Microbial Production of L-Glutamic Acid by Glycerol Auxotrophs

Part I. Induction of Glycerol Auxotrophs and Production of L-Glutamic Acid from n-Paraffins

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To establish a novel process for the production of L-glutamic acid from n-paraffins, a glycerol auxotroph GL-21, a new type mutant, was successfully obtained from Corynebacterium alkanolyticum No. 314 by treatment with N-methyl-N±N±nitro-N-nitrosoguanidine. This auxotroph required glycerol for its growth regardless of the carbon source used.

At 72 hr, this mutant GL-21 produced about 40 mg/ml of L-glutamic acid from n-paraffins in the culture broth at 0.01 per cent addition of glycerol in the absence of penicillin.

A thiamine auxotroph, a biotin auxotroph and an oleic acid auxotroph were also obtained by a similar technique, but these auxotrophs were found to be inapplicable for the production of L-glutamic acid from n-paraffins.

While L-glutamic acid has been produced from carbohydrates on an industrial scale, a suitable fermentation process for the production of L-glutamic acid from n-paraffins has not been successfully established.

When each of carbohydrates such as glucose, acetate and molasses was employed as the sole source of carbon, bacterial strains belonging to genus Corynebacterium or genus Brevibacterium, which were isolated from natural sources as biotin auxotrophs, accumulated a large amount of L-glutamic acid in the culture broth under limited supply of biotin1) or oleic acid2,3) (or unsaturated fatty acids) or by the addition of penicillin4) or surfactants5±7).

On the basis of the results of intensive studies concerning the relationship between L-glutamic acid excretion and limited supply of biotin or oleic acid, the cellular content of unsaturated fatty acids, especially of oleic acid, is understood to play an important role in the control of L-glutamic acid excretion through the bacterial cell membrane.

On the other hand, advancement in our knowledge of L-glutamic acid production from n-paraffins was made by the development of the process involving the addition of penicillin8,9) or limited supply of thiamine10,11) using Corynebacterium sp. or Arthrobacter sp., while the mechanism of action of these substances has remained unknown.

In the course of our studies to clarify the function of penicillin on L-glutamic acid production from n-paraffins,12) it was found that the excretion of L-glutamic acid by the addition of penicillin or cephalosporin was accompanied by the excretion of both phospholipids and N-acetylglucosamine, which are

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1) Presented at 19th Symposium on Amino Acid and Nucleic Acid, Tokyo, December 1, 1970.
known to be components of the cell membrane
and the cell wall. The close relationship
between the extracellular accumulation of
L-glutamic acid and the excretion of phospho-
lipids led to the suggestion that phospholipids
might be operative in the regulation of L-
glutamic acid excretion through the bacterial
cell membrane.

Based on this suggestion, to remove the per-
meability barrier to L-glutamic acid, a glycerol
auxotroph GL-21 in which cellular phos-
pholipid synthesis is regulated by an amount
of glycerol supplied was obtained from Coryne-
bacterium alkanolyticum No. 314 by N-methyl-
N'-nitro-N-nitrosoguanidine treatment.13) This
mutant required glycerol for its growth and
accumulated a large amount of L-glutamic
acid under limited supply of glycerol.

This first success in the accumulation of L-
glutamic acid by this mutant will present
new information for investigating the mecha-
nism of L-glutamic acid excretion through the
cell membrane.

In the present paper, emphasis was placed
on the induction of the glycerol auxotroph.

MATERIALS AND METHODS

Microorganisms. From among many n-paraffins-
asimilating microorganisms which were isolated from
natural sources in our laboratory, the strain No. 314
was selected on the basis of high productivity of L-
glutamic acid from n-paraffins. The strain No. 314
and the mutant GL-21, which was derived from the
strain No. 314 as a glycerol auxotroph, were mainly
used throughout this study unless otherwise stated.

Induction of various auxotrophs. Auxotrophs were
obtained by treatment of bacterial cells, grown for
24 hr on bouillon agar slant, with 1.5 mg of N-methyl-
N'-nitro-N-nitrosoguanidine14) in 5 ml of 1/20 M Tri-
maleate buffer (pH 7.0) at 28°C for 25 min with
shaking. Auxotrophs were selected by the replica
method15) using various media shown below.

Media for mutant selection. Complete medium for
biotin or thiamine auxotroph selection contained, per
liter: peptone, 10 g; yeast extract, 10 g; and NaCl, 5 g.

To this complete medium, 0.3 g of sodium oleate
and 0.6 g of Tween 80 for oleic acid auxotroph selection and 0.5 g of glycerol for glycerol auxotroph selection were supplemented, respectively.

Minimum medium contained, per liter: glucose, 10 g; Na2SO4, 1.0 g; MgSO4·7H2O, 0.1 g; Na-glutamate, 6.5 g; KH2PO4, 1.0 g; K2HPO4, 3.0 g; FeSO4·
7H2O, 0.05 g; and vitamin free casamino acid (Difco),
0.01 g; pH 7.0.

To this minimum medium, 30 μg of biotin, 0.3 g
of sodium oleate and 0.6 g of Tween 80, 200 μg of
vitamin B1·HCl or 0.5 g of glycerol was added as the
supplement medium for the selection of a biotin, an
oleic acid, a thiamine or a glycerol auxotroph,
respectively.

Solid media were prepared by adding 20 g of agar
to each medium.

Culture media and conditions. To detect the bacterial
growth, the organisms were inoculated into 4 ml of a
medium in a test tube which contained, per liter:
carbon source, 30 g; (NH4)2SO4, 3 g; NH4NO3, 3 g;
(NH4)2CO, 0.5 g; KH2PO4, 2 g; K2HPO4, 2 g; MgSO4·
7H2O, 1 g; FeSO4·7H2O, 0.003; MnSO4, 20 mg;
ZnSO4·7H2O, 10 mg; CoSO4·7H2O, 10 mg; CaCl2.

<table>
<thead>
<tr>
<th>TABLE 1. CULTURE MEDIA</th>
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<tbody>
<tr>
<td>Seed (%)</td>
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</tr>
<tr>
<td>α-Paraffins</td>
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<tr>
<td>(NH4)2SO4</td>
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<tr>
<td>NH4NO3</td>
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<tr>
<td>(NH4)2CO</td>
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<tr>
<td>KH2PO4</td>
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<td>FeSO4·7H2O</td>
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<td>MnSO4</td>
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<tr>
<td>ZnSO4·7H2O</td>
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<tr>
<td>CoSO4·7H2O</td>
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<tr>
<td>Corn steep liquor</td>
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<tr>
<td>Phenol red</td>
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<tr>
<td>CaCO3</td>
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<tr>
<td>Glycerol</td>
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<tr>
<td>Thiamine-HCl[a]</td>
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<tr>
<td>Biotin[a]</td>
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<td>Na-oleate[a]</td>
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[a] For thiamine, biotin, oleic acid auxotrophs, glycerol was replaced by thiamine-HCl, biotin or Na-oleate, respectively.
2H₂O, 10 mg; and various amounts of nutrient; pH 7.0 and incubated for 2 or 3 days at 28°C with shaking.

The media employed in L-glutamic acid production are shown in Table I. The test strain was used to inoculate 20 ml of the seed medium in a 200 ml flask and incubated at 28°C for 24 hr on a rotary shaker (200 rpm). One and a half ml of culture broth thus obtained was transferred into 30 ml of the main medium in a 200 ml creased flask. Incubation was carried out on the rotary shaker (200 rpm) at 28°C for 72 hr. Nine hundred μg of penicillin G was added to the main culture at 21 hr, when necessary.

Measurement of bacterial growth. Bacterial growth in glucose medium was estimated by measuring the optical density of the diluted culture broth at 660 μm. For the measurement of growth in n-paraffin medium, culture broth was diluted 15-fold with ethanol : n-butanol : chloroform mixture according to the method of Nakahara et al.16) and its optical density at 660 μm was measured.

Assay of extracellular L-glutamic acid. Extracellular L-glutamic acid was estimated by a microbiological assay method using Lactobacillus arabinosus 17-5.

Materials. N-Methyl-N'-nitro-N-nitrosoguanidine and n-paraffins (C₁₁-C₁₇) were purchased from Aldrich Chemical Co., Ltd. and Nikko Petrochemical Co., Ltd., respectively.

RESULTS AND DISCUSSION

Taxonomical studies of the bacterial strain No. 314

The morphological and physiological characteristics of n-paraffins-assimilating bacterium No. 314 which was isolated from soil as a strain having an excellent ability to produce L-glutamic acid are summarized in Table II. The strain was curved or branched and non-motile. Gram positive rods with metachromatic granules did not form endospores.

According to the descriptions in Bergey's Manual,17) it is appropriate to conclude that this organism belongs to genus Corynebacterium. However, characteristics of this organism were not identical with those of any known Corynebacterium species described in Bergey's Manual.17) This organism did not change litmus milk, could grow at 37°C, reduced nitrate to nitrite and required no growth factor. As these properties were different from those of any other strains belonging to genus Corynebacterium, isolated as the strains capable of producing L-glutamic acid from n-paraffins, this organism was named Corynebacterium alkanolyticum as a new species, from the ability to assimilate an alkane.

Growth of various mutants and their productivities of L-glutamic acid from n-paraffins

As will be seen from the introduction, possible types of mutants asked for L-glutamic acid production from n-paraffins might be as
TABLE III. Growth and L-Glutamic Acid Productivity of Various Mutants

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Growth</th>
<th>L-Glutamic acid production from n-paraffins</th>
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<tbody>
<tr>
<td></td>
<td>n-Paraffins</td>
<td>Glucose</td>
</tr>
<tr>
<td>Biotin^-</td>
<td>+</td>
<td>±</td>
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<tr>
<td>Oleic acid^-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Thiamine^-</td>
<td>+</td>
<td>-</td>
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</table>

Parent strain: Corynebacterium alkanolyticum No. 314.

follows: 1) a biotin auxotroph or an oleic acid auxotroph: the success in L-glutamic acid fermentation using carbohydrates as a carbon source by a biotin or an oleic acid auxotroph should be applied to one using n-paraffins.

2) a thiamine auxotroph: much remains to be solved on the function of thiamine in L-glutamic acid production from n-paraffins.

3) a new type mutant: on the basis of studies on penicillin action on L-glutamic acid excretion, other mutants should be artificially derived. As demonstrated in Table III, a biotin, an oleic acid and a thiamine auxotroph obtained from C. alkanolyticum No. 314 were examined on their growth responses towards respective growth factors and on the abilities of L-glutamic acid production from n-paraffins. A biotin auxotroph, which required biotin for its growth in glucose medium but not in n-paraffins, could not produce L-glutamic acid from n-paraffins. Such a fact could not be expected from the studies on L-glutamic acid production from carbohydrates.

On the basis of this phenomenon and the results obtained from the analysis of cellular fatty acid components in L-glutamic acid producing bacteria, it seems reasonable to assume that unsaturated fatty acids might be directly synthesized from n-paraffins via saturated fatty acids. This hypothesis is shown in Fig. 1. In this case, the cellular content of unsaturated fatty acids, especially of oleic acid, could not be controlled by a biotin auxotroph, in which the biosynthetic pathway from acetyl-CoA to malonyl-CoA is regulated by a biotin level in the cells. Therefore, L-glutamic acid appears not to be produced from n-paraffins by this mutant. This estimation is supported by the results obtained from experiments using an oleic acid auxotroph, in which an enzyme participating in the biosynthesis from capric acid (C10) to oleic acid (C18) is believed to be lacking. This auxotroph required oleic acid for its growth in glucose medium but took the same attitude as a prototroph in a n-paraffins medium.

Accordingly, the application of the biotin or the oleic acid auxotroph to L-glutamic acid production from n-paraffins proved to be unsuccessful.

On the other hand, a thiamine auxotroph required thiamine regardless of any kind of carbon sources and produced about 15 mg/ml of L-glutamic acid from n-paraffins. Although

![Fig. 1. Biosynthetic Pathway of Fatty Acid.](attachment:image)
an interesting problem concerning the relationship between thiamine deficiency and L-glutamic acid excretion appears to remain, the thiamine auxotroph will not be suitable for the production of a large amount of L-glutamic acid.

Induction of glycerol auxotroph

As is clear in the descriptions above, the possibility for the production of L-glutamic acid from \( n \)-paraffins by the biotin auxotroph, the oleic acid auxotroph or the thiamine auxotroph is small.

In the course of our studies on penicillin action on L-glutamic acid excretion, a close relationship between the cellular content of phospholipids and the excretion of L-glutamic acid was observed. In view of this fact, the limitation of the cellular phospholipid content is expected to destroy the permeability barrier in the cell membrane to L-glutamic acid.

To control the amount of cellular phospholipids using a biochemical mutant, there may be four methods in consideration of the structure of phospholipids shown in Fig. 2.

![Fig. 2. Structure of Phospholipid.](image)

The mutants expected are as follows: 1) mutants requiring phospholipids themselves. 2) mutants requiring the side chain \( R \). 3) mutants requiring fatty acids. 4) mutants requiring glycerol or its derivatives. Methods 1) and 2) are not always appropriate because of the vagueness of the kinds of phospholipids participating in L-glutamic acid excretion. Method 3) would be impossible or very difficult on account of the reasons indicated above.

On the basis of these reasons, the glycerol auxotroph GL-21 was successfully obtained from \( C. \) *alkanolyticum* No. 314. Glycerol auxotrophs could be induced from strains belonging to other species of genus *Corynebacterium* and moreover from strains belonging to genus *Brevibacterium*, *Arthrobacter* and *Candida*. Although there is much literature on biochemical mutants requiring amino acids, vitamins, nucleic acid bases and fatty acids, little attention has been paid to carbohydrate-requiring mutants. However, our success in the induction of glycerol auxotrophs confirms the possibility of deriving and utilizing this kind of auxotroph.

Recently, glycerol auxotrophs have been obtained from *Bacillus subtilis* and *Escherichia coli* by other investigators independently.

Glycerol requirement of the glycerol auxotroph GL-21 grown on various carbon sources

As stated, the biotin or the oleic acid auxotroph grown in glucose medium took a dif-

<table>
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<th>TABLE IV. GLYCEROL REQUIREMENT ON VARIOUS CARBON SOURCES</th>
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<tr>
<td><strong>Carbon sources</strong></td>
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<tr>
<td>Hexadecane</td>
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<td>Gluconate</td>
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<td>Pyruvate</td>
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<td>Ethanol</td>
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Strains; \( C. \) *alkanolyticum* No. 314 (Prototroph).
\( C. \) *alkanolyticum* GL-21 (Glycerol auxotroph).
different attitude from one grown in \( n \)-paraffins medium in the requirement of biotin or oleic acid. The glycerol requirement of this glycerol auxotroph GL-21 was studied using various carbon sources. As shown in Table IV, this auxotroph GL-21 required glycerol for its growth regardless of the kind of carbon sources employed. This finding supports the possibility of extensive utilization of this mutant for L-glutamic acid fermentation using any carbon sources.

**Growth response of the glycerol auxotroph**

Growth responses of the prototroph No. 314 and the glycerol auxotroph GL-21 towards glycerol concentrations were examined using glucose or \( n \)-paraffins medium. As can be understood from Fig. 3, the parent strain No. 314 grew regardless of the glycerol concentration employing either glucose or \( n \)-paraffins as the carbon source. However, the growth of the mutant GL-21 varied in proportion to the glycerol concentration and maximal growth was obtained at 0.02% concentration of glycerol in both carbon sources used.

**Extracellular accumulation of L-glutamic acid by the glycerol auxotroph**

As indicated in Fig. 4, the parent strain No. 314 had no ability to accumulate L-glutamic acid in the culture broth in the absence of penicillin, while the glycerol auxotroph GL-21 accumulated about 40 mg/ml of L-glutamic acid by the addition of 0.01% glycerol even in the absence of penicillin. Over this glycerol concentration, the extracellular L-glutamic acid decreased gradually according to the increase of glycerol concentration.

![Graph showing growth response of Corynebacterium alkanolyticum to glycerol concentration](image)

**Fig. 3. Growth Response of Corynebacterium alkanolyticum to Glycerol Concentration.**

- \( \times \times \) C. alkanolyticum No. 314
- \( \bigcirc \bigcirc \) C. alkanolyticum GL-21 (Glycerol-)

![Graph showing extracellular L-glutamic acid production](image)

**Fig. 4. Effects of Penicillin and Glycerol Concentration on L-Glutamic Acid Production.**

- \( \bigcirc \bigcirc \) None
- \( \times \times \) Penicillin added at 21 hr (30 \( \mu \)g/ml)
supplied. This is the first success that the extracellular accumulation of a large quantity of L-glutamic acid was recognized by a glycerol auxotroph derived from a wild strain which required no growth factor. Penicillin was effective for the excretion of L-glutamic acid using the parent strain No. 314 or under the excess supply of glycerol to the glycerol auxotroph GL-21. These results indicate that L-glutamic acid excretion was triggered by the alteration of permeability in this glycerol auxotroph.

In the case of L-glutamic acid fermentation from carbohydrates, all of the strains employed were isolated from natural sources as biotin auxotrophs, which genetically require biotin, i.e. unsaturated fatty acids for their growth. On account of this background, a controlling factor in L-glutamic acid fermentation has been commonly attributed to the cellular content of unsaturated fatty acids, especially of oleic acid.

Nevertheless, it was shown that this concept was not always true, because the glycerol auxotroph GL-21, in which unsaturated fatty acid biosynthesis is not regulated, could accumulate a large amount of L-glutamic acid under limited supply of glycerol.

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REFERENCES


