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Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains

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Summary. The *hom-thr*B operon (homoserine dehydrogenase/homoserine kinase) and the *thr*C gene (threonine synthase) of *Corynebacterium glutamicum* ATCC 13032 and the hom_{FBR} (homoserine dehydrogenase resistant to feedback inhibition by threonine) alone as well as hom_{FBR}-*thr*B operon of *C. glutamicum* DM 368-3 were cloned separately and in combination in the *Escherichia coli*/*C. glutamicum* shuttle vector pEK0 and introduced into different corynebacterial strains. All recombinant strains showed 8- to 20-fold higher specific activities of homoserine dehydrogenase, homoserine kinase, and/or threonine synthase compared to the respective host. In wild-type *C. glutamicum*, amplification of the threonine genes did not result in secretion of threonine. In the lysine producer *C. glutamicum* DG 52-5 and in the lysine-plus-threonine producer *C. glutamicum* DM 368-3 overexpression of *hom-thr*B resulted in a notable shift of carbon flux from lysine to threonine whereas cloning of hom_{FBR}-*thr*B as well as of hom_{FBR} in *C. glutamicum* DM 368-3 led to a complete shift towards threonine or towards threonine and its precursor homoserine, respectively. Overexpression of *thr*C alone or in combination with that of hom_{FBR} and *thr*B had no effect on threonine or lysine formation in all recombinant strains tested.

Introduction

L-Threonine is one of the essential amino acids and, after lysine and methionine, in many cases the next limiting amino acid in animal and human foodstuffs (Kleemann et al. 1985). Therefore, there is an interest in microbial L-threonine production for food supplementation. Until a few years ago microbial threonine production was primarily dependent on mutant strains of *Serratia marcescens*, or of the coryneform species *Corynebacterium glutamicum*, *Brevibacterium flavum*, or *B. lactofermentum*, which were all obtained by classical mutagenesis (Kleemann et al. 1985; Kinoshita 1985). Only recently have powerful cloning systems been developed for coryneform bacteria (for a review see Martin et al. 1987) thus opening the possibility of studying the molecular biology of these organisms and of applying recombinant DNA techniques for strain development and for overcoming possible limiting steps in amino acid production.

L-Threonine biosynthesis from aspartate in coryneform bacteria has been well characterized. It consists of five enzymatic steps (Fig. 1), the initial two being common in threonine and lysine biosynthesis. In contrast to *E. coli* only one aspartate kinase is present (Shiio et al. 1970). This enzyme is feedback inhibited when both lysine and threonine are present in excess (Shiio and Miyajima 1969). Homoserine dehydrogenase (HDH) is feedback inhibited by threonine and both HDH and homoserine kinase (HK) are weakly repressed by methionine (Miyajima et al. 1968; Miyajima and Shiio

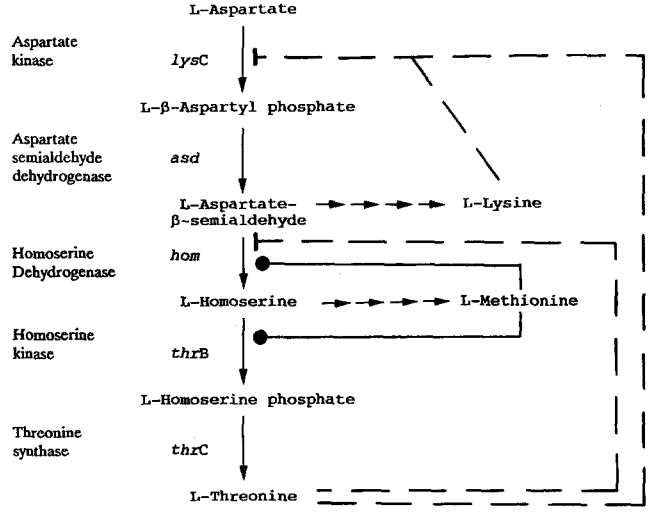

Fig. 1. Biosynthetic pathway of L-threonine and its regulation in *Corynebacterium glutamicum*: ——▮ inhibition; ——● repression; lys C, asd, hom, thr B, and thr C are the genes coding for the respective enzymes

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1970, 1971). The other two enzymes involved in L-threonine biosynthesis of coryneform bacteria seem not to be subjected to inhibition or repression (Miyajima et al. 1968; Miyajima and Shiio 1971).

Recently, the organization of the *hom*, *thr*B, and *thr*C genes coding for HDH, HK and threonine synthase (TS), respectively, from *C. glutamicum* (Katsumata et al. 1986; Follettie et al. 1988; Peoples et al. 1988; Han et al. 1990) and from *B. lactofermentum* (Mateos et al. 1987a, b) have been described. Also, attempts have been made to improve L-threonine production by amplification of hom_{FBR}-*thr*B coding for HDH released from feedback inhibition and HK in mutant strains of *C. glutamicum* or *B. lactofermentum* (Katsumata et al. 1986; Morinaga et al. 1987) or by amplification of the *E. coli* threonine operon in a *B. flavum* mutant strain (Ishida et al. 1989).

In order to obtain information on the limiting steps in carbon flux to threonine and in order to construct a well-defined recombinant threonine-producing strain we studied the separate and/or combined amplification of the threonine biosynthesis genes *hom*, hom_{FBR}, *thr*B, and *thr*C and its influence on amino acid formation in wild-type, in a lysine-producing strain, and in a lysine- and threonine-producing strain of *C. glutamicum*.

Materials and methods

Strains, plasmids, and media. All bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* CGSC strains were obtained from Bachmann (New Haven, Conn., USA). The minimal medium used for *C. glutamicum* contained per litre: 5 g $(NH_4)_2SO_4$, 5 g urea, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 20.9 g 3-(*N*-morpholino)-propane-sulphonic acid (MOPS), 0.25 g $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot H_2O$, 10 mg $MnSO_4 \cdot H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 0.2 mg $CuSO_4$, 0.2 mg biotin. The pH was adjusted to 6.5 and 4% (w/v) glucose was added after sterilization. For determination of amino acid production the $(NH_4)_2SO_4$ concentration was increased to 20 g/l, urea and MOPS were omitted, and 20 g/l $CaCO_3$ was added after sterilization. The media were inoculated with washed cells from an overnight culture to give an initial optical density at 600 nm (OD_{600}) of about 0.7. All cultivations were done in 500-ml baffled erlenmeyer flasks with 60 ml medium at 30° C on a rotary shaker at 140 rpm. M9 medium (Miller 1972) was the minimal medium for *E. coli*, and LB medium (Lennox 1955) without glucose was the complex medium used for all organisms. When appropriate, ampicillin (50 mg/l) or kanamycin (15 mg/l or 50 mg/l) were added to the medium. *E. coli* cells were grown aerobically at 37° C.

Preparation of DNA and transformation. Chromosomal DNA and plasmid DNA from *C. glutamicum* were isolated and purified as described by Follettie and Sinskey (1986). Plasmid DNA from *E. coli* was isolated by the method of Birnboim and Doly (1979) followed by a CsCl density gradient. *C. glutamicum* was transformed via electroporation of whole cells as described by Liebl et al. (1989). *E. coli* was transformed by the $CaCl_2$ method of Cohen et al. (1973).

DNA manipulations. All restriction enzymes, T4 DNA ligase, Klenow polymerase and calf intestine alkaline phosphatase were obtained from New England Biolabs (Schwalbach, FRG) or from Boehringer (Mannheim, FRG) and used as instructed by the manufacturer. Restriction-generated fragments were separated in 0.8% agarose gels and isolated and purified using the Geneclean™-Kit (Dianova, Hamburg, FRG) from BIO101.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Source or reference
Corynebacterium glutamicum		
13032	Type strain	ATCC
DG 52-5	AEC^R, aspartate kinase deregulated	Cremer et al. (1988), Degussa AG
DM 368-3	AEC^R; AHV^R, aspartate kinase and homoserine dehydrogenase deregulated	Degussa AG
Escherichia coli		
Gif102 CGSC 5075	*thrA1015, thi-1, lysC1004, metLM1005, relA1*, lambda$^-$, *spoT1*	Theze and Saint-Girons (1974)
Gif41 CGSC 5077	*thrC1001, thi-1, relA1*, lambda$^-$, *spoT1*	Theze and Saint-Girons (1974)
YA73 CGSC 5076	*thrB1000, thi-1, relA1*, lambda$^-$, *spoT1*	Theze and Saint-Girons (1974)
Plasmids		
pEK0	*C. glutamicum/E. coli* shuttle vector: Km^R, 6.3 kb	Kleinertz and Eikmanns, unpublished
pUC18	Ap^R, *lac*P (polylinker), *lacZ'*, replicon ColE1	Vieira and Messing (1982)

AEC^R, *S*-(2-aminoethyl)-L-cysteine resistance; AHV^R, 2-amino-3-hydroxy-valerate resistance; ATCC, American Type Culture Collection, Rockville, Md., USA

Enzyme assays. For determination of enzyme activities cells were grown in 60 ml minimal medium to late exponential phase, washed twice in 20 ml of 50 mM TRIS/HCl buffer, pH 7.6, containing 50 mM NaCl, and resuspended in 2 ml of 100 mM HEPES buffer, pH 7.6. The cells were disrupted by sonication with a microtip-equipped Branson (Danbury, Conn., USA) sonifier at maximal setting (2 × 2 min at 0° C). After centrifugation for 30 min at 13,000 *g* the supernatant was used for assays. The protein concentration of the extracts was determined by the method of Bradford (1976) using egg albumin as standard.

HDH (EC 1.1.1.3) was assayed as described by Follettie et al. (1988) at 30° C in 1 ml of 66 mM potassium phosphate buffer (pH 7.0), 0.25 mM NADPH, and 2.5 mM aspartate semialdehyde as substrate. The activity was determined by following the decrease in absorbance at 340 nm.

HK (EC 2.7.1.39) was assayed as described by Follettie et al. (1988) at 30° C in 1 ml of 100 mM HEPES buffer (pH 7.8) containing 250 mM KCl, 3.3 mM ATP, 0.25 mM NADH, 5 mM phosphoenolpyruvate, 10 mM $MgCl_2$, 10 units (U) pyruvate kinase (Boehringer), 25 U lactate dehydrogenase (Boehringer), and 5 mM L-homoserine as substrate. The activity was determined by following the decrease in absorbance at 340 nm.

TS (EC 4.2.99.2) activity was determined by the amount of inorganic phosphate released using a method described by Shames and Wedler (1984). The test was performed at 37° C for 30 min in 1 ml 20 mM HEPES buffer (pH 7.5) containing 200 mM KCl, 0.1 mM pyridoxalphosphate, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol, and 0.4 mM homoserine phosphate as substrate. The inorganic phosphate released was determined by the method of Lanzetta et al. (1979). Homoserine phosphate was prepared by the method of Skarstedt and Greer (1973) using cell free extracts of *E. coli* mutant Gif 41.

Amino acid analysis. The concentration of threonine, lysine, homoserine, isoleucine, and glycine in the culture filtrate was determined using a Biotronic (Maintal, FRG) amino acid analyser.

Results and discussion

Cloning of hom-thrB, hom$_{FBR}$-thrB, hom$_{FBR}$, and of thrC

The *hom-thr*B operon coding for HDH and HK and the *thr*C gene coding for TS were cloned from wild-type *C. glutamicum* ATCC 13032. The *hom*$_{FBR}$-*thr*B operon coding for HDH released from feedback inhibition by threonine and for HK was isolated from a *C. glutamicum* mutant strain DM 368-3 which is resistant to the L-threonine analogue 2-amino-3-hydroxy-valerate (9 mg/ml).

It was shown by Follettie et al. (1988) and by Peoples et al. (1988) that the *C. glutamicum hom* and *thr*B genes are expressed as an operon (*hom-thr*B) and can be located on a 3.6-kb *Sal*I chromosomal fragment. Therefore, we attempted to isolate both the *hom-thr*B operon from *C. glutamicum* ATCC 13032 and the *hom*$_{FBR}$-*thr*B operon from strain DM 368-3 as a *Sal*I fragment. *Sal*I-generated and size-fractionated (2.5 to 4.5 kb) chromosomal DNA fragments of both strains were ligated separately into the *Sal*I site of the *E. coli*/*C. glutamicum* shuttle vector pEK0, transformed into the *E. coli* HDH mutant Gif102 (*thr*A), and transformants were screened for complementation of the *thr*A marker (phenotype of homoserine requirement during growth on glucose minimal medium). Several colonies in both experiments showed a *thr*A$^+$ phenotype. Plasmid DNA of these strains was isolated and restriction analysis with *Sal*I, *Hind*III, *Pst*I, *Sma*I, *Xmn*I and *Eco*RI revealed that both the *thr*A-complementing *Sal*I fragment from the wild-type *C. glutamicum* as well as the one from strain DM 368-3 are 3.6 kb in size and show identical restriction patterns. The recombinant plasmids containing the *Sal*I fragment of wild-type *C. glutamicum* (in both orientations with respect to the vector) were designated pEK-hom-thrB1 and pEK-hom-thrB2; those containing the *Sal*I fragment from strain DM 368-3 were designated pEK-hom$_{FBR}$-thrB1 and pEK-hom$_{FBR}$-thrB2.

For subcloning of the *hom*$_{FBR}$ gene without *thr*B, a 1.7-kb *Sma*I/*Xmn*I fragment of pEK-hom$_{FBR}$-thrB1 was isolated and ligated into *Sal*I/Klenow-treated pEK0. The resulting plasmid was designated pEK-hom$_{FBR}$.

According to Follettie et al. (1988) a 2.1-kb *Bcl*I/*Sph*I chromosomal fragment of *C. glutamicum* is able to complement an *E. coli thr*C mutant. Therefore, *C. glutamicum* chromosomal *Bcl*I/*Sph*I DNA fragments of 1.5 to 3.0 kb were isolated and ligated into *Bam*HI/*Sph*I-restricted pUC19, transformed into *E. coli* Gif41 (*thr*C) and transformants were screened for complementation of the *thr*C marker (phenotype of threonine requirement during growth) by replica-plating onto glucose minimal medium. Plasmid DNA (designated pUC-thrC) of a *thr*C$^+$ phenotype colony was isolated and the *thr*C-containing fragment integrated into the shuttle vector pEK0. For this purpose the *thr*C-gene-carrying fragment was isolated from pUC-thrC as a *Hind*III/*Sma*I fragment, blunt-ended, and ligated into *Eco*RI/Klenow-treated pEK0, resulting in pEK-thrC1 and pEK-thrC2 (different orientation of the fragment). Interestingly, pEK-thrC2 but not pEK-thrC1 was able to complement the *E. coli* mutant Gif41. Together with the fact that in *C. glutamicum thr*C is expressed from both pEK-thrC1 and pEK-thrC2 (see below), this result indicates that the cloned fragment contains a *C. glutamicum* promoter that does not function in *E. coli* and that expression of *thr*C in *E. coli* from pUC-thrC and from pEK-thrC2 might be due to read-through transcription from the *lacZ* promoter located in front of the cloning site in both vectors.

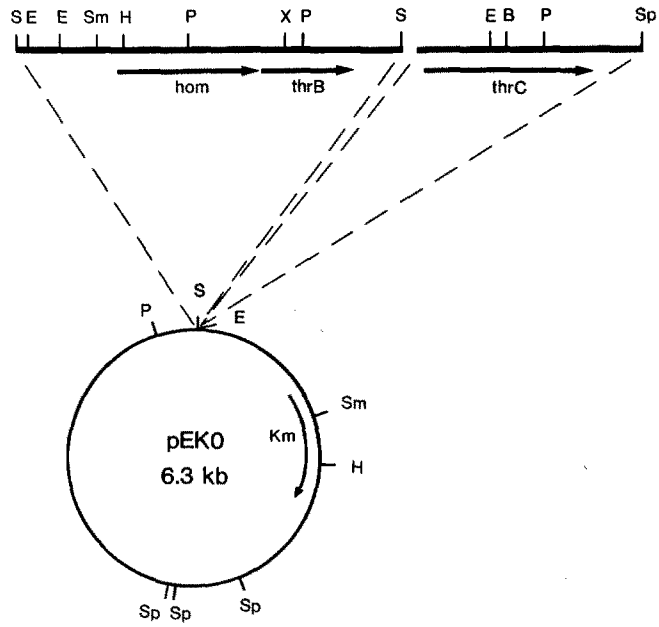

Fig. 2. Restriction map of vector pEK-hom$_{FBR}$-thrB+C: *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dIII; *P*, *Pst*I; *S*, *Sal*I; *Sm*, *Sma*I; *Sp*, *Sph*I; *X*, *Xmn*I; *Km*, kanamycin resistance

The *thr*C-gene-carrying fragment from pUC-thrC was also cloned into pEK-hom$_{FBR}$-thrB1 for study of combined overexpression of HDH/HK and TS in *C. glutamicum*. The resulting plasmid, containing *hom*$_{FBR}$, *thr*B, and *thr*C in this order was designated pEK-hom$_{FBR}$-thrB+C and is shown in Fig. 2.

Expression of cloned genes in C. glutamicum

The plasmids pEK-hom-thrB (1 and 2) and pEK-thrC (1 and 2) were introduced into *C. glutamicum* ATCC 13032 (wild-type), DG 52-5 (lysine producer) and into DM 368-3 (lysine and threonine producer). Despite several attempts to clone pEK-hom$_{FBR}$, pEK-hom$_{FBR}$-thrB1, and pEK-hom$_{FBR}$-thrB+C in all three strains, only stable transformants of strain DM 368-3 could be obtained. With both *C. glutamicum* ATCC 13032 and

Table 2. Specific activities of homoserine dehydrogenase (HDH), homoserine kinase (HK), and threonine synthase (TS) in *C. glutamicum* 13032, DG 52-5, DM 368-3, and in recombinant derivatives grown on minimal medium

Strain	HDH (μmol min^{-1} mg^{-1} protein)		HK (μmol min^{-1} mg^{-1} protein)	TS (μmol min^{-1} mg^{-1} protein)
	− Threonine	+ Threonine		
13032	0.83	0.02	0.022	0.006
13032 pEK-hom-thrB1	9.48	0.21	0.588	n.d.
13032 pEK-thrC1	n.d.	n.d.	n.d.	0.049
DG 52-5	0.60	0.02	0.017	0.008
DG 52-5 pEK-hom-thrB1	4.40	0.05	0.376	n.d.
DG 52-5 pEK-thrC1	n.d.	n.d.	n.d.	0.057
DM 368-3	0.84	0.82	0.015	0.005
DM 368-3 pEK-hom-thrB1	7.60	0.72	0.220	n.d.
DM 368-3 pEK-hom_{FBR}	9.72	8.40	0.017	n.d.
DM 368-3 pEK-hom_{FBR}-thrB1	9.40	9.58	0.250	n.d.
DM 368-3 pEK-hom_{FBR}-thrB+C	10.70	10.30	0.272	0.063
DM 368-3 pEK-thrC1	n.d.	n.d.	n.d.	0.067

n.d., not determined

DG 52-5 only transformants carrying plasmids with altered structures could be isolated. Apparently there is a strong pressure against retention of plasmids containing *hom_{FBR}*. *C. glutamicum* DM 368-3 transformed with either of the three plasmids showed significantly slower growth (doubling time of 200 min instead of 100 min during exponential growth) indicating also a negative effect of overexpressed *hom_{FBR}*. At present we are not able to give a plausible explanation for these results.

In order to assess the expression of the cloned threonine genes the specific activities of HDH, HK and TS in cell-free extracts of the recombinant *C. glutamicum* strains were determined (Table 2). All strains carrying pEK-hom-thrB1, pEK-hom_{FBR}, pEK-hom_{FBR}-thrB1, or pEK-hom_{FBR}-thrB+C showed 8- to 12-fold higher specific HDH activity and, except the one carrying pEK-hom_{FBR}, 15- to 25-fold higher specific HK activity compared to the host. The HDH activity of *C. glutamicum* DM 368-3 and of the recombinant strains carrying *hom_{FBR}*, *hom_{FBR}*-thrB1 or *hom_{FBR}-thr*B+C was not affected by the presence of threonine (up to 25 mM) in the assay mixture whereas the HDH expressed from cloned *hom-thr*B1 was completely inhibited by 5 mM L-threonine. All strains carrying pEK-thrC1 or pEK-hom_{FBR}-thrB+C showed also 8- to 12-fold higher specific TS activity compared to the host. The TS activity was not inhibited by threonine, lysine, methionine, homoserine, or aspartate (up to 50 mM) in the assay buffer (data not shown).

The orientation of *hom-thr*B and of *thr*C in pEK0 had no effect on the expression of the respective genes. The specific HDH activities in *C. glutamicum* carrying pEK-hom-thrB1 and pEK-hom-thrB2 as well as specific TS activities in strains carrying pEK-thrC1 and pEK-thrC2 were essentially the same.

Threonine and lysine production by recombinant strains

To test the effect of the overexpressed threonine genes on the carbon flux from aspartate to threonine and lysine, the formation of these amino acids by recombinant *C. glutamicum* strains was determined in minimal medium plus glucose (Table 3). *C. glutamicum* ATCC 13032 and all derivatives of this strain excreted neither threonine nor lysine. *C. glutamicum* DG 52-5, possessing an aspartate kinase released from feedback inhibition, excreted 22.7 mM lysine but no threonine due to feedback regulation of the HDH by threonine (Sano and Shiio 1970; Tosaka et al. 1979). Overexpression of HDH and HK in this strain resulted in decreased formation of lysine (7 mM) and simultaneously in production of 14 mM threonine. No effect on threonine or lysine formation could be observed in pEK-thrC-carrying (TS overexpressing) recombinants of *C. glutamicum* DG 52-5. *C. glutamicum* DM 368-3, possessing an aspartate kinase and an HDH, both released from feedback inhibition, excreted about the same amounts of threonine (6.3 mM) and lysine (7.2 mM). Overexpression of *hom-thr*B (HDH/HK) resulted in an increase in threonine (8.3 mM) and a concomitant decrease in lysine (3.8 mM) formation. When *hom_{FBR}* was amplified

Table 3. Threonine and lysine concentrations in cultures of *C. glutamicum* 13032, DG 52-5, 368-3, and recombinant derivatives after 24 h incubation

Strain	Threonine (mM)	Lysine (mM)
13032	<0.1	<0.1
13032 pEK-hom-thrB1	<0.1	<0.1
13032 pEK-thrC1	<0.1	<0.1
DG 52-5	<0.1	22.7
DG 52-5 pEK-hom-thrB1	14.0	7.0
DG 52-5 pEK-thrC1	<0.1	20.0
DM 368-3	6.3	7.2
DM 368-3 pEK-hom-thrB1	8.3	3.8
DM 368-3 pEK-hom_{FBR}	7.5	0.1
DM 368-3 pEK-hom_{FBR}-thrB	14.1	0.1
DM 368-3 pEK-hom_{FBR}-thrB+C	14.2	0.1
DM 368-3 pEK-thrC1	7.3	8.4

in *C. glutamicum* DM 368-3 lysine formation was negligible, threonine formation was about the same as in the host, but 12.3 mM homoserine, a precursor of threonine, was present in the fermentation medium. Obviously the HK reaction is a limiting step in this case. However, on amplification of the *hom*$_{FBR}$-*thr*B operon in strain DM 368-3 neither lysine nor homoserine but 14.1 mM threonine was formed by the organisms. Overexpression of *thr*C (TS) alone or in combination with that of *hom*$_{FBR}$-*thr*B had no effect on the amino acid excretion pattern in *C. glutamicum* DM 368-3.

All recombinant strains producing threonine also excreted up to 3 mM isoleucine and up to 6 mM glycine (data not shown) indicating a drain-off of the threonine formed. Isoleucine might be formed via 2-oxobutyrate by the normal isoleucine biosynthesis pathway; glycine might be formed by degradation of threonine via threonine dehydrogenase and 2-amino-3-oxobutyrate-CoA ligase, which were shown to exist as threonine-inducible enzymes in coryneform bacteria (Bell and Turner 1976).

The data given in Table 3 were obtained after growth of the organisms on minimal glucose medium for 24 h. The total amount of each amino acid produced increased four- to fivefold while incubating the bacteria for 70 h on medium containing glucose plus molasses. The results obtained with *C. glutamicum* ATCC 13 032 reflect the probable key role of aspartate kinase, which in this strain is feedback-inhibited by threonine plus lysine and thus limits the carbon flow into the overall pathway. This control is omitted in both *C. glutamicum* DG 52-5 and DM 368-3. The results obtained with these two strains demonstrate (i) that carbon flux from aspartate to lysine could be partially directed to threonine by elevating the content of HDH and HK and even completely by elevating that of deregulated HDH and HK, (ii) that both HK and deregulated HDH have to be amplified for efficient threonine formation and (iii) that carbon flux to threonine is not limited by the TS reaction. However, the recombinant strains carrying *hom-thr*B, *hom*$_{FBR}$, *hom*$_{FBR}$-*thr*B, or *hom*$_{FBR}$-*thr*B + C did not excrete considerably more amino acids in total (more threonine plus isoleucine plus glycine but less lysine) than the respective host strain, again indicating the limitation of carbon flux into the overall pathway at the level of aspartate kinase. To overcome this limitation and also in order to construct a well-defined recombinant threonine-producing strain of *C. glutamicum*, introduction of a plasmid containing *lys*C$_{FBR}$, coding for a feedback-released aspartate kinase, together with *hom*$_{FBR}$-*thr*B might be useful.

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