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RESEARCH PAPER

Development of a 3-hydroxypropionate resistant *Escherichia coli* strain

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ABSTRACT

3-hydroxypropionate (3HP) is an important platform chemical, and its biosynthesis is severely restricted by the toxicity of 3HP on cell growth and survival. To improve *Escherichia coli* resistance to 3HP and reduce the total production cost in industrial applications, we have identified variations in protein expression level exposed to sub-lethal concentration of this chemical using 2-dimensional gel electrophoresis. Under 3HP stress, the amount of 46 proteins was increased while the amount of 23 proteins was reduced. According to the proteomic results, overexpression of some identified proteins significantly increased the *E. coli* survival rate under 3HP stress. This study shed light on clues for developing *E. coli* strains with higher resistance to 3HP toxicity and lower production cost for industrial applications.

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Introduction

3-hydroxypropionate (3HP) is an industrially attractive chemical, and listed as one of top value added chemicals from biomass by US Department of Energy.[1] 3HP can be readily converted to acrylate, acrylamide, and 1,3-propanediol, and those have a combined current market value of about $10 billion. This important platform chemical can be produced either by chemical or biological processes, and the biological production of 3HP is more feasible from technical and environmental aspects. Several attempts were reported to produce 3HP in engineered *Escherichia coli* strains.[2-5]

Despite the promising progress, 3HP bioproduction still has many problems. The toxic effect of 3HP on cell growth and survival was considered as a key barrier to commercialization.[6] It is mainly resulted from the increase in both proton and associated anion concentrations during the process of 3HP production. In general, the concentrations of some organic acids including 3HP have already shown toxicity before reaching commercial levels.[7] For 3HP production, toxicity starts only at the concentration of 200 mM, which is too low for its commercial production.[8,9] *E. coli* has the ability to remain viable under abroad range of pH conditions between pH= 2-8. It is due to the different acid resistance (AR) systems that have been identified to counteract the acid stress.[10] Although most AR systems are activated in stationary phase, the percent survival is still too low.[11] In industrial fermentations, 3HP production is usually performed under pH controlled conditions. Large volumes of base titrant are needed to maintain a constant pH, leaving the final acid molecule in the undissociated form. Following production under this condition, large amounts of acid must be added to recover the acid in the protonated form.[12] Metabolic engineering of 3HP production strains, making fermentation at low pH, will avoid the additional consumption of acid and base titrants and lower the total production cost. So it is valuable to develop *E. coli* with higher resistance to 3HP toxicity.

In this study, we analyzed the differentially expressed proteins of *E. coli* BL21(DE3) strain under 3HP stress by 2DE. Some up-regulated proteins were overexpressed to improve the bacterial resistance to 3HP toxicity, and the function of identified proteins were also discussed.

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Results and discussion

Effects of 3HP on E. coli *proteome*

Cell cultures of *E. coli* BL21(DE3) strain, in the exponential phase of growth in minimal medium, were challenged with different concentration of 3HP, and growth status was monitored using OD600. Compared with the strain without challenge, 3HP with the concentration of 1g/L and 2g/L slightly repressed the growth of *E. coli* strain, and the cell growth was significantly inhibited by 10 g/L of 3HP. When the cells were challenged with 5 g/L 3HP, only slight increase of culture OD600 was observed in the following several hours (Fig. 1). So 5 g/L 3HP which led to the pH of medium to about 4.0 was selected and used in proteomic study.

To obtain insights into the *E. coli* global response to 3HP, the proteomes of *E. coli* cells with and without 3HP stress were compared using 2-dimensional gel electrophoresis (2DE). The amount of 80 protein spots varied following 3HP challenge, and those spots were identified using trypsin digestion and MS analysis. The expression level of 46 proteins increased, and 23 proteins corresponding to 25 spots were down-regulated under 3HP stress. A minimal threshold variation of 2- (for upregulated) and 0.5-fold (for downregulated) was considered significant only when the observed differences were consistently observed in 3 independent experiments. The identified proteins were clustered into functional groups, according to the biological processes in which they are involved, and are presented and discussed below (Fig. 2 and Table S1).

Proteins involved in amino acids metabolism

During adaptation to 3HP, the amount of 16 proteins involved in amino acid biosynthesis changed: 13 proteins were up-regulated while 3 proteins were down-regulated. Five enzymes in superpathway of chorismate metabolism including AroF, TrpB, TrpC, TyrA and TyrB were upregulated under 3HP stress, in line with the previous study,[13] Chorismate is the principle common precursor of the aromatic amino acids tryptophan, tyrosine and phenylalanine, as well as the essential compounds tetrahydrofolate, ubiquinone-8, etc.

An increased quantity of key enzyme DapD in lysine biosynthesis and the enzymes ArgG and ArgH associated with arginine metabolism were also observed in response to 3HP, probably resulting in elevated cellular concentrations of lysine and arginine. Interestingly, *E. coli* has proton-consuming AR systems dependent on extracellular arginine and lysine, respectively.[14] However, no arginine and lysine was supplemented in minimal medium in our study. It was presumed that *E. coli* can also lower internal pH by decarboxylation of endogenous amino acids.

CysJ and CysN belonging to the cysteine biosynthetic pathway were also found to be up-regulated

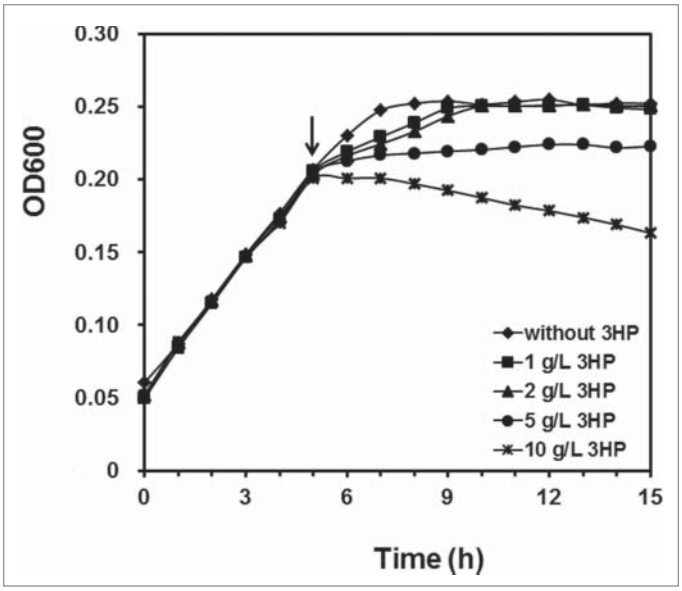

Figure 1. Growth curve of *E. coli* BL21(DE3) in minimal medium with different concentration of 3HP. The growth curves presented are representative of at least 3 independent experiments. The arrow indicate the time of 3HP addition.

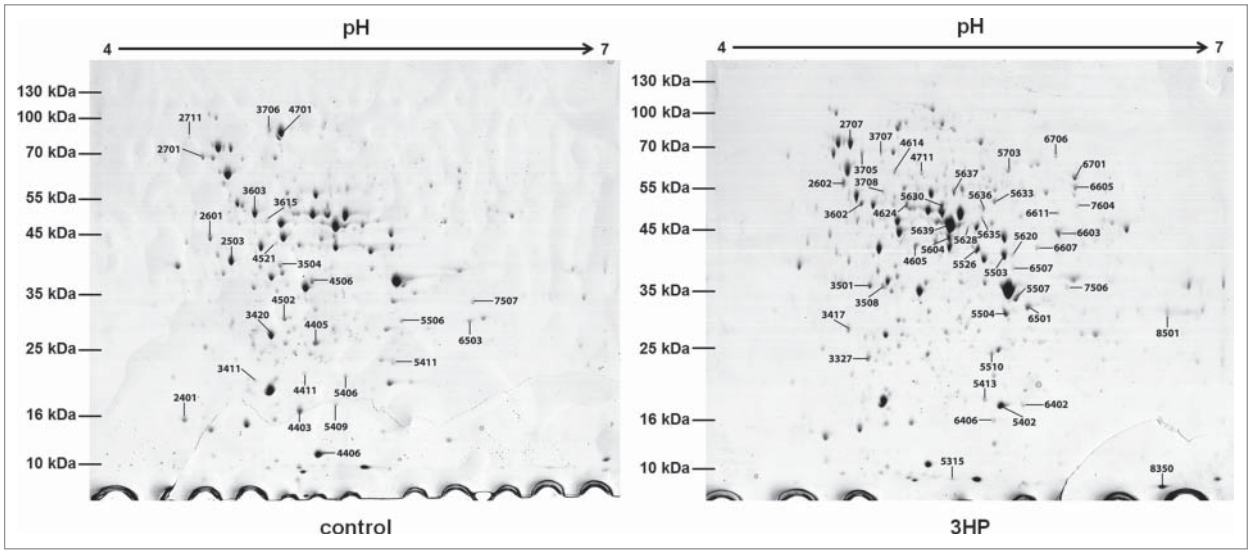

Figure 2. 2D gel images of cell extracts from *E. coli* BL21(DE3) cells without and with 3HP challenge. The proteins spots varied under different conditions were labeled and identified by mass spectrum analysis.

under 3HP stress. The MetA, MetF and MetK involved in methionine metabolism were prominently downregulated, indicating decreased intracellular methionine level. Downregulation of methionine synthesis genes was also observed in *E. coli* under butanol stress.[15] As methionine is the first amino acid residue in any protein, *E. coli* could control the global protein expression by depressing methionine biosynthesis under stress conditions.

Proteins involved in energy metabolism

The enzymes involved in energetic metabolism that were upregulated under 3HP stress included Mdh, SdhA, SucD, NuoB, and NuoE. The malate dehydrogenase, Mdh, catalyzes the reversible oxidation of malate to generate oxaloacetate as part of the TCA cycle, glyoxylate cycle, and gluconeogenesis.[16] Mdh is also believed to play a role in promoting the stress-induced mutagenesis response of *E. coli*.[17] SucD is the α subunit of succinyl-CoA synthetase, which catalyzes the only substrate-level phosphorylation reaction in the TCA cycle.[18]

Some components of the electron transport chain, including NADH dehydrogenase I subunits NuoB and NuoE and succinate dehydrogenase subunit SdhA, presented an increased expression level under 3HP stress. The NADH dehydrogenase I oxidizes NADH to NAD, concomitantly pumping $4H^+$ into periplasm and transferring 2 electrons to the quinone pool.[19] The succinate dehydrogenase complex catalyzes the oxidation of succinate to fumarate and transfers 2 electrons to the quinone pool,[20] from which the cytochrome *bo* oxidase accepts electrons and pumps out 2 protons for each electron.[21] This observation is consistent with previous reports,[22,23] and the up-regulation of enzymes in electron transport chain would help the cells to counteract increased internal acidity by directly exporting protons.

Proteins involved in ATP biosynthesis

The abundance of 6 enzymes involved in ATP biosynthesis, including AtpA, PurB, PurD, PurH, PurK, and purT, was found to increase with 3HP supplementation. It was reported that ATP was required for acidic resistance in *E. coli*. Mutation of genes in ATP biosynthetic pathway resulted in decreased cellular content of ATP and decreased *E. coli* survival rate at pH 2.5.[24,25] ATP participates in many metabolic processes as a major substrate for energy supply, and an ATP-dependent DNA repair system was believed to contribute to *E. coli* acid resistance under extremely acidic conditions.[24]

Testing of gene-specific 3HP tolerance

To improve *E.coli* tolerance to 3HP, 12 genes were selected and cloned into vector pTrcHis2B according to the above proteomic results, and these recombinant plasmids and empty vector were transformed into BL21(DE3) and DH5α strains, respectively. SDS-PAGE showed that the

recombinant proteins were clearly expressed and noted distinct bands of the expected size in bacterial extracts of the recombinant strains when compared to the control strain (Fig. S1). The resultant strains were used to determine the survival rate under 3HP stress. It is clearly obvious in the early stage after 3HP supplementation to culture, the cell growth will be resumed after a period of adaptation to the presence of 3HP.[26] So 30 min 3HP shock was selected and used in testing of gene-specific 3HP tolerance. As shown in Fig. 3 and Table S3, most BL21(DE3) strains showed increased tolerance to 3HP, and the survival rates were between 67% and 85%, while the strain carrying empty vector presented a survival rate of 47% after 30-min challenge of 5 g/L 3HP at pH4. DH5α strains showed similar increased tolerance to 3HP as BL21(DE3) strains. However, the strain with *proA* overexpression was more susceptible to 3HP. The gene *proA* encodes glutamate semialdehyde dehydrogenase catalyzing the second reaction in proline biosynthesis pathway.[27] This enzyme interacts with γ-glutamyl kinase ProB, the first enzyme in proline pathway, to form a multimeric enzyme complex,[28] explaining the uselessness of *proA* overexpression. So, overexpression of some specific genes can significantly improve the survival rate of *Escherichia coli* under 3HP stress. In addition, the expression level of some genes is down-regulated under 3HP stress, knockout of these genes may be another way to enhance 3HP resistance. Besides that, there are other effective acid resistance systems, such as the Ni-dependent urease system of *Helicobacter*.[29] It may be helpful to improve 3HP resistance if the system is introduced into *Escherichia coli*. Future work will focus on these aspects to further improve *E.coli* tolerance to 3HP.

Regulation of 3HP-induced genes

Transcriptional regulation of genes responding to 3HP stress was reviewed and summarized in Table 1. There are several global regulators involved in the 3HP stress response, including CRP, Fis, ArcAB, Fnr, and Fur. The cAMP receptor protein CRP regulates many genes associated with catabolism of secondary carbon sources,[30] and functions in AR1 system along with alternative signal factor σ^{s10}. The Fis protein can organize and maintain the nucleoid structure through direct DNA binding and by modulating synthesis of gyrase, topoisomerase I and other proteins, and transcription of approximately 21% of *E. coli* genes is modulated directly or indirectly by Fis.[31] The two-component system ArcAB and the regulatory protein Fnr are both related with gene expression under anaerobic conditions. Some Arc- and Fnr-repressed genes were up-regulated in this study, implying that the oxidative level was elevated under 3HP stress. The Fur protein is a sensor of intracellular iron concentration, and activates transcription of genes involved in ferric uptake when the cellular Fe^{2+} concentration is below physiological requirement. All these regulation systems would be ideal targets in further development of 3HP-resistant *E. coli* strain.

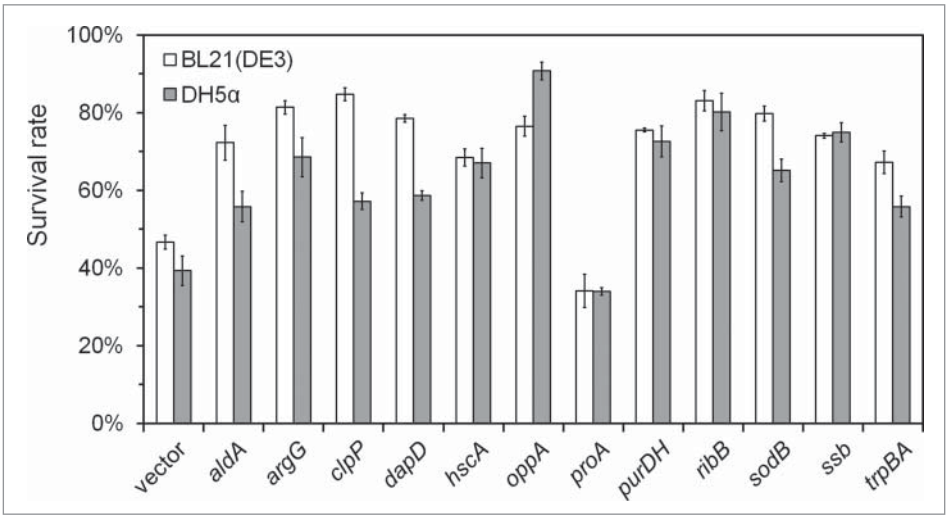

Figure 3. The survival rates of *E.coli* BL21(DE3) and DH5α strains with overexpression of identified individual genes under 3HP stress.

Table 1. Regulation of gene changed under 3HP stress.

	CRP	Fis	ArcAB	Fnr	Fur
Up-regulated					
aldA	A		R	R	
argG	A				
fbaA	A				
gatA	A		R		
gatZ	A		R		
glnA	A/R	A			
glpK	A				
glpQ	A	A		R	
manX	A				
mdh	A		R		
nuoB		A	R	R	
nuoE		A	R	R	
oppA			R		R
rbsK	A				
sdhA	A		R	R	A
sodB	R			R**	A
ssb			R		
sucD	A		R	R	A
Down-regulated					
crr	A/R				
fepA					R
fhuE					R
gnd					R
gpmA				R**	R
luxS	R*				
metK	R				
ompX	R*			R	
osmY	R	R			
ptsI	A/R				

A, transcription of this gene is activated by corresponding regulator; R, transcription of this gene is repressed by corresponding regulator.
* Mediated by small regulatory RNA CyaR.
**Mediated by small regulatory RNA FnrS.

Conclusion

In this study, variations in protein expression level in *E. coli* under 3HP stress were identified by comparative proteomics. Exposed to sub-lethal 3HP concentration, *E. coli* increased the expression level of 46 proteins while the amount of 23 proteins was repressed. The upregulated proteins were classified into several categories based on their functions, and the 3 largest categories are amino acids metabolism, energy metabolism, and ATP biosynthesis. According to the proteomic results, overexpression of some identified proteins significantly increased the survival rate of *E. coli* under 3HP stress. This study provides valuable information for developing *E. coli* strains with higher resistance to 3HP, which will significantly reduce the production cost and promote 3HP biosynthesis in the future.

Materials and methods

Determination of sub-lethal concentration of 3HP

E. coli BL21(DE3) was incubated with shaking at 37°C in minimal medium, which contains 10 g glucose, 1.5 g KH_2PO_4 , 3 g $(NH_4)_2SO_4$, 1 g citric acid, 1 g citrate sodium, 1.9 g KCl, 3 g $MgSO_4$, 0.138 g $FeSO_4{\cdot}7H_2O$, 45 mg vitamin B_1, and 1 ml of trace element solution per liter. The trace element solution contained (per liter): 0.37 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 2.47 g H_3BO_4, 1.58 g $MnCl_2{\cdot}4H_2O$, 0.29 g $ZnSO_4{\cdot}7H_2O$, 0.25 g $CuSO_4{\cdot}5H_2O$. The different concentrations of 0g/L, 1g/L, 2g/L, 5g/L, 10g/L of 3HP were added to the bacterial culture after incubated 6 h. The OD_{600} of the culture was measured and determined the sub-lethal concentration of 3HP.

Preparation of protein extracts

The strain was grown overnight with shaking at 37°C in minimal medium. Then it was diluted 1:50 into fresh medium and grown until OD_{600}~0.4. 3HP was added to final concentration of 5 g/L, and culture without 3HP addition was used as control. The cells were grown for another for 4 h, and collected by centrifugation. The cell pellet was washed twice with 30 mM phosphate potassium buffer pH7, and cellular proteins from the pellet were solubilized as described previously.[32]

Two-dimensional electrophoresis and protein identification

Protein sample of 0.5 mg was loaded at the anodic end of IPG gel strip (pH4–7, 24 cm, GE Healthcare) by sample cup, and the isoelectrofocusing was carried out in an Ettan IPGphor 3 electrophoresis unit (GE Healthcare) for 100 kVh at 16°C according to manufacturer's instructions. For the second dimension, vertical slab SDS-PAGE (12%) was run in an Ettan DALTsix electrophoresis unit (GE Healthcare). Gels were stained with colloidal Coomassie blue G250,[33] and scanned with an Image Scanner III (GE Healthcare). PDQuest (Bio-Rad Laboratories) was used for image analysis. Protein spots of interest were excised from the gels and subjected to in-gel trypsin digestion and a maXis UHR-TOF (Bruker Daltonics) analysis. For database search, an in-house Mascot server (http://www.matrixscience.com) was used. Scores calculated by the Mowse scoring algorithm in Mascot ($p<0.05$) were considered as positive identifications.

Clone construction

PCR was used to amplify the genes from *E. coli* BL21 (DE3) genomic DNA, and the PCR product was digested with restriction enzymes and ligated into the multiple cloning site of pTrcHis2B vector (Invitrogen). The ligation product was then transformed into chemically component *E. coli* DH5α cells, plated on LB plate containing ampicillin, and incubated at 37°C overnight. To confirm the insertion of positive transformants, plasmids were isolated and sequenced. All primers used in this study were listed in Table S2.

Recombinant proteins expression and gel electrophoresis analysis

E. coli BL21(DE3) strains carrying pTrcHis2B vector or other recombinant plasmids were grown overnight in minimal medium at 37°C. These cultures were diluted 1:50 into fresh medium, grown at 37°C to an OD_{600} of 0.3–0.4, and induced with 0.5 mM IPTG. After 1 h incubation, recombinant proteins were extracted and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Killing assay

Killing assay was carried out in *E. coli* BL21(DE3) and DH5α strains. The strains cultivation and recombinant proteins expression were the same as mentioned above. When the cells were induced with 0.5 mM IPTG and further incubated after 1 h, 3HP was added into the medium to final concentration of 5 g/L, and the cells were incubated for 30 min. Then the cultures were diluted and plated onto LB plate with ampicillin. The number of colony-forming units (CFU) was counted after overnight incubation at 37 °C. The percentage survival was calculated as follows: survival (%) = CFU of treated culture/CFU of untreated culture × 100.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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