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Tailoring of global transcription sigma D factor by random mutagenesis to improve *Escherichia coli* tolerance towards low-pHs

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ABSTRACT

Bioconversion processes of organic acid or acid hydrolysis of raw material for microbial metabolism often suffer limitations as a result of microbial sensitivity in low-pH conditions. We adopted a three-step method called RAndom Insertional-deletional Strand Exchange mutagenesis (RAISE) to engineer the components of global regulator Sigma D factor (RpoD) of *Escherichia coli* to improve its acid tolerance. The best strain Mutant VII was identified from random mutagenesis libraries based on the growth performance, which exhibited much higher growth rate than the control (0.22 h^{-1} vs. 0.15 h^{-1}) at pH as low as 3.17. Combined transcriptome and phenome analysis of *E. coli* was carried out to better understand the global effects of RpoD on the regulatory networks. Our analysis showed that 95 (2.1%) of all *E. coli* genes were induced and 178 (4.0%) genes were repressed, including those for trehalose biosynthesis, nucleotides biosynthesis, carbon metabolism, amino acid utilization, except for acid resistance. Also regulated were the master regulators (ArcA, EvgA, H-NS and RpoS) and gene/operon-specific transcription factors (GadX, GadW, AppY, YdeO, KdgR). These results demonstrated that RpoD acts as global regulator in the growth phase of *E. coli* and consequently improves acid tolerances.

1. Introduction

Biorefining promises the development of efficient biocatalysis processes for the conversion of renewable biomass resources into building block chemicals that are amenable to further conversions to higher value products as well as large volume commodity chemicals. Nevertheless, many challenges still remain for the economical bio-production of these chemicals. For example, product toxicity issue associated with the bioconversion processes of organic acid is one of the primary challenges based on the use of platform organisms, such as *Escherichia coli*. *E. coli* has been chosen as a model organism for the production of high-value organic acids like succinic acid (Chan et al., 2012), 3-hydroxypropionic acid (Kwak et al., 2013), lactic acid (Mazumdar et al., 2013), and so on, due to its clear genetic background, convenient to be genetically modified as well as good growth property with low nutrient requirements. However, high titers of organic acid in the free acid form during fermentation inhibits both cell growth and acid production of *E. coli* (Chun et al., 2014). While the control of fermentation broth pH by addition of large volumes of alkaline substances (e.g., $CaCO_3$, NaOH and NH_4OH) could reduce the negative effects of free acid accumulation but strongly foul downstream processing (Abdel-Rahman et al., 2013). Meanwhile, acid-hydrolysis-mediated pretreatment of raw material to release its component sugars which are suitable for microbial metabolism, in turn impair viability of microbes because of the low pH circumstance (Huang et al., 2009). One model for addressing such challenges involves the improvement of cellular phenotype under low pH or acid stress conditions.

Measures have previously been taken to elevate *E. coli* acid tolerance through classical strain engineering approaches such as UV/chemical mutagen treatment, adaptive evolution, wihch are often time and labour-intensive (Dragosits and Mattanovich, 2013). Metabolic engineering tools, including deletion of *glpK*, *yqhD*, *gldA*, *pta*, *ldhA*, and individual over-expression of *cacKB*, *cscA*, *dhaB*, *dhaR*, *aldH* have also been employed, which are, however, difficult to reach a global phenotype optimum due to the complexity of metabolic landscapes (Chan et al., 2012; Kwak et al., 2013). More recently, transcriptional engineering starts to emerge as a alternative strategy for the improvement of strain tolerance and production phenotypes through multiple, simultaneous perturbations regulated by reprogramming the transcriptome

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(Alper et al., 2006). A successful case is reported by Jiang et al. (2013a,b). that the global regulator cAMP receptor protein (CRP) has been engineered by error-prone PCR to improve the tolerance of *E. coli* towards low-pH and acetate stresses (Basak and Geng, 2014). The mutant strain almost doubled ($0.113\,h^{-1}$) the growth rate of the control ($0.062\,h^{-1}$) at pH 4.24. However, the growth rate needs to be further improved to better meet the requirements for industrialized production. It has been pointed out that the pattern of genome transcription in bacteria is mainly determined by controlling the utilization of RNA polymerase, the promoter specificity of which was further controlled by several species of RNA polymerase sigma factor (Ishihama, 2010). Earlier work revealed that the global regulator CRP in *E. coli* played an important regulatory role in synthesis of the stationary-phase specific sigma S factor (RpoS), which was actually responsible for many different stress conditions (Hengge-Aronis, 2002). Most analyses have, however, paid full attention to the strains entrying into stationary phase accompanied with a reduction or cessation of growth, and ignored the influence of exponential-phase global regulators on the evolution of host regulatory networks. The sigma D factor, encoded by the *rpoD* gene, plays a primary and major role in transcription of most of the genes that is expressed in exponentially growing phase of *E. coli*, and can partially replace the sigma factor RpoS under many stress conditions (Alper and Stephanopoulos, 2007). In particular, it is reported that *E. coli* is surprisingly acid sensitive in exponential phase when cultured planktonically (Richard and Foster, 2004).

In this work, the *rpoD* gene was subjected to random mutagenesis by RAndom Insertional-deletional Strand Exchange mutagenesis (RAISE) method, which is based on gene shuffling except the tailing by terminal deoxynucleotidyl transferase (TdT) before the self-priming PCR step (Fujii et al., 2006). The RAISE product was inserted into a low-copy expression vector pKSC by overlap extension PCR (Basak and Geng, 2014). By taking the approach of mutagenesis and phenotype selection on the *rpoD* gene, the major goal is to elicite a desired cellular phenotype with elevated low pH tolerance. Furthermore, transcriptome and phenome properties as well as quantitative real-time PCR (qRT-PCR) measurement were carried out on the best mutant to investigate the effects of the global regulator RpoD on the changes in gene expression in the heterologous *E. coli* host.

2. Materials and methods

2.1. Strains and media

E. coli DH5α was applied to routine transformations, as well as RNase-free DNase I, TdT, and restriction enzymes were purchased from TaKaRa (Shanghai, China). Strains were cultured in improved Luria–Bertani (LB) broth at 37 °C with 200 rpm orbital shaking or on improved LB agar at 37 °C. Earlier studies reported that acid sensitivity of cells is enhanced by incorporation of more than 150 mM sodium chloride (Rowbury et al., 1994), a therefore, 5 g/l NaCl (86 mM) was utilized in LB here. Culture was supplemented with 30 μg/ml of kanamycin for low-copy-number (~5 copies/cell) host plasmid breeding (pKSC plasmid). The DH5α strain containing plasmid pKSC-*rpoD* is denoted as a control strain in this study. All remaining chemicals were purchased from Sigma-Aldrich and primers were from Invitrogen.

2.2. Library construction

A low-copy-numberplasmid (pKSC) was structured by Rongrong Jiang according to a previously reported protocol with improvement (Zhang et al., 2012). The PCR primers used in this work are displayed in Table 1. Sigma D factor was inspected for a target of RAISE method (Fujii et al., 2006). The *rpoD* operon was amplified from *E. coli* genomic DNA with primers *rpoD*-sense-*Eco*RI and *rpoD*-anti-*Hind*III. The purified PCR products (2075 bp) were digested with RNase-free DNase I in the buffer containing Tris–HCl (pH 7.0) and $MnCl_2$. To avoid introduction of too many point mutations in the ensuing self-priming PCR step, $MnCl_2$ was utilized as a DNase I co-factor to control the size of the digestion products. Determined by agarose gel electrophoresis, the fragments of 100–300 bp were terminated by adding Ethylene Diamine Tetraacetic Acid (EDTA). The addition of TdT was randomly attached to the 3′ terminus of the fragments in the solution containing buffer and dNTPs. With a DNA polymerase possessing no proofreading activity, the 3′-tailed purified fragments were reassembled by self-priming PCR.

The full-length of the mutated *rpoD* gene (RAISE product) was amplified by PCR, utilising the M13 universal primes. The purified RAISE products were digested by *Eco*RI and *Hind*III overnight, ligated into a digested pKSC, and transformed into *E. coli* DH5α. The transformed cells were plated onto LB agar containing 30 μg/ml of kanamycin and scraped off white colonies to create a liquid library. The same procedure (and library size) was used for the three rounds of mutagenesis used in the low pH experiment.

2.3. Phenotype selection

Phenotype selection and evaluation of samples from the liquid library were chosen based on acid shock method. The random mutagenesis library was inoculated into fresh 50-ml LB broth at 37 °C with 200 rpm orbital shaking. When strains grew up to the beginning of the exponential phase (OD_{600}~0.3), 36% (w/w) HCl was added to the medium to regulate its pH decreasing from 4.0 to 3.0 for 4 generation and the strains were cultured at 37 °C with 200 rpm orbital shaking for 2 h. The strains were then washed with fresh LB broth thrice and spread onto LB-kanamycin agar plates after incubation. After culturing at 37 °C, 20 colonies was picked up randomly and their plasmids were isolated by miniprep. DNA sequencing was used to indentify the mutations in *rpoD*. To remove any possible interfrence, the mutant *rpoD* was backcloned into fresh pKSC plamid, and then transformed back to fresh *E. coli* DH5α. The strain showing the best improvement of acid tolerance was chosen as the greatest mutant from Round 1 and was respectively cultured under pH decreasing from 7.0 to 3.0. The above procedure was repeated for the three rounds of mutagenesis used in selecting a mutant strain of highest enhancement of acid resistence.

After above experiments, each overnight colony (both mutant and control) was inoculted into fresh improved LB-kanamycin broth to grow up to OD_{600}~0.3, and then pH of the mediums were regulated from 3.17, 3.62, 4.15–6.86. Strain growth were recorded by monitering its absorbance at 600 nm.

2.4. Transcriptome analysis by RNA-seq

Overnight strains (both the control and Mutant VII) were inoculated into imprved LB-kanamycin broth to grow up to OD_{600}~0.3. pH of the mediums were regulated to 4.15 for 2 h and then the strains were incubated into fresh LB-kanamycin broth (pH ~7.0). When they were grown to an OD of 0.6–0.8, their total RNA were extracted with RNeasy Mini Kit (Qiagen, Germany). RNA-Seq services were provied by BGI tech utilising Illumina HiSeq2000 (Marioni et al., 2008). Gene expression difference analysis was mainly represented by the analysis of Gene Ontology and KEGG Pathway. Cluster software and Java Treeview software were utilized to perform cluster analysis of gene expression patterns. The P-values were corrected by FDR(False Discovery Rate) control. To permit statistical confidence in differential gene expression, each sample had three biological replicates.