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Gene Amplification of *aceA* and *aceB* in Lysine-producing *Corynebacterium glutamicum* ssp. *lactofermentum* ATCC21799

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The role of glyoxylate bypass in lysine production by *Corynebacterium glutamicum* ssp. *lactofermentum* ATCC21799 was analyzed by using cloned *aceA* and *aceB* genes which encode enzymes catalyzing the bypass. Introduction of a plasmid carrying *aceA* and *aceB* to the strain increased enzyme activities of the bypass to approximately 5 fold on acetate minimal medium. The strain with amplified glyoxylate bypass excreted 25% more lysine to the growth medium than the parental strain, apparently due to the increased availability of intracellular oxaloacetate. The final cell yield was lower in the strain with amplified glyoxylate bypass. These changes were specific to the lysine-producing *C. glutamicum* ssp. *lactofermentum* ATCC21799, since the lysine-nonproducing wild type *Corynebacterium glutamicum* strain grew faster and achieved higher cell yield when the glyoxylate bypass was amplified. These findings suggest that the lysine producing *C. glutamicum* ssp. *lactofermentum* ATCC21799 has the ability to efficiently channel oxaloacetate, the TCA cycle intermediate, to the lysine biosynthesis pathway whereas lysine-nonproducing strains do not. Our results show that amplification of the glyoxylate bypass efficiently increases the intracellular oxaloacetate in lysine producing *Corynebacterium* species and thus results in increased lysine production.

Corynebacterium species have been widely used for the industrial production of amino acids (9, 11). Increasing and optimizing the final yield of metabolites by strain manipulation has long been a major interest in the food and feed industry. The availability of genetic and molecular biological tools developed for *Corynebacterium* and related species has made possible designing and controlling novel pathways at the molecular level (for reviews see 7, 12, 19). The goal of these studies is to understand the biochemical backgrounds of production strains and the subsequent use of the information in designing a strain with enhanced capability of metabolite production.

The biosynthesis of lysine by *Corynebacterium* species begins at oxaloacetate (OAA). OAA which is an intermediate of the TCA cycle is continuously replenished by several routes. These include the phosphoenolpyruvate-pyruvate-OAA triangle and glyoxylate bypass (Fig. 1). The glyoxylate bypass of *Corynebacterium glutamicum* and related species consists of two enzymes (Fig. 1; 5, 16). Isocitrate lyase (ICL) which is encoded by *aceA* catalyzes the conversion of the TCA cycle intermediate, isocitrate, to glyoxylate and succinate (17). Malate synthase (MS), the product of *aceB*, catalyzes subsequent condensation of the glyoxylate with acetyl-CoA to produce malate, which in turn enters the TCA cycle (18). Expression of the glyoxylate bypass enzymes is essential for growth on two-carbon compounds, such as acetate, as the sole source of carbon, since it prevents the net loss of the acetate carbon as CO_2 through the TCA cycle. As is true for *E. coli*, the glyoxylate bypass enzymes of *C. glutamicum* strains are normally repressed when glucose is provided as the carbon and energy source (5, 21).

The glyoxylate bypass of *Corynebacterium* species has been assumed to be important in carbon flux control. *C. glutamicum* ssp. *flavum* mutant strains showing increased ICL activity produced significantly higher amounts of glutamate than the parental strain did (21). This suggests a role of the glyoxylate bypass as one of the routes of carbon supply in the strain. Thus, efficient use of the glyoxylate cycle may minimize the entry of carbon into the CO_2-generating steps of the TCA cycle and increase the production of other amino acids as well. Based on this information, in this study, we preferentially channeled carbon to the glyoxylate bypass using cloned *aceA* and *aceB* genes and analyzed the effect

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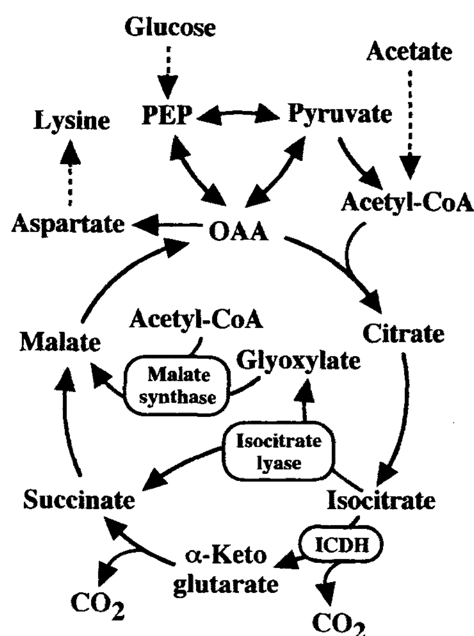

Fig. 1. The glyoxylate bypass and associated pathways of *Corynebacterium*.

The glyoxylate bypass is carried out by isocitrate lyase and malate synthase. Abbreviations: ICDH, isocitrate dehydrogenase; OAA, oxaloacetate; PEP, phosphoenol pyruvate. Dashed arrows imply multiple steps.

on lysine production.

C. glutamicum ssp. *lactofermentum* ATCC21799 is one of the most well characterized lysine-producing strains (1, 4-6). However, information on the role of glyoxylate bypass as one of the routes of carbon supply in the strain is still limited. Furthermore, the biochemical background of the organism as a lysine-producing strain are still largely unknown. Since glyoxylate bypass enzymes are normally repressed by glucose, at least, the bypass has been assumed to play no major role in supplying carbon to lysine in a medium containing glucose as the carbon source. However, in this report, we differentially expressed the glyoxylate bypass on a medium containing acetate as the carbon source and analyzed the effect on lysine production and the physiological changes. The information obtained in this study indicates that the glyoxylate bypass has the potential to be manipulated to increase lysine production even in a medium containing glucose as the carbon source.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *C. glutamicum* strains were routinely grown in MB (2) and *E. coli* strains were grown in LB (13). Minimal medium for *Corynebacterium* was a modified MCGC (5). Unless otherwise specified, glucose or acetate was added to the final concentration of 2%. The fermentation medium for *Corynebacterium* species was modified MCGC containing 2% sodium acetate as the carbon source. Antibiotics were added in the following amounts (μg per milliliter): ampicillin, 50; kanamycin, 25. *Corynebacterium* species and *E. coli* cells were grown at 30°C and 37°C, respectively.

Electroporation

Electroporation of *C. glutamicum* strains was performed as follows. *C. glutamicum* cells were grown in 100 ml of MB broth. When the optical density at 600 nm reached 0.6, ampicillin was added to the final concentration of 1.5 μg/ml. After incubating the culture for

Table 1. Bacterial strains and plasmids.

Strains or plasmids	Relevant genotypes or phenotypes[a]	Sources or references
C. glutamincum		
ASO19	Spontaneous rifampin resistant mutant of ATCC13059	23
ASO19-E12	Restriction-deficient varient of ASO19	3
SL033	ASO19-E12 carrying plasmid pMT1, Km^r	10
SL036	ASO19-E12 carrying plasmid pSL05, Km^r	10
C. glutamicum ssp. *lactofermentum*		
ATCC21799	L-lysine production strain *S*-(aminoethyl)-L-cysteine(ACE) resistant	ATCC[b]
SL073	ATCC21799 carrying plasmid pMT1, Km^r	This work
SL077	ATCC21799 carrying plasmid pSL05, Km^r	This work
Plasmids		
pMT1	Shuttle vector; Ap^r (*E. coli*), Km^r (*C. glutamicum*)	2
pSL08	pMT1 with 4.3-kb insert carrying *aceB*; Ap^r, Km^r	10
pSL05	pMT1 with 5.3-kb insert carrying *aceA* and *aceB*; Ap^r, Km^r	10

[a] r superscripts indicate resistance. Ap, ampicillin, Km, kanamycin. [b] ATCC, American Type Culture Collection, Rockville, MD, USA.

1.5 h, cells were collected by centrifugation at 5,000 × *g* for 10 min. The cell pellet was washed with 10 ml of EPB1 (20 mM Hepes pH 7.2, 5% glycerol) 3 times and resuspended with 1.5 ml of EPB2 (5 mM Hepes pH 7.2, 15% glycerol). The cell suspension (150 μl) was mixed with plasmid DNA (1~2 μl), incubated on ice for 5 min, and electroporated at 2.5 kV in a 2-mm cuvette. Immediately after the pulse, 1 ml of recovery broth (per liter, 40 g brain heart infusion, 30 g sorbitol, 10 g sucrose) was added and the mixture was incubated at 30°C for 1.5 h. After incubation, aliquots were spread on to a plate (per liter, brain heart infusion 40 g, sorbitol 40 g, sucrose 10 g, kanamycin 15 mg, agar 16 g).

Preparation of Cell-free Extracts

Cell-free extracts were prepared as described previously (8). Cells were harvested by centrifugation and washed with 100 mM Tris-HCl (pH 7.5), containing 20 mM KCl, 33 mM $MgCl_2$, 10 mM $MnCl_2$, 5% glycerol, and resuspended in 5 ml of the same buffer. Cells were disrupted by shaking vigorously with glass beads (212~300 microns, Sigma, U.S.A.) in a mini-bead beater (Biospec Products, U.S.A.). The cell debris was removed by centrifugation for 30 min at 15,000 × *g*. The supernatant was used as crude extract.

Enzyme Assays

Malate synthase [EC 4.1.3.2] was assayed in 1 ml containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.2 mM acetyl-CoA, 2 mM glyoxylate, and 0.5 mM DTNB (5,5'-dithio-bis[2-nitrobenzoic acid]). Optical changes were monitored at 412 nm. Specific activities were calculated based on ε_{412}=14.1 mM/cm. Isocitrate lyase [EC 4.1.3.1] was assayed in a 1 ml mixture containing 200 mM KH_2PO_4 (pH 6.8), 10 mM $MgCl_2$, 100 mM phenylhydrazine-HCl, 200 mM cysteine, and 100 mM isocitrate. The rate of glyoxylate phenylhydrazine formation was monitored at 324 mm and specific activities were calculated using the extinction coefficient ε_{324}=17.0 mM/cm. Isocitrate dehydrogenase [EC 1.1.1.42] was assayed in 1 ml containing 50 mM Tris-HCl (pH 7.5), 1 mM β-NADP, and 2 mM isocitrate. Changes in optical density were monitored at 340 nm. Specific activities were calculated based on ε_{412}=6.2 mM/cm. All enzyme activities were determined at room temperature.

Fermentation and Determination of Lysine

C. glutamicum strains were cultivated in 500-ml baffled flasks containing 50 ml of fermentation medium. Samples of 1 ml were taken and analyzed for cell growth and amino acids. L-lysine was measured enzymatically by the method described by Nakatani *et al.* (14). The reaction mixture (1 ml) contained 150 mM KH_2PO_4 (pH 6.8), 0.13 mM β-NADH, and 4 mM α-ketoglutarate. The reaction was started by adding 0.2 units of yeast saccharopine dehydrogenase [EC 1.5.1.7]. Changes in the optical density were measured at 340 nm.

RESULTS AND DISCUSSION

Amplification of Glyoxylate Bypass

Plasmid pSL05 carries cloned *aceA* and *aceB* (Fig. 2). The genes were previously isolated by the complementation of *E. coli* CGSC5236, an *aceB* mutant strain (10). As shown in Fig. 2, the *aceA* and *aceB* genes transcribed in the opposite direction and were contained in the 5.3-kb DNA fragment. To amplify the glyoxylate bypass, the plasmid was introduced into *C. glutamicum* ssp. *lactofermentum* ATCC21799 by electroporation. Crude extracts were prepared from cells grown on MCGC minimal medium containing 2% sodium acetate as the carbon and energy source. As shown in Table 2, introduction of the plasmid into *C. glutamicum* ssp. *lactofermentum* ATCC21799 increased MS and ICL activities to 5.9 fold and 4 fold, respectively, accomplishing 5 fold amplification of the bypass. SDS-PAGE analysis of *C. glutamicum* strain carrying the plasmid suggested that the increase in activities is due to the increase in the amount of expressed enzymes as judged by the expressed protein bands (Fig. 3). As expected, the expression of isocitrate dehydrogenase (ICDH) was minimal, indicating the preferential flow of carbon through the glyoxylate bypass on the medium (Table 2).

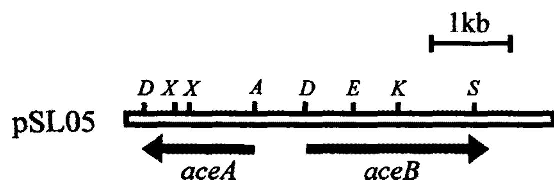

Fig. 2. The *aceA* and *aceB* locus of *C. glutamicum* ASO19-E12.

The boxed region indicates segment of DNA carried in plasmid pSL05. Vector pMT1 is not shown. Arrows indicate *aceA* and *aceB* coding regions. Some major restriction sites are shown. Abbreviations: A, *AflII*; D, *DraI*; E, *EcoRI*; K, *KpnI*; S, *SalI*; X, *XhoI*.

Table 2. Expression of glyoxylate bypass enzymes in *C. glutamicum* ssp. *lactofermentum* ATCC21799[a].

Strain	Plasmid[b]	Specific activity[c] (nmol min^{-1} mg^{-1})		
		MS	ICL	ICDH
C. glutamicum ssp. *lactofermentum*	pMT1[d]	140	294	10
ATCC21799	pSL05[e]	826	1178	3

[a]The glyoxylate bypass enzymes were induced by growth to the stationary phase on modified (5) MCGC broth (15) containing 2% sodium acetate. [b]Plasmids were introduced into *Corynebacterium* by electroporation. [c]The activities of malate synthase (MS), isocitrate lyase (ICL), and isocitrate dehydrogenase (ICDH) were measured as described in the Materials and Methods. Cell extracts were prepared as described in the Materials and Methods. [d]Plasmid pMT1 (2) carries no insert. [e]Plasmid pSL05 carries *aceA* and *aceB* coding region.

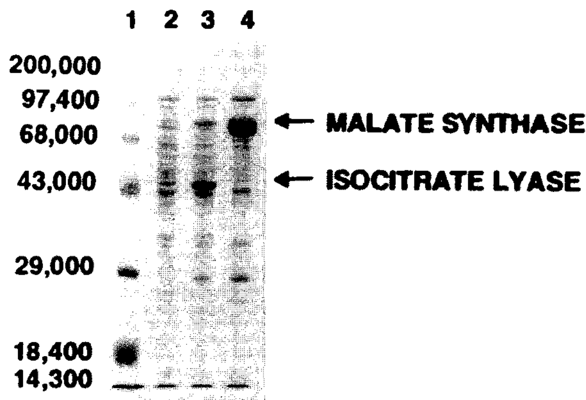

Fig. 3. Expression of isocitrate lyase and malate synthase from plasmid pSL05.

Crude extracts were prepared from cells grown on MB media containing acetate. Proteins were separated on 12% SDS-PAGE. Plasmid pSL08 overexpresses malate synthase. Lanes: 1, Molecular weight markers; 2, *C. glutamicum* ASO19-E12 (pMT1); 3, *C. glutamicum* ASO19-E12 (pSL05); 4, *C. glutamicum* ASO19-E12 (pSL08).

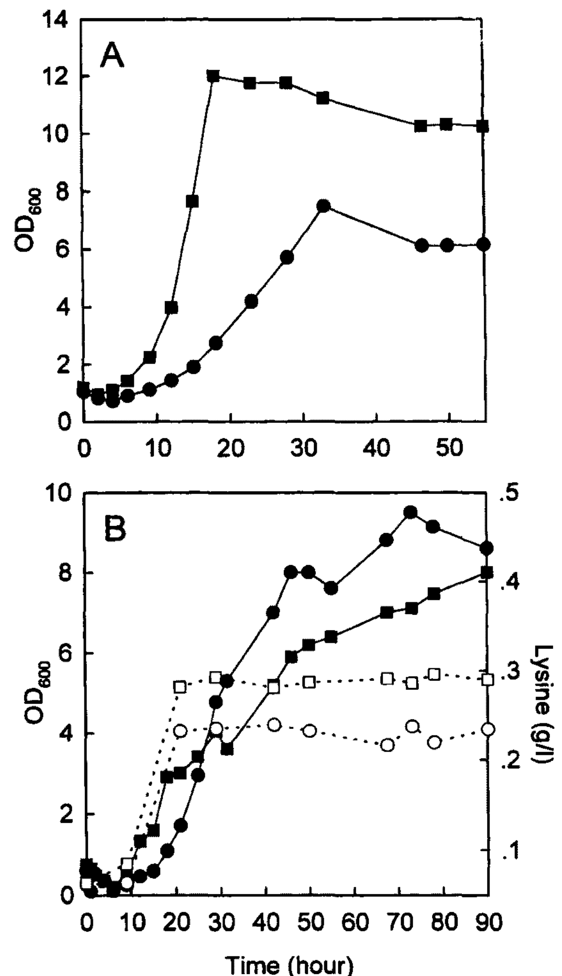

Fig. 4. Comparison of growth and lysine production in batch fermentations of *Corynebacterium* species harboring plasmid pMT1 (circles) or plasmid pSL05 (rectangles).

Plasmid pMT1 carries no insert. Fermentation broth was the modified (5) MCGC (15). Acetate was used as the carbon source and supplemented to the final concentration of 2%. Lysine was measured by enzymatic assay developed by Nakatani *et al.* (14). (A) Growth patterns of *C. glutamicum* ASO19-E12. (B) Growth (filled symbols) and lysine production (open symbols) patterns of *C. glutamicum* ssp. *lactofermentum* ATCC21799.

Growth Characteristics

The physiological effects of the amplification of glyoxylate bypass in *C. glutamicum* ssp. *lactofermentum* ATCC21799 were analyzed. We assumed that increased glyoxylate bypass enzyme activities would result in an increase in the amount of intracellular malate and subsequently OAA, and this might in turn result in increased lysine production (see Fig. 1). To test this, the lysine producing strain *C. glutamicum* ssp. *lactofermentum* ATCC21799 carrying either empty vector pMT1 or plasmid pSL05 was grown in baffled flasks containing modified MCGC minimal medium (5). The medium was supplemented with 2% sodium acetate as the carbon source. As shown in Fig. 4 (Panel B), the growth rates were similar for both strains. Calculation from the mid-log phase of growth revealed a doubling time of approximately 4 h for both strains. Interestingly, the strain carrying plasmid pSL05 produced lower cell yield than the one carrying pMT1 (Fig. 4, Panel B). This phenomenon was specific to lysine-producing *C. glutamicum* ssp. *lactofermentum* ATCC21799, since the lysine-nonproducing *C. glutamicum* ASO19-E12 strain grew faster and produced higher cell yields with amplified glyoxylate bypass than the parental strain did (Fig. 4, Panel A). In the minimal medium containing glucose as the carbon source the strains carrying either pSL05 or pMT1 showed identical growth characteristics (data not shown): glyoxylate bypass enzymes are repressed when glucose is provided as the carbon source (5).

Lysine Production

The accumulation of lysine by *C. glutamicum* ssp. *lactofermentum* ATCC21799 carrying either pSL05 or PMT1 was monitored (Fig. 4, Panel B). After 20 h of fermentation, the two strains accumulated lysine which was approximately 0.2~0.3 g/l. The total amount of lysine accumulated in the medium was small due to the use of minimal medium with acetate as the carbon source. Apparently, the strain carrying plasmid pSL05 accumulated 25% more lysine in the growth medium than the strain carrying plasmid pMT1. The degree of increse in lysine production roughly matched that of the decrease in cell yield. These results indicate that the amplification of glyoxylate bypass in the lysine producing *C. glutamicum* ssp. *lactofermentum* ATCC21799 resulted in the preferential flow of carbon through the glyoxylate bypass

and might have resulted in an increase in intracellular malate and subsequently intracellular OAA. The increased OAA would be preferentially converted into aspartate which is eventually transformed into lysine. However, the increase in lysine production was relatively small as compared to the increase in the enzyme activities which was 5 fold. This could be due to the presence of other metabolic pathways which might limit the flow of carbon in the strain. There is also a possibility that increased amount of OAA could have been used to make other amino acids. Metabolic flux analysis based on the concentration of metabolites during batch fermentation also indicated that the regulation of the PEP branch point, where PEP is either converted to pyruvate by pyruvate kinase or carboxylated to OAA by PEP carboxylase, is the limiting factor in lysine production, suggesting the importance of intracellular OAA (22). It has been shown that amplification of the PEP carboxylase which converts PEP to OAA also improves the production of certain amino acids, such as threonine which belongs to the same biosynthetic group as lysine (20). These previous findings and our data strongly suggest that lysine-producing *C. glutamicum* ssp. *lactofermentum* ATCC21799 can efficiently convert OAA to lysine. It also indicates that maintaining high intracellular concentration of OAA is crucial for the efficient production of lysine in the strain. The efficient flow of carbon to lysine from OAA was apparently made possible by the feedback-insensitive aspartokinase in the strain. Since many lysine-producing strains have feedback-insensitive aspartokinase (7, 11), amplification of the glyoxylate bypass in the strains may also show similar effects. In contrast to the lysine-producing *C. glutamicum* ssp. *lactofermentum* ATCC21799, lysine-nonproducing *C. glutamicum* ASO19-E12 appeared to redistribute the increased OAA to make other essential precursor metabolites, such as phosphoenol pyruvate and pyruvate rather than preferentially transform it into aspartate to form lysine. This situation may be translated into an increased growth rate and yield, as shown in Fig. 4, Panel A.

The information that we present here evaluates the role of the glyoxylate bypass in lysine production. Lysine fermentation using acetate as the carbon source can be greatly enhanced by amplifying the glyoxylate bypass. Similar to the findings of Shiio *et al.* who noted that the expression of glyoxylate bypass enzymes increases glutamate production (21), introduction of constitutively expressed genes into a lysine-producing strain might also be effective in increasing lysine production even on a medium containing glucose as the carbon source.

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