〈論　文〉

Depression of L-cystathionine accumulation caused by over-expression of *metB* gene in a *metC* deficient mutant of *Escherichia coli**

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Running title：Depression of L-cystathionine production in *metB* transformant

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It was previously reported (Nishi et al., 2002) that an *Escherichia coli* CAG18475, which was deficient in cystathionine β-lyase (*metC* gene product, EC 4.4.1.8) due to Tn10 insertion (Tc) and required methionine for growth, could accumulate L-cystathionine. However, the amount was low (around 1 mg per ml) in comparison with some other amino acids (Araki, K. and Oseki, T., 1992) and it is desired to enhance its production and maintain stable supply for the medical research on the prophylactic and therapeutic effects of this amino acid.

It is known that L-cystathionine is a precursor for L-methionine biosynthesis in *E. coli* and formed by transsulfuration of O-succinyl-L-homoserine by L-cysteine catalyzed by cystathionine γ-synthase (*metB* gene product, EC 4.2.99.9) (Rowbury, 1983). Expecting to increase the L-cystathionine accumulation, we tried to clone *metB* gene from *E. coli* K12 and express it in *E. coli* CAG18475.

Amplification of *metB* gene by polymelase chain reaction was carried out with the genomic DNA of a wild-type *E. coli* K-12 strain EMG2 (kindly supplied by Dr. Bachman of Yale University) as the template and the primers:5'-GTAATGCACCTGTCGGCGTGATA-3' and 5'-TATCACGCCGACAGGTGCATTAC-3'. The DNA fragment of 1.38 Kbp obtained was ligated to "pDrive cloning vector" (3.8 Kbp, Qiagen, Tokyo)

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possessing a cloning site within *lacZ* gene and marker genes resistant to ampicillin (Ap) and kanamycin (Km), and *E. coli lacZ* mutant E2 (Qiagen) was transformed with the ligation products and then tranformants with Ap^r, Km^r, Lac^- traits were selected. Among these one strain was ascertained to have a plasmid of 5.2 Kbp (designated pMO3-b) which complemented the methionine auxotrophy of an *E. coli metB* mutant strain CGSC4946 (kindly supplied by Dr. Berlyn of Yale University). We transformed the L-cystathionine producing strain CAG18475 with this plasmid, and a transformant with the traits of Ap^r, Km^r, Tc^r and Met^- was selected.

The cystathionine γ -synthase activity of the transformant was measured by the method of Kaplan and Flavin (Kaplan and Flavin, 1966). The enzyme extract was prepared as follows. Cell was cultured in 300-ml Erlenmeyer flasks basically according to the previously described method (Nishi et al., 2002) under the condition with 125 μ g/ml of L-methionine in 7 ml of a fermentation medium containing 6 % fructose (3 % initially and another 3 % at 24 hr of the culture) and 1 % ammonium sulfate as carbon, nitrogen and sulfur sources, respectively. The L-methionine concentration was favorable to L-cystathionine production by the host strain CAG18475 as will be described below. After culturing for 27 h, cells were harvested by centrifugation, washed once with 10 mM potassium phosphate buffer (pH 7) and disrupted by sonication. To the cell free extract obtained therefrom after centrifugation at 12,000 rpm for 30 min, ammonium sulfate was added to make 80 % saturation, and the resulted insoluble fraction was dialyzed against the buffer described above and used for the enzyme assay. Protein concentration was determined by the method of Lowry et al. (Lowry et al., 1951) using bovine serum albumin as the standard. As can be seen in Table 1, the cystathionine γ -synthase activity of the transformant was 31 times as much as that of the host strain.

Table 1. Cystathionine γ -synthase activity in *metB* gene transformant

Strain	Enzyme activity (μmol/min/mg-protein)	Ratio
CAG18475	0.04	1.0
CAG18475/pMO3-b	1.24	31.0

The enzyme extracts prepared after 80% ammonium sulfate precipitation and dialysis against 20 mM potassium phosphate buffer (pH 7) of the cell-free extracts from the cells obtained by culturing for 27 hr in the fermentation medium favorable to L-cystathionine production (Nishi et al., 2002) were used for the enzyme assay. The enzyme assay was carried out by the method of Kaplan and Flavin (Kaplan and Flavin, 1966). Strain and plasmid: CAG18475, *E. coli* host strain (*metC* : Tc); pMO3-b, recombinant plasmid carrying *metB* gene. Details for the experiment, see the text.

Then we carried out the fermentation test for L-cystathionine production with the transformant CAG18475/pMO3-b. The fermentation was done as described above except that L-methionine concentrations in the medium were varied among 0 to 500 μg per ml and the culturing was continued to 48hr. On the contrary to our expectation, the transformant did not accumulate L-cystathionine at all, while the reference culture with the host strain accumulated 0.6-0.8 mg per ml of L-cystathionine at the L-methionine concentrations within 100-200 μg per ml (Fig. 1). The transformant grew finally to a constant level irrespective of the L-methionine concentrations added initially. This indicates that the transformant lost an auxotrophic nature of the host strain. On the other hand, the growth of the host strain clearly depended on the L-methionine concentrations, that is to say, the growth increased linearly up to 200 μg per ml of L-methionine and leached a plateau of the same level with that of the transformant

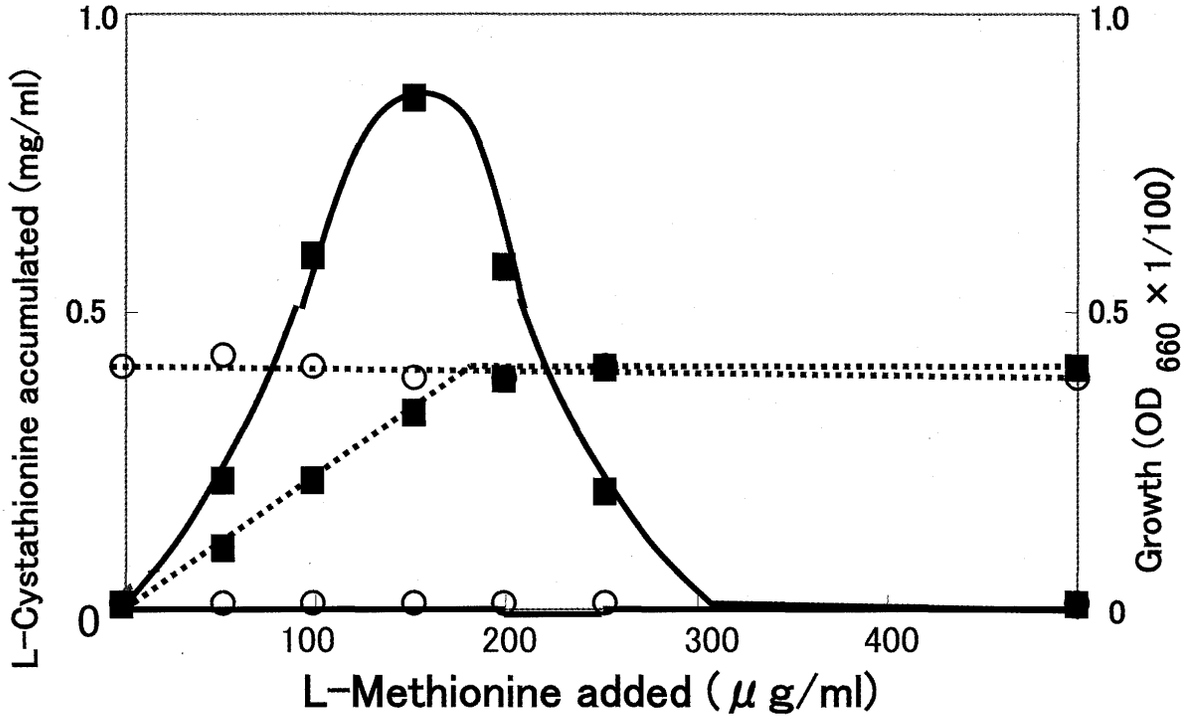

Fig.1

at the higher L-methionine concentrations. The results obtained with the host strain were in good accordance with those observed in the previous study (Nishi et al., 2002).

The growth behavior of the transformant was further examined with a chemically defined minimal medium according to the previously described method (Araki et al., 1999). In an agar plate test, the transformant gave a much broader growing zone around a pit filled with L-methionie solution than that with the host strain (Fig. 2). This shows the leaky type nature of methionine auxotrophy for the transformant. The sodium sulfite in place of L-methionine supported the growth of transformant at the molar concentrations six times as much higher than that of L-methionine while it did not affect the growth of the host strain (Fig. 3). In a liquid culture, the transformant showed a delayed growth without exogeneous L-methionine and the growth was stimulated by L-methionine. The host strain did not grow without L-methionine (Fig. 4). The transformant was comfirmed to retain the Tc^r trait, indicating Tn10 element remains in *metC* gene. These results suggest that the enhanced expression of *metB* gene and hence the increase in cystathionine γ-synthase activity leaked L-methionine synthesis in the transformant strain, leaving the genetic block at *metC* gene.

Two pathways have been reported for methionine synthesis in bacteria (Hacham, Y. et al., 2003). One is the transsulfuration operated in *E. coli* as described above. L-Homocysteine, the terminal intermediate, is formed via L-cystathionine. The other is the direct sulfhydrylation pathway which is catalyzed by acetylhomoserine (or succinylhomoserine) sulfhydrylase (EC 4.2.99.10), which is found in several bacteria. In this reaction, O-acetyl-L-homoserine or O-succinyl-L-homoserine and sulfite serve as substrates to form L-homocysteine. Both pathways have been found to be cooperative in some bacteria such as *Corynebacterium glutamicum* (Hwang, B. J. et al., 2002; Ruckert et al., 2003).

Simon and Hong (Simon and Hong, 1983) found, during the study on prototrophic revertants, mutations which enabled *E. coli* to form L-homocysteine in the absence of cystathionine β-lyase but required cystathionin γ-synthase and homoserine succinyltransferase (EC 2.3.1.46). They proposed a direct L-homocysteine biosynthesis from O-succinyl-L-homoserine, bypassing L-cystathionine, although the sulfur donor had not been specified.

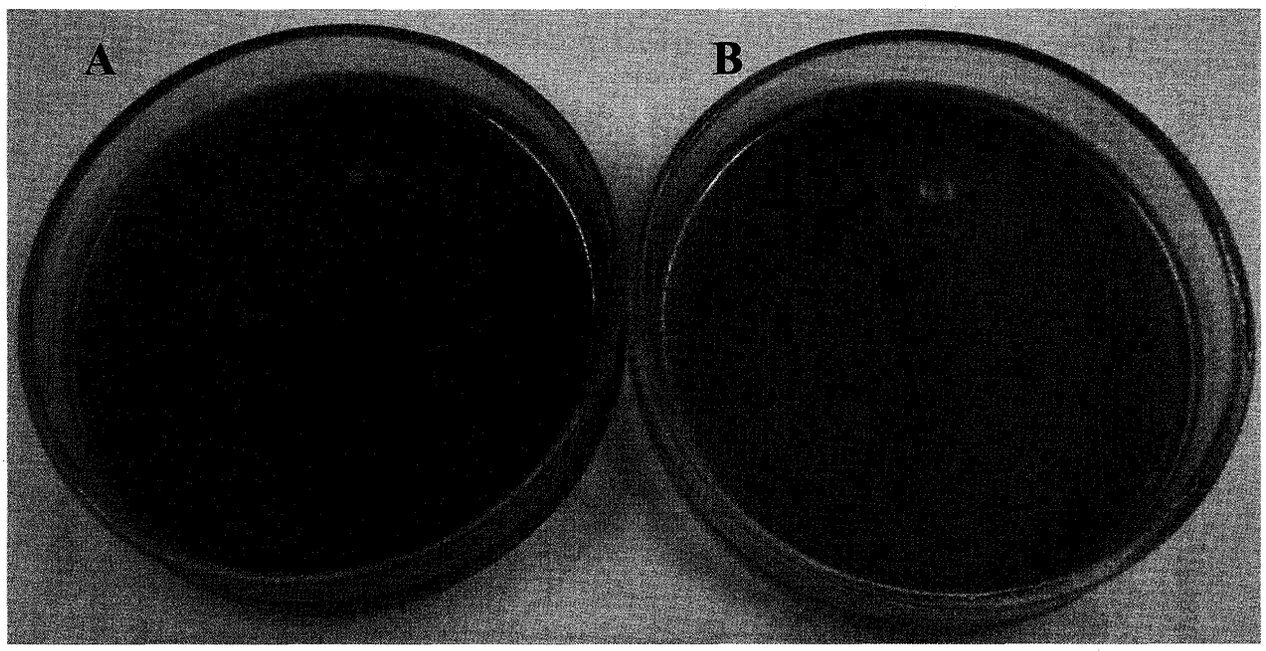

Fig.2

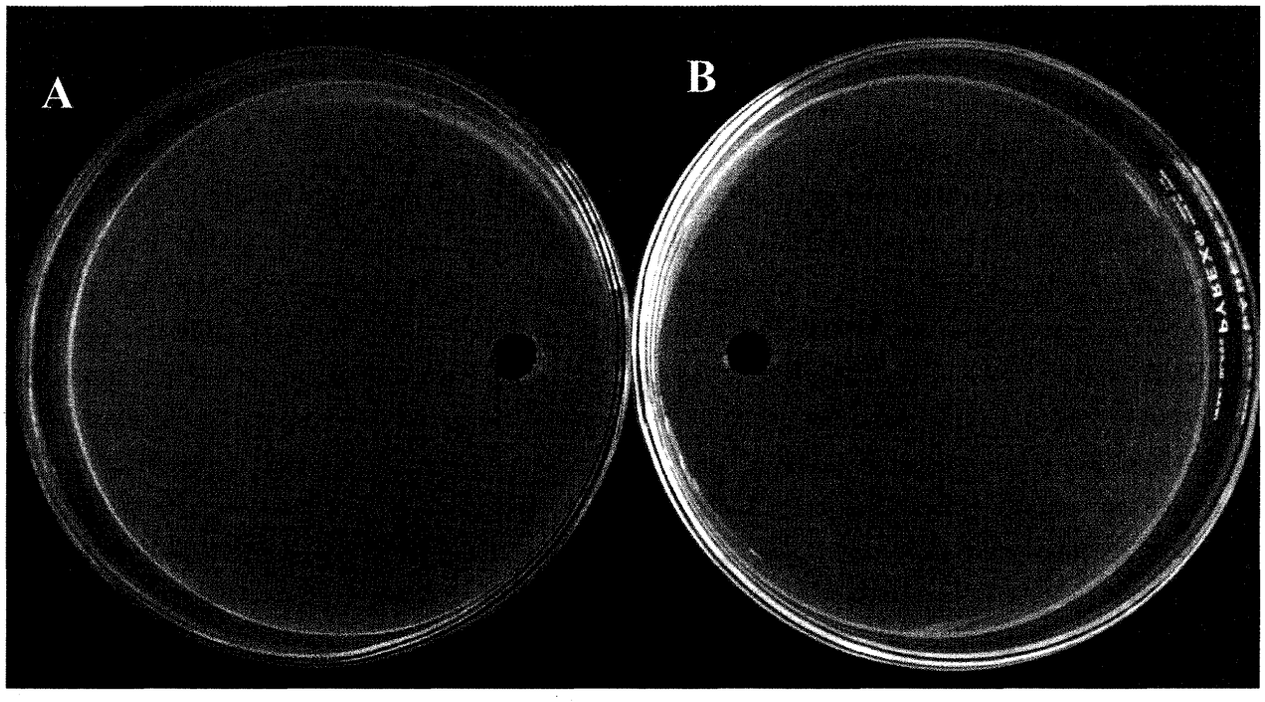

Fig.3

Just before our preliminary report (Motomatsu et al., 2003), Hacham et al. (Hacham et al., 2003) reported that a transformation with *metB* gene complemented the methionine auxotrophy of a *metB* and *metC* double mutant of *E. coli* and, on the basis of this fact, proposed that cystathionine γ -synthase has the activity of O-succinylhomoserine sulfhydrylase as well as its intrinsic one as a vestige on the evolution of methinine biosynthetic pathway. We tried to detect O-succinylhomoserine sulfhydrylase activity in the enzyme extract of the transformant CAG18475/pMO3-b, but it has been unsuccessful at present. However, we observed that sodium sulfite weakly but significantly complemented the methionine auxotrophy of this strain. Moreover, Flavin and Slaughter (Flavin and Slaughter, 1967) have shown that cystathionine γ -synthase of *E. coli* catalyzes L-homocysteine formation *in vitro*

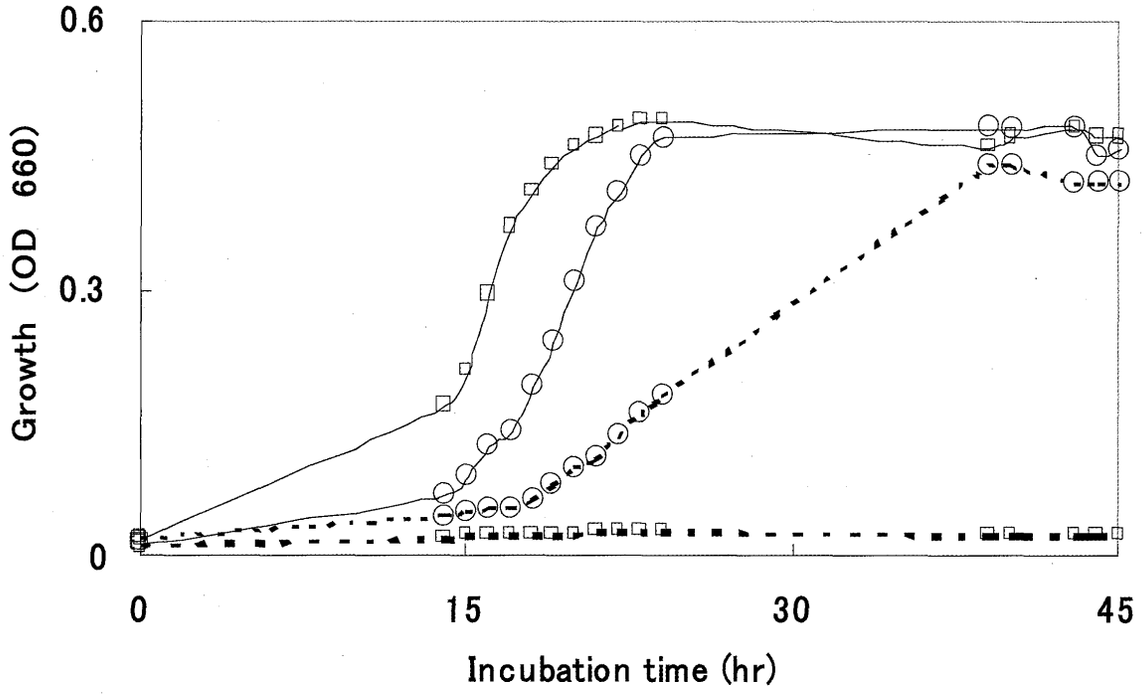

Fig.4

by direct condensation of sulfite with O-succinyl-L-homoserine.

The results of our present study could be explained by these facts as follows. The natural level of the sulfhydrylation activity of cystathionine γ -synthase in *E. coli* might be too small to support the bacterial growth, because the *metC* mutant CAG18475 exhibited clearly methionine auxotrophy (Figs. 1, 2 and 4). However, the sulfhydrylase activity could act to a certain extent to partially complement the methionine auxotrophy in the *metB* transformant as the result of the enhanced expression of *metB* gene. The depression in L-cystathionine accumulation would be explained by feedback regulation by the L-methionine, formed through this bypassing, exerting on the L-cystathionine synthetic pathway.

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LEGENDS FOR FIGURES

Fig. 1. Depression of L-cystathionine production by transformation with *metB* gene in *E. coli* CAG18475.

Fermentation was carried out in 300 ml-Erlenmeyer flasks with the host strain { *E.coli* CAG18475 (*metC* : Tc) } (■) and *metB* gene transformant CAG118475/pMO3-b (○) under various L-methionine concentrations as given in the 7 ml medium containing 6 % (3 % initially and another 3 % at 24 h) fructose and 1 % ammonium sulfate as carbon, and nitrogen and sulfur sources, respectively, for 48 h. Amount of L-cystathionine accumulated (————) was assayed by high performance liquid chromatography and the bacterial growth (----------) was measured the absorbance at 660nm of the culture after 100-fold dilution with 0.02 N HCl through a light path of 10 mm. For the other details of the experiment, see the previous paper (Nishi et al., 2002).

Fig. 2. Characterization of methionine auxotrophy in the *metB* gene transformant CAG18475/pMO3-b strain by plate culture.

The cells (10^6 cells per plate) of the host strain { *E.coli* CAG18475 (*metC* : Tc) }(A) and the transformant strain CAG18475/ pMO3-b (B) were spread on the minimal agar plates (Araki et al., 1999) supplemented with 200 μ g/ml of bromothymol blue, and 50 $\mu\ell$ of 22 mM L-methionine was poured in the pits on the corners of the plates and incubated for 42 hr at 30℃.

Fig. 3. Complementation of methionine auxotrophy of the *metB* gene transformamant CAG18475/ pMO3-b with sodium sulfite.

The cells of the host strain (B) and the transformant strain CAG18475/ pMO3-b (A) were incubated on the agar plates by the same method with that described in the legend to Fig. 2 except that 128 mM sodium sulfite was used in place of L-methionine and incubation time was 48 hr.

Fig. 4. Characterization of methionine auxotrophy in the *metB* gene transformant CAG18475/pMO3-b strain by liquid culture.

Bacterial cells, inoculated at 10^7 cell concentration per ml, were cultured in an L-shaped test tube (ϕ 20mm) containing 8 ml of a minimal liquid medium (Araki et al., 1999) supplemented with (————) or without (----------) L-methionine (30 μ g/ml) on a Monod shaker (TAITEC Co., Saitama) operated at 30℃ and 60 rpm and the bacterial growth was determined by the measurement of the absorbance at 660 nm using a photometer (ANA-7A, Tokyo Photoelectric Co.) through the light path of 20 mm. Bacterial strains: CAG118475, *E. coli* host strain (*metC* : Tc) (□); CAG118475/pMO3-b, *metB* transformant (○).