Effect of Pyruvate Kinase Deficiency on L-Lysine Productivities of Mutants with Feedback-resistant Aspartokinases

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The cultural conditions were investigated for a *Brevibacterium flavum* mutant, No. 2-190, with a low level of citrate synthase (CS) and with feedback-resistant phosphoenolpyruvate (PEP) carboxylase and aspartokinase (AK). The productivity was increased from 28 to 38 g/l (as the HCl salt) with a medium containing 10% glucose. From this strain, pyruvate kinase (PK)-defective mutants were derived and selected as to the inability to grow on ribose. Among them, strain KL-18 showed higher lysine productivity than the parent under all cultural conditions tested, and produced 43 g/l of lysine, at maximum. A lysine-producing mutant, No. 536-4, with a feedback-resistant AK was derived from PK-defective strain KH-21 which had low CS activity and a feedback-resistant PEP carboxylase. The mutant was isolated by a new selection method, that is, on the basis of resistance to α -amino- β -hydroxyvaleric acid, a threonine analogue plus lysine. In this strain, HD had been altered so as to become feedback-resistant at the same time, resulting in the byproduction of threonine and isoleucine. The total amount of these aspartate family amino acids was higher on molar basis than that of lysine produced by strain No. 2-190.

The following three types of lysine-producing mutants of *Brevibacterium flavum* have been reported¹⁾: mutants with aspartokinases (AK) resistant to feedback inhibition by lysine plus threonine (AK^R); threonine- or methionine-sensitive mutants with low homoserine dehydrogenase (HD) activities (HD^L); threonine auxotrophs lacking HD or homoserine kinase (HK) (HD⁻ or HK⁻). The lysine productivities of these mutants were further improved by mutational increases in their abilities to supply raw materials for lysine biosynthesis, including aspartate, pyruvate and acetyl-CoA (Fig. 1). The effects of these mutations were not the same among the above three types of lysine producers.^{2,3)} For example, a decrease in the activity of citrate synthase (CS) and resistance of phosphoenolpyruvate (PEP) carboxylase to the feedback inhibition by aspartate, which were considered

to increase the intra-cellular levels of acetyl-CoA and aspartate, enhanced the lysine productivities of AK^R mutants but not those of HD^L or HD⁻ mutants. On the other hand, pyruvate kinase (PK) deficiency, which was expected to further increase the flux to aspartate, promoted the productivities of HD^L and HD⁻ mutants. The effects on those of AK^R mutants still remained to be tested, because there was no selection marker other than resistance to *S*-(2-aminoethyl)-L-cystein (AEC), a lysine analogue, for the isolation of mutants with a PK defect or AK^R character, thus making it difficult to obtain mutants having these two characters simultaneously.

In this study, the possibilities of the following two selection methods were investigated: isolation of mutants with AK^R character by a method other than resistance to AEC; screening of PK-defective strains with a new method

Abbreviations: AEC, *S*-(2-aminoethyl)-L-cysteine; AHV, α -amino- β -hydroxyvaleric acid; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; CTAB, cetyltrimethylammonium bromide; PEP, phosphoenolpyruvate; SMH, soybean-meal hydrolysate; CS, citrate synthase; PK, pyruvate kinase; AK, aspartokinase; HD, homoserine dehydrogenase; AK^R, feedback-resistant aspartokinase.

FIG. 1. Enzymes Related to Lysine Biosynthesis in *Brevibacterium flavum*.

Abbreviations: PK, pyruvate kinase; PC, phosphoenolpyruvate carboxylase; PD, pyruvate dehydrogenase; CS, citrate synthase; AK, aspartokinase; HD, homoserine dehydrogenase; DPS, dihydrodipicolinate synthase.

developed from characteristics of PK-less mutants so far obtained.⁴⁾ As a result, the enhancing effect of PK deficiency on lysine productivities was also found in AK^R mutants.

MATERIALS AND METHODS

Bacterial strains and media. *Brevibacterium flavum* No. 2247 (ATCC 14067, wild strain) and its lysine-producing mutants, No. 22²⁾ with PK and HD defects and No. 2-190^{2,3)} with a feedback-resistant AK, both of which were derived from strain 15-8 with a low CS level and a feedback-resistant PEP carboxylase, were used. Medium T-2⁵⁾ contained 100 g of glucose, 30 g of (NH₄)₂SO₄, 1.5 g of KH₂PO₄, 0.4 g of MgSO₄·7H₂O, 10 mg of FeSO₄·7H₂O, 8 mg of MnSO₄·4H₂O, 200 µg of *d*-biotin, 300 µg of thiamine·HCl, 4 ml of Mielki (a mixture of amino acids) and 50 g of CaCO₃ (sterilized separately) in a total volume of 1 l, and the pH was adjusted to 7.2 with KOH. Medium 36⁶⁾ was composed of 100 g of glucose, 60 g of (NH₄)₂SO₄, 1 g of KH₂PO₄, 0.4 g of MgSO₄·7H₂O, 10 mg of FeSO₄·7H₂O, 8 mg of MnSO₄·4H₂O, 300 µg of *d*-biotin, 200 µg of thiamine·HCl, 35 ml of soybean-meal acid hydrolysate (SMH, 32 g total-N/l), 50 mg of L-methionine, 50 g of CaCO₃ (sterilized separately) and NaOH, to adjust the pH 7.0, per liter. Medium 7,⁷⁾ Medium 8,⁷⁾ Medium 10,⁷⁾ Medium 13,⁷⁾ Medium 33²⁾ and

Medium 41⁴⁾ were reported previously.

Chemicals. α-Amino-β-hydroxyvaleric acid (AHV) and ribose were purchased from Sigma. DL-Aspartate-β-semialdehyde was prepared by ozonolysis of DL-allylglycine by the method of Black and Wright.⁸⁾ The other reagents were as described previously.

Production of lysine. Method A: A loopful of cells grown on Medium 7 supplemented with 10 g/l of monosodium glutamate for 24 hr was inoculated into 3 ml of Medium T-2 in a test tube and then cultured at 30°C for 72 hr. The cultivation temperature was 30°C throughout this study. Method B: Two loopfuls of cells obtained as in method A were inoculated into 20 ml of Medium 36 without L-methionine in a 500-ml shaking flask and then cultured for 72 hr. Amino acids produced were detected by thin-layer chromatography using a solvent system of phenol: H₂O=7:3. Lysine in the culture broth was assayed as described previously.⁹⁾ Isoleucine was determined microbiologically using *Leuconostoc mesenteroides* ATCC 8042.

Enzyme preparation. As a culture medium for cell preparation, Medium 33 supplemented with 5 g/l of yeast extract was used for AK assay, while Medium T-2 was employed for HD measurement. The cells were cultured in 500-ml shaking flasks containing 20 ml of the above media for 40 hr and washed twice with 0.2% KCl solution. The

crude extract for HD assay was prepared by sonic disruption of the cells in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5 M KCl, followed by centrifugation at $30,000 \times g$, 0°C , for 30 min. The supernatant solution was passed through a column of Sephadex G-50 with the same buffer to eliminate low molecular weight contaminants. The crude extract for AK measurement was prepared by sonic disintegration in 0.05 M potassium phosphate buffer, pH 7.0, containing 0.01 M MgSO_4 and 0.8 M $(\text{NH}_4)_2\text{SO}_4$, followed by centrifugation. To the extract, five volumes of a saturated $(\text{NH}_4)_2\text{SO}_4$ solution was added. The precipitate that had formed at 0°C after 30 min was collected by centrifugation at $14,000 \times g$, 0°C , for 20 min. PK was assayed with a crude extract²⁾ or CTAB-treated cells,⁴⁾ both prepared as described previously from cells grown in 50 ml of Medium 8 in 500-ml shaking flasks for 24 hr.

Enzyme assay. HD was assayed as reported in the previous paper¹⁰⁾ in a reaction mixture containing 0.2 mM aspartate- β -semialdehyde (as the L-form). The methods for the determination of AK¹¹⁾ and PK¹²⁾ with crude extracts were described previously. The PK activities of CTAB-treated cells were assayed as mentioned in the preceding paper.⁴⁾

Isolation of homoserine dehydrogenase revertants. PK- and HD-defective strain No. 22 cultured in 4 ml of Medium 7 supplemented with 10 g/l of monosodium glutamate, but without agar for 15 hr, was harvested, washed twice with 0.1 M sodium phosphate buffer, pH 7.0, resuspended in the same buffer, and then treated with 300 $\mu\text{g}/\text{ml}$ of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) at 30°C for 15 min. The treated cells were washed and then spread on agar plates of Medium 10 supplemented with 300 $\mu\text{g}/\text{l}$ of *d*-biotin and 500 mg/l of L-methionine at the inoculum size of 10^4 to 10^6 cells per plate (diameter, 9 cm). After cultivation for 4 days, colonies that had appeared on the plates were picked up and then their growth was further confirmed on the agar plates described above. From the strains, mutants unable to produce lysine were selected by method A (with the exception that Medium 36 was used). The above selection methods made it possible to eliminate most of the mutants with partially reverted HDs, which were sensitive to methionine and produced lysine. Then, the HD activities of these selected strains were measured and strain KH-21, which showed HD activity as high as that of strain No. 2247, was obtained.

Derivation of mutants resistant to AHV or AHV plus lysine. Washed cells of strain KH-21 were prepared by the same method as described above except that Medium 13 supplemented with 300 $\mu\text{g}/\text{l}$ of *d*-biotin was used. They were then treated with 150 $\mu\text{g}/\text{ml}$ of NG at 30°C for 15 min, and then spread on agar plates of the same medium supplemented with AHV (5 to 20 g/l) or AHV plus lysine (each 5 g/l) at the inoculum size of 5 to 8×10^7 cells per

plate (diameter, 9 cm). Colonies that appeared during cultivation for 8 days were picked up.

Derivation of pyruvate kinase-defective mutants. The method used was basically the same as that described in the preceding paper.⁴⁾ Cells of strain No. 2-190 were treated with NG in the same way as for strain KH-21, except that the NG concentration was 400 $\mu\text{g}/\text{ml}$, and then spread on Medium 7 plates supplemented with 10 g/l of monosodium glutamate. Colonies that appeared on 3-day cultivation were replicated onto agar plates of Medium 41 supplemented with 0.1% of Casamino acids, and the same medium containing ribose instead of glucose. After cultivation for 24 hr, colonies which grew on the former but not on the latter were picked up. Among them, strains which showed pyruvate auxotrophy on the latter ribose plate were assayed for their PK activities, using CTAB-treated cells. Those without detectable PK activities were selected as PK-defective mutants, and the absence of activities was further confirmed using their crude extracts.

RESULTS

Derivation of mutants with feedback-resistant aspartokinases from a pyruvate kinase-defective strain, KH-21

Mutants with feedback-resistant AKs are selected on the basis of their resistance to AEC, a lysine analogue plus threonine.¹⁾ However, it was impossible to select such mutants from strain KH-21 by means of AEC plus threonine-resistance, because strain No. 22, the parent of strain KH-21, was derived from strain No. 1-231,³⁾ which is resistant to AEC plus threonine. Therefore, an attempt was made to select them as to the resistance to AHV, a threonine analogue plus lysine, which was the reverse combination of AEC plus threonine. As reported already with *Brevibacterium lactofermentum*,¹³⁾ AEC plus threonine-resistant strain KH-21 was still sensitive to AHV plus lysine. The mutants resistant to AHV plus lysine were cultured by method A and the amino acids produced were detected by thin-layer chromatography. As a control experiment, selection of AHV-resistant mutants, which was commonly used as a method for breeding threonine producers, was also performed, and the mutants were cultured under the same conditions. As shown in Table

TABLE I. DERIVATION OF α -AMINO- β -HYDROXYVALERIC ACID (AHV) OR AHV PLUS LYSINE RESISTANT MUTANTS FROM STRAIN KH-21

The derivation conditions were given under MATERIALS AND METHODS. Resistant strains were cultured by method A, except that the $(\text{NH}_4)_2\text{SO}_4$ concentration in the production medium was 20 g/l. Amino acids produced in the culture broth were detected by thin-layer chromatography.

Selection by resistance to	Number of mutants tested	Number of threonine producers	Number of lysine producers
AHV ^a + lysine at each 5 g/l	270	20	2 ^b
AHV at 5 to 20 g/l	2600	344	0

^a α -Amino- β -hydroxyvaleric acid.

^b Strain Nos. 422-1 and 536-4.

I, it was found that whereas the frequency of the appearance of threonine producers was almost the same between the two phenotypes, lysine producers were specifically found at a frequency of 2 out of 260 strains of AHV plus lysine-resistant mutants, but there were none among 2600 strains of AHV-resistant mutants. The two strains, Nos. 422-1 and 536-4, produced 17 and 14 g/l of lysine, respectively, with the additional production of threonine, less than 4 g/l, after 72 hr cultivation in 20 ml of Medium T-2 containing 20 g/l of $(\text{NH}_4)_2\text{SO}_4$ in 500-ml shaking flasks.

In order to clarify the mechanism of lysine production in strain Nos. 422-1 and 536-4, their AK and HD activities were assayed. As shown in Table II, both AKs and HDs of the strains became resistant to feedback inhibition by threonine plus lysine and by threonine,

TABLE II. ENZYMIC PROPERTIES OF MUTANTS RESISTANT TO α -AMINO- β -HYDROXYVALERIC ACID PLUS LYSINE

Enzyme activities were measured as described under MATERIALS AND METHODS.

Enzymatic properties	Strain			
	2247	KH-21	536-4	422-1
Aspartokinase				
Specific activity ^a	13.1	11.5	10.4	7.6
Relative activity in the presence of effectors				
None	100%	100	100	100
Lysine, 1 mM	82	71	84	76
Threonine, 1 mM	105	57	103	309
Lysine + Threonine, each 1 mM	4	9	96	277
Homoserine dehydrogenase				
Specific activity ^a	192	229	238	116
Relative activity in the presence of threonine at				
0 mM	100%	100	100	100
0.05	— ^b	67	93	100
0.1	—	15	93	105
0.5	—	3	91	94
1.0	4	3	88	101
5.0	—	—	—	66
10.0	—	—	82	35
20.0	—	—	71	11
40.0	—	—	84	—
80.0	—	—	51	—

^a nmol/min/mg protein.

^b Not determined.

TABLE III. EFFECTS OF THE SOYBEAN-MEAL HYDROLYSATE AND $(\text{NH}_4)_2\text{SO}_4$ CONCENTRATIONS ON LYSINE PRODUCTION IN STRAIN 536-4

Each strain was cultured by method B described under MATERIALS AND METHODS for 72 hr, except that the culture medium contained the indicated concentrations of soybean-meal hydrolysate (SMH) and $(\text{NH}_4)_2\text{SO}_4$. The underlines indicate the concentration of each component in the basal medium, *i.e.*, Medium 36 without L-methionine.

Strain	Culture conditions			Amino acids production		
	SMH (ml/l)	$(\text{NH}_4)_2\text{SO}_4$ (g/l)	Growth (A_{562})	Lys·HCl (g/l)	Ile (g/l)	Lys·HCl + Ile (as Lys·HCl, g/l)
536-4	25	30	0.89	30.1	3.7	35.3
		40	0.94	34.2	5.4	41.7
		50	0.92	32.8	5.2	40.0
	30	30	0.92	31.2	3.6	36.2
		40	0.93	35.4	5.2	42.6
		50	0.92	33.3	5.2	40.5
		60	0.82	27.3	4.4	33.3
	<u>35</u>	30	0.94	32.0	3.7	37.1
		40	0.92	35.2	5.2	42.4
		50	0.95	34.0	5.4	41.5
		<u>60</u>	0.87	32.3	4.8	39.0
2-190	45	100	0.99	38.0	0	38.0

respectively. Thus, it was confirmed that the lysine production by these strains was due to the resistance of AKs to concerted feedback inhibition. It was also observed that the specific activity of AK in strain No. 422-1 was decreased to a half of that of wild type strain No. 2247, but was increased by 1 mM each of threonine and lysine to a level comparable to that of strain No. 2247.

Lysine production by strain No.536-4

To determine whether or not a PK deficiency was effective for the improvement of the lysine productivities of AK^R mutants, the cultural conditions for lysine production by strain No. 536-4 with a feedback-resistant AK and a PK defect were examined using Medium 36 without L-methionine as the standard medium, and the productivities were compared with those of AK^R lysine producer No. 2-190, which had a normal PK and was derived directly from strain No. 15-8,²⁾ the original strain of mutant No. 536-4. Strain No. 422-1 was not employed because of its poor growth

in the standard medium. As shown in Table III, strain No. 536-4 produced 35.4 g/l of lysine, at maximum, in the medium containing lower concentrations of SMH (30 ml/l) and $(\text{NH}_4)_2\text{SO}_4$ (40 g/l) than those in the standard medium (SMH 35 ml/l, $(\text{NH}_4)_2\text{SO}_4$ 60 g/l). This productivity was not more than that of strain No. 2-190, 38 g/l, under the optimum conditions (SMH 45 ml/l, $(\text{NH}_4)_2\text{SO}_4$ 100 g/l, see Table IV). However, as mentioned above, No. 536-4 had a feedback-resistant HD, and thus produced 5.2 g/l of isoleucine in addition to lysine. Here, we have estimated the total production of lysine and isoleucine as that of lysine, under the assumption that aspartate- β -semialdehyde, a common intermediate of lysine and isoleucine biosynthesis, was exclusively converted to lysine. As shown in Table III, the estimated lysine productivity of strain No. 536-4 was 42.6 g/l under the optimum conditions, *i.e.*, higher than that of strain No. 2-190. These results suggest that a PK deficiency might be effective for the improvement of the lysine productivities of AK^R

TABLE IV. DERIVATION OF PYRUVATE KINASE-DEFECTIVE MUTANTS FROM STRAIN NO. 2-190

PK-Defective mutants were derived as described under MATERIALS AND METHODS. The growth response was tested by culturing the parent and mutant strains for 24 hr on Medium 41 supplemented with 0.1% of Casamino acids, and on the same medium containing ribose or ribose plus sodium pyruvate (5 g/l) instead of glucose. PK activities were assayed using CTAB-treated cells and expressed relative to that of the parent strain. Lysine production was conducted by method B given under MATERIALS AND METHODS, except that the culture medium contained 70 g/l of $(\text{NH}_4)_2\text{SO}_4$.

Strain	Growth			Pyruvate kinase activity (%)	Lys·HCl production (g/l)
	Glucose	Ribose	Ribose + pyruvate		
2-190	+++	+++	++	100	28.9
18	+++	—	+	0	37.8
21	++	±	++	128	— ^a
22	+++	—	+	0	35.9
48	+++	±	++	119	—
98	+++	±	++	103	—
105	+++	—	+	122	—
117	++	—	+	106	—
128	+++	±	++	100	—
166	++	±	++	32	15.1
168	++	—	+	133	—
174	+++	±	++	96	—
202	++	—	+	5	31.5
251	++	—	+	100	—
275	++	±	+	169	—

^a Not determined.

type mutants. However, in order to confirm more clearly the effect of a PK defect on lysine productivity, it seemed necessary to derive AK^R type lysine producers with a normal HD but a PK defect.

Derivation of pyruvate kinase-defective mutants from strain No. 2-190 with feedback-resistant aspartokinase

For the above purpose, PK-defective mutants were derived from the AK^R type lysine producer, No. 2-190, and selected by a new screening method,⁴⁾ i.e., the inability to grow on ribose in the absence of pyruvate. From among 20000 NG-treated cells, 14 strains which showed no or only poor growth and pyruvate auxotrophy on ribose were selected, and their PK activities were assayed using CTAB-treated cells. Three PK-defective mutants (KL-18, 22 and 202) and one mutant (KL-166) with low PK activity were found and

examined as to their lysine productivities. As shown in Table IV, all the three defective mutants showed higher productivity than that of the parent strain, 28.9 g/l. The two best strains, KL-18 and KL-22, had low but detectable levels of PK activity, 2 to 4% of that of the parent strain, No. 2-190, when assayed using crude extracts, as shown in Table V. Furthermore, no significant changes were observed both in the specific activities of AK and HD and in the feedback inhibition of AK between strain KL-18 and its parent strain, No. 2-190. These results indicate that the improvement of lysine productivity was due to, the PK deficiency. It was also found that the specific AK activity of strain No. 2-190 had been decreased to one tenth of that of the original wild strain, No. 2247.

Lysine production by strain KL-18

Table VI shows the effects of the SMH and

TABLE V. ENZYMATIC PROPERTIES OF STRAINS KL-18 AND KL-22

The experimental conditions were given under MATERIALS AND METHODS.

Strain	Pyruvate kinase activity ^a	Aspartokinase		Homoserine dehydrogenase activity ^a
		Activity ^a	Inhibition ^b	
2247 (wild)	1243	13.1	96.0	192
2-190 (parent)	1312	1.3	29.7	175
KL-18	31	1.4	23.1	205
KL-22	56	— ^c	—	—

^a nmol/min/mg protein.

^b % Inhibition by 1 mM L-lysine plus L-threonine.

^c Not tested.

TABLE VI. EFFECTS OF THE SOYBEAN-MEAL HYDROLYSATE AND (NH₄)₂SO₄ CONCENTRATIONS ON THE LYSINE PRODUCTION IN STRAINS 2-190 AND KL-18

Each strain was cultured for 72 hr as in Table III except that the culture medium contained the indicated concentrations of soybean-meal hydrolysate (SMH) and (NH₄)₂SO₄. The underlines show the concentrations in Table III.

Culture conditions		2-190		KL-18	
SMH (ml/l)	(NH ₄) ₂ SO ₄ (g/l)	Growth (<i>A</i> ₅₆₂)	Lysine · HCl (g/l)	Growth (<i>A</i> ₅₆₂)	Lysine · HCl (g/l)
35	70	1.16	25.9	1.08	35.6
	80	1.06	32.0	1.08	38.2
	90	1.00	35.6	0.93	39.2
	100	0.96	37.8	0.88	39.0
40	70	1.16	26.2	1.06	35.6
	80	1.08	31.3	1.01	38.1
	90	1.02	36.1	0.92	39.2
	100	0.97	36.1	0.88	39.9
	110	0.76	24.0	— ^a	—
<u>45</u>	70	—	—	1.07	37.8
	80	—	—	0.98	39.7
	90	1.03	35.6	0.94	40.9
	<u>100</u>	0.99	38.0	0.88	42.7
	<u>110</u>	0.80	25.1	0.74	26.2
50	90	1.02	36.6	0.98	38.3
	100	0.98	36.2	0.94	41.2
	110	0.79	25.2	0.82	33.6

^a Not done.

(NH₄)₂SO₄ concentrations on the lysine production by strain KL-18. Strain KL-22 was not used because of the decrease in lysine productivity in the later experiments. The maximum amount of lysine produced, 42.7 g/l, was obtained with 45 ml/l of SMH and 100 g/l

of (NH₄)₂SO₄. This yield was higher than that of the parent strain, No. 2-190, 38 g/l, at maximum, obtained under the same conditions. Moreover, strain KL-18 showed higher productivity than the parent strain under all the conditions tested. These results confirmed that

the PK deficiency was effective for the improvement of the lysine productivities of AK^R mutants.

In strain KL-18, the optimum concentration of $(\text{NH}_4)_2\text{SO}_4$ for lysine production was markedly different from that in the case of strain No. 536-4. In both strains, higher concentrations of $(\text{NH}_4)_2\text{SO}_4$ were favorable for lysine production. However, severe growth inhibition occurred under the conditions of excess $(\text{NH}_4)_2\text{SO}_4$, resulting in decreases in the lysine yields. In strain KL-18, the maximum amount of lysine was obtained when the $(\text{NH}_4)_2\text{SO}_4$ concentration was high enough to cause slight growth inhibition. Thus, the difference in the optimum $(\text{NH}_4)_2\text{SO}_4$ concentration for the two strains seemed to be due to the difference in the degree of resistance to $(\text{NH}_4)_2\text{SO}_4$.

DISCUSSION

In order to test the effects of a PK deficiency on the lysine productivities of AK^R type producers, mutants with both feedback-resistant AKs and a PK defect were derived by two new methods.

As reported in the previous paper,⁶⁾ PK-defective mutants had already acquired resistance to AEC, a lysine analogue plus threonine. Thus, it was impossible to isolate mutants with AKs resistant to feedback inhibition by lysine plus threonine from the above PK-defective strains by means of the conventional selection marker of resistance to AEC plus threonine. Tosaka *et al.*¹³⁾ reported that a strain of *Brevibacterium lactofermentum* resistant to AEC plus threonine was still sensitive to AHV, a threonine analogue plus lysine, and that the AK of the lysine-producing resistant strain thus obtained became insensitive to the feedback inhibition by lysine alone, but no change was seen in the sensitivity to the concerned feedback inhibition by lysine plus threonine. In accordance with these findings, strain KH-21, a mutant of *Brevibacterium flavum* with a PK defect and AEC plus threonine resistance, was sensitive to AHV

plus lysine. However, unlike the results of Tosaka *et al.*, the AKs of the lysine-producing resistant mutants obtained, strain Nos. 536-4 and 422-1, were normally sensitive to the feedback inhibition by lysine alone but altered so as to be insensitive to the feedback inhibition by threonine and that by lysine plus threonine. These properties of AKs were the same as those of the AK of the AEC plus threonine-resistant lysine producer derived from the wild strain of *B. flavum* No. 2247.¹⁾ Thus, in spite of the results of Tosaka *et al.*, we obtained mutants for the purpose of this study, that is, with both feedback-resistant AKs and a PK defect. However, these mutants had acquired another character which was unfavorable for lysine production, *i.e.*, resistance of HD to feedback inhibition by threonine. A HD of this type converts considerable amounts of aspartate- β -semi-aldehyde, a common intermediate for aspartate family amino acids biosynthesis, to threonine or isoleucine, resulting in a decrease in the lysine productivity. The above character, which was in common with AHV-resistant threonine or isoleucine producers, would naturally be expected from the selection method used for mutants, *i.e.*, resistance to AHV plus lysine.

Next, to obtain AK^R type lysine producers with a PK defect but without the above unfavorable character for lysine production, we tried to isolate PK-defective mutants from AK^R type strain No. 2-190. Since the parent strain No. 2-190 had resistance to AEC plus threonine, the conventional selection method, involving resistance to AEC plus threonine, was not applicable in this case to obtain PK-defective mutants. Thus, to solve the problem, some properties of the PK-defective strains so far obtained were studied. Among them, the inability to grow on ribose was confirmed as a possible selection marker for the isolation of mutants with a PK defect. The details were published in a separate paper.⁴⁾ The results described in this paper were the first application of this new method to the breeding of amino acid producers. All the three PK-

defective mutants obtained produced more lysine than the parent strain, No. 2-190. In particular, strain KL-18 showed better productivity than that of the parent strain under all cultural conditions tested, with the maximum accumulation of 43 g/l (yield, 43%).

From these results, it will be concluded that the lysine productivities of AK^R mutants can be improved by endowing them with a PK deficiency.

In addition to the above conclusion, through investigation of the cultural conditions, the lysine productivity of parent strain No. 2-190 was increased to 38 g/l (yield, 38%) in an industrially applicable medium. This yield was higher than that obtained with HD-defective lysine producer H-3-4, derived from the same parent strain, No. 15-8.^{2,3)}

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REFERENCES

- 1) I. Shiio, *J. Ferment. Technol.*, **52**, 62 (1974).
- 2) H. Ozaki and I. Shiio, *Agric. Biol. Chem.*, **47**, 1569 (1983).
- 3) I. Shiio, H. Ozaki and K. Ujigawa-Takeda, *Agric. Biol. Chem.*, **46**, 101 (1982).
- 4) M. Mori and I. Shiio, *Agric. Biol. Chem.*, **51**, 129 (1987).
- 5) I. Shiio and S. Nakamori, *Agric. Biol. Chem.*, **34**, 448 (1970).
- 6) I. Shiio, S. Sugimoto and Y. Toride, *Agric. Biol. Chem.*, **48**, 1551 (1984).
- 7) I. Shiio and K. Ujigawa, *J. Biochem.*, **84**, 647 (1978).
- 8) S. Black and N. Wright, *J. Biol. Chem.*, **213**, 39 (1955).
- 9) F. O. Chinard, *J. Biol. Chem.*, **199**, 91 (1952).
- 10) R. Miyajima and I. Shiio, *J. Biochem.*, **68**, 311 (1970).
- 11) I. Shiio and R. Miyajima, *J. Biochem.*, **65**, 894 (1969).
- 12) H. Ozaki and I. Shiio, *J. Biochem.*, **66**, 297 (1969).
- 13) O. Tosaka, K. Takinami and Y. Hirose, *Agric. Biol. Chem.*, **42**, 745 (1978).