Stabilization of an Unstable L-Leucine Producing Strain of *Corynebacterium glutamicum* by Enhancement of L-Valine Biosynthesis[†]

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Received December 28, 1987

The L-leucine productivity of an L-leucine producer, *Corynebacterium glutamicum* H-1204, was unstable due to reversion, which was assumed to be caused by the depletion of intracellular L-valine. The genetic enhancement of L-valine biosynthesis was attempted for stabilization using L-valine accumulation as an index. Strain AB-47, derived as an α -aminobutyrate resistant mutant, accumulated L-valine as a by-product besides L-leucine. The L-leucine productivity of strain AB-47 was remarkably stabilized under conditions under which that of parental strain H-1204 became unstable. These results indicate that the balanced biosynthesis of metabolites is important for the breeding of a stable amino acid producer.

In our previous paper,¹⁾ we reported that the L-leucine production by an L-leucine producer, *Corynebacterium glutamicum* H-1204, was unstable due to reversion. Since the original L-leucine producer (L-type) was found to be a valine leaky mutant, it was strongly suggested that α -ketoisovaleric acid, a common precursor of L-leucine and L-valine, was preferentially utilized for L-leucine biosynthesis. This distribution imbalance induced depletion of the intracellular L-valine pool and allowed revertants with higher growth rates to increase in the number.

We also showed that the addition of L-valine to the medium was effective for stabilization.²⁾ L-Valine not only promoted the growth of the L-type strain but also inhibited the growth of the revertant (leucine auxotroph), as an antagonist as to its leucine uptake.

In this study, we examined whether or not the genetic enhancement of L-valine biosynthesis through mutagenesis improves the stability of the L-leucine fermentation.

MATERIALS AND METHODS

Microorganisms. An *S*-(2-aminoethyl)-L-cysteine resistant mutant, H-1204, derived from *Corynebacterium glutamicum*, ATCC 13032, was used as the L-leucine producing strain throughout this work. Strain AB-47 was derived from H-1204 as an α -aminobutyrate resistant mutant by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment.

Media and culture conditions. The compositions of the media and the culture conditions for test tube and jar fermentor cultivations were given previously.¹⁾

Isolation of valine analog resistant mutants. Bacterial cells cultured overnight at 30°C in test tubes containing 8 ml of the seed medium were inoculated into 8 ml of the same medium and then cultured for 2 hr at 30°C. The cells were harvested, washed twice with 50 mM Tris-maleate buffer (pH 6.0) and then resuspended at a concentration of about 10^9 cells/ml in an equal volume of the same buffer containing 250 μ g/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). After incubation for 30 min at 30°C, the cells were washed twice and then resuspended in the same buffer. After appropriate dilution, the cell suspension was spread on minimal medium plates containing an analog at a concentration inhibitory for the parental strain. The plates were incubated for 5 to 10 days at 30°C. The colonies appearing on the plates were isolated as analog resistant mutants and then tested for L-leucine production.

[†] Instability and Stabilization of an L-Leucine Producing Strain of *Corynebacterium glutamicum*. Part III.

Analysis. The measurement of bacterial growth (OD₆₆₀) and the amount of L-leucine produced was carried out as described previously.¹⁾ The amount of L-valine was determined by the same method as that for L-leucine.

Chemicals. α -Aminobutyrate, β -hydroxyvaline, DL-norleucine and DL-valine hydroxamate were purchased from Sigma Chemical Co. Amino acids were the products of Kyowa Hakko Kogyo Co., Ltd.

RESULTS AND DISCUSSION

Enhancement of L-valine biosynthetic activity

The instability of L-leucine production by strain H-1204 was speculated to be caused by depletion of the intracellular L-valine pool. It was considered essential for stabilization to increase the pool through enhancement of the L-valine biosynthetic activity (Fig. 1).

The biosynthesis is controlled primarily by the first step enzyme, α -acetoxy acid synthetase, which is sensitive to branched chain amino acids.³⁾ *Escherichia coli*⁴⁾ and *Serratia marcescens*⁵⁾ have been reported to

FIG. 1. Metabolic Pathway of L-Leucine and L-Valine Biosynthesis.

Abbreviations: AHAS, α -acetoxy acid synthetase; IPMS, α -isopropylmalate synthetase.

TABLE I. SUMMARY OF THE SCREENING FOR L-VALINE PRODUCERS AMONG MUTANTS RESISTANT TO VARIOUS VALINE ANALOGS

Analog	Conc. (mg/ml)	Number tested	Number of strains producing L-valine as a by-product
α -Aminobutyrate	20	97	13
β -Hydroxyvaline	10	85	1
Norleucine	10	45	2
Val hydroxamate	5	50	1

Cultivations for the screening were performed for 72 hr at 30°C in test tubes.

excrete L-valine into the media because of the acquirement of valine analog resistance. It has also been reported that α -acetoxy acid synthetase was derepressed or desensitized as to branched chain amino acids in L-valine producing strains of *S. marcescens*⁵⁾ and *Brevibacterium lactofermentum*.⁶⁾

Mutants resistant to various valine analogs were derived from strain H-1204 by NTG treatment. These mutants were tested for L-leucine production. Then, it was determined, from the L-valine accumulation as a by-product, whether or not the ability of a mutant to synthesize L-valine had been enhanced.

FIG. 2. Distribution of the α -Aminobutyrate Resistant Mutants with Respect to L-Leucine and L-Valine Production.

The hatched parts show the numbers of strains producing L-valine as a by-product.

TABLE II. RELATIONSHIPS BETWEEN THE PRODUCTION PATTERNS AND THE PHENOTYPES OF REPRESENTATIVE STRAINS

Strain	Accumulation (mg/ml)		Phenotype
	L-Leucine	L-Valine	Valine
H-1204	10.1	0	L
AB-39	10.8	0	L
AB-47	10.2	1.1	+

Symbols: L, leaky; +, prototrophic. Strains AB-39 and AB-47 were derived as α -aminobutyrate resistant mutants. The phenotypes were determined by replicating each strain on minimal medium with and without L-valine supplementation.

The results are summarized in Table I. Strains exhibiting the simultaneous accumulation of L-leucine and L-valine were found at a higher frequency among the mutants resistant to α -aminobutyrate. Figure 2 shows the distribution of the α -aminobutyrate resistant mutants with respect to the accumulation of these two amino acids. Among the total 97 mutants tested, two strains showed similar L-leucine productivity to that of the parental strain, H-1204, and 13 strains produced both L-leucine and L-valine.

Relationships between the production patterns and the phenotypes of α -aminobutyrate resistant mutants

Strains AB-39 and AB-47 were selected as representatives as to the production patterns and phenotypes (Table II). Strain AB-39, which accumulated an increased amount of L-leucine, still had the same phenotype as the parental strain H-1204 (valine leaky). On the other hand, the phenotype changed from valine leaky to prototrophic in the case of strain AB-47, which accumulated both L-leucine and L-valine. This indicates that the phenotype reflects the ability to synthesize L-valine.

Stability of α -aminobutyrate resistant mutants during subculturing

The stability of the L-leucine productivity of various strains was examined by subculturing (Table III). The L-leucine production by strains H-1204 and AB-39 decreased remarkably with subculturing. In contrast, strain AB-47 showed quite stable productivity. The change to prototrophy was found to be effective for stabilization.

Comparison of L-leucine production by a stable mutant, AB-47, and the parental strain, H-1204

The time courses of L-leucine production were compared for AB-47 and H-1204, in 5 l jar fermentors (Fig. 3). Strain H-1204 showed higher growth but only produced 5.2 mg/ml of L-leucine. Strain AB-47 produced 10.8 mg/ml

TABLE III. COMPARISON OF STABILITY DURING SUBCULTURING

Number of subcultures	L-Leucine produced (mg/ml)		
	H-1204	AB-39	AB-47
0	10.1	10.8	10.2
1	7.5	8.0	10.0
3	6.4	6.2	10.5
5	3.7	3.9	10.3

Subculturing was performed on complete medium slants. The L-leucine production was carried out in test tubes.

FIG. 3. Comparison of the Time Courses for Strains H-1204 and AB-47.

Symbols: ○●, L-leucine produced; □■, growth (OD₆₆₀); △▲, residual sugar concentration. Open and closed symbols denote H-1204 and AB-47, respectively. The fermentations were carried out in 5 l jar fermentors.

of L-leucine stably on 40 hr cultivation under the same conditions. No revertants appeared in the culture broth.

Very few studies have dealt with the stabilization of an amino acid producer on the basis of metabolic analysis. Sugiura and Kisumi⁷⁾ reported that the depletion of intracellular ATP, which is utilized for histidine biosynthesis, caused the appearance of revertants with reduced L-histidine productivity in *S. marcescens*. They showed that the genetic enhancement of the ATP pool was effective for stabilization of the production and suppression of reversion.

In this study, we also stabilized the L-leucine productivity of strain H-1204. These results

suggest that one of the reasons for the instability is the metabolic imbalance accompanied by overproduction of metabolites. Therefore, it seems that the maintenance of a metabolic balance is important for the breeding of a stable amino acid producer.

Acknowledgment. The authors are grateful to Mr. Sadao Noguchi for his encouragement throughout this study.

REFERENCES

- 1) T. Azuma, T. Nakanishi and H. Hagino, *Agric. Biol. Chem.*, **51**, 3245 (1987).
 - 2) T. Azuma, T. Nakanishi and H. Hagino, *Agric. Biol. Chem.*, **52**, 1129 (1988).
 - 3) J. M. Blett, W. J. Pledger and H. E. Umbarger, *Biochem. Biophys. Res. Commun.*, **48**, 444 (1972).
 - 4) O. Karlstrom, *Biotechnol. Bioeng.*, **7**, 245 (1965).
 - 5) M. Kisumi, S. Komatsubara and I. Chibata, *Amino Acid and Nucleic Acid*, **19**, 1 (1969).
 - 6) T. Tsuchida and H. Momose, *Agric. Biol. Chem.*, **39**, 2193 (1975).
 - 7) M. Sugiura and M. Kisumi, *Appl. Environ. Microbiol.*, **48**, 43 (1984).
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