Production of Nucleic Acid-Related Substances by Fermentative Processes

Part XXIII. Derivation of IMP-Producing Mutants of *Brevibacterium ammoniagenes*

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In attempts to obtain GMP producing strains, *Brevibacterium ammoniagenes* was treated with UV, N.T.G. or D.E.S. as a mutagen. Adenine-guanine requiring mutants were obtained from an adenine-requiring mutant of *Brev. ammoniagenes*, KY 3482-9 and two of them, presumably adenine-xanthine requiring mutants, were then reverted to mutants which required only adenine for their growth.

Although these revertants were not able to accumulate a copious amount of GMP, most of them and of adenine-guanine requiring mutants produced larger amounts of IMP than the parent adenine-requiring strain.

Effects of Mn^{2+} and purine bases in the medium on IMP production by these mutants were examined and IMP productivities of these mutants were compared with the parent strain under optimal conditions.

These mutagenic treatments were thus proved to be effective for the increase of *de novo* IMP production by *Brev. ammoniagenes* mutants.

Demain *et al.*¹⁾ reported that a revertant derived from an adenine and xanthine auxotroph of *Corynebacterium glutamicum* accumulated GMP in a fermentation medium. It was suggested that IMP dehydrogenase in the revertant might be desensitized against the inhibition by GMP.

In order to obtain GMP-accumulating strains, *Brev. ammoniagenes* was selected as a parental strain for mutation, since the organism was previously shown capable of produc-

ing a large amount of IMP through *de novo*²⁾ or *salvage*³⁾ synthesis.

Numerous mutants of *Brev. ammoniagenes* were obtained by induced mutation techniques. None of them produced a copious amount of GMP, but many mutants derived from an adenine-requiring mutant of *Brev. ammoniagenes* KY 3482, one of natural isolates, accumulated larger amounts of IMP and/or XMP in their culture fluid.

These mutagenic treatments were thus found more effective for obtaining a strain capable of accumulating larger amounts of IMP in the medium.

Abbreviation. IMP: 5'-Inosine monophosphate, GMP: 5'-Guanosine monophosphate, XMP: 5'-Xanthosine monophosphate, Hx: Hypoxanthine, Ad: Adenine, Gu: Guanine, Xa: Xanthine, S-AMP: Adonylosuccinate, DCW: Dried cell weight (mg/ml), N.T.G.: N-methyl-N'-nitro-N-nitrosoguanidine, D.E.S.: Diethyl sulfate, UV: Ultraviolet.

1) A. L. Demain, M. Jackson, R. A. Vitali, D. Hendlin and T. A. Jacob, *Appl. Microbiol.*, **14**, 821 (1966).

2) T. Nara, M. Misawa and S. Kinoshita, *Agr. Biol. Chem.*, **31**, 1351 (1967).

3) T. Nara, M. Misawa and S. Kinoshita, *ibid.*, **32**, 561 (1968).

MATERIALS AND METHODS

Microorganisms used. An adenine-requiring mutant, KY 3482-9, derived from *Brevibacterium ammoniagenes* KY 3482 by irradiation with ultraviolet light⁴⁾ was used as a parent strain to obtain mutants. Strain KY 3482, one of the natural isolates, is able to accumulate a large amount of IMP from hypoxanthine in the medium.⁴⁾

Media. Three media were used: (1) a nutrient medium ("N" medium) consisting of glucose 2%, yeast extract 0.5%, peptone 1.0%, meat extract 1.0%, and NaCl 0.25%, (2) a complete medium ("C" medium) consisting of glucose 1.0%, (NH₄)₂SO₄ 0.4%, K₂HPO₄ 0.1%, MgSO₄·7aq 0.03%, casamino acids "Nissan" 0.25%, thiamine·HCl 5 mg/liter, Ca-D-pantothenate 10 mg/liter, trace elements 1.0 ml/liter, urea 0.1%, L-cysteine 50 mg/liter, L-tryptophan 50 mg/liter, biotin 30 µg/liter, adenine 30 mg/liter, and guanine 30 mg/liter and (3) a minimal medium ("M" medium) consisting of the same composition as "C" medium with the exception of purine bases.

pH of each medium was adjusted to 7.3 with 5N-NaOH and 2% of agar were added for a solid medium.

For the examination of nucleotides fermentation, a seed medium, whose composition is the same as "N" medium, and two fermentation media were used: (1) natural fermentation medium ("Y" medium) consisting of glucose 10.0%, KH₂PO₄ 1.0%, K₂HPO₄ 1.0%, MgSO₄·7aq 1.0%, CaCl₂·2aq 0.01%, yeast extract 1.0%, and biotin 30 µg/liter and (2) a semi-defined medium ("D" medium) consisting of glucose 10.0%, KH₂PO₄ 1.0%, K₂HPO₄ 1.0%, MgSO₄·7aq 1.0%, CaCl₂·2aq 0.01%, meat extract 0.2%, thiamine·HCl 5 mg/liter, Ca-D-pantothenate 10 mg/liter, biotin 30 µg/liter, and MnSO₄·4aq 40 µg/liter.

pHs of both fermentation media were adjusted to 8.2 with 5N-NaOH, and 0.6% of urea (autoclaved separately) was supplemented to both media before inoculation.

Detailed fermentation techniques were reported previously.⁵⁾

Induction of mutation. To obtain adenine and guanine doubleless mutants from an adenineless mutant (KY 3482-9) of *Brev. ammoniagenes*, UV, N-

methyl-N'-nitro-N-nitrosoguanidine (abbreviated as N.T.G.) and diethyl sulfate (D.E.S.) were used as mutagens. Revertants which required adenine alone were derived spontaneously from the adenine-guanine-requiring mutants, KY 3482-9-26 and KY 3482-9-54.

Mutation techniques were the same as those of Lichstein *et al.*⁶⁾ KY 3482-9 was incubated in "M" medium for 1 day at 30°C, harvested by centrifugation, suspended in and irradiated with UV light (Toshiba Electric Co. Ltd., GL-15) or treated with N.T.G. or D.E.S. In the case of N.T.G. treatment, cells were mixed with 100 µg/ml of N.T.G. solution for 15 or 30 min.⁷⁾ Treatments with D.E.S. solution were made by the modified method of Udagawa.⁸⁾ A reaction mixture for D.E.S. treatment consisted of cell suspension (*ca.* 10⁸ cells/ml) 0.5 ml, M/10 phosphate buffer (pH 7.0) 4.3 ml, and 16% D.E.S. solution in ethanol 0.2 ml. Reaction mixture was incubated for 1, 2 and 4 hr at 30°C.

The cells treated with each mutagen were washed twice with saline and spread on "N" agar medium directly, or incubated in "C" liquid medium overnight and transferred into "M" liquid medium containing 100 u/ml of penicillin G for enrichment.⁹⁾

Mutants were isolated by replica method from "C" and "M" agar plate media.

Analytical method. Determination of IMP, XMP and purine bases was made by the paperchromatography⁵⁾ with a solvent system of isobutyric acid, 1 N-ammonia, and acetic acid (10:5:1). Yields of IMP and XMP were expressed as mg/ml of IMP·2Na·7½H₂O and of XMP·2Na, respectively. Those of purine bases were shown as mg per ml of the broth.

Glucose and bacterial growth (shown as D.C.W.) were determined by the same methods as reported previously.¹⁰⁾

Materials used. Casamino acids (vitamin-free acid hydrolyzed casein) were obtained from Nissui Seiyaku Co., Tokyo; diethyl sulfate was purchased from Wakō Jyunyaku Co. Ltd., Tokyo, and N.T.G. was from

6) H. C. Lichstein and E. L. Oginsky, "Experimental Microbial Physiology", W. H. Freeman Co., p. 72 (1965).

7) E. A. Adelberg, M. Mandel and G. C. C. Chen, *Biochem. Biophys. Res. Comm.*, **18**, 788 (1965).

8) K. Udagawa, a personal communication.

9) B. D. Davis, *J. Am. Chem. Soc.*, **70**, 4267 (1948).

4) T. Nara, unpublished data.
5) K. Nakayama, T. Suzuki, Z. Sato and S. Kinoshita, *J. Gen. Appl. Microbiol.*, **10**, 133 (1964).

10) M. Misawa, T. Nara, K. Udagawa, S. Abe and S. Kinoshita, *Agr. Biol. Chem.*, **28**, 688 (1964).

Aldrich Chem. Co. Inc., Milwaukee, Wis., U.S.A.

RESULTS

Isolation of adenine-guanine requiring mutants from an adenine requiring mutant

An adenine requiring mutant (KY 3482-9) of *Brev. ammoniagenes* KY 3482, one of natural isolates, accumulates a large amount of IMP in a culture medium.⁴⁾ To obtain adenine-guanine requiring mutants, KY 3482-9 strain was treated with three kinds of mutagens.

The growth responses to purine bases of purine base requiring mutants obtained from KY 3482-9 are shown in Table I.

TABLE I. GROWTH RESPONSES OF MUTANTS TO PURINE BASES

Mutants	Addition Growth				
	"N"	Ad·Hx	Gu·Ad	Gu·Hx	none
Ad ⁻ Gu	+	—	+	—	—
Ad ⁻ (parent)	+	+	+	—	—
Gu ⁻	+	—	+	+	—
Pur ⁻	+	+	+	+	—
Wild type or Revertant	+	+	+	+	+

+: growth —: no growth

Basal medium: "M" medium (agar plate).

"N": Nutrient medium described in Materials and Methods. Growth on plate media was checked after 1 day.

Strict adenine-guanine requiring mutants grow only on the "M" medium supplemented with adenine and guanine, whereas non-strict purine auxotrophs are able to grow on the "M" medium supplemented with adenine and guanine, adenine and hypoxanthine, guanine and hypoxanthine, or hypoxanthine, but not on adenine or guanine alone. All of the mutants derived from KY 3482-9 were examined for their growth on those media and strict adenine-guanine mutants were isolated and stored on slant agar media.

The typical examples of mutations with

TABLE II. TYPICAL EXAMPLES OF MUTATION WITH THREE MUTAGENS TO OBTAIN ADENINE-GUANINE REQUIRING MUTANTS

Mutagen	Conditions	Killing ratio (%)	Mutation* rate (%)
UV	51 cm, 3'	99.998	0.044
N.T.G.	100 µg/ml, 30'	66.40	0.318
D.E.S.	0.32%, 2 hr	99.46	2.11

* Mutation rate is expressed as the percentage of the number of strict adenine-guanine requiring mutants against that of isolated cells.

three mutagens are shown in Table II. D.E.S. was usually the most effective mutagen for obtaining adenine-guanine requiring mutants from KY 3482-9.

Nucleotide productivity of adenine-guanine requiring mutants

Adenine-guanine requiring mutants (545 strains) were incubated both in "Y" medium (see Materials and Method) and "Y" supplemented with 1 mg/ml of Mn²⁺, for 5 days at 30°C and the kinds and yields of fermentation products were studied.

The results are shown in Tables III and IV.

As reported previously, in the presence of an excessive level of Mn²⁺ in the medium, IMP production was inhibited markedly,¹¹⁾ but XMP production was stimulated by guanineless mutants.¹²⁾ However, several mutants were found to be stimulated IMP production in the "Y" medium containing

TABLE III. RELATIONS BETWEEN PRODUCTS AND POSSIBLE NUTRITIONAL REQUIREMENTS

Products	No. of mutants	Requirements
XMP	262	ad ⁻ , gu ⁻
IMP	117*	ad ⁻ , xa ⁻
XMP+IMP	156	ad ⁻ , gu ⁻
None	10	?

* All of these mutants were phenotypically adenine-guanine requiring strains.

11) T. Nara, M. Misawa and S. Kinoshita, *Agr. Biol. Chem.*, **32**, 1153 (1968).

12) M. Misawa, T. Nara, K. Udagawa, S. Abe and S. Kinoshita, *ibid.*, **33**, 370 (1969).

TABLE IV. NUCLEOTIDE PRODUCTIVITY OF SOME ADENINE-GUANINE REQUIRING MUTANTS DERIVED FROM KY 3482-9

Strains	Requirements	Mutagen	5-Day				
			pH	DCW mg/ml	IMP mg/ml	Hx mg/ml	XMP mg/ml
Parent*	ad		6.2	8.3	4.63	0.62	0
2	ad. gu (xa)	UV	6.3	10.8	7.48	0.83	0
26	"	D.E.S.	6.3	10.4	8.60	0.40	0
28	"	"	6.2	9.7	7.93	0.64	0
54	"	N.T.G.	6.2	10.3	8.78	0.34	0
66	"	"	6.1	8.9	8.55	0.65	0
99	"	"	6.0	8.6	8.50	0.44	0
207	"	"	6.1	8.8	8.44	0.54	0
402	ad. gu	D.E.S.	6.3	8.7	0.57	0.41	6.39
527	"	"	6.2	9.9	0.55	0.58	5.90

Fermentation medium: "D" medium supplemented with 150 μ g/ml each of adenine and guanine was used for the IMP producing strains. "Y" medium supplemented with 4 mg/liter of $\text{MnSO}_4 \cdot 4\text{aq}$ was used for XMP producing strains.

* KY 3482-9.

TABLE V. EFFECTS OF MEDIUM AND Mn^{2+} ON IMP PRODUCTION BY KY 3482-9 AND KY 3482-9-54

Strains	Medium	Mn^{2+} 1 mg/liter	5-Day				
			pH	DCW mg/ml	IMP mg/ml	Hx mg/ml	
Parent (ad ⁻) KY 3482-9	Y	{	-	5.9	10.0	3.10	2.37
			+	5.8	14.7	1.23	2.26
	D	{	-	6.2	8.4	4.63	0.62
			+	"	13.4	0.92	1.11
KY 3482-9-54 (ad ⁻ , gu ⁻)	Y	{	-	6.7	7.1	1.38	1.34
			+	6.2	8.3	2.20	2.95
	D	{	-	6.1	10.2	8.78	0.34
			+	"	13.1	2.03	1.22

"Y" and "D" media described in Materials and Methods were supplemented with both adenine 150 μ g/ml and guanine 150 μ g/ml.

excessive levels of Mn^{2+} KY 3482-9-54 is one of these strains as shown in Table V.

From the results in Table III, it appears that the mutants accumulating XMP or XMP and IMP are the adenine-guanine doubleless mutants; namely, both S-AMP synthetase (EC 6.3.4.4) and XMP aminase (EC 6.3.5.2) of these mutants might be lost simultaneously. In contrast to these mutants, 117 strains accumulated only IMP and they seem to be blocked in two reactions which are catalyzed by S-AMP synthetase and IMP dehydrogenase, respectively. These mutants are probably

adenine-xanthine requiring mutants. However, these mutants and a non-strict purineless mutants of *Br. ammoniagenes* were not able to grow on the "M" medium supplemented with adenine and xanthine, since it has been reported that the XMP-pyrophosphorylase of *Br. ammoniagenes* was lacking or its activity was of low level.¹²⁾

The IMP productivity of most of these 117 mutants was higher than that of the parent strain as shown in Table IV. This fact also supports that these strains were the adenine-xanthine requiring mutants, since it is rea-

sonable to suppose that the deficiency of IMP dehydrogenase (xanthine-less) in addition to that of S. AMP synthetase (adenine-less) would favor the enhancement of IMP accumulation.¹³⁾

Optimal concentrations of adenine and guanine

Optimal concentrations of adenine and guanine in the fermentation medium for the IMP production by an adenine-guanine (or strictly speaking, adenine-xanthine) requiring mutant, KY 3482-9-28, were compared with those by a parent strain, KY 3482-9. (Fig. 1).

(A) "D" medium, Mn^{2+} -deficient ($10 \mu\text{g Mn}^{2+}/\text{ml}$).

(B) "D" medium, Mn^{2+} -excess ($1000 \mu\text{g Mn}^{2+}/\text{ml}$).

FIG. 1. Effect of Adenine and Guanine Levels on IMP Accumulation.

○—○ KY 3482-9 (ad⁻).
 △—△ KY 3482-9-28 (ad⁻, gu⁻)
 after 5 days cultivation.

By acquisition of guanine requirement, the optimal concentrations of both bases were lowered to $150 \mu\text{g}$ of each base per ml from $200 \mu\text{g/ml}$ for an adenine-requiring mutant.

Inhibition of IMP production by an excess amount of Mn^{2+} with KY 3482-9-28 was lower than that with the parent strain.

Reversion to adenine requiring mutant from adenine-guanine requiring mutant

To obtain a mutant with altered IMP dehydrogenase, an adenine-guanine requiring mutant (adenine-xanthine requiring mutant, strictly speaking) was reverted to adenine-requiring mutants.

In these experiments two strains, KY 3482-9-26 and KY 3482-9-54 were selected as the parents since they had higher productivity of IMP.

Three hundred and twenty seven revertants were obtained spontaneously from these two strains and they were shown to have only adenine requirement.

Productivities of nucleotides by revertants

The adenine-requiring mutants (revertants)

FIG. 2. IMP Productivities of Mutants.

Medium: "D" medium.

○—○ KY 3482-9 (ad⁻) ad $150 \mu\text{g/ml}$.
 ●—● KY 3482-9-54 (ad⁻, gu⁻) ad $150 \mu\text{g/ml}$
 and gu $150 \mu\text{g/ml}$.
 △—△ KY 3482-9-54-252 (Revertant, ad⁻) ad $150 \mu\text{g/ml}$.

13) A. L. Demain, M. Jackson, R. A. Vitali, D. Handlin and T. A. Jacob, *Appl. Microbiol.*, **13**, 757 (1965).

(A) KY 3482-9 (ad^-). (B) KY 3482-9-54-252 (ad^- Revertant).

derived from the adenine-guanine requiring mutants were cultured in the "D" medium supplemented with 150 $\mu\text{g/ml}$ of adenine.

None of them produced a copious amount of guanine nucleotides, but the amounts of IMP accumulated were higher than those accumulated by a parent, KY 3482-9 as shown in Fig. 2.

From the results the order of the IMP productivity of these mutants was shown as follows:

$$Ad^- Xa^- > Ad^- \text{ Revertant} > Ad^-$$

(C) KY 3482-9-54 ($ad^- gu^-$).

FIG. 3. Time Courses of IMP Fermentation by Three Mutants of *Brev. ammoniagenes*.

Media: "D" supplemented with 150 $\mu\text{g/ml}$ of adenine for (A) and (B), and with 150 $\mu\text{g/ml}$ of adenine and of guanine for (C).

○—○ IMP (mg/ml). □—□ Glucose (%).
△—△ Hx (mg/ml). ×—× pH.
●—● DCW (mg/ml).

Time courses of IMP fermentation by these mutants are shown in Fig. 3. Although dried cell weights of both adenineless mutants, KY 3482-9 and KY 3482-9-54-252, were constant from 2 to 5-day of incubation, that of an adenine-guanine requiring mutant, KY 3482-9-54, was decreased gradually after 2 days of incubation.

DISCUSSION

Revertants (from $ad^- + xa^-$ to ad^-) of *Brev. ammoniagenes* did not accumulate a large amount of guanine nucleotides in the fermentation medium. This was different from the results obtained with *Corynebacterium glutamicum*.¹⁾

Possible reasons to explain the difference might be as follows: (1) difference of strains used, (2) IMP dehydrogenase of revertants was sensitive against GMP inhibition, (3)

repression of this enzyme by guanine, (4) effect of cultural conditions and (5) degradation of guanine nucleotides by phosphatases or other enzymes.

However, the amounts of IMP produced by these mutants were increased markedly, since two enzymes, S-AMP synthetase and presumably IMP dehydrogenase, which catalyze two reactions from IMP to AMP and GMP were blocked by the mutation. It is interesting that the level of IMP accumulated by a

revertant was higher than that accumulated by the parent strain. Activity of IMP dehydrogenase of the revertant may be changed by reversion.

It is concluded that these mutagenic treatments are effective to obtain a strain which accumulates larger amounts of IMP.

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