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Proteomic Analysis of L-isoleucine Production by *Corynebacterium glutamicum*

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***Corynebacterium glutamicum* JHI3-156 is an L-isoleucine producing strain, but its metabolic mechanism is not fully understood. In this work, total proteins extracted from JHI3-156 and the wild type *Corynebacterium glutamicum* ATCC 13869 were comparatively analyzed by two-dimensional electrophoresis and MALDI-TOF mass spectrometry. About 750 protein spots were visualized on two-dimensional gels from both samples. Compared with the wild type ATCC13869, 82 proteins were up-regulated and 123 proteins were down-regulated in JHI3-156. There were 181 proteins observed only in ATCC13869 and 197 proteins observed only in JHI3-156. 13 protein spots from the ATCC13869 gel and 18 protein spots from the JHI3-156 gel were chosen and further analyzed by MALDI-TOF mass spectrometry. These proteins are involved in cell growth, L-isoleucine biosynthesis and stress response. This study provided some clues to understand the molecular and metabolic mechanisms that lead to the increased L-isoleucine production in *Corynebacterium glutamicum*.**

Key words: *Corynebacterium glutamicum*; proteomic analysis; L-isoleucine; two-dimensional electrophoresis.

L-isoleucine is one of three branched-chain amino acids produced industrially by *Corynebacterium glutamicum* and used as food additives, pharmaceuticals, and animal feed supplements[1]. L-isoleucine producing *C. glutamicum* strains were first obtained by multiple rounds of random mutagenesis[2,3], and modified by metabolic engineering and fermentation engineering to increase the yield of L-isoleucine[4-6]. Since whole-cell random mutagenesis accumulates unknown mutations which might impair the performance of the organism, the wild type *C. glutamicum* has been improved by metabolic engineering to produce L-isoleucine[7-14]. Overexpression of key enzymes in the biosynthesis pathway of L-isoleucine usually increases the L-isoleucine production in wild-type *C. glutamicum*[15], but sometime it does not work. For example, simultaneous overexpression of homoserine dehydrogenase, homoserine kinase and threonine dehydrogenase could increase L-isoleucine production by 385%[11], but overexpression homoserine dehydrogenase alone was lethal to the cell[16,17] possibly because of the intracellular accumulation of L-threonine and L-homoserine. To improve the yield of L-isoleucine production in wild type *C. glutamicum* is not easy.

Analyzing the globe genetic changes in industrial L-isoleucine producing strains by using comparative proteomics would provide novel target proteins for strain improvement. These target proteins might not be involved in L-isoleucine biosynthesis pathway but play important roles for L-isoleucine production. Several industrial amino acids production strains have been analyzed by

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proteomic methods[18-21], however, systematic analysis on L-isoleucine production strains of *C. glutamcium* has not been carried out.

In this study, a comparative proteomics analysis on an L-isoleucine producer *C. glutamicum* JHI3-156 and its wild type strain *C. glutamicum* ATCC13869 was performed. The results provided useful information for increasing L-isoleucine production in *C. glutamicum*.

MATERIALS AND METHODS

Fermentation of *C. glutamicum* ATCC13869 and JHI3-156

C. glutamicum ATCC13869 and JHI3-156 were grown at 30 °C. Cells for inoculation were grown for 36 hours on agar plates containing rich medium (5 g/L glucose, 10 g/L beef extract, 10 g/L protein, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0). One loop of colonies was served as the inoculum for the precultures. The precultures were grown for 18 hours in 500-mL baffled shake flasks conatining 50 mL seed medium (25 g/L glucose, 1.25 g/L urea, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.01 g/L $MnSO_4 \cdot H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.001 g/L biotin, and 0.001 g/L thiamine, pH 7.0), and used as the inoculum for the batch cultures with an initial optical density at 562 nm (OD_{562}) adjusted to 0.2. The batch fermentations were performed in a 3-L fermentor (New Brunswick Scientific BioFlo 110, USA) containing 1.5 L fermentation medium (120 g/L glucose, 40 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.3 g/L $MgSO_4$, 0.01 g/L $MnSO_4 \cdot H_2O$, 0.01 g/L, $FeSO_4 \cdot 7H_2O$, 0.001 g/L biotin, 0.001 g/L thiamine, 1 g/L yeast extract and 1 g/L). The pH was controlled at 7.0 by automatic addition of 50% NH_4OH solution. The dissolved oxygen level was controlled by adjusting the agitation speeds (400 rpm for 4 hours and 600 rpm afterwards) and the aeration rate (1.5 vvm).

Samples were taken every 4 hours to determine cell density, residual glucose, and amino acid concentrations. The cell density was determined by measuring the OD_{562} with UV-1800 spectrophotometer (Shimadzu, Japan). The residual glucose was measured by glucose-glutamate analyzer SBA-40C (Jinan, China). The levels of L-isoleucine and other amino acids were analyzed by method[22].

Extraction of total proteins from *C. glutamicum* ATCC13869 and JHI3-156

When reached the late exponential phase, cells were harvested and washed three times with 40 mM Tris-HCl, pH 7.5. The pellets were grounded to fine powder in liquid N_2, and suspended in phosphate buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 10 mg/ml dithiothreitol (DTT). The suspension was shaken at 150 rpm at 4 °C for 30 min. The supernatants containing proteins were collected by centrifugation at 14000 rpm for 30 min. Proteins were precipitated overnight at -20 °C in 5 volumes of ice-cold solvent of trichloroacetic acid and acetone (v/v; 1:1), and then collected by centrifugation at 14000 rpm for 30 min. The precipitates were rinsed twice with 90% acetone, air-dried and resuspended in the rehydration buffer (8 M urea, 2 M thiourea, 0.5% CHAPS, 0.52% immobilized pH gradient buffer, pH 4–7, 0.02% bromphenol blue, 1% DTT). The concentrations of proteins were determined by using Bio-Rad protein assay kit.

Dimensional electrophoresis of proteins from *C. glutamicum* ATCC13869 and JHI3-156

Firstly, 1 mg protein samples were loaded on 17-cm strips (pH gradient 4-7) and rehydrated for 12 hours at 20 °C. The isoelectric focusing was performed by using Multiphor II (Amersham) at 20 °C. The volts were applied as the following: 2 hours increasing from 0 to 500 V, 5 hours staying at 500 V, 5 hours increasing from 500 to 3500 V, 14.3 hours staying at 3500 V, and 4 hours increasing from 3500 to 500 V. When the isoelectric focusing was done, strips were incubated for 15 min in 10 ml of 50 mM Tris-HCl buffer (pH 6.8) containing 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate, 2% DTT, and 0.02% bromphenol blue, and then for 15 min in the above buffer solution in which DTT was replaced by 2.5% iodoacetamide. Next, the strips were placed on top of the 12% polyacrylamide gels. Protein electrophoresis was performed by using Protein II Xi cell (Bio-Rad). The current used was 15 mA for the first 30 min and 30 mA afterwards. The protein gels were stained by coomassie brilliant blue G-250 solution (10% $(NH_4)_2SO_4$, 10% H_3PO_4, 0.12% G-250, and 20% methanol), and destained in 3% glacial acetic acid overnight. The protein gels were scanned by GS-800 Calibrated Densitometer (Bio-Rad). The image analysis was carried out by PDQuest version 7.3.0 software (Bio-Rad). After

spot detection and background subtraction, 2-dimensional gels were aligned and matched. The value assigned to a protein spot was calculated as a percentage of the total volumes of all detectable spots[23]. To search for the protein spot variations, the 2-dimensional electrophoresis images of the control and the sample were systematically compared, and the differentially expressed protein spots with twofold or above in magnitude and statistically significant were recorded.

MALDI-TOF MS analysis and identification of proteins

The protein spots of interest were excised from the 2-dimensional gels and transferred into 1.5-ml Eppendorf tubes. The gels were rinsed with water, destained three times with 100 µl acetonitrile solution (50% acetonitrile in 25 mM ammonium bicarbonate, pH 8.0), dehydrated in 30 µl acetonitrile for 5 min, and dried by a speed vacuum concentrator. The dry gels were mixed with 30 µl of 50 mM ammonium bicarbonate and 8 µl of 0.1 mg/ml trypsin solution, and incubated at 37 °C for 12 hours. The digested peptide fragments were extracted from the gel pieces by adding 100 µl of solution containing 50% acetonitrile and 5% trifluoroacetic acid (TFA), and vortexing for 1 hour. After centrifugation, the supernatant was transferred into a new Eppendorf tube, and the remaining peptide fragments were extracted again by the same solution. The supernatants were combined, dried, and dissolved in 0.5% TFA for further analysis.

For MALDI-TOF MS analysis, 0.3 µl tryptic pepeides were desalted using ZipTipC18 (Millipore, Bedford, MA, USA), and eluted directly onto AnchorChip plate using 0.3 µl of 4 mg/ml CHCA matrix solution containing 70% CH_3CN and 0.1% TFA (Sigma, St. Louis, USA). The AnchorChip plate with peptides samples was injected to a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). The mass spectrometer was operated under 20 kV accelerating voltage in positive reflection mode at m/z range of 700–4000 controlled by 4000 Series software (ABI, USA).

MS data were searched against the latest releases of the Swiss-Prot and non-redundant NCBI protein databases using the MASCOT search engine (version 2.0, Matrix Science, London, UK). For MALDI data, Applied Biosystems' GPS Explorer 3.5 software was employed to generate peak lists and automate MASCOT searches. The following search parameters were chosen: maximum missed cleavages of 1, peptide mass tolerance of ±0.1 Da, cysteine carbamidomethylation as fixed modification, and methionine oxidation as optional. Positive identifications were accepted when protein score confidence index was more than 95%, when the candidate agreed with the estimated isoelectric point and molecular weight from the 2-dimensional gel, and when more than five peptides matched and the sequence coverage were larger than 20%.

RESULTS

Comparison of the 2-dimensional electrophoresis of total proteins from *C. glutamicum* JHI3-156 and ATCC13869

C. glutamicum JHI3-156 is an L-isoleucine production strain obtained by multiple rounds random mutagenesis. After 72 h of bath fermentation, L-isoleucine reached 102.7 mM in JHI3-156, but only 2.1 mM in wild type ATCC13869 (Fig. 1A). JHI3-156 produced more L-aspartic acid, L-lysine, L-threonine, and L-glycine than ATCC13869 (Fig. 1A). L-aspartic acid and L-threonine are precursors for L-isoleucine biosynthesis, while L-glycine is a decomposition product of L-threonine. The levels of the other two branched-chain amino acids, L-leucine and L-valine, decreased in JHI3-156 compared with ATCC13869. Except for L-lysine and L-glycine, the major amino acid products in ATCC13869 (L-alanine, L-glutamic acid, L-proline, and L-phenylalanine) dramatically decreased in JHI3-156 (Fig. 1A). Even though they have different patterns of amino acid production, ATCC13869 and JHI3-156 showed similar growth curves (Fig. 1B). Both strains grew slowly in the first 12 hours, grew rapidly in the second 12 hours, and reached the transition phase in the third 12 hours. The final cell concentration of JHI3-156 was slightly lower than that of ATCC13869. JHI3-156 consumed glucose slightly slower than ATCC13869 in the first 36 h, but faster thereafter (Fig. 1C).

ATCC13869 and JHI3-156 cells for proteomic analysis were harvested in the late exponential phase which is believed the onset of L-isoleucine production in the fermentative processes[24]. Total proteins were extracted from the

cell and used for isoelectric focusing electrophoresis and SDS-PAGE. As shown in Fig. 2, approximately 750 spots were visualized on each gel. When compared with the control strain ATCC13869, 82 protein spots were up-regulated, and 123 protein spots were down-regulated in JHI3-156. In addition, there were 181 protein spots only observed in ATCC13869, and 197 protein spots only observed in JHI3-156.

13 protein spots from ATCC13869 and 18 spots from JHI3-156 were selected from the gel and further analyzed by MS. In JHI3-156, 4 of the 13 proteins chosen from ATCC13869 were not detected, 4 of them were upshifted, and 5 of them were downshifted. In ATCC13869, 9 of the 18 proteins chosen from JHI3-156 were not detected, 5 of them were upshifted, and 4 of them were downshifted (Fig. 3). The predicted functions of these proteins are listed in Table 1.

Predicted functions of proteins down-regulated in JHI3-156

Among the 9 proteins down-regulated in JHI3-156, 4 proteins (spots 1-4 in Fig. 2) were not detected. These 4 proteins are homologue to cytochrome c oxidase subunit II (CtaC), homoserine acetyltransferase (HAT), short chain dehydrogenase (SDH), and pyrimidine regulatory protein (PyrR), respectively (Table 1). CtaC participates in the electron transfer to oxygen for ATP formation; HAT encoded by the gene *metX* synthesizes acetyl-homoserine from acetyl-CoA and homoserine[25]; SDH catalyzes NADP(H)-dependent oxidation/reduction reactions; PyrR negatively control the expression of *pyrH* gene whose product is involved in pyrimidine biosynthesis[26]. Among the other 5 proteins down-regulated in JHI3-156 (spots 5-9 in Fig. 2), 3 proteins are homologue to inositol monophosphatase family protein (IMP), phosphoribosyl-formylglycinamidine synthase subunit I (PurQ),

Table 1. Differentially expressed proteins identified in L-isoleucine producer *C. glutamicum* JHI3-156, compared with the control strain ATCC13869

Protein Spot No.[a]	Proposed Genes	Description	MW (Da)/pI	Accession No.
1	*ctaC*	Cytochrome c oxidase subunit II (CtaC)	39722/4.77	gi|15430575
2	*metX*	Homoserine acetyltransferase (HAT)	41419/5.39	gi|37515389
3	*ltdh*	Short chain dehydrogenase (SDH)	25414/4.9	gi|62391042
4	*pyrR*	Pyrimidine regulatory protein (PyrR)	20939/4.93	gi|19552825
5	*suhB*	Inositol monophosphatase family protein (IMP)	27550/5.06	gi|19552072
6	*cgR-0973*	Hypothetical protein cgR_0973	24344/5.94	gi|145295029
7	*cgR-2165*	Hypothetical protein cgR_2165	18769/4.85	gi|145296248
8	*purQ*	Phosphoribosylformylglycinamidine synthase subunit I (PurQ)	23639/5.07	gi|19553785
9	*sdhB/frdB*	Succinate dehydrogenase/fumarate reductase iron-sulfur subunit (SdhB/FrdB)	27258/5.4	gi|19551618
10	*aph*	Aminoglycoside 3'-phosphotransferase (APH)	29769/5.16	gi|67476481
11	*metE*	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MHM)	85104/5.71	gi|836660
12	*PrpC2*	Methylcitrate synthase (PrpC2)	42625/5.77	gi|19551889
13	*sod*	Superoxide dismutase (SOD)	21320/5.58	gi|281178728
14	*rplI*	50S ribosomal protein L9 (L9)	15761/5.78	gi|82779401
15	*yodA*	Metal-binding protein yodA (YodA)	23399/5.98	gi|170172434
16	*gnd*	6-Phosphogluconate dehydrogenase (6-PGD)	51563/5.06	gi|16129970
17	*cysK*	Cysteine synthase A (CysA)	34525/5.83	gi|15802947
18	*serS*	Seryl-tRNA synthetase (SerRS)	48673/5.28	gi|82777585
19	*yfiA*	Ribosome-associated protein Y (pY)	24558/5.41	gi|19551984
20	*fusA*	Elongation factor G (EF-G)	77690/5.24	gi|194435123
21	*cgR-2826*	Hypothetical protein cgR_2826	54839/4.65	gi|145296926
22	*zenU*	High-affinity zinc transporter (ZnuA)	33942/5.52	gi|157156162

[a]Protein spot No. The same numbers as shown in Fig. 2 and Fig. 3.

and succinate dehydrogenase/fumarate reductase iron-sulfur subunit (SdhB/FrdB), respectively (Table 1). IMP dephosphorylates inositol 3-phosphate to generate inositol whose derivatives are related to cell growth[27]; PurQ is involved in the purine pathway (fourteh step of the pathway); SdhB/FrdB is a part of catalytic domain of succinate-ubiquinone oxidoreductase (SQR) and menaquinol-fumarate oxidoreductase (QFR). SQR participates in tricarboxylic acid cycle and QFR is used for anaerobic respiration. The other two proteins down-regulated in JHI3-156 (spots 6-7 in Fig. 2) are encoded by cgR-0973 and cgR-2165, and do not show significant homology to any known proteins. Their roles in relation to JHI3-156 L-isoleucine overproduction need to be elucidated.

Predicted functions of proteins up-regulated in JHI3-156

There were 13 of the chosen proteins up-regulated in JHI3-156, in which 9 proteins were not detected in ATCC13869 (spots 10-18 in Fig. 2). These 9 proteins are homologue to aminoglycoside 3'-phosphotransferase (APH), 5-methyltetrahydropteroyltriglutamate-homocysteine methyl transferase (MHM), methylcitrate synthase (PrpC2), superoxide dismutase (SOD), 50S ribosomal protein L9, metal-binding protein YodA, 6-phosphogluconate dehydrogenase (6-PGD),

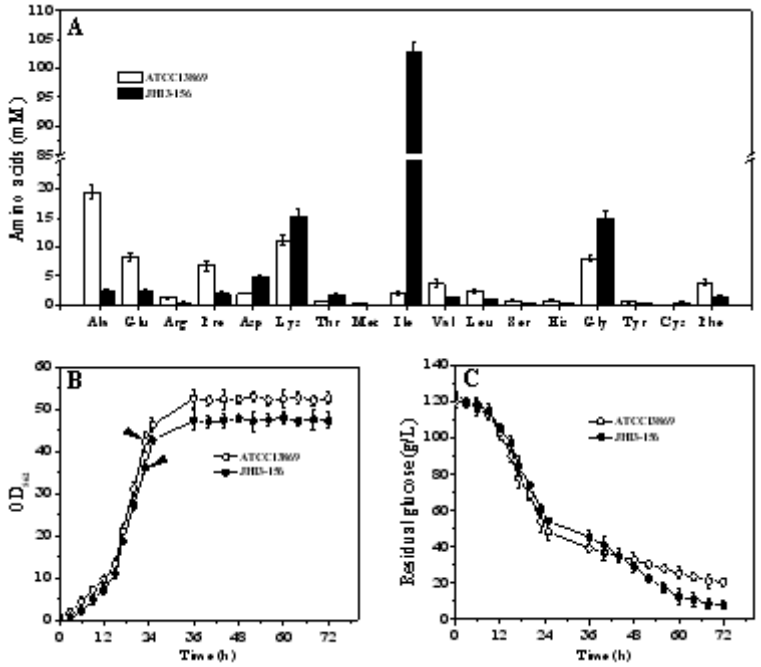

Fig. 1. Comparison of amino acids levels(A), biomasses(B), and residual glucose (C) in batch fermentation of the L-isoleucine producer *C. glutamicum* JHI3-156 and the control strain ATCC13869. The harvest point for the cells are indicated by black arrows. Ala, L-alanine; Glu, L-glutamic acid; Arg, L-argnine; Pro, L-proline; Asp, L-aspartate; Lys, L-lysine; Thr, L-threonine; Met, L-methionine; Ile, L-isoleucine; Val, L-valine; Leu, L-leucine; Ser, L-serine; His, L-histidine; Gly, L-glycine; Tyr, L-tyrosine; Cys, L-cysteine; Phe, L-phenylalanine. Error bars indicate the standard deviations from three parallel samples

cysteine synthase A (CysA), and seryl-tRNA synthetase (SerRS), respectively (Table 1). APH confers resistance to many aminoglycoside antibiotics; MHM encoded by *metE* catalyzes the terminal step in methionine biosynthesis and in the alternative pathway from methionine to homocycteine[28]; PrpC2 condenses propionyl-CoA and oxaloacetate to 2-methylcitrate which is further degraded to pyruvate and succinate[29,30] that can be used to synthesize oxaloacetate through the trichloroacetic acid cycle or the anaplerosis reaction; SOD is a key enzyme in oxygen defense systems for disrupting superoxide anion into oxygen and H_2O[31]; L9 is involved in protein folding

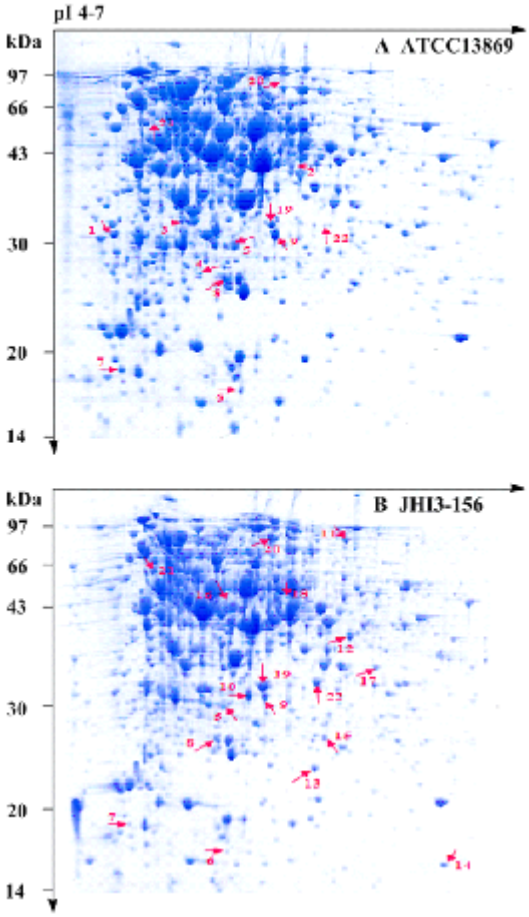

Fig. 2. 2-DE comparison of the total proteins from wild type *C. glutamicum* ATCC13869 (A) and the L-isoleucine producer JHI3-156 (B). The numbers refer to the spot identification used in Table 1 and Fig. 3

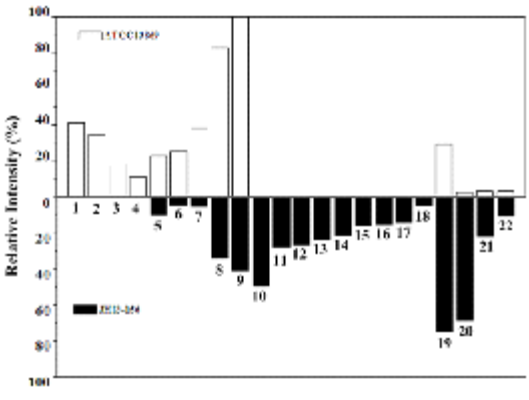

Fig. 3. Relative expression levels of 22 proteins identified by proteomic analysis.

and stability; YodA might be related to stress response; 6-PGD encoded by *gnd* catalyzes the third step in pentose phosphate pathway coupled with NADPH regeneration; CysA encoded by *cysK* catalyzes the reaction from O-acetylserine to cysteine in cysteine biosynthetic pathway; SerRS plays an essential role for aminoacylating the cognate tRNASer with serine. The other 4 proteins up-regulated in JHI3-156 were detected in ATCC13869 (spots 19-22 in Fig. 2); 3 of them are homologue to ribosome-associated protein Y (pY), elongation factor G (EF-G), and high-affinity zinc transporter periplasmic component (ZnuA), respectively (Table 1). The 4th protein encoded by cgR-2826 does not show significant homology to any known proteins. pY inhibits protein synthesis at the elongation stage of translation[32]; EF-G accelerates the translocation step of the elongation cycle[33, 34]; ZnuA participates in uptake the zinc from surrounding environment.

DISCUSSION

In *C. glutamicum*, L-isoleucine biosynthesis from L-aspartate involves 10 reactions[9]. Five rate-limited enzymes in this pathway are regulated by feedback inhibition. Aspartate kinase, homoserine dehydrogenase, and homoserine kinase are inhibited by L-threonine[7, 35, 36], threonine dehydrogenase is inhibited by L-isoleucine[9], and acetohydroxy acid synthase is inhibited by all three branched-chain amino acids. Sequence comparison of these rate-limited enzymes from JHI3-156 and ATCC13869 revealed point mutations. One amino acid mutation of threonine dehydratase (Phe383Val) in JHI3-156 showed not only enhanced activity but also completely resistance to L-isoleucine inhibition[6]. Since there were L-aspartic acid, L-lysine and L-threonine accumulated in JHI3-156 (Fig. 1A), overexpression of the rate-limited enzymes in the L-isoleucine pathway such as the feedback-resistant threonine dehydratase or acetohydroxy acid synthase in JHI3-156 led to further increase of L-isoleucine production[6]. Apart from the enzymes evolved in the biosynthesis pathway of L-isoleucine, little is known about other enzymes important for the L-isoleucine production in *C. glutamicum* JHI3-156. Therefore, the proteomic analysis was performed to identify differentially

expressed proteins which might be important for L-isoleucine production.

Several differentially expressed enzymes in JHI3-56 could lead the carbon flux to L-isoleucine production. HAT which could draw the carbon flux from homoserine to L-methionine was down-regulated; while MHM was up-regulated in order to produce enough methionine for cell growth. PrpC2 which could metabolize propionate to oxaloacetate for L-isoleucine biosynthesis was up-regulated. SdhB/FrdB was down-regulated, which could reduce the rate of tricarboxylic acid cycle, decrease the production of L-glutamic acid, L-argnine and L-proline (Fig. 1A), and shift the cellular activity from growth to L-isoleucine production.

The up-regulated 6-PGD and ZnuA in JHI3-156 may provide more cofactors NADPH and zinc to increase the production of L-isoleucine. NADPH is required by several enzymes in L-isoleucine biosynthesis pathway, it can be generated by dehydrogenases such as 6-PGD. Overexpression of these dehydrogenases could increase the supply of NADPH for the production of L-lysine[37, 38], L-glutamate[39], L-valine[40] and L-isoleucine[41]. Therefore, the up-regulated 6-PGD in JHI3-156 might generate sufficient NADPH to strength carbon flux for L-isoleucine production. Zinc serves as a cofactor for many enzymes in the cell[42-44], such as ribosomal proteins, DNA and RNA polymerases[45], DNA primases[46], RNA and protein synthesis, as well as some β-lactamases[43] that is essential for cell growth under stress conditions. Therefore, the up-regulated ZnuA in JHI3-156 might supply adequate zinc to meet the requirement for cell growth.

Bacterial mutants constructed for overproducing amino acids usually grew significantly slow[18, 47, 48], but JHI3-156 only showed slightly cell growth retard (Fig. 1B). As an aerobic bacterium, *C. glutamicum* requires oxygen as exogenous electron acceptor for respiration. The electrons are passed via the cytochrome bc_1 complex either to the aa_3-type cytochrome c oxidase with low oxygen affinity and high energetic efficiency[49], or to the cytochrome bd-type menaquinol oxidase with high oxygen affinity and low energetic efficiency[50]. The bc_1-aa_3 branch is the major electron channel for aerobic growth[49]. The cytochrome aa_3 oxidase consists of four subunits encoded by the genes *ctaD*, *ctaC*, *ctaE*, and *ctaF*[49]. Higher amino acids synthesis is required for overexpressing the cytochrome aa_3[51]. Since CtaC was not detected in JHI3-156, suggesting the respiration is a factor affecting the cell growth. In addition, the up-regulated 6-PGD, CysA, YodA, and down-regulated SdhB/FrdB in JHI3-156 might also contribute to the retarded cell growth[52, 53]. CysA and YodA could form reactive oxygen species during fermentation[54] to induce oxidative stress[55,56]. The up-regualted SOD in JHI3-156, however, might play an important role to repair the oxidative damages[31]. More detailed investigations are required to optimize flux distribution within metabolism to achieve higher production of L-isoleucine without growth impairment in *C. glutamicum*.

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REFERENCES

1. Park, J.H., Lee, S.Y. Metabolic pathways and fermentative production of L-aspartate family amino acids. *Biotech. J.*, 2010; **5**: 560-577.
2. Ikeda, S., Fujita, I., Yoshinaga, F. Screening of L-isoleucine producers among ethionine resistant mutants of L-threonine producing bacteria. *Agric. Biol. Chem.*, 1976; **40**: 511-516.
3. Kase, H., Nakayama, K. L-Isoleucine production by analog-resistant mutants derived from threonine-producing strain of *Corynebacterium glutamicum*. *Agric. Biol. Chem.*, 1977; **41**: 109-116.
4. Kelle, R., Hermann, T., Weuster-Botz, D., Eggeling, L., Krämer, R., Wandrey, C. Glucose-controlled L-isoleucine fed-batch production with recombinant strains of *Corynebacterium glutamicum*. *J. Biotechnol.*, 1996; **50**: 123-136.
5. Peng, Z.J., Fang, J., Li, J.H., Liu, L., Du G.C., Chen, J., Wang X.Y., Ning, J.F., Cai, L.M. Combined dissolved oxygen and pH control strategy to improve the fermentative production of L-isoleucine by *Brevibacterium lactofermentum*. *Bioproc. Biosyst. Eng.*, 2010;

33: 339-345.

6. Yin, L., Hu, X., Xu, D., Ning, J., Chen, J., Wang, X. Co-expression of feedback-resistant threonine dehydratase and acetohydeoxy acid synthase increase L-isoleucine production in *Corynebacterium glutamicum*. *Metab*. *Eng.*, 2012; **14**: 542-550.
7. Colon, G.E., Jetten, M.S.M., Nguyen, T.T., Gubler, M.E., Follettie, M.T., Sinskey, A.J., Stephanopoulos, G. Effect of inducible *thrB* expression on amino acid production in *Corynebacterium lactofermentum* ATCC 21799. *Appl*. *Environ*. *Microbiol*., 1994; **61**: 74-78.
8. Colon, G.E., Nguyen, T.T., Jetten, M.S.M., Sinskey, A.J., Stephanopoulos, G. Production of isoleucine by overexpression of *ilvA* in a *Corynebacterium lactofermentum* threonine producer. *Appl*. *Microbiol*. *Biotechnol*., 1995; **43**: 482-488.
9. Morbach, S., Sahm, H., Eggeling, L. Use of feedback-resistant threonine dehydratases of *Corynebacterium glutamicum* to increase carbon flux towards L-isoleucine. *Appl*. *Environ*. *Microbiol*. 1995; **61**: 4315-4320.
10. Morbach, S., Kelle, R., Winkels, S., Sahm, H., Eggeling, L. Engineering the homoserine dehydrogenase and threonine dehydratase control points to analyse flux towards L-isoleucine in *Corynebacterium glutamicum*. *Appl*. *Microbiol*. *Biotechnol*., 1996; **45**: 612-620.
11. Morbach, S., Sahm, H., Eggeling, L. L-Isoleucine Production with *Corynebacterium glutamicum*: Further Flux Increase and Limitation of Export. *Appl*. *Environ*. *Microbiol*., 1996; **62**: 4345-4351.
12. Guillouet, S., Rodal, A.A., Sinskey, A.J. Expression of the *Escherichia coli* catabolic threonine dehydratase in *Corynebacterium glutamicum* and its effect on isoleucine production. *Appl*. *Environ*. *Microbiol*., 1999; **65**: 3100-3107.
13. Guillouet, S., Rodal, A.A., An, G.H., Gorret, N., Lessard, P.A., Sinskey, A.J. Metabolic redirection of carbon flow toward isoleucine by expressing a catabolic threonine dehydratase in a threonine-overproducing *Corynebacterium glutamicum*. *Appl*. *Microbiol*. *Biotechnol*., 2001; **57**: 667-673.
14. Krömer, J.O., Heinzle, E., Schróder, H., Wittmann, C. Accumulation of Homolanthionine and Activation of a Novel Pathway for Isoleucine Biosynthesis in *Corynebacterium glutamicum* McbR Deletion Strains. *J*. *Bacteriol*., 2006; **188**: 609-618.
15. Ohnishi, J., Mitsuhashi, S., Hayashi, M., Ando, S., Yokoi, H., Ochiai, K., Ikeda, M. A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. *Appl*. *Microbiol*. *Biotechnol*., 2002; **58**: 217-223.
16. Archer, J.A.C., Solow-Cordero, D.E., Sinskey, A.J. A C-terminal deletion in *Corynebacterium glutamicum* homoserine dehydrogenase abolishes allosteric inhibition by L-threonine. *Gene*, 1991; **107**: 53-59.
17. Eikmanns, B., Kleinertz, E., Liebl, W., Sahm. H. A family of *Corynebacterium glutamicum*/*Escherichia coli* shuttle vectors for gene cloning, controlled gene expression, and promoter probing. *Gene*, 1991; **102**: 93-98.
18. Kim, Y.H., Park, J.S., Cho, J.Y., Cho, K.M., Park, Y.H., Lee, J. Proteomic response analysis of a threonine-overproducing mutant of *Escherichia coli*. *Biochem*. *J*., 2004; **381**: 823-829.
19. Li, L.Y., Wada, M., Yokota, A. Cytoplasmic proteome reference map for a glutamic acid-producing *Corynebacterium glutamicum* ATCC 14067. *Proteomics*, 2007; **7**: 4317-4322.
20. Schluesener, D., Rögnr, M., Poetsch, A. Evaluation of two proteomics technologies used to screen the membrane proteomes of wild-type *Corynebacterium glutamicum* and an L -lysine-producing strain. *Anal*. *Bioanal*. *Chem*., 2007; **389**: 1055-1064.
21. Fränzel, B., Poetsch, A., Trötschel, C., Persicke, M., Kalinowski, J., Wolters, D.A. Quantitative proteomic overview on the *Corynebacterium glutamicum* L-lysine producing strain DM1730. *J*. *Proteomics*, 2010; **73**: 2336-2353.
22. Körös, ., Varga, Z.S., Molnr, P. Simultaneous analysis of amino acids and amines as their ophthalaldehyde-ethanethiol-9-ûuorenylmethyl chloroformate derivatives in cheese by high-performance liquid chromatography. *J*. *Chromatogr*. *A*., 2008; **1203**: 146-152.
23. Choe, L.H., Lee, K.H. Quantitative and qualitative measure of intralaboratory two-dimensional protein gel reproducibility and the effects of sample preparation, sample load, and image analysis. *Electrophoresis*, 2003; **24**: 3500-3507.
24. Pfefferle, W., Möckel, B., Bathe, B., Marx, A. Biotechnological manufacture of lysine. *Adv*. *Biochem*. *Eng*. *Biotechnol*., 2003; **79**: 59-112.
25. Park, S.D., Lee, J.Y., Kim, Y., Kim, J.H., Lee, H.S. Isolation and analysis of *metA*, a methionine biosynthetic gene encoding homoserine acetyltransferase in *Corynebacterium glutamicum*. *Mol*. *Cells*, 1998; **8**: 286-294.
26. Lee, H.H., Chung, S.S., Jo, J.H. Expression of *pyrH* gene encoding UMP-kinase is regulated by direct binding of PyrR to the promoter in

Corynebacterium glutamicum. *Genet*. *Ind*. *Microorganism*, 2006; **10**: 91.

27. Brennan, P.J., Lehane, D.P. The phospholipids of *corynebacteria*. *Lipids*, 1971; **6**: 401-409.
28. Nagahara, N., Katayama, A. The mercaptopyruvate pathway in cysteine catabolism: a physiologic role and related disease of the multifunctional 3-mercaptopyruvate sulfurtransferase. *Curr*. *Med*. *Chem*., 2006; **13**: 1219-1230.
29. Brock, M., Darley, D., Textor, S., Buckel, W. 2-Methylisocitrate lyase from the bacterium *Escherichia coli* and the filamentous fungus *Aspergillus nidulans*. Characterization and comparison of both enzymes. *Eur*. *J*. *Biochem*. 2001; **268**: 3577-3586.
30. Tabuchi, T., Uchiyama, H. Methylcitrate condensing and methylisocitrate cleaving enzymes; evidence for the pathway of oxidation of propionyl-CoA to pyruvate via C7-tricarboxylic acids. *Agric*. *Biol*. *Chem*., 1975; **39**: 2035-2042.
31. Fridovich, I. Superoxide radical and superoxide dismutases. *Annu*. *Rev*. *Biochem*., 1995; **64**: 97-112.
32. Agafonov, D.E., Kolb, V.A., Spirin, A.S. Ribosome-associated protein that inhibits translation at the aminoacyl-tRNA binding stage. *EMBO Rep*., 2001; **2**: 399-402.
33. Gavrilova, L.P., Kostiashkina, O.E., Koteliansky, V.E., Rutkevitch, N.M., Spirin, A.S. Factor-free ("non-enzymic") and factor-dependent systems of translation of polyuridylic acid by *Escherichia coli* ribosomes. *J*. *Mol*. *Biol*., 1976; **101**: 537-552.
34. Southworth, D.R., Brunelle, J.L., Green, R. EFG-independent translocation of the mRNA: tRNA complex is promoted by modification of the ribosome with thiol-specific reagents. *J*. *Mol*. *Biol*., 2002; **324**: 611-623.
35. Morinaga, Y., Takagi, H., Ishida, M., Miwa, K., Sato, T., Nakamori, S., Sano, K. Threonine production by co-existence of cloned genes coding homoserine dehydrogenase and homoserine kinase in *Brevibacterium lactofermentum*. *Agric*. *Biol*. *Chem*., 1987; **51**: 93-100.
36. Dong, X., Quinn, P.J., Wang, X. Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for the production of L-threonine. *Biotechnol*. *Adv*., 2011; 29: 11-23.
37. Becker, J., Klopprogge, C., Herold, A., Zelder, O., Bolten, C.J. Wittmann, C. Metabolic flux engineering of L-lysine production in *Corynebacterium glutamicum* over expression and modification of G6P dehydrogenase. *J*. *Biotechnol*., 2007; **132**: 99-109.
38. Takeno, S., Murata, R., Kobayashi, R., Mitsuhashi, S., Ikeda, M. Engineering of *Corynebacterium glutamicum* with an NADPH-generating glycolytic pathway for L-lysine production. *Appl*. *Environ*. *Microb*., 2010; **76**: 7154-7160.
39. Georgi, T., Rittmann, D., Wendisch, V.F. Lysine and glutamate production by *Corynebacterium glutamicum* on glucose, fructose and sucrose: roles of malic enzyme and fructose-1,6-bisphosphatase. *Metab*. *Eng*., 2005; **7**: 291-301.
40. Bartek, T., Blombach, B., Zonnchen, E., Makus, P., Lang, S., Eikmanns, B.J., Oldiges, M. Importance of NADPH supply for improved L-valine formation in *Corynebacterium glutamicum*. *Biotechnol*. *Progr*., 2010; **26**: 361-371.
41. Shi, F., Huan, X.J., Wang, X.Y., Ning, J.F. Overexpression of NAD kinases improves the L-isoleucine biosyntheis in *Corynebacterium glutamicum* ssp. *lactofermentum*. *Enzyme*. *Microb*. *Tech*., 2012; **51**: 73-80.
42. Berg, J.M., Shi, Y. The galvanization of biology: a growing appreciation of the roles of zinc. *Science*, 1996; 271: 1081-1085.
43. Andreini, C., Banci, L., Bertini, I., Rosato, A. Zinc through the three domains of life. *J*. *Proteome Res*., 2006; **5**: 3173-3178.
44. Haas, C.E., Rodionov, D.A., Kropat, J., Malasarn, D., Merchant, S.S., de Crécy-Lagard, V. A subset of the diverse COG0523 family of putative metal chaperones is linked to zinc homeostasis in all kingdoms of life. *BMC Genomics*, 2009; **10**: 470.
45. Blaby-Haas, C.E., Furman, R., Rodionov, D.A., Artsimovitch, I., de Crécy-Lagard, V. Role of a Zn-independent DksA in Zn homeostasis and stringent response. *Mol*. *Microbiol*., 2011; **79**: 700-715.
46. Pan, H., and Wigley, D.B. Structure of the zinc-binding domain of *Bacillus stearothermophilus* DNA primase. *Structure*, 2000; **8**: 231-239.
47. Kisumi, M., Komatsubara, S., Chibata, I. Enhancement of isoleucine hydroxamate-mediated growth inhibition and improvement of isoleucine-producing strains of *Serratia marcescens*. *Appl*. *Environ*. *Microbiol*., 1977; **34**: 647-653.
48. Eggeling, L., Morbach, S., Sahm, H. The fruits of molecular physiology: engineering the L-isoleusine biosynthesis pathway in *Corynebacterium glutamicum*. *J*. *Biotechnol*., 1997; **56**: 167-182.
49. Niebisch, A., Bott, M. Purification of a

cytochrome bc_1-aa_3 supercomplex with quinol oxidase activity from *Corynebacterium glutamicum*. Identification of a fourth subunit of cytochrome aa_3 oxidase and mutational analysis of diheme cytochrome c_1. *J. Biol. Chem.*, 2003; **278**: 4339-4346.

50. Kusumoto, K., Sakiyama, M., Sakamoto, J., Noguchi, S., Sone, N. Menaquinol oxidase activity and primary structure of cytochrome *bd* from the amino-acid fermenting bacterium *Corynebacterium glutamicum*. *Arch. Microbiol.*, 2000; **173**: 390-397.
51. Kabus, A., Niebisch, A., Bott, M. Role of cytochrome bd oxidase from *Corynebacterium glutamicum* in growth and lysine production. *Appl. Environ. Microbiol.*, 2007; **73**: 861-868.
52. Kamada, N., Yasuhara, A., Takano, Y., Nakano, T., Ikeda, M. Effect of transketolase modifications on carbon flow to the purine-nucleotide pathway in *Corynebacterium ammoniagenes*. *Appl. Microbiol. Biotechnol.*, 2001; **56**: 710-717.
53. Gallo, G., Alduina, R., Renzone, G., Thykaer, J., Bianco, L., Eliasson-Lantz, A., Scaloni, A., Puglia, A.M. Differential proteomic analysis highlights metabolic strategies associated with balhimycin production in *Amycolatopsis balhimycina* chemostat cultivations. *Microb. Cell. Fact.*, 2010; **9**: 95-109.
54. Storz, G., Imlay, J.A. Oxidative stress. *Curr. Opin. Microbiol.*, 1999; **2**: 188-194.
55. Barriuso-Iglesias, M., Schluesener, D., Barreiro, C., Poetsch, A., Martín, J.F. Response of the cytoplasmic and membrane proteome of *Corynebacterium glutamicum* ATCC13032 to pH changes. *BMC Microbiol.*, 2008; **8**: 225.
56. Puškarova, A., Ferianc, P., Kormanec, J., Homerova, D., Farewell, A., Nyström, T. Regulation of *yodA* encoding a novel cadmium-induced protein in *Escherichia coli*. *Microbiol.*, 2002; **148**: 3801-3811.