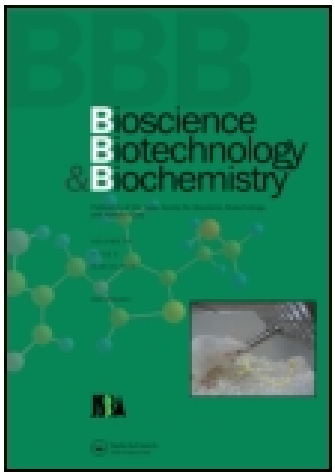

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

High Fermentative Production of L-Threonine from Acetate by a Brevibacterium flavum Stabilized Strain Transformed with a Recombinant Plasmid Carrying the Escherichia coli thr Operon

Masaaki Ishida[a], Katsuaki Sato[a], Ken-ichi Hashiguchi[a], Hisao Ito[a], Hitoshi Enei[a] & Shigeru Nakamori[a]

[a] Central Research Laboratories of Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki, Kanagawa 210, Japan

Published online: 12 Jun 2014.

To cite this article: Masaaki Ishida, Katsuaki Sato, Ken-ichi Hashiguchi, Hisao Ito, Hitoshi Enei & Shigeru Nakamori (1993) High Fermentative Production of L-Threonine from Acetate by a Brevibacterium flavum Stabilized Strain Transformed with a Recombinant Plasmid Carrying the Escherichia coli thr Operon, Bioscience, Biotechnology, and Biochemistry, 57:10, 1755-1756, DOI: 10.1271/bbb.57.1755

To link to this article: http://dx.doi.org/10.1271/bbb.57.1755

Biosci. Biotech. Biochem., **57** (10), 1755–1756, 1993

Note

High Fermentative Production of L-Threonine from Acetate by a *Brevibacterium flavum* Stabilized Strain Transformed with a Recombinant Plasmid Carrying the *Escherichia coli thr* Operon†

Masaaki ISHIDA, Katsuaki SATO, Ken-ichi HASHIGUCHI, Hisao ITO, Hitoshi ENEI, and Shigeru NAKAMORI

Central Research Laboratories of Ajinomoto Co., Inc., 1–1 Suzuki-cho, Kawasaki-ku, Kawasaki, Kanagawa 210, Japan
Received February 3, 1993

Decrease in L-threonine productivity caused not only by plasmid-free segregation but also by plasmid deletion was observed in a *Brevibacterium flavum* L-threonine producer when transformed with a recombinant plasmid carrying *Escherichia coli thr* operon. However, a recombinant strain, HT-16, was stabilized by the addition of trimethoprim (a selective marker on the vector) at concentration of 1000 μg/ml, which was rather higher than the minimum inhibitory one, into the stock culture medium. Strain HT-16 produced 64.4 g/liter of L-threonine, 30% higher than that of the host strain, after 92 h of cultivation in a small jar fermentor using acetic acid as a carbon source without trimethoprim.

Novel host-vector systems have been developed for commercially important amino acid-producing coryneform bacteria such as *Corynebacterium glutamicum* and *Brevibacterium lactofermentum* (for a review, see reference 1). Using these systems, several trials of molecular breeding of L-threonine producers have been successfully carried out by the amplifications of indigenous[2-5] or foreign genes[6,7] encoding L-threonine biosynthetic enzymes.

In the previous paper,[7] we reported on availability of an amplification of the *Escherichia coli thr* operon in *Brevibacterium flavum* BBIB-19 for an improvement of L-threonine production. The maximum yield of L-threonine production from glucose by a recombinant strain, HT-16, carrying pAJ514 was 27%, and 2.3 times higher than that of the host strain at the flask level. Then, L-homoserine, a precursor of L-threonine, disappeared. This result suggests that the effective conversion of L-homoserine to L-threonine was caused by the amplification of the *thr* operon.

Plasmid instability is a serious problem often encountered in the case of scaling up amino acid fermentation. In this report, we describe the successful L-threonine production by the stabilized recombinant strain in a small jar fermentor.

Plasmid pAJ220, a vector used for the construction of pAJ514, was derived from a cryptic plasmid, pAM330, in *B. lactofermentum*. High plasmid stability of pAM330 derivatives was confirmed even in the absence of trimethoprim, an antibacterial substance as a selection marker on the vectors, by their use for cloning of several indigenous genes.[3,9,10] We also investigated stability of L-threonine productivity using single colonies isolated from strain HT-16 stored at −70°C on a plate of medium CM2G (1% peptone, 1% yeast extract, 0.5% NaCl and 0.5% glucose, pH 7.0)[8] without trimethoprim. Then, difference in the colony size was observed. An L-threonine production test was done by aerobic cultivation in a 500 ml flask containing 20 ml of L-threonine production medium TP-2 (10% glucose, 3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 10 ppm $FeSO_4 \cdot 7H_2O$, 10 ppm $MnSO_4 \cdot 4H_2O$. 5 μg/ml thiamine·HCl, 0.1 μg/ml *d*-biotin, 300 μg/ml L-isoleucine, 320 μg of soybean-meal hydrolysate as the total nitrogen per milliliter, and separately sterilized 5% $CaCO_3$, pH 7.0)[7] without trimethoprim at 30°C for 72 h, and followed by the estimation with HPLC. In the larger colonies, loss of the entire plasmid was observed and their L-threonine productivities were reduced to the host strain level (11.5 g/liter). On the other hand, various deleted plasmids were detected in the smaller colonies, of which L-threonine productivities were slightly higher (approximately 12–17 g/liter) than that of host strain.

The effect of trimethoprim in the stock medium on plasmid stabilization, and therefore, the maintenance of the L-threonine productivity was investigated. In recombinant strain HT-16 cells grown on CM2G-plate containing 300 μg/ml trimethoprim, at which host strain BBIB-19 was scarcely able to grow, deleted plasmids were yet observed although fewer than the case without trimethoprim, and their L-threonine productivity decreased to 15–16 g/liter. When the recombinant cells grown at 1000 μg/ml trimethoprim were used as an inoculant for the L-threonine production test, they maintained the original productivity (23.3 g/liger) of strain HT-16, and no enhanced instability of the plasmid was observed during the cultivation in L-threonine production medium TP-2 without trimethoprim at 30°C for 72 h. The initial L-threonine productivity in the cells after the 6 successive subcultures (approximately 20 cell doublings) was also maintained without increase of deleted plasmids in medium CM2G supplemented with 1000 μg/ml trimethoprim. Thus, we adopted this as the stock medium of strain HT-16. Addition of trimethoprim at 1000 μg/ml, which seems to be rather strong as compared with the minimum inhibitory concentration (300 μg/ml) of the host strain, led to the repression of segregation of plasmid-free cells, and interestingly the effective elimination of the deleted plasmids. The latter phenomenon suggests the existence of hot spots for deleted regions including, partially or wholly, not only the *thr* operon but also the trimethoprim resistance gene. This spontaneous deletion mechanism might be explained by the slipped misparing model[11] based on the slippage of DNA polymerase between sites of short sequence matching during replication of the hybrid plasmid. Because no enlarged plasmids were detected in the course of this study, and when the *thr* operon was cloned onto another vector derived from pAJ1844[8] which was compatible with pAM330 derivatives, no deleted plasmids were observed although plasmid-free segregants occurred (data not shown). Further investigation at the DNA sequence level is necessary to clarify this.

We investigated L-threonine production by recombinant strain

† This work was presented in part at the Annual Meetings of the Agricultural Chemical Society of Japan, in Tokyo (1987) and the Society of Fermentation Technology, Japan, in Osaka (1987).

Abbreviation: AHV^R, resistant to α-amino-β-hydroxyvaleric acid.

Table L-Threonine Production by Strain HT-16 in a Small Jar Fermentor

L-Threonine fermentation was carried out in the following manner: 50 ml of the pre-culture medium in a 500 ml flask was inoculated with one loopful of the tested strain grown on a CM2G-agar slant. After 14–16 h of aerobic cultivation at 31.5°C, 15 ml of the pre-culture was transferred to 285 ml of L-threonine fermentation medium concentrated from 300 ml of its original volume in a 1-liter fermentor. The L-threonine fermentation medium consisted of 10% glucose, 1% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 10 ppm $FeSO_4 \cdot 7H_2O$, 10 ppm $MnSO_4 \cdot 4H_2O$, 0.5% urea, 5 μg/ml thiamine·HCl, 0.05 μg/ml *d*-biotin, 0.41% CH_3COONa, 0.4% CH_3COONH_4, 0.35 mg/ml L-isoleucine, 0.47 mg of soybean-meal hydrolysate as the total nitrogen per milliliter. The cultivation temperature was 27°C. Oxygen was supplied at 1200 rpm of agitation speed and 1/2 vvm of air rate during the glucose consumption, followed by the aeration of 1/1 vvm. The pH of the culture medium was automatically controlled at 7.7 with gaseous ammonia in the fermentation assimilating glucose, and then by the addition of feeding solution which consisted of CH_3COONH_4 and CH_3COOH. The molecular ratio of CH_3COONH_4 to CH_3COOH was 0.2, and total acetate concentration was 700 g/liter L-Amino acid analysis of the culture broth was done with an amino acid analyzer.

Strain	Amino acid produced (g/liter)		Cultivation time (h)	Growth ($O.D._{562}$ ×26)
	L-Thr	L-Hse		
BBIB-19	49.2 (14.3)[a]	5.5	97	1.50
HT-16	64.4 (19.9)	0.4	92	1.45

[a] Yield (%) of L-threonine produced.

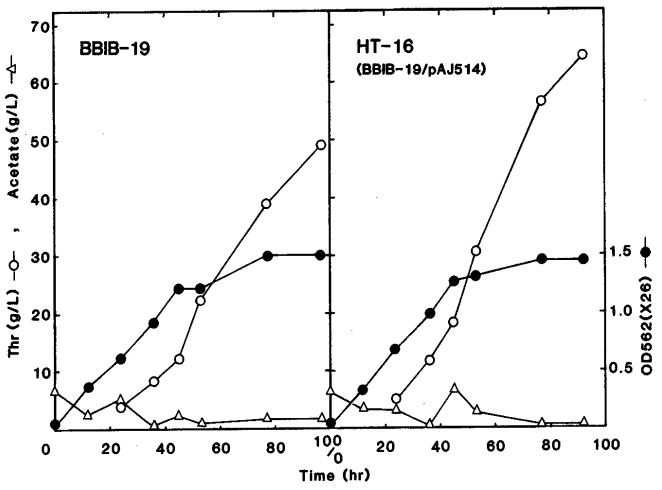

Fig. Time Course of L-Threonine Fermentation Using Strain HT-16 in a Small Jar Fermentor.

HT-16 from acetate in a fed-batch culture, since Akashi *et al.* reported a higher yield of L-threonine production by a *B. flavum* AHV^R mutant from acetate as a carbon source than that from glucose and then a problem of a by-product, L-homoserine, formation caused by regulated homoserine kinase.[12] After 16 h of cultivation at 30°C using the plasmid-stabilized strain in the pre-culture medium for the small jar fermentor (3% glucose, 0.15% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 2 ppm $FeSO_4 \cdot 7H_2O$, 2 ppm $MnSO_4 \cdot 4H_2O$, 0.3% urea, 0.3 μg/ml thiamine·HCl, 0.2 μg/ml *d*-biotin, and 2 mg of soybean-meal hydrolysate as the total nitrogen per milliliter, pH 7.0), 98% of the tested colonies carried intact pAJ514. This result shows the stability of pAJ514 is guaranteed during the pre-culture in the absence of trimethoprim, when approximately 7.0 cell doublings occurred. As shown in Table, the effective conversion of L-homoserine to L-threonine in recombinant HT-16 was demonstrated in a small jar fermentor. Figure shows a typical time course of the improved L-threonine production by strain HT-16. This result shows that the L-threonine production was successfully carried out in the absence of trimethoprim during approximately 7.0 generations by using the intact pAJ514-carrying strain as an inoculant.

Acknowledgments. The authors are indebted to Drs. R. Tsugawa and M. Dazai for their encouragement. They are grateful to Messrs. Y. Nishimoto, N. Kurihara, and H. Kawashima, and Drs. K. Akashi, T. Tsuchida, and H. Kojima for their useful advice, and Drs. H. Matsui and K. Miwa for their valuable comments on this manuscript.

References

1) T. Beppu, in "Biotechnology of Amino Acid Production," ed. by K. Aida, I. Chibata, K. Nakayama, K. Takinami, and H. Yamada, Kodansha, Tokyo, and Elsevier Science Publishers, Amsterdam, 1986, pp. 24–35.
2) S. Nakamori, M. Ishida, H. Takagi, K. Ito, K. Miwa, and K. Sano, *Agric. Biol. Chem.*, **51**, 87–91 (1987).
3) Y. Morinaga, H. Takagi, M. Ishida, K. Miwa, T. Sato, S. Nakamori, and K. Sano, *Agric. Biol. Chem.*, **51**, 93–100 (1987).
4) R. Katsumata, T. Mizukami, Y. Kikuchi, and K. Kino, in "Genetics of Industrial Microorganisms," Part B, ed. by M. Alacevic, Z. Hranueli, and Z. Toman, Pliva Publishing Co., Zagreb, Yugoslavia, 1987, pp. 217–226.
5) B. J. Eikmanns, M. Metzger, D. Reinscheid, M. Kircher, and H. Sahm, *Appl. Microbiol. Biotechnol.*, **34**, 617–622 (1991).
6) R. Katsumata, M. Hara, T. Mizukami, T. Oka, and A. Furuya, Abstracts of Papers, the Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, Japan, April, 1984, p. 248.
7) M. Ishida, E. Yoshino, R. Makihara, K. Sato, H. Enei, and S. Nakamori, *Agric. Biol. Chem.*, **53**, 2269–2271 (1989).
8) K. Miwa, K. Matsui, M. Terabe, K. Ito, M. Ishida, H. Takagi, S. Nakamori, and K. Sano, *Gene*, **39**, 281–286 (1985).
9) H. Takagi, Y. Morinaga, K. Miwa, S. Nakamori, and K. Sano, *Agric. Biol. Chem.*, **50**, 2597–2603 (1986).
10) H. Ito, K. Sato, K. Matsui, K. Sano, H. Enei, and Y. Hirose, *Appl. Microbiol. Biotechnol.*, **33**, 190–195 (1990).
11) A. M. Albertini, M. Hofer, M. P. Calos, and J. H. Miller, *Cell*, **29**, 319–328 (1982).
12) K. Akashi, H. Shibai, and Y. Hirose, *Agric. Biol. Chem.*, **43**, 1563–1566 (1979).