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A novel L-isoleucine metabolism in *Bacillus thuringiensis* generating (2*S*,3*R*,4*S*)-4-hydroxyisoleucine, a potential insulinotropic and anti-obesity amino acid

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Abstract 4-Hydroxyisoleucine (HIL) found in fenugreek seeds has insulinotropic and anti-obesity effects, and is expected to be a novel orally-active drug for insulin-independent diabetes. Here we show that newly isolated strain *Bacillus thuringiensis* 2e2 and the closely related strain *B. thuringiensis* ATCC 35646 operates a novel metabolic pathway for L-isoleucine (L-Ile) via HIL and 2-amino-3-methyl-4-ketopentanoic acid (AMKP). The HIL synthesis was catalyzed stereoselectively by an α-ketoglutaric acid-dependent dioxygenase and to be useful for efficient production of a naturally occurring HIL isomer, (2*S*,3*R*,4*S*)-HIL. The (2*S*,3*R*,4*S*)-HIL was oxidized to (2*S*,3*R*)-AMKP by a NAD^+-dependent dehydrogenase. The metabolic pathway functions as an effective by-pass pathway that compensates for the incomplete TCA cycle in *Bacillus* species and also explains how AMKP, a vitamin B_{12} antimetabolite with antibiotic activity, is synthesized. These novel findings pave a new way for commercial production of HIL and also for AMKP.

Keywords L-Isoleucine dioxygenase · 4-Hydroxyisoleucine ·NAD^+-dependent dehydrogenase · 2-amino-3-methyl-4-ketopentanoic acid · *Bacillus thuringiensis* · TCA cycle

Introduction

4-Hydroxyisoleucine (HIL) is a polar non-charged amino acid structurally related to branched chain amino acids that is contained in fenugreek seeds (Fowden et al. 1973), which are used in traditional medicines as a remedy for diabetes and hypercholesterolemia (Al-Habori and Raman 1998). In 2000, Broca C. et al. discovered that the major HIL isomer in fenugreek seeds, (2*S*,3*R*,4*S*)-HIL (Fig. 1a), enhances glucose-induced insulin secretion through a direct effect on pancreatic β cells in rats and humans (Broca et al. 2000). Furthermore, this compound was found to be active and partly corrected hyperglycemia and glucose tolerance in a new rat model of type II diabetes (Broca et al. 1999). In 2005, Handa T. et al. reported the effects of HIL in obese mice fed a high-fat diet and suggested that HIL ought to be accepted as a useful anti-obesity food (Handa et al. 2005).

Several methods of chemo enzymatic synthesis of HIL have been reported. Wang Q. et al. reported an eight-step synthesis process involving a stereoselective bioreduction step catalyzed by *Geotrichum candidum* (Wang et al. 2002), and Rolland-Fulcrand V. et al. reported a six-step synthesis process involving an enzymatic chiral resolution step catalyzed by immobilized penicillin acylase G (Rolland-Fulcrand et al. 2004).

However, these processes require complex multistep reactions and have difficulties in controlling the stereochemistry of HIL that has three chiral centers in its molecule (Fig. 1). An enzymatic method through aldol-condensation of acetaldehyde and α-ketobutyric acid catalyzed by an aldolase and consecutive amination by transaminase was reported, and resulted in the generation of two kinds of stereoisomers, (2*S*,3*R*,4*S*)-HIL and (2*S*,3*R*,4*R*)-HIL (Fig. 1a and 1c, respectively), with low yield (Ogawa et al. 2007; Smirnov et al. 2007). One paper described an enzyme involved in the biosynthesis of HIL in fenugreek. In 1996, Haefele C. et al. reported that a α-ketoacid-dependent dioxygenase plays a key role in the biosynthetic pathway of HIL, although the enzyme has not been purified yet and not characterized in detail (Haefele et al. 1997).

We recently found a microorganism, *Bacillus thuringiensis* strain 2e2, that is able to catalyze the hydroxylation of L-Ile to HIL. From the lysate supernatant of *B. thuringiensis* strain 2e2, the L-Ile hydroxylating enzyme was partially purified and analyzed (Kodera et al. 2009). Because this enzyme needed α-ketoglutaric acid (αKG), Fe^{2+}, and ascorbic acid as cofactors for the hydroxylating reaction, it turned out to be novel type of αKG-dependent dioxygenase. In this paper, we describe that the metabolic fate of L-Ile in two related *B. thuringiensis* species, 2e2 and ATCC 35646, including the conversion of L-Ile to (2*S*,3*R*,4*S*)-HIL and further oxidation to (2*S*,3*R*)-2-amino-3-methyl-4-ketopentanoic acid (AMKP) catalyzed by a novel $NAD(P)^+$-dependent dehydrogenase. The physiological role of this novel pathway, to effectively compensate for the transformation of αKG to succinic acid, a missing TCA cycle reaction in *B. thuringiensis*, is also discussed.

Materials and methods

Screening for strains containing L-Ile hydroxylase

Stock or soil-isolated microorganisms were inoculated into culture medium comprising 0.4% (w/v) soluble starch, 0.4% (w/v) yeast extract, 1% (w/v) malt extract, and 0.2% (w/v) L-Ile (pH 7.0). After shaking the culture at 28°C for 2 days, HIL was analyzed by amino acid analysis of the centrifuged supernatant. To preserve the isolated microorganisms, a 2% (w/v) agar plate of preservation medium comprising 0.1% (w/v) KH_2PO_4, 0.1% (w/v) K_2HPO_4, 0.03% (w/v) $MgSO_4 \cdot 7H_2O$, 0.01% (w/v) yeast extract, 0.2% (w/v) NH_4Cl, and 0.2% (w/v) L-Ile (pH 7.0) was used.

Isolation and identification of strain 2e2

By using L-Ile as a substrate, screening was performed for microorganisms with HIL producing ability. Five hundred and twelve strains isolated from soil and 597 strains from stock cultures were tested. After shaking the culture at 28°C for 2 days, the HIL produced during the cultivation was analyzed by amino acid analysis of the centrifugation supernatant. As a result, one of the bacterial strains, strain 2e2, was found to have the ability to produce HIL.

Strain 2e2 underwent molecular phylogenetic analysis based on the nucleotide sequence of 16S rDNA. As a result of a homology search of the bacterial strain database using BLAST, a partial nucleotide sequence of the 16S rDNA of strain 2e2 (AB560665) was found to match that of the 16S rDNA of the *Bacillus thuringiensis* ATCC 10792 strain with a homology of 100%. As a result of a homology search of GenBank/DDBJ/EMBL, the 16S rDNA of the strain 2e2 was found to show a high homology with that of *B. thuringiensis*. Furthermore, in a simplified molecular phylogenetic analysis using the 16S rDNA of strain 2e2 and 16S rDNA of the 30 most homologous strains retrieved from the bacterial strain database, strain 2e2 was found to belong to the same phylogenetic branch as that of the 16S rDNA of *B. thuringiensis*, showing that they are very closely related. In a simplified morphological observation, strain 2e2 showed common properties of *Bacillus* bacteria, e.g., cell morphology: bacillus (size: 1.0 to 1.2 x 2.0 to 3.0 μm); Gram staining: positive; endospore: positive; attitude to oxygen: aerobic; growth temperature: favorable growth at 20 to 35°C; optimum pH: pH 7.0 to 7.5, and the results of the analysis of the partial 16S rDNA sequence also showed that the strain 2e2 belonged to *B. thuringiensis*. Strain *B. thuringiensis* 2e2 was deposited as AKU 251 in Culture Collection of the Faculty of Agriculture, Kyoto University (Kyoto, Japan), and as FERM BP-10688 in International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Tsukuba, Japan).

Amino acid analysis conditions

HIL, AMKP, and L-Ile were derivatized using the AccQ-Tag method (Waters, Milford, MA, USA). The amino acid derivatives were analyzed using a high-performance liquid chromatography (HPLC) system equipped with a fluorescent detector. The XBridge C_{18} column (5 μm, 2.1 × 150 mm; Waters) was used for the separation at 40°C. The mobile phases were Waters AccQ-Tag Eluent A (eluent A) and methanol (eluent B), and the flow rate of the eluent was 0.3 ml/min. The eluent gradients were 20-40% (v/v) B in 0-15 min and 80% (v/v) B in 15.1-18 min.

Analysis of 4 pairs of HIL enantiomers

As representatives of each enantiomer pair, (2*R*,3*R*,4*R*)-HIL (HIL1) and (2*S*,3*R*,4*R*)-HIL (HIL3) were obtained according to the method of a previous report (Inghardt et al. 1991), and (2*R*,3*R*,4*S*)-HIL (HIL2) and (2*S*,3*R*,4*S*)-HIL (HIL4) were obtained according to that of another report (Wang et al. 2002). When HIL1 to HIL4 obtained by chemical synthesis were analyzed under the conditions described above, they appeared at different retention times: HIL1: 4.4 min; HIL2: 12.9 min; HIL3: 9.8 min; HIL4: 10.6 min.

Analysis of 8 stereoisomer of HIL and 4 stereoisomer of AMKP by HPLC

Amino acid solutions (0.5 mg/ml amino acids and 4 mg/ml triethylamine in 50% acetonitrile) were mixed in equal amount with 2 mg/ml 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) in acetonitrile, and were reacted for 30 min at RT. The amino acid derivatives were analyzed using a HPLC system equipped with a UV detector. The CAPCELL PAK C18 column (5 μm, 4.6 × 250 mm; Shiseido, Tokyo, Japan) was used for the separation at 45°C. The mobile phases were 10 mM KH_2PO_4, pH 2.8 (eluent A) and acetonitrile (eluent B), and the flow rate of the eluent was 0.3 ml/min. The eluent gradients were 20-25% (v/v) B in 0-60 min and 25% (v/v) B in 60.1-70 min. The diastereomers were detected spectrophotometrically at 250 nm.

Nuclear magnetic resonance (NMR) and mass-spectrometric (MS) analysis of metabolites in *B. thuringiensis* 2e2 culture

Naturally occurring HIL was extracted and isolated from fenugreek seeds according to a previously described HIL purification method (Alcock et al. 1989). AMKP in the culture media of *B. thuringiensis* 2e2 was purified and a synthesized AMKP was obtained according to previously reported methods (Perlman et al. 1977). These compounds were dissolved in D_2O, and their ^{1}H-NMR spectra were recorded on an Avance 400 (Bruker, Billerica, MA, USA). MS-analysis of AMKP was performed using a high-resolution mass-spectrometer (Q-TofMS).

Assay of L-Ile hydroxylation, HIL dehydrogenation, and deamination activities in *B. thuringiensis* 2e2

B. thuringiensis 2e2 cells were cultivated in the culture medium until an $O.D._{660}$ of 3.2 was achieved, harvested by centrifugation, and washed in physiological saline twice. The washed cells were resuspended in a buffer consisting of 10% (w/v) of glycerol, Complete Mini (Roche, Basel, Switzerland), and 20 mM Tris buffer (pH 7.5); disrupted 3 times using French Pressure Cells and Press (Thermo Spectronic, Ronchester, USA); and centrifuged to obtain the cell lysate with a protein concentration of about 10 mg/ml, and the precipitates. The centrifugation precipitates were suspended in physiological saline of the same volume as the cell lysate. Similarly, resting cells used for the preparation of the cell lysate were washed and suspended in physiological saline at a concentration 10 times higher than that of the culture broth. For the standard L-Ile hydroxylation activity assay, the lysate, the precipitate suspension, and the resting cells were each mixed with equal volumes of a 2 × hydroxylation reaction mixture comprised of 10 mM L-Ile, 2 mM $FeSO_4 \cdot 7H_2O$, 10 mM αKG, 10 mM ascorbic acid, and 200 mM TES (pH 7.0), and the reaction was allowed to proceed at 28°C for 1 hour. Enzymatic activity was determined by measuring the amount of HIL produced in the reaction mixture.

For the standard HIL dehydrogenation activity assay, the lysate, the precipitate suspension, and the resting cells were each mixed with equal volumes of a 2 ×dehydrogenation reaction mixture comprised of 10 mM HIL, 10 mM NAD^+ or $NADP^+$, and 200 mM TES (pH 7.0), and the reaction was allowed to proceed at 28°C for 1 hour. Enzymatic activity was determined by measuring the amount of AMKP produced in the reaction mixture.

For the dehydrogenating deamination activity assay, the lysate was mixed with an equal volume of a 2 × dehydrogenating deamination reaction mixture comprised of 5 mM substrate, 5 mM $NAD(P)^+$, and 20 mM Tris (pH 7.5), and the reaction was allowed to proceed at 37°C for 1 hour. Enzymatic activity was determined by measuring the absorbance at 340 nm in the reaction mixture.

For the αKG-dependent transamination activity assay, the lysate was mixed with an equal volume of a 2 ×transamination reaction mixture comprised of 5 mM substrate, 20 mM αKG, 0.1 mM pyridoxal-5-phosphate, and 20 mM Tris buffer (pH 7.5) was used at 28°C. Enzymatic activity was determined by measuring the amount of glutamic acid produced in the reaction mixture.

Assay of L-Ile hydroxylation with *B. thuringiensis* 2e2 lysate under various conditions

The standard L-Ile hydroxylation activity assay as above was performed with some modifications. In order to examine effects of various cofactors on the reaction, 5 mM of each cofactor was used in various combinations. In order to examine the pH dependency of the reaction, MES (pH 4.0-6.0), MOPS (pH 6.0-7.0), TES (pH 7.0-8.0), Boric acid (pH 8.0), and Tris (pH 9.0) were used as buffers to adjust the pH. In order to examine the temperature dependency of the reaction, the reaction temperature was varied from 15 to 50°C in 100 mM GTA (pH 6.0). To measure temperature stability, the lysate was incubated at 0 to 50°C for 1 hour, and then L-Ile hydroxylation activity was measured in the reaction mixture buffered with 100 mM HEPES (pH 7.0).

Construction of the *Escherichia coli* strains expressing two proteins of *B. thuringiensis* ATCC 35646 and assay for the enzyme activities by use of the lysates

B. thuringiensis ATCC 35646 was purchased from American Type Culture Collection and pre-cultured overnight at 28°C in 5 ml of LB medium. By using 1.5 ml of the culture broth as a seed, a main culture was carried out in 50 ml of LB medium. After cultivation up to a logarithmic growth phase, cells were harvested from 50 ml of the culture broth by centrifugation (12,000 × *g*, 4°C, 15 min). From these cells, chromosomal DNA was prepared according to an ordinary method. Based on published genomic sequence information about *B. thuringiensis* ATCC 35646 (GenBank accession No. NZ_AAJM01000012), the following primer sets were synthesized: CATATGGAGGTTTTTATAATGACGTTTGTT and CTCGAGTTTTGTCTCCTTATAAGAAAATGT for ZP_00738910; CATATGAGAGAGAATAAAATAATTATGATTTCT and CTCGAGCTACAAGTTTTTCCCAGCAGTCCAA for ZP_00738909. By using the prepared primers and chromosomal DNA of *B. thuringiensis* ATCC 35646 as a template, amplification by PCR was carried out with PrimeSTAR (Takara, Kyoto, Japan) under the following condition: 30 cycles for 10 seconds at 98°C, 15 seconds at 52°C and 1 minute at 72°C. The obtained PCR products were digested with *Nde*I and *Xho*I endonucleases, and cloned into an expression vector pET21b (Novagen, CA, USA) which has been digested with the same endonucleases. The plasmids was introduced into *E. coli* Rosetta2 (DE3), and the resulting transformants were pre-cultured with shaking in LB medium supplemented with 50 μg/ml ampicilin. By seeding the pre-cultured broth in 50 ml LB medium at 1%, the main culture was carried out at 37°C. At 2 hours after the start of the cultivation, IPTG was added at a final concentration of 1 mM, and the cultivation was carried out for further 3 hours. After completion of the cultivation, cells were harvested, washed, suspended in 1 ml of 20 mM Tris-HCl (pH 7.6) and disrupted with a sonicator, Insonator 201M (Kubota, Tokyo, Japan). The lysates were centrifuged at 15,000 rpm for 10 minutes to obtain a supernatant which was used as crude enzyme solutions. Using the lysate (12 mg/ml of protein concentration) including the recombinant protein of ZP_00738910, the optimized L-Ile hydroxylation activity assay was performed in 15 mM L-Ile, 50 mM αKG, 0.5 mM Fe^{2+}, 5 mM ascorbic acid at 28°C, and pH 6.0, and then the produced HIL was determined by HPLC. Also produced succinic acid was determined by F-kit (Roche Diagnostics,. Basel, Switzerland). By using the lysate including the recombinant protein of ZP_00738909, HIL dehydrogenating activity assay was performed as described above.

Results

B. thuringiensis 2e2 produced HIL and also accumulated another amino acid in the culture medium containing L-Ile

B. thuringiensis 2e2, was found to have the ability to produce a substance with the same retention time on amino acid analysis as authentic HIL (Product 1 in Fig. 2). This product was purified from the culture media of *B. thuringiensis* 2e2, and NMR analysis was performed. As a result, this compound showed similar chemical shifts to HIL extracted from fenugreek seeds (Table 1). From the above results, it was revealed that *B. thuringiensis* 2e2 produced HIL in culture medium containing L-Ile. Additionally, in the culture supernatant of *B. thuringiensis* 2e2, another amino acid was also observed as a major product during HPLC analysis as well as HIL (Product 2 in Fig. 2).

A (2*S*,3*R*,4*S*)-HIL was the sole HIL stereoisomer produced from L-Ile by *B. thuringiensis* 2e2

The steric configuration of the HIL produced by *B. thuringiensis* 2e2 was determined. HIL has asymmetric carbons at 3 sites, and 8 types of diastereomers and 4 pairs of enantiomers exist. The 4 pairs of enantiomers are (2*S*,3*S*,4*S*) and (2*R*,3*R*,4*R*) (henceforth referred to as HIL1), (2*S*,3*S*,4*R*) and (2*R*,3*R*,4*S*) (HIL2), (2*S*,3*R*,4*R*) and (2*R*,3*S*,4*S*) (HIL3), and (2*S*,3*R*,4*S*) and (2*R*,3*S*,4*R*) (HIL4). These pairs of HIL enantiomers were well separated under the conditions of the amino acid analysis. The retention time of the HIL produced was the same as that of HIL4 when the HIL-containing sample prepared from the culture of the *B. thuringiensis* 2e2 was analyzed. When the sample was mixed with a HIL4 standard and analyzed, the peaks matched completely. Because, when L-Ile, which has a steric configuration of (2*S*,3*S*), was used as a substrate in this study, the HIL produced by the hydroxylation reaction was either the (2*S*,3*R*,4*S*) or (2*S*,3*R*,4*R*) enantiomer. Putting together these facts, it was shown that, when L-Ile was used as a substrate, the HIL produced by *B. thuringiensis* 2e2 was entirely in the (2*S*,3*R*,4*S*) diastereomer form, which is the main component of the naturally occurring HIL existing in fenugreek. Further confirmation of the stereostructure of HIL was obtained using a newly established HPLC method for separating all 8 stereoisomers.

2-Amino-3-methyl-4-ketopentanoic acid (AMKP) accumulated in the L-Ile converting culture of *B. thuringiensis* 2e2

Next, the other amino acid produced by *B. thuringiensis* 2e2 (Product 2 in Fig. 2) when L-Ile was used as a substrate was identified. The molecular weight of the product was analyzed by MS and found to be 145, which was lighter than that of HIL by 2. Furthermore, when its molecular formula was estimated by precise mass measurement using a high resolution mass spectrometer (Q-TofMS), $C_6H_{11}NO_3$ was obtained, which has 2 fewer hydrogen atoms than HIL. The above results suggested that the other substance produced by *B. thuringiensis* 2e2 was 2-amino-3-keto-4-methylpentanoic acid (AMKP). The product was purified from the culture media of *B. thuringiensis* 2e2, and NMR analysis was performed. As a result, this compound showed similar chemical shifts to chemically synthesized AMKP (Table 1). From the above findings, it was confirmed that *B. thuringiensis* 2e2 produces AMKP. In HPLC analysis, which can differentiate among AMKP diastereomers, the produced AMKP appeared as a single peak, indicating that only one isomer, (2*S*,3*R*)-AMKP, which retains the stereostructure of L-Ile had accumulated.

Metabolic analysis of HIL and AMKP synthesis with *B. thuringiensis* 2e2 lysate

The lysate of *B. thuringiensis* 2e2 was prepared to characterize L-Ile hydroxylation activity. The amounts of HIL and AMKP produced by the resting cells and the lysate fractions are shown in Fig. 3. Using the lysate supernatant, its HIL production activity was confirmed using L-Ile as a substrate (Fig. 3a). The AMKP production found in the resting cells of *B. thuringiensis* 2e2 was not detected in any fraction of the lysates without any cofactors.

Next, the dehydrogenation activity of HIL in the lysate fractions of *B. thuringiensis* 2e2 was determined. AMKP production was observed in the lysate fractions containing HIL as a substrate as well as in the resting cells in the presence of NAD^+ but $NADP^+$ (Fig. 3b). This result indicated that AMKP is a dehydrogenated product of HIL made in a reaction involving a NAD^+-dependent dehydrogenase.

Using the lysate supernatant of *B. thuringiensis* 2e2, the optimal reaction conditions for HIL production were investigated. First, the effects of cofactors on the HIL production reaction involving the hydroxylation of L-Ile were examined. The reaction was performed in a reaction mixture containing various cofactors, and the amount of HIL produced was measured. In previous paper, we showed that Fe^{2+} and αKG were essential for the production of HIL, and the amount of HIL produced was maximized further by adding ascorbic acid (Kodera et al. 2009). Therefore, it was strongly suggested that an αKG-dependent dioxygenase is involved in the production of HIL through the hydroxylation of L-Ile. Second, the pH dependency of the HIL production activity was evaluated. Activity was detected at pH 5.0 to 8.0 with maximal activity at pH 6.0. Third, the temperature dependency of the HIL production activity was evaluated. The maximum activity was found at 30°C. Forth, the temperature stability of the HIL production activity was evaluated. The enzyme was inactivated at 50°C or higher in 1 hour incubation at pH 7.5. Under the optimum reaction conditions; i.e., pH 6.0 and 28°C in the presence of 5 mM αKG, 1 mM Fe^{2+}, and 5 mM ascorbic acid, the lysate supernatant produced 1.6 mM of HIL from 5 mM L-Ile in 1 hour.

Further deamination of HIL and AMKP was mainly catalyzed by aminotransferase in *B. thuringiensis* 2e2

B. thuringiensis lacks a component of the citric acid cycle (TCA cycle) reactions, succinic acid generation from αKG (Aronson et al. 1975). This is compensated for by transaminase-decarboxylase coupling amino acid reactions (Fig. 4b). To investigate the involvement of the novel L-Ile metabolic pathway (Fig. 4a) in the TCA-cycle by-pass reaction, HIL and AMKP deamination activity was measured in the supernatant of the lysate of *B. thuringiensis* 2e2 (Table 2). In a reaction mixture containing $NAD(P)^+$ for amino acid dehydrogenase reactions, the deamination of L-Ile, HIL, and

AMKP were detected only weakly. On the other hand, the amino acids were strongly deaminated in a reaction mixture containing αKG as a substrate for transaminase reactions. These results suggested that the deamination of HIL and AMKP is mainly catalyzed by transaminases with αKG as an amino acceptor rather than amino acid dehydrogenases and possibly compensates for the absent component of the TCA cycle reaction (Fig. 4c).

Identification of L-Ile dioxygenase in *B. thuringiensis* ATCC 35646 and its application to the HIL production

From the lysate supernatant of *B. thuringiensis* strain 2e2, the dioxygenase was partially purified and the N-terminal amino acid sequence of the purified protein with molecular weight of about 31 kDa was analyzed (Kodera et al. 2009). The N-terminal first 20-amino acid sequence was subjected to BLAST search and a hypothetical protein, ZP_00738910, of *B. thuringiensis* ATCC 35646 was found to have 100% homology in the N-terminal region.

Here ZP_00738910 was expressed in *E. coli* and the lysate supernatant of the transformed *E. coli* was evaluated as to HIL production from L-Ile. The *E. coli* lysate produced 13 mM (1.9 g/L) HIL and about the same amount of succinic acid in 3 h from 15 mM L-Ile, and 50 mM αKG. The stereostructure of the produced HIL was again confirmed as (2*S*,3*R*,4*S*)-HIL. It was demonstrated that ZP_00738910 of *B. thuringiensis* ATCC 35646 was L-Ile dioxygense and was about the same protein found in *B. thuringiensis* 2e2. The amino acid sequence of ZP_00738910 contained three metal ligands and an αKG binding residue (Fig. 5a), which were conserved in the αKG-dependent dioxygenase family (Hausinger 2004). Thus ZP_00738910 was confirmed to be an L-Ile dioxygenase (IDO) from both the catalytic properties and sequence information. Homology analysis of the dioxygenase was conducted with four dioxygenases known to hydroxylate free amino acids, L-proline, L-asparagine, and L-arginine (Mori et al. 1997; Shibasaki et al. 1999; Strieker et al. 2007; Yin and Zabriskie 2004). The IDO was not similar to these dioxygenases, and had only 18.6 % homology to L-proline 3-hydroxylase at a maximum (Fig. 5b).

Gene cloning for the identification of HIL dehydrogenase from *B. thuringiensis* ATCC 35646

By searching the genomic sequence of *B. thuringiensis* ATCC 35646, it was found that one gene was found next to the gene of IDO with a 4 base-overlap (Fig. 5a). This gene was encoded ZP_00738909 that was annotated as 3-ketoacyl-ACP (acyl carrier protein) reductase, which was a kind of NAD(P)H/NAD(P)$^+$-dependent oxidoreductase. Then ZP_00738909 was expressed in *E. coli* and the lysate supernatant of the transformed *E. coli* was applied to the assay for HIL dehydrogenation activity. The *E. coli* lysate showed the activity of dehydrogenating 10 mM (2*S*,3*R*,4*S*)-HIL into 4.9 mM AMKP in the presence of 10 mM NAD$^+$. The stereostructure of produced AMKP was confirmed as (2*S*,3*R*)-AMKP. Thus it was demonstrated that ZP_00738909 was a NAD$^+$-dependent HIL dehydrogenase (HILDH).

Discussion

The (2*S*,3*R*,4*S*)-HIL, a diastereomer coincident with the main component of the naturally occurring HIL present in fenugreek seeds, was enzymatically produced from L-Ile through direct hydroxylation using *B. thuringiensis* 2e2 cells or the recombinant IDO derived from *B. thuringiensis* ATCC 35646. IDO belonging to αKG-dependent dioxygenase family showed homology less than 20 % with other free amino acid hydroxylases classified into this family (Fig. 5b). Together with the fact that IDO was the first enzyme possessing C4-hydroxylation activity towards a free aliphatic amino acid, the IDO is a novel type of αKG-dependent dioxygenase.

The amount of HIL obtained by using L-Ile hydroxylation of IDO was enough comparable to that extracted from fenugreek seeds (0.6 g of HIL need 100 g of the seeds) (Broca et al. 2000). Additionally, the enzymatic hydroxylation of L-Ile has an advantage for stereoselective HIL production over the extraction method from fenugreek, because it leads to a single diastereomer of a (2*S*,3*R*,4*S*)-HIL with potential insulinotropic and anti-obesity activities; whereas, a mixture of (2*S*,3*R*,4*S*)- and (2*R*,3*R*,4*S*)-HIL diastereomers is present in fenugreek seeds.

In *B. thuringiensis* 2e2 culture containing L-Ile, AMKP also accumulated. AMKP, a C4-keto form of Ile, was first found in *B. cereus* 439, a strain closely related to *B. thuringiensis*, and acts as the antimetabolite for vitamin B_{12} but its physiological function is still unknown (Perlman et al. 1977). It has not been determined how AMKP is biosynthesized in *B. cereus* 439. As AMKP was found to be a dehydrogenated product of a (2*S*,3*R*,4*S*)-HIL diastereomer in *B. thuringiensis* 2e2, a (2*S*,3*R*)-AMKP diastereomer was produced. The amount of AMKP produced by *B. thuringiensis* 2e2 culture was 3.06 mM. On the other hand, the estimated amount of

AMKP produced by the *Bacillus* bacteria was previously described to be 0.04 mM (Perlman et al. 1977), and thus, it was confirmed that *B. thuringiensis* 2e2 shows high AMKP production activity.

In this paper, furthermore, HILDH of *B. thuringiensis* ATCC 35646 was found to dehydrogenate (2*S*,3*R*,4*S*)-HIL into (2*S*,3*R*)-AMKP in the presence of NAD^+. HILDH was annotated to a 3-ketoacyl-ACP reductase, FabG. FabG is involved in type II fatty acid biosynthesis mainly present in bacteria and plants, and catalyzes reduction of 3-ketoacyl-ACP to 3-hydroxyacyl-ACP (Chan and Vogel 2010). It was found that the homologous enzymes of FabG could be used for the production of chiral alcohols like 4-chloro-3-hydroxybutanoate and ethyl 4-chloroacetoacetate (Holsch et al. 2008; Yamamoto et al. 2003), but no homologue had been reported to react with keto amino acids. In the genome survey of *B. thuringiensis* ATCC 35646, IDO and HILDH genes were found to be adjacent and partially overlapped (Fig. 5a). In addition, it was also found that a gene of putative transporter, ZP_00738908, located behind HILDH gene and formed an operon together with IDO and HILDH genes. It is very likely that the transporter exchanges L-Ile, HIL, and/or AMKP across the cell membrane.

In our preliminary observation, AMKP was gradually degraded in *B. thuringiensis* 2e2 culture as one can see the different amounts of HIL decomposition and AMKP accumulation in Fig. 3b. AMKP must have been deaminized to α-keto acid because no other amino acid metabolites were detected. Indeed, strong transaminase activities were detected for HIL and AMKP in the lysate of *B. thuringiensis* 2e2. Microorganisms belonging to *B. thuringiensis* usually have an incomplete TCA cycle that lacks the ability to dehydrogenate αKG to form succinic acid (Aronson et al. 1975). Instead of this pathway, they perform a γ-aminobutyric acid bypass reaction via L-glutamic acid (Fig. 4b). The conversion of αKG into L-glutamic acid is accompanied by the transamination of HIL and AMKP into the corresponding α-keto acids (Table 2). HIL and AMKP behave as amino group donors in order to prepare αKG for the γ-aminobutyric acid bypass. Furthermore, the direct conversion of αKG into succinic acid is accompanied by the hydroxylation of L-Ile. Overall, two equivalents of αKG are converted into succinic acid from one equivalent of L-Ile via HIL or AMKP. In contrast, only one equivalent of αKG is consumed without such hydroxylation (Fig. 4b and c). It was suggested that the hydroxylation of L-Ile and further dehydrogenation to AMKP facilitate the carbon flux of the incomplete TCA cycle of *B. thuringiensis*.

The results of the present study provided the following five important scientific findings: 1) novel microbial methods for the production of (2*S*,3*R*,4*S*)-HIL were established; 2) a novel αKG-dependent dioxygenase for the aliphatic amino acid L-Ile was found; 3) a novel NAD^+-dependent dehydrogenase for HIL was found; 4) the unknown biosynthetic pathway of a vitamin B_{12} antimetabolite, AMKP, was revealed, and 5) a novel effective TCA-cycle by-pass pathway involving coupling reactions with a dioxygenase, dehydrogenase, transaminase, and decarboxylase was found. In particular, the hydroxylation of L-Ile with the predicted αKG-dependent dioxygenase should be useful for the industrial production of its (2*S*,3*R*,4*S*)-HIL diastereomer, which is the major diastereomer component of natural occurring HIL extracted from fenugreek seeds and has potential insulinotropic and anti-obesity activities.

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Figure legends

Fig. 1 Eight diastereomers of 4-hydroxyisoleucine (HIL). **a-h** HIL has asymmetric carbons at 3 sites, and 8 types of diastereomers and 4 pairs of enantiomers exist. The 4 pairs of enantiomers are **a** and **b**, **c** and **d**, **e** and **f**, and **g** and **h**.

Fig. 2 Detection of two amino acids produced from L-Ile in *B. thuringiensis* 2e2 culture by amino acid analysis. The amino acids present in the culture after 2 days cultivation were derivatized with AccQ-tag and analyzed. Gray line shows a chromatogram of amino acid analysis from the medium without *B. thuringiensis* 2e2, and black line shows that from *B. thuringiensis* 2e2 culture. The arrows show two novel amino acids produced by *B. thuringiensis* 2e2.

Fig. 3 Determination of HIL and AMKP production activity in *B. thuringiensis* 2e2. **a** L-Ile transforming activity. Using 10 mM L-Ile as a substrate, HIL (open bars) and AMKP (filled bar) production for 1 hour were assayed in resting cells, the cell lysate, and cell precipitates in the presence of 5 mM αKG, 1 mM Fe^{2+}, and 5 mM ascorbic acid. **b** HIL transforming activity. Using 10 mM HIL as a substrate, HIL reduction (gray bars) and AMKP production (filled bars) for 1 hour were assayed in resting cells, the cell lysate, and cell precipitates in the presence of 5 mM NAD^+.

Fig. 4 Effect of the novel metabolic pathway of L-Ile on the incomplete TCA cycle of *B. thuringiensis* 2e2. **a** Stereoselective enzymatic conversion of L-Ile into HIL and AMKP found in *B. thuringiensis* 2e2. **b** and **c** Prospective pathways for the conversion of αKG into succinic acid in the presence of L-Ile. **b** A pathway without αKG-dependent hydroxylation of L-Ile. **c** A reaction involving a novel L-Ile metabolic pathway found in this study that occurs via HIL and AMKP. L-Glu: L-glutamic acid, GABA: γ-aminobutyric acid.

Fig. 5 L-Ile dioxygenase (IDO) and HIL dehydrogenase (HILDH) of *B. thuringiensis* ATCC 35646. **a** Amino acid sequences of IDO (upper) and HILDH (lower), and close gene arrangement of them (center, in italic). In IDO sequence, filled boxes show three metal ligands, and gray box shows an αKG-binding residue. Partially overlap of IDO and HILDH genes in the genome was shown as underlined. **b** Molecular phylogenetic analysis of IDO with known dioxygenases hydroxylating free amino acids: NP_627448 of *Streptomyces coelicolor* A3(2); AAP92493 of *Streptomyces vinaceus*; BAA20094 of

Dactylosporangium sp.; and BAA22406 of *Streptomyces* sp.

Table 1 NMR analysis of metabolites in *B. thuringiensis* 2e2 culture

Compound	^{1}H NMR spectrum (400 MHz; D_2O)
HIL in fenugreek	δ = 0.78 (3H, d, *J* = 7.0 Hz, $CHCH_3$), 1.07 (3H, d, *J* = 6.3 Hz, δCH_3), 1.75 (1H, m, βCH), 3.68 (1H, m, γCH), 3.72 (1H, d, *J* = 4.3 Hz, αCH).
HIL in *B. thuringiensis* 2e2	δ = 0.79 (3H, d, *J* = 7.0 Hz, $CHCH_3$), 1.07 (3H, d, *J* = 6.3 Hz, δCH_3), 1.76 (1H, m, βCH), 3.68 (1H, m, γCH), 3.73 (1H, d, *J* = 4.4 Hz, αCH)
Racemic AMKP[a]	δ = 1.08 (3H, d, *J* = 7.6 Hz, $CHCH_3$, **A**), 1.13 (3H, d, *J* = 7.5 Hz, $CHCH_3$, **B**), 2.12 (3H, s, δCH_3, **B**), 2.14 (3H, s, δCH_3, **A**), 3.18 (1H, m, βCH, **AB**), 3.76 (1H, d, *J* = 4.5 Hz, αCH, **B**), 3.92 (1H, d, *J* = 3.3 Hz, αCH, **A**)
AMKP in *B. thuringiensis* 2e2	δ = 1.11 (3H, d, *J* = 7.3 Hz, $CHCH_3$), 2.11 (3H, s, δCH_3), 3.15 (1H, m, βCH), 3.73 (1H, d, *J* = 4.7 Hz, αCH)

Abbreviations for NMR: s: singlet; d: doublet; m: multiplet.

[a]A mixture of two diastereomers (**A**: major and **B**: minor)

Table 2 Deamination activity of the metabolites of L-Ile in *B. thuringiensis* 2e2 cell lysate

Substrate	Cofactor	Specific activity[a]
Amino acid dehydrogenase activity		
L-Ile	NAD^+	0.20 (0.02)
	$NADP^+$	n.d.
HIL	NAD^+	0.48 (0.01)
	$NADP^+$	n.d.
AMKP	NAD^+	0.03 (0.01)
	$NADP^+$	n.d.
αKG-dependent transaminase activity		
L-Ile	αKG	4.94 (0.42)
HIL	αKG	16.02 (1.63)
AMKP	αKG	7.43 (0.74)

[a]nmol/min per milligram of total protein in *B. thuringiensis* 2e2 cell lyaste.

Table 3 Deamination activity of the metabolites of L-Ile in *B. thuringiensis* 2e2 cell lysate

Substrate	Cofactor	Specific activity[a]
Amino acid dehydrogenase activity		
L-Ile	NAD^+	0.20 (0.02)
	$NADP^+$	n.d.
HIL	NAD^+	0.48 (0.01)
	$NADP^+$	n.d.
AMKP	NAD^+	0.03 (0.01)
	$NADP^+$	n.d.
αKG-dependent transaminase activity		
L-Ile	αKG	4.94 (0.42)
HIL	αKG	16.02 (1.63)
AMKP	αKG	7.43 (0.74)

[a]nmol/min per milligram of total protein in *B. thuringiensis* 2e2 cell lyaste.

Fig. 1

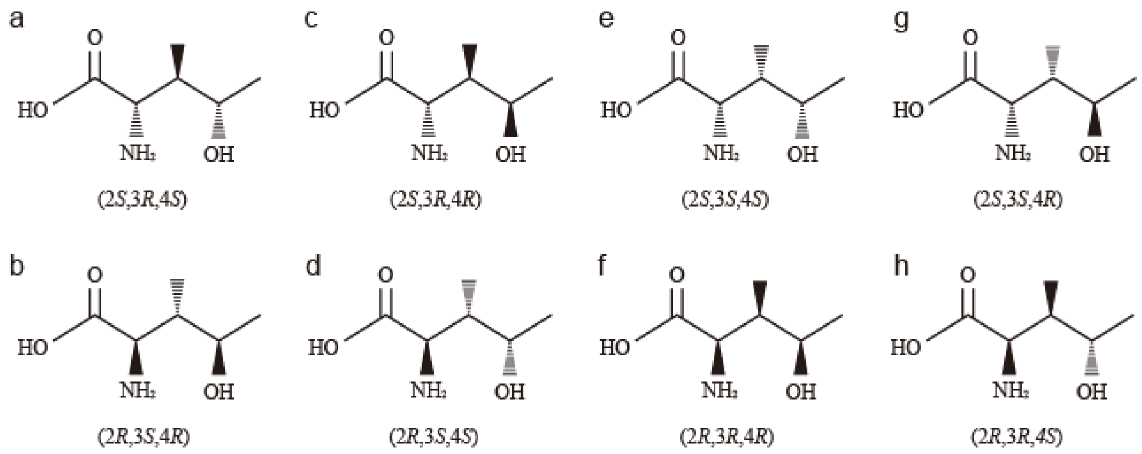
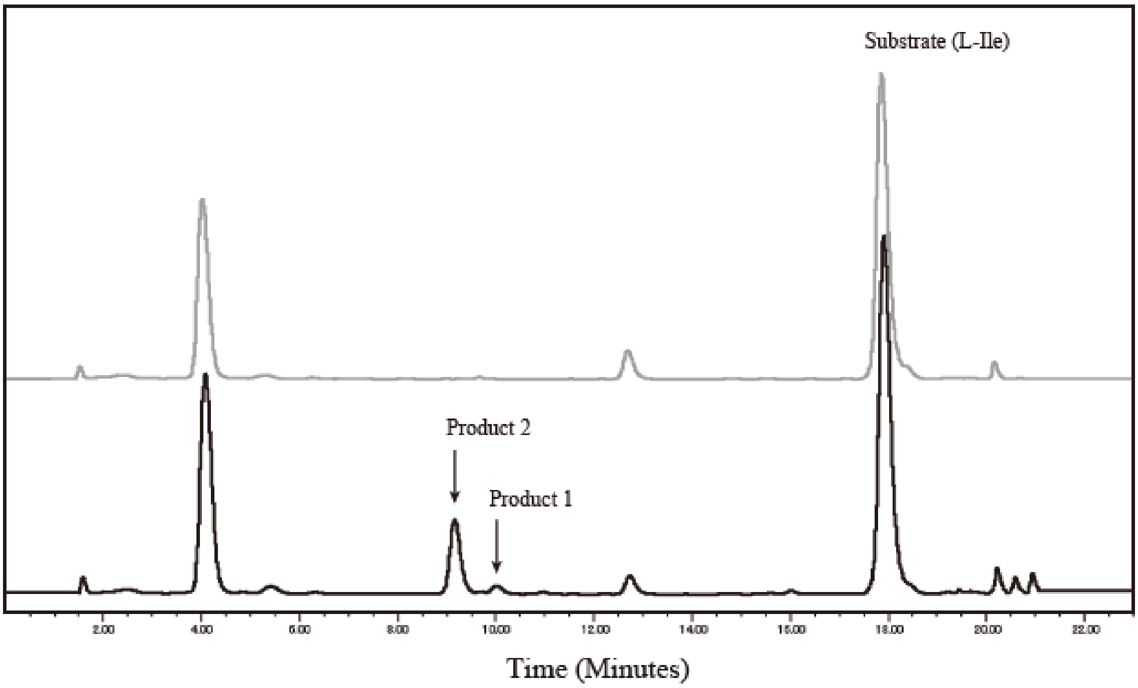

a
(2S,3R,4S)
c
(2S,3R,4R)
e
(2S,3S,4S)
g
(2S,3S,4R)
b
(2R,3S,4R)
d
(2R,3S,4S)
f
(2R,3R,4R)
h
(2R,3R,4S)

Fig. 2



Substrate (L-Ile)
Product 2
Product 1
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
Time (Minutes)

Fig. 3

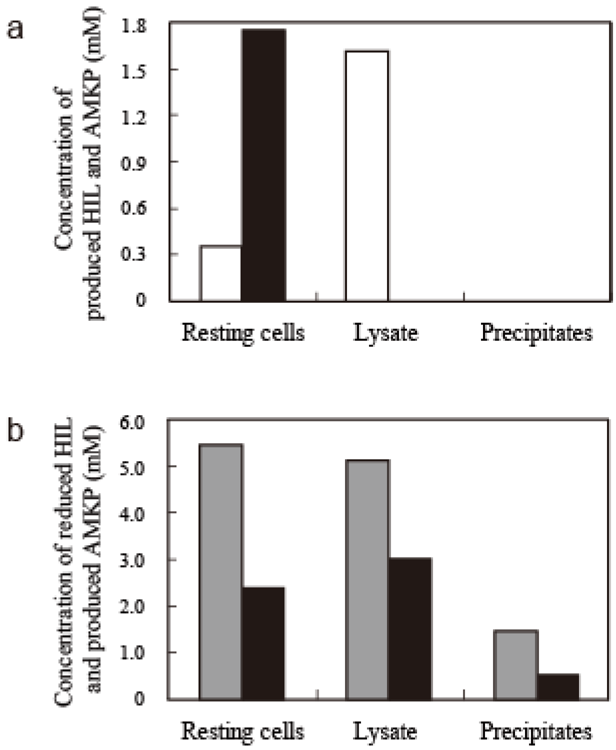

a
Concentration of produced HIL and AMKP (mM)
1.8
1.5
1.2
0.9
0.6
0.3
0
Resting cells
Lysate
Precipitates
b
Concentration of reduced HIL and produced AMKP (mM)
6.0
5.0
4.0
3.0
2.0
1.0
0
Resting cells
Lysate
Precipitates

Fig. 4

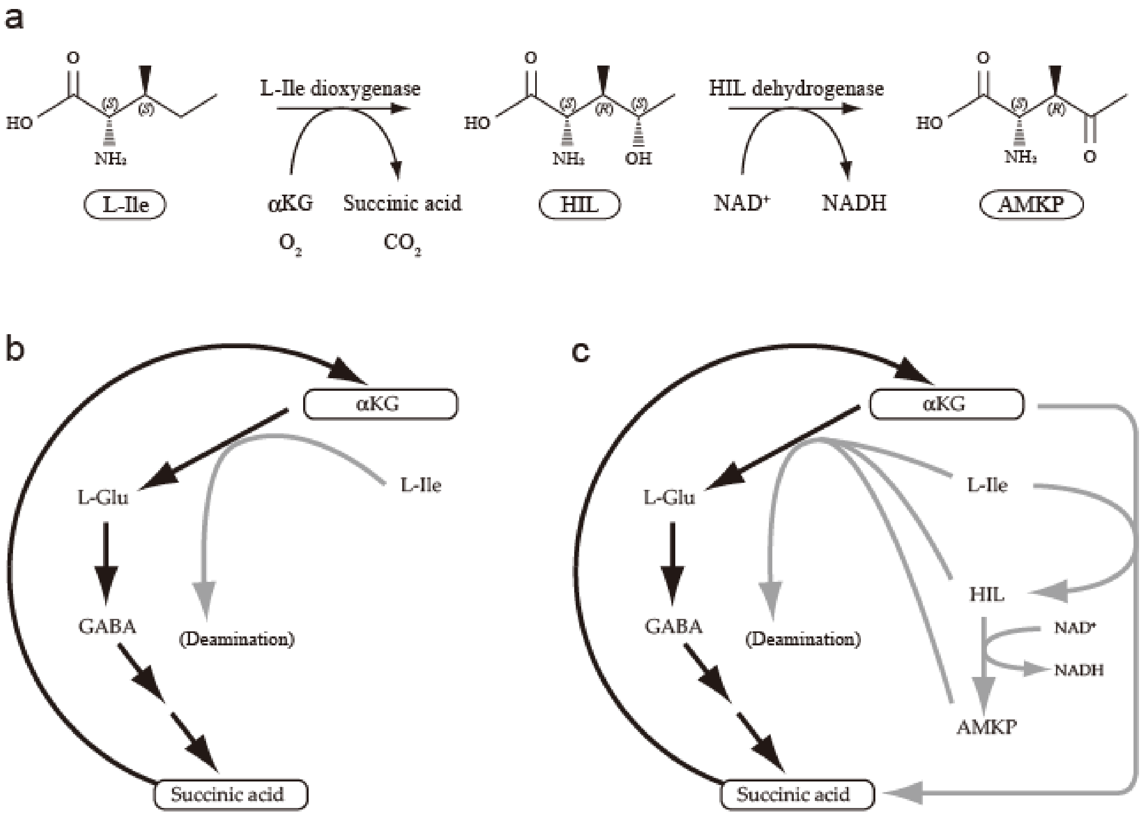

a
L-Ile dioxygenase
HIL dehydrogenase
L-Ile
αKG
Succinic acid
O_2
CO_2
HIL
NAD^+
NADH
AMKP
b
αKG
L-Glu
L-Ile
GABA
(Deamination)
Succinic acid
c
αKG
L-Glu
L-Ile
HIL
GABA
(Deamination)
NAD^+
NADH
AMKP
Succinic acid

Fig. 5

a

```
MTFVLSKMSGFSIEEKVHEFESKGFLEISNEIFLQEEENHRLLTQAQLDYYNLEDDAYGE    60 |
CRARSYSRYIKYVDSPDYILDNSNDYFQSKEYNYDDGGKVRQFHSINDSFLYNPLIQNIV   120 |      IDO
RFDTEFAFKTNIIDTSKDLIIGLHQVRYKATKERPSFSSPIWLHKDDEPVVFLHLMNLSN   180 | (ZP_00738910)
TAIGGDNLIANSPREINQFISLKEPLETLVFGQKVFHAVTPLGTECSTEAFRDILLVTFS   240 |
Y  K  E  T  K  *                                               245 |
```

tataaggagacaaa<u>atga</u>gagagaataaaataattatgatttctggagccaatagcggta

```
               M  R  E  N  K  I  I  M  I  S  G  A  N  S  G  I   16 |
GHACIKYFLEKSFHVIALDINNNNLIDYMKTDMPLKVVQIDLSNSEAIRNLFTQLDLEKL    76 |      HILDH
SPDILINAAGIREITPVLNLSDDMFKKVIDVNLVAPFILSREVAKRWCESKIKGCIVNIA   136 | (ZP_00738909)
SVSGLMAEPERAAYVASKHALIGLTKQMAMEFGKQNIRVNSISPGVIRTELTEEYFSNKA   196 |
LMSMIKSNQSLDTWGLPQDIVSCIEYLISDQARFITGSNFVIDGGWTAGKNL*          248 |
```

b