Biosci. Biotechnol. Biochem., **63** (4), 672-679, 1999

Construction of an L-Isoleucine Overproducing Strain of *Escherichia coli* K-12

Ken-ichi HASHIGUCHI,* Hiroko TAKESADA,** Eiichiro SUZUKI,** and Hiroshi MATSUI**

**Fermentation & Biotechnology Laboratories, and*
***Central Research Laboratories, Ajinomoto Co. Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210-8681, Japan*

Received October 13, 1998; Accepted December 9, 1998

The genes for a threonine deaminase that is resistant to feedback inhibition by L-isoleucine and for an active acetohydroxyacid synthase II were introduced by a plasmid into a L-threonine-producing recombinant strain of *Escherichia coli* K-12. Analysis of culture broth of the strain using ^{13}C nuclear magnetic resonance suggested that α, β-dihydroxy-β-methylvalerate (DHMV) and α-keto-β-methylvalerate (KMV), the third and the fourth intermediates in the L-isoleucine biosynthetic pathway from L-threonine, respectively, accumulated in the medium in amounts comparable to that of L-isoleucine. The ratio of accumulated L-isoleucine:DHMV:KMV were approximately 2:1:1. The concentration of accumulated L-isoleucine increased by twofold after the additional introduction of the genes for dihyroxyacid dehydratase (DH) and transaminase-B (TA-B), and the intermediates no longer accumulated. The resultant strain TVD5 accumulated 10 g/l of L-isoleucine from 40 g/l of glucose.

Key words: L-isoleucine; overproduction; *Escherichia coli*; intermediates, NMR

The biosynthetic pathway of L-isoleucine and the regulation of that pathway have been well studied in *E. coli*,[1,2] and several studies on the breeding of L-isoleucine-overproducing strains of coryneform bacteria[3-5,11,20] and *Serratia marcescens*[5-7] have also been done. These strains were raised to be resistant mainly to such drugs as analogs of L-isoleucine or to be genetically engineered, and the yield of these strains from a carbon source ranged from 15% to over 20%. Through these studies it has been discovered that the limiting step in L-isoleucine biosynthesis involves primarily threonine deaminase (TD) and, secondarily, acetohydroxyacid synthase (AHAS). The activity of TD is inhibited by L-isoleucine, and that of AHAS is inhibited by L-valine, which is synthesized through common enzymes with L-isoleucine biosynthesis, following TD (Fig. 1). In *E. coli*, there are three isozymes of AHAS: AHAS I, II, and III.[1] Although AHAS II is resistant to inhibition by L-valine, it is inactive in *E. coli* K-12 because of a frameshift mutation in the *ilvG* gene,[14] which encodes the large subunit of AHAS II. The small subunit of AHAS II is encoded by the *ilvM* gene. The genes for TD, AHAS II, dihydroxyacid dehydratase (DH) and transaminase-B (TA-B) constitute the *ilvGMEDA* operon, and its expression is controlled by multivarent attenuation by L-isoleucine, L-valine, and L-leucine.[1,2]

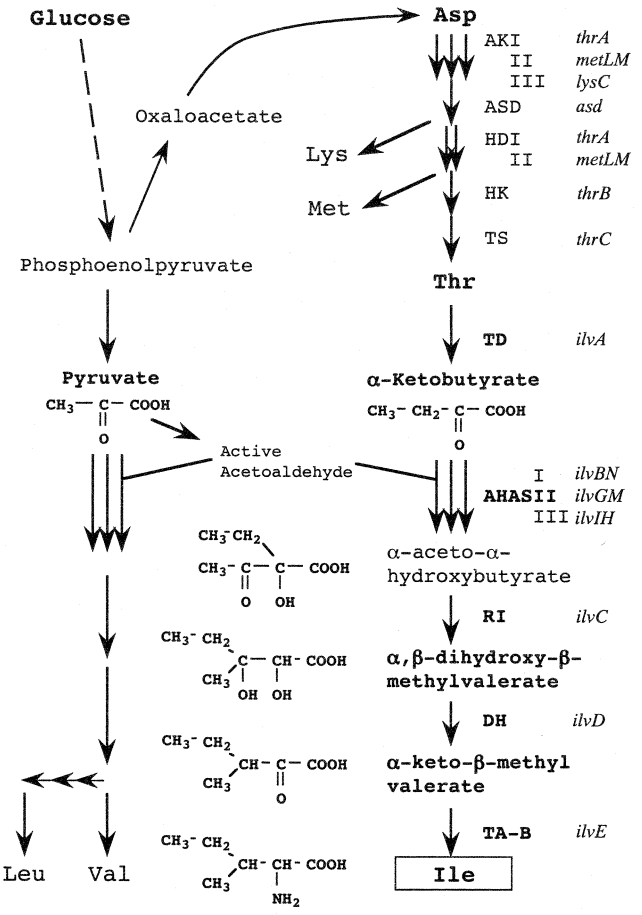

Fig. 1. Biosynthetic Pathway of L-Isoleucine in *E. coli*.
AK, Aspartokinase; ASD, Aspartatesemialdehyde dehydrogenase; HD, Homoserine dehydrogenase; HK, Homoserine kinase; TS, Threonine synthase; TD, Threonine deaminase; AHAS, Acetohydroxyacid synthase; RI, Dihydroxyacid reductoisomerase; DH, Dihydroxyacid dehydratase; TA-B, Transaminase-B.

In this paper, we report the phenomenon of intermediate accumulation that occurs under the condition where the genes for TD that is resistant to feedback inhibition and for active AHAS II are introduced into an L-threonine producer of *E. coli* K-12 by a plasmid, and the construction of an L-isoleucine over-producing strain.

To whom correspondence should be addressed. Ken-ichi HASHIGUTI, Fax: +81-44-245-1548; E-mail:bldhasiguti@te2.ajinomoto.co.jp
Abbreviations: DHMV, α, β-dihydroxy-β-methylvalerate; KMV, α-keto-β-methylvalerate; TD, Threonine deaminase; AHAS, Acetohydroxyacid synthase; RI, Dihydroxyacid reductoisomerase; DH, Dihydroxyacid dehydratase; TA-B, Transaminase-B

Materials and Methods

Strains and plasmids. The bacterial strains and plasmids used in this study are summarized in Table 1 and Table 2, respectively.

Medium. MSI medium comprises 16 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 1 g/l $MgSO_4$, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 0.01 g/l $MnSO_4 \cdot 7H_2O$, 2 g/l yeast extract (Difco), 40 g/l glucose and 30 g/l $CaCO_3$. The pH was adjusted to 7.0 using KOH. Sterilization was done for 10 min at 115°C. Glucose and $CaCO_3$ were sterilized separately from everything else. Each flask contained 20 ml of medium.

Cultivation conditions. Tested strains were cultivated on agar plates containing 10 g/l bactotryptone (Difco), 5 g/l yeast extract (Difco), 5 g/l NaCl, 15 g/l agar, and antibiotics for 18–24 h at 37°C, and then inoculated into each flask by one eighth of a plate. Antibiotics were used at the following concentrations: 0.1 mg/ml ampicillin; 0.1 mg/ml streptomycin; and 0.03 mg/ml chloramphenicol. Cultivation in the flask was done at 37°C.

Table 1. List of Bacterial Strains

Strain	Genotype or Description	Reference
MI162	*thr-10, car-94, thi-1, ilvG603, relA1,* λ^-	8)
MI262	*leuB6, ilvI614, ilvH612, ilvB619, ilvG603, ilvG605*(am), *thi-1, relA1, spoT1,* λ^-	8)
AB2070	*proA2, trp-3, hisG4, ilvE12, metE46, thi-1, ara-9, lacY1* or *lacZ4, galK2, malA1, mtl-1, rpsL, ton-1, tsx-3, supE44,* λ^R, λ^-	9)
AB1419	*thi-1, ilvC49, argH1, metB1, hisG1, mtl-2, xyl-7, lacY1* or *lacZ4, malA1, ara-13, tonA2, rpsL,* λ^R, λ^-, *supE44*	9)
TDH6	*thr*[Ra)], *thrC, tdh::Tn5, ilvA*	10)
ACS3T	*thr*[Ra)], *thrC, tdh::Tn5, ilvG603, Δatt(ilvG)*[b)]	This work
B-3996	TDH6 carrying pVIC40	10)
TDGA	TDH6 carrying pDGA5	This work
TVDGA	TDH6 carrying both pVIC40 and pDGA5	This work
TVMGA	TDH6 carrying both pVIC40 and pMGA2	This work
TD5	TDH6 carrying pMWD5	This work
TDV5	TDH6 carrying both pVIC40 and pMWD5	This work
TVDC	TDH6 carrying pVIC40, pMWD5 and pHSGC12	This work
AVMGA	ACS3T carrying both pVIC40 and pMGA2	This work
AVD5	ACS3T carrying both pVIC40 and pMWD5	This work

[a)] thrR, normal growth in the presence of 5 mg/ml L-threonine.
[b)] represents deletion of the leader-attenuator region in front of the *ilvG* gene.

Table 2. List of Plasmids

Plasmid	Vector	Confering genes	Reference
pVIC40	RSF1010 deriv.	*thrA*BC*[b)]	10)
pDRIA4	pDR720 deriv.	*ilvA**[b)]	11)
pBRGM7	pBR322	*ilvGM*[c)]	This work
pBRGMA1	pBR322	*ilvGM*[c)], *ilvA**[b)]	This work
pDGA5	pBR322	*ilvGM*[c)], *ilvA**[b)], *Δatt(ilvG)*[a)]	This work
pMGA2	pMW119	*ilvGM*[c)], *ilvA**[b)], *Δatt(ilvG)*[a)]	This work
pMWD5	pMW119	*ilvGMEDA**[b)c)], *Δatt(ilvG)*[a)]	This work
pHSGC12	pHSG399	*ilvC*	This work
pUCE1	pUC18	*ilvE*	This work

[a)] represents deletion of the leader-attenuator region in front of the *ilvG* gene. [b)]*, resistant to feedback inhibition. [c)] *ilvG603* allele.

^{13}C NMR analysis. The medium and the supernatants of the sonicated cells were freeze-dried and then dissolved in D_2O. They were then analyzed by nuclear magnetic resonance. Cultivation of the strain TDGA for ^{13}C NMR analysis was done using MSI medium containing 100 mg/l ampcillin, 7.5 g/l L-threonine (^{12}C), and 0.7 g/l uniform-^{13}C-L-threonine.

Measurement of amino acids. The concentrations of amino acids were measured by high-performance liquid chromatography.

Cloning of ilv genes. The cloned segments for *ilv* genes are summarized in Fig. 2. The *ilvGM* genes (*ilvG603* allele) were cloned on pBR322 from *E. coli* MI162[8)] as a 4.8-kb *Hin*dIII fragment, using MI262[8)] as a recipient, by the shotgun method (pBRGM7). The nucleotide sequence of approxymately 200 base pairs from both ends of the cloned fragment was identical with the reported sequence.[15,16)] The *ilvE* gene was cloned on pUC18 from W3110 as a 2.3-kb *Sal*I-*Pst*I fragment, using AB2070[9)] as a recipient, by the shotgun method (pUCE1). This *Sal*I-*Pst*I fragment also contained a large *N*-terminal part of the *ilvD* gene. The *ilvA* gene had been cloned on pBR328 as a 2.8-kb *Cla*I fragment (pILVA1),[11)] and been mutated to cause resistance to feedback inhibition by L-isoleucine (*ilvA**).[11)] After the *Cla*I fragment had been sub-cloned as a *Bam*HI fragment using a *Bam*HI linker, it was inserted into the *Bam*HI site on a plasmid vector derived from pDR720 (Pharmacia Co., Uppsala, Sweden) to make pDRIA4.[11)] The *ilvC* gene was cloned into the *Sma*I site of pHSG399 from a 1.7-kb polymerase chain reaction (PCR) product. The PCR product was responsible for nucleotide numbers from 1088 to 2827 of GenBank accession number M11689 or M14492, and contained from the putative ribosomal binding site to the end of the coding region of the *ilvC* gene. Synthetic oligonucleotides 5′GTGAATTCAATATCGCAAACG3′ and 5′CGCCAGGGTCAGATGGTTC3′ were used as primers. Plas-

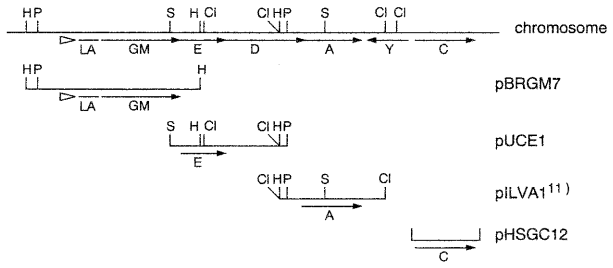

Fig. 2. Summary of the Cloned Region.
LA, leader-attenuator region. GM, *ilvGM*; E, *ilvE*; D, *ilvD*; A, *ilvA*; Y, *ilvY*; C, *ilvC* genes. Concerning pILVA1 see reference 11. H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Cl, *Cla*I. ▷, *ilv* promoter.

mids that had the *ilvC* gene in the same direction as the *lac* promoter were selected by restriction analysis (pHSGC12). Plasmid pHSGC12 complemented the growth deficiency of AB1419[9] (*ilvC*) on minimal medium without L-isoleucine.

Removing the attenuator region in front of the ilvG gene. According to the reported DNA sequences around the attenuator region in front of the *ilvG* gene,[15,16] the fragments in the upstream region of the leader peptide (between primer 1 and primer 2 in Fig. 3), and in the downstream region of the attenuator (between primer 3

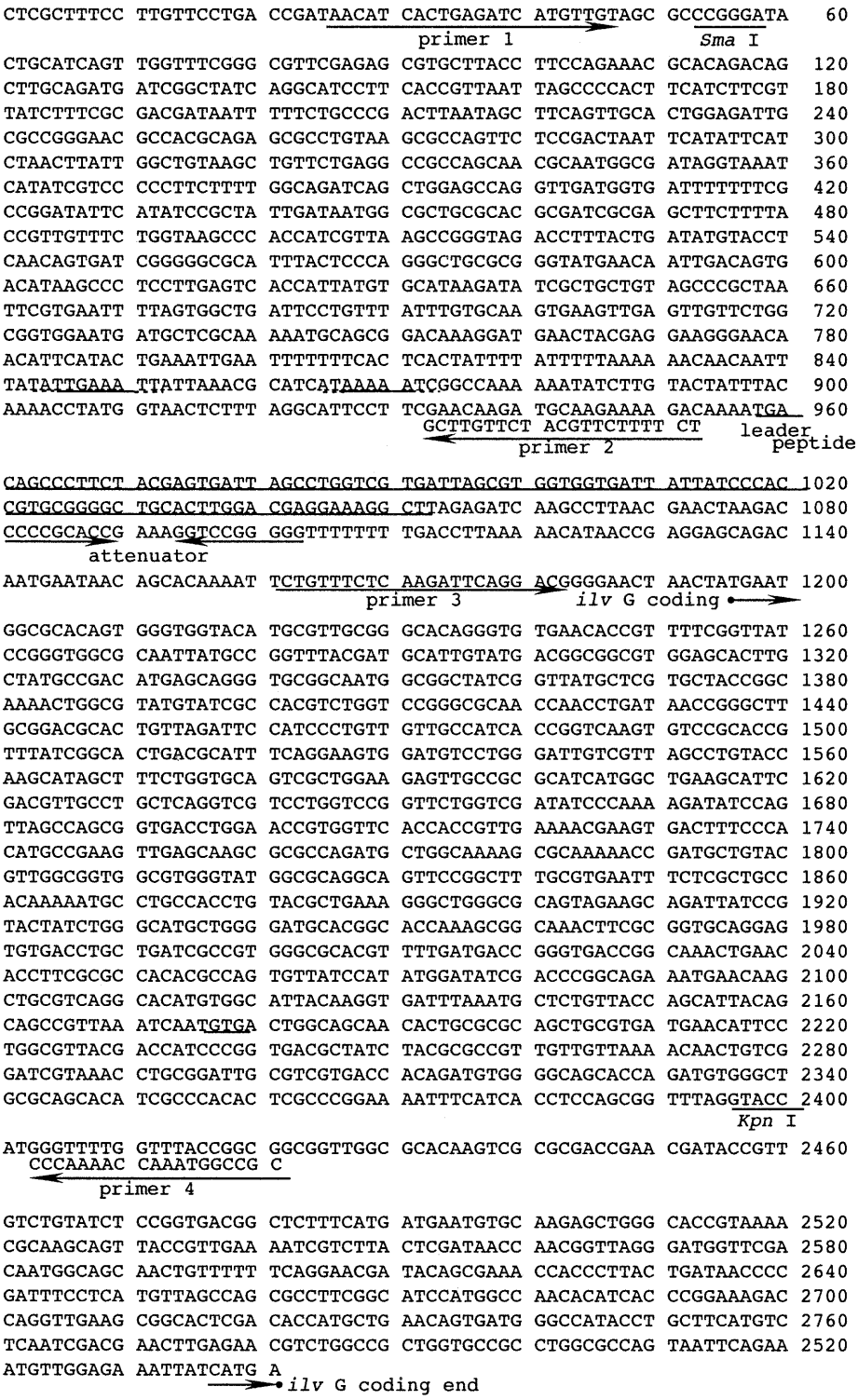

Fig. 3. DNA Sequence around the *ilvG* Gene.

The sequence consists of the data from the Genbank file accession No. M37337 (complement), No. M10313, and reference 8. The region between primer 2 and primer 3 was deleted to make the *ilvG* gene without leader-attenuator region. Underlined TGTG at position 2176-2179 is TG in the wild K12 allele, and insertion of TG or GT is the mutational point of the *ilvG603* allele.[8]

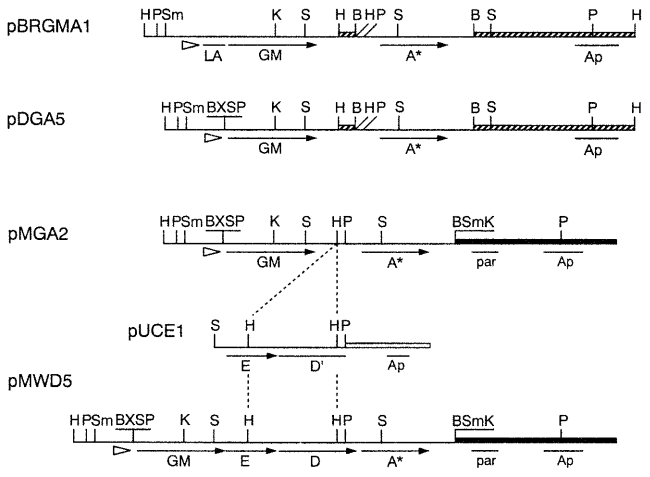

Fig. 4. Structures of the Plasmids.
LA, leader-attenuator region. GM, *ilvGM*; E, *ilvE*; D, *ilvD* genes respectively. A*, *ilvA* gene that encodes feedback-resistant threonine deaminase. Ap, ampicillin-resistant gene; par, region for stabilization. H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; K, *Kpn*I; B, *Bam*HI; X, *Xba*I; ▨, pBR322; □, pUC18; ■, pMW119; ▷, *ilv* promoter.

and primer 4 in Fig. 3) were prepared by PCR using the synthetic oligomers shown in Fig. 3 as primers. The upstream fragment was cloned into the *Sma*I site of pUC18 so that the direction of the *ilv* promoter was the same as the *lac* promoter of pUC18 (pUCU1). The downstream fragment was digested with *Kpn*I and cloned into the *Kpn*I-*Hin*cII site of pHSG399 (pHD1). Plasmid pUCU1 was digested with *Kpn*I, blunted with T4 DNA polymerase, then digested with *Pst*I. The resulting fragment containing the upstream region was inserted into the *Hin*dIII(blunted)-*Pst*I site of pHD1 to join the fragments containing the upstream region and the downstream region with an intervention sequence comprising *Bam*HI, *Xba*I, *Sal*I, and *Pst*I sites derived from the multiple cloning site of pUC18. With the resultant *Sma*I-*Kpn*I fragment comprising the promoter region and *N*-terminal region of the *ilvG* gene, the corresponding fragment on pBRGMA1 was replaced to make pDGA5 (Fig. 4). On pDGA5 there are the *ilvA** gene encoding feedback-resistant TD and the *ilvGM* genes (*ilvG603* allele) without leader-attenuator region.

Construction of the strain ACS3T. The *Hin*dIII fragment containing the *ilvGM* genes on pMWD5 was subcloned into pHSG399. The resultant plasmid was named pHSGM1. Strain TDH6 (*ilvA*) were transduced to Ile$^+$ by a P1 phage grown on W3110 (wild). The resultant strain was infected with No. 557 phage of Kohara's λ library[12] (comprising an *ilvGMEDA* region) prepared from pHSGM1-harboring W3110 by the method of Berg.[13] Among the Val^r transductants, a strain that was co-transduced with the deletion of the leader-attenuator region was selected as follows. PCR was done using primers 1 and 4 shown in Fig. 3 and the strain ACS3T was selected as the transductant, the PCR product of which was smaller than the wild one, and could be digested by *Bam*HI.

Assay of AHAS activity. The AHAS activity was measured as described by Felice *et al.*[19] with the exception that 100 mM Tris HCl (pH 8.0) was used as a buffer.

Measurement of plasmid stability. Cells were harvested at the end of cultivation, diluted appropriately, then spread onto agar plates without antibiotics. Colonies were picked and put onto agar plates with and without antibiotic, and the ratio of the number of colonies formed on antibiotic-containing agar plates versus the number of colonies formed on agar plates without antibiotics was scored as plasmid stability.

Results

Construction of plasmid pDGA5 containing the ilvA and the ilvGM genes*

Because AHAS II, which is resistant to the inhibition by L-valine, is inactive in *E. coli* K-12, we considered that it would be favorable for the overproduction of L-isoleucine in *E. coli* K-12 to introduce the active *ilvGM* genes together with the *ilvA** gene encoding TD that is resistant to feedback inhibition by L-isoleucine.

There are several mutants in which the frame-shift mutation in the *ilvG* gene, the wild type *E. coli* K-12 has, is recovered.[8] We cloned *ilvGM* genes from one of such mutant MI162[8] (*ilvG603* allele), as described in Materials and Methods. The plasmid was named pBRGM7.

The *Bam*HI fragment of pDRIA4,[11] comprising the *ilvA** gene encoding TD that is resistant to feedback inhibition by L-isoleucine, was inserted to the *Bam*HI site of pBRGM7 to construct pBRGMA1 (Fig. 4). On pBRGMA1, the *ilvA** gene was inserted in the same direction as the *ilvGM* genes. To avoid the attenuation of both the *ilvGM* and the *ilvA** genes (expressed by readthrough from the *ilvGM* genes) on pBRGMA1, the attenuator region in front of the *ilvG* gene was removed as described in Materials and Methods, and pDGA5 was constructed (Fig. 4).

Effects of pDGA5 on metabolic flow in a recombinant L-threonine producer

Strain B-3996[10] is a recombinant L-threonine producer of *E. coli* K-12, and it consists of the host strain TDH6 and the plasmid pVIC40 comprising the *thrABC* operon. The *thrA* gene on pVIC40 encodes aspartokinase I and homoserine dehydrogenase I, which are resistant to inhibition by L-threonine.[10] Strain B-3996 accumulated 15 g/l L-threonine after cultivation with MSI medium.

Since pVIC40 is a derivative of RSF1010, pDGA5 can be harbored together with pVIC40. We introduced pDGA5 into B-3996 to make the strain TVDGA (TDH6 carryng both pVIC40 and pDGA5). As expected, L-isoleucine accumulated in the medium during cultivation of TVDGA (Fig. 5), and no L-threonine accumulated. However, the productivity of TVDGA was low (the yield from glucose was 6.8%). Unexpectedly, the accumulation of L-isoleucine continued to increase for several hours after almost all of the glucose was con-

sumed, as shown in Fig. 5.

Table 3 demonstrates the conversion of L-threonine to L-isoleucine by strain TDGA (TDH6 carrying only pDGA5). Because strain TDH6 is deficient in threonine synthase (*thrC*), threonine dehydrogenase (*tdh*) and TD (*ilvA*), it cannot sythesize L-threonine from glucose, and does not degrade excess L-threonine in the medium. So in strain TDGA it was expected that almost all of the L-threonine added to the medium would be degraded through TD on pDGA5, and all of the synthesized L-isoleucine would be derived from the added L-threonine. As indicated in Table 3, although the added L-threonine had disappeared by 25 h, the concentration of L-isoleucine continued to increase until 30 h. The conversion rate was only around 45% on a molar basis.

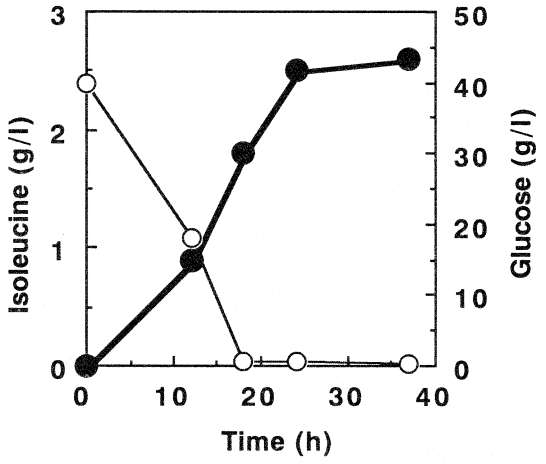

Fig. 5. Course of Isoleucine Production by Strain TVDGA.
Strain TVDGA was cultivated with MSI medium at 37°C. ●, isoleucine; ○, residual glucose.

Table 3. Conversion of Threonine to Isoleucine by Strain TDGA

Time (h)	Conc. (g/l)	
	Thr	Ile
0	10	n.d.[a)]
25	n.d.	3.9
27	n.d.	4.7
30	n.d.	5.0

Strain TDGA was cultivated with MSI medium containing 10 g/l L-threonine in flask at 37°C. [a)]n.d., not detected.

These two results seemed to imply the accumulation of intermediate(s), at least between L-threonine and L-isoleucine. It was assumed that a large amount of intermediate(s) between L-threonine and L-isoleucine accumulated during the cultivation of TVDGA or TDGA, and a part of the accumulated intermediate(s) were converted to L-isoleucine by residual enzymatic activity after all of the glucose was consumed.

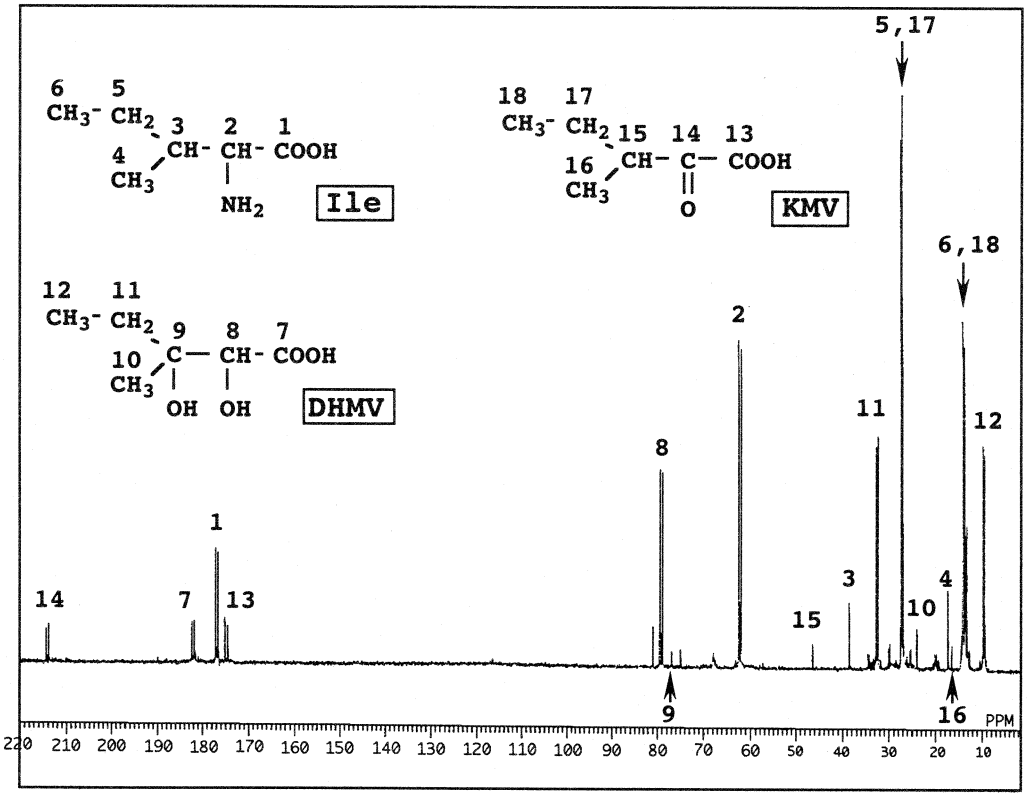

Fig. 6. ^{13}C-NMR Chart of the Culture Broth of Strain TDGA.
Strain TDGA was cultivated with the MSI medium containing UL-^{13}C-threonine and ampicillin for 28 h at 37°C. The medium was analyzed by ^{13}C-NMR as described in Material and Methods. Numbers represent the asigned carbons. Carbons No. 3, 4, 9, 10, 15, and 16 derive from pyruvate (all of them are ^{12}C), others derive from threonine (containing ^{13}C). Ile,isoleucine; DHMV,α, β-dihydroxy-β-methylvalerate; KMV, α-keto-β-methylvalerate.

Detection of intermediates in L-isoleucine biosynthesis using ^{13}C-L-threonine

For detection of intermediates, strain TDGA was cultivated in the medium containing uniform-^{13}C-L-threonine, and then the culture broth and the supernatant of sonicated cells were analyzed by ^{13}C-NMR.

A peculiar result was obtained from this analysis of the culture broth (Fig. 6). The major peaks in the chart were doublets, not more than triplets, indicating that the major compounds containing ^{13}C had a ^{13}C-^{13}C bond, but did not have a ^{13}C-^{13}C-^{13}C-^{13}C bond which uniform-^{13}C-L-threonine has. According to the biosynthetic pathway of L-isoleucine, the carbon bonds of L-threonine are conserved in acetohydroxybutyrate, the product of AHAS, then four continuous carbons derived from L-threonine are separated to two sets of two continuous carbons by pinacol rearrangement at the next step, dihydroxyacid reductoisomerase (RI) (Fig. 1). So, it was quite possible that the detected compounds were the intermediates downstream of RI or their derivatives. L-Isoleucine was, of course, thought to be one of them.

After two dimensional NMR analyses and ^{1}H-NMR analysis, the detected compounds having ^{13}C were assumed to be the intermediates α, β-dihydroxy-β-methylvalerate (DHMV) and α-keto-β-methylvalerate (KMV), and L-isoleucine (Ile). The molar ratio of these substances was estimated to be approximately DHMV:KMV:Ile=1:1:2. This ratio was almost in accordance with the conversion rate of L-threonine to L-isoleucine by TDGA (approxymately 45% in molar basis). No significant peak was detected from the supernatant of the sonicated cells.

These results indicate that α, β-dihydroxyacid dehydratase (DH, *ilvD*) and transaminase-B (TA-B, *ilvE*) became the new limiting step for L-isoleucine overproduction when the TD that is resistant to feedback inhibition (*ilvA**) and the AHAS II (*ilvGM*) were enhanced, but the RI (*ilvC*), the next step of AHAS, did not become the limiting step.

Construction of an L-isoleucine overproducing strain

To improve the *ilvD* and the *ilvE* genes together with the *ilvA** and the *ilvGM* genes, we were tempted to reconstruct the *ilvGMEDA* operon without the leader-attenuator region and with the *ilvA** gene which encoded feedback-resistant TD. Since pDGA5 was not stable in strain TVDGA (only 6% of colonies were resistant to ampcillin at the end of the cultivation with MSI medium), the *ilvGM* genes without the leader-attenuator region and the *ilvA** gene on pDGA5 were put onto a stable low-copy plasmid vector pMW119 (purchased from Nippon Gene Co., Tokyo, Japan) to construct pMGA2 (Fig. 4). The 1.7-kb *Hin*dIII fragment on pUCE1 (see Materials and Methods and Fig. 2) was inserted into the *Hin*dIII site between the *ilvGM* genes and the *ilvA** gene on pMGA2 in the direction to re-generate the *ilvE* and the *ilvD* genes. The resultant plasmid pMWD5 comprised the *ilvGMEDA* operon without the leader-attenuator region and with the *ilvA** gene which encoded feedback-resistant TD.

Plasmid pMGA2 or pMWD5 was introduced into the L-threonine producer B-3996. Strain TVMGA (TDH6 carrying both pVIC40 and pMGA2) accumulated 5 g/l L-isoleucine and TVD5 (TDH6 carrying both pVIC40 and pMWD5) accumulated 10 g/l L-isoleucine from 40 g/l glucose, and no L-threonine accumulation was observed in either case (Table 4). The L-isoleucine productivity of the strain using pMWD5 (*ilvGMEDA**) was twofold more than that using pMGA2 (*ilvGMA**). It was assumed that accumulation of DHMV and KMV occurred during cultivation of TVMGA from glucose, similarly to the case of conversion from L-threonine by TDGA (DHMV:KMV:Ile=1:1:2), and that the substrates were converted to L-isoleucine efficiently without accumulation of those intermediates in the case of TVD5. Indeed, DHMV was hardly detected and less than one tenth of KMV, compared to the culture broth of TVDGA, was detected in the culture broth of TVD5. The yield from glucose by TVD5 reached 25%.

The introduction of plasmid pHSGC12 (see Materials and Methods and Fig. 2), conferring RI (*ilvC*), to TVD5 had no great effect on L-isoleucine production (Table 4). This result concurred with those of the NMR analyses, showing that accumulated intermediates occur downstream of RI.

Table 4. L-Isoleucine Productivity

Strain	Host/Plasmid	Genes Confered by Plasmid	Time[a] (h)	Plasmid Stability[b] (%)	Accumulation (g/l)		Yield (%)
					Thr	Ile	
TVMGA	TDH6/pVIC40, pMGA2	thrABC ilvGM, A	24	>95 95	n.d.[c]	5.0	12.5
TVD5	TDH6/pVIC40, pMWD5	thrABC ilvGMEDA	24	>95 95	n.d.	10.2	25.5
TVDC	TDH6/pVIC40, pMWD5, pHSGC12	thrABC ilvGMEDA ilvC	24	>95 95 95	n.d.	9.1	22.8
AVMGA	ACS3T/pVIC40, pMGA2	thrABC ilvGM, A	24	>95 90	n.d.	5.3	13.3
AVD5	ACS3T/pVIC40, pMWD5	thrABC ilvGMEDA	23	>95 90	n.d.	10.2	25.5

Strains were cultivated with MSI medium in flasks at 37°C.

[a]Time, Culture time; [b]Measured at the end of cultivation, as described in Materials and Methods; [c]n.d., not detected.

Table 5. Acetohydroxyacid Synyhase (AHAS) Activity

Strain	AHAS activity (nmol/min/mg[a])	
	Without Val	Val 1 mM
TDH6	36.0	4.0
ACS3T	45.7	11.9

Strains were cultivated with MSI medium containing 100 mg/l each of L-threonine, L-isoleucine, L-valine and L-leucine for 16 h at 37°C. Cells were harvested and AHAS activities of crude extracts were measured with or without the presence of 1 mM L-valine, as described in Materials and Methods. [a]The protein concentration crude extract was measured using bovine serum albumin as a standard.

Effects of Activation of the Chromosomal ilvDE Genes

It is known that the expression level of the *ilvGMEDA* operon of *E. coli* K-12 is reduced because of the polar effect caused by the frame-shift mutation in the *ilvG* gene, and the expression level is elevated by the recovery of this mutation.[14] It was expected that the recovery of the frame-shift mutation in the chromosomal *ilvG* gene would elevate the expression level of the *ilvDE* genes on the chromosome, and would result in at least a partial release of the limiting step in the case using pMGA2. For the recovery of this mutation on the chromosomal *ilvG* gene, we constructed a strain named ACS3T that has the same *ilvG* gene as pMWD5 (*ilvG603* allele without the leader-attenuator region) as described in Materials and Methods.

The valine-resistant AHAS activity of strain ACS3T elevated by three-fold compared to that of the parental strain after cultivation in the presence of excess L-isoleucine, L-valine, and L-leucine (Table 5). This was thought to indicate activation of the *ilvG* gene in ACS3T, and it was expected from previous information[14] that the expression levels of *ilvDE* genes were also elevated.

Strain AVMGA (ACS3T carrying both pVIC40 and pMGA2) accumulated a slightly larger amount of L-isoleucine than TVMGA, showing that the activation of chromosomal *ilvDE* genes was somewhat effective (Table 4). However, this effect was masked in the case of using pMWD5, where the L-isoleucine productivity by AVD5 (ACS3T carring both pVIC40 and pMWD5) was the same as that by TVD5 (Table 4).

Discussion

This is the first report that shows the accumulation of the intermediates between L-threonine and L-isoleucine directly and quantitatively under the conditions for L-isoleucine overproduction. The limiting step between L-threonine and L-isoleucine shifted to DH and TA-B by amplification of the genes for feedback-resistant TD and active AHAS II by using a plasmid.

TD and AHAS are the primary and the secondary limiting steps of L-isoleucine biosynthesis because of their inhibitory regulation. In addition, they are also the first and the second steps from L-threonine to L-isoleucine. So, the introduction of the *ilvA** and the *ilvGM* genes by plasmid (pDGA5 or pMGA2) caused not only the release of inhibitory regulation, but also the increase of the first and the second reaction activities from L-threonine. It may be natural that the rate-limiting step shift to the third step (*i.e.* RI) under such conditions. However, the limiting step shifted to the fourth and the fifth steps. It is known that the expression of the *ilvGMEDA* operon is reduced by the polar effect caused by a frame-shift mutation in the *ilvG* gene in *E. coli* K-12[14] while the expression of the *ilvC* gene is induced by acetolactate or acetohydroxybutyrate, which are the substrates of RI (encoded by the *ilvC* gene itself), and are the products of AHAS.[17,18] This may be the reason why the increase of the first and the second reaction activities from L-threonine caused the shift of the limiting step to the fourth and the fifth step, not to the third one.

The amplification of the genes responsible for DH and TA-B on the plasmid (pMWD5) led to a doubling of the accumulation of L-isoleucine, as expected from the amount of accumulated intermediates. L-threonine became efficiently converted to L-isoleucine by using pMWD5. In the L-threonine conversion experiment using MSI medium with 10 g/l L-threonine, the amount of accumulated L-isoleucine was approximately 95% of the amount of L-threonine that disappeared on a molar basis in the case of TD5 (TDH6 carring pMWD5, data not shown). The trial to increase the activities of DH and TA-B by activation of the chromosomal *ilvDE* genes (using ATS3T as the host) was not so efficient. The activity of [TD+AHAS] and [DH+TA-B] might be still unbalanced in this case although the copy number of pMGA2 seems to be low (approximately 5).

The strain B-3996 accumulates 15 g/l L-threonine from 40 g/l glucose using MSI medium. If 15 g/l L-threonine was converted to L-isoleucine with an efficiency of 95% on a molar basis, the accumulation of L-isoleucine should be 15.7 g/l (39% in yield). The yield of L-isoleucine from glucose by the finally constructed L-isoleucine producer in this report, TVD5 (TDH6 carrying both pVIC40 and pMWD5), reached 25%. To the best of our knowledge, this is the highest yield of L-isoleucine among the reports from which we can define the yield from glucose or other sugars. However, the yield is lower than that expected from the combination of L-threonine productivity of B-3996 and the convertion efficiency of TD5. The L-threonine conversion experiment using MSI medium with added L-threonine includes direct addition of one of substrate (L-threonine) for L-isoleucine synthesis, and pyruvate is supplied from glucose independently. Pyruvate is needed for L-isoleucine synthesis, but not for L-threonine synthesis (Fig. 1). Further more, the *thrABC* operon on pVIC40 is under the control by attenuation which reduces its expression with the existence of L-isoleucine and L-threonine. Although L-threonine did not accumulate in the culture broth of TVD5, intracellular L-threonine and L-isoleucine may be enough to partial attenuation of the *thrABC* operon on pVIC40. The supply of L-threonine may be reduced in L-isoleucine fermentation by TVD5 than in L-threonine fermentation by B-3996 because of the substrate sharing between L-threonine and pyruvate, and/or the reduction of L-threonine synthetic activity.

Acknowledgment

We thank Dr. Kainosho for his kind offer of uniform-^{13}C-L-threonine.

References

1) Umbarger, H. E., Biosynthesis of the Branched-Chain Amino Acids. In "Escherichia coli and Salmonella typhimurium" Vol. 1, eds. Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umbarger, H. E., American Society for Microbiology, Washington, D.C., pp. 352–367 (1987).
2) Chen, J., Bennett, D. C., and Umbarger, H. E., Specificity of attenuation control in the *ilvGMEDA* operon of *Escherichia coli* K-12. *J. Bacteriol.*, **173**, 2328–2340 (1991).
3) Shiio, I., Sasaki, A., and Nakamori, S., Production of L-isoleucine by AHV resistant mutants of *Brevibacterium flavum*. *Agric. Biol. Chem.*, **37**, 2053–2061 (1973).
4) Ikeda, S., Fujita, I., and Yoshinaga, F., Screening of L-isoleucine producers among ethionine resistant mutants of L-threonine producing bacteria. *Agric. Biol. Chem.*, **40**, 511–516 (1976).
5) Komatsubara, S. and Kisumi, M., Fermentation of Isoleucine, Valine and Leucine. In "Aminosan Hakko" (in Japanese), eds. Aida, H., Takinami, K., Chibata, I., Nakayama, K., and Yamada, H., Gakkai Shuppan Center, Tokyo, pp. 397–422 (1986).
6) Kisumi,M., Komatsubara,S., and Chibata,I., Enhancement of isoleucine hydroxamate-mediated growth inhibition and improvement of isoleucine-producing strains of *Serratia marcescens*. *Appl. Environ. Microbiol.*, **34**, 647–653 (1977).
7) Komatsubara, S., Kisumi, M., and Chibata, I., Transductional construction of an isoleucine-producing strain of *Serratia marcescens*. *J. Gen. Microbiol.*, **119**, 51–61 (1980).
8) Lawther, R. P., Calhoun, D. H., Gray, J., Adams, C. W., Hauser, C. A., and Hatfield, G. W., DNA Sequence fine-structure analysis of *ilvG* (IlvG$^+$) Mutations of *Escherichia coli* K-12. *J. Bacteriol.*, **159**, 294–298 (1982).
9) Marsh, N. J. and Duggan, D. E., Ordering of mutant sites in the isoleucine-valine genes of *Escherichia coli* by use of merogenotes derived from F^{14}: a new procedure for fine-structure mapping. *J. Bacteriol.*, **109**, 730–740 (1972).
10) V. G. Debabov, PCT Application, WO90/04636 (May 3, 1990).
11) Hashiguchi, K., Kojima, H., Sato, K., and Sano, K., Effects of an *Escherichia coli ilvA* mutant gene encoding feedback-resistant threonine deaminase on L-isoleucine production by *Brevibacterium flavum*. *Biosci. Biotechnol. Biochem.*, **61**, 105–108 (1997).
12) Kohara, Y., Akiyama, K., and Isono, K., The physical map of the whole *E. coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell*, **50**, 495–508 (1987).
13) Kulakauskas, S., Wikstrom, P. M., and Berg, D. E., Efficient introduction of cloned mutant alleles into the *Escherichia coli* chromosome. *J. Bacteriol.*, **173**, 2633–2638 (1991).
14) Lawther, R. P., Calhoun, D. H., Adams, C. W., Hauser, C. A., Gray, J., and Hatfield, G. W., Molecular basis of valine resistance in *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA*, **78**, 922–925 (1981).
15) Coppola, G., Huang, F., Riley, J., Cox, J. L., Hantzopoulos, P., Zhou, L., and Calhoun, D. H., Sequence and transcriptional activity of the *Escherichia coli* K-12 chromosome region between *rrnC* and *ilvGMEDA*. *Gene*, **97**, 21–27 (1991).
16) Lawther, R. P., Wek, R. C., Lopes, J. M., Pereira, R., Taillon, B. E., and Hatfield, G. W., The complete nucleotide sequence of the *ilvGMEDA* operon of *Escherichia coli* K-12. *Nuc. Aci. Res.*, **15**, 2137–2155 (1987).
17) Arfin, S. M., Ratzkin, B., and Umbarger, H. E., The metabolism of valine and isoleucine in *Escherichia coli* XVII, The role of induction in the derepression of acetohydroxy acid isomeroreductase. *Biochem. Biophys. Res. Commun.*, **37**, 902–908 (1969).
18) Wek, R. C. and Hatfield, G. W., Nucleotide Sequence and *in vivo* expression of the *ilvY* and *ilvC* genes in *Escherichia coli* K12. *J. Biol. Chem.*, **261**, 2441–2450 (1986).
19) Felice, M. D., Griffo, G., Lago, C. T., Limauro, D., and Ricca, E., Detection of the Acetolactate Synthase Isozymes I and III of *Escherichia Coli* K12. In "Methods in Enzymology" Vol. 166, eds. Harris, R. A. and Sokatch, J. R., Academic Press, San Diego, California, pp. 241–244 (1988).
20) Morbach, S., Sahm, H., and Eggeling, L., L-isoleucine production with *Corynebacterium glutamicum*: Further flux increase and limitation of export. *Appl. Environ. Microbiol.*, **62**, 4345–4351 (1996).