Appl Microbiol Biotechnol (1994) 42:67–72

ORIGINAL PAPER

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Effects of the amplification of the genes coding for the L-threonine biosynthetic enzymes on the L-threonine production from methanol by a gram-negative obligate methylotroph, *Methylobacillus glycogenes*

Received: 3 February 1994 / Received last Revision: 14 April 1994 / Accepted: 3 May 1994

Abstract We constructed recombinant plasmids carrying the genes coding for the L-threonine biosynthetic enzymes, the *hom* gene, the *hom-thrC* genes, and the *thrB* genes, of a gram-negative obligate methylotroph, *Methylobacillus glycogenes*, and examined the effects of them on the production of L-threonine from methanol. The *hom* gene, which encodes the homoserine dehydrogenase, and the *hom-thrC* genes, containing the gene coding for threonine synthase together with the *hom* gene, were cloned from a wild-type strain, and the *thrB* gene encoding the desensitized homoserine kinase was cloned from an L-threonine-producing mutant, ATR80. The recombinant plasmids were transferred into ATR80 and its L-isoleucine auxotroph, A513, by conjugation. Amplification of the genes coding for the L-threonine biosynthetic enzymes elevated the activities of the L-threonine biosynthetic enzymes of the transconjugants 10- to 30-fold over those of the strains containing only vectors. The L-threonine production from methanol in test-tube cultivation was increased about 30% and 40% by the amplification of the *hom* gene and the *hom-thrC* gene respectively, and it was slightly increased by that of the *thrB* gene. The effects of gene amplification were confirmed by the cultivation in 5-l jar fermentors. The best producer, an A513 transconjugant containing the plasmid carrying the *hom-thrC* genes, produced 16.3 g/l L-threonine for 72 h.

Introduction

Methanol, a compound chemically synthesized from natural gas, is an attractive raw material for fermentation industries because of its high purity and low prices. We isolated L-glutamic-acid-hyperproducing mutants from gram-negative obligate methylotrophs, *Methylobacillus glycogenes* ATCC 21276 and ATCC 21371, and further derived L-threonine-producing and L-lysine-producing mutants from these mutants (Motoyama et al. 1993a).

Recombinant techniques were successfully applied to the improvement of amino acid production in various bacteria (Beppu 1986). We tried to improve L-threonine productivities of *M. glycogenes* mutants by recombinant techniques. We have cloned and sequenced the *hom-thrC* genes, which encoded homoserine dehydrogenases and threonine synthases of wild-type *M. glycogenes* (Motoyama et al. 1994), and the *thrB* gene, which encoded a partially deregulated homoserine kinase of an L-threonine-producing mutant, ATR80 (Motoyama et al. in preparation). In this paper we describe the effects of the amplification of the *hom*, *thrC*, and *thrB* genes on L-threonine production from methanol by *M. glycogenes*.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

Media and culture methods

Culture methods and culture media for *M. glycogenes* have been described previously (Motoyama et al. 1993a). *Escherichia coli* strains were cultivated as described previously (Maniatis et al.1982). Amino acids required for auxotrophic mutants of *M. glycogenes* and *E. coli* were added to media at a concentration of 50 mg/l. Liquid or agar media were supplemented with 50 mg/l kanamycin, 100 mg/l ampicillin or 10 mg/l tetracycline to cultivate *M. glycogenes* or *E. coli* strains containing plasmids.

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Table 1 Bacterial strains and plasmids used in this study. *AEC+Thr*^{R} *S*-(2-aminoethyl)-L-cysteine plus L-threonine resistance, *Amp*^{R} ampicillin resitance, *Cm*^{R} chloramphenicol resistance, *HD* homoserine dehydrogenase, *HK* homoserine kinase, *Km*^{R} kanamycin resistance, *Tc*^{R} tetracycline resistance, *Tp*^{R} trimethoprim resistance, *TS* threonine synthase

Strain or plasmid	Genotype or relevant characteristics	Phenotype	References
Methylobacillus glycogenes			
ATR80	An L-threonine-producing mutant derived from *M. glycogenes* ATCC21371	AEC+Thr^R, phe$^-$	Motoyama et al. (1993a)
A513	An L-isoleucine auxotroph derived from *M. glycogenes* ATR80	AEC+Thr^R, Km^R, phe$^-$, Ile$^-$	This study
Escherichia coli			
S17-1	*recA*, chromosomally integrated RP4 derivative	pro$^-$, Tp^R	Simon (1984)
Vector			
pUC19	2.7 kb	Amp^R	Yanisch-Perron et al. (1985)
pLA2905	20 kb	Tc^R, Km^R	Allen and Hanson (1985)
pMFY42	10.9 kb	Tc^R, Km^R	Fukuda (1990)
pSUP5011	pBR325-Tn5-Mob.	Amp^R, Cm^R, Km^R	Simon (1984)
Recombinant plasmids containing the L-threonine biosynthetic genes of *M. glycogenes*			
pTHD-1	Derivative of pBR322 carrying 4.15-kb *Pst*I fragment cloned from *M. glycogenes* ATCC21371	Tc^R, HD$^+$, TS$^+$	Motoyama et al. (1994)
pTHD-30	Derivatives of pLA2905 carrying 4.15-kb *Pst*I fragment of pTHD-1	Tc^R, HD$^+$, TS$^+$	This study
pTHD-31	Derivative of pLA2905 carrying 2.1-kb *Sma*I fragment of pTHD-1	Tc^R, HD$^+$	This study
pTHD-41	Derivative of pUC19 carrying 2.1-kb *Sma*I fragment of pTHD-1	Amp^R, HD$^+$	This study
pAHK-14-2	Derivative of pUC19 carrying 2.2-kb *Eco*RI-*Nco*I fragment cloned from *M. glycogenes* ATR80	Amp^R, HK$^+$	Motoyama et al. (in preparation)
pMAHK-1	Derivatives of pMFY42 carrying 2.2-kb *Eco*RI-*Nco*I fragment of pAHK-1	Tc^R, HK	This study

DNA manipulation

DNA digestion by restriction enzymes, separation of DNA fragments by gel electrophoresis, DNA ligation and transformation of *E. coli* strains were performed according to the methods described previously (Maniatis et al.1982). Restriction enzymes and DNA ligase were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan).

Enzyme assay

Preparation of crude enzymes of *M. glycogenes* strains and measurements of activities of homoserine dehydrogenase and homoserine kinase were done as described previously (Motoyama et al. 1993b). One unit of homoserine dehydrogenase was defined as the amount of enzyme that oxidized 1 μmol NADH in 1 min. One unit of homoserine kinase was defined as the amount of enzyme that converted 1 μmol ATP to ADP in 1 min. Threonine synthase activities were measured according to the method described previously (Skarstedt and Greer 1973). One unit was defined as the amount of enzyme that liberated 1 μmol inorganic phosphate from homoserine phosphate in 1 min.

Construction of recombinant plasmids

A plasmid, pTHD-30, was constructed by introducing the 4.15x10^3–base (4.15-kb) *Pst*I fragment of pTHD-1 (Motoyama et al. 1994) containing the *hom-thrC* gene of *M. glycogenes* ATCC 21371 into the *Pst*I site of pLA2905. The 2.1-kb *Sma*I fragment of pTHD-1, containing the *hom* gene of *M. glycogenes* ATCC 21371, was inserted into the *Sma*I site of pUC19 to yield pTHD-41. The 2.1-kb *Bam*HI-*Pst*I fragment excised from pTHD-41 was introduced into the *Bgl*II-*Pst*I site of pLA2905 to construct pTHD-31. The 2.6-kb *Pvu*II fragment of pAHK 14–2 (Motoyama et al. in preparation) containing the *thrB* gene of ATR80 was inserted into the *Pvu*II site of pMFY42 to yield pMAHK-1.

Conjugation

E. coli S17-1, containing a mobilizable plasmid (donor), was cultivated in L-broth supplemented with an antibiotic overnight at 30° C. An *M. glycogenes* mutant (recipient) was cultivated in PY medium (Motoyama et al. 1993a) overnight at 30° C. Cells were collected by centrifugation, washed once with 0.9% sodium chloride, and suspended in 0.9% sodium chloride to adjust the absorbances (660 nm) of the suspensions to 0.5. Both suspensions (2.5 ml) were mixed and cells were fixed onto a 0.45-μm Millipore filter with a sterile syringe. The filter was put on a PY/agar me-

dium, incubated overnight at 30° C, then cells were collected by washing the filter with 3 ml 0.9% sodium chloride. The cell suspension was incubated at 30° C for 1 h in the presence of 0.5 mg/l mitomycin C (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) to reduce *E. coli* donor strains. Cells were washed twice with 0.9% sodium chloride, suspended with 0.9% sodium chloride, and plated onto a selection medium, M1 medium (a minimal medium containing methanol as a sole carbon source, Motoyama et al. 1993a), supplemented with amino acids required for auxotrophs and an antibiotic according to the plasmid. Plates were cultivated for 3–7 days at 30° C, and colonies that appeared on the plates were replica-plated onto the selection medium and L-broth. Colonies that could grow only on the selection medium were picked up as transconjugants.

Results

Construction of *M. glycogenes* transconjugants

There are few reports on the transformation of gram-negative methylotrophs. Usually gene transfer into methylotrophs is done by conjugation, and vectors derived from broad-host-range plasmids, such as RK2 (IncP1) or RSF1010 (IncQ), are widely used in genetic studies (Lidstrom and Tsygankov 1991). We examined whether pLA2905, a derivative of RK2 (Allen and Hanson 1985) and pMFY42, a derivative of RSF1010, (Fukuda 1990) could be mobilized from *E. coli* S17-1 to ATR80 by conjugation. The drug-resistant markers of the plasmids, tetracycline and kanamycin resistance, were mobilized from *E. coli* S17-1 into ATR80 at a frequency of 10^{-2} per recipient. Restriction-enzyme analysis of the plasmids isolated from transconjugants of ATR80 revealed that the plasmids from the transconjugants had the same structures as those from *E. coli* transformants (data not shown).

Removal of donor *E. coli* S17-1 strains from *M. glycogenes* transconjugants after conjugation was usually troublesome. Repetitive mono-colony isolation was always necessary. *E. coli* S17-1 is a *recA* mutant, therefore it shows a high sensitivity to mitomycin C (Finch et al. 1986). We tested minimal inhibitory concentrations of mitomycin C against *E. coli* S17-1, *M. glycogenes* ATCC 21276 and ATCC 21371. The minimal inhibitory concentrations were 0.5 mg/l for *E. coli* S17-1 and 5 mg/l for the *M. glycogenes* strains. We expected that a treatment of a cell suspension of *E. coli* S17-1 and *M. glycogenes* ATR80 after conjugation with mitomycin C should selectively kill the donor *E. coli* strains. The suspension was incubated for 1 h at 30° C in the presence or absence of 0.5 mg/l mitomycin C, then cells were washed and plated onto L-broth and M1 medium containing L-phenylalanine to examine the effects on the viabilities of both strains by the treatment. The viability of *E. coli* S17-1 was reduced to 10^{-3} to 10^{-4}, whereas that of *M. glycogenes* was hardly affected by the treatment, suggesting that the mitomycin C treatment could effectively remove *E. coli* S17-1 from conjugation mixtures. Thereafter the mitomycin C treatment was included in our conjugation procedure.

Isolation of an L-isoleucine auxotroph from ATR80

Derivation of an L-isoleucine mutant is important in breeding an L-threonine-producing mutant to prevent a leakage of carbon flow from L-threonine to L-isoleucine. ATR80 was conjugated with *E. coli* S17-1 containing pSUP5011, a suicide vector for delivery of Tn5 (Simon 1984), and an L-isoleucine auxotroph was screened among kanamycin-resistant clones on which Tn5 transposed. An L-isoleucine auxotroph, A513, was isolated among 3000 kanamycin-resistant clones. The growth of A513 was recovered by *2*-oxobutyrate as well as L-isoleucine, suggesting it was defective in threonine deaminase, which converts L-threonine to 2-oxobutyrate. The L-threonine productivity of A513 was 13% higher than that of the parental strain, ATR80, in test-tube cultivation.

Effects of the amplification of the genes coding for the L-threonine biosynthetic enzymes on the L-threonine production from methanol

Preliminary experiments showed that the homoserine dehydrogenase activity was enhanced 5- to 10-fold in *E. coli* if the promoter of the kanamycin resistance gene of pLA2905 or the β-galactosidase gene of pUC19 was present upstream of the *hom* gene. In this way, recombinant plasmids containing the genes coding for the L-threonine biosynthetic enzymes of *M. glycogenes* were constructed so that the genes could be read by the promoter of kanamycin resistance gene or the *lac* promoter (Fig. 1). The plasmids were mobilized into L-threonine-producing mutants, ATR80 and A513, and the resulting transconjugants were cultivated in test-tubes.

Amplification of the genes coding for the L-threonine biosynthetic enzymes resulted in great elevation of the biosynthetic enzyme activities of *M. glycogenes* transconjugants. The specific activities of homoserine dehydrogenase and threonine synthase of the transconjugants were enhanced about 10-fold by the introduction of pTHD-31 or pTHD-30, and those of homoserine kinase of the transconjugants containing pMAHK-1 were 20- or 28-fold those of the strains containing the vectors (Table 2).

Effects of the gene amplification were also reflected in L-threonine production. L-Threonine production of ATR80 or A513 was increased about 30% and 40% by the introduction of pTHD-31 carrying the *hom* gene and pTHD-30 carrying the *hom–thrC* gene respectively (Table 3). The growth of the transconjugants was almost the same as that of the recipients. The transconjugants of A513 produced more L-threonine than those of ATR80, probably because the biosynthetic pathway from L-threonine to L-isoleucine was blocked in A513. ATR80 was derived from an L-glutamic-acid-producing mutant, and therefore ATR80 produced considerable amounts of L-glutamic acid and L-aspartic acid (Motoyama et al. 1993a). Accumulation of L-aspartic acid

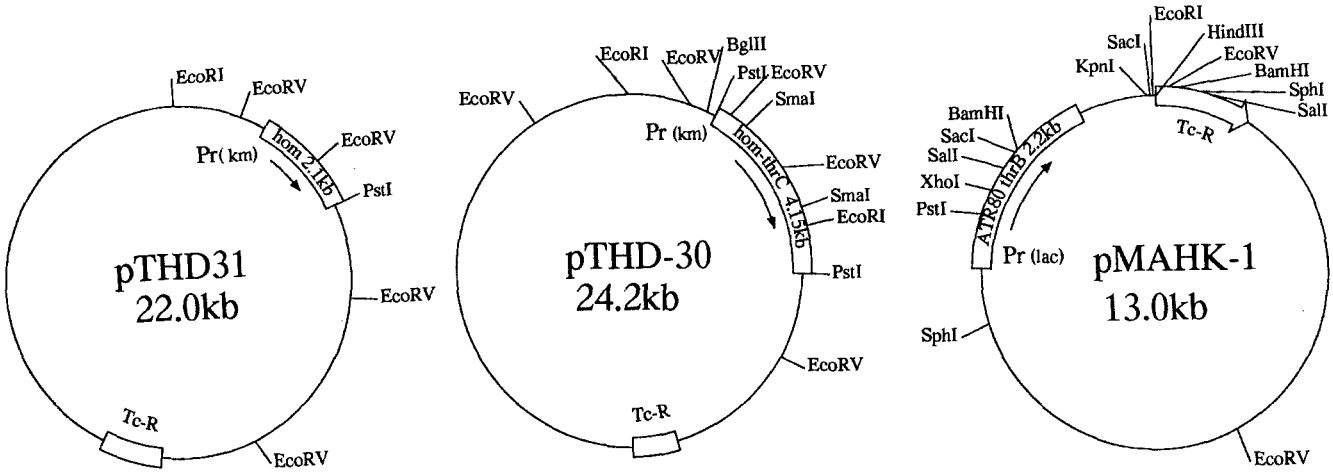

Fig. 1 Physical maps of recombinant plasmids carrying the genes coding for the L-threonine biosynthetic enzymes of *M. glycogenes*. *Pr(km),(lac)* the positions of promoter of kanamycin resistance gene of pLA2905 and that of *lac* promoter of pUC19 respectively. *Arrows* indicate the orientation of transcription

and L-glutamic acid was not affected by the introduction of the recombinant plasmids. L-Threonine production was slightly enhanced by the introduction of pMAHK-1 carrying the *thrB* gene of ATR80, and the growth of the transconjugants was greatly reduced.

The transconjugants were cultivated in 5-l jar fermentors (Table 4, Fig. 2). L-Threonine production was elevated by the introduction of pTHD-30 or pTHD-31 as in test-tube cultivation. The best producer, the transconjugant of A513 containing pTHD-30, produced 16.3 g/l of L-threonine for 72 h. This value was the highest in the fermentative process of L-threonine production from methanol.

Discussion

Two-broad-host range plasmids, pLA2905 derived from RK2 and pMFY42 derived from RSF1010, could be easily mobilized from *E. coli* to *M. glycogenes*, and the structures of the plasmids were not modified in *M. glycogenes*. Mobilization of plasmids occurred at very low frequency in a gram-negative facultative methylotroph, *Paracoccus denitrificans*, because of severe restriction, and derivation of restriction-negative mutants was necessary to mobilize plasmids efficiently (de Vries et al. 1989). RSF1010-derived plasmids could not replicate in *Methylobacterium organophilum* DSM 760, and mobilized plasmids were found to be integrated in the chromosomal DNA (Mazodier et al. 1988). An efficient and easy gene transfer into *M. glycogenes* is a great advantage for breeding amino acid producers by recombinant technology.

Usually purification of transconjugants after conjugation is troublesome, and selection procedures should be employed according to the combination of donors and recipients. In this study we devised an easy and efficient method using mitomycin C to kill the donor, the *recA* strain of *E. coli*, selectively after conjugation. This method can be easily applicable to any conjugation with a *recA* strain as a donor.

L-Threonine production by *M. glycogenes* mutants was improved by the amplification of the *hom* gene, and it was further increased by that of the *hom-thrC* genes, suggesting that homoserine dehydrogenase and

Table 2 Specific activities of the L-threonine biosynthetic enzymes of the *M. glycogenes* transconjugants containing the genes coding for the L-threonine biosynthetic enzymes. Numbers in parentheses show relatives activities. *NT* not tested

Recipient	Plasmid	Gene	Specific activities (μmol min^{-1} mg $protein^{-1}$)		
			Homoserine dehydrogenase	Homoserine kinase	Threonine synthase
ATR80	pLA2905	–	1.2×10^{-2} (1.0)	6.4×10^{-3} (1.0)	5.9×10^{-3} (1.0)
	pTHD-31	*hom*	1.2×10^{-1} (10.0)	4.2×10^{-3} (0.7)	6.4×10^{-3} (1.1)
	pTHD-30	*hom-thrC*	1.5×10^{-1} (12.0)	5.3×10^{-3} (0.8)	6.4×10^{-2} (11.0)
	pMFY42	–	1.8×10^{-2} (1.0)	1.6×10^{-2} (1.0)	1.1×10^{-2} (1.0)
	pMAHK-1	*thrB*	2.4×10^{-2} (1.4)	3.2×10^{-1} (20.0)	NT
A513	pLA2905	–	1.9×10^{-2} (1.0)	9.7×10^{-3}	4.4×10^{-3} (1.0)
	pTHD-30	*hom-thrC*	2.0×10^{-1} (9.8)	NT	5.2×10^{-2} (11.0)
	pMFY42	–	2.2×10^{-2} (1.0)	2.0×10^{-2} (1.0)	NT
	pMAK-1	*thrB*	2.4×10^{-2} (1.1)	5.5×10^{-1} (28.0)	NT

Table 3 Amino acid production by the *M. glycogenes* transconjugants containing the genes coding for the L-threonine biosynthetic enzymes in test tube cultivation. The transconjugants were cultivated at 30° C for 48 h in test-tubes. *A* absorbance

Recipient	Plasmid	Gene	A_{660}	Amino acid (g/l)		
				Asp	Glu	Thr
ATR80	–	–	6.5	0.7	4.7	1.4
	pLA2905	–	6.1	0.8	4.0	1.5
	pTHD-31	*hom*	6.7	1.0	4.8	2.0
	pTHD-30	*hom-thrC*	6.5	0.8	4.4	2.2
	pMFY42	–	6.4	0.8	3.7	1.4
	pMAHK-1	*thrB*	4.9	0.8	2.8	1.6
A513	–	–	5.9	1.0	6.9	1.6
	pLA2905	–	6.0	1.0	4.9	1.7
	pTHD-31	*hom*	6.2	0.9	4.8	2.1
	pTHD-30	*hom-thrC*	6.1	0.8	4.2	2.4
	pMFY42	–	5.3	0.7	3.5	1.5
	pMAHK-1	*thrB*	4.4	0.6	3.4	1.6

Table 4 Amino acid production by the *M. glycogenes* transconjugants containing the genes coding for the L-threonine biosynthetic enzymes in 5-l jar fermentors. Values absorbances and amino acids at 72 h are shown

Recipient	Plasmid	Gene	A_{660}	Amino acid (g/l)		
				Asp	Glu	Thr
ATR80	–	–	30.0	7.2	24.1	8.5
	pLA2905	–	26.4	5.8	20.2	10.2
	pTHD-31	*hom*	25.6	6.1	20.9	11.1
	pTHD-30	*hom-thrC*	27.4	4.8	23.4	12.3
A513	pLA2905	–	30.2	5.8	21.6	11.9
	pTHD-31	*hom*	30.2	5.2	15.5	15.4
	pTHD-30	*hom-thrC*	28.4	5.8	22.4	16.3
	pMAHK-1	*thrB*	19.0	3.0	28.0	15.0

threonine synthase were limiting enzymes in the L-threonine biosynthetic pathway.

L-Threonine production was only slightly increased by the introduction of the plasmid containing the *thrB* gene, and the growth of the transconjugants was greatly reduced. The growth inhibition might be caused by accumulation of homoserine phosphate overproduced by the *thrB* gene product. If this were true, the accumulated homoserine phosphate could be converted to L-threonine by the co-introduction of the *thrB* gene and the *thrC* gene, and the growth inhibition might be overcome. We are now attempting to study the effects of the combination of both genes.

The *hom* gene used in this study was derived from the wild-type strain, and its product, homoserine dehydrogenase, was strongly inhibited by L-threonine (Motoyama et al. 1994). No mutants of *M. glycogenes* with deregulated homoserine dehydrogenase have yet been isolated. Mutation of the *hom* gene should be attempted to desensitize the gene product for the further improvement of L-threonine production. Marchenko and Tsygankov (1992) succeeded in the desensitization of the homoserine dehydrogenase of *M. flagellatum* by *in vitro* mutagenesis of the *hom* gene. A *C. glutamicum* transformant that had a plasmid containing the *hom-thrB* was treated with a mutagen, and a derivative containing the mutated *hom* gene directing the synthesis of deregulated homoserine dehydrogenase was isolated from an L-threonine-analog-resistant transformant (Katsumata et al. 1986). The *hom* gene product of *M. glycogenes* could be desensitized by similar procedures, and L-threonine production could be improved by use of a desensitized *hom* gene .

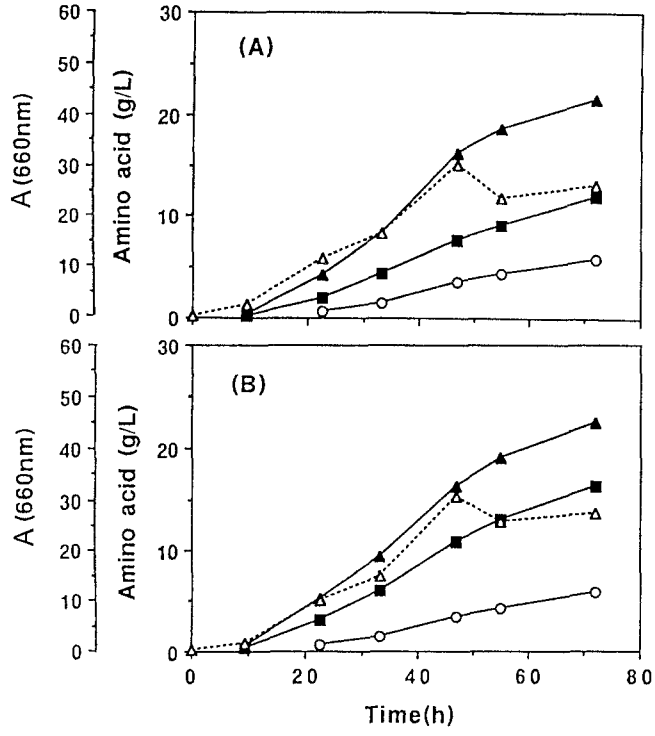

Fig. 2A, B Time course of amino acid production by strain *M. glycogenes* A513 containing pLA 2905 (**A**) or pTHD-30 (**B**). △ Absorbance, ■ Thr; ○ Asp, ▲ Glu

The L-threonine-producing mutants employed in the present study produced considerable amounts of L-glutamic acid. It might be necessary to derive mutants that have lesions in the tricarboxylic acid cycle enzymes to change the carbon flow from the biosynthesis of L-glutamic acid to that of the L-aspartic acid family amino acids. Accumulation of L-aspartic acid suggests that aspartokinase, which converts L-aspartic acid to L-aspartate phosphate, is limited in the strain. Cloning of the gene encoding aspartokinase from *M. glycogenes* and amplification of the gene in the L-threonine-producing mutants might convert accumulated L-aspartic acid to L-threonine or L-lysine.

Acknowledgements We thank Dr. R. Simon for sending us *E. coli* S17-1 and pSUP5011, Dr. M. Fukuda for the kind gift of pMFY42, and Ms. K. Hasegawa, Mr. K. Maki, and Ms. E. Honma for technical assistance.

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