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Site-specific Disruption of Glyoxylate Bypass and Its Effect in Lysine-producing *Corynebacterium lactofermentum* Strain

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The role of glyoxylate bypass in a lysine-producing *Corynebacterium lactofermentum* strain was analyzed. Unlike the wild type, the strain expressed enzymes of glyoxylate bypass during growth in the fermentation broth containing glucose as the carbon source. To evaluate the importance of glyoxylate bypass in the strain, we disrupted chromosomal *aceA* by using a cloned fragment of the gene. Site-specific disruption of *aceA* which codes for the isocitrate lyase, the first enzyme of the bypass, was confirmed by Southern blot analysis. The *aceA* mutant strain completely lost isocitrate lyase activity and ability to grow in a minimal medium containing acetate as the sole carbon source. The mutant strain was similar to its parental strain in growth characteristics and produced comparable amounts of lysine in shake flasks containing glucose as the carbon source. The amount of oxaloacetate accumulated in the fermentation medium was similar for both strains, suggesting that expression of glyoxylate bypass does not necessarily lead to the increase in intracellular oxaloacetate. These data clearly demonstrate that glyoxylate bypass does not function as one of the routes of carbon supply for lysine production in the strain. It appears that the leakiness of the glyoxylate bypass in the strain might be the result of a secondary mutation which arose during previous strain development by random mutagenesis.

Corynebacterium glutamicum and related species have been widely used for the industrial production of amino acids (9, 12). Increasing and optimizing the final yield of metabolites by strain manipulation has long been a major interest of the food and feed industry. The availability of genetic and molecular biological tools developed for *Corynebacterium* and related species has made possible the analysis of the biochemical pathways of production strains at a molecular level (1, 5, 6, 15, 16, 18). The goal of these studies is to understand the biochemical backgrounds of production strains and subsequent use of the information to design a novel strain with increased metabolite production capability.

The biosynthetic pathway of lysine begins at oxaloacetate (OAA). OAA which is an intermediate of the TCA cycle is continuously replenished by several routes. These include the phosphoenolpyruvate(PEP)-pyruvate-OAA triangle and glyoxylate bypass (Fig. 1). The glyoxylate bypass of *C. glutamicum* and related species consists of two enzymes (6, 14) (Fig. 1). Isocitrate lyase (ICL) which is encoded by *aceA* catalyzes the conversion of the TCA cycle intermediate, isocitrate to glyoxylate and succinate. 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. Expression of the glyoxylate bypass enzymes is essential for growth on two-carbon compounds, such as acetate, as the sole carbon source, since it prevents the net loss of the acetate carbon as CO_2 in the TCA cycle. As is true for *E. coli*, the glyoxylate bypass enzymes of *C. glutamicum* and *C. lactofermentum* strains are normally repressed when grown in a minimal medium containing glucose as the sole carbon and energy source (6, 10, 25).

The glyoxylate bypass of *Corynebacterium* species has been assumed to be of major importance in carbon flux control. However, reports on the role of the glyoxylate bypass in amino acid production have been contradictory. *C. glutamicum* subsp. *flavum* mutant strains exhibiting increased ICL activity were found to form significantly higher amounts of glutamate than the parental strain did, demonstrating the key position of ICL in the carbon flux (23). Thus, bypassing the CO_2 generating

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steps of the TCA cycle may contribute to the production of other amino acids, as well. On the other hand, some recent evidence indicated the relatively minor role of the glyoxylate bypass in lysine production (6). These conclusions, however, were based on indirect observations and direct evidence has been lacking.

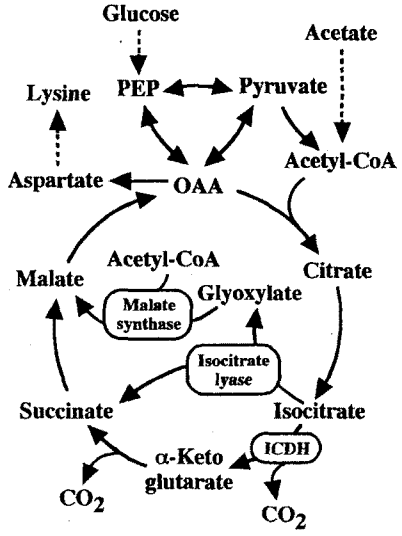

Fig. 1. The glyoxylate bypass and associated pathways of *Corynebacterium*.

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.

C. lactofermentum ATCC21799 is one of the most well characterized lysine-producing strains (1, 5, 6, 7). However, much of its biochemical background is still unknown. In this report, we directly analyzed the glyoxylate bypass of the strain. We blocked the bypass by introducing a mutation into the chromosomal copy. We also analyzed the effect of the mutation on lysine production.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in Table 1. *C. glutamicum* and *C. lactofermentum* were routinely grown in MB (3), and *E. coli* strains were grown in LB (13). The fermentation medium for *Corynebacterium* species was prepared as described (5). Minimal medium for *Corynebacterium* was a modified MCGC (6). Unless otherwise specified, glucose or acetate was added to the final concentration of 2% for minimal medium and 8% for fermentation medium. Antibiotics were added in the following amounts (μg per milliliter): ampicillin, 50; kanamycin, 25; nalidixic acid, 25. *Corynebacterium* species and *E. coli* cells were grown at 30°C and 37°C, respectively.

Plasmids

The plasmids used in this study are listed in Table 1. Plasmid pSL18 was made by deleting the 2.8 kb *Hin*dIII-

Table 1. Bacterial strains and plasmids.

Strains or plasmids	Relevant genotypes or phenotypes[a]	Sources or references
Escherichia coli		
S17-1	*tra* from RP4-2 integrated in the chromosome	22, 24
DH5α	F⁻ Φ80d*lacZ*DM15 Δ(*lacZYA-argF*)*U*169 *deoR endA*1 *hsdR*17 *supE*44 *thi*-1 *recA*1 *gyrA*96 *relA*1 λ⁻	BRL
Corynebacterium glutamicum		
ASO19	Spontaneous rifampin resistant mutant of ATCC13059	27
ASO19E12	Restriction-deficient varient of ASO19	3
Corynebacterium lactofermentum		
ATCC21799	L-lysine production strain S-(aminoethyl)-L-cysteine(AEC) resistant	ATCC[b]
799ICL1	*aceA* derivative of ATCC21799, Km^r	This work
Plasmids		
pUC19	Ap^r	26
pMT1	Shuttle vector; Ap^r (*E. coli*), Km^r (*C. glutamicum, C. lactofermentum*)	3
pSUP301	pACYC177 derivative, Ap^r, Km^r, *mob*	22
pMG107	pMT1 derivative, Ap^r, Km^r, *mob*	This work
pSL18	Delection derivative of pMG107, Ap^r, Km^r, *mob*, 4.8 kb in size, no replication in *Corynebacterium*, Corynebacterial gene-disruption vector	This work
pSL05	pMT1 with 5.3 kb insert carrying *aceA* and *aceB*, Ap^r, Km^r	This work
pSL65	pSL18 with 0.7 kb *Afl*II-*Msc*I fragment of pSL05, Ap^r, Km^r, harbouring internal fragment of *aceA*	This work

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

*Xho*I fragment of pMG107 which was made by ligating the 1.5 kb *Aat*II-*Bst*EII fragment of plasmid pSUP301 (22) into the *Aat*II-*Sca*I site of plasmid pMT1 (3). Plasmid pSL65 which was used to disrupt the chromosomal copy of *aceA* was constructed by inserting the 0.7 kb *Afl*II-*Msc*I fragment (17) of plasmid pSL05 (11) into the *Sma*I site of pSL18.

Electroporation and Transconjugations

Electroporation of *Corynebacterium* strains was performed as described previously (3). Mobilization of plasmids from *E. coli* to *Corynebacterium* was done as described by Schäffer *et al.* (21). The donor-recipient ratio was 1:5 and heat treatment of recipients was performed at 49°C for 9 min. *Corynebacterium* transconjugants were selected on MB agar containing nalidixic acid and kanamycin.

DNA Manipulations and Southern Blot Analysis

Standard molecular cloning, transformation, and electrophoresis procedures were used. Genomic DNA from *Corynebacterium* strains was isolated as described (6). For Southern blot analysis, chromosomal DNA digested with restriction enzymes, *Eco*RI and *Xho*I, was electrophoretically separated in agarose gels, denatured, and transferred to nitrocellulose filters as described by Sambrook *et al.* (19). The probe was isolated as the 1.5 kb *Aat*II-*Bam*HI fragment from plasmid pSL18. The probe was labeled with Dioxygenin-11-dUTP (Bohringer, Mannheim, Germany) in a random primed DNA-labeling reaction as described by the manufacturer. Hybridization, washing, and colorimetric detection for the Southern blot was done using the Genius-System™ from Boehringer following their users guide for filter hybridization.

Preparation of Cell Extracts and Enzyme Assays

Assays were carried out with cell-free extracts, which were prepared as described by Jetten and Sinskey (8). All enzyme activities were determined spectrophotometrically at room temperature. Malate synthase and isocitrate lyase were assayed as described (6).

Fermentation and Analysis of Fermentation Products

Fermentation and analysis of the fermentation products were performed as described by Gubler *et al.* (5). *C. lactofermentum* strains were cultivated in 250-ml baffled flasks containing 45 ml of fermentation medium. Inoculation was performed with 5 ml of an overnight culture in MB containing 1% glucose. Samples of 1 ml were taken every 24 h and analyzed for cell growth, amino acids, and organic acids. Glucose was quantified enzymatically with the Trinder (Sigma Diagnostics, USA) assay. Amino acids, such as lysine, aspartic acid, threonine, alanine, and valine, were analyzed as orthophthal-dialdehyde derivatives by reversed-phase chromatography by using the Aminoquant (Hewlett-Packard Analytical Dividion) method on a Hewlett packard 1050 high-performance liquid chromatography (HPLC) with a Bio-Rad HPX-87H column at 45°C, with a mobile phase consisting of 5 mM H_2SO_4. Compounds were detected by both A_{206} and refractive index.

RESULTS AND DISCUSSION

Expression of Glyoxylate Bypass

As is true for *E. coli*, the wild type *C. glutamicum* strain expressed glyoxylate bypass enzymes only when a two-carbon compound, such as acetate, was present in the growth medium (Table 2). Use of the pathway which bypasses the CO_2 generating steps of the TCA cycle apparently benefits the cell by saving carbon for the synthesis of cellular material. As with *E. coli*, the presence of glucose in the growth medium almost completely repressed the expression of isocitrate lyase (ICL) and malate synthase (MS). This pattern of regulation was also apparent in the lysine-producing *C. lactofermentum* ATCC21799 strain. However, the activity of ICL on an acetate medium was 2.7 fold higher in the lysine-producing strain than in the wild type (Table 2). Furthermore, glucose did not completely repress the expression of ICL and MS. The level of leaky expression reached almost 25% of the fully expressed level. To test the possibility that the leakiness was caused by acetate which might have been produced during fermentation as a

Table 2. Expression of glyoxylate bypass enzymes[a].

Strain	Phenotype	Carbon	Specific activity[b] (nmol min^{-1} mg^{-1})	
			ICL	MS
C. glutamicum ASO19E12	-	Glucose	8	5
		Acetate	260	440
C. lactofermentum ATCC21799	Lysine producer	Glucose	135	110
		Acetate	710	400
C. lactofermentum 799ICL1	*aceA*[c] mutant	Glucose	-	75
		Acetate	-	-

[a]The glyoxylate bypass enzymes were induced by growth to the stationary phase on the fermentation broth containing 8% sodium acetate or 8% glucose. [b]The activities of isocitrate lyase (ICL) and malate synthase (MS) were measured as described in Materials and Methods. [c]The mutant strain does not have the ability to grow in a medium containing acetate as the sole carbon source.

byproduct, we measured the amount of accumulated acetate by HPLC (see below). No accumulation of acetate was observed (data not shown) during the fermentation period, indicating that the leakiness is probably an intrinsic property of the strain. However, we were unable to rule out the possibility that the leakiness was caused by the unidentified ingredient of the fermentation medium which contained corn steep liquor of undefined composition. Since the expression of ICL and MS saves carbon by bypassing the CO_2 generating steps of the TCA cycle, we speculated that the situation might be beneficial to lysine production and that it might be one of the reasons why the strain produces lysine. Bypassing the CO_2 generating steps of the TCA cycle will minimizie the accumulation of cell mass due to lack of energy and this will in turn maximize the availability of carbon for the synthesis of other metabolites. Thus, channeling carbon through the glyoxylate bypass may lead to increased synthesis of oxaloacetate (OAA) and subsequently the increased synthesis of lysine (Fig. 1).

Disruption of Glyoxylate Bypass

To evaluate the importance of the glyoxylate bypass on lysine production in the *C. lactofermentum* ATCC 21799 strain, we disrupted *aceA* which expresses ICL, the first enzyme of the glyoxylate bypass, and analyzed the mutational effect on lysine production. To disrupt the chromosomal copy of *aceA*, an internal fragment of cloned *ace*A was used (Fig. 2A, designated as *aceA'*). Plasmid pSL65 (see Materials and Methods for construction), an *aceA*-disruption vector, was transferred by conjugation from *E. coli* S17-1 to *C. lactofermentum* ATCC21799. Since plasmid pSL65 confers resistance against kanamycin and cannot replicate in *Corynebacterium*, plating on kanamycin will select for clones which had integrated pSL65 into the genome via a single crossover between the fragment of *aceA* on the plasmid and the genomic *aceA* locus (Fig. 2B). This event disrupts the chromosomal copy of *aceA* due to the integration of the plasmid. Site-specific disruption of *aceA* was confirmed by Southern blot analysis. As shown in Fig. 3, a single hybridization band of approximately 7 kb in size was detected. This was in good agreement with the expected value which was deduced from the size of the plasmid and digested *aceA* locus.

The mutant strain was grown in the fermentation broth containing glucose as the carbon source and cell extracts were prepared and assayed for isocitrate lyase activity. As shown in Table 2, the isolated *aceA* mutant strain showed no activities of the target enzyme. As expected, the strain also lost the ability to grow in acetate minimal medium (data not shown).

Fermentation Analysis

The effect of blockage of the glyoxylate bypass on lysine production was analyzed. The lysine producing *C. lactofermentum* ATCC21799 and *aceA* mutant derivative

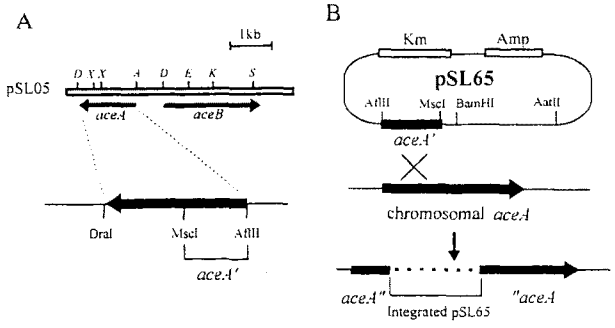

Fig. 2. Schematic illustration of the event leading to the inactivation of *aceA* gene.

(A) Plasmid pSL05 carries *aceA* and *aceB*. The *aceA'* fragment was used to disrupt chromosomal *aceA*. (B) A single crossover between regions of homology (designated as chromosomal *aceA* and *aceA'*) results in chromosomal integration of the plasmid into the *aceA* locus and produces progeney clones that are resistant to kanamycin. This event results in the disruption of chromosomal *aceA* (designated as *aceA"* and *"aceA*). Gene disruption vector pSL65 is not replicable in *Corynebacterium*. Vectors are not drawn in scale. The 1.5 kb *Bam*HI-*Aat*II fragment was used to detect chromosomal integration of the plasmid (see Fig. 3). Not all restriction sites are shown. Abbreviations: D, *Dra*I; X, *Xho*I; A, *Afl*II; E, *Eco*RI; K. *Kpn*I; S, *Sal*I.

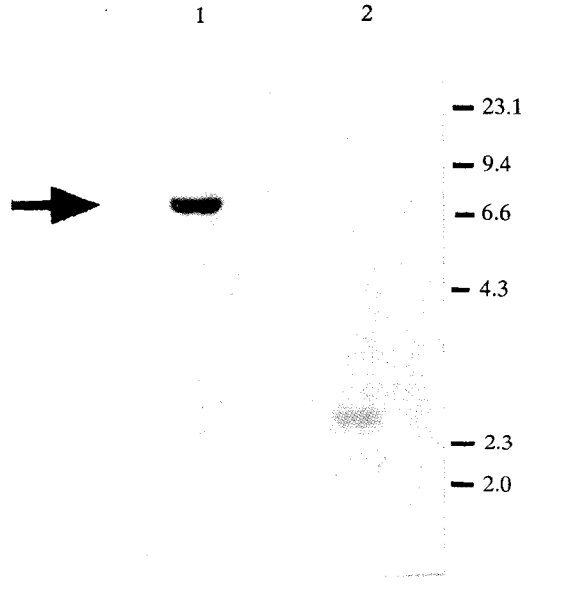

Fig. 3. Southern blot analysis of the constructed mutant.

Genomic DNA from the parent strain (lane 2) and from the *aceA* mutant (lane 1) was cleaved with *Eco*RI and *Xho*I, subjected to agarose gel electrophoresis, blotted onto a nitrocellulose filter, and hybridized with a labeled probe which detects integrated plasmid. The probe was 1.5 kb *Bam*HI-*Aat*II fragment of plasmid pSL18 (see Fig. 2). The band indicated with an arrow is approximately 7 kb in size. The band includes *aceA* locus and integrated plasmid (Fig. 2). Size makers (kb) of λ DNA cleaved with *Hin*dIII are shown on the right.

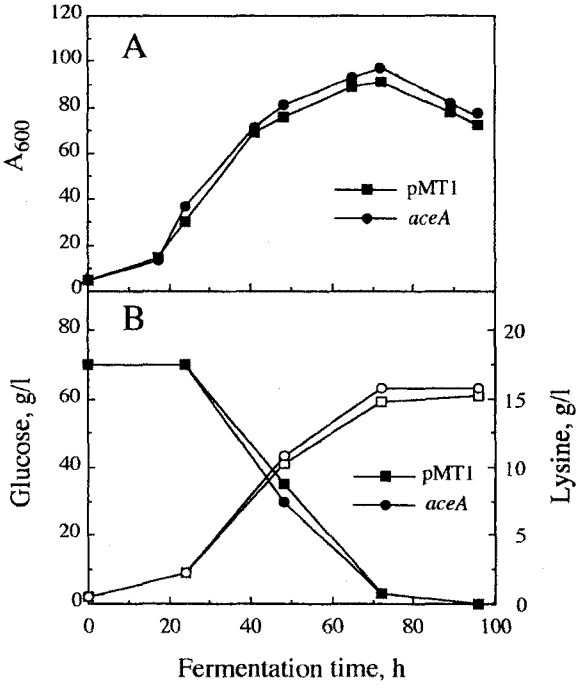

Fig. 4. Comparison of growth, glucose consumption, and lysine production in batch fermentations of *C. lactofermentum* ATCC21799 harboring plasmid pMT1 (rectangulars) and *aceA* mutant (circles).

Plasmid pMT1 carries no insert. (A) Biomass accumulation as measured by optical density. (B) Decrease of glucose concentration (filled symbols) and increase in lysine concentration (open symbols) in the fermentation broth. Fermentations and analysis of fermentation products were done as previously reported (6).

of the strain were individually grown in shake flasks containing the fermentation medium. The accumulation of lysine as well as the consumption of glucose was monitored by HPLC (Fig. 4B). After 24, 48, 72 and 96 h of fermentation, the two strains accumulated similar amounts of lysine which nearly peaked at 72 h with approximately 15 g/l. Similarly, no major differences were found with respect to glucose consumption rate. Within 72 h of fermentation, the glucose concentration dropped from an initial 80 g/l to nearly 0. As shown in Fig. 4A, the growth rate was also very similar for the two strains. The concentration of excreted OAA was also measured by HPLC to see if there was any decrease in the amount of OAA produced by the mutant strain. Although the amount of OAA detected in the medium was small, no differences were found for both strains (data not shown). These results indicate that the glyoxylate bypass plays no major role in lysine production by the *C. lactofermentum* ATCC21799.

Even though *C. lactofermentum* ATCC21799 expressed glyoxylate bypass even on glucose-containing fermentation medium, that did not appear to contribute to the lysine-producing characteristics of the strain. However, there is a possibility that the level of leaky expression might not be high enough to be reflected as a detectible increase in lysine. Conversely, the mutational effect might not be big enough to be detected in these experiments. It is possible that expression of the glyoxylate bypass may in fact result in a transient increase in the intracellular concentration of OAA. However, the increased amount of OAA could be rapidly redistributed in the cell rather than being channeled through the biosynthetic pathway leading to lysine. Since OAA is a major precursor metabolite required for the synthesis of many cellular materials, the intracellular concentration of OAA is probably tightly regulated to maintain it at an appropriate level. If the expression of the glyoxylate bypass is enhanced enough to overcome the cellular redistribution rate, the extra OAA may be used for the synthesis of lysine. Congruent with this hypothesis, it is known that increasing the OAA by amplifying the PEP carboxylase which converts PEP to OAA results in the increased biosynthesis of lysine (see Fig. 1; 2, 20). By the same logic, amplifying the glyoxylate bypass by introducing multiple copies of constitutively expressed *aceA* and *aceB* may show a similar effect.

Like most lysine producing strains, *C. lactofermentum* ATCC21799 was constructed by classical mutagenesis. The leakiness of glyoxylate bypass enzyme activities as shown in Table 1 could be the result of a secondary mutation which arose during strain development.

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