
b4197	<i>ulaE</i>	L-xylulose 5-phosphate 3-epimerase	0.442	0.484
b2805	<i>fucR</i>	FucR transcriptional activator	0.380	0.482
b1620	<i>mall</i>	MalI DNA-binding transcriptional repressor	0.428	0.467
b3414	<i>nfuA</i>	iron-sulfur cluster scaffold protein	0.467	0.450
b3905	<i>rhaS</i>	RhaS transcriptional activator	0.475	0.437
b4194	<i>ulaB</i>	L-ascorbate-specific enzyme IIB component of PTS	0.456	0.436
b0113	<i>pdhR</i>	PdhR DNA-binding transcriptional dual regulator	0.217	0.414
b4196	<i>ulaD</i>	3-keto-L-gulonate 6-phosphate decarboxylase	0.269	0.404
b4213	<i>cpdB</i>	2',3'-cyclic nucleotide 2'-phosphodiesterase / 3'-nucleotidase	0.435	0.398
b3236	<i>mdh</i>	malate dehydrogenase	0.396	0.390
b2799	<i>fucO</i>	L-1,2-propanediol oxidoreductase	0.244	0.384

b1594	<i>dgsA</i>	DgsA DNA-binding transcriptional repressor	0.454	0.370
b1205	<i>ychH</i>	stress-induced protein	0.246	0.360
b2147	<i>preA</i>	NADH-dependent dihydropyrimidine dehydrogenase subunit	0.099	0.352
b3868	<i>glnG</i>	NtrC transcriptional dual regulator	0.422	0.350
b1415	<i>aldA</i>	aldehyde dehydrogenase A, NAD-linked	0.159	0.339
b4003	<i>zraS</i>	ZraS sensory histidine kinase	0.418	0.335
b3566	<i>xylF</i>	xylose ABC transporter - periplasmic binding protein	0.217	0.330
b1002	<i>agp</i>	3-phytase / glucose-1-phosphatase	0.327	0.323
b2801	<i>fucP</i>	FucP fucose MFS transporter	0.433	0.313
b2146	<i>preT</i>	NADH-dependent dihydropyrimidine dehydrogenase subunit	0.123	0.310
b4268	<i>idnK</i>	D-gluconate kinase, thermosensitive	0.221	0.298
b4460	<i>araH</i>	arabinose ABC transporter - membrane subunit	0.211	0.285
b3091	<i>uxaA</i>	D-altronate dehydratase	0.379	0.281
b3113	<i>tdcF</i>	predicted L-PSP (mRNA) endoribonuclease	0.283	0.275
b2803	<i>fucK</i>	L-fuculokinase	0.383	0.274
b2614	<i>grpE</i>	phage lambda replication; host DNA synthesis; heat shock protein; protein repair	0.201	0.271
b3221	<i>yhcH</i>	conserved protein	0.220	0.268
b4266	<i>idnO</i>	5-keto-D-gluconate 5-reductase	0.267	0.267
b3092	<i>uxaC</i>	D-glucuronate isomerase / D-galacturonate isomerase	0.347	0.262
b3565	<i>xylA</i>	xylose isomerase	0.249	0.253
b0679	<i>nagE</i>	N-acetylglucosamine PTS permease	0.489	0.243
b4323	<i>uxuB</i>	D-mannonate oxidoreductase	0.082	0.239
b4069	<i>acs</i>	acetyl-CoA synthetase (AMP-forming)	0.014	0.236
b2365	<i>dsdX</i>	DsdX Gnt transporter	0.348	0.234
b1817	<i>manX</i>	mannose PTS permease - ManX subunit	0.369	0.220
b3134	<i>agaW</i>	PTS system N-acetylgalactosamine-specific IIC component 2	0.360	0.218
b3133	<i>agaV</i>	PTS system, cytoplasmic, N-acetylgalactosamine-specific IIB component 2 (EIIB-AGA)	0.258	0.215
b3575	<i>yiaK</i>	2,3-diketo-L-gulonate reductase	0.197	0.202
b2841	<i>araE</i>	AraE arabinose MFS transporter	0.245	0.198

b4322	<i>uxuA</i>	D-mannonate dehydratase	0.186	0.196
b2802	<i>fucI</i>	L-fucose isomerase	0.270	0.180
b3114	<i>tdcE</i>	2-ketobutyrate formate-lyase/pyruvate formate-lyase 4, inactive	0.249	0.167
b4311	<i>nanC</i>	N-acetylneuraminic acid outer membrane channel	0.153	0.166
b2092	<i>gatC</i>	galactitol-specific enzyme IIC component of PTS	0.424	0.164
b3528	<i>dctA</i>	DctA dicarboxylate DAACS transporter	0.179	0.162
b0598	<i>cstA</i>	peptide transporter induced by carbon starvation	0.063	0.161
b2091	<i>gatD</i>	galactitol-1-phosphate dehydrogenase	0.302	0.147
b4118	<i>melR</i>	MelR DNA-binding transcriptional dual regulator	0.308	0.144
b2093	<i>gatB</i>	galactitol-specific enzyme IIB component of PTS	0.457	0.136
b2151	<i>galS</i>	GalS DNA-binding transcriptional dual regulator	0.124	0.127
b3927	<i>glpF</i>	GlpF glycerol MIP channel	0.119	0.123
b3576	<i>yiaL</i>	conserved protein	0.339	0.115
b3670	<i>ilvN</i>	acetolactate synthase I, small subunit	0.301	0.114
b4321	<i>gntP</i>	GntP Gluconate Gnt transporter	0.267	0.105
b3077	<i>ebgC</i>	evolved β -D-galactosidase, β subunit	0.390	0.103
b3222	<i>nanK</i>	N-acetylmannosamine kinase	0.109	0.102
b2957	<i>ansB</i>	asparaginase II	0.473	0.101
b0759	<i>galE</i>	UDP-glucose 4-epimerase	0.001	0.099
b3581	<i>sgbH</i>	3-keto-L-gulonate 6-phosphate decarboxylase	0.186	0.099
b3926	<i>glpK</i>	glycerol kinase	0.227	0.098
b4267	<i>idnD</i>	L-idonate 5-dehydrogenase	0.167	0.093
b3671	<i>ilvB</i>	acetolactate synthase I, large subunit	0.212	0.079
b3115	<i>tdcD</i>	propionate kinase	0.026	0.077
b4032	<i>malG</i>	maltose ABC transporter - membrane subunit	0.283	0.076
b3116	<i>tdcC</i>	TdcC threonine STP transporter	0.051	0.074
b3076	<i>ebgA</i>	evolved β -D-galactosidase, α subunit	0.210	0.071
b4239	<i>treC</i>	trehalose-6-phosphate hydrolase	0.395	0.069
b0596	<i>entA</i>	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	0.096	0.063
b2705	<i>srlD</i>	sorbitol-6-phosphate dehydrogenase	0.496	0.060

b1900	<i>araG</i>	arabinose ABC transporter - ATP binding subunit	0.131	0.056
b4240	<i>treB</i>	fused trehalose(maltose)-specific PTS enzyme: IIB component/IIC component	0.248	0.054
b4033	<i>malF</i>	maltose ABC transporter - membrane subunit	0.306	0.052
b3224	<i>nanT</i>	NanT sialic acid MFS transporter	0.078	0.050
b1901	<i>araF</i>	arabinose ABC transporter - periplasmic binding protein	0.136	0.049
b4037	<i>malM</i>	maltose regulon periplasmic protein	0.308	0.042
b2704	<i>srlB</i>	glucitol/sorbitol-specific enzyme IIA component of PTS	0.381	0.041
b2148	<i>mglC</i>	galactose ABC transporter - membrane subunit	0.198	0.036
b4034	<i>malE</i>	maltose ABC transporter - periplasmic binding protein	0.284	0.034
b2149	<i>mglA</i>	galactose ABC transporter - ATP binding subunit	0.090	0.032
b3118	<i>tdcA</i>	TdcA DNA-binding transcriptional activator	0.122	0.030
b4036	<i>lamB</i>	phage lambda receptor protein; maltose high-affinity receptor	0.264	0.026
b2150	<i>mglB</i>	galactose ABC transporter - periplasmic binding protein	0.030	0.026
b2239	<i>glpQ</i>	glycerophosphoryl diester phosphodiesterase, periplasmic	0.227	0.024
b4035	<i>malK</i>	maltose ABC transporter - ATP binding subunit	0.266	0.022
b3225	<i>nanA</i>	N-acetylneuraminate lyase	0.132	0.020
b3223	<i>nanE</i>	predicted N-acetylmannosamine-6-phosphate epimerase	0.035	0.014
b2240	<i>glpT</i>	GlpT glycerol-3-P MFS transporter	0.220	0.014
b3709	<i>tnaB</i>	TnaB tryptophan ArAAP transporter	0.039	0.012
b3117	<i>tdcB</i>	catabolic threonine dehydratase	0.040	0.008
b3708	<i>tnaA</i>	L-cysteine desulphydrase / tryptophanase	0.019	0.004

^aFrom the EcoCyc database (<http://ecocyc.org>)

Table 10: Genes that are up-regulated OR down-regulated in both iE2 and E2 as compared to their controls in the presence of ethanol stress.

b-number	Gene	Function^a	Fold-change (E2/WT)	Fold-change (iE2/BW25113)
b0759	<i>galE</i>	UDP-glucose 4-epimerase	265.076	22.301
b0431	<i>cyoB</i>	cytochrome bo terminal oxidase subunit I	2.641	3.661
b1531	<i>marA</i>	MarA DNA-binding transcriptional dual regulator	2.700	3.072
b0429	<i>cyoD</i>	cytochrome bo terminal oxidase subunit IV	3.050	2.561
b1112	<i>bhsA</i>	protein involved in stress resistance and biofilm formation	4.061	2.528
b0583	<i>entD</i>	phosphopantetheinyl transferase	2.089	2.387
b1819	<i>manZ</i>	mannose PTS permease - ManZ subunit	0.251	0.477
b2092	<i>gatC</i>	galactitol-specific enzyme IIC component of PTS	0.197	0.459
b4266	<i>idnO</i>	5-keto-D-gluconate 5-reductase	0.280	0.447
b4118	<i>melR</i>	MelR DNA-binding transcriptional dual regulator	0.379	0.389
b2663	<i>gabP</i>	GabP APC transporter	0.281	0.373
b3367	<i>nirC</i>	NirC nitrite FNT transporter	0.476	0.373
b4194	<i>ulaB</i>	L-ascorbate-specific enzyme IIB component of PTS	0.453	0.281
b1101	<i>ptsG</i>	fused glucose-specific PTS enzymes: IIB component/IIC component	0.202	0.233
b2703	<i>srlE</i>	glucitol/sorbitol-specific enzyme IIB component of PTS	0.132	0.062
b2702	<i>srlA</i>	glucitol/sorbitol-specific enzyme IIC component of PTS	0.072	0.028
b2705	<i>srlD</i>	sorbitol-6-phosphate dehydrogenase	0.136	0.026
b2704	<i>srlB</i>	glucitol/sorbitol-specific enzyme IIA component of PTS	0.088	0.025

^aFrom the EcoCyc database (<http://ecocyc.org>)

Chapter 4: Improving acetate tolerance of *Escherichia coli* by rewiring its global regulator cAMP receptor protein (CRP)

4.1. Introduction

High cell density fermentation often results in the formation of byproducts, including acetate, formate, lactate and ethanol, especially in the presence of excess glucose. Among these byproducts, acetate is the most prominent one and has been widely studied (Kirkpatrick et al. 2001; Lin et al. 2005; Polen et al. 2003; Tomar et al. 2003). The presence of acetate exceeding 5g/L is a major concern during *E. coli* fermentation due to its inhibitory effect on cell growth, thereby limiting high-density cell growth and recombinant protein production (Aristidou et al. 1994; De Mey et al. 2007; Gilbert et al. 2009; Kleman and Strohl 1994; Luli and Strohl 1990). The unprotonated form of acetate can penetrate cell membrane, resulting in the reduction of intracellular pH (Kleman and Strohl 1994; Luli and Strohl 1990), and an accumulation of anion in the cytoplasm (Roe et al. 1998; Russell 1992), both of which could contribute to the inhibition of cell growth (De Mey et al. 2007).

In order to resolve these undesirable effects, process conditions such as controlling glucose feed rate in fed-batch cultivations, managing dissolved oxygen level in bioreactors, and using fructose instead of glucose as sole carbon source, are optimized to reduce the formation of acetate (Han et al. 1993; Lee 1996). Acetate toxicity can also be alleviated *via* the addition of methionine (Han et al. 1993; Mordukhova et al. 2008), glycine (Han et al. 1993), arginine, threonine, lysine, cytosine and uracil (Sandoval et al. 2011).

Reduction in acetate production can also be achieved through the adoption of metabolic engineering tools. Genes related to acetate producing pathways can either be knocked out (Chou et al. 1994; Contiero et al. 2000; Diazricci et al. 1991; Kang et al. 2009; Wong et al. 2008), or heterologous genes can be introduced to *E. coli* to convert acetate to other less harmful byproducts (Aristidou et al. 1994; Ingram and Conway 1988). Moreover, directed evolution of homoserine *o*-succinyltransferase involved in methionine biosynthesis was also used to improve acetate tolerance of *E. coli* (Mordukhova et al. 2008). The choice of heterologous genes does not limit to only enzymes, the exogenous global regulator IrrE from *Deinococcus radiodurans* was also overexpressed in *E. coli* to confer acetic acid tolerance (Chen et al. 2011).

Besides metabolic engineering approaches, classical strain engineering methods using UV mutagen and evolutionary engineering strategy have also been used to improve microbial tolerance towards acetate stress. UV mutagenesis was performed on *Clostridium thermoaceticum* and *Clostridium thermoautophicum* to improve their acetate tolerance (Reed et al. 1987), while acetate-tolerant *E. coli* and *S. cerevisiae* mutants were generated through evolutionary engineering strategy (Gilbert et al. 2009; Treves et al. 1998; Wright et al. 2011).

Since classical strain engineering approaches are often time- and labour-intensive, while metabolic engineering tools are limited to very few microorganisms with well-studied metabolic pathways (Patnaik 2008), new strain engineering approaches such as genome shuffling (Patnaik et al. 2002) and transcriptional engineering have been developed to improve various cell phenotypes. Examples of transcription factors that had been engineered so far

include zinc-finger artificial transcription factors (Park et al. 2005a; Park et al. 2003), Spt15 (Alper et al. 2006), sigma factors (Alper and Stephanopoulos 2007), H-NS and Hha (Hong et al. 2010a; Hong et al. 2010b), and IrrE (Chen et al. 2011; Wang et al. 2012). Genome shuffling was used to increase acetic acid tolerance in *Candida krusei* (Wei et al. 2008), and IrrE had been engineered to improve tolerance to acetic acid as aforementioned. To date, there is no report on engineering native global transcription factor to improve acetate tolerance.

Our lab has successfully improved the tolerance of *E. coli* towards 1-butanol, organic solvent and osmotic stress in the past by rewiring its global regulator cAMP receptor protein (Zhang et al. 2012a; Zhang et al. 2012b). In this work, we have also chosen to engineer cAMP-receptor protein (CRP) in *Escherichia coli* to improve its acetate tolerance. CRP is able to regulate over 400 genes in *E. coli*, especially the genes involved in carbon metabolism (Keseler et al. 2011). In addition, the production and assimilation of acetate were found to be largely controlled by the reactions in the tricarboxylic acid (TCA) cycle, where the genes were mainly regulated by CRP (De Mey et al. 2007; Oh et al. 2002; Peng and Shimizu 2003; Phue et al. 2005; Polen et al. 2003), which makes CRP an attractive native global regulator to engineer. Here, we have introduced random mutations to CRP by error-prone PCR and isolated four variants (A1-A4) with improved tolerance to acetate. The best mutant was further characterized in terms of its cross-tolerance to other fermentative byproducts, extracellular acetate measurement and cell morphology studies. The changes in the expression level of 444 CRP-regulated genes in A2 were also assessed by quantitative real-time reverse transcription PCR using OpenArray®.

4.2. Methods and materials

4.2.1. Materials

The host strain *E. coli* DH5 α Δ *crp* was constructed by knocking out *crp* from *E. coli* DH5 α (Invitrogen, San Diego, US) according to previously established protocols (Baba et al. 2006; Datsenko and Wanner 2000). Overnight cultures were prepared in Luria-Bertani (LB) medium containing 1 % tryptone (Oxoid, Hampshire, UK), 0.5 % yeast extract (Merck, Damstadt, Germany), and 1 % sodium chloride (Merck, Damstadt, Germany). M9 minimal medium was used for cells cultured under acetate stress, which composed of the following (per liter): 6.78 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl, 0.49 g MgSO₄·7H₂O, 0.011 g CaCl₂, 2 g glucose and 1 ml of trace metal stock solution. The trace metal stock solution contained the following (per liter): 0.8 g CoCl₂, 0.4 g ZnSO₄·7H₂O, 2 g MnCl₂·4H₂O, 0.2 g Na₂MoO₄·2H₂O, 0.2 g CuCl₂, 2.5 g FeSO₄·7H₂O and 1 g thiamine. Sodium acetate was added to M9 minimal medium accordingly when required. All chemicals were purchased from either Merck or Sigma-Aldrich. Restriction enzymes were from Fermentas (Burlington, Canada), while T4 DNA ligase was purchased from New England Biolabs (Ipswich, MA, US). Gel purification and plasmid extraction were performed using QIAquick gel extraction kit and QIAprep spin miniprep kit (QIAGEN, Germany), respectively.

4.2.2. Library construction

Error-prone PCR was carried out using GeneMorph® II Random Mutagenesis Kit (Stratagene, La Jolla, CA, US) with the primers A and B (Table 1), with 30 ng template. The PCR condition is as follows: 2 min at 95 °C, 30 cycles of 95 °C for 1 min, 62 °C for 1 min, followed by 72 °C for 1 min, and

finally 10 min at 72 °C. The resulting *crp* products were digested and cloned into self-constructed plasmid pKCP (section 4.6, Figure 18). The mutant libraries were then transformed into *E. coli* DH5a Δcrp cells by electroporation using Eppendorf multiporator (Hamburg, Germany).

4.2.3. Mutant selection

To select mutants with improved acetate tolerance, the transformants were first cultured in 50 ml M9 minimal medium supplemented with 5 g/L sodium acetate. The cell culture was cultivated for 20-30 h after inoculation and this process was repeated with two subcultures containing 10 g/L sodium acetate. The cell culture was plated onto LB agar plates after each round of selection, and individual clones were randomly picked for sequencing. In order to exclude false positives, the mutant plasmids were re-transformed into fresh *E. coli* DH5a Δcrp background.

4.2.4. Mutant growth under acetate stress

The growth profile of the CRP mutants isolated from the error-prone PCR libraries was determined at the following sodium acetate concentrations: 0, 10 and 15 g/L. One percent (v/v) overnight cell culture was inoculated into 10-ml M9 medium supplemented with certain concentration of sodium acetate, and cultured at 37 °C with reciprocal shaking at 200 rpm. Their absorbance at 600 nm was recorded at certain time interval.

4.2.5. Measurement of extracellular acetate concentration

One percent (v/v) overnight cell culture was inoculated either in M9 medium (0 g/L sodium acetate) or M9 medium supplemented with 10 g/L sodium

acetate. 200- μ l cell culture samples were collected at certain intervals. For analysing extracellular acetate concentration, the samples were centrifuged at 13,000 rpm for 5 min, and their supernatant was collected and stored at -20 °C before measurement. The acetate concentration was determined enzymatically using R-biopharm acetic acid test kit (Damstadt, Germany) according to manufacturer's instructions.

4.2.6. Cross-tolerance to other fermentative products

One percent (v/v) of overnight cell culture was inoculated into 10-ml M9 medium supplemented with either 5 g/L sodium formate or 18 g/L sodium propionate. Cells were incubated at 37 °C with reciprocal shaking at 200 rpm, and their absorbance was measured at 600 nm.

4.2.7. RNA isolation

One percent (v/v) overnight cell culture was inoculated into M9 medium supplemented with or without 10 g/L sodium acetate. Cells were cultured for 8 h in the absence of sodium acetate, or for 18 h in 10 g/L sodium acetate. Total RNA was isolated using PureLink RNA mini kit (Ambion, Carlsbad, CA, USA), and treated with PureLink DNase (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The quality and integrity of the isolated RNA was determined through spectrophotometer and agarose gel electrophoresis. 800 ng total RNA was converted into cDNA by reverse transcription in 20- μ l reaction mixture using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with random primer mix following manufacturer's protocol.

4.2.8. Quantitative real-time reverse transcription PCR (RT-PCR) using OpenArray®

Quantitative real-time reverse transcription PCR for CRP-regulated genes was conducted in OpenArray® Real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) in duplicates, using Lightcycler® FastStart DNA Master SYBR Green I (Roche, Germany) in reformatted OpenArray® real-time PCR plates (Foster City, CA, USA). OpenArray is a high throughput real-time PCR platform, which allows 224 genes to be assessed on a single plate. 33 nl reaction mixture was loaded into each through-hole on the OpenArray® real-time PCR plates with the OpenArray® AccuFill™ system (Applied Biosystems, Foster City, CA, USA). The real-time PCR conditions were carried as follows: 1 cycle at 90 °C for 10 min, 36 cycles at 48 °C for 18 s, 54 °C for 1 min, 77 °C for 22 s, 72 °C for 70 s, and 95 °C for 20 s, followed by 1 cycle at 70 °C for 5 min. The bacterial 16S rRNA (*rrsG*) was used as the internal standard and the sequence of the primers are given in Table 4 (see supplementary information). The values of cycle threshold (C_t) were provided by the OpenArray® Real-time qPCR Analysis Software Version 1.0.4, and $2^{-\Delta\Delta C_t}$ method of relative quantification was utilized to compute the relative expression level. The p -value for each gene was calculated by student's t -test using IBM SPSS Statistics Software Version 19.

4.2.9. Effects of gene overexpression on acetate tolerance

The ASKA library was used for overexpression of genes (Kitagawa et al. 2006) in *E. coli* DH5 α . 1 % (v/v) overnight culture was inoculated into either 0 g/L or 5 g/L sodium acetate in 10-ml M9 medium. 25 μ g/ml chloramphenicol was

used to maintain the pCA24N-based plasmids, while 0.1 mM IPTG was used to induce overexpression of genes from pCA24N. Cell cultures were incubated at 37 °C with reciprocal shaking at 200 rpm, and their absorbance was measured at 600 nm.

4.3. Results

4.3.1. Mutant isolation

The mutated *crp* products obtained via error-prone PCR were cloned into previously constructed pKSC plasmids. *E. coli* DH5 α Δ *crp* strain harboring pKCP (containing native *crp* operon) is denoted as wild type (WT) in this study. Six error-prone PCR libraries with a total library size of $\sim 3 \times 10^6$ were subjected to 5 g/L sodium acetate for variant selection, followed by two subcultures in 10 g/L sodium acetate. At the end of the selection process, 32 colonies were randomly picked and sequenced to determine their amino acid modifications in CRP. Four mutants (A1- A4) with better growth under acetate stress were finally identified and their amino acid mutations are listed in Table 11.

Table 11: Amino acid substitutions in error-prone PCR mutants (A1-A4)

Mutant	Amino Acid Substitution(s)			
A1	Y63F			
A2	D138Y			
A3	V176I			
A4	Y99C	A151V	V176A	L195M

4.3.2. Mutant growth profile under acetate stress

The growth profiles of the mutants, A1-A4, and WT were determined in the absence and presence of acetate stress. When the cells were grown in M9 medium without any sodium acetate supplement (0 g/L sodium acetate, Figure 13a), it was observed that all mutants grew better than WT, with A2 as the fastest among the mutants. The growth rate of A2 and WT were calculated to be 0.19 h^{-1} and 0.13 h^{-1} respectively. The presence of 10 g/L (Figure 13b) and 15 g/L sodium acetate (Figure 13c) led to strong growth inhibition of WT, but did not influence the mutants significantly. It was evident that A2 grew faster than other mutants under acetate stress. With 10 g/L sodium acetate, the growth rate of A2 was 0.10 h^{-1} , compared to that of WT at 0.026 h^{-1} . When sodium acetate concentration was increased to 15 g/L, the growth rate of A2 was reduced to 0.083 h^{-1} , whereas that of WT was even lowered to 0.016 h^{-1} .

4.3.3. Measurement of extracellular acetate concentration

The amount of acetate produced by A2 and WT were measured to investigate on their acetate-producing ability because previous publications have indicated that strain with less acetate production usually grows faster (Phue and Shiloach 2002; van de Walle and Shiloach 1998; Wolfe 2005). Due to the growth difference between A2 and WT, the measured acetate concentration was normalized based on OD600. Without sodium acetate supplement, it was observed that the acetate produced by A2 was nearly double that of WT over the sampling period of 14 h (Figure 14). The average acetate concentration per OD600 over 14h for A2 was about 0.15 g/L, while that of WT was 0.07 g/L. In contrast, when A2 and WT were exposed to 10 g/L sodium

acetate, the concentration of acetate for both A2 and WT were almost the same (Figure 15), indicating that acetate production or assimilation is similar between A2 and WT.

Figure 13: Growth profiles of WT and CRP mutant strains in M9 medium at 37 °C. **(a)** 0 g/L sodium acetate. **(b)** 10 g/L sodium acetate. **(c)** 15 g/L sodium acetate. Values shown represent average of triplicate experiments.

Figure 14: Concentration of acetate in A2 and WT in M9 medium (0 g/L sodium acetate) at different time intervals. The acetate concentration was normalized based on the absorbance at 600 nm.

Figure 15: Concentration of acetate in A2 and WT in M9 minimal medium supplemented with 10 g/L sodium acetate at different time intervals.

4.3.4. Cross-tolerance to other fermentative byproducts

In addition to acetate, formate and propionate are common metabolic byproducts during *E. coli* fermentation (Kirkpatrick et al. 2001; Polen et al. 2003), hence the tolerance of A2 towards these two byproducts was also studied.

The growth pattern of A2 and WT cultivated in M9 medium supplemented with 5 g/L sodium formate or 18 g/L sodium propionate was presented in Figure 16a and Figure 16b respectively. A2 exhibited slightly better tolerance (0.16 h^{-1}) than WT (0.14 h^{-1}) in formate, whereas its performance was much enhanced in the presence of propionate—the growth rate of A2 was 0.07 h^{-1} , nearly twice that of WT at 0.03 h^{-1} .

Figure 16: Comparison between A2 and WT in the presence of metabolic byproducts in M9 medium at 37 °C. **(a)** sodium formate, 5 g/L. **(b)** sodium propionate, 18 g/L.

4.3.5. Quantitative real-time reverse transcription PCR using OpenArray®

We investigated on the relative gene expression level of 444 genes regulated by CRP (Keseler et al. 2011) between the best mutant A2 and WT in the presence or absence of sodium acetate stress. Without stress, 407 CRP-regulated genes were differentially expressed. Among these genes, 290 genes were up-regulated and 14 genes were down-regulated in A2 by more than 2-fold ($p < 0.05$) as presented in Table 14 (see supplementary information). The two highest up-regulated genes were *yfiD* and *pflB*, 17- and 15-fold respectively, while *galE* displayed the biggest down-regulation of 143-fold. On the other hand, the presence of sodium acetate stress resulted in the differential expression of 432 CRP-regulated genes in A2, with 6 up-regulated and 406 genes down-regulated by more than 2-fold at a p -value threshold < 0.05 (see supplementary information Table 15). To be more specific, the gene expression of *galE* had the largest increase by nearly 14-fold in A2, whereas *lsrB* and *lsrD*, part of transporter for signal molecule autoinducer-2 (AI-2) and involved in chemotaxis, showed the largest decrease of 27- and 23-fold respectively (Pereira et al. 2009).

It was found that five of the 444 assessed genes (*prpB*, *prpC*, *prpD*, *prpE*, and *paaJ*) had low expression level such that C_t value could not be measured in A2 and WT with or without stress. Interestingly, *aroA*, which encodes for 3-phosphoshikimate-1-carboxyvinyltransferase, was without C_t value in the absence of sodium acetate stress for both A2 and WT. However, the gene expression level of *aroA* increased under acetate stress such that C_t value could be provided, with A2 showing decreased expression level by 1.9-fold than WT.

aroA is known to increase the tolerance to herbicide glyphosate (Rogers et al. 1983), but there have been no reports so far on the relationship between *aroA* and acetate stress.

In addition to genes playing roles in acetate assimilation, the presence of sodium acetate in the culture medium also resulted in the down-regulation of genes from various metabolic pathways, such as tricarboxylic acid cycle (TCA cycle) (*sdhCDAB*, *sucABCD*, and *mdh*), phosphotransferase system (PTS) (*ptsG*, *ptsHI-crr*, *nagE*, and *malX*), ATP binding cassette (ABC) transporters (*malEFG*, *mglBAC*, *flhCD*, and *lsrB*), two-component system (*ompF*, *dctA*), amino acid metabolism (*tnaA*, *gadA*), and amino sugar and nucleotide sugar metabolism (*nanE*), whose fold-change were presented in Table 12. Many of these genes have been demonstrated previously to be associated with adaptation to acetate stress (Arnold et al. 2001; Kirkpatrick et al. 2001; Oh et al. 2002; Polen et al. 2003). Interestingly, except for the aforementioned genes involved in the TCA cycle, the rest of genes were actually up-regulated when sodium acetate was not added to the culture medium.

Table 12: Genes with differential expression level in A2 as compared to WT associated with acetate stress adaptation in various metabolic pathways.

Metabolic Pathway	Gene or operon	Fold-change	
		-sodium acetate	+sodium acetate
TCA cycle	<i>sdhCDAB</i>	0.26	0.073-0.080
	<i>sucABCD</i>	0.2-0.37	0.13-0.23
	<i>mdh</i>	0.46	0.33
Galactose metabolism	<i>galE</i>	0.007	14

Glyoxylate pathway	<i>aceB</i> and <i>aceA</i>	0.38-0.49	0.14-0.15
	<i>aceK</i>	2	0.11
	<i>acnB</i>	0.31	0.15
Phosphotransferase system (PTS)	<i>ptsG</i>	1.1	0.18
	<i>ptsHI-crr</i>	1.3-1.9	0.34
	<i>nagE</i>	2.4	0.097
	<i>malX</i>	1.9	0.0067
Pyruvate metabolism	<i>acs</i>	-	0.13
	<i>pflB</i>	15	4.4
	<i>yfiD</i>	17	2
ABC Transporters	<i>malEFG</i>	2.2-3.9	0.082-0.11
	<i>mglBAC</i>	2-2.1	0.070-0.085
	<i>flhCD</i>	2-2.1	0.079-0.088
	<i>lsrB</i>	2.4	0.042
Two-component system	<i>ompF</i>	0.25	0.095
	<i>dctA</i>	2.6	0.099
Tryptophan metabolism	<i>tnaA</i>	3.3	0.12
Alanine, aspartate and glutamate metabolism	<i>gadA</i>	2.6	3.1
Amino sugar and nucleotide sugar metabolism	<i>nanE</i>	-	0.16

4.3.6. Effects of gene overexpression on acetate tolerance

Eight genes (*pflB*, *yfiD*, *galE*, *gadA*, *uxaB*, *aroA*, *feaR* and *araG*) that displayed large increment or decline in their expression levels in A2 under acetate stress were chosen to be overexpressed in *E. coli* to determine their effects on acetate tolerance, with *E. coli* DH5 α harboring pCA24N as the control. It was observed that the overexpression of *pflB*, *yfiD*, *aroA*, *galE* and

gadA did not improve the growth of *E. coli* DH5 α under acetate stress, while overexpressing *feaR* and *araG* did not result in acetate sensitivity. In contrast, the growth difference was observed between the control and UxaB overexpression when facing acetate stress. Both had similar growth rate of 0.09-0.1 h⁻¹ when cultured without sodium acetate (see Figure 17a). The presence of 5g/L sodium acetate reduced the growth rate of the control to about 0.05 h⁻¹, but exerted a greater deleterious effect on the cells overexpressing *uxaB* (see Figure 17b), about 60% to 0.021 h⁻¹.

Figure 17: Growth profiles of *E. coli* DH5α overexpressing *uxaB* in M9 medium at 37 °C. *E. coli* DH5α harbouring pCA24N was used as control. **(a)** 0 g/L sodium acetate. **(b)** 5 g/L sodium acetate.

4.4. Discussion

In this study, we have successfully isolated four mutants (A1 – A4) with much enhanced tolerance towards acetate stress (10 g/L and 15 g/L sodium acetate) by rewiring its global regulator CRP. Error-prone PCR was applied to introduce random mutations to CRP and the selection process for improved

mutants required about 5-7 days. This is the first study whereby a native regulator is engineered for enhancing cell growth under acetate stress.

The four mutants, A1-A4, had amino acid substitutions in various positions: Y63F (β -strand 5), Y99C (β -strand 8), D138Y (Hinge region), A151V (D-helix), V176I/V176A (F-helix) and L195 (β -strand 11) (Weber and Steitz 1987). The position D138 is of particular interest as our previous studies on CRP mutants with either improved osmotolerance or 1-butanol tolerance also had mutations in this position (Zhang et al. 2012a; Zhang et al. 2012b). D138 is known to play essential roles during the hinge reorientation and helical adjustments upon allosteric activation by cAMP molecule (Sharma et al. 2009; Yu and Lee 2004) through formation of hydrogen bonds with G141 (Popovych et al. 2009). Although D138 is not located in the DNA binding domain (residue 140-209), any mutations to this position can affect the DNA binding affinity of CRP through involving the F-helix (Yu and Lee 2004).

It was found before that strains that produce less acetate would be able to grow faster (Phue and Shiloach 2002), thus many strategies have focused on reducing the production of acetate to improve cell tolerance to acetate (Eiteman and Altman 2006). Interestingly, we found that although A2 was able to grow faster than WT in minimal medium, A2 produced more acetate than WT. It was known that there are two acetate producing pathways in *E. coli*: the first pathway is catalyzed by acetate kinase (*ackA*) and phosphotransacetylase (*pta*) to produce acetate from acetyl-CoA with acetyl-phosphate as the intermediate, while pyruvate kinase (*poxB*) converts pyruvate to acetate directly in the second pathway (De Mey et al. 2007; Wolfe 2005). From our RT-PCR results on these three genes, it was revealed that A2 had higher expression level of *ackA*, *pta*

and *poxB* than WT (see supplementary, Figure 19). Therefore, increased acetate production in A2 is likely to be attributed to the higher gene expression of the genes involved in acetate production. The presence of more acetate in A2 cultivation probably induced acetate tolerance response in A2, which in turn resulted in A2 growing faster than WT in minimal medium without sodium acetate supplement.

In order to determine how mutations in A2 could affect the transcript profile of CRP-regulated genes, RT-PCR analysis was performed on 444 CRP-regulated genes with OpenArray® platform. It was revealed that most of the CRP-regulated genes were differentially expressed with or without the addition of sodium acetate, due to the mutation in A2. Although the expression levels of most CRP-regulated genes were increased in the absence of sodium acetate, many of the genes involved in the TCA cycle were down-regulated, which may lead to reduced flux of the TCA cycle and resulted in higher acetate accumulation in A2 (De Mey et al. 2007; Eiteman and Altman 2006). In contrast, many of the CRP-regulated genes were actually down-regulated when sodium acetate was present, including genes involved in acetate uptake (*acs*) and glyoxylate bypass (*aceBAK*). Acetate can also be utilised as sole carbon source, whereby the uptake of acetate is performed by acetyl-CoA synthetase (*acs*), and metabolized through the glyoxylate cycle (*aceBAK* operon, *mdh*, *gltA* and *acnB*). Many studies investigating on the responses of *E. coli* towards acetate stress had reported that *acs* and *aceBAK* were induced by the presence of acetate (Oh et al. 2002; Peng and Shimizu 2003; Phue and Shiloach 2002). However, as presented in Table 2, the gene expression of *acs* was down-regulated by about 7-fold, while the genes involved in glyoxylate pathway had

lower expression in A2 than WT under acetate stress. Although higher expression of these genes is expected to aid in acetate adaption, yet A2 is able to tolerate higher concentration of sodium acetate despite the transcript level of these genes being lower in A2 than WT.

Acetate is also known to result in internal acidification whereby the intracellular pH decreases due to dissociation of acetic acid (weak acid) in the cytoplasm (Roe et al. 1998; Russell 1992). Genes such as *yfiD*, *pflB*, *gadA* and *gadB* were found to increase their expression in order to protect *E. coli* against internal acidification (Arnold et al. 2001; Blankenhorn et al. 1999; Kirkpatrick et al. 2001), which concurs with our findings in this study. In particular, *yfiD*, which encodes stress-induced alternate pyruvate formate lyase subunit, is up-regulated by almost 17-fold even without the addition of sodium acetate. The expression of *pflB* (encode pyruvate formate lyase), *gadA* and *gadB* (encode glutamate decarboxylase) also increase in A2, which implies that A2 is probably protected against internal acidification by the pre-programmed up-regulation of the aforementioned genes. The up-regulation of *yfiD*, *pflB* and *gadA* by approximately 2-3 folds in the presence of sodium acetate ensures the continual protection of A2 against internal acidification caused by acetate anion. Yet, we found that overexpression of individual genes *pflB*, *yfiD*, or *gadA* did not confer the acetate resistance phenotype in *E. coli* DH5 α . It seems that coordinated changes in expression levels of multiple genes are required to bring about the tolerance phenotype (Gonzalez et al. 2003).

Previous findings on microarray analysis of *E. coli* after long-term adaption to acetate have demonstrated that many genes involved in the uptake and catabolism of carbon and energy sources apart from glucose decreases in

mRNA abundances, such as maltose (*malXY*, and *malEFG*), galactose (*galETKM*, *galS*, and *mglBAC*), amino sugars (*nanATEK-yhcH*), sugar alcohols (*srlA1A2BD-gutM*, and *srlR-gutQ*), amino acids (*tnaA*), and dicarboxylic acids (*dctA*) (Polen et al. 2003). This is in agreement with our OpenArray® findings whereby many of the genes involved in carbon catabolism are down-regulated in A2. In addition, similar to our findings, the expression of transport genes such as *malE* and *ompF* were also repressed in the presence of acetate (Arnold et al. 2001; Kirkpatrick et al. 2001; Polen et al. 2003). Interestingly, the expression levels of these genes increase in WT, but decrease in A2 when comparing the expression levels with sodium acetate to without sodium acetate (data not shown). This implies that A2 is already pre-programmed for long-term adaption to acetate, while WT is require more time to adapt to the stress condition, which may explain for the growth differences observed between A2 and WT.

We have also observed that the overexpression of *uxaB* would decrease the growth of *E. coli* under acetate stress. *uxaB* encodes for altronate oxidoreductase responsible for the reversible NADH-dependent reduction of D-tagaturonate to D-altronate in galacturonate catabolism pathway (Portalier 1972). Currently, there are no known findings on the relationship between *uxaB* and acetate metabolism or tolerance. The relationship between *uxaB* and acetate tolerance requires further investigation in the future.

Scanning electron micrographs of A2 and WT (Figure 20) when cultivated with or without sodium acetate stress has revealed that A2 is not different from WT in terms of shapes or surface roughness. It was previously reported that *E. coli* could suffer from surface indentation and lose surface integrity when

exposed to osmotic stress (Taylor et al. 1993), which were not observed in A2 and WT in this study, implying osmotic stress to the cells was negligible at the concentration of sodium acetate used although there was an increase in medium osmolality.

In conclusion, genetic modifications can be achieved through directed evolution of the global regulator CRP, resulting in changes in transcript profile of CRP-regulated genes involved in various metabolic pathways. In addition to existing approaches of process optimization or genetic modifications through gene overexpression or gene knockouts, this method can also provide an effective alternative to enhance the growth of *E. coli* in the presence of inhibitory metabolic byproducts such as acetate.

4.5. Acknowledgement

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4.6. Supplementary Information

Figure 18: Vector map of plasmid pKCP containing native *crp* operon with the sequences provided below.

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1  TCGCGCGTTT  CGGTGATGAC  GGTGAAAACC  TCTGACACAT  GCAGTCCCG  GAGACGGTCA
61  CAGCTTGTCT  GTAAGCGGAT  GCCGGGAGCA  GACAAGCCCG  TCAGGGCGCG  TCAGCGGGTG
121  TTGGCGGGTG  TCGGGGCTGG  CTTAACTATG  CGGCATCAGA  GCAGATTGTA  CTGAGAGTGC
181  ACCATATGCG  GTGTGAAATA  CCGCACAGAT  GCGTAAGGAG  AAAATACCGC  ATCAGGCGCC
241  ATTCGCCATT  CAGGCTGCGC  AACTGTTGGG  AAGGGCGATC  GGTGCGGGCC  TCTTCGCTAT
301  TACGCCAGCT  GGCGAAAGGG  GGATGTGCTG  CAAGGCGATT  AAGTTGGGTA  ACGCCAGGGT
361  TTTCCAGTC  ACGACGTTGT  AAAACGACGG  CCAGTGCCAA  GCTTAGAGTA  CGCGTACTAA
421  CCAATCGCG  CAACGGAAGG  CGACCTGGGT  CATGCTGAAG  CGAGACACCA  GGAGACACAA
481  AGCGAAAGCT  ATGCTAAAA  AGTCAGCATG  CTACAGTAAT  ACATTGATGT  ACTGCATGTA
541  TGCAAAGGAC  GTCACATTAC  CGTGCACTAC  AGTTGATAGC  CCCTTCCCAG  GTAGCGGGAA
601  GCATATTTTC  GCAATCCAGA  GACAGCGGCG  TTATCTGGCT  CTGGAGAGGA  TCCATAACAG
661  AGGATAACCG  CGCATGGTGC  TTGGCAAACC  GCAAACAGAC  CCGACTCTCG  AATGGTTCTT
721  GTCTCATTGC  CACATTCATA  AGTACCCATC  CAAGAGCACG  CTTATTCACC  AGGGTGAAAA
781  AGCGGAAACG  CTGTACTACA  TCGTTAAAGG  CTCTGTGGCA  GTGCTGATCA  AAGACGAAGA
841  GGGTAAAGAA  ATGATCCTCT  CCTATCTGAA  TCAGGGTGAT  TTTATTGGCG  AACTGGGCCT
901  GTTTGAAGAG  GGCCAGGAAC  GTAGCCCATG  GGTACGTGCG  AAAACCGCCT  GTGAAGTGGC
961  TGAAATTTTC  TACAAAAAAT  TTCGCCAATT  GATTCAGGTA  AACCCGGACA  TTCTGATGCG
1021  TTTGTCTGCA  CAGATGGCGC  GTCGTCTGCA  AGTCACTTCA  GAGAAAGTGG  GCAACCTGGC
1081  GTTCTCGAC  GTGACGGGCC  GCATTGCACA  GACTCTGCTG  AATCTGGCAA  AACACACAGA
1141  CGCTATGACT  CACCCGGACG  GTATGCAAA  CAAAATTACC  CGTCAGGAAA  TTGGTCAGAT
1201  TGTCGGCTGT  TCTCGTGAAA  CCGTGGGACG  CATTCTGAAG  ATGCTGGAAG  ATCAGAACCT
1261  GATCTCCGCA  CACGTAAAA  CCATCGTCGT  TTACGGCACT  CGTTAATCCC  GTCGGAGTGG
1321  CGCGTTACCT  GGTAGCGCCC  CATTTTGTTC  GGTACCGAAG  AACCGTGGTA  TGTGGTGGAC
1381  AAAGACGTCG  AAAACAACAT  TCTGGTTGTC  GCTCAGGGCC  ATGAACACCC  GCGGCTGATG
1441  TCTGTCGGGT  TGATTGCCCA  GCAGTTGCAC  TGGGTGATC  GCGAACCATT  CACCGGCACT

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1501 ATGCGTTGCA CGGTAAAAAC CCGCTATCGC CAGACCGATA TCCCTTGCAC CGTCAAGGCG
1561 CTGGACGATG ATCGCATTGA AGTGATTTTC GATGAACCGG TTGCCGCCGT GACGCCGGGC
1621 CAGTCTGCCG TCTTCTATAA CCGTGAAGTG TGCCTCGGTG GCGGTATTAT TGAGCAGCGT
1681 CTGCCGCTGC CCGTCTGATT ATTATCTTTA CTTAAAACAA GGAAGCAGTG AACGTGGCAA
1741 AGAATTACTA TGACATCACC CTCGCCCTGG CCGGTATTTG TCAGTCGGCA CGCCTGGTGC
1801 AACAACTCGC TCACCAGGGG CATTGTGATG CCGATGCGCT ACACGTCTCA CTCAACAGTA
1861 TTATTGATAT GAACCCAGC TCGACGCTGG CGGTTTTTGG CGGTAGCGAA GCCAACCTGC
1921 GCGTCGGGCT GGAAACCCTG CTCGGCGTGC TCAATGCCAG CAGTCGCCAG GGCTTAAACG
1981 CCGAATTAAC CCGCTACACA CTCAGCTTGA TGGTGCTTGA GCGCAAATC TCCTCAGCGA
2041 AAGGCGCGCT CGACACTCTG GGCAACCGGA TCAACGGCCT GCAACGCCAG CTCGAACACT
2101 TCGATTACCA GTCCGAAACG CTGATGAGCG CGATGGCTGC TATCTATGTT TATCTGATTA
2161 CCCCGCTTGG CCCGCGCATT CAGGTCACCG GTTCCCCTGC TGTACTGCAA AGCCACAAAG
2221 TGCAGGCGAA AGTTCGCGCA ACCCTGCTGG CAGGCATTTC GCGCGCCGTG CTCTGGCACC
2281 AGGTGCGCGG CGGACGTCTG CAACTGATGT TTTCTCGTAA TCGCCTGACC ACTCAGGCAA
2341 AACAAATCTT TGCTCATTTA ACCCCGGAGT TGTGATCTAT GGAATTATCC TCACTGACCG
2401 CCGTTTCCCC TGTCGATGGA CGCTACGGCG ATAAAGTCAG CCGCTGCGC GGGATTTTCA
2461 GCGAATATGG TTTGCTGAAA TTCCGTGTAC AAGTTGAAGT ACGTTGGCTG CAAAAACTGG
2521 CCGCGCACGC AGCGATCAAG GAAGTTCCGT CTTTGTGCTG CGACGCAATC GGTTACCTTG
2581 ATGCAATCGT CGCCAGTTTC AGCGAAGAAG ATGCGGCGCG CATCAAAACT ATCGAGCGTA
2641 CCACTAACCA CGACGTTAAA GCGGTTGAGT ATTTCTGAA AGAAAAAGTG GCGGAGATCC
2701 CGGAAGTGA CCGCGTTTCT AAATTCATCC ACTTTGCCTG TACTTCGGAA GATATCAATA
2761 ACCTCTCCCA CGCATTAATG CTGAAAACCG CGCGTGATGA AGTGATCCTG CCATACTGCG
2821 GTCAACTGAT TGATGGCATT AAAGATCTCG CCGTTCAGTA TCGCGATATC CCGCTGCTGT
2881 CTCGTACCCA CCGTCAGCCA GCCACGCCGT CAACCATCGG TAAAGAGATG GCAAACGTGCG
2941 CCTACCGTAT GGAGCGCCAG TACCGCCAGC TTAACCAAGT GGAGATCCTC GGCAAAATCA
3001 ACGGCGCGGT CCGTAACTAT AACGCCCACA TCGCCGCTTA CCCGGAAGTT GACTGGCATC
3061 AGTTCAGCGA AGAGTTCGTC ACCTCGCTGG GTATTGAGTG GAACCCGTAC ACCACCAGA
3121 TCGAACCGCA CGACTACATT GCCGAAGTGT TTGATTGCGT TCGCGCTTTC AACACTATTC
3181 TGATCGACTT TGACCGTGAC GTCTGGGGTT ATATCGCCCT TAACCACTTC AAACAGAAAA
3241 CCATTGCTGG TGAGATTGGT TCTTCCACCA TGCCGCATAA AGTTAACCCG ATCGACTTCG
3301 AAAACTCCGA AGGGAATCTG GGCCTTTCCA ACGCGGTATT GCAGCATCTG GCAAGCAAAC
3361 TGCCGCTTTC CCGCTGGCAG CGTGACCTGA CCGACTCTAC CGTGCTGCGT AACCTCGGCG
3421 TGGGTATCGG TTATGCCTTG ATTGCATATC AATCCACCCT GAAAGGCGTG AGCAAAGTGG
3481 AAGTGAACCG TGACCATCTG CTGGATGAAC TGGATCACA CTGGGAAGTG CTGGCTGAAC
3541 CAATCCAGAC AGTTATGCGT CGCTATGGCA TCGAAAAACC GTACGAGAAG CTGAAAGAGC
3601 TGACTCGCGG TAAGCGCGTT GACGCCGAAG GCATGAAGCA GTTTATCGAT GGTCTGGCGT
3661 TGCCAGAAGA AGAGAAAAGC CGCCTGAAAG CGATGACGCC GGCTAACTAT ATTGGTTCGAG
3721 CTATACGAT GGTGTATGAG CTGAAAATAA CCTCGTATCA GTGCCGGATG GCGATGCTGT
3781 CCGGCTGCT TATTAAGATT ATCCGCTTTT TATTTTTCCT CTTTACCTCC CCTCCCGCT
3841 GGTTTATTTA ATGTTTACCC CCATAACCAC ATAATCGCGT TACACTATTT TAATAATTAA
3901 GACAGGGAGA AATAAAAATG CGCGTACTGG TTGTTGAAGA CAATGCGTTG TTACGTCACC
3961 ACCTTAAAGT TCAGATTGAG GATGCTGGTC ATCAGGTGCA TGACGCAGAA GATGCCAAAG
4021 AAGCCGATTA TTATCTCAAT GAACATATAC CGGATATTGC GATTGTCTGAT CTCGGATTGC
4081 CAGACGAGGA CCGTCTGTCA CTGATTGCGC GCTGGCGTAT CAACGATGTT TCACTGCCGA
4141 TTCTGGTATT AACC GCCCGT GAAAGTGGC AGGACAAAGT CGAAGTATTA AGTCCCGGTG
4201 CTGATGATTA TGTGACTAAA CCGTTTCATA TTGAAGAGGT GATGGCGCGA ATGCAGGCAT
4261 TAATGCGCGG TAATAGCGGT CTGGCTTCAC AGGTCAATTC GCTCCCCCGG TTTTCAGTTG
4321 ATCTCTCTCG CCGTGAATTA TCTATTAATG ACGAAGTGAT CAAACTGACC GCGTTCGAAT
4381 ACACCATAT GGAACCGTTG ATACGCAATA ATGGCAAAGT GGTCAGCAAA GATTCGTTAA
4441 TGCTCCAAT CTATCCGAT GCGGAGCTGC GGGAAAGCCA TACCATTGAT GTACTGATGG
4501 GACGTCTGCG CAAAAAATT CAGGCACAAT ATCCCAAGA AGTGATTACC ACCGTTCGCG
4561 GCCAGGGCTA TCTGTTTCGAA TTGCGCTGAT GAAAAAATTA CTGCGTCTTT TTTTCCCGCT
4621 CTCGCTGCGG GTACGTTTTT TGTGGCAAC GGCAGCGGTA GTACTGGTGC TTTTCGTTGC
4681 CTACGGAATG GTCGCGCTGA TCGGTATAG CGTCAGTTTC GATAAACTA CGTTTCGGCT
4741 GTTACGTGGC GAGAGCAATC TGTTCTATAC CCTTGCAGAG TGGGAAAAACA ATAAGTTGCA
4801 TGTCGAGTTA CCCGAAAAA TCGACAAGCA AAGCCCCAC ATGACGCTAA TTTATGATGA
4861 GAACGGGCGA CTTTTATGGG CGCAACGTGA CGTGCCCTGG CTGATGAAGA TGATCCAGCC
4921 TGACTGGCTG AAATCGAATG GTTTTCATGA AATTGAAGCG GATGTAAACG ATACCAACCT
4981 CTTGCTGAGT GGAGATCATT CGATACAGCA ACAGTTGCAG GAAGTGCGGG AAGATGATGA
5041 CGACGCGGAG ATGACCCACT CCGTGGCAGT AAACGTCTAC CCGCAACAT CGCGGATGCC
5101 AAAATTAAAC ATTGTGGTGG TGGATACCAT TCCGGTGGAG CTAAAAAGTT CCTATATGGT
5161 CTGGAGCTGG TTTATCTATG TGCTCTCAG CAATCTGCTG TTAGTGATCC CGCTGCTGTG
5221 GGTGCGCGCC TGGTGGAGTT TACGCCCATC CGAAGCCCTG GCAAAAGAG TCCGCGAATC
5281 GGAAGAACAT AACC GCGAAT TGCTCAATCC AGCCACAACG CGAGACTGA CCAGTCTGGT
5341 ACGAAACCTG AACC GATTGT TAAAAAGTGA ACGCGAACGT TACGACAAAT ACCGTACGAC
5401 GCTCACCGAC CTGACCCATA GTCTGAAAAC GCCACTGGCG GTGCTGCAAA GTACGCTGCG
5461 TTCTCTGCGT AGTGAAAAGA TGAGCGTCAG TGATGCTGAG CCGATAATGC TGGAGCAAAT
5521 CAGCCGCATT TCACAGCAAA TTGGCTACTA CCTGCATCGT GCCAGTATGC GCGGCGGGAC

5581 ATTGCTCAGC CGCGAGCTGC ATCCGGTCCG CCCACTGCTG GACAATCTCA CCTCAGCGCT
5641 GAACAAAGTG TATCAACGCA AAGGGGTCAA TATCTCTCTC GATATTTCGC CAGAGATCAG
5701 CTTTGTCCGGT GAGCAGAACG ATTTTGTCTGA GGTGATGGGC AACGTGCTGG ATAATGCCTG
5761 TAAATATTGC CTCGAGTTTG TCGAAATTCG TGCAAGGCAA ACCGACGAGC ATCTCTATAT
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5881 TGGTCAACGG GTTGATACTT TACGCCCTGG GCAAGGTGTA GGGCTGGCGG TAGCCCGCGA
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6181 ACGCCCGGTG GTGTTAAAC GCGGCTTTAA TAATTTTATT GACCCGATCT CTCCAGACGA
6241 GTTGGCGGGT CTGGCGATGG AAAGCGAAGT TGACAGTCGA CTGGTCAGTC ACCAGGATGG
6301 CAAATGGCAG GTCAGCCACG GCGCGTTCTGA AAGCTACGAT CATCTCGGTG AAACCAACTG
6361 GTCATTACTG GTACAGGCAG TGAACCACTG GCATGAGCCG ACCGCCGCGC TGATGCGACC
6421 GTTCCGTGAA CTACCGGACT GGCGTATTGA TGATCTGATG ATTTCTTTTT CTGTACCCGG
6481 CGGCGGCGTC GGCCCGCATC TCGATCAGTA CGACGTGTTT ATCATTCAGG GTACCCAGGC
6541 ATCAATAAAA ACGAAAGGCT CAGTCGGAAG ACTGGGCCTT TCGTTTTATC TGTGTTTGT
6601 CGGTGCAACG TCTCCTGAGT AGGACAAATC CGCCGGGAGC GGATTTGAAC GTTGCGAAGC
6661 AACGGCCCGG AGGGTGGCGG GCAGGACGCC CGCCATAAAC TGCCAGGCAT CAAATTAAGC
6721 AGAAGGCCAT CCTGACGGAT GGCTTTTTTG CGTTTCTACA AACTCTTCCT GTCGTCATAT
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7141 AACATGGACG TGTCAGCGGC TTTCCAGAAC TCGGACGGTT TATGCGCTGC CCACGCCGGC
7201 ATATTGCCGG ACTCCTGTG CTCAAGGTG GAGTCTTTTT CACGGGCATA CTTTCCTCA
7261 CGCGCAATAT AATCGGCATG AGGAGAGGCA CTGCCTTTTC CGCCGGTTTT TACGCTGAGA
7321 TGATAGGATG CCATCGTGTT TTATCCCGCT GAAGGGCGCA CGTTTCTGAA CGAAGTGAAG
7381 AAAGTCTAAG TCGCCCTGA TAAATAAAG AGTTATCAGG GATTGTAGT GGAATTTGACC
7441 TCCTCTGCCA TCATGAGCGT AATCATTTCCG TTAGCATTCA GGAGGTAAAC AGCATGAATA
7501 AAAGCGAAAA AACAGGAACA ATGGGCAGCA GAAAGAGTGC AGTATATTCG CGGCTTAAAG
7561 TCGCCGAATG AGCAACAGAA ACTTATGCTG ATACTGACGG ATAAAGCAGA TAAACAGCA
7621 CAGGATATCA AAACGCTGTC CCTGCTGATG AAGGTGAAC AGGCAGCAGA GAAAGCGCAG
7681 GAAGCCAGAG CGAAAGTCAT GAACCTGATA CAGGCAGAAA AGCGAGCCGA AGCCAGAGCC
7741 GCCGTAAAG CCCGTGACCA TGCTCTGTAC CAGTCTGCCG GATTGCTTAT CCTGGCGGGT
7801 CTGGTTGACA GTAAGACGGG TAAGCCTGTT GATGATACCG CTGCCTTACT GGGTGCATTA
7861 GCCAGTCTGA ATGACCTGTC ACGGGATAAT CCGAAGTGGT CAGACTGGAA AATCAGAGGG
7921 CAGGAAGTGC TGAACAGCAA AAAGTCAGAT AGCACCACAT AGCAGACCCG CCATAAAACG
7981 CCCTGAGAAG CCCGTGACGG GCTTTTCTTG TATTATGGGT AGTTTCCTTG CATGAATCCA
8041 TAAAAGGCGC CTGTAGTGCC ATTTACCCCC ATTCACTGCC AGAGCCGTGA GCGCAGCGAA
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8221 TTTGTAGAGG AGCAAACAGC GTTTGCGACA TCCTTTTGTA ATACTGCGGA ACTGACTAAA
8281 GTAGTGAGTT ATACACAGGG CTGGGATCTA TTCTTTTAT CTTTTTTTAT TCTTTCTTTA
8341 TTCTATAAAT TATAACCACT TGAATATAAA CAAAAAAAC ACACAAAGGT CTAGCGGAAT
8401 TTACAGAGGG TCTAGCAGAA TTTACAGT TTTCCAGCAA GGTCTAGCAG AATTACAGA
8461 TACCCACAAC TCAAAGGAAA AGGACTAGTA ATTATCATTG ACTAGCCCAT CTCAATTGGT
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8581 CAAATGAAC AGCGATTAGT CGCTATGACT TAACGGAGCA TGAACCAAG CTAATTTTAT
8641 GCTGTGTGGC ACTACTCAAC CCCACGATTG AAAACCCCTAC AAGGAAAGAA CGGACGGTAT
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8821 TTAAAGGCTT TGAGATTTTC CAGTGGACAA ACTATGCCAA GTTCTCAAGC GAAAAATTAG
8881 AATTAGTTTT TAGTGAAGAG ATATTGCCTT ATCTTTTCCA GTTAAAAAA TTCAAAAAAT
8941 ATAATCTGGA ACATGTTAAG TCTTTTGAAA ACAAATACTC TATGAGGATT TATGAGTGGT
9001 TATTAAAGA ACTAACACAA AAGAAAATC ACAAGGCAAA TATAGAGATT AGCCTTGATG
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9121 GGGTTTTGAA ACCAATAAGT AAAGATTTAA ACACTTACAG CAATATGAAA TTGGTGGTTG
9181 ATAAGCGAGG CCGCCGACT GATACGTTGA TTTTCCAAGT TGAAC TAGAT AGACAAATGG
9241 ATCTCGTAAC CGAACTTGAG AACAACCAGA TAAAAATGAA TGGTGACAAA ATACCAACAA
9301 CCATTACATC AGATTCCCTAC CTACATAACG GACTAAGAAA AACACTACAC GATGCTTTAA
9361 CTGCAAAAAT TCAGCTCACC AGTTTGTAGG CAAAATTTTT GAGTGACATG CAAAGTAAGT
9421 ATGATCTCAA TGGTTCGTTT TCATGGCTCA CGCAAAAAACA ACGAACCACA CTAGAGAACA
9481 TACTGGCTAA ATACGGAAGG ATCTGAGGTT CTTATGGCTC TTGTATCTAT CAGTGAAGCA
9541 TCAAGACTAA CAAACAAAAG TAGAACAACT GTTCACCGTT ACATATCAAA GGGAAAACCTG
9601 TCCATATGCA CAGATGAAAA CGGTGTAAAA AAGATAGATA CATCAGAGCT TTTACGAGTT

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9661 TTTGGTGCAT TCAAAGCTGT TCACCATGAA CAGATCGACA ATGTAACAGA TGAACAGCAT
9721 GTAACACCTA ATAGAACAGG TGAAACCAGT AAAACAAAGC AACTAGAACA TGAAATTGAA
9781 CACCTGAGAC AACTTGTTAC AGCTCAACAG TCACACATAG ACAGCCTGAA ACAGGCGATG
9841 CTGCTTATCG AATCAAAGCT GCCGACAACA CGGGAGCCAG TGACGCCTCC CGTGGGGAAA
9901 AAATCATCTC GAGGAAGATC CTTTGATCTT TTCTACGGGG TCTGACGCTC AGTGGAACGA
9961 AAATCACCCT TAAGGGATTT TGGTCATGAA CAATAAAACT GTCTGCTTAC ATAAACAGTA
10021 ATACAAGGGG TGTTATGAGC CATATTCAAC GGGAAACGTC TTGCTCTAGG CCGCGATTAA
10081 ATTCCAACAT GGATGCTGAT TTATATGGGT ATAAATGGGC TCGCGATAAT GTCGGGCAAT
10141 CAGGTGCGAC AATCTATCGA TTGTATGGGA AGCCCGATGC GCCAGAGTTG TTTCTGAAAC
10201 ATGGCAAAGG TAGCGTTGCC AATGATGTTA CAGATGAGAT GGTGAGACTA AACTGGCTGA
10261 CGGAATTTAT GCCTCTTCCG ACCATCAAGC ATTTTATCCG TACTCCTGAT GATGCATGGT
10321 TACTCACCAC TGCGATCCCC GGGAAACACG CATTCCAGGT ATTAGAAGAA TATCCTGATT
10381 CAGGTGAAAA TATTGTTGAT GCGCTGGCAG TGTTCCTGCG CCGGTTGCAT TCGATTCTTG
10441 TTTGTAATTG TCCTTTTAAAC AGCGATCGCG TATTTCTGCT CGCTCAGGCG CAATCACGAA
10501 TGAATAACGG TTTGGTTGAT GCGAGTGATT TTGATGACGA GCGTAATGGC TGGCCTGTTG
10561 AACAAGTCTG GAAAGAAATG CATAAACTTT TGCCATTCTC ACCGGATTCA GTCGTCACTC
10621 ATGGTGATTT CTCACTTGAT AACCTTATTT TTGACGAGGG GAAATTAATA GGTGTGATTG
10681 ATGTTGGACG AGTCGGAATC GCAGACCGAT ACCAGGATCT TGCCATCCTA TGGAACTGCC
10741 TCGGTGAGTT TTCTCCTTCA TTACAGAAAC GGCTTTTTTCA AAAATATGGT ATTGATAATC
10801 CTGATATGAA TAAATTGCAG TTTCATTTGA TGCTCGATGA GTTTTTCTAA GAATTAATTC
10861 ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC AAATAGGGGT TCCGCGCACA
10921 TTTCCCGGAA AAGTGCCACC TGAAATTGTA AGCGTTAATA TTTTGTAAA ATTCCGCTTA
10981 AATTTTTGTT AAATCAGCTC ATTTTTTAAAC CAATAGGCCG AAATCGGCAA AATCCCTTAT
11041 AAATCAAAG AATAGACCGA GATAGGGTTG AGTGTGTTC CAGTTTGAA CAAGAGTCCA
11101 CTATTAAAGA ACGTGGACTC CAACGTCAAA GGGCGAAAAA CCGTCTATCA GGGCGATGGC
11161 CCACTACGTG AACCATCACC CTAATCAAGT TTTTGGGGT CGAGGTGCCG TAAAGCACTA
11221 AATCGGAACC CTAAAGGGAG CCCCCGATTT AGAGCTTGAC GGGGAAAGCC GGCGAACGTG
11281 GCGAGAAAGG AAGGGAAGAA AGCGAAAGGA GCGGGCGCTA GGGCGCTGGC AAGTGTAGCG
11341 GTCACGCTGC GCGTAACCAC CACACCCGCC GCGCTTAATG CGCCGCTACA GGGCGCGTCC
11401 CATTCGCCAA TCCGGATATA GTTCCTCCTT TCAGCAAAAA ACCCCTCAAG ACCCGTTTAG
11461 AGGCCCCAAG GGGTTATGCT AGTTATTGCT CAGCGGTGGC AGCAGCCAAC TCAGGTTTCT
11521 TTCGGGCTTT GTTTAGCAGC CTAGGTATTA ATCAAGACGT CTAAGAAACC ATTATTATCA
11581 TGACATTAAC CTATAAAAAAT AGGCGTATCA CGAGGCCCTT TCGTC

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4.6.1. RT-PCR for genes involved in acetate production

The gene expression levels for genes involved in acetate production (*ackA*, *pta* and *poxB*) were measured in StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA, USA) using Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The real-time PCR conditions were carried as follows: 10 min at 95 °C, 40 cycles of 95 °C for 15 s, followed by 60 °C for 1 min. The bacterial 16S rRNA (*rrsG*) was used as the internal standard and the sequences of the primers used were provided in Table 13. The values of cycle threshold (C_t) were provided by the software, and $2^{-\Delta\Delta C_t}$ method of relative quantification was utilized to compute the relative expression level. The *p*-value for each gene was calculated by student's *t*-test.

Table 13: Primers used in RT-PCR for genes involved in acetate production

Gene	Forward sequence (5'→3')	Reverse sequence (5'→3')
<i>ackA</i>	TACGCCCTGCCTTACAACCTGT	CGGATAGCAGAAACGGAACCAC
<i>pta</i>	TGCTGAACGTGGTATCGCAACT	GGTTTCGGTCATGCCTTTGTTC
<i>poxB</i>	CAGCAAGGTGGATATGGCACTG	TATTGCGGGTGAATGGCTTTCT

Figure 19: Fold-changes in expression levels of selected genes for A2 (grey) and WT (white) when cultivated in M9 minimal medium supplemented with **(A)** 0 g/L sodium acetate and **(B)** 10 g/L sodium acetate. * $p < 0.05$, # $p < 0.01$ and ** $p < 0.001$, compared to WT using t -test (mean $\pm$ standard deviation, $n = 3$)

The relative gene expression levels of three genes (*ackA*, *pta* and *poxB*) that were responsible for acetate production in *E. coli* were determined in this study. There are two acetate production pathways in *E. coli*. In the first pathway, acetate kinase (*ackA*) and phosphotransacetylase (*pta*) would work together to convert acetyl-CoA to acetate via acetyl-phosphate. Acetate can also be produced through conversion of pyruvate to acetate, which is catalysed by

pyruvate kinase (*poxB*) (De Mey et al. 2007; Wolfe 2005). From Figure 19, it was observed that all three genes were differentially expressed between A2 and WT in both the presence and absence of sodium acetate. In 0 g/L sodium acetate, *ackA*, *pta* and *poxB* were up-regulated in A2 by 11.4-, 5.8- and 2.5-fold respectively. However, the expression of these three genes were reduced by about 3-5.5 fold when cultivated in 10 g/L sodium acetate.

4.6.2. Determination of cell morphology using scanning electron microscopy (SEM)

Morphological changes of the mutants and the control under low temperature were determined via a JSM-6700F field emission scanning electron microscope (JEOL, Japan). Cells were either grown for 16 h without acetate stress (0 g/L sodium acetate) or for 30 h under acetate stress (10 g/L sodium acetate), and harvested by centrifugation. Cell pellets were washed with cold 0.9 % NaCl solution thrice before resuspending in the same solution. Cell samples were fixed with 2 % glutaldehyde by incubating at 4 °C for 3 h. Ethanol concentrations from 10 % to 100 % (v/v) were gradually applied to the cells for dehydration. Samples were then mounted onto SEM stubs and coated with platinum at 20 mA for 60 s using a gold sputter.

Figure 20: Scanning electron micrographs of WT and MT6 in M9 minimal medium at 37 °C. (A) A2, 0 g/L sodium acetate (B) WT, 0 g/L sodium acetate (C) A2, 10 g/L sodium acetate (D) WT, 10 g/L sodium acetate

Cell morphology of A2 and WT was studied in the absence/presence of acetate using scanning electron microscope. The micrographs of A2 and WT under different stress conditions were illustrated in Figure 20. Cellular size was measured based on more than 50 cells. The average size of A2 and WT grown in 0 g/L sodium acetate was $1.56 \pm 0.36 \times 0.53 \pm 0.07 \mu\text{m}$ and $1.84 \pm 0.30 \times 0.53 \pm 0.04 \mu\text{m}$ respectively. When cultured in 10 g/L sodium acetate, both the length and the diameter of A2 were increased to $2.18 \pm 0.57 \mu\text{m}$ and $0.78 \pm 0.06 \mu\text{m}$ respectively, but the size of WT remained almost the same at $1.89 \pm 0.42 \times 0.52 \pm 0.03 \mu\text{m}$. The surface roughness of A2 and WT was almost the same in the absence or presence of acetate stress.

Table 14: Genes with >2-fold change in expression level in A2 as compared to WT in the absence of sodium acetate stress, using a *p*-value threshold less than 0.05.

b-number	Gene	Function^a	Fold-change^b	<i>p</i>-value
b2579	<i>yfiD</i>	stress-induced alternate pyruvate formate-lyase subunit	17.682	4.96E-04
b0903	<i>pflB</i>	pyruvate formate-lyase (inactive)	15.511	3.01E-04
b0904	<i>focA</i>	FocA formate transporter	6.872	7.16E-08
b3172	<i>argG</i>	argininosuccinate synthase	5.528	6.10E-09
b3870	<i>glnA</i>	glutamine synthetase	5.089	4.61E-05
b1272	<i>sohB</i>	predicted inner membrane peptidase	5.068	2.94E-03
b3868	<i>glnG</i>	NtrC transcriptional dual regulator	5.053	1.31E-05
b3721	<i>bglB</i>	6-phospho- β -glucosidase B	4.383	3.61E-06
b3366	<i>nirD</i>	nitrite reductase, small subunit	4.338	1.16E-10
b3906	<i>rhaR</i>	RhaR transcriptional activator	4.236	8.55E-06
b1593	<i>ynfK</i>	predicted dethiobiotin synthetase	4.026	3.89E-06
b3902	<i>rhaD</i>	rhamnulose-1-phosphate aldolase	4.022	1.08E-05
b4197	<i>ulaE</i>	L-xylulose 5-phosphate 3-epimerase	3.991	1.53E-05
b4033	<i>malF</i>	maltose ABC transporter - membrane subunit	3.898	1.81E-05
b1779	<i>gapA</i>	glyceraldehyde 3-phosphate dehydrogenase-A complex	3.876	4.56E-08
b3452	<i>ugpA</i>	glycerol-3-phosphate/glycerol-2-phosphate ABC transporter - putative membrane subunit	3.872	2.34E-07
b3571	<i>malS</i>	α -amylase	3.784	2.98E-06
b3413	<i>gntX</i>	protein involved in utilization of DNA as a carbon source	3.759	2.88E-06
b3221	<i>yhcH</i>	conserved protein	3.728	6.27E-06
b4122	<i>fumB</i>	fumarase B	3.662	7.64E-07
b2241	<i>glpA</i>	glycerol-3-phosphate dehydrogenase (anaerobic), large subunit	3.598	4.14E-05
b3076	<i>ebgA</i>	evolved β -D-galactosidase, α subunit	3.570	5.47E-06
b3451	<i>ugpE</i>	glycerol-3-phosphate/glycerol-2-phosphate ABC transporter - putative membrane subunit	3.566	4.18E-07
b2469	<i>narQ</i>	NarQ sensory histidine kinase	3.554	2.10E-03
b0063	<i>araB</i>	L-ribulokinase	3.542	2.79E-06
b3565	<i>xylA</i>	xylose isomerase	3.537	4.84E-08
b3367	<i>nirC</i>	NirC nitrite FNT transporter	3.536	1.82E-04
b4194	<i>ulaB</i>	L-ascorbate-specific enzyme IIB component of PTS	3.534	1.75E-06
b3365	<i>nirB</i>	dimer of large subunit of nitrite reductase	3.532	5.19E-07
b3575	<i>yiaK</i>	2,3-diketo-L-gulonate reductase	3.524	4.32E-07
b3512	<i>gadE</i>	GadE DNA-binding transcriptional activator	3.523	1.14E-05
b2488	<i>hyfH</i>	hydrogenase 4, component H	3.475	2.12E-04
b3904	<i>rhaB</i>	L-rhamnulose kinase	3.471	1.38E-04
b2702	<i>srlA</i>	glucitol/sorbitol-specific enzyme IIC component of PTS	3.454	3.13E-03
b0037	<i>caiC</i>	carnitine-CoA ligase	3.438	1.01E-02
b1388	<i>paaA</i>	ring 1,2-phenylacetyl-CoA epoxidase, monooxygenase subunit	3.437	1.88E-05

b4035	<i>malK</i>	maltose ABC transporter - ATP binding subunit	3.426	3.02E-05
b4139	<i>aspA</i>	aspartate ammonia-lyase	3.425	1.78E-05
b3395	<i>hofM</i>	protein involved in utilization of DNA as a carbon source	3.400	1.27E-05
b2242	<i>glpB</i>	glycerol-3-phosphate dehydrogenase (anaerobic), membrane anchor subunit	3.398	4.35E-07
b3225	<i>nanA</i>	N-acetylneuraminate lyase	3.394	1.66E-04
b3564	<i>xylB</i>	xylulokinase	3.388	1.60E-04
b4037	<i>malM</i>	maltose regulon periplasmic protein	3.363	1.61E-04
b3241	<i>aaeA</i>	AaeAB Hydroxylated, Aromatic Carboxylic Acid Efflux Transport System Protein A	3.354	9.66E-06
b4266	<i>idnO</i>	5-keto-D-gluconate 5-reductase	3.349	1.44E-05
b3093	<i>exuT</i>	ExuT hexuronate MFS transporter	3.348	5.44E-06
b2802	<i>fucI</i>	L-fucose isomerase	3.308	1.10E-06
b4004	<i>zraR</i>	ZraR transcriptional activator	3.307	7.21E-06
b3708	<i>tnaA</i>	L-cysteine desulphydrase / tryptophanase	3.306	1.98E-04
b4288	<i>fecD</i>	ferric dicitrate ABC transporter - membrane subunit	3.305	3.19E-03
b0106	<i>hofC</i>	protein transport protein HofC	3.298	3.00E-03
b3569	<i>xylR</i>	XylR transcriptional activator	3.274	3.88E-05
b1622	<i>malY</i>	bifunctional β -cystathionase, PLP-dependent and regulator of maltose regulon	3.272	1.39E-03
b3925	<i>glpX</i>	fructose 1,6-bisphosphatase II	3.269	1.13E-05
b2482	<i>hyfB</i>	hydrogenase 4, component B	3.264	2.91E-03
b4032	<i>malG</i>	maltose ABC transporter - membrane subunit	3.263	2.78E-04
b3574	<i>yiaJ</i>	YiaJ DNA-binding transcriptional repressor	3.259	7.38E-06
b2535	<i>csiE</i>	stationary phase inducible protein	3.238	7.67E-08
b1521	<i>uxaB</i>	altronate oxidoreductase	3.236	1.02E-03
b4291	<i>fecA</i>	outer membrane receptor; citrate-dependent iron transport, outer membrane receptor	3.220	8.89E-04
b4123	<i>dcuB</i>	DcuB dicarboxylate Dcu transporter	3.215	2.07E-04
b3514	<i>mdtF</i>	MdtEF-TolC multidrug efflux transport system - permease subunit	3.170	2.92E-06
b3405	<i>ompR</i>	DNA-binding response regulator in two-component regulatory system with EnvZ	3.169	6.02E-05
b4324	<i>uxuR</i>	UxuR DNA-binding transcriptional repressor	3.159	1.13E-05
b3588	<i>aldB</i>	acetaldehyde dehydrogenase	3.143	3.63E-05
b3583	<i>sgbE</i>	L-ribulose-5-phosphate 4-epimerase	3.131	1.21E-06
b3566	<i>xylF</i>	xylose ABC transporter - periplasmic binding protein	3.130	2.40E-06
b3831	<i>udp</i>	uridine phosphorylase	3.107	2.32E-05
b3666	<i>uhpT</i>	UhpT-hexose phosphate MFS transporter	3.101	3.52E-06
b4068	<i>yjcH</i>	conserved inner membrane protein	3.098	1.30E-06
b3115	<i>tdcD</i>	propionate kinase	3.090	1.12E-03
b3430	<i>glgC</i>	glucose-1-phosphate adenylyltransferase	3.073	8.72E-10
b2483	<i>hyfC</i>	hydrogenase 4, component C	3.059	2.60E-05
b0330	<i>prpR</i>	PrpR DNA-binding transcriptional dual regulator	3.059	2.16E-02
b2926	<i>pgk</i>	phosphoglycerate kinase	3.054	6.29E-07
b3580	<i>lyxK</i>	L-xylulose kinase	3.046	2.75E-03
b3240	<i>aaeB</i>	AaeAB Hydroxylated, Aromatic Carboxylic Acid Efflux Transport System Protein B	3.038	4.68E-07
b3723	<i>bglG</i>	BglG transcriptional antiterminator	3.036	3.71E-04

b2801	<i>fucP</i>	FucP fucose MFS transporter	3.033	9.96E-06
b0107	<i>hofB</i>	protein involved in plasmid replication	3.033	5.97E-06
b3428	<i>glgP</i>	glycogen phosphorylase	2.999	1.55E-04
b0062	<i>araA</i>	L-arabinose isomerase	2.994	3.61E-04
b4323	<i>uxuB</i>	D-mannonate oxidoreductase	2.994	3.22E-04
b1901	<i>araF</i>	arabinose ABC transporter - periplasmic binding protein	2.990	1.92E-04
b4264	<i>idnR</i>	IdnR-5-ketogluconate DNA-binding transcriptional dual regulator	2.979	2.32E-06
b3135	<i>agaA</i>	predicted truncated N-acetylgalactosamine-6-phosphate deacetylase	2.973	5.66E-08
b3224	<i>nanT</i>	NanT sialic acid MFS transporter	2.969	3.63E-06
b2805	<i>fucR</i>	FucR transcriptional activator	2.965	9.43E-07
b4003	<i>zraS</i>	ZraS sensory histidine kinase	2.960	3.96E-05
b4119	<i>melA</i>	α -galactosidase	2.957	1.25E-04
b2660	<i>lhgO</i>	L-2-hydroxyglutarate oxidase	2.955	3.83E-06
b3577	<i>yiaM</i>	predicted transporter	2.949	9.72E-05
b4382	<i>deoA</i>	thymidine phosphorylase	2.929	1.27E-03
b3418	<i>malT</i>	MalT transcriptional activator	2.917	1.61E-06
b0110	<i>ampD</i>	N-acetyl-anhydromuramyl-L-alanine-amidase	2.913	2.97E-05
b3567	<i>xylG</i>	xylose ABC transporter - ATP binding subunit	2.876	2.94E-05
b1182	<i>hlyE</i>	hemolysin E	2.872	5.76E-03
b0039	<i>caiA</i>	crotonobetainyl-CoA reductase	2.852	4.66E-03
b2614	<i>grpE</i>	phage lambda replication; host DNA synthesis; heat shock protein; protein repair	2.851	8.35E-06
b4067	<i>actP</i>	acetate / glycolate transporter	2.845	2.39E-04
b3599	<i>mtlA</i>	mannitol PTS permease	2.830	8.51E-06
b3081	<i>fadH</i>	2,4-dienoyl-CoA reductase	2.815	3.55E-05
b4240	<i>treB</i>	fused trehalose(maltose)-specific PTS enzyme: IIB component/IIC component	2.803	1.71E-04
b4138	<i>dcuA</i>	DcuA dicarboxylate Dcu transporter	2.801	8.58E-04
b4287	<i>fecE</i>	ferric dicitrate ABC transporter - ATP binding subunit	2.788	7.01E-06
b4196	<i>ulaD</i>	3-keto-L-gulonate 6-phosphate decarboxylase	2.786	3.64E-06
b2092	<i>gatC</i>	galactitol-specific enzyme IIC component of PTS	2.782	9.45E-05
b1392	<i>paaE</i>	ring 1,2-phenylacetyl-CoA epoxidase, reductase subunit	2.779	4.86E-05
b3423	<i>glpR</i>	GlpR DNA-binding transcriptional repressor	2.777	7.26E-06
b4120	<i>melB</i>	MelB GPH transporter	2.767	2.74E-04
b1492	<i>gadC</i>	GadC GABA APC transporter	2.765	1.28E-03
b4198	<i>ulaF</i>	L-ribulose 5-phosphate 4-epimerase	2.752	8.77E-04
b3091	<i>uxaA</i>	D-altronate dehydratase	2.752	1.62E-03
b0190	<i>yaeQ</i>	conserved protein	2.749	4.84E-06
b4265	<i>idnT</i>	L-idonate / 5-ketogluconate / gluconate transporter	2.741	3.23E-04
b3077	<i>ebgC</i>	evolved β -D-galactosidase, β subunit	2.738	2.95E-07
b2943	<i>galP</i>	GalP - galactose MFS transporter	2.734	9.84E-07
b3404	<i>envZ</i>	EnvZ sensory histidine kinase	2.727	8.89E-04
b0061	<i>araD</i>	L-ribulose 5-phosphate 4-epimerase	2.724	2.36E-08
b2663	<i>gabP</i>	GabP APC transporter	2.723	6.31E-06
b3709	<i>tnaB</i>	TnaB tryptophan ArAAP transporter	2.720	2.73E-07
b3907	<i>rhaT</i>	rhamnose RhaT transporter	2.716	8.68E-05

b1384	<i>feaR</i>	FeaR DNA-binding transcriptional activator	2.712	7.91E-05
b2799	<i>fucO</i>	L-1,2-propanediol oxidoreductase	2.710	9.63E-05
b1517	<i>lsrF</i>	predicted class I aldolase	2.708	6.78E-05
b0343	<i>lacY</i>	LacY lactose MFS transporter	2.708	3.55E-07
b4321	<i>gntP</i>	GntP Gluconate Gnt transporter	2.705	3.84E-05
b3392	<i>hofP</i>	protein involved in utilization of DNA as a carbon source	2.695	4.83E-05
b4233	<i>mpl</i>	UDP-N-acetylmuramate:L-alanyl- γ -D-glutamyl-meso-diaminopimelate ligase	2.693	3.94E-04
b3513	<i>mdtE</i>	MdtEF-TolC multidrug efflux transport system - membrane fusion protein	2.691	8.96E-07
b3368	<i>cysG</i>	uroporphyrin III C-methyltransferase [multifunctional]	2.691	4.31E-06
b1389	<i>paaB</i>	ring 1,2-phenylacetyl-CoA epoxidase subunit	2.682	1.72E-02
b2957	<i>ansB</i>	asparaginase II	2.682	6.43E-06
b3214	<i>glfF</i>	periplasmic protein	2.676	2.63E-06
b1391	<i>paaD</i>	phenylacetate degradation protein	2.676	1.72E-02
b2468	<i>aegA</i>	putative oxidoreductase, Fe-S subunit	2.666	2.89E-03
b3600	<i>mtlD</i>	mannitol-1-phosphate 5-dehydrogenase	2.663	9.36E-06
b4213	<i>cpdB</i>	2',3'-cyclic nucleotide 2'-phosphodiesterase / 3'-nucleotidase	2.662	2.86E-03
b4193	<i>ulaA</i>	L-ascorbate-specific enzyme IIC component of PTS	2.657	1.75E-03
b0344	<i>lacZ</i>	β -galactosidase	2.643	2.18E-05
b2841	<i>araE</i>	AraE arabinose MFS transporter	2.642	2.29E-05
b3722	<i>bglF</i>	β -glucoside PTS permease	2.636	7.96E-05
b3601	<i>mtlR</i>	MtlR DNA-binding transcriptional repressor	2.631	4.56E-04
b3133	<i>agaV</i>	PTS system, cytoplasmic, N-acetylgalactosamine-specific IIB component 2 (EIIB-AGA)	2.631	1.33E-03
b3517	<i>gadA</i>	glutamate decarboxylase A	2.623	2.85E-06
b2481	<i>hyfA</i>	hydrogenase 4, component A	2.621	2.00E-05
b3528	<i>dctA</i>	DctA dicarboxylate DAACS transporter	2.612	1.98E-04
b4381	<i>deoC</i>	2-deoxyribose-5-phosphate aldolase, NAD(P)-linked	2.611	1.19E-04
b4384	<i>deoD</i>	purine-nucleoside phosphorylase	2.605	1.39E-04
b3869	<i>glnL</i>	NtrB sensory histidine kinase	2.603	2.67E-05
b2708	<i>gutQ</i>	D-arabinose 5-phosphate isomerase	2.593	8.20E-05
b1615	<i>uidC</i>	membrane-associated protein	2.573	3.93E-05
b2009	<i>sbmC</i>	DNA gyrase inhibitor	2.562	2.98E-06
b2803	<i>fucK</i>	L-fuculokinase	2.560	3.27E-07
b2925	<i>fbaA</i>	fructose bisphosphate aldolase class II	2.556	3.33E-08
b0342	<i>lacA</i>	galactoside O-acetyltransferase	2.543	3.30E-05
b2800	<i>fucA</i>	L-fuculose-phosphate aldolase	2.542	3.14E-07
b1390	<i>paaC</i>	ring 1,2-phenylacetyl-CoA epoxidase, structural subunit	2.529	3.93E-03
b3092	<i>uxaC</i>	D-glucuronate isomerase / D-galacturonate isomerase	2.524	9.98E-05
b4195	<i>ulaC</i>	L-ascorbate-specific enzyme IIA component of PTS	2.519	5.66E-05
b1656	<i>sodB</i>	superoxide dismutase (Fe)	2.518	4.38E-07
b2487	<i>hyfG</i>	hydrogenase 4, large subunit	2.516	3.96E-04
b2243	<i>glpC</i>	glycerol-3-phosphate dehydrogenase (anaerobic),	2.516	5.63E-05

		small subunit		
b4118	<i>melR</i>	MeIR DNA-binding transcriptional dual regulator	2.514	6.03E-05
b3113	<i>tdcF</i>	predicted L-PSP (mRNA) endoribonuclease	2.508	1.17E-04
b2707	<i>srlR</i>	GutR DNA-binding transcriptional repressor	2.506	2.54E-07
b2715	<i>ascF</i>	β -glucoside PTS permease	2.489	6.92E-05
b4036	<i>lamB</i>	phage lambda receptor protein; maltose high-affinity receptor	2.476	4.51E-04
b3117	<i>tdcB</i>	catabolic threonine dehydratase	2.475	5.64E-06
b2147	<i>preA</i>	NADH-dependent dihydropyrimidine dehydrogenase subunit	2.471	1.03E-03
b2964	<i>nupG</i>	NupG nucleoside MFS transporter	2.464	2.50E-04
b0349	<i>mhpC</i>	2-hydroxy-6-ketono-2,4-dienedioate hydrolase	2.464	2.54E-04
b3114	<i>tdcE</i>	2-ketobutyrate formate-lyase/pyruvate formate-lyase 4, inactive	2.461	4.16E-05
b1805	<i>fadD</i>	fatty acyl-CoA synthetase	2.461	1.96E-05
b1530	<i>marR</i>	MarR DNA-binding transcriptional repressor	2.456	4.23E-03
b3393	<i>hofO</i>	protein involved in utilization of DNA as a carbon source	2.455	3.22E-05
b3905	<i>rhaS</i>	RhaS transcriptional activator	2.447	1.33E-04
b1395	<i>paaH</i>	3-hydroxyadipyl-CoA dehydrogenase (NAD ⁺)	2.445	5.77E-05
b1514	<i>lsrC</i>	AI-2 ABC transporter - membrane subunit	2.442	3.69E-03
b1516	<i>lsrB</i>	AI-2 ABC transporter - periplasmic binding protein	2.428	2.09E-03
b0108	<i>ppdD</i>	prepilin peptidase dependent protein	2.428	3.16E-07
b1738	<i>chbB</i>	N,N'-diacetylchitobiose-specific enzyme IIB component of PTS	2.425	4.21E-05
b2485	<i>hyfE</i>	hydrogenase 4, component E	2.423	4.39E-03
b2091	<i>gatD</i>	galactitol-1-phosphate dehydrogenase	2.418	1.95E-03
b1900	<i>araG</i>	arabinose ABC transporter - ATP binding subunit	2.413	2.48E-02
b1002	<i>agp</i>	3-phytase / glucose-1-phosphatase	2.410	1.58E-04
b3243	<i>aaeR</i>	quorum sensing transcriptional regulator of LYSR-type	2.410	8.18E-05
b2491	<i>hyfR</i>	HyfR DNA-binding transcriptional activator	2.401	3.62E-07
b3222	<i>nanK</i>	N-acetylmannosamine kinase	2.400	1.76E-05
b1518	<i>lsrG</i>	Autoinducer 2-degrading protein	2.397	1.03E-05
b3429	<i>glgA</i>	glycogen synthase	2.387	4.20E-05
b0677	<i>nagA</i>	N-acetylglucosamine-6-phosphate deacetylase	2.384	2.28E-05
b3903	<i>rhaA</i>	L-rhamnose isomerase	2.381	1.94E-04
b0765	<i>modC</i>	molybdate ABC transporter - ATP binding subunit	2.380	4.89E-06
b3578	<i>yiaN</i>	L-dehydroascorbate transporter	2.379	1.91E-04
b4239	<i>treC</i>	trehalose-6-phosphate hydrolase	2.377	3.26E-05
b0679	<i>nagE</i>	N-acetylglucosamine PTS permease	2.371	8.52E-03
b0064	<i>araC</i>	AraC DNA-binding transcriptional dual regulator	2.370	2.03E-05
b1421	<i>trg</i>	methyl-accepting chemotaxis protein III, ribose and galactose sensor receptor	2.364	8.70E-03
b2492	<i>focB</i>	FocB formate FNT transporter	2.363	3.79E-03
b4268	<i>idnK</i>	D-gluconate kinase, thermosensitive	2.360	8.58E-06
b0111	<i>ampE</i>	predicted inner membrane protein	2.354	5.92E-04
b1736	<i>chbA</i>	N,N'-diacetylchitobiose-specific enzyme IIA component of PTS	2.351	5.74E-05
b1387	<i>paaZ</i>	oxepin-CoA hydrolase/3-oxo-5,6-dehydrosueryl-	2.351	6.31E-03

		CoA semialdehyde dehydrogenase		
b4383	<i>deoB</i>	phosphopentomutase	2.348	2.12E-04
b4124	<i>dcuR</i>	DcuR transcriptional activator	2.330	1.36E-05
b1519	<i>tam</i>	trans-aconitate methyltransferase	2.329	2.75E-04
b0043	<i>fixC</i>	flavoprotein (electron transport), possibly involved in anaerobic carnitine metabolism	2.320	4.79E-03
b2705	<i>srlD</i>	sorbitol-6-phosphate dehydrogenase	2.317	1.34E-03
b4267	<i>idnD</i>	L-idonate 5-dehydrogenase	2.316	5.59E-04
b3424	<i>glpG</i>	intramembrane serine protease GlpG	2.303	1.35E-06
b1617	<i>uidA</i>	β -D-glucuronidase	2.303	6.06E-05
b2703	<i>srlE</i>	glucitol/sorbitol-specific enzyme IIB component of PTS	2.297	2.29E-06
b0113	<i>pdhR</i>	PdhR DNA-binding transcriptional dual regulator	2.295	1.23E-06
b2965	<i>speC</i>	ornithine decarboxylase, biosynthetic	2.293	1.90E-05
b1493	<i>gadB</i>	glutamate decarboxylase B	2.293	5.20E-07
b3934	<i>cytR</i>	CytR DNA-binding transcriptional repressor	2.285	3.14E-04
b2486	<i>hyfF</i>	hydrogenase 4, component F	2.285	2.50E-05
b3167	<i>rbfA</i>	30S ribosome binding factor	2.282	4.81E-05
b2146	<i>preT</i>	NADH-dependent dihydropyrimidine dehydrogenase subunit	2.281	9.08E-04
b4476	<i>gntU</i>	GntU gluconate Gnt transporter	2.271	2.17E-07
b0191	<i>yaeJ</i>	peptidyl-tRNA hydrolase, ribosome rescue factor	2.270	2.02E-04
b1039	<i>csgE</i>	curli production assembly/transport component	2.269	4.22E-03
b1205	<i>ychH</i>	stress-induced protein	2.265	1.95E-05
b0035	<i>caiE</i>	predicted acyl transferase	2.261	1.20E-07
b1737	<i>chbC</i>	N,N'-diacetylchitobiose-specific enzyme IIC component of PTS	2.256	5.96E-06
b0763	<i>modA</i>	molybdate ABC transporter - periplasmic binding protein	2.254	1.60E-05
b3394	<i>hofN</i>	protein involved in utilization of DNA as a carbon source	2.250	4.45E-04
b1190	<i>dadX</i>	alanine racemase 2, PLP-binding	2.247	3.97E-05
b1513	<i>lsrA</i>	AI-2 ABC transporter - ATP binding subunit	2.246	4.77E-04
b1393	<i>paaF</i>	predicted 2,3-dehydroadipyl-CoA hydratase	2.244	1.72E-03
b4311	<i>nanC</i>	N-acetylneuraminic acid outer membrane channel	2.240	1.20E-03
b3582	<i>sgbU</i>	predicted L-xylulose 5-phosphate 3-epimerase	2.240	4.21E-04
b3072	<i>aer</i>	aerotaxis sensor receptor, flavoprotein	2.234	4.88E-05
b3415	<i>gntT</i>	GntT Gluconate Gnt transporter	2.232	1.89E-05
b3568	<i>xylH</i>	xylose ABC transporter - membrane subunit	2.226	7.86E-06
b3753	<i>rbsR</i>	RbsR DNA-binding transcriptional repressor	2.226	2.95E-05
b4034	<i>malE</i>	maltose ABC transporter - periplasmic binding protein	2.221	1.29E-03
b4322	<i>uxuA</i>	D-mannonate dehydratase	2.220	1.14E-02
b1620	<i>malI</i>	MalI DNA-binding transcriptional repressor	2.220	4.60E-04
b3516	<i>gadX</i>	GadX DNA-binding transcriptional dual regulator	2.215	4.24E-04
b2365	<i>dsdX</i>	DsdX Gnt transporter	2.214	1.02E-02
b0396	<i>araJ</i>	putative arabinose efflux transporter	2.212	2.55E-04
b0146,	<i>sfsA</i>	predicted DNA-binding transcriptional regulator of maltose metabolism	2.212	2.01E-06
b1761	<i>gdhA</i>	glutamate dehydrogenase	2.205	1.94E-05
b0352	<i>mhpE</i>	4-hydroxy-2-ketovalerate aldolase	2.202	3.50E-04
b1038	<i>csgF</i>	curli assembly component	2.193	5.78E-05

b2149	<i>mglA</i>	galactose ABC transporter - ATP binding subunit	2.189	2.95E-05
b0040	<i>caiT</i>	L-carnitine/ γ -butyrobetaine antiport	2.188	7.29E-06
b3134	<i>agaW</i>	PTS system N-acetylgalactosamine-specific IIC component 2	2.185	2.85E-04
b1819	<i>manZ</i>	mannose PTS permease - ManZ subunit	2.181	3.27E-04
b1891	<i>flhC</i>	DNA-binding transcriptional dual regulator with FlhD	2.179	7.44E-03
b3118	<i>tdcA</i>	TdcA DNA-binding transcriptional activator	2.179	1.54E-03
b1818	<i>manY</i>	mannose PTS permease - ManY subunit	2.175	2.53E-03
b3453	<i>ugpB</i>	glycerol-3-phosphate/glycerol-2-phosphate ABC transporter - putative periplasmic binding protein	2.170	3.25E-05
b1512	<i>lsrR</i>	LsrR DNA-binding transcriptional repressor	2.167	1.69E-04
b1616	<i>uidB</i>	UidB glucuronides GPH transporter	2.164	1.96E-02
b4460	<i>araH</i>	arabinose ABC transporter - membrane subunit	2.161	3.14E-03
b2508	<i>guaB</i>	IMP dehydrogenase	2.155	4.44E-04
b3450	<i>ugpC</i>	glycerol-3-phosphate/glycerol-2-phosphate ABC transporter - putative ATP binding subunit	2.153	3.47E-04
b3356	<i>yhfA</i>	conserved protein	2.150	6.48E-07
b3260	<i>dusB</i>	tRNA dihydrouridine synthase	2.140	9.67E-04
b4471	<i>tdcG</i>	L-serine deaminase III	2.134	2.77E-05
b3576	<i>yiaL</i>	conserved protein	2.119	9.52E-04
b1396	<i>paal</i>	hydroxyphenylacetyl-CoA thioesterase	2.119	9.91E-03
b0348	<i>mhpB</i>	2,3-dihydroxyphenylpropionate 1,2-dioxygenase	2.114	1.74E-05
b1015	<i>putP</i>	proline:sodium symporter	2.108	1.24E-04
b3579	<i>yiaO</i>	L-dehydroascorbate transporter, periplasmic binding protein	2.106	4.23E-05
b2804	<i>fucU</i>	L-fucose mutarotase	2.104	3.52E-06
b2716	<i>ascB</i>	6-phospho- β -glucosidase	2.093	6.75E-05
b2489	<i>hyfI</i>	hydrogenase 4, small subunit	2.088	1.78E-04
b3116	<i>tdcC</i>	TdcC threonine STP transporter	2.084	6.94E-05
b0678	<i>nagB</i>	glucosamine-6-phosphate deaminase	2.077	1.83E-04
b1734	<i>chbF</i>	diacetylchitobiose-6-phosphate hydrolase	2.073	6.20E-05
b4310	<i>nanM</i>	N-acetylneuraminate mutarotase	2.073	2.54E-04
b2231	<i>gyrA</i>	DNA gyrase, subunit A	2.071	3.46E-04
b2659	<i>csiD</i>	predicted protein	2.070	2.04E-04
b2148	<i>mglC</i>	galactose ABC transporter - membrane subunit	2.057	1.86E-03
b2706	<i>gutM</i>	GutM DNA-binding transcriptional activator	2.057	9.45E-06
b2661	<i>gabD</i>	succinate semialdehyde dehydrogenase, NADP ⁺ -dependent	2.048	6.65E-03
b1511	<i>lsrK</i>	autoinducer-2 kinase	2.045	3.64E-06
b1394	<i>paaG</i>	predicted ring 1,2-epoxyphenylacetyl-CoA isomerase (oxepin-CoA forming)	2.043	4.99E-03
b1735	<i>chbR</i>	ChbR DNA-binding transcriptional dual regulator	2.040	1.52E-02
b3749	<i>rbsA</i>	ribose ABC transporter - putative ATP binding subunit	2.038	2.86E-03
b3132	<i>kbaZ</i>	tagatose 6-phosphate aldolase 1, kbaZ subunit	2.031	2.31E-06
b1892	<i>flhD</i>	DNA-binding transcriptional dual regulator with FlhC	2.024	1.02E-05
b4290	<i>fecB</i>	ferric dicitrate ABC transporter - periplasmic binding protein	2.010	1.60E-03
b2171	<i>yeiP</i>	predicted dehydrogenase, NAD-dependent	2.005	6.33E-04
b3581	<i>sgbH</i>	3-keto-L-gulonate 6-phosphate decarboxylase	2.004	1.27E-03

b4016	<i>aceK</i>	isocitrate dehydrogenase phosphatase / isocitrate dehydrogenase kinase	2.000	7.03E-05
b4015	<i>aceA</i>	isocitrate lyase	0.491	1.22E-02
b3236	<i>mdh</i>	malate dehydrogenase	0.458	5.09E-04
b4014	<i>aceB</i>	malate synthase A	0.377	6.60E-03
b0729	<i>sucD</i>	succinyl-CoA synthetase, α subunit	0.368	2.32E-05
b0118	<i>acnB</i>	bifunctional aconitate hydratase 2 and 2-methylisocitrate dehydratase	0.314	4.55E-08
b0727	<i>sucB</i>	dihydrolipoyltranssuccinylase	0.292	1.23E-07
b0722	<i>sdhD</i>	succinate dehydrogenase membrane protein	0.264	1.02E-04
b0721	<i>sdhC</i>	succinate dehydrogenase membrane protein	0.261	9.56E-08
b0723	<i>sdhA</i>	succinate dehydrogenase flavoprotein	0.260	3.33E-05
b0929	<i>ompF</i>	outer membrane porin F	0.252	5.50E-07
b0728	<i>sucC</i>	succinyl-CoA synthetase, β subunit	0.232	2.04E-06
b0720	<i>gltA</i>	citrate synthase	0.203	4.21E-10
b0726	<i>sucA</i>	2-oxoglutarate decarboxylase, thiamin-requiring	0.196	1.74E-08
b0759	<i>galE</i>	UDP-glucose 4-epimerase	0.007	4.30E-02

^aFrom the EcoCyc database (<http://ecocyc.org>)

^bFold-change in gene expression between A2 and WT (average of duplicate experiments)

Table 15: Genes with >2-fold change in expression level in A2 as compared to WT in the presence of sodium acetate stress, using a *p*-value threshold less than 0.05.

b-number	Gene	Function^a	Fold-change	<i>p</i>-value
b0759	<i>galE</i>	UDP-glucose 4-epimerase	14.296	3.04E-03
b0903	<i>pflB</i>	pyruvate formate-lyase (inactive)	4.405	5.38E-09
b3517	<i>gadA</i>	glutamate decarboxylase A	3.128	1.72E-05
b2925	<i>fbaA</i>	fructose bisphosphate aldolase class II	2.117	1.30E-06
b2579	<i>yfiD</i>	stress-induced alternate pyruvate formate-lyase subunit	2.067	5.65E-05
b3870	<i>glnA</i>	glutamine synthetase	2.016	1.49E-04
b3430	<i>glgC</i>	glucose-1-phosphate adenylyltransferase	0.493	6.10E-04
b2093	<i>gatB</i>	galactitol-specific enzyme IIB component of PTS	0.478	8.62E-03
b3461	<i>rpoH</i>	RNA polymerase, sigma 32 (sigma H) factor	0.474	5.24E-03
b0931	<i>pncB</i>	nicotinate phosphoribosyltransferase	0.469	1.90E-07
b0904	<i>focA</i>	FocA formate transporter	0.465	1.04E-05
b3168	<i>infB</i>	protein chain initiation factor IF-2	0.461	3.86E-03
b0907	<i>serC</i>	phosphohydroxythreonine aminotransferase / 3-phosphoserine aminotransferase	0.447	2.93E-06
b3512	<i>gadE</i>	GadE DNA-binding transcriptional activator	0.427	1.62E-02
b2094	<i>gatA</i>	galactitol-specific enzyme IIA component of PTS	0.402	3.03E-03
b3172	<i>argG</i>	argininosuccinate synthase	0.397	1.55E-03
b4384	<i>deoD</i>	purine-nucleoside phosphorylase	0.391	2.52E-02
b3165	<i>rpsO</i>	30S ribosomal subunit protein S15	0.389	5.33E-03
b4376	<i>osmY</i>	periplasmic protein	0.388	3.38E-02
b2231	<i>gyrA</i>	DNA gyrase, subunit A	0.380	9.05E-06
b3670	<i>ilvN</i>	acetolactate synthase I, small subunit	0.358	3.08E-02
b1818	<i>manY</i>	mannose PTS permease - ManY subunit	0.351	1.48E-05
b3357	<i>crp</i>	CRP transcriptional dual regulator	0.344	5.87E-03
b2417	<i>crr</i>	glucose-specific enzyme IIA component of PTS	0.341	6.90E-06
b3236	<i>mdh</i>	malate dehydrogenase	0.333	1.25E-02
b3166	<i>truB</i>	tRNA pseudouridine 55 synthase	0.331	7.95E-03
b3429	<i>glgA</i>	glycogen synthase	0.325	2.26E-03
b3363	<i>ppiA</i>	peptidyl-prolyl cis-trans isomerase A (rotamase A)	0.323	5.87E-03
b3405	<i>ompR</i>	DNA-binding response regulator in two-component regulatory system with EnvZ	0.322	1.55E-03
b3671	<i>ilvB</i>	acetolactate synthase I, large subunit	0.320	1.55E-02
b4381	<i>deoC</i>	2-deoxyribose-5-phosphate aldolase, NAD(P)-linked	0.319	3.66E-04
b3868	<i>glnG</i>	NtrC transcriptional dual regulator	0.318	2.49E-03
b0683	<i>fur</i>	DNA-binding transcriptional dual regulator of siderophore biosynthesis and transport	0.307	6.88E-04
b0819	<i>ybiS</i>	L,D-transpeptidase YbiS	0.298	2.08E-04
b3414	<i>nfuA</i>	iron-sulfur cluster scaffold protein	0.294	1.20E-03
b3600	<i>mtlD</i>	mannitol-1-phosphate 5-dehydrogenase	0.287	2.12E-04
b1817	<i>manX</i>	mannose PTS permease - ManX subunit	0.285	1.02E-03

b3781	<i>trxA</i>	thioredoxin 1	0.281	1.31E-02
b2741	<i>rpoS</i>	RNA polymerase, sigma S (sigma 38) factor	0.280	1.37E-05
b2507	<i>guaA</i>	GMP synthetase	0.276	8.25E-04
b0115	<i>aceF</i>	pyruvate dehydrogenase, dihydrolipoyltransacetylase component E2	0.276	8.76E-10
b3449	<i>ugpQ</i>	glycerophosphodiester phosphodiesterase, cytosolic	0.272	1.07E-03
b0594	<i>entE</i>	2,3-dihydroxybenzoate-holo-EntB ligase [multifunctional]	0.269	1.69E-03
b0125	<i>hpt</i>	hypoxanthine phosphoribosyltransferase	0.268	5.07E-09
b3167	<i>rbfA</i>	30S ribosome binding factor	0.257	2.72E-04
b2508	<i>guaB</i>	IMP dehydrogenase	0.251	1.11E-04
b3514	<i>mdtF</i>	MdtEF-TolC multidrug efflux transport system - permease subunit	0.251	1.05E-02
b4324	<i>uxuR</i>	UxuR DNA-binding transcriptional repressor	0.250	1.27E-02
b0440	<i>hupB</i>	transcriptional dual regulator HU- β , NS1 (HU-1)	0.250	4.85E-04
b3806	<i>cyaA</i>	adenylate cyclase	0.249	7.42E-04
b0820	<i>ybiT</i>	putative ATP-binding component of a transport system	0.244	7.53E-04
b4139	<i>aspA</i>	aspartate ammonia-lyase	0.233	5.07E-04
b0728	<i>sucC</i>	succinyl-CoA synthetase, β subunit	0.232	6.62E-04
b3366	<i>nirD</i>	nitrite reductase, small subunit	0.229	6.50E-03
b3601	<i>mtlR</i>	MtlR DNA-binding transcriptional repressor	0.223	5.22E-03
b3869	<i>glnL</i>	NtrB sensory histidine kinase	0.221	6.24E-03
b4035	<i>malK</i>	maltose ABC transporter - ATP binding subunit	0.218	1.42E-03
b0727	<i>sucB</i>	dihydrolipoyltranssuccinylase	0.217	3.72E-07
b3748	<i>rbsD</i>	ribose pyranase	0.217	2.84E-03
b3516	<i>gadX</i>	GadX DNA-binding transcriptional dual regulator	0.216	8.45E-05
b3583	<i>sgbE</i>	L-ribulose-5-phosphate 4-epimerase	0.215	9.92E-05
b3365	<i>nirB</i>	dimer of large subunit of nitrite reductase	0.214	3.80E-03
b2614	<i>grpE</i>	phage lambda replication; host DNA synthesis; heat shock protein; protein repair	0.213	2.62E-04
b0114	<i>aceE</i>	pyruvate dehydrogenase	0.211	2.08E-04
b0432	<i>cyoA</i>	cytochrome bo terminal oxidase subunit II	0.211	8.36E-04
b3425	<i>glpE</i>	thiosulfate sulfurtransferase	0.208	7.15E-03
b3905	<i>rhaS</i>	RhaS transcriptional activator	0.206	1.52E-02
b4290	<i>fecB</i>	ferric dicitrate ABC transporter - periplasmic binding protein	0.204	2.12E-03
b2662	<i>gabT</i>	4-aminobutyrate transaminase	0.204	2.08E-07
b0729	<i>sucD</i>	succinyl-CoA synthetase, α subunit	0.204	8.37E-05
b3415	<i>gntT</i>	GntT Gluconate Gnt transporter	0.203	9.36E-03
b0351	<i>mhpF</i>	acetaldehyde dehydrogenase 2	0.203	9.87E-05
b3927	<i>glpF</i>	GlpF glycerol MIP channel	0.199	4.08E-03
b0116	<i>lpd</i>	lipoamide dehydrogenase	0.197	1.92E-07
b4289	<i>fecC</i>	ferric dicitrate ABC transporter - membrane subunit	0.196	1.56E-02
b3368	<i>cysG</i>	uroporphyrin III C-methyltransferase [multifunctional]	0.194	9.21E-03
b3450	<i>ugpC</i>	glycerol-3-phosphate / glycerol-2-phosphate ABC transporter - putative ATP binding subunit	0.193	1.68E-05

b4311	<i>nanC</i>	N-acetylneuraminic acid outer membrane channel	0.192	1.44E-02
b3599	<i>mtlA</i>	mannitol PTS permease	0.190	8.46E-03
b4310	<i>nanM</i>	N-acetylneuraminate mutarotase	0.189	5.56E-03
b0676	<i>nagC</i>	NagC DNA-binding transcriptional dual regulator	0.188	1.16E-07
b4267	<i>idnD</i>	L-idonate 5-dehydrogenase	0.188	1.46E-02
b3750	<i>rbsC</i>	ribose ABC transporter - membrane subunit	0.185	3.84E-03
b2784	<i>relA</i>	GDP pyrophosphokinase / GTP pyrophosphokinase	0.183	4.59E-05
b3513	<i>mdtE</i>	MdtEF-TolC multidrug efflux transport system - membrane fusion protein	0.183	6.13E-03
b1101	<i>ptsG</i>	fused glucose-specific PTS enzymes: IIB component/IIC component	0.182	1.13E-03
b3072	<i>aer</i>	aerotaxis sensor receptor, flavoprotein	0.182	7.35E-03
b3578	<i>yiaN</i>	L-dehydroascorbate transporter	0.180	4.44E-03
b2661	<i>gabD</i>	succinate semialdehyde dehydrogenase, NADP+-dependent	0.179	4.13E-07
b3393	<i>hofO</i>	protein involved in utilization of DNA as a carbon source	0.178	3.10E-04
b3394	<i>hofN</i>	protein involved in utilization of DNA as a carbon source	0.175	1.38E-02
b2659	<i>csiD</i>	predicted protein	0.174	5.50E-04
b4195	<i>ulaC</i>	L-ascorbate-specific enzyme IIA component of PTS	0.174	1.77E-02
b4382	<i>deoA</i>	thymidine phosphorylase	0.173	5.41E-03
b2782	<i>chpA</i>	MazF toxin of the MazF-MazE toxin-antitoxin system that exhibits ribonuclease activity	0.172	2.69E-04
b3222	<i>nanK</i>	N-acetylmannosamine kinase	0.171	6.34E-03
b2965	<i>speC</i>	ornithine decarboxylase, biosynthetic	0.171	4.61E-03
b3356	<i>yhfa</i>	conserved protein	0.171	2.40E-03
b2964	<i>nupG</i>	NupG nucleoside MFS transporter	0.170	2.60E-03
b0145	<i>dksA</i>	RNA polymerase-binding transcription factor	0.169	2.60E-04
b3961	<i>oxyR</i>	OxyR DNA-binding transcriptional dual regulator	0.169	8.46E-03
b3243	<i>aaeR</i>	quorum sensing transcriptional regulator of LYSR-type	0.168	6.91E-03
b0428	<i>cyoE</i>	heme O synthase	0.167	7.14E-07
b0583	<i>entD</i>	phosphopantetheinyl transferase	0.165	5.86E-04
b3223	<i>nanE</i>	predicted N-acetylmannosamine-6-phosphate epimerase	0.165	3.74E-02
b0411	<i>tsx</i>	nucleoside channel; receptor of phage T6 and colicin K	0.164	9.50E-09
b3666	<i>uhpT</i>	UhpT-hexose phosphate MFS transporter	0.163	5.83E-03
b3423	<i>glpR</i>	GlpR DNA-binding transcriptional repressor	0.162	1.92E-03
b4266	<i>idnO</i>	5-keto-D-gluconate 5-reductase	0.162	1.77E-02
b3902	<i>rhaD</i>	rhamnulose-1-phosphate aldolase	0.161	3.20E-04
b3426	<i>glpD</i>	glycerol 3-phosphate dehydrogenase, aerobic	0.161	1.16E-02
b0191	<i>yaeJ</i>	peptidyl-tRNA hydrolase, ribosome rescue factor	0.161	1.41E-05
b3428	<i>glgP</i>	glycogen phosphorylase	0.161	3.68E-03
b2143	<i>cdd</i>	cytidine deaminase	0.161	5.22E-04

b3413	<i>gntX</i>	protein involved in utilization of DNA as a carbon source	0.160	7.20E-03
b3751	<i>rbsB</i>	ribose ABC transporter - putative periplasmic binding protein	0.158	3.13E-04
b3424	<i>glpG</i>	intramembrane serine protease GlpG	0.157	5.73E-03
b4014	<i>aceB</i>	malate synthase A	0.157	1.69E-03
b3581	<i>sgbH</i>	3-keto-L-gulonate 6-phosphate decarboxylase	0.156	2.03E-02
b2943	<i>galP</i>	GalP - galactose MFS transporter	0.156	8.71E-04
b3076	<i>ebgA</i>	evolved β -D-galactosidase, α subunit	0.155	9.11E-03
b0118	<i>acnB</i>	bifunctional aconitate hydratase 2 and 2-methylisocitrate dehydratase	0.154	2.65E-05
b0429	<i>cyoD</i>	cytochrome bo terminal oxidase subunit IV	0.154	1.08E-05
b3437	<i>gntK</i>	D-gluconate kinase, thermostable	0.153	2.56E-03
b3752	<i>rbsK</i>	ribokinase	0.153	8.66E-03
b4233	<i>mpl</i>	UDP-N-acetylmuramate:L-alanyl- γ -D-glutamyl-meso-diaminopimelate ligase	0.152	2.75E-03
b0678	<i>nagB</i>	glucosamine-6-phosphate deaminase	0.151	5.43E-04
b3240	<i>aaeB</i>	AaeAB Hydroxylated, Aromatic Carboxylic Acid Efflux Transport System Protein B	0.151	7.86E-03
b1656	<i>sodB</i>	superoxide dismutase (Fe)	0.150	1.37E-07
b3404	<i>envZ</i>	EnvZ sensory histidine kinase	0.150	2.00E-03
b0597	<i>entH</i>	proofreading thioesterase in enterobactin biosynthesis	0.150	2.99E-06
b3214	<i>gltF</i>	periplasmic protein	0.149	2.06E-03
b4119	<i>mela</i>	α -galactosidase	0.149	9.40E-03
b1205	<i>ychH</i>	stress-induced protein	0.148	5.99E-04
b3118	<i>tdcA</i>	TdcA DNA-binding transcriptional activator	0.147	1.44E-05
b4287	<i>fecE</i>	ferric dicitrate ABC transporter - ATP binding subunit	0.146	3.35E-03
b0192	<i>nlpE</i>	outer membrane lipoprotein NlpE, involved in surface sensing	0.145	3.30E-05
b4003	<i>zraS</i>	ZraS sensory histidine kinase	0.144	8.31E-03
b3092	<i>uxaC</i>	D-glucuronate isomerase / D-galacturonate isomerase	0.144	2.71E-03
b3093	<i>exuT</i>	ExuT hexuronate MFS transporter	0.144	8.08E-04
b3133	<i>agaV</i>	PTS system, cytoplasmic, N-acetylglactosamine-specific IIB component 2 (EIIB-AGA)	0.143	2.69E-03
b3395	<i>hofM</i>	protein involved in utilization of DNA as a carbon source	0.142	5.26E-05
b3582	<i>sgbU</i>	predicted L-xylulose 5-phosphate 3-epimerase	0.142	2.47E-03
b4015	<i>aceA</i>	isocitrate lyase	0.142	5.26E-03
b3749	<i>rbsA</i>	ribose ABC transporter - putative ATP binding subunit	0.141	8.54E-04
b3135	<i>agaA</i>	predicted truncated N-acetylglactosamine-6-phosphate deacetylase	0.141	3.47E-03
b4124	<i>dcuR</i>	DcuR transcriptional activator	0.141	2.41E-04
b4240	<i>treB</i>	fused trehalose(maltose)-specific PTS enzyme: IIB component/IIC component	0.141	5.59E-04
b3241	<i>aaeA</i>	AaeAB Hydroxylated, Aromatic Carboxylic Acid Efflux Transport System Protein A	0.140	2.86E-03
b3574	<i>yiaJ</i>	YiaJ DNA-binding transcriptional repressor	0.140	2.97E-05

b4268	<i>idnK</i>	D-gluconate kinase, thermosensitive	0.140	2.58E-04
b2708	<i>gutQ</i>	D-arabinose 5-phosphate isomerase	0.140	5.02E-03
b0109	<i>nadC</i>	quinolinate phosphoribosyltransferase	0.140	4.04E-07
b3418	<i>malT</i>	MalT transcriptional activator	0.139	7.40E-04
b4004	<i>zraR</i>	ZraR transcriptional activator	0.138	5.09E-03
b4239	<i>treC</i>	trehalose-6-phosphate hydrolase	0.138	3.92E-03
b0431	<i>cyoB</i>	cytochrome bo terminal oxidase subunit I	0.138	5.70E-05
b0726	<i>sucA</i>	2-oxoglutarate decarboxylase, thiamin-requiring	0.137	5.58E-04
b4476	<i>gntU</i>	GntU gluconate Gnt transporter	0.137	4.15E-06
b0756	<i>galM</i>	galactose-1-epimerase	0.137	1.11E-03
b4030	<i>psiE</i>	predicted phosphate starvation-inducible protein	0.137	3.38E-03
b4265	<i>idnT</i>	L-idonate / 5-ketogluconate / gluconate transporter	0.136	2.11E-05
b3564	<i>xylB</i>	xylulokinase	0.136	5.71E-03
b2703	<i>srlE</i>	glucitol/sorbitol-specific enzyme IIB component of PTS	0.136	3.56E-03
b2509	<i>xseA</i>	exonuclease VII, large subunit	0.136	3.53E-04
b4069	<i>acs</i>	acetyl-CoA synthetase (AMP-forming)	0.135	2.41E-03
b3577	<i>yiaM</i>	predicted transporter	0.135	9.06E-03
b4068	<i>yjcH</i>	conserved inner membrane protein	0.134	5.70E-03
b0584	<i>fepA</i>	FepA, outer membrane receptor for ferric enterobactin (enterochelin) and colicins B and D	0.134	9.11E-04
b3392	<i>hofP</i>	protein involved in utilization of DNA as a carbon source	0.134	4.16E-04
b4118	<i>melR</i>	MeIR DNA-binding transcriptional dual regulator	0.133	3.25E-04
b3567	<i>xylG</i>	xylose ABC transporter - ATP binding subunit	0.133	1.64E-03
b4323	<i>uxuB</i>	D-mannonate oxidoreductase	0.133	2.95E-04
b2171	<i>yeiP</i>	predicted dehydrogenase, NAD-dependent	0.132	3.16E-06
b4138	<i>dcuA</i>	DcuA dicarboxylate Dcu transporter	0.132	2.22E-04
b3117	<i>tdcB</i>	catabolic threonine dehydratase	0.131	3.49E-03
b0593	<i>entC</i>	isochorismate synthase 1	0.131	1.46E-03
b3451	<i>ugpE</i>	glycerol-3-phosphate / glycerol-2-phosphate ABC transporter - putative membrane subunit	0.131	3.68E-03
b4122	<i>fumB</i>	fumarase B	0.130	4.76E-03
b3722	<i>bglF</i>	β -glucoside PTS permease	0.130	2.91E-03
b2841	<i>araE</i>	AraE arabinose MFS transporter	0.130	3.29E-05
b3565	<i>xylA</i>	xylose isomerase	0.129	8.45E-05
b0677	<i>nagA</i>	N-acetylglucosamine-6-phosphate deacetylase	0.129	2.91E-06
b0598	<i>cstA</i>	peptide transporter induced by carbon starvation	0.128	2.34E-06
b4264	<i>idnR</i>	IdnR-5-ketogluconate DNA-binding transcriptional dual regulator	0.128	4.72E-05
b3576	<i>yiaL</i>	conserved protein	0.127	6.50E-03
b2980	<i>glcC</i>	GlcC transcriptional dual regulator	0.127	6.69E-03
b3132	<i>kbaZ</i>	tagatose 6-phosphate aldolase 1, kbaZ subunit	0.126	3.04E-03
b0430	<i>cyoC</i>	cytochrome bo terminal oxidase subunit III	0.126	4.19E-04
b2707	<i>srlR</i>	GutR DNA-binding transcriptional repressor	0.126	3.67E-03
b3225	<i>nanA</i>	N-acetylneuraminase lyase	0.125	4.09E-03
b3224	<i>nanT</i>	NanT sialic acid MFS transporter	0.125	9.47E-03
b1276	<i>acnA</i>	aconitate hydratase 1	0.124	1.13E-03
b3588	<i>aldB</i>	acetaldehyde dehydrogenase	0.124	2.99E-04
b3077	<i>ebgC</i>	evolved β -D-galactosidase, β subunit	0.123	3.56E-03

b2799	<i>fucO</i>	L-1,2-propanediol oxidoreductase	0.123	8.73E-04
b3708	<i>tnaA</i>	L-cysteine desulfhydrase / tryptophanase	0.123	4.02E-03
b0675	<i>nagD</i>	ribonucleotide monophosphatase	0.123	4.68E-09
b3906	<i>rhaR</i>	RhaR transcriptional activator	0.123	6.28E-03
b4037	<i>malM</i>	maltose regulon periplasmic protein	0.123	4.43E-03
b2705	<i>srlD</i>	sorbitol-6-phosphate dehydrogenase	0.122	4.46E-04
b4321	<i>gntP</i>	GntP Gluconate Gnt transporter	0.122	1.79E-04
b3903	<i>rhaA</i>	L-rhamnose isomerase	0.121	1.34E-03
b3907	<i>rhaT</i>	rhamnose RhaT transporter	0.121	3.01E-03
b2957	<i>ansB</i>	asparaginase II	0.121	2.24E-03
b3367	<i>nirC</i>	NirC nitrite FNT transporter	0.120	3.33E-03
b4067	<i>actP</i>	acetate / glycolate transporter	0.120	2.39E-03
b2009	<i>sbmC</i>	DNA gyrase inhibitor	0.120	3.48E-03
b4036	<i>lamB</i>	phage lambda receptor protein; maltose high-affinity receptor	0.119	8.73E-03
b0113	<i>pdhR</i>	PdhR DNA-binding transcriptional dual regulator	0.118	5.58E-05
b0124	<i>gcd</i>	glucose dehydrogenase	0.118	2.15E-03
b1819	<i>manZ</i>	mannose PTS permease - ManZ subunit	0.118	2.02E-03
b3926	<i>glpK</i>	glycerol kinase	0.117	6.41E-03
b0595	<i>entB</i>	apo-EntB multimer	0.117	4.62E-04
b4291	<i>fecA</i>	outer membrane receptor; citrate-dependent iron transport, outer membrane receptor	0.117	1.10E-04
b3569	<i>xylR</i>	XylR transcriptional activator	0.117	5.07E-03
b2715	<i>ascF</i>	β -glucoside PTS permease	0.117	3.88E-03
b2535	<i>csiE</i>	stationary phase inducible protein	0.117	1.31E-03
b3568	<i>xylH</i>	xylose ABC transporter - membrane subunit	0.116	4.68E-03
b3721	<i>bglB</i>	6-phospho- β -glucosidase B	0.115	2.72E-03
b4016	<i>aceK</i>	isocitrate dehydrogenase phosphatase / isocitrate dehydrogenase kinase	0.115	7.39E-03
b3091	<i>uxaA</i>	D-altronate dehydratase	0.115	8.31E-05
b0757	<i>galK</i>	galactokinase	0.114	1.25E-06
b2660	<i>lhgO</i>	L-2-hydroxyglutarate oxidase	0.114	9.25E-04
b2800	<i>fucA</i>	L-fuculose-phosphate aldolase	0.114	8.79E-04
b3113	<i>tdcF</i>	predicted L-PSP (mRNA) endoribonuclease	0.114	2.80E-03
b0146,	<i>sfsA</i>	predicted DNA-binding transcriptional regulator of maltose metabolism	0.113	8.68E-10
b2803	<i>fucK</i>	L-fuculokinase	0.113	1.04E-03
b4196	<i>ulaD</i>	3-keto-L-gulonate 6-phosphate decarboxylase	0.113	4.37E-03
b3579	<i>yiaO</i>	L-dehydroascorbate transporter, periplasmic binding protein	0.113	3.55E-03
b2805	<i>fucR</i>	FucR transcriptional activator	0.113	6.31E-04
b2706	<i>gutM</i>	GutM DNA-binding transcriptional activator	0.113	7.84E-07
b3081	<i>fadH</i>	2,4-dienoyl-CoA reductase	0.112	1.49E-02
b3452	<i>ugpA</i>	glycerol-3-phosphate / glycerol-2-phosphate ABC transporter - putative membrane subunit	0.112	3.26E-03
b2241	<i>glpA</i>	glycerol-3-phosphate dehydrogenase (anaerobic), large subunit	0.112	1.14E-03
b3134	<i>agaW</i>	PTS system N-acetylgalactosamine-specific IIC component 2	0.112	6.81E-03
b3453	<i>ugpB</i>	glycerol-3-phosphate / glycerol-2-phosphate ABC transporter - putative periplasmic binding	0.111	2.20E-03

		protein		
b3566	<i>xytF</i>	xylose ABC transporter - periplasmic binding protein	0.111	3.36E-03
b3753	<i>rbsR</i>	RbsR DNA-binding transcriptional repressor	0.111	6.81E-05
b3925	<i>glpX</i>	fructose 1,6-bisphosphatase II	0.111	2.73E-04
b0043	<i>fixC</i>	flavoprotein (electron transport), possibly involved in anaerobic carnitine metabolism	0.111	4.46E-08
b3116	<i>tdcC</i>	TdcC threonine STP transporter	0.111	5.67E-03
b3934	<i>cytR</i>	CytR DNA-binding transcriptional repressor	0.110	3.47E-03
b2234	<i>nrdA</i>	ribonucleoside diphosphate reductase 1, α subunit dimer	0.109	5.96E-04
b2393	<i>nupC</i>	NupC nucleoside NUP transporter	0.109	3.13E-03
b1272	<i>sohB</i>	predicted inner membrane peptidase	0.108	3.99E-05
b4198	<i>ulaF</i>	L-ribulose 5-phosphate 4-epimerase	0.108	1.04E-02
b3571	<i>malS</i>	α -amylase	0.108	4.07E-03
b4123	<i>dcuB</i>	DcuB dicarboxylate Dcu transporter	0.108	5.63E-03
b4194	<i>ulaB</i>	L-ascorbate-specific enzyme IIB component of PTS	0.108	1.45E-02
b4033	<i>malF</i>	maltose ABC transporter - membrane subunit	0.108	4.21E-04
b4213	<i>cpdB</i>	2',3'-cyclic nucleotide 2'-phosphodiesterase / 3'-nucleotidase	0.108	1.43E-04
b4032	<i>malG</i>	maltose ABC transporter - membrane subunit	0.107	2.92E-03
b4322	<i>uxuA</i>	D-mannonate dehydratase	0.107	1.37E-04
b3904	<i>rhaB</i>	L-rhamnulose kinase	0.107	5.19E-03
b0758	<i>galT</i>	galactose-1-phosphate uridylyltransferase	0.107	1.33E-04
b0061	<i>araD</i>	L-ribulose 5-phosphate 4-epimerase	0.106	4.14E-07
b3221	<i>yhch</i>	conserved protein	0.106	2.10E-03
b0038	<i>caiB</i>	γ -butyrobetainyl-CoA:carnitine CoA transferase	0.106	3.39E-04
b2663	<i>gabP</i>	GabP APC transporter	0.106	7.27E-04
b0110	<i>ampD</i>	N-acetyl-anhydromuramyl-L-alanine-amidase	0.105	4.72E-07
b0041	<i>fixA</i>	probable flavoprotein subunit required for anaerobic carnitine metabolism	0.105	2.96E-04
b2232	<i>ubiG</i>	bifunctional 3-demethylubiquinone-8 3-O-methyltransferase and 2-octaprenyl-6-hydroxyphenol methylase	0.104	1.15E-05
b2155	<i>cirA</i>	outer membrane receptor involved in uptake of ferric dihydroxybenzoylserine	0.104	5.32E-04
b3709	<i>tnaB</i>	TnaB tryptophan ArAAP transporter	0.104	2.47E-03
b3580	<i>lyxK</i>	L-xylulose kinase	0.103	1.07E-03
b2365	<i>dsdX</i>	DsdX Gnt transporter	0.102	8.39E-05
b4288	<i>fecD</i>	ferric dicitrate ABC transporter - membrane subunit	0.102	9.20E-03
b4197	<i>ulaE</i>	L-xylulose 5-phosphate 3-epimerase	0.102	8.56E-03
b4120	<i>melB</i>	MelB GPH transporter	0.102	5.93E-03
b2801	<i>fucP</i>	FucP fucose MFS transporter	0.102	1.45E-04
b4193	<i>ulaA</i>	L-ascorbate-specific enzyme IIC component of PTS	0.101	7.13E-03
b0034	<i>caiF</i>	CaiF transcriptional activator	0.101	2.68E-04
b3723	<i>bglG</i>	BglG transcriptional antiterminator	0.100	1.19E-04
b0042	<i>fixB</i>	probable flavoprotein subunit required for anaerobic carnitine metabolism	0.100	1.77E-05
b2481	<i>hyfA</i>	hydrogenase 4, component A	0.100	4.33E-06

b0720	<i>gltA</i>	citrate synthase	0.099	6.65E-07
b2716	<i>ascB</i>	6-phospho- β -glucosidase	0.099	4.44E-03
b3528	<i>dctA</i>	DctA dicarboxylate DAACS transporter	0.099	4.88E-03
b2802	<i>fucI</i>	L-fucose isomerase	0.098	3.29E-04
b0679	<i>nagE</i>	N-acetylglucosamine PTS permease	0.097	5.15E-07
b0039	<i>caiA</i>	crotonobetainyl-CoA reductase	0.097	1.18E-06
b0929	<i>ompF</i>	outer membrane porin F	0.095	1.23E-03
b1111	<i>ycfQ</i>	predicted DNA-binding transcriptional regulator	0.095	4.18E-04
b0040	<i>caiT</i>	L-carnitine/ γ -butyrobetaine antiport	0.094	4.21E-04
b1040	<i>csgD</i>	CsgD DNA-binding transcriptional dual regulator	0.094	6.96E-07
b0805	<i>fiu</i>	putative outer membrane receptor for iron transport	0.094	7.15E-07
b1593	<i>ynfK</i>	predicted dethiobiotin synthetase	0.094	6.39E-04
b2804	<i>fucU</i>	L-fucose mutarotase	0.093	2.37E-06
b1519	<i>tam</i>	trans-aconitate methyltransferase	0.093	1.59E-04
b3575	<i>yiaK</i>	2,3-diketo-L-gulonate reductase	0.092	5.81E-03
b2468	<i>aegA</i>	putative oxidoreductase, Fe-S subunit	0.091	2.48E-04
b2091	<i>gatD</i>	galactitol-1-phosphate dehydrogenase	0.090	5.05E-04
b1015	<i>putP</i>	proline:sodium symporter	0.089	1.14E-04
b1394	<i>paaG</i>	predicted ring 1,2-epoxyphenylacetyl-CoA isomerase (oxepin-CoA forming)	0.089	6.28E-05
b0062	<i>araA</i>	L-arabinose isomerase	0.089	8.82E-06
b0036	<i>caiD</i>	crotonobetainyl-CoA hydratase	0.089	1.66E-06
b1892	<i>flhD</i>	DNA-binding transcriptional dual regulator with FlhC	0.089	8.74E-04
b0106	<i>hofC</i>	protein transport protein HofC	0.087	7.43E-08
b1622	<i>malY</i>	bifunctional β -cystathionase, PLP-dependent and regulator of maltose regulon	0.087	5.84E-04
b0064	<i>araC</i>	AraC DNA-binding transcriptional dual regulator	0.087	1.13E-10
b0108	<i>ppdD</i>	prepilin peptidase dependent protein	0.087	2.19E-04
b2488	<i>hyfH</i>	hydrogenase 4, component H	0.086	1.57E-05
b2148	<i>mglC</i>	galactose ABC transporter - membrane subunit	0.085	8.57E-04

^aFrom the EcoCyc database (<http://ecocyc.org>)

^bFold-change in gene expression between A2 and WT (average of duplicate experiments)

Chapter 5: CONCLUSIONS AND FUTURE WORKS

Strain engineering has long been applied for the improvement of cell phenotypes that are industrially essential for many microorganisms. However, cell phenotypes rarely depend on simple genotype-phenotype relationship; therefore it is challenging to engineer cell phenotypes even for simple microbe like *E. coli*. In this study, I have employed transcriptional engineering to enhance the tolerance of *E. coli* towards stresses that are commonly encountered by microorganisms during fermentation, including ethanol (product toxicity) and acetate (byproduct toxicity). The global transcription factor CRP has been chosen as our target regulatory protein due to its essentiality as one of the key regulatory protein in *E. coli*. CRP can regulate more than 400 genes belonging to various functional classes in *E. coli*.

We had successfully isolated three ethanol tolerant mutants (E1-E3) and four acetate tolerant mutants (A1-A4) with better growth at low temperature as discussed in Chapter 3 and 4 of this dissertation respectively. Their improvements in stress tolerance were confirmed through investigation on their growth profiles, and the best mutant in each study was subjected to further characterization based on their cross-tolerance and changes in transcript profile of CRP-regulated genes using OpenArray® and cell morphology by scanning electron microscopy (SEM).

As mentioned in Chapter 3, the *crp* operon of the best ethanol-tolerant mutant E2 was integrated into the chromosome to create the strain iE2. The mutant strain iE2 not only displayed better growth than parent strain BW25113 in ethanol, but also in other short-chain alcohols, namely 1-propanol, 1-butanol

and 1-pentanol. iE2 also illustrated significantly higher cell viability than BW25113 when exposed to lethal concentration of ethanol. The cell surface of iE2 was also relatively smoother and less wrinkled than its control when cultivated in the presence of ethanol, as verified by SEM. Due to noticeable growth differences between E2 and iE2 under ethanol stress, we have compared the transcript profile of the CRP-regulated genes using OpenArray® real-time PCR, and found that only a handful of genes were up-regulated or down-regulated in both iE2 and E2 under ethanol stress.

With respect to improving acetate tolerance in Chapter 4, the best mutant, A2, was shown to grow faster in minimal medium, with or without sodium acetate supplements. The growth of A2 was also better in the presence of other byproducts, such as formate and propionate. The cellular size of A2 increased when grown under acetate stress, whereas that of WT remained the same. Differences in the transcription profile of CRP-regulated genes between A2 and WT were determined through OpenArray® real-time PCR, where more than 68% and 90% of the 444 CRP-regulated genes assessed were differentially expressed by more than 2-fold in A2 in the absence and presence of sodium acetate respectively.

The property improvements for the mutants are summarized in Table 16 below:

Table 16: Summary of property improvements observed in ethanol-tolerant mutants (E2 and iE2) and acetate-tolerant mutant (A2) over wild-type, and comparison between engineering of CRP and classical strain improvement approaches.

Properties	Ethanol tolerance				Acetate tolerance	
	E2	WT	iE2	BW25113	A2	WT
Growth rate					0 g/l sodium acetate: 0.19 h ⁻¹	0 g/l sodium acetate: 0.13 h ⁻¹
	62 g/l ethanol: 0.08 h ⁻¹	62 g/l ethanol: 0.06 h ⁻¹	62 g/l ethanol: 0.07 h ⁻¹	62 g/l ethanol: 0.05 h ⁻¹	10 g/l sodium acetate: 0.1 h ⁻¹	10 g/l sodium acetate: 0.026 h ⁻¹
					15 g/l sodium acetate: 0.083 h ⁻¹	15 g/l sodium acetate: 0.016 h ⁻¹
Maximum OD600 reached					0 g/l sodium acetate: 2.22	0 g/l sodium acetate: 1.36 after 14 h
	0.19	0.1	0.13	0.09	10 g/l sodium acetate: 2.15	10 g/l sodium acetate: 0.524 after 30 h
					15 g/l sodium acetate: 2.16	15 g/l sodium acetate: 0.368 after 35 h
Cross-tolerance			3.1 % (v/v) 1-propanol: 0.129 h ⁻¹	3.1 % (v/v) 1-propanol: 0.087 h ⁻¹	5 g/ sodium formate: 0.16 h ⁻¹	5 g/ sodium formate: 0.14 h ⁻¹
			0.45 % (v/v) 1-pentanol: 0.062 h ⁻¹	0.45 % (v/v) 1-pentanol: 0.047 h ⁻¹	18 g/l sodium propionate: 0.07 h ⁻¹	18 g/l sodium propionate: 0.03 h ⁻¹

Comparison to classical methods	Adaptive evolution method requires about 1000 h to select for mutant (Horinouchi et al. 2010), while this method needs 3-5 days to isolate 3 ethanol-tolerant mutants.	Adaptive evolution method requires 1750 generations (~18-20 days) to select mutants (Treves et al. 1998), while this method needs 5-7 days to isolate acetate-tolerant mutant.
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In summary,

1. Transcriptional engineering can provide an efficient alternative approach in strain engineering due to its ability to perturb the expression of multiple genes simultaneously, as verified by the RT-PCR analysis in these studies.
2. Transcriptional engineering is less time-consuming and labor-intensive than conventional strain engineering approaches, since several mutants can be selected from error-prone PCR libraries of $> 10^6$ within 3-7 days. Conventional strain engineering requires selection period of weeks to months (~1000 hours), and library size of $> 10^8$.
3. Engineering of CRP can improve a variety of phenotypes, such as ethanol and acetate tolerance, which are some commonly encountered stress conditions during fermentation. In this dissertation, the ethanol tolerance was improved in *E. coli* BW25113, while acetate tolerance was enhanced in *E. coli* DH5 α . The successful isolation of mutants for each type of stress proved that directed evolution of CRP is also applicable to different strains of *E. coli*, and possibly to other microorganisms with CRP as well.

4. When comparing the amino acid substitutions among the variants (E1-E3, A1-A4), it is observed that only mutation at V176 is repeated in A3 and A4. However, the role of this amino acid in CRP is currently not known. In contrast, amino acid substitution at D138 in A2 is of considerable interest, as mutation to this residue also appears in 1-butanol-tolerant and osmotolerant mutants in previous reports. D138 is found in the hinge region, and mutation to this position is thought to affect CRP activation upon cAMP binding.
5. RT-PCR analysis of CRP-regulated genes using OpenArray® verifies that CRP mutation(s) can induce changes in transcript level of its regulated genes, particularly in acetate tolerance. CRP is known to be the main regulator of carbon metabolism, which includes acetate production and assimilation. Therefore, more than 90 % of CRP-regulated genes were differentially expressed by more than 2-fold in A2 under acetate stress, as compared to 20 % and 36 % in iE2 and E2 under ethanol stress respectively. Comparisons of the transcript profiles between chromosomally integrated CRP mutant iE2 and plasmid-based CRP mutant E2 are also made. It is found that about 18 genes are commonly differentially expressed both in iE2 and E2.
6. Attempts to find the trait-conferring genotypes are made through overexpression of genes from the ASKA library. The gene *uxaB* was found to confer poor growth under acetate stress in *E. coli* DH5 α , while none of the investigated genes was discovered to affect ethanol tolerance in *E. coli* BW25113.

7. Cells can adapt to stress through modification of their cell morphology.

For instance, the cell surface of *E. coli* BW25113 becomes rough and wrinkled in response to ethanol stress, but iE2 illustrates a relatively smoother and less wrinkled surface. This implies that CRP mutation can help in counteracting the impact of ethanol on membrane integrity.

Despite the many advantages that transcriptional engineering can provide, this method is not without its drawbacks. One major drawback of using transcriptional engineering is that the choice of phenotype is limited to stress tolerance or improving yields and titers of products with colorimetric properties. In this dissertation, the improvement in stress tolerance is based on cell growth. However, if the phenotype of interest cannot be differentiated by cell growth, it would be impossible to select for improved mutants. Furthermore, although microarray and RT-PCR analysis on the mutants can provide insights on trait-conferring genotype, it is not possible to identify the essential genes without performing overexpression or knockout experiments.

The future works recommended for further investigation on the mechanisms that CRP mutants engaged in conferring tolerance phenotype are as follows:

1. Since only changes in transcript profile of CRP-regulated genes are determined by RT-PCR, microarray analysis may be used to determine changes in the expression level of other genes not regulated by CRP. However, since microarray and RT-PCR analysis only provide correlational relationships between genotypes and phenotypes, overexpression of genes from ASKA library is still required to track the gene(s) that are essential in resulting stress tolerance or sensitivity.

2. The effect of the mutated CRP on promoter activity, namely Class I and Class II promoters, as well as the binding affinity of CRP to cAMP can also be investigate to provide insights on how CRP mutation can affect CRP activation and binding to promoter, and thus result in changes to expression level of its regulated genes.
3. This approach can also be extended to microorganisms containing CRP as its regulatory protein in order to find common genes that can confer the tolerance phenotype.

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