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Rational Design of *Escherichia coli* for L-Isoleucine Production

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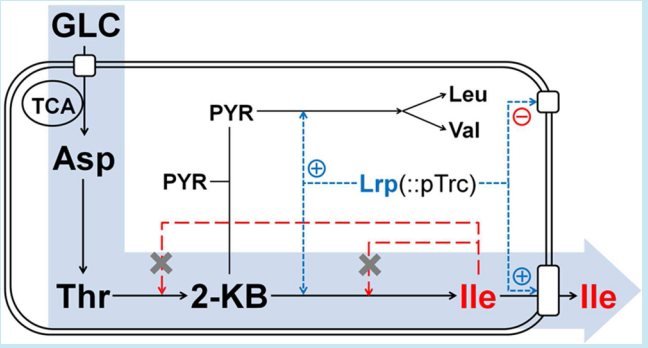
ABSTRACT: Metabolic engineering of *Escherichia coli* was performed to construct a 100% rationally engineered strain capable of overproducing L-isoleucine, an important branched-chain amino acid. The *thrABC* (encoding L-threonine biosynthetic enzymes), *ilvA* (encoding feedback-resistant threonine dehydratase), *ilvIH* (encoding feedback-resistant acetohydroxy acid synthase III), and *ygaZH* (encoding branched-chain amino acid exporter) genes were amplified by plasmid-based overexpression. The *ilvCED* (encoding L-isoleucine biosynthetic enzymes) and *lrp* (encoding global regulator Lrp) genes were also amplified by chromosomal promoter replacement in order to further increase the flux toward L-isoleucine. The final engineered *E. coli* strain was able to produce 9.46 g/L of L-isoleucine with a yield of 0.14 g/g of glucose by fed-batch culture. The overall design principles described here for the production of highly regulated product should be useful in designing strains for the production of other similar bioproducts.



KEYWORDS: *L-isoleucine, branched chain amino acid, metabolic engineering, fed-batch fermentation, regulatory network, feedback inhibition*

Amino acids are commercially important as they are widely used in food, pharmaceutical, cosmetics and animal feed industries[1−3] with annual market growth rate of 5−7% worldwide.[4] L-Isoleucine, an essential hydrophobic and branched-chain amino acid (BCAA) is currently utilized as a constituent of infusions and functional products, such as dietary products and beverage.[5] Therefore, L-isoleucine has been drawing much attention for its biotechnological overproduction. So far, most amino acid producing strains have conventionally been constructed by random mutagenesis. Despite the development of high performance producers by taking such approaches, traditional random mutagenesis causes several problems such as retarded cell growth and difficulty of changing carbon substrates when needed. Also, because of the unknown and sometimes unwanted mutations, it is rather difficult to perform subsequent strain improvement by rational engineering. Recent advances in systems metabolic engineering that integrates systems biology and synthetic biology with metabolic engineering now provides a new avenue toward the development of 100% genetically defined production strains, enabling further strain improvement easily when needed.[6,7]

Escherichia coli has been widely used for amino acid production, as demonstrated in the case of L-threonine,[8,9] L-valine,[10] L-phenylalanine,[11] L-alanine[12] and L-tyrosine.[13] *E. coli* has also been used to produce L-isoleucine. In one report, the *ilvA*, *ilvGM*, *ilvD*, and *ilvE* genes, which encode feedback-resistant threonine dehydratase, an active AHAS II, dihydroxy acid dehydratase, and BCAA transaminase B, respectively, were amplified by plasmid-based overexpression in the L-threonine production strain of *E. coli*.[14] The resulting strain was able to produce 10.0 g/L of L-isoleucine from 40 g/L of glucose.[14] This strain, with additional overexpression of feedback-resistant aspartate kinase III (encoded by mutant *lysC*), was able to produce 12.0 g/L of L-isoleucine from 40 g/L of glucose.[15] However, these strains were derived from a classic industrial L-threonine production strain, which was constructed by random mutagenesis. So far, there has been no report on the development of 100% rationally engineered *E. coli* strain for the production of L-isoleucine.

In *E. coli*, L-isoleucine is synthesized through five enzymatic reactions from L-threonine as a precursor (Figure 1).[16,17] Threonine dehydratase (encoded by *ilvA*), which is involved in the first step toward L-isoleucine synthesis, is subject to feedback inhibition by L-isoleucine.[18] Three acetohydroxy acid synthase (AHAS) isoenzymes, each of which has distinct biochemical properties and regulatory mechanisms, are key enzymes in the biosynthesis of BCAAs. Each isoenzyme encoded by *ilvBN*, *ilvGM*, and *ilvIH*, respectively, is controlled by regulatory mechanisms, such as feedback inhibition and

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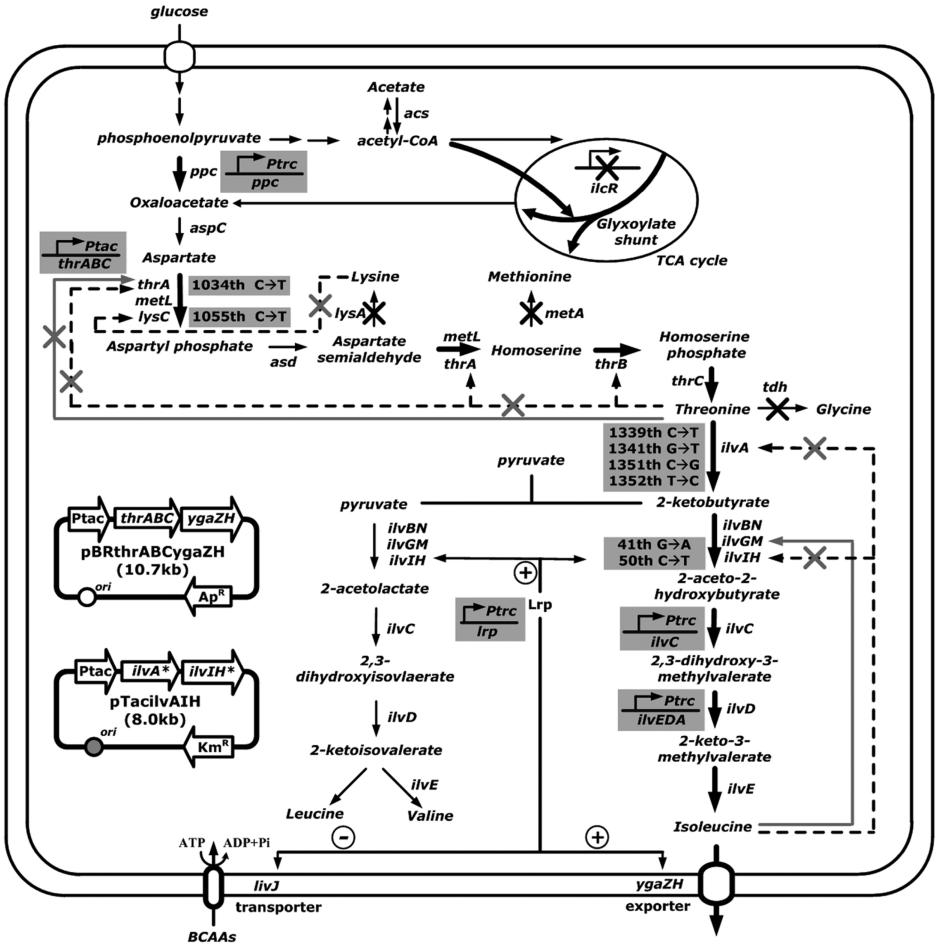

Figure 1. The biosynthetic pathways of branched-chain amino acids in *E. coli*, regulations involved, and the strategies for constructing the L-isoleucine production strain. The shaded boxes represent mutations introduced into the genome. The black Xs indicate the deletion of genes and the gray Xs indicate the removal of inhibition or repression. Dotted lines indicate the feedback inhibition. Gray lines indicate the transcriptional attenuation regulation. The thick arrows indicate the increased flux or activity by directly overexpressing the corresponding genes. The plus (+) and minus (−) symbols indicate activation and repression of gene expression by Lrp, respectively.

transcriptional attenuation regulation by BCAAs.[19] Expression of the *ilvGM* genes is controlled by transcriptional attenuation mediated by L-isoleucine.[20,21] In particular, AHAS I, encoded by the *ilvBN* genes, has a much higher affinity for pyruvate than for 2-ketobutyrate compared with the isoenzymes AHAS II and AHAS III.[22] In contrast, AHAS II and AHAS III encoded by *ilvGM* and *ilvIH*, respectively, have much higher affinity to 2-ketobutyrate, an important precursor only for L-isoleucine, than pyruvate, which is a common precursor for BCAAs.[22] It has been known that AHAS II is not expressed due to the frameshift mutation in *ilvG* in *E. coli*[23] Thus, the AHAS III seems to be more suited for L-isoleucine biosynthesis than AHAS II. Furthermore, *ilvC* encoding acetohydroxy acid isomeroreductase, *ilvD* encoding dihydroxy acid dehydratase, and *ilvE* encoding BCAAs aminotransferase are involved in the biosynthetic pathway of BCAAs including L-isoleucine.

As L-isoleucine is derived from L-threonine, the biosynthetic pathway and regulations involved for the biosynthesis of L-threonine are also important to be considered for L-isoleucine production (Figure 1). *E. coli* has three aspartate kinase (AK) isoenzymes, which catalyze the first step in L-threonine biosynthesis. The three isoenzymes AK I, II, and III, which are encoded by the *thrA*, *metL*, and *lysC* genes, respectively, are again subject to different regulations.[24] Expression of the *thrA* gene is controlled by transcriptional attenuation mediated by L-threonine.[24] The AK I and III encoded by *thrA* and *lysC* genes, respectively, are subject to feedback inhibition by L-threonine and L-lysine, respectively.[24] The homoserine kinase encoded by *thrB* is subject to feedback inhibition by L-threonine.[24]

Apparently from the complex metabolic and regulatory information described above, it is necessary to take systematic metabolic engineering approaches to develop an L-isoleucine overproducing *E. coli* strain. Here we report the development of a 100% genetically defined strain of *E. coli* efficiently producing L-isoleucine by metabolic engineering.

RESULTS AND DISCUSSION

Construction of Engineered *E. coli* Strains for the Production of L-Isoleucine. The *E. coli* TH20 strain (Table 1), which was previously constructed for the overproduction of L-threonine,[9] was further engineered to construct an L-isoleucine producing ILE01 strain. In TH20 strain, feedback inhibitions of aspartokinase I and III by L-threonine and L-lysine, respectively, were removed, and the native promoter containing the transcriptional attenuator leader region of the *thrABC* operon was replaced with the *tac* promoter. The *metA*, *lysA*, *tdh* and *iclR* genes were deleted to make more precursors available for L-threonine formation. The *ilvA* gene encoding

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Table 1. Bacterial Strains and Plasmids Used in This Study

strain/plasmid	descriptions[a]	reference/ source
Strains		
E. coli W3110	Coli Genetic Stock Center strain (CGSC) No. 4474	CGSC[b]
E. coli Top10	F- *mcrA* Δ*(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139* Δ*(ara-leu)7697galU galK rpsL (StrR) endA1 nupG*	Invitrogen[c]
WL	W3110 (Δ*lacI*)	10
TH20	W3110 (Δ*lacI, thrA*^{C1034T}, *lysC*^{C1055T}, P*thrABC*::P*tac*, Δ*lysA*, Δ*metA*, *ilvA*^{C290T}, Δ*tdh*, Δ*iclR*, P*ppc*::P*trc*)	9
ILE01	TH20 (*ilvA*$^{C1339T, G1341T, C1351G, T1352C}$)	this study
ILE02	ILE01 (P*ilvEDA*::P*trc*, P*ilvC*::P*trc*)	this study
ILE03	ILE01 (P*ilvEDA*::P*trc*, P*ilvC*::P*trc*, P*lrp*::P*trc*)	this study
WygaZH	W3110 (*ygaZH*::Km^R)	10
Plasmids		
pKD46	Ap^R, Red recombinase expression plasmid, temperature-sensitive origin	34
pJW168	Ap^R, repA(Ts), pSC101 based vector expressing cre-recombinase, 5.5-kb	35
pTac15K	Km^R, p15A ori, tac promoter-containing overexpression vector, 4.4-kb	26
pSacHR06	Km^R, homologous recombination vector, 4.3-kb	10
pSacilvA	Km^R, mutant *ilvA* cloned in the *Bam*HI *and PstI* site of pSacHR06, 5.8-kb	this study
pTacilvIH	Km^R, mutant *ilvIH* cloned in the *Sac*I and *Xba*I site of pTac15K, 6.4-kb	this study
pTacilvAIH	Km^R, mutant *ilvAIH* cloned in the *Sac*I site of pTacilvIH, 8.0-kb	this study
pBRthrABC	Ap^R, *thrABC* overexpression vector, 9.4kb	9
pBRthrABCygaZH	Ap^R, *ygaZH* cloned in the *Hin*dIII site of pBRthrABC, 10.7-kb	this study
pmtrc9	*Cm*^{R}, lox66-*Cm*^{R}-lox71, 4.7-kb	lab stock
pTrc184ygaZH	*Cm*^{R}, *ygaZH* overexpression vector, 6.1-kb	10

[a]Abbreviations: Ap, ampicillin; *Cm*, chloramphenicol; Km, kanamycin; Tc, tetracycline; R, resistance. [b]Coli Genetic Stock Center. [c]Invitrogen, Corp., Carlsbad, CA.

threonine dehydratase was mutated by replacing the 290th C with T (Ser 97 → Phe) to decrease the activity of the corresponding enzyme. The native promoter of the *ppc* gene was replaced with the *trc* promoter in the chromosome to increase the pool of oxaloacetate, a starting precursor of L-threonine biosynthesis. This engineered TH20 strain was used for developing the L-isoleucine production strain, as L-isoleucine is synthesized through five enzymatic steps from L-threonine. Further rational metabolic engineering was performed to develop an L-isoleucine producer.

First, feedback inhibition of threonine dehydratase (encoded by *ilvA*), which catalyzes the first step in L-isoleucine biosynthesis, should be removed. Notably, the threonine dehydratase in TH20 strain is not active due to the point mutation introduced into the *ilvA* gene. Thus, the inactive threonine dehydratase was restored by the active and feedback-resistant one by using the *sacB* homologous recombination system. It was expected that restored threonine dehydratase catalyzes the metabolic reaction toward the formation of 2-ketobutyrate, which is an important precursor of L-isoleucine biosynthesis. On the basis of the previous studies,[25,26] feedback inhibition of threonine dehydratase was removed by replacing the 1339th base C with T, 1341th G with T, 1351th C with G and 1352th T with C in the chromosome by site-directed mutagenesis. The key genotype of the resulting IlE01 (Table 1) includes removal of the feedback inhibitions of aspartokinase I, III and threonine dehydratase by L-threonine, L-lysine and L-isoleucine, respectively, and also the removal of the transcriptional attenuation regulation of the *thrABC* operon. The *lysA* and *metA* genes were deleted to make more aspartate semialdehyde and homoserine, respectively, available for L-isoleucine formation. The *tdh* gene was also deleted to make more L-threonine available for L-isoleucine formation. The native promoter of the *ppc* gene was replaced with the *trc* promoter in the chromosome to increase the pool of oxaloacetate, a starting precursor of L-threonine, and consequently L-isoleucine, biosynthesis. The *iclR* gene was also deleted to increase the pool of oxaloacetate.

In case of AHAS, *E. coli* possesses three isoenzymes differing in their biochemical properties and regulation mechanisms. Among them, AHAS III, encoded by *ilvIH*, has a much higher affinity for 2-ketobutyrate, while the AHAS I, encoded by *ilvBN*, has a higher affinity for pyruvate than 2-ketobutyrate. Thus, the *ilvIH* operon was selected for amplification. It has been known that the activity of AHAS III is inhibited by L-valine. We further reasoned that AHAS III might also be inhibited by L-isoleucine because L-valine and L-isoleucine have similar chemical structures, all of which possess nonpolar side chains. Thus, we decided to remove the feedback inhibition of AHAS III by L-isoleucine. However, the specific feedback inhibition site of AHAS III by L-isoleucine has not been known. Thus, assumption was made that the feedback inhibition sites of AHAS III by L-isoleucine and L-valine are similar; the potential feedback inhibition of AHAS III by L-isoleucine was eliminated by engineering the same site responsible for the feedback inhibition by L-valine.[10] After this manipulation on the *ilvIH* genes, it was cloned into pTac15K[27] by overlapping PCR to make pTacilvIH (see Methods). To examine whether the mutant *ilvIH* can truly confer resistance to inhibition by L-isoleucine in *E. coli* W3110, the effect of L-isoleucine analogue, DL-4-thiaisoleucine, on cell growth was examined by growing WL (pTac15K) and WL (pTacilvIH) strains in the presence of varying concentrations of DL-4-thiaisoleucine (Figure 2). The control WL (pTac15K) strain did not grow in the presence of 0.1, 0.5, and 1.0 mM of DL-4-thiaisoleucine, while the WL (pTacilvIH) strain overexpressing the feedback-resistant AHAS III showed normal growth even in the presence of 1.0 mM of DL-4-thiaisoleucine. Taken together, it could be concluded that the mutant *ilvIH* employed here conferred good resistance to L-isoleucine analogue, DL-4-thiaisoleucine, in *E. coli*, and thus the mutant *ilvIH* will be beneficial for the enhanced production of L-isoleucine in *E. coli*. This is the first experimental proof that the AHAS III is subject to feedback inhibition by L-isoleucine, and this inhibition could also be removed by employing the mutant *ilvIH* described here.

Next, the mutant *ilvA* gene, encoding the feedback-resistant threonine dehydratase, was cloned into pTacilvIH to make pTacilvAIH, which allows constitutive expression of feedback-resistant threonine dehydratase (encoded by *ilvA*) and AHAS III (encoded by *ilvIH*) under the strong *tac* promoter. Finally, plasmid pBRthrABC, constructed for the overproduction of L-threonine in the previous study[9] was transformed into the ILE01 strain together with pTacilvAIH. The resulting ILE01 (pTacilvAIH, pBRthrABC) strain was cultured in NM16

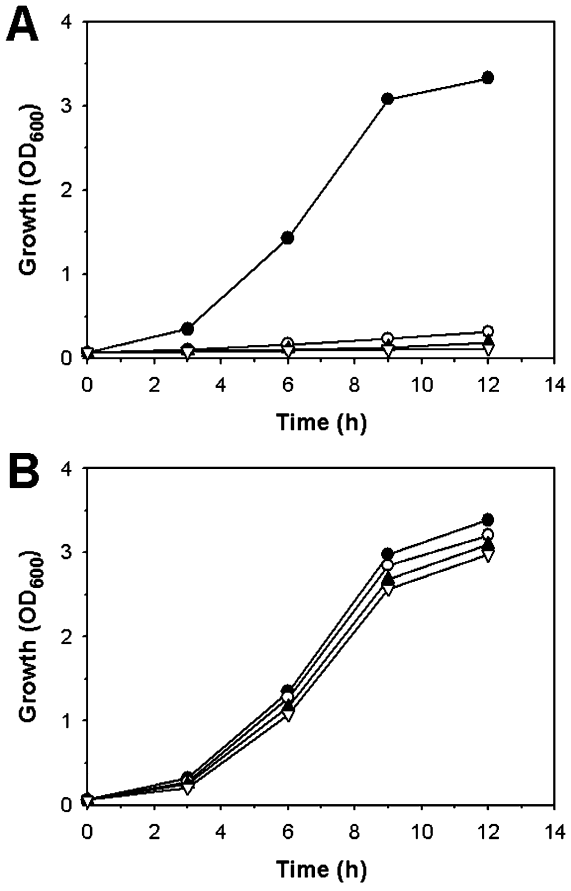

Figure 2. Growth profiles of WL (pTac15K) (A) and WL (pTacilvIH) (B) strains treated with various concentrations of DL-4-thiaisoleucine. Cells were grown at 37 °C in the R/2 medium (pH 6.80) supplemented with 5 g/L of glucose. Symbols are as follows: solid circles, control; open circles, 0.1 mM; solid triangles, 0.5 mM; open inverse triangles, 1.0 mM.

medium containing 20 g/L of glucose. This base strain was able to successfully produce 0.322 g/L of L-isoleucine. Further stepwise metabolic engineering was performed on this base strain as follows.

Engineering of L-Isoleucine Export System. Efficient export of L-isoleucine is important for further enhancing its production. Our previous work on L-valine production experimentally demonstrated that the *ygaZH* genes encode L-valine exporter in *E. coli*.[10] In addition, the *Corynebacterium glutamicum brnFE* genes, which are homologous to *ygaZH*, were reported to encode a two-component permease exporting all three BCAAs in a process depending on the proton motive force.[28]

On the basis of these previous results, we reasoned that the *ygaZH* genes might also function as an L-isoleucine exporter. To examine whether this is true, cell growth inhibition test was performed using the L-isoleucine analogue, DL-4-thiaisoleucine, in three different strains, including W3110, the *ygaZH*-deleted WygaZH, and WL harboring pTrc184ygaZH. As the concentration of analogue becomes higher, WL harboring pTrc184ygaZH grows comparatively well even though the growth by measuring the absorbance at 600 nm was negatively affected by high concentration of L-isoleucine analogue (Figure 3). On the other hand, the *ygaZH*-deficient WygaZH and W3110 did not grow even in the presence of 0.1 mM DL-4-thiaisoleucine (Figure 3B). These results suggest that amplified YgaZH seem to export DL-4-thiaisoleucine, thereby maintaining good growth even in the presence of 1.0 mM DL-4-thiaisoleucine. This is the first experimental proof that YgaZH functions not only as the exporter of L-valine but also as an export of L-isoleucine. On the basis of these findings, it can be strongly suggested that the overexpression of the *ygaZH* genes can increase the production of L-isoleucine.

To examine whether amplified YgaZH can truly increase L-isoleucine production, the *ygaZH* genes were cloned into pBRthrABC to make pBRthrABCygaZH. The ability to produce L-isoleucine was examined by culturing the ILE01 (pTacilvAIH, pBRthrABCygaZH) strain in NM16 medium containing 20 g/L of glucose. The final L-isoleucine concentration obtained was 1.11 g/L, which is 245% higher than that (0.322 g/L) obtained without *ygaZH* amplification. Taken together, it is concluded that YgaZH is one of the L-isoleucine exporters in *E. coli*, which, upon amplification, is beneficial for the enhanced production of L-isoleucine.

Increasing L-Isoleucine Biosynthesis by the Amplification of the *ilvCED* Genes. The *ilvCED* genes encoding acetohydroxy acid isomeroreductase (*ilvC*), BCAA aminotransferase (*ilvE*) and dihydroxy acid dehydratase (*ilvD*) are involved in the L-isoleucine biosynthetic pathway. Looking at the metabolic pathways, it was reasoned that amplification of these genes could further increase L-isoleucine production. However, amplification of too many genes by plasmid-based overexpression might hinder both cell growth and L-isoleucine production. Thus, the native promoters of the *ilvEDA* operon and the *ilvC* gene were replaced with the strong *trc* promoter by homologous recombination in the chromosome; this strain was named ILE02. It is notable that the feedback-resistant *ilvA* gene was already amplified by plasmid-based overexpression. As the *ilvA* gene forms an operon with the *ilvED* genes, the substitution of the native promoter of this operon could additionally amplify the *ilvA* gene. When ILE02 (pTacilvAIH, pBRthrABCygaZH) strain was cultured in NM16 medium containing 20 g/L of glucose, the final L-isoleucine concentration obtained was 2.11 g/L, which is 90% higher than that (1.11 g/L) obtained without changing the native promoter of the *ilvC* and *ilvEDA* genes. Thus, the amplification of the *ilvC* and *ilvEDA* genes by chromosomal promoter replacement was critical for the enhanced production of L-isoleucine.

Engineering of a Global Regulator to Enhance L-Isoleucine Production. It has been known that Lrp, a global regulatory protein in *E. coli*, activates the expression of the *ilvIH* operon which encodes the AHAS III involved in the biosynthesis of BCAAs.[29] It was also found that Lrp activates the expression of the *ygaZH* genes.[10] Furthermore, the effect of amplifying Lrp and YgaZH on L-valine production was more than additive because Lrp activates the expression of *ygaZH*.[10] Lrp is also known to repress the expression of the *livJ* gene, encoding the L-isoleucine transporter (importer).[30] On the basis of this regulatory information, the *lrp* gene was selected as the next target for overexpression. Because of the same reason applied in the overexpression of the *ilvC* and *ilvEDA* genes, we decided to amplify the expression of the *lrp* gene by replacing the native promoter with a strong *trc* promoter in ILE02 strain. The resulting strain was named ILE03 strain.

To see whether amplification of Lrp increases L-isoleucine production, the ILE03 (pTacilvAIH, pBRthrABCygaZH) strain was cultured in NM16 medium containing 20 g/L of glucose. The final L-isoleucine concentration obtained was 2.83 g/L,

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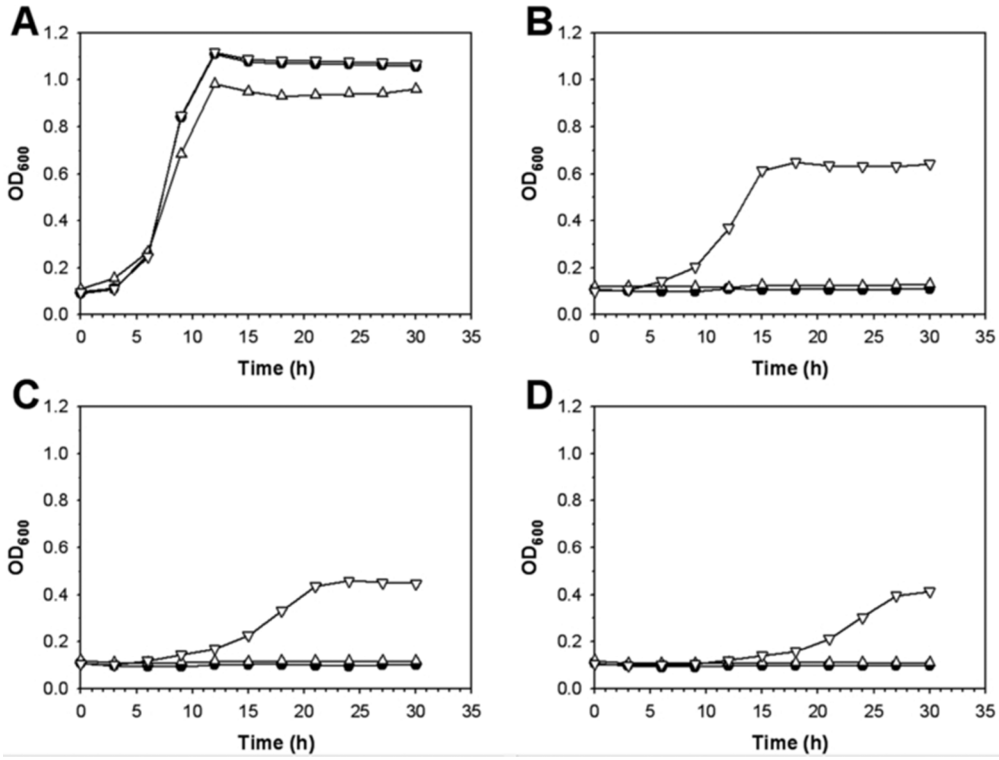

Figure 3. Growth profiles of W3110, *ygaZH*-deficient WygaZH, and WL (pTrc184ygaZH) strains treated with DL-4-thiaisoleucine at 0 mM (A), 0.1 mM (B), 0.5 mM (C), 1 mM (D). Cells were grown at 37 °C in the R/2 medium (pH 6.80) supplemented with 5 g/L of glucose. Symbols are as follows: solid circles, W3110; open triangles, WygaZH; open inverse triangles, WL (pTrc184ygaZH).

which is 34% higher than that (2.11 g/L) obtained without the amplification of the *lrp* gene.

Fed-batch Culture for Enhanced L-Isoleucine Production. For the high level production of L-isoleucine, fed-batch culture of the ILE03 (pTacilvAIH, pBRthrABCygaZH) strain was carried out. When the initial glucose was consumed, feeding solution was intermittently fed into the bioreactor (see Methods). Figure 4A shows fed-batch culture profiles of the ILE03 (pTacilvAIH, pBRthrABCygaZH) strain. Cell growth stopped when the concentrations of L-isoleucine and acetic acid reached 6.92 and 2.50 g/L, respectively, at 36.5 h, after which cells continued to produce L-isoleucine and acetic acid up to 8.72 and 12.61 g/L, respectively. Thus, it was obvious that a high concentration of acetic acid inhibited both cell growth and L-isoleucine production during the fed-batch culture. The overall volumetric L-isoleucine productivity obtained was 0.121 g/L/h. It is reasoned that considerable amount of pyruvate is required for L-isoleucine production, as pyruvate is an important intermediate precursor for L-isoleucine biosynthesis as well as biomass formation. Under normal growth condition, L-isoleucine production might be hindered due to the depletion of pyruvate. Thus, we thought that addition of pyruvate might improve L-isoleucine production.

Figure 4B shows the fed-batch culture profiles of the ILE03 (pTacilvAIH, pBRthrABCygaZH) strain with the feeding of 1.0 mM pyruvate. The final L-isoleucine concentration reached 8.95 g/L in 61 h, with a volumetric productivity of 0.147 g/L/h, which is 21.5% higher than that obtained without addition of pyruvate. However, the concentration of acetic acid increased by 8.4% up to 13.67 g/L. Another fed-batch fermentation of the ILE03 (pTacilvAIH, pBRthrABCygaZH) strain was carried out in the presence of 0.5 mM pyruvate as shown in Figure 4C. The final L-isoleucine concentration reached 9.46 g/L in 60 h, with a volumetric productivity of 0.158 g/L/h, which is 7.5% higher than that obtained with the addition of 1.0 mM pyruvate. The final L-isoleucine yield per glucose obtained with the 100% rational designed *E. coli* in this study was 0.14 g/g (19% of the theoretical yield), while that of the strain developed on the basis of a random mutant reported in the literature is 0.30 g/g (41% of the theoretical yield).[15] The final acetic acid concentration was 11.34 g/L, which is 17.0% lower than that obtained with the addition of 1.0 mM pyruvate. These results clearly suggest that there is pyruvate shortage in the cell, which should be considered for further metabolic engineering to enhance L-isoleucine production.

The present study was aimed at constructing a 100% genetically defined *E. coli* strain capable of producing L-isoleucine. To produce L-isoleucine, L-threonine overproducing strain of TH20[9] was utilized as a starting strain, as L-isoleucine is synthesized from L-threonine through five enzymatic steps. The TH20 strain was metabolically engineered to construct an L-isoleucine production strain by following strategies: (i) identification and removal of feedback inhibitions of threonine dehydratase (encoded by *ilvA*) and AHAS III (encoded by *ilvH*) by L-isoleucine; (ii) amplification of L-isoleucine biosynthetic *ilvC* and *ilvEDA* genes; (iii) amplification of global regulator Lrp; (iv) identification and amplification of L-isoleucine exporter YgaZH. Also, we suggested a strategy for further increasing L-isoleucine production by showing that supplementing an optimal level of pyruvate during the fed-batch culture reduced fermentation time and acetic acid accumulation, resulting in an increased L-isoleucine productivity.

The L-isoleucine concentration obtained in this study is 9.46 g/L, which is less than that (12.0 g/L) obtained with the classically mutagenized strain.[15] However, this is the only the

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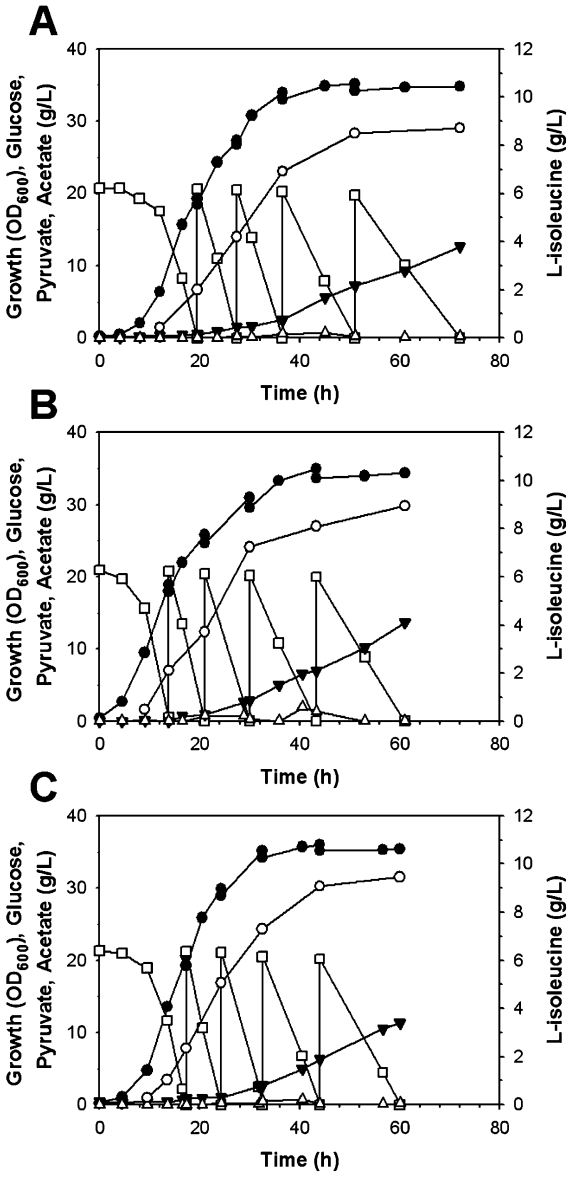

Figure 4. Fed-batch culture profiles of the ILE 03 (pBRthrABCygaZH, pTacilvAIH) strain in the presence of 0 mM pyruvate (A), 1.0 mM pyruvate (B), and 0.5 mM pyruvate (C). Symbols are as follows: solid circles, cell growth measured by OD_{600} value; open squares, glucose concentration; open triangles, pyruvate concentration; solid reverse triangles, acetate concentration; open circles, L-isoleucine concentration.

first step of the development of an industrially applicable strain. The importance of 100% genetically defined amino acid producer has already been verified in our previous works on L-valine production.[10,30,31] Using this strain, further metabolic engineering effort will be exerted to develop a strain with optimal pyruvate pool and much reduced acetate formation.

In conclusion, here we presented a strategy for metabolic engineering of *E. coli* strain capable of producing L-isoleucine. The resulting 100% rationally designed *E. coli* strain is expected to serve as a platform strain for the enhanced production of L-isoleucine. We adhered to the systems biological perspective throughout this metabolic engineering study. Specifically, whole *E. coli* metabolic pathways, starting from the consumption of glucose toward the L-isoleucine biosynthesis, were considered as a network. By doing so, it was possible to design a rational strategy for targeting genes to knock out or amplify at various critical metabolic steps. As a consequence, the target genes manipulated included those involved beyond the conventionally handled central carbon metabolism, and the engineering outcome was noteworthy. Additional engineering based on systems biology approaches including *in silico* metabolic simulation and multiomics studies can further improve this engineered strain. Furthermore, the general strategies reported here will be useful for developing strains for the overproduction of other amino acids and bioproducts relying on complex metabolic pathways and regulatory mechanisms.

METHODS

Bacterial Strains, Plasmids and Oligonucleotide Primers. The strains and plasmids used in this study are listed in Table 1. The primers used for gene cloning and site-directed mutagenesis are listed in Table 2. All DNA manipulations were carried out by following standard protocols.[32]

Site-Directed Mutagenesis. The removal of feedback inhibition of the threonine dehydratase (encoded by *ilvA*) by L-isoleucine was performed using the *sacB* homologous recombination system.[33] The substitution of the four bases in *ilvA* encoding the threonine dehydratase responsible for feedback inhibition was performed as follows. The oligonucleotide primers ilvA1 and ilvA2, containing four mutated bases (1339th C → T, 1341st G → T, 1351st C → G, 1352nd T → C), were used to amplify a 1592-bp DNA fragment. Another DNA fragment of 577-bp was amplified using the primers ilvA3, containing four mutated bases (1339th G → A, 1341st C → A, 1351st G → C, 1352nd A → G), and ilvA4. Two DNA fragments were purified and mixed, and the complete 2115-bp fragment was amplified by overlapping PCR using the primers ilvA1 and ilvA4. The *Bam*HI-*Pst*I-digested 2115-bp PCR fragment was ligated into the *Bam*HI-*Pst*I-digested pSacHR06 to make pSacilvA. Successful substitution of the four bases was confirmed by sequencing. The resultant construct was digested with *Nhe*I and self-ligated to remove the 855-bp fragment containing the pMB1 origin of replication. The final construct was then transformed into the strain of TH20 (W3110 Δ*lacI*, $thrA^{C1034T}$, $lysC^{C1055T}$, P*thr*::P*tac*, Δ*lysA*, Δ*metA*, $ilvA^{C290T}$, Δ*tdh*, Δ*iclR*, P*ppc*::P*trc*), which was constructed in our previous study[9] by electroporation, and clones in which double homologous recombination occurred were identified by positive selection based on the conditional lethal effect of the *sacB* gene in TH20.

Chromosomal Promoter Replacement. Replacement of the native promoter in *ilvEDA* genes with *trc* promoter was performed by PCR-based λ-red recombination, which is a slightly modified one-step inactivation method.[34] Recombinant *E. coli* harboring pKD46 was cultivated at 30 °C, and the expression of λ recombinase was induced by adding 10 mM L-arabinose. Then electrocompetent cells were prepared by standard protocol.[32] PCR was performed using plasmid pmtrc9, which contains the fused lox71-chloramphenicol marker-lox66 DNA fragment, with primers EDAtrcf and EDAtrcr. The 1822-bp PCR product was electroporated into the ILE01 strain carrying λ-Red recombinase expression plasmid pKD46. Cells, in which double homologous recombination occurred, were selected on the agar plate containing chloramphenicol and screened by direct colony PCR. Replacement of the promoter of the *ilvEDA* gene with the *trc* promoter was confirmed by DNA sequence analysis. The antibiotic marker was eliminated by using a helper plasmid pJW168 encoding the cre 25

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Table 2. Oligonucleotides Used for Gene Cloning and Site-Directed Mutagenesis

name	sequence (5′ → 3′)[a]
ilvA1	ATAC*GGATCC*TGGTGACCTGATCGCTATCG
ilvA2	**TGT**TGGCGAAGC**GC**AGAAACGCGCCCGGTGATTCCGGGAATTCGAAGCTGTAGA
ilvA3	TCTACAGCTTCGAATTCCCGGAATCACCGGGCGCGTTTCT**GC**GCTTCGCCAA**CA**
ilvA4	AGTC*CTGCAGG*TGGTTTCGACGCAATAAAA
ilvIH1	AGTC*GAGCTC*AAATTGCTGTAAGTTGTGGG
ilvIH2	GGAAAAAAGGCCAATCACGCGGA**A**TAACGCG**T**CTGATTCATTTTCGAGTAAG
ilvIH3	CTTACTCGAAAATGAATCAG**A**CGCGTTAT**T**CCGCGTGATTGGCCTTTTTTCC
ilvIH4	ACTG*TCTAGA*AAGATCACTAGTTCAACGCATTATTTTATCGC
EDAtrcf	TTTCCACGTCTGCTCAATGAATATGGCCGCCGCCAGCGATGCACAAAATACGCGTCATACACATACGATT
EDAtrcr	CAGCGAACCATCTCCCCATTGAACCAAATGTAATCAGCTTTCTTCGTGGTCATGGTCTGTTTCCTGTGTG
ilvCtrcf	GCGGCTTTCCGCCAGATGCAGGAAGGTTTTCAGATCGCGTAAATCCACAGCGCGTCATACACATACGATT
ilvCtrcr	CCCAGCTGTGCCAGCTGCTGGCGCAGATTCAGTGTATTGAAGTAGTTAGCCATGGTCTGTTTCCTGTGTG
ygaZHup	ATG*CAAGCTT*CTAATTTCAGCCTCAGCCTG
ygaZHdo	AGT*CAAGCTT*GAAAAATGATTCTTGTGGGT
lrp1st	GTTTTGCTTTGACAATCCCCTGGTGTTTTGCGAAAACATTCGAGGAAGAACGCGTCATACACATACGATT
lrp2nd	TTACGATCGATACGGTCGAGATCTTTGCCAGGGCGCTTCTTGCTATCTACCATGGTCTGTTTCCTGTGTG

[a]Mutated bases are in boldface and restriction sites are in italic.

recombinase.[35] The elimination of antibiotic marker was verified by PCR.

Substitution of a native promoter of the *ilvC* gene by the *trc* promoter was performed in the same manner as described for the *ilvEDA* operon. A 1646-bp fused lox71-chloramphenicol marker-lox66 DNA fragment was obtained by the PCR reaction with primers ilvCtrcf and ilvCtrcr using pmtrc9 as a template. The PCR product was electroporated into the ILE01 strain carrying λ-Red recombinase expression plasmid pKD46. Double homologous recombinants were selected on the agar plate containing chloramphenicol and screened by direct colony PCR. Replacement of the promoter of the *ilvC* gene with the *trc* promoter was confirmed by DNA sequence analysis. The antibiotic marker was eliminated by using a helper plasmid pJW168 encoding the cre 25 recombinase.[35] Elimination of antibiotic marker was verified by PCR.

Substitution of a native promoter of *lrp* by *trc* promoter was carried out by λ-Red recombination in the same approach as depicted in *ilvC* and *ilvEDA* genes. A 1782-bp fused lox 71 chloramphenicol marker-lox66 DNA fragment was electroporated into the ILE02 strain harboring pKD46 plasmid, which expresses λ-Red recombinase. Then, cells in which double homologous recombination occurred were selected on the agar plate containing chloramphenicol and confirmed by PCR analysis. The antibiotic resistant marker was eliminated by using a helper plasmid pJW168 encoding the cre 25 recombinase and verified by PCR analysis.

Construction of Plasmids. The plasmid vector for overexpression of feedback-resistant AHAS III encoded by mutant *ilvIH* was constructed as follows. The oligonucleotide primers ilvIH1 and ilvIH2, containing two mutated bases (41st G → A, 50th C → T), were used to amplify a 2009-bp DNA fragment using the genomic DNA of *E. coli* W3110 as a template. Another DNA fragment of 492-bp was amplified with the primers ilvIH3, containing two mutated bases (41st C → T, 50th G → A), and ilvIH4 using the genomic DNA of *E. coli* W3110 as a template. Two DNA fragments were purified and mixed, and the complete 2439-bp fragment was amplified by overlapping PCR using the primers ilvIH1 and ilvIH4. The *Sac*I-*Xba*I-digested 2439-bp PCR fragment was ligated into the *Sac*I-*Xba*I-digested pTac15K to make pTacilvIH. Successful substitution of the two bases was confirmed by sequencing. To overexpress mutant *ilvA* (*ilvA*$^{C1339T, G1341T, C1351G, T1352C}$) gene encoding feedback-resistant threonine dehydratase, PCR was performed with primers ilvASac1 and ilvASac2 using the genomic DNA of IlE01 strain as a template. The PCR product was digested with *Sac*I and ligated into the *Sac*I-digested pTacilvIH to make pTacilvAIH.

Likewise, the plasmid vector for *ygaZH* overexpression encoding BCAA exporter was constructed. The primers ygaZHup and ygaZHdo were used to amplify a 1351-bp DNA fragment using the genomic DNA of *E. coli* W3110. The PCR fragment was digested with *Hin*dIII and ligated into the *Hin*dIII-digested pBRthrABC to construct pBRthrABCygaZH. The direction of the *ygaZH* gene in the plasmid pBRthrABCygaZH was confirmed by sequencing.

DL-4-Thiaisoleucine Tolerance Test. DL-4-Thiaisoleucine tolerance was examined for WL (pTac15K) and WL (pTacilvIH) strains at 37 °C in flasks containing R/2 medium in a shaking incubator at 250 rpm. The R/2 medium (pH 6.8) contains the following per liter: $(NH_4)_2HPO_4$, 2 g; KH_2PO_4, 6.75 g; citric acid, 0.85 g; and $MgSO_4{\cdot}7H_2O$, 0.7 g; trace metal solution, 5 mL. The trace metal solution contains the following per liter: $FeSO_4{\cdot}7H_2O$, 10 g; $CaCl_2$, 1.35 g; $ZnSO_4{\cdot}7H_2O$, 2.25 g; $MnSO_4{\cdot}4H_2O$, 0.5 g; $CuSO_4{\cdot}5H_2O$, 1 g; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.106 g; $Na_2B_4O_7{\cdot}10H_2O$, 0.23 g; 35% HCl, 10 mL. The seed culture was prepared by inoculating a small aliquot of cell glycerol stock into 10 mL of Luria−Bertani (LB; 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) medium and cultured overnight at 250 rpm at 37 °C. One milliliter of this seed culture was used to inoculate a 250 mL Erlenmeyer flask containing 50 mL of the R/2 medium supplemented with 5 g/L of glucose. Cells were treated with DL-4-thiaisoleucine at a final concentration of 0.1, 0.5, or 1.0 mM. The control experiments were performed without the addition of DL-4-thiaisoleucine. Samples were taken periodically for monitoring cell growth.

Cell Growth Inhibition Test. *E. coli* W3110, the *ygaZH*-deleted WygaZH and WL harboring pTrc184ygaZH were first cultured in R/2 medium at 37 °C until the absorbances at 600 nm (OD_{600}) were between 0.10 to 0.15. Then 10 μL of each cells were inoculated into 100-well plate carrying DL-4-thiaisoleucine, which is an L-isoleucine analogue. The working medium of the 100-well plate containing R/2 medium and 5 g/

L glucose was adjusted at 200 μL by adding DL-4-thiaisoleucine at 0, 0.1, 0.5, 1.0 mM respectively.[36] Then, bacterial cells were harvested for 30 h using Bioscreen C (Oy Growth Curves Ab Ltd., Finland) by measuring the absorbance at 600 nm (OD_{600}).

Fermentation. Batch and fed-batch fermentation of the engineered *E. coli* strains were carried out in the semidefined NM16 medium containing specific amount of glucose at 31 °C and the initial pH of 6.0. The NM16 medium contains the following per liter: glucose 20 g; yeast extract, 3 g; $(NH_4)_2SO_4$, 15 g; $MnSO_4{\cdot}5H_2O$, 0.02 g; $MgSO_4{\cdot}7H_2O$, 2 g; KH_2PO_4, 2 g; trace metal element 5 mL; betaine hydrochloride 1 g; nicotinic acid 0.1 g; thiamine hydrochloride 0.1 g. The culture medium was supplemented with 0.2 g/L of L-lysine and 0.2 g/L of L-methionine. Kanamycin (40 μg/mL) and ampicillin (50 μg/mL) were added to the medium when necessary. Seed cultures were prepared by transferring 500 μL of 10 mL overnight cultures prepared in Luria−Bertani (LB) medium into 250-mL Erlenmeyer flasks containing 50 mL of LB medium and culturing at 31 °C with 250 rpm of shaking incubator (SI-900; Jeio Tech, Daejeon, Korea). Cultured cells were used to inoculate a 6.6-L bioreactor (Bioflo 3000, New Brunswick Scientific Co., Edison, NJ) containing 2 L of NM16 medium to give an initial optical density of 0.2−0.3, and cultivated by batch mode at 31 °C. Feeding solution for fed-batch cultivation was made of 400 g/L of glucose, 20 g/L of KH_2PO_4, 2.92 g/L of L-lysine, and 2.98 g/L of L-methionine. When the glucose concentration in the culture broth fell below 1 g/L, 100 mL of feeding solution (thus, equivalent to 40 g of glucose, 2 g of KH_2PO_4, 0.292 g of L-lysine, and 0.298 g of L-methionine) was added. The pH was controlled at 6.0, and the dissolved oxygen concentration was controlled at 40% of air saturation by automatically increasing the agitation speed up to 1000 rpm.

Analytical Procedures. The concentrations of glucose and organic acids (acetic acid and pyruvic acids) were measured by high-performance liquid chromatography (ProStar 210; Varian, Palo Alto, CA) equipped with UV/visible light (ProStar 320; Varian, Palo Alto, CA) and refractive index (Shodex RI-71, Tokyo, Japan) detectors. A MetaCarb 87H column (300 by 7.8 mm; Varian) was eluted isocratically with 0.01 NH_2SO_4 at 60 °C at a flow rate of 0.6 mL/min. Cell growth was monitored by measuring the OD_{600} using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Amino acids were analyzed at Science Lab Center Co. (Daejeon, Korea) after centrifugation and filtration of the sampled culture broth. The supernatant obtained by the centrifugation and filtration of the sampled culture broth (5 mL) was appropriately diluted (100−500 fold diluted) and was injected into the cation separation column (LCA K06/Na 1.6 × 150 mm; Sykam GmbH, Eresing, Germany) and analyzed with the Sykam S433 amino acid analyzer.

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Author Contributions

§J.H.P. and J.E.O. contributed equally to this work. J.H.P., J.E.O., and S.Y.L. designed research; J.H.P., J.E.O., and K.H.L. performed research; and J.H.P., J.E.O., J.Y.K., and S.Y.L. wrote the paper.

Notes

The authors declare no competing financial interest.

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