STUDIES ON LYSINE FERMENTATION I. THE CONTROL MECHANISM ON LYSINE ACCUMULATION BY HOMOSERINE AND THREONINE

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A preliminary report on L-lysine production using microbial auxotroph was previously published by the present authors (1). Lysine production by this method is now going on both in this country and in U.S.A. The finding of lysine accumulation by microbial auxotrophs was reported for the first time, though accumulation of metabolic precursor or related substance by microbial auxotroph had already been well recognized along with recent progress of microbial genetics. In this paper, the lysine accumulation by various auxotrophs derived from various microorganisms is shown. Studies embodying high lysine producer, homoserine-less mutant of glutamic acid producing bacterium *Micrococcus glutamicus* (2) and especially the influence of growth factor concentration on lysine accumulation and specific inhibition of lysine accumulation by homoserine or threonine by intact cell are also described.

METHODS

Microorganisms: Auxotrophs of various types were obtained (3) both by UV-light or Co⁶⁰ irradiation and by penicillin method employing glutamic acid producing bacteria, *Micrococcus glutamicus* (2), *Escherichia coli*, and *Bacillus subtilis*. *E. coli* 177-184 (homoserine-less) were kindly supplied by the laboratory of Dr. B. D. DAVIS. *B. subtilis* 186x. (methionine and threonine-less), 190 (homoserine-less) were kindly supplied by Dr. P. R. BURKHOLDER. *Micrococcus glutamicus*, the glutamic acid producer isolated in our laboratories and used in this experiment was initially recognized as *Micrococcus* but later found to be a new group of bacteria which can be called "glutamic acid bacteria".

Incubation: The seed medium is composed of 2.0% glucose, 1.0% peptone, 0.5% meat extract, 0.3% sodium chloride, 10 µg/L biotin, pH 7.2. Ten per cent of the seed grown on the medium at 28°C for 24 hours with shaking was used as the inoculum to the fermentation medium. Sterilization

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conditions of the media used were 1 atm 10 minutes with a synthetic medium and 1 atm 15 minutes with other media. Fermentation was conducted at 28°C in a 250 ml Erlenmeyer flask in which 30 ml of the medium was dispensed. The flasks were shaken for 3-4 days on a rotary shaker operated at 220 r.p.m. Growth of cells for washed cell suspension experiments was accomplished by shaking a 2 L-flask containing 500 ml medium (composition is shown in footnote of Table 6) for 24 hours on a rotary shaker operated at 150 r.p.m. The composition of the fermentation medium was shown in the footnote of Tables 1 and 2. Basal medium of the following composition was used for testing the effect of nitrilite concentration on metabolite accumulation: 10% glucose, 2.0% $(\text{NH}_4)_2\text{SO}_4$, 0.05% K_2HPO_4 , 0.05% KH_2PO_4 , 0.025% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0% CaCO_3 , pH 7.2. Twenty micrograms of biotin per liter were added to this basal medium in experiment shown in Table 3.

Analysis: L-Lysine was estimated by manometric method using lysine decarboxylase (5) and then verified by paperchromatography. Glutamic acid was estimated by manometric method using a glutamic acid decarboxylase of *Escherichia coli* C-6. A method described by BARKER and SUMMERSON (6) was used for lactic acid assay. Cell amount was shown in terms of dry weight by use of a previously prepared relation curve between dry cell weight and optical density. An aliquot of culture broth was centrifuged and cells were resuspended in distilled water. Residual calcium carbonate was dissolved in a proper amount of dilute hydrochloric acid where necessary. After proper dilution, the optical density at 650 m μ was taken using Beckman model DU spectrophotometer.

Experiment using washed cell suspension: Cells were collected by centrifuge from the culture broth described above, washed 2 times with saline water, and suspended in M/15 phosphate buffer (pH 7.5). The composition of reaction mixture was shown in the footnote of Table 6. In the case shown in Figs. 1-3, experiment was accomplished in 4-fold scale but 400 μ moles of glucose in 2 ml was used and homoserine or threonine solution or water (control) was added at a specified time. The reaction mixture was incubated in a large test tube at 28°C on test tube shaker.

RESULTS

1. Lysine Accumulation by Various Auxotrophs

Lysine accumulation by many microorganisms including newly isolated strains was tested for the purpose of lysine production by fermentative method. But it was very difficult to obtain lysine producer by this screening, though some strains which produce glutamic acid, alanin or valine were found. So the method using microbial auxotroph was considered hopeful in view of the practical application of diaminopimelic acid accumulation by *Escherichia coli* auxotroph to lysine production (7). Induction of microbial auxotrophs and their screening of lysine producer were performed. As a re-

Table 1. Lysine production by auxotrophs from various species.

Microorganism	Nutritional requirement	Lysine produced Lys·HCl mg/ml*
<i>Micrococcus glutamicus</i> 901	Homoserine	12.9
" 602	Methionine	1.6
" 702	Homoserine	11.4
" 703	Methionine	1.6
" 701	Leucine + Isoleu.	2.3
<i>Escherichia coli</i> 177-184**	Homoserine	0.9
" 628	Methionine	0.7
<i>Bacillus subtilis</i> 186×***	Methionine + Thre.	3.9
" 190***	Homoserine	3.6
" B2-101	Methionine + Thre.	3.9
" B2-102	Homoserine	4.3

* Value in initial medium was 0.4 mg/ml.

Fermentation medium: Cerelose 10%, $(\text{NH}_4)_2\text{SO}_4$ 2.0%, NZ-amine 1.0%, KH_2HO_4 0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.025%, K_2HPO_4 0.05%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001%, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.001%, Biotin 15 $\mu\text{g/L}$, CaCO_3 1.0% pH 7.2.

** Supplied by Dr. B. D. DAVIS.

*** Supplied by Dr. P. R. BURKHOLDER.

Table 2. Lysine production by various auxotrophs of *Micrococcus glutamicus*.

Strain (No. of isolation)	Nutritional requirement	NZ-amine added (%)	Lysine produced (Lys·HCl mg/m)
534-Co 155	Homoserine (Meth. + Thr.)	0.5	7.4
615-338-345	Homoserine (Meth. + Thr.)	0.2	4.1
534-13	Threonine	1.5	1.8
534-62	Threonine	0.5	2.6
615-313	Isoleucine	0.5	3.2
615-396	Isoleucine	0.2	2.3
534-75	Leucine	0.5	3.4
534-16	Proline	0	1.1
615-388-439	Leu. + Isoleu.	1.0	1.4
534-Co 127	Arg./Cit./Orn.	1.0	0.9
534-6	Adenine/Xanthine	1.5	1.2
534-28	Uracil	1.5	1.6
534-78	Tryptophan	1.5	1.7
534-1	Phenylalanine + Tyros.	1.5	1.4
588-219	Phenylalanine	1.5	1.7

Fermentation medium: Cerelose 7.5%, $(\text{NH}_4)_2\text{SO}_4$ 1.5%, K_2HPO_4 0.05%, KH_2PO_4 0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.025%, Biotin 30 $\mu\text{g/L}$, CaCO_2 1.0%.

sult high lysine producer was obtained from mutants of glutamic acid producing bacterium *M. glutamicus*. Besides, considerable lysine accumulation was recognized in various types of auxotrophs (Tables 1 and 2).

Lysin accumulation was highest in homoserine (or both methionine and threonine) requiring mutant and threonine-less, isoleucine-less, leucine-less, and both isoleucine- and leucine-less mutants were high producers of the next grade. This order of accumulativity seems to be very interesting as viewed from the information of biosynthetic pathway of amino acid and feed back mechanism (9, 10) in *E. coli*. Homoserine-less mutant of *Bacillus subtilis* had easily accumulated 4 mg lysine per milliliter. Small but significant lysine accumulation by mutants which require substances such as arginine, tryptophane, and adenine apparently not related to lysine biosynthesis seems to indicate complexity of interrelationships of metabolic pathways.

Over 20 mg lysine per milliliter can be easily produced under certain conditions by these homoserine-less mutant of *M. glutamicus*. They are now being employed in commercial production of lysine.

The following experiments were accomplished by use of homoserine-less mutant No. 901 of *M. glutamicus*.

2. Effect of Isoleucine

During the studies on the effect of various amino acids upon lysine accumulation in a synthetic medium it was found that isoleucine improved considerably both growth and lysine accumulation of strain No. 901. Higher effect of isoleucine than that of threonine or methionine in increasing the growth (Table 3) indicates that isoleucine is required relatively by this strain rather than threonine is saved by this amino acid.

Table 3. Effect of isoleucine on growth and lysine accumulation.

DL-Threonine	L-Methionine	DL-Isoleucine	72 hr		96 hr
$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	Lysine·HCl mg/ml	Cell mg/ml	Lysine·HCl mg/ml
200	200	600	11.1	12.6	12.2
200	200	400	10.5	13.6	12.6
200	200	100	10.1	15.5	8.8
200	200	50	6.0	8.6	7.2
200	200	25	6.1	8.5	6.9
200	200	12.5	5.4	6.7	6.9
200	200	6.3	5.4	6.6	6.6
200	200	0	6.0	6.1	6.3
400	200	0	8.2	8.2	8.9
600	200	0	7.6	6.6	9.8
200	400	0	6.6	6.1	7.0
200	600	0	4.9	4.4	7.9

3. Change of Fermentation Type According to the Requiring Substance Concentration in Medium

The fact that the accumulation of metabolites by microbial auxotroph occurs only in a limited concentration of requiring substance was reported in many cases. This tendency was recognized by present authors at valine accumulation in isoleucine-less and leucine-less mutants (11). This principle was also experienced in lysine accumulation by homoserine-less mutant as shown in Table 4. The strain required biotin in addition to homoserine (or

Table 4. Effect of nucurilite concentration on metabolite accumulation.

DL-Homoserine μg/ml	Nutrite added				Metabolite accumulation		
	DL-Threonine μg/ml	L-Methionine μg/ml	DL-Isoleucine μg/ml	Biotine μg/L	Lysine* HCl·salt mg/ml	Glutamic acid* mg/ml	Lactic acid** mg/ml
	200	200	200	20	12.5	trace	±
	400	200	200	20	7.9	0.27	±
	1000	200	200	20	1.7	trace	±
	400	400	200	20	10.6		
	400	1000	200	20	9.0		+
	400	200	200	20	5.2		+
	200	400	1000	20	6.3		
	1000	1000	1000	20	0.3	trace	±
	1000	1000	1000	5	trace	trace	±
	1000	1000	1000	2.5	trace	trace	39.8
	1000	1000	1000	12.5	trace	0.8	35.5
	1000	1000	1000	0.6	trace	7.3	2.3
	1000	1000	1000	0.3	trace	6.7	0.8
400			200	20	11.1	0.4	0.9
600			200	20	8.9	0.3	3.3
1000			200	20	6.3	trace	26.7
1000			1000	20	2.9	trace	32.5

* Meane value of 2 flasks at 4th day.

** Maximum value during 4 days.

both methionine and threonine) like its parent strain M. 534 (ATCC 13032) of *M. glutamicus*. So various amounts of biotin were added in media with various amounts of homoserine, threonine and isoleucine. In excessive concentration of homoserine or threonine and 20 μg per L of biotin, lactic acid accumulated in place of lysine. The effect of homoserine and threonine was decisive while the effect of methionine and isoleucine was little. In

excessive concentration of homoserine or threonine and suitably little amount of biotin, glutamic acid accumulated in place of lactic acid. This change of fermentation type from glutamic acid to lactic acid fermentation by the increase of biotin in media was reported by TANAKA *et al.* (8) at the parent strain. The same phenomenon was confirmed in our mutant. Both lysine and glutamic acid accumulated in the case where the amount of requiring amino acids suitable for lysine production, and limited amounts of biotin were added in media. But in this case the accumulation of each amino acid was low compared with those when one amino acid was accumulated under the conditions described above.

4. Specific Inhibition of Lysine Accumulation by Homoserine or Threonine

The influence of the addition of various amino acids on lysine accumulation from glucose and ammonia by washed cell suspension was investigated. As shown in Table 5, homoserine and threonine inhibited specifically lysine

Table 5. Specific inhibition of L-Lysine production by homoserine and threonine.

Amino acid added (1) 10^{-2} M	Lysine produced*		Amino acid added	Lysine produced*	
	μ moles	%**		μ moles	%**
L-Lysine	12.3***	64	DL- α -AB	17.0	88
L-Arginine	14.2	74	L-Cysteine	8.5	44
L-Methionine	15.9	83	α -KB	23.1	119
L-Cystine	15.9	83	DL-Homoserine	0	0
L-Leucine	16.9	88	Not added	19.3	100
DL-Isoleucine	14.1	73	(2) 10^{-3} M		
DL-Valine	16.9	88	L-Lysine	28.0***	145
L-Phenylalanine	17.4	90	L-Methionine	21.8	113
DL-Tyrosine	15.0	78	L-Leucine	16.6	86
L-Tryptophan	12.7	66	DL-Isoleucine	12.9	67
DL-Histidine	19.5	100	DL-Valine	11.2	58
DL-Threonine	0	0	DL-Threonine	0	0
L-Glutamic acid	40.4	209	α -KB	17.0	88
L-Proline	19.9	103	DL-Homoserine	0	0
L-Aspartic acid	26.7	138	(3) 10^{-4} M		
DL-Alanine	20.7	107	L-Methionine	16.6	55
Glycine	15.6	81	DL-Threonine	14.2	74
L-Serine	14.7	76	DL-Homoserine	2.4	12.4

* Subtracted the value of blank (neither amino acid nor glucose were added).

** Ratio to control (no amino acid was added).

*** Subtracted the added lysine value.

DL- α -AB=DL- α -Amino-*n*-butyric acid, α -KB=DL- α -Keto-*n*-butyric acid, incubation: 7 hrs.

production. Lysine production stopped completely at the concentration of $10^{-3}M$ of these amino acids. Inhibiting effect of homoserine was stronger than that of threonine and the inhibition was almost complete (88%) at $10^{-4}M$. Practically, inhibition by methionine and isoleucine did not occur in the present experiment.

In order to clarify these inhibition of lysine accumulation by homoserine

Fig. 1. Inhibition of lysine accumulation by threonine. DL-Threonine was added to $10^{-3}M$ (on L-form basis).

Fig. 2. Inhibition of lysine accumulation by homoserine. DL-Homoserine was added to $10^{-3}M$ (on L-form basis).

and threonine is caused whether by inhibition of enzyme formation of lysine producing system or by inhibition of enzyme action, homoserine or threonine was added to the system in the way of accumulating lysine. The results of this experiment were shown in Figs. 1 and 2. Lysine accumulation was stopped by the addition of these amino acids even into the system which is accumulating lysine actively. Inhibition of enzyme activity is suggested from these results. Cell suspension which stopped lysine accumulation by initial addition of inhibiting amino acids was washed and transferred to fresh

Fig. 3. Reproduction of lysine on washing by cells which stopped lysine production with homoserine or threonine.

- amino acids were not added.
- threonine (10^{-3} M) was added at 0 hr.
- homoserine (10^{-3} M) was added at 4 hr.
- △, ▲ incubated with amino acid during first 3 hr. then washed and resuspended in fresh medium.
- (△---△ homoserine ▲---▲ threonine).

reaction mixture and incubated. Lysine accumulation occurred in the manner similar to the case where no inhibiting amino acids were added to the initial stage. This observation also suggests the inhibition of enzyme action.

Possibility of the inhibition of enzyme formation was tested. Lysine accumulating activity of the cells harvested from the medium containing excessive ($1000 \mu\text{g DL-homoserine per ml}$) homoserine was compared with that of the cells used in previous experiments. As shown in Table 6, difference was scarcely recognized. In this experiment amounts of lysine accumulated after incubation for 7 hours was assayed but the values may represent accumulating velocity, namely enzyme concentration, considering the time-course indicated in Figs. 1 to 3.

Table 6. Lysine production by cells grown on medium containing excessive amount of homoserine.

Harvesting medium	Lysine produced
A*	27.4 μ moles
A + Homoserine 1000 μ g/ml	26.0 μ moles

* A: Cerelose 2.5%, NZ-amine 1.0%, $(\text{NH}_4)_2\text{SO}_4$ 0.5%, K_2HPO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.025%, trace element soln. 1 ml/L, Biotin 20 μ g/L pH 7.2.

Reaction mixture: N/15 phosphate buffer (pH 7.5) 1 ml, NH_4Cl (200 μ moles) 1 ml, glucose (100 μ moles) 1 ml, MgCl_2 (10 μ moles) 1 ml, cell (dry weight 77 mg) 1 ml, total 5 ml.

was incubated with shaking at 30°C for 7 hrs.

DISCUSSION

From the above observations homoserine and threonine seem to inhibit the enzyme action of lysine accumulating system, but it is not clear whether the action of these amino acids is direct or side effect caused after transformation to the other amino acid inside the cell. Inhibition of homoserine-kinase by threonine in *E. coli* reported by WORMER and PARDEE (9) suggests the secondary action of threonine through homoserine accumulation inside the cell. Delay of inhibition of lysine accumulation by threonine indicated in Fig. 1 appears to warrant this possibility. On the other hand, lysine accumulation by threonine-less mutant described in this paper and inhibition of the enzyme activity of homoserine producing system reported by NARA *et al.* (12) of our laboratories in the same threonine-less mutant indicate the direct action of threonine. Also the possibility of the indirect action of homoserine is not eliminated. These problems can not be answered at present because neither the biosynthetic pathway nor inhibiting step is as yet clarified.

Lysine accumulation by homoserine-less and threonine-less mutants may be understandable as the result of breaking of inhibition (regulation) mechanism described above by mutation. Lysine accumulation by isoleucine-requiring strain (which response also to α -aminobutyrate and α -ketobutyrate) may be understandable as breakage of control mechanism through threonine accumulation inside the cell. But exact mechanism must be clarified by further studies. Lysine accumulation by leucine-less mutant and by strain requiring both isoleucine and leucine can not be explained by present knowledge. Lysine-less mutant which accumulates diaminopimelic acid was obtained by the present authors (3). In this strain diaminopimelic acid did not accumulate in excessive lysine concentration in media. Therefore control mechanism of lysine synthesis by lysine may be suggested. Consequently, the explanation of a large quantity of lysine accumulation is now still difficult.

This exciting and provocative problem seems to await further exploration.

SUMMARY

1. Lysine accumulation by various auxotrophs was found. Especially a large quantity of lysine was accumulated by homoserine-less mutant of glutamic acid producer, previously named *Micrococcus glutamicus*. All homoserine-less mutants tested including *Bacillus subtilis* and *E. coli* showed lysine accumulation. Considerable lysine accumulation occurred with mutants such as threonine-less, isoleucine-less, leucine-less and both isoleucine and leucine-less mutants.

2. Lysine accumulation by homoserine-less mutant of *M. glutamicus* was studied rather in detail. Lactic acid accumulated in place of lysine in excess of homoserine or threonine and biotin in media. In sufficient concentration of biotin and a proper amount of requiring amino acids, lysine accumulated. In low biotin concentration the tendency of glutamic acid accumulation was recognized.

3. Homoserine and threonine inhibited specifically the lysine accumulation from glucose and ammonia by washed cell suspension of homoserine-less mutant of *M. glutamicus*. The action of these amino acids was suggested to be inhibition of enzyme action rather than enzyme formation.

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