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Improvement of L-Lysine Production by *Methylophilus methylotrophus* from Methanol *via* the Entner-Doudoroff Pathway, Originating in *Escherichia coli*

Kohei ISHIKAWA,[1,3,†] Yoshiya GUNJI,[1] Hisashi YASUEDA,[2] and Kozo ASANO[3]

[1]*Fermentation and Biotechnology Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210-8681, Japan*
[2]*Life Science Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210-8681, Japan*
[3]*Graduate School of Agriculture, Hokkaido University, Kita 9, Nishi 9, Sapporo 060-8589, Japan*

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To improve the amino acid production by metabolic engineering, eliminating the pathway bottleneck is known to be very effective. The metabolic response of *Methylophilus methylotrophus* upon the addition of glucose and of pyruvate was investigated in batch cultivation. We found that the supply of pyruvate is a bottleneck in L-lysine production in *M. methylotrophus* from methanol as carbon source. *M. methylotrophus* has a ribulose monophosphate (RuMP) pathway for methanol assimilation, and consequently synthesized fructose-6-phosphate is metabolized to pyruvate *via* the Entner-Doudoroff (ED) pathway, and the ED pathway is thought to be the main pathway for pyruvate supply. An L-lysine producer of *M. methylotrophus* with an enhanced ED pathway was constructed by the introduction of the *E. coli edd-eda* operon encoding the enzyme involving the ED pathway. In this strain, the overall enzymatic activity of ED pathway, which is estimated by measuring the activities of 6-phosphogluconate dehydrogenase plus 2-keto-3-deoxy-6-phosphogluconate aldolase, was about 20 times higher than in the parent. This strain produced 1.2 times more L-lysine than the parent producer. Perhaps, then, the supply of pyruvate was a bottleneck in L-lysine production in the L-lysine producer of *M. methylotrophus*.

Key words: L-lysine production; *edd-eda* (ED); methanol; *Methylophilus methylotrophus*

L-Lysine is important amino acid used mostly as a feed additive for livestock above 7×10^5 tons/year. It is exclusively produced in a bioprocess employing coryneform bacteria, usually *Corynebacterium glutamicum*. The substrates for amino acid fermentation are generally sugars from agricultural crops, but in the future such agricultural crops will be needed to meet the increase in demand for human food, which is expected to cause a rise in sugar prices. Methanol has received considerable attention from the fermentation industry as an alternative substrate, since it is relatively inexpensive, of high purity, and is easily handled during transportation and storage. In addition, because it is produced from natural gas, which can be obtained cheaply on a large scale in some parts of the world, it does not compete directly with the raw materials for human food.

Methylophilus methylotrophus AS1 is an obligate methylotroph originally isolated from activated sludge.[1)] The organism was extensively studied in the 1970s, and was industrialized for the production of a single-cell protein.[2,3)] It has several advantages for industrial exploitation. Its growth rate is the highest of any methylotroph, and many genetic tools have been developed for it.[4)] These properties make *M. methylotrophus* a potentially useful host in the production of many chemicals, including amino acids from methanol. We chose *M. methylotrophus* AS1 as a host strain to produce L-lysine from methanol.

M. methylotrophus has a characteristic central metabolic pathway that is different from that of *E. coli*. Methanol is oxidized to formaldehyde *via* an oxygen-dependent respiratory chain, the methanol oxidase system, and the formaldehyde is subsequently assimilated into the cell material *via* the ribulose monophosphate (RuMP) pathway, or dissimilated to CO_2 and NAD(P)H, by either the RuMP pathway or a linear route in which formate is an intermediate (Fig. 1).[5–7)] It has been reported that the TCA cycle, the main energy production pathway for many aerobes, is incomplete in this bacterium due to the absence of 2-oxoglutarate dehydrogenase.[8)] The RuMP pathway and the form-

† To whom correspondence should be addressed. Tel: +81-44-244-7168; Fax: +81-44-211-7833; E-mail: kouhei_ishikawa@ajinomoto.com

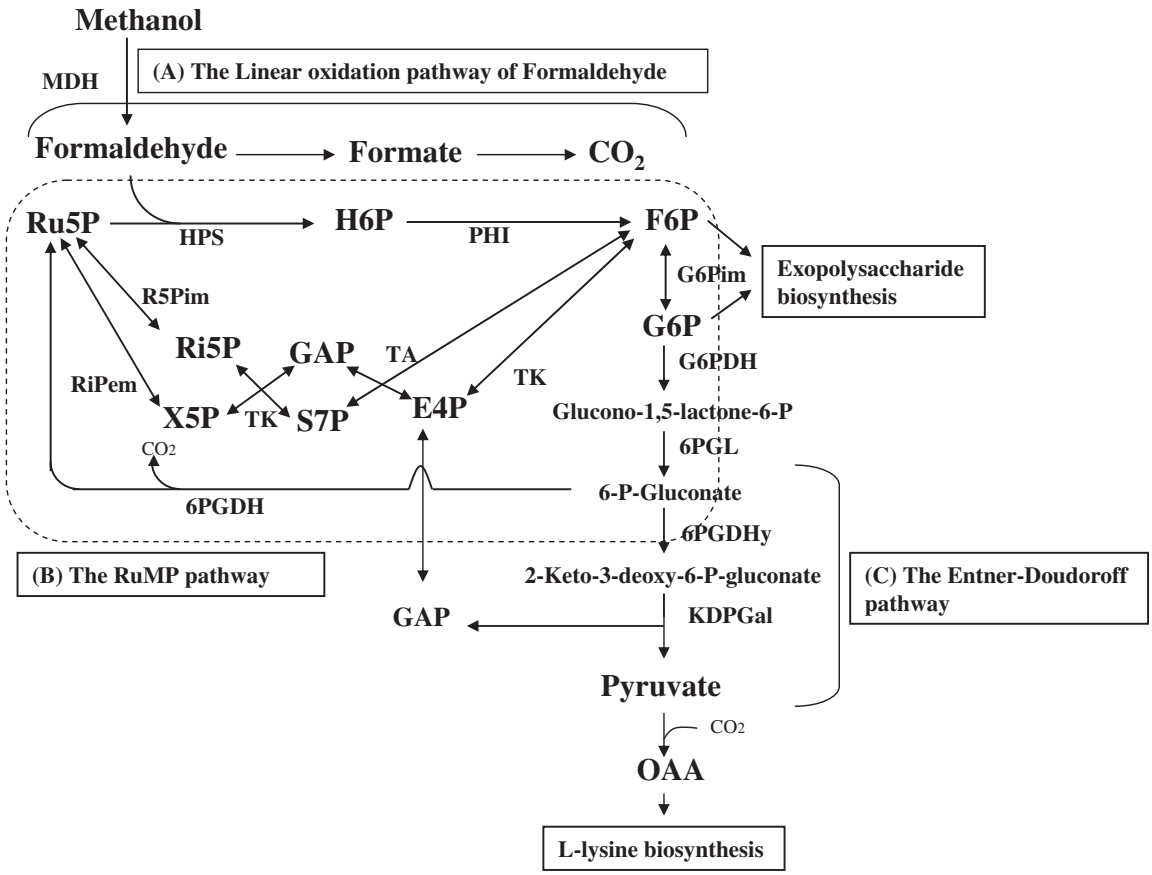

Fig. 1. Methanol Metabolic Pathways in *M. methylotrophus*.

Methanol oxidized by corresponding oxidative systems to produce formaldehyde, which is oxidized in the linear oxidation pathway (A) or assimilated in the RuMP pathway (B). Pyruvate is supplied *via* Entner-Doudroff pathway (C). *Abbreviations*: MDH, methanol dehydrogenase; HPS, 3-hexulose-6-phosphate synthase; PHI, 6-phospho-3-hexuloisomerase; G6Pim, glucose-6-phosphate isomerase; G6PDH, glucose-6-phosphate 1-dehydrogenase; 6PGL, 6-phosphogluconolactonase; 6PGDH, 6-phosphogluconate dehydrogenase; 6PGDHy, 6-phospho-gluconate dehydratase; KDPGal, 2-keto-3-deoxy-6-phosphogluconate aldolase; R5Pim, ribose-5-phosphate isomerase; Ripem, ribulose-5-phosphate 3-epimerase; TA, transaldolase; TK, transketolase; TPI, triosephosphate isomerase; Ru5P, ribulose 5-phosphate; H6P, 3-hexulose 6-phosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; E4P, erythrose 4-phosphate; S7P, sedoheptulose 7-phosphate; Ri5P, ribose 5-phosphate; X5P, xylulose 5-phosphate; OAA, oxaloacetate.

aldehyde oxidation pathway are thought to be the main energy and CO_2 production pathways in *M. methylotrophus*.[9] Extracellular polysaccharide (EPS) is also produced from a metabolic intermediate on the RuMP pathway using sugar phosphate, glucose-6-phosphate, and fructose-6-phosphate.[10] Formaldehyde is assimilated into ribulose-5-phosphate and converted to pyruvate through the RuMP pathway and the Entner-Doudoroff (ED) pathway (Fig. 1). The ED pathway consists of two enzymes, 6-phosphogluconate (6PG) dehydrogenase, encoded by the *edd* gene, and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, encoded by the *eda* gene. The ED pathway is thought to be the main pathway for pyruvate supply from methanol in *M. methylotorophus*.[7] We have reported that *M. methylotrophus* possesses the diaminopimelic acid (DAP) pathway for L-lysine biosynthesis.[11,12] Oxaloacetate, a precursor of L-lysine, is produced from pyruvate by pyruvate carboxylase, and pyruvate is also utilized in the DAP pathway. Hence, pyruvate supply appears to be very important to L-lysine production in *M. methylotrophus*.

The breeding of the L-lysine producer by metabolic engineering has been examined in some bacteria, especially in *C. glutamicum*. Avoiding feedback inhibition and increasing intracellular metabolic precursors in the L-lysine biosynthetic pathway are known to have positive effects on L-lysine production.[13–17] From the standpoint of avoiding feedback inhibition, we reported an increase in L-lysine production due to the introduction of *dapA24*, which encodes a dihydrodipocolinate synthase desensitized to feedback inhibition by L-lysine, in combination with *lysE24*, a mutated *lysE* gene originating from *Corynebacterium glutamicum* 2256.[18] Furthermore, we have reported that the disruptant strain of *metF*, encoding 5,10-methylene-tetra-hydrofolate reductase, leads to increases in L-lysine production by avoiding feedback inhibition of threonine against aspartokinase.[19] In this study, we focused on increasing the intracellular metabolites in the L-lysine biosynthetic

Table 1. Bacterial Strains and Plasmids Used in This Study

Strain or plasmid	Description	Phenotype	Source of reference
Strains			
E. coli JM109	recA1, endA1, tyrA1, gyrA96, thi-1, hsdR17, supE44, relA1, Δ(lac-proAB)/F′[traD36, proAB+, lacIq, lacZΔM15]		Takara Bio. Inc.
E. coli K-12 W3110	F$^-$IN(*rrn-rrn* E)		Bachmann *et al.*[22]
M. methylotrophus AS1	Wild type, NCIMB 10515		NCIMB
M. methylotrophus 102	*metF* disruptant strain derived from *M. methylotrophus* AS1	Met$^-$	Ishikawa *et al.*[19]
Plasmids			
pML122	Broad-host-range vector derived from RSF1010	Gm^R	Labes *et al.*[24]
pBHR1	Broad-host-range vector derived from pML122	Km^R	Antonie *et al.*[23]
pBHREA	pBHR1 derivative contain *lysE24* and *dapA24*	Km^R	This study
pSEA10	pRSt derivative contain *lysE24* and *dapA24*	Sm^R	Gunji *et al.*[18]
pBGEA10	pBHREA derivative contain gentamycine resistant gene	Km^R, Gm^R	This study
pRSt	pRS derivative plasmid carrying *Ptac* promoter and cloning sites	Sm^R	Gunji *et al.*[18]
pRSED	pRS derivative contain *edd-eda* gene from *E. coli*	Sm^R	This study

Abbreviations: *lysE24*, mutated *lysE* gene encoding L-lysine exporter, which is originated from *Corynebacterium glutamicum* 2256; *dapA24*, mutated *dapA* gene encoding a dihydrodipicolinate synthase desensitized from feedback inhibition by L-lysine; *Ptac*, *tac* promoter; NCIMB, National Collections of Industrial and Marine Bacteria; Met$^-$, met required; Km^R, kanamycin resistance; Sm^R, streptomycine resistance; Gm^R, gentamycine resistance.

pathway to improve L-lysine production in *M. methylotrophus*. We examined biomass and product yield in *M. methylotrophus*, and identified the target to increase L-lysine yield. We constructed the L-lysine producer of *M. methylotrophus* by the enhanced ED pathway. The strain produced more L-lysine than the parent producer, and both CO_2 and EPS production in this strain decreased as compared with the parent.

Materials and Methods

Bacteria strains, plasmids, and culture conditions. The bacterial strains and plasmids used or created in this work are shown in Table 1. *M. methylotrophus* and derived strains were grown in a modified mineral salt medium (SEIIa), as described previously.[12] *M. methylotrophus* was grown at 37 °C, and 50 mg/l of streptomycin, 50 mg/l of gentamycin, and 20 mg/l of kanamycin were used to maintain the respective plasmids. *Escherichia coli* strains were grown at 37 °C in L broth with appropriate antibiotics.[20]

Batch cultivation using Jar fermentation was performed in 1-liter glass vessels (Able, Tokyo) using SEIIa medium, which contains 500 mM methanol.[11] Fermentation of *M. methylotrophus* was initiated by a 1:10 dilution of a flask culture at an OD660 of 1.0. The cells were cultured at 34 °C, and the pH of the medium was maintained at 6.6 with ammonia gas. Dissolved oxygen (DO) was controlled at more than 25% saturation using a DO control system (DL-1033, Able), and cell growth was monitored by measuring OD660. On average, an OD660 of 1.0 resulted in a cell dry weight of 0.56 g/l.

Fed-batch cultivation using Jar fermentation was performed in 1-liter glass vessels (Able) using SEIIc medium.[11] The initial concentration of methanol in the culture was 500 mM, and a solution containing a mixture of 12.3 M methanol and 4.5 g/l ammonium sulfate was fed during growth to maintain a methanol concentration of 50 mM using an automatic feed controller with an alcohol sensor probe (Able). Dissolved oxygen (DO) was controlled at 25% saturation using a DO control system (DL-1033, Able).

DNA manipulation and transformation. The standard procedures were as described by Sambrook and Russell.[20] Plasmid DNA was isolated from *M. methylotrophus* using Wizard Plus Minipreps DNA Purification Systems (Promega, Madison, WI). Restriction enzymes, DNA ligase, Klenow enzymes, and Pyrobest DNA polymerase were purchased from Takara-Bio (Kyoto, Japan). PCR amplification was performed as indicated by the manufacturer (Takara-Bio). Transformation was done by electroporation using a Gene Pulser II electroporation system (Bio-Rad, Le Jolla, CA).[21] DNA was sequenced with a BigDyeTM Terminator Cycle Sequencing Kit with an ABI model 310A sequencer. DNA sequence analysis was carried out with GENETYX software (Software Development, Tokyo).

Construction of plasmids and strains. The fragments of DNA containing the *edd-eda* operon were amplified by PCR from genomic DNA of *E. coli*. The PCR primers ED-1 5′-CGCTAGTCGACCCAATTTTTACACTTTC-AGGCCTCG-3′ and ED-2 5′-GGGGGGGATCCAGT-CAGAATGTCACGTTTGATAAT-3′ were used to amplify the *edd-eda* operon from *E. coli* W3110,[22] and this DNA fragment was blunted and kinated using a BKL kit (Takara-Bio) and ligated into plasmid vector pRStac[11] digested with Sse8387I and SapI (Takara Bio) to construct pRSED. Plasmid pBGEA10 was constructed to express *lysE24* and *dapA24* in *M. methylotrophus*.[18] The fragment of DNA containing *lysE24* and *dapA24* was collected from pSEA10 digested with EcoRI and BglII (Takara Bio), and this DNA fragment was blunted, kinated, and ligated into plasmid vector pBHR1,[23] which was digested with DraI using a BKL kit (Takara-Bio) to construct plasmid pBHR-EA. The DNA fragment encoding the gentamicin resistance gene was amplified by PCR

using PCR primers GM-1: 5′-CGCCAGCCAGGACAGAAATGC-3′ and GM-2: 5′-GTCCAGCGGTTTTTCTTGGGCT-3′ using plasmid pML122 as a template.[24] To construct pBGEA, this DNA fragment was blunted, kinated, and ligated into pBHR-EA digested with NcoI using a BKL kit (Takara-Bio).

Enzyme assays. M. methylotrohus cells were grown at 37 °C for 16 h in SEIIa medium, at which time it reached late-log phase. The cells were harvested, washed, and suspended in 100 mM Tris–HCl buffer (pH 7.6). Cell extracts were prepared by sonication at 4 °C. Cell debris was removed by centrifugation at 75,000 × *g* for 10 min. Clarified supernatants were used as crude cell extracts.[11]

The ED pathway consists of two enzymes, 6-phosphogluconate dehydrogenase and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, and these enzyme reactions proseed, continuously. These two enzymatic reactions, the overall activity of the ED pathway, can be estimated by measuring the activities of the 6-PGD dehydrogenase and KDPG aldolase enzymes, as described previously.[25,26] The total proteins in the crude extract were determined in triplicate following Lowry *et al.*[27]

Analysis. The concentrations of methanol, L-lysine, and glutamate in culture media were in most cases measured with a BioFlow BF5 (Oji Scientific Instruments, Hyogo, Japan) using alcohol oxidase sensor, and with a Biotech-Analyzer AS210 (Sakura Seiki, Tokyo) using a lysine oxidase sensor or glucose oxidase sensor. Organic acids containing citrate, 2-keto-gluconate, malate, succinate, lactate, formate, pyruvate, and acetate in the culture medium were determined using a high-performance liquid chromatograph (L8500, Hitachi, Tokyo). The concentrations of amino acids in the culture supernatants were measured with an automatic amino acid analyzer (Hitachi). The concentration of extracellurar polysaccharide (EPS) in the culture supernatants was determined by the phenol/sulphuric acid method.[28] Intracellular metabolites ribulose-5-phosphate, fructose-6-phosphate, glucose-6-phosphate, 6-phosphogluconate, sedoheptulose-7-phosphate, pyruvate, alanine, valine, isoleucine and leucine were measured by capillary electrophoresis mass spectrometry.[29]

Online analysis of the oxygen and carbon dioxide content of the exhaust gas was performed using an exhaust gas analyzer (Able). The amount of carbon dioxide evolution (given in g/l) was determined by the following equation:

$$\Delta[CO_2]_n = 44 \cdot \Delta T\left(\left(\frac{[CO_2]_{n-1} + [CO_2]_n}{200}\right) \cdot Q_{out} - \frac{PC_{in}}{100} \cdot Q_{in}\right) \cdot \frac{273}{(T + 273)} \cdot \frac{1}{VL \cdot 22.4},$$

where $\Delta[CO_2]_n$ is the concentration of CO_2 evolution (g/l); ΔT is time between measurements (h); $[CO_2]_{n-1}$ and $[CO_2]_n$ are the concentrations of CO_2 in the exhaust gas at n and at n-1 times (g/l); Q_{out} is air flow rate from the inlet (ml/min); Q_{in} is air flow rate from the outlet (ml/min); PC_{in} is the concentration of CO_2 in the supply air; T is the temperature (°C); and VL is working volume of the bioreactor (ml).

Determination of the ratio DCW per OD_{660}. For determination of dry cell weight (DCW) per OD660, cells of a SEII plate culture of *M. methylotrophus* AS1 and 102 were washed with saline and inoculated into SEIIa medium. The cells were harvested by centrifugation (8,000 rpm, 4 °C, 20 min) in the exponential phase (OD660 of about 9). After two washing steps with saline, the cells were dried for 24 h at 105 °C, and DCW was determined. For both AS1 and the 102 strain, an OD660 of 1 corresponded to 0.56 g, DCW/l. According to Goldberg *et al.*,[30] the C content of the *M. methylotrophus* DCW amounts to 45% (*w*/*w*).

Analysis of product formation. For analysis of the biomass and product formation, *M. methylotrophus* was grown in batch and in fed-batch cultivation using a jar fermentor. The Cmols were calculated by the carbon number of each product to the mol of each product, cell, amino acid, EPS, and of evolved CO_2 after complete consumption of all carbon sources was measured. The EPS produced from *M. methylotrophus* constituted of glucose, mannose, and galactose, and the ratio was 1:3:1.[10] We calculated that molecular weight of EPS is 180.13 and the C number of EPS was 6.

Results

Biomass and L-lysine yield in batch cultivation

To determine the target for further improvement of L-lysine production, we examined biomass and product yield using both wild-type strain, *M. methylotrophus* AS1, and the L-lysine producer of *M. methylotrophus* 102, a methionine auxotroph, with plasmid pBGEA expressing *lysE24* and *dapA24*. Biomass and product accumulation, including extracellular polysaccharide (EPS), amino acid, organic acid, and evolved CO_2, were examined in both *M. methylotrophus* AS1 and *M. methylotrophus* 102 harboring pBGEA in batch cultivation using a jar fermentor. It was found that almost all the carbon of methanol against methanol added to the medium (500 Cmmol), 93.8% Cmmol/Cmmol, was incorporated into cells, CO_2, and EPS in *M. methylotrophus* AS1 (Table 2). The L-lysine in *M. methylotrophus* 102/pBGEA was 45.3 Cmmol (9.06% Cmmol/Cmmol). The total yield of cells, CO_2, and EPS added to the medium was 79.8% Cmmol/Cmmol in *M. methylotrophus* 102/pBGEA. These products of *M. methylotrophus* 102/pBGEA were lower than those of *M. methylotrophus* AS1 (Table 2). No organic acids were detected in either strains.

Table 2. Cell and Product Formation in *M. methylotrophus* AS1 and *M. methylotrophus* 102/pBGEA

Strain	Cells and product (Cmmol)[a]					
	L-lysine	Cell	CO_2	EPS	Glu	Other amino acid[b]
M. methylotrophus AS1	N.D.[c]	221	207	41.0	6.80	6.89
M. methylotrophus 102/pBGEA	45.3	176	193	29.7	2.04	11.5

[a]Cell and product concentrations were determined after complete consumption of methanol. Values are means from at least three independent cultivations. Standard deviations were in all experiments below 5%.
[b]Other amino acid means total amount of asparaginic acid, glutamine, threonine, serine, glycine, alanine, cysteine, valine, leucine, isoleucine, tyrosine, phenylalanine, histidine, and proline.
[c]N.D., not detected.

Table 3. Effect of Glucose and Pyruvate Addition in Batch Cultivation Using *M. methylotrophus* AS1 and *M. methylotrophus* 102/pBGEA

Strain	Carbon source (Cmmol)			Cells and product (Cmmol)[a]				
	Methanol	Glucose	Pyruvate	Cells	CO_2	EPS	L-lysine	Glutamic acid
M. methylotrophus AS1	500	0	0	200	207	40.3	N.D.[b]	5.44
	500	90	0	218	248	55.3	N.D.[b]	7.48
	500	0	90	205	218	39.7	N.D.[b]	15.6
M. metylotrophus 102/pBGEA	500	0	0	174	195	33.0	45.4	4.01
	500	90	0	176	261	42.7	50.5	6.56
	500	0	90	179	230	34.0	61.8	7.51

[a]Cell and product concentrations were determined after complete consumption of methanol. Values are means from at least three independent cultivations. Standard deviations were in all experiments below 5%.
[b]N.D., not detected.

Effects of glucose and pyruvate addition on L-lysine production

To identify the bottleneck in L-lysine production, the additional effects of pyruvate and of glucose in *M. methylotrophus* AS1 and *M. methylotophus* 102/pBGEA strain were examined. Batch cultivation was carried out in SEIIa medium containing 500 Cmmol methanol supplemented with 90 Cmmol glucose or 90 Cmmol pyruvate. Glucose and pyruvate were completely consumed during 30 h of cultivation. Glutamate significantly increased in the medium with pyruvate, and L-lysine was not detected under any condition using *M. methylotrophus* AS1 (Table 3). EPS and CO_2 under the condition of glucose added increased especially. These results indicate that the consumed carbon of glucose mainly transferred to EPS and CO_2 *via* the RuMP pathway, and that of pyruvate mainly transferred to CO_2 and glutamate.

In *M. methylotrophus* 102/pBGEA, L-lysine production was significantly increased by the addition of pyruvate. The total cell amount was maintained at same level under all conditions using methionine auxotroph. The amounts of CO_2 and EPS were the highest under the condition of glucose added in *M. methylotrophus* 102/pBGEA (Table 3). In both strains, it appears that the addition of glucose was mainly transferred to CO_2 and EPS production. In *M. methylotrophus* 102/pBGEA, pyruvate addition enhanced L-lysine production. Based on these results, it was speculated that the pyruvate supply is bottleneck in L-lysine production.

Effects of overexpression of the edd-eda *operon in fed-batch cultivation*

In order to increase the pyruvate supply, we decided to enhance the ED pathway in *M. methylotrophus*, the main pathway for pyruvate supply. We constructed plasmid pRSED, containing the *edd-eda* operon from *E. coli*. pRSED was introduced into *M. methylotrophus* 102/pBGEA, and we obtained *M. methylotrophus* 102/pBGEA/pRSED.

The effect of the *edd-eda* operon on L-lysine production from methanol was determined in fed-batch cultivation using *M. methylotrophus* 102/pRSED and *M. methylotrophus* 102/pRSED/pBGEA. The overall enzymatic activity of the ED pathway was about 20 times higher than that in the parent (Table 4). Cell growth stopped at 20 h under both conditions, because methionine was limited (Fig. 2A), but L-lysine production in *M. methylotrophus* 102/pBGEA/pRSED was higher than in *M. methylotrophus* 102/pBGEA through the culture (Fig. 2B). The yields of CO_2 and of EPS in *M. methylotrophus* 102/pBGEA/pRSED were 20.0 Cmmol and 10.0 Cmmol lower than those in *M. methylotrophus* 102/pBGEA. The yields of valine, leucine, isoleucine, and glutamate in *M. methylotrophus* 102/pBGEA/pRSED were higher than those in *M. methylotrophus* 102/pBGEA (Table 4). The result in this experiment was similar to that under the added pyruvate condition, and hence it was suggested that the L-lysine producer of *M. methylotrophus* with enhanced ED pathway is capable of producing L-lysine more effectively than the parental strain.

Table 4. Product Formation and ED-Activity in a Fed-Batch Culture

Host	plasmid-1	plasmid-2	Cell and products (Cmmol)[a]								ED activity
			Cell	CO_2	EPS	L-lysine	Valine	Leucine	Isoleucine	Glutamate	(U/mg·protein)[b]
M. methylotrophus 102	pBGEA	—	267	418	33.3	92.5	13.3	5.79	8.61	12.8	0.09
	pBGEA	pRSED	276	398	23.3	112	15.6	6.31	10.1	18.4	1.93

[a]Cell and product concentrations were determined after complete consumption of methanol. Values are means from at least three independent cultivations. Standard deviations were in all experiments below 5%.
[b]Acitivities were examined in the crude extracts. One units of activity is defined as the amount of enzyme which produces 1 μmol of products per min. Three independent enzyme assays were performed, all three showing comparable results.

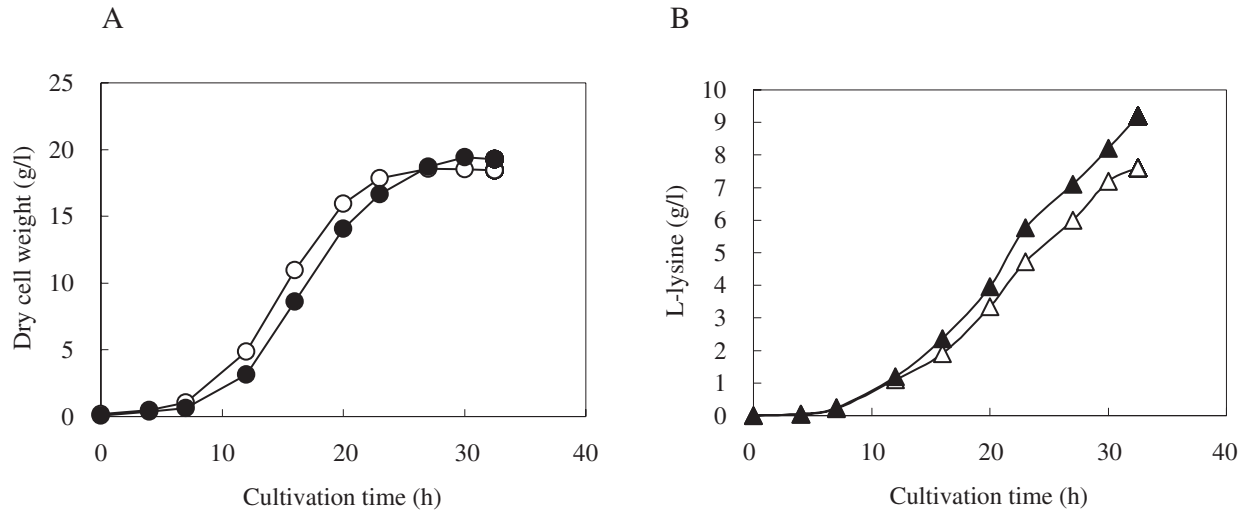

Fig. 2. Profile of Cell Growth and L-Lysine Production.
A, Cell growth. Open circles (○) show *M. methylotrophus* 102/pBGEA, closed circles (●) show *M. methylotrophus* 102/pBGEA/pRSED. The X-axis shows cultivation time (h). The Y-axis shows the amounts of dry cells. B, L-lysine production. Open triangles (△) show *M. methylotrophus* 102/pBGEA, and closed triangles (▲) showed *M. methylotrophus* 102/pBGEA/pRSED. The X-axis shows cultivation time (h). The Y-axis shows L-lysine production. Three independent fermentations were performed and all three showed comparable results.

Table 5. Analysis of Intracelular Metabolites in *M. methylotrophus* 102/pBGEA/pRSED

Host	plasmid-1	plasmid-2	Intracellular metabolites (μM)									
			R5P	F6P	G6P	6PG	S-7P	Pyr	Ala	Val	Ile	Leu
M. methylotrophus 102	pBGEA	—	15.0	40.7	18.6	7.23	13.4	173	29.2	214	80.1	68.8
	pBGEA	pRSED	9.53	14.0	7.14	6.91	9.73	298	49.9	417	164	139

Abbreviations: R5P, ribulose-5-phosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; S7P, sedoheputulose-7-phosphate; Pyr, pyruvate; Ala, alanine; Val, valine; Ile, isoleucine; Leu, leucine

Analysis of overexpression of the ED pathway by intracellular metabolites analysis

In order to confirm the effect of overexpression of the ED pathway, we examined some intracellular metabolites in *M. methylotrophus* 102/pBGEA and *M. methylotrophus* 102/pBGEA/pRSED at 27 h in fed-batch culture (previous section). The amounts of sugar phosphates, including ribulose-5-phosphate, fructose-6-phosophate, glucose-6-phosphate, 6-phosphogluconate, and sedoheputulose-7-phosophate, in *M. methylotrophus* 102/pBGEA/pRSED decreased as compared with *M. methylotrophus* 102/pBGEA (Table 5). On the other hand, pyruvate, alanine, valine, isoleucine and leucine in *M. methylotrophus* 102/pBGEA/pRSED increased compared with those in *M. methylotrophus* 102/pBGEA (Table 5). Sugar phosphates were produced *via* RuMP pathway, and alanine, valine, isoleucine, and leucine were synthesized using pyruvate. Perhaps, enhancement of the ED pathway contributed to the decrease in intracellular metabolites in the RuMP pathway and to the increase in pyruvate.

Discussion

In this study, we confirmed the effectiveness of the addition of pyruvate for improvement of L-lysine

production. Under the condition of glucose added, the amounts of cells, CO_2, and EPS increased, but the amounts of L-lysine and glutamate did not increase (Table 3). It suggested that the bottleneck in L-lysine production is pyruvate supply, and so we constructed an L-lysine producer of *M. methylotrophus* with an enhanced ED pathway in order to increase the intracellular pyruvate supply. The amounts of valine, leucine, and isoleucine in the L-lysine producer of *M. methylotrophus* with the enhanced ED pathway, *M. methylotrophus* 102/pBGEA/pRSED, was higher than in the reference strain in fed-batch culture (Table 4). Moreover, this showed that the amount of L-lysine in *M. methylotrophus* 102/pBGEA/pRSED was higher than that in the reference strain. These results confirmed our speculation, that the pyruvate supply is the bottleneck in L-lysine production, and indicate that an increase in pyruvate supply is useful for improvement of L-lysine production in *M. methylotrophus*. On the other hand, the cell growth rate in *M. methylotrophus* 102/pBGEA/pRSED was slightly lower than in the parent producer (Fig. 2). It was speculated that cell growth depends on the amount of metabolites in the RuMP pathway.

It is known that the NADPH supply in *M. methylotrophus* is important to L-lysine production, because the L-lysine biosynthetic pathway and diaminopimalate pathway require 4 mol NADPH per mol L-lysine from oxaloacetate. Kabus *et al.*[14] reported that the expression of *E. coli pntAB* genes encoding a membrane-bound transhydrogenase in *C. glutamicum* was an efficient way to improve L-lysine production. Becker *et al.* focused on engineering of the pentose phosphate pathway, the main NADPH production pathway, and reported that overexpression of the *zwf* gene, encoding glucose-6-phosphate dehydrogenase, resulted in increased L-lysine production. However, *M. methylotrophus* does not have the *pntAB* gene from the genomic sequence (our unpublished data). Hence, we attempted to improve the NADPH supply by expressing *E. coli pntAB* genes in the L-lysine producer of *M. methylotrophus*, but the L-lysine yield did not increase (data not shown). In this study, we found that the pyruvate supply was bottleneck in L-lysine production, so we thought that the NADPH supply would not limit the yield of L-lysine in *M. methylotrophus*.

The overall activity of the ED pathway in *M. methylotrophus* 102/pBGEA/pRSED was 20 times higher than that in the parent due to the introduction of the *edd-eda* operon from *E. coli* (Table 4), but L-lysine production in *M. methylotrophus* 102/pBGEA/pRSED was only 1.2 times higher than in the parent. Judging by the results of intracellular metabolite analysis in *M. methylotrophus* 102/pBGEA/pRSED, the concentrations of intracellular pyruvate, valine, leucine, and isoleucine were even higher than that of metabolites in the RuMP pathway (Table 5). In this strain, perhaps that the next target should be pyruvate carboxylase reaction, as in *C. glutamicum*.[31]

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