L-Threonine Production by a Mutant of *Arthrobacter paraffineus*[†]

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An isoleucine leaky auxotroph of *Arthrobacter paraffineus*, which was isolated by Takayama *et al.*⁽³⁾ as a mutant producing L-threonine and L-valine from *n*-paraffin, was subjected to further mutagenesis in an attempt to obtain better L-threonine producers. Some of the double auxotrophs derived from the isoleucine auxotroph and some of their revertants with respect to isoleucine requirement produced more L-threonine than the original isoleucine auxotroph. In contrast to the original isoleucine auxotroph, a revertant derived from a methionine plus isoleucine double auxotroph, KY7135, produced an increased amount of L-threonine and a decreased amount of L-valine. The optimum level of L-methionine for L-threonine production in KY7135 was much higher (1000~2000 μ g/ml) with *n*-paraffin medium than with sorbitol or mannitol medium (10~50 μ g/ml). L-Threonine production reached a maximum level (11.5 mg/ml) in 7 days incubation with the medium containing 10% *n*-paraffin (C_{12} ~ C_{14} rich). Several mutants which produce L-threonine more than 12 mg/ml were obtained from KY 7135 by monoclonal isolation procedure.

L-Threonine production from hydrocarbon has recently been reported by Takayama *et al.*⁽³⁾ in our laboratory. They found that an isoleucine leaky auxotroph, KY7104, derived from *Arthrobacter paraffineus*, a hydrocarbon utilizer, produced a large amount of both L-threonine and L-valine from *n*-paraffin at the same time. In a favorable condition, the maximum amount of L-threonine and L-valine production was about 9 mg/ml each.

In the course of the studies on L-threonine fermentation using microbial mutants, KY7104 was subjected to further mutagenesis in an attempt to obtain better L-threonine producers from hydrocarbon. Thus, many double auxotrophs and their revertants with respect to isoleucine requirement were derived and screened for their ability to produce an increased amount of L-threonine. In the present paper, the results obtained with one of the revertants which produced more L-threonine but much less L-valine than the original strain are described.

MATERIALS AND METHODS

Microorganisms. *Arthrobacter paraffineus* KY7104 (Ile⁻/aAB⁻/aKB⁻/Thr⁻/Hom⁻) isolated by Takayama *et al.*⁽³⁾ was employed as a starting strain.

Culture medium. Minimal and complete medium of the following compositions were used for mutational experiments. Minimal medium; 10 g fructose, 1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 5 mg thiamine and 1 ml of trace element solution in a liter of distilled water (pH 7.2). Composition of the trace element solution has been previously described.⁽²⁾ Complete medium; 10 g peptone, 10 g meat extract, 5 g yeast extract and 3 g NaCl in a liter of water (pH 7.2). Seed medium contained 5% sorbitol, 1% meat extract, 1% peptone, 0.5% yeast extract and 0.3% NaCl (pH 7.4). The composition of the fermentation medium is described in the legend of each Table and Figure.

Mutation. Mutations were induced by treatment of the bacterial cells with ultraviolet light or N-methyl-N'-nitro-N-nitrosoguanidine (NTG), as previously described.^(1,2)

For the selection of double auxotrophs, NTG treated cells were tested by replica method, and those which grew well on the minimal medium supplemented with L-isoleucine, L-methionine, L-lysine and α , ϵ -diaminopimelic acid, but hardly on the minimal medium supplemented with L-isoleucine were selected.

[†] Studies on L-Threonine Fermentation Part III, see Reference 1, 2).

Culture method. Thirty milliliters of a seed

medium in a 250 ml Erlenmeyer flask was inoculated with one loopful of an organism grown on a bouillon agar slant, and incubated on a rotary shaker operated at 220 rpm. After incubation for 24 hr at 30°C, 1 ml of the seed culture was transferred into a 250 ml Erlenmeyer flask containing 10 ml of a fermentation medium. The flask was incubated at 30°C on a rotary shaker for 4~10 days.

Analysis. L-Threonine and L-valine were estimated by colorimetry with ninhydrin after paperchromatographical separation. The paperchromatography was performed on "Toyo Roshi No. 50" paper by use of the following solvent system; methylethylketone—*n*-butanol—conc. ammonia—water (100 : 100 : 5 : 60 v/v) or *n*-butanol—acetic acid—water (4 : 1 : 2 v/v).

RESULTS

1. Isolation of mutant

Figure 1 shows the genealogy of *Arthrobacter paraffineus* KY7135 which was used to study on some cultural conditions for L-threonine production. Some of the double auxotrophs including lysine or α,ϵ -diaminopimelic acid plus isoleucine and methionine plus isoleucine auxotrophs, and some of their revertants with respect to isoleucine requirement were found to produce more L-threonine than the original strain. For example, KY7135, a revertant derived from an isoleucine plus methionine auxotroph KY7137, produced more L-threonine than the original strain KY7104. In contrast to KY7104 or KY7137, the amount of L-valine produced by some revertants including KY7135 was much reduced.

2. Cultural conditions for L-threonine production with KY7135

a) *Effect of carbon source and L-methionine concentration on L-threonine production.* Sorbitol, mannitol and *n*-paraffin were compared with respect to L-threonine production at various levels of L-methionine (Table I). *n*-Paraffin gave much higher yield than sorbitol or mannitol. With mannitol or sorbitol, the optimal level of L-methionine for L-threonine production was 10~50 $\mu\text{g/ml}$, and higher levels of L-methionine (above

FIG. 1. Genealogy of *Arthrobacter paraffineus* KY 7135.

* see Reference 3)

** α,ϵ -diaminopimelic acid

FIG. 2. Effect of L-Methionine on L-Threonine Production in *A. paraffineus* KY 7135.

Basal medium; *n*-paraffin 10%, $(\text{NH}_4)_2\text{SO}_4$ 2%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, trace element solution 1 ml/liter thiamine 1 $\mu\text{g/ml}$, CaCO_3 2% (pH 7.4).

Incubation time; 8 days.

100 $\mu\text{g/ml}$) suppressed the production of L-threonine. In contrast, high level (above 200 $\mu\text{g/ml}$) of L-methionine was favorable for L-threonine production in the case of *n*-paraffin. Figure 2 shows the effect of high level of L-methionine on L-threonine production in 8 days incubation with *n*-paraffin. As L-methionine level increased, L-threonine yield increased steadily to a maximum (at 1000~2000 $\mu\text{g/ml}$ of L-methionine) and then slowly decreased.

D-Isomer of methionine was proved to be as effective as L-isomer on L-threonine production (Table II).

b) *Effect of incubation time on L-threonine production.* As Fig. 3 shows, L-threonine concentration slowly increased and reached to a maximum level in about 8 days. Slight

TABLE I. EFFECT OF CARBON SOURCE AND L-METHIONINE ON L-THREONINE PRODUCTION IN *A. paraffineus* KY7135

Incubation time; 98 hr

Basal medium; $(\text{NH}_4)_2\text{SO}_4$ 2%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, trace element solution 1 ml/liter, thiamine 1 $\mu\text{g/ml}$, CaCO_3 2% (pH 7.4).

Carbon source	%	Concn. of L-methionine $\mu\text{g/ml}$	pH	Growth (OD* 1/10)	L-Threonine found mg/ml
Mannitol	5	0	6.7	0.359	±
		10	6.6	0.320	0.5 (±)
		50	6.6	0.428	±
		100	6.4	0.458	±
		200	6.5	0.459	±
Sorbitol	5	0	5.9	0.745	0.6 (± ~ +)
		10	5.8	0.890	± ~ +
		50	5.9	0.650	± ~ +
		100	6.4	0.720	±
		200	6.8	0.725	±
<i>n</i> -Paraffin	5	50	5.7	++	3.8
		100	6.1	++	4.4
		200	6.0	++	5.0
		500	6.1	++	5.0
	10	50	6.1	++	1.9
		100	6.2	++	2.7
		200	6.2	++	4.0
		500	6.0	++	3.4

* Optical density at 660 $m\mu$ TABLE II. EFFECT OF METHIONINE ISOMERS ON L-THREONINE PRODUCTION IN *A. paraffineus* KY7135

Incubation time; 7 days

Basal medium; *n*-paraffin 10%, $(\text{NH}_4)_2\text{SO}_4$ 2%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, trace element solution 1 ml/liter, thiamine 2 $\mu\text{g/ml}$, CaCO_3 2%.

Methionine isomers ^{a)}	pH	Growth	L-Threonine found mg/ml
L-isomer	6.8	+++	7.5
D-isomer	6.7	+++	7.6
DL-isomer	6.1	+++	7.2
— ^{b)}	7.6	±	3.4

^{a)} The concentration of methionine isomers was each 2 mg/ml.^{b)} Control, no methionine was added to the fermentation medium.

degradation of L-threonine was noted in 10 days incubation.

c) *Effect of thiamine concentration on L-threonine production.* KY7135 requires thiamine for growth. Effect of thiamine FIG. 3. Effect of Incubation Time on L-Threonine Production in *A. paraffineus* KY 7135.

The medium described in the footnote of Fig. 2 was supplemented with 2 mg/ml of DL-methionine.

concentration on L-threonine production was investigated. The result is presented in Fig. 4. Unexpectedly, L-threonine production was slightly affected by thiamine level. Even if thiamine was not added to the fermentation medium, the amount of L-threonine production

FIG. 4. Effect of Thiamine on L-Threonine Production in *A. paraffineus* KY 7135.

Basal medium; *n*-paraffin 10%, $(\text{NH}_4)_2\text{SO}_4$ 2%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, trace element solution 1 ml/liter, CaCO_3 2% (pH 7.4). Incubation time; 7 days.

DL-Methionine concentration; ●—●, 100 µg/ml; ▲—▲, 500 µg/ml; ○—○, 2000 µg/ml.

was comparable to that in high level of thiamine.

d) *Effect of ammonium sulfate on L-threonine production.* Effect of ammonium sulfate concentration on L-threonine production was tested. The optimal level of ammonium sulfate was 2%. Higher concentration of it decreased L-threonine production (Fig. 5).

e) *Effect of inorganic salts on L-threonine*

FIG. 5. Effect of Ammonium Sulfate on L-Threonine Production in *A. paraffineus* KY 7135.

Basal medium; *n*-paraffin 10%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, trace element solution 1 ml/liter, thiamine 1 µg/ml, DL-methionine 500 µg/ml (pH 7.4). Incubation time; 7 days

TABLE III. EFFECT OF INORGANIC SALTS ON L-THREONINE PRODUCTION IN *A. paraffineus* KY7135

Incubation time; 7 days
Basal medium; *n*-paraffin 10%, $(\text{NH}_4)_2\text{SO}_4$ 2%, trace element solution 1 ml/liter, thiamine 1 µg/ml, DL-methionine 500 µg/ml, CaCO_3 2% (pH 7.4).

KH_2PO_4 and K_2HPO_4 % ^{a)}	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ %	L-Threonine found mg/ml
0.05	0.05	7.7
	0.1	8.2
	0.2	7.5
	0.4	6.8
0.1	0.05	9.2
	0.1	10.0
	0.2	7.9
	0.4	6.7
0.2	0.05	11.5
	0.1	8.6
	0.2	8.3
	0.4	6.1
0.4	0.05	11.4
	0.1	10.0
	0.2	7.9
	0.4	7.1

^{a)} KH_2PO_4 and K_2HPO_4 were used in 1:1 ratio on weight basis. The concentration in the table represents the level of each phosphate.

production. Effect of the concentration of phosphate and magnesium ion on L-threonine production was tested (Table III). Lower concentrations of magnesium sulfate (0.025~0.05%) were favorable for L-threonine production. The proper ranges of the concentrations of dipotassium hydrogen phosphate and potassium dihydrogen phosphate were 0.2~0.4% each. The amount of L-threonine production at the optimal concentrations of the inorganic salts was 11.5 mg/ml.

f) *Effect of amino acids, vitamins and precursors on L-threonine production.* The effect of 23 natural amino acids including glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-serine, L-glutamic acid, L-aspartic acid, L-proline, L-ornithine, L-arginine, L-tryptophan, L-tyrosine, L-phenylalanine, L-cystine, L-cysteine, L-homoserine, DL- α -amino-butyric acid, L-lysine, L-histidine, L-glutamine,

TABLE IV. EFFECT OF AMINO ACIDS AND FUMARIC ACID ON L-THREONINE PRODUCTION IN *A. paraffineus* KY7135

Incubation time; 7 days
 Basal medium; *n*-paraffin 10%, (NH₄)₂SO₄ 4%, KH₂PO₄ 0.2%, K₂HPO₄ 0.2%, MgSO₄·7H₂O 0.05%, trace element solution 1 ml/liter, thiamine 2 µg/ml, DL-methionine 2 mg/ml, CaCO₃ 2% (pH 7.4).

Amino acid or precursor	mg/ml	L-Threonine found mg/ml
Val	0.2	3.5
	1.0	6.3
	10.0	6.7
	20.0	9.0
Leu	0.2	5.4
	1.0	4.2
	10.0	5.9
	20.0	6.2
Asp	0.2	5.6
	1.0	4.2
	10.0	7.5
	20.0	8.1
Glu	0.2	3.8
	1.0	3.3
	10.0	7.2
	20.0	7.5
Homoserine	0.2	5.6
	1.0	5.5
	5.0	6.2
	10.0	6.6
Fumaric acid	10.0	7.0
	20.0	8.8
Control	—	5.9

L-asparagine and α,ϵ -diaminopimelic acid on L-threonine production was investigated. A part of the results is presented in Table IV. Addition of L-valine or L-aspartic acid at high level (1~2%) enhanced L-threonine production to some extent. High concentration of L-leucine stimulated the growth, whereas its effect on L-threonine production was slight. Addition of L-homoserine, a threonine precursor, gave no prominent effect on L-threonine production, whereas addition of it at high level reduced the bacterial growth.

Addition of fumaric acid stimulated L-threonine production to some extent (Table IV).

With addition of riboflavine, or biotin,

TABLE V. EFFECT OF VITAMINES ON L-THREONINE PRODUCTION IN *A. paraffineus* KY7135

The medium described in the legend of Table IV was used for the basal medium.

Incubation time; 7 days

Vitamines	µg/ml	L-Threonine found mg/ml
Riboflavine	0.4	0.3
	4.0	2.7
Biotin	0.4	4.7
	4.0	0.6
Control	—	6.0

TABLE VI. EFFECT OF NATURAL NUTRIENTS ON L-THREONINE PRODUCTION IN *A. paraffineus* KY7135

The medium described in the legend of Table IV was used for the basal medium.

Incubation time: 7 days

Natural nutrients	%	L-Threonine found mg/ml
Yeast extract	0.05	8.3
	0.1	9.6
	0.25	9.3
	0.5	7.9
Peptone	0.05	5.4
	0.1	8.8
	0.25	7.6
	0.5	5.9
	0.05	7.7
Corn steep liquor	0.1	6.2
	0.25	6.6
	0.5	4.8
Meat extract	0.05	7.3
	0.1	7.3
	0.25	8.1
	0.5	6.5
Control	—	5.3

both L-threonine production and the growth were repressed (Table V). The other vitamins including *p*-aminobenzoic acid, pyridoxine, pantothenic acid, folic acid, cyanocobalamine, inositol and nicotinic acid (0.4 and 4 µg/ml) showed slight effect at best.

g) *Effect of various natural nutrients on L-threonine production.* Yeast extract, peptone, meat extract and corn steep liquor were tested for their effect on L-threonine

production. As shown in Table VI, all of them showed stimulatory effects on L-threonine production. Yeast extract was proved to be the most favorable nutrient for L-threonine production.

h) Effect of various sugars, organic acid and alcohols on L-threonine production. The effect of various sugars, organic acids and alcohols on L-threonine production was studied in attempts not only to increase L-threonine production but also to shorten the fermentation time. Results with the compounds effective on L-threonine production are summarized in Tables VII and VIII. Among monocarboxylic acids, including myristic, lauric, capric, pelargonic, enanthic, caprylic, caproic, palmitic, stearic and linoleic acids, myristic acid (C₁₄), *n*-capric acid (C₁₀), pelargonic acid (C₉) and enanthic acid (C₇) showed stimulatory effect on L-threonine production (Table VII). As for dicarboxylic acids including succinic, glutaric, adipic, pimelic, suberic, azelaic and sebacic acids addition of sebacic acid or glutaric acid at low level (60~125 µg/ml) enhanced

TABLE VII. EFFECT OF ORGANIC ACIDS ON L-THREONINE PRODUCTION IN *A. paraffineus* KY7135

Incubation time; 7 days

Basal medium; *n*-paraffine 10%, (NH₄)₂SO₄ 2%, KH₂PO₄ 0.2%, K₂HPO₄ 0.2%, MgSO₄·7H₂O 0.05%, trace element solution 1 ml/liter, thiamine 2 µg/ml, DL-methionine 2 mg/ml, CaCO₃ 2% (pH 7.4).

Organic acids	%	L-Threonine found mg/ml
Myristic acid	0.006	10.4
	0.012	10.0
<i>n</i> -Capric acid	0.006	10.6
	0.012	9.4
Pelargonic acid	0.006	11.5
	0.012	6.9
Enanthic acid	0.006	10.4
	0.012	8.3
<i>n</i> -Caproic acid	0.006	6.9
	0.012	4.3
Palmitic acid	0.006	4.8
	0.012	7.9
Control	—	9.2

TABLE VIII. EFFECT OF DICARBOXYLIC ACIDS ON L-THREONINE PRODUCTION IN *A. paraffineus* KY7135

The medium described in the legend of Table VII was used for the basal medium.

Incubation time; 7 days

Dicarboxylic acids	%	L-Threonine found mg/ml
Succinic acid	0.006	6.0
	0.012	8.4
Glutaric acid	0.006	8.6
	0.012	9.1
Suberic acid	0.006	5.5
	0.012	5.4
Sebacic acid	0.006	9.8
	0.013	7.6
	0.025	6.2
	0.05	6.0
Azelaic acid	0.006	5.3
	0.012	7.9
Control	—	7.2

TABLE IX. L-THREONINE PRODUCTION BY MONOCOLONY ISOLATES OBTAINED FROM *A. paraffineus* KY7135

The medium described in the legend of Table VII was used.

Incubation time; 7 days

Isolate No.	L-Threonine found mg/ml	L-Valine found
42-7B-7	14.5	± ~ +
42-7B-13	14.0	± ~ +
42-7B-19	12.6	± ~ ±
42-7C-2	12.5	± ~ +
42-7C-8	13.0	trace
KY7135	9.8	± ~ +

L-threonine production (Table VIII). Addition of sugars (glucose, mannitol, sorbitol, or fructose at 1 or 2%) was found to decrease L-threonine production.

An attempt to shorten the fermentation time has not been successful so far.

i) Monocolony isolates from KY7135. Fifty isolates were obtained from KY7135 by monocolony procedure. Among them, five isolates produced above 12 mg/ml of L-threonine with the medium containing 10% *n*-

paraffin and 2000 $\mu\text{g/ml}$ of DL-methionine (Table IX).

DISCUSSION

An isoleucine leaky auxotroph of *Arthrobacter paraffineus*, which produced a large amount of both L-threonine and L-valine from *n*-paraffin at the same time, was mutagenized to obtain better L-threonine producers. In a previous paper,¹⁾ it was found that some double auxotrophs increased L-threonine productivity as compared with the parent single auxotroph and some isoleucine revertants of triple-less auxotrophs of *Escherichia coli* produced more L-threonine than the parent strains. In view of these findings, many double auxotrophs and its revertants with respect to isoleucine requirement were derived from the mutant of *A. paraffineus*. It has now been found that some of the double auxotrophs and the revertants increased in threonine productivity also in *A. paraffineus*. Threonine producing mutants described here may be regulatory mutants as discussed by Takayama *et al.*³⁾ although evidence supporting this speculation awaits to be obtained. Double auxotrophs might be able to release from the control by corresponding end products and favorable for L-threonine production. The cause of the increase of L-threonine production in the revertants has not been clarified so far.

The amount of L-valine produced in some isoleucine revertants was largely decreased.

Takayama *et al.*³⁾ reported that excessive addition of L-isoleucine in the medium inhibited the production of L-valine in the isoleucine auxotroph of *A. paraffineus*. Hence, it is assumed that the decrease of L-valine production in isoleucine revertants may be due to the suppression of L-valine biosynthesis by L-isoleucine readily synthesized in the revertants.

With carbohydrate medium such as mannitol or sorbitol, higher levels of L-methionine (above 100 $\mu\text{g/ml}$) suppressed L-threonine production, whereas with *n*-paraffin medium high levels (1000~2000 $\mu\text{g/ml}$) of it were favorable for L-threonine production (Table I). Similar phenomena were also reported in L-ornithine,⁴⁾ L-citrulline⁵⁾ or L-homoserine⁶⁾ production in hydrocarbon utilizer. Presumably, the difference in the level of requirement between carbohydrate medium and *n*-paraffin medium was due to the difference in the uptake rate of the requiring amino acids.

REFERENCES

- 1) H. Kase, H. Tanaka and K. Nakayama, *Agr. Biol. Chem.*, **35**, 2089 (1971).
- 2) H. Kase and K. Nakayama, *ibid.*, **36**, 1611 (1972).
- 3) K. Takayama, S. Abe and S. Kinoshita, *Amino Acid and Nucleic Acid*, No. **19**, 121 (1969).
- 4) K. Tanaka, K. Oshima, Y. Tokoro and S. Kinoshita, *ibid.*, No. **18**, 57 (1968).
- 5) Y. Tokoro, K. Oshima, K. Tanaka and S. Kinoshita, *ibid.*, No. **19**, 115 (1969).
- 6) K. Oshima, Y. Tokoro, S. Okii, K. Tanaka and S. Kinoshita, *ibid.*, No. **20**, 1 (1969).