Studies on 5'-Nucleotidase-Lacking Mutants Derived from *Bacillus subtilis*

Part I. Derivation of Mutants from an Adenine Auxotroph and their Some Properties

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Two 5'-nucleotidase-lacking mutants, R-42 and A-1, were derived from an adenine-requiring mutant, *B. subtilis* 1145-2-83, which has productivity of both inosine and hypoxanthine. Strain A-1 accumulated 5'-IMP as well as inosine and hypoxanthine, and strain R-42 accumulated 5'-IMP and 5'-GMP as well as inosine and hypoxanthine in their culture fluids. These mutants responded to either adenine or adenosine, but did not to 5'-AMP. This fact suggests that adenine or adenosine may be incorporated into the cells, but 5'-AMP may neither be incorporated into the cells nor be degraded during culture. 5'-GMP was converted to 5'-IMP, and 5'-AMP was phosphorylated to ADP in the growing culture of strain A-1.

**INTRODUCTION**

There are many reports indicating the accumulation of nucleosides or purine-bases such as inosine, xanthosine and hypoxanthine by various kinds of auxotrophic mutants of bacteria. Among these mutants, adenine-requiring mutants of *B. subtilis* have been found to have a remarkable productivity of inosine. On the other hand, there are only a few reports on direct fermentation of 5'-nucleotides: Uchida et al. and Nakayama et al. reported the accumulation of 5'-IMP using an adenine-requiring mutant of *B. subtilis* and that of *Micrococcus glutamicus* respectively. Misawa et al. reported the accumulation of 5'-XMP using a guanine-requiring mutant of *Micrococcus glutamicus*. For the accumulation of 5'-nucleotides it is essential that phosphatase activity of a used strain is low. Therefore, it is reasonable to select a wild strain low in phosphatase activity as a starting strain for the artificial mutation, or to derive a mutant lacking phosphatase activity secondarily from a strain having productivity of nucleosides or purine- or pyrimidine-bases. From this viewpoint, Misawa et al. investigated on phosphatase activity of type cultures, and Momose et al. reported that a mutant lacking 5'-nucleotide-degrading activity induced from *Micrococcus glutamicus*.
*B. subtilis* accumulated a small amount of 5'-IMP in the culture fluid. The authors also attempted to derive mutants lacking phosphatase activity from an adenine-requiring mutant, *B. subtilis* 1145-2-83, which has high productivity of inosine and high phosphatase activity. As a result, two nucleotidase-lacking mutants, strain A-1 and strain R-42, were isolated. The present paper describes the accumulation of 5'-IMP and 5'-GMP by these mutants and other interesting properties of these mutants: incorporation of adenine or adenosine into the cells, conversion of 5'-GMP to 5'-IMP, and phosphorylation of 5'-AMP to ADP in the growing culture.

**EXPERIMENTALS**

**Organism.** *B. subtilis* 1145-2-83 was used as the starting strain for the artificial mutation. Strain 1145-2-83 is one of the adenine-requiring mutants derived from *B. subtilis* IAM 1145 and has high productivity of inosine.

**Medium.** The following three media were employed. C medium (complete medium); beef extract 10g, peptone 10g, yeast extract 5g, NaCl 5g, and adenine 30mg in 1000ml of distilled water. The pH was 7.0 after sterilization. M medium (minimal medium); the composition thereof was the same as that of Gray and Tatum's minimal medium.10) S medium; sodium citrate 11.7g, (NH₄)₂HPO₄ 10g, KCl 1.5g, MgSO₄·7H₂O 0.5g, CaCl₂·2H₂O 0.15g, and Glucose 120g in 1000ml of the soybean extract*. The pH was 6.5 after sterilization. This medium was used for both the accumulation of ultraviolet-absorbing substances and the assay of nucleotidase activity.

**Measurement of Growth.** Growth was estimated by measuring the turbidity at the wavelength of 660nm.

**Cultivation.** All cultivations were carried out in a 500ml Erlenmeyer flask containing 50ml of medium on a rotary shaker at 28°C.

**Induction of Mutation and Isolation of Mutants**

Spores from 7-days' culture of *B. subtilis* 1145-2-83 on the potato agar were suspended in 0.85% NaCl solution. The suspension was exposed to ultraviolet light (Toshiba sterilizing lamp, 15W, 300 mA, at 40 cm above the suspension, for 7 minutes). The survival ratio was about 0.02%. The survivals were plated on to C medium and replica plating technique was applied to the colonies appeared on a plate for isolation of mutants responded to adenine but not to 5'-AMP. This technique was convenient to select “5'-nucleotidase-lacking mutants” Total isolation method was also carried out, in which colonies on a plate of C medium were isolated at random and, then their nucleotidase activities were assayed.

**Assay of Nucleotidase Activity.** The reaction mixture for the assay of nucleotidase activity contained 2ml of M/7 sodium veronal-sodium acetate-hydrochloric acid buffer (pH 7.0), 1ml of 40mM substrate and 1ml of the sample (the dialyzed culture fluid* or the suspension of the washed cells). The reaction mixture was incubated at 37°C, and inorganic phosphate liberated was determined according to Allen method.11)

**Determination of Ultraviolet-Absorbing Substances.** Samples were subjected to paper chromatography or paper electrophoresis, and the spots separated on the paper were detected by illumination with an ultraviolet lamp (National Gl 15W) with filter-2537A. The spots detected were extracted with 0.1N HCl at 37°C overnight, and their amounts in the extracts were determined spectrophotometrically. The identification of 5'-IMP, 5'-GMP and ADP will be described in detail in “Results”

**Paper Electrophoresis.** Paper electrophoresis was carried out in 10% acetic acid with 400V and 0.4 mA/cm for four hours, the starting line being at the middle between the anode side and the cathode side. The distance from one end to the other was 31 cm.

**Paper Chromatography.** The following solvent systems were employed for paper chromatography:


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*The soybean extract was prepared as follows: fifty grams of the defatted soybeans were steamed for fifty minutes in 1000ml of 0.025N NaOH, and filtered.


RESULTS

Phosphatase Activities of Mutants Derived from B. subtilis 1145-2-83

Strain 1145-2-83 is capable of accumulating a large amount of inosine and a small amount of hypoxanthine. However, as this strain has high phosphatase activity, it is difficult to accumulate nucleotides by this strain. Therefore the derivation of “5’-nucleotidase-lacking mutants” by exposing this strain to ultraviolet light was attempted. As shown in Table I, several mutants low in nucleotidase activity were isolated. Especially in the cases of strain

**TABLE I. 5’-IMP-DEGRADING ACTIVITY OF VARIOUS MUTANTS DERIVED FROM B. subtilis 1145-2-83.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1145-2-83</td>
<td></td>
</tr>
<tr>
<td>(original strain)</td>
<td>6.80 22.56</td>
</tr>
<tr>
<td>A-1*</td>
<td>0.43 0</td>
</tr>
<tr>
<td>R-42**</td>
<td>0.19 0</td>
</tr>
<tr>
<td>R-47**</td>
<td>1.14 3.78</td>
</tr>
<tr>
<td>R-218**</td>
<td>3.96 8.64</td>
</tr>
<tr>
<td>R-1125**</td>
<td>2.56 2.96</td>
</tr>
<tr>
<td>R-3681**</td>
<td>3.79 9.92</td>
</tr>
<tr>
<td>R-6325**</td>
<td>2.11 12.12</td>
</tr>
</tbody>
</table>

Intact cells and culture fluid were prepared from 48 hr. and 96 hr. cultures in S medium respectively. In the case of strain R-42 S medium supplemented with 200 mg of adenine per litter was used.

* A strain isolated at random by total isolation method.
** Strains isolated by replica isolation method.
1) P(mole) liberated by 1 ml of cell suspension, whose O.D. 660 mµ is 10, for 30 min.
2) P(mole) liberated by 1 ml of culture fluid for 30 min.

R-42 and strain A-1, most of 5’-IMP-degrading activity in the original strain was not detected. Actually 5’-IMP added to their cultures exogenously was maintained without degradation during cultivation (Fig. 1). The phosphatase activities to various substrates were tested. As shown in Table II, different nucleotides were hydrolyzed at different rates. It is interesting that strain R-42 had not significant activity to both 5’-nucleotide and 3’-nucleotide, while strain A-1 still had the activity to only 3’-AMP. The activity of alkaline phosphatase of strain R-42 or strain A-1 was not so low in comparison with that of strain 1145-2-83. From these results, it may be concluded that strain R-42 is a mutant lacking both 3’- and 5’-nucleotidases, and strain A-1 is a 5’-nucleotidase-lacking mutant. It was also observed that only strain R-42 grew little in S medium alone, but did in S medium supplemented with adenine.

**Accumulation of Ultraviolet-Absorbing Substances**

To check changes in the accumulation of ultraviolet-absorbing substances, various nucleotidase-lacking mutants obtained were cultivated in S medium and the culture fluids were subjected to paper chromatography. The results are shown in Table III. Strain A-1 accumulated 5’-IMP as well as inosine and hypoxanthine, and strain R-42 accumulated 5’-IMP and 5’-GMP as well as inosine.

![Fig. 1. Degradation of 5’-IMP Added to S-medium during Cultivation.](image)
and hypoxanthine in their culture fluids (Fig. 2). These results suggest that the accumulation of 5'-IMP and 5'-GMP was caused by the lack of 5'-nucleotidase.

The isolation and purification of 5'-IMP accumulated in the culture medium of strain A-I were carried out as shown in Fig. 3. Needle-like crystals were obtained. The crystals (Sample I) were compared with authentic 5'-IMP·2Na as follows.

1) Ultraviolet absorption spectra: Ultraviolet absorption spectra were measured in 0.1 N HCl and 1 N NaOH (Fig. 4).

2) Paper electrophoresis: The sample was observed to include one ultraviolet-absorbing component. The distance migrated to the anode side of the Sample I and authentic 5'-IMP·2 Na was 5.4 cm and 5.4 cm, respectively.

3) Paper chromatography: $R_F$ values of the Sample I coincided with those of authentic 5'-IMP·2 Na in Solvent I and Solvent II (Fig. 5).

4) Pentose-orcinol reaction: 1 ml of the Sample I equivalent to 0.2 mM of nucleotide was heated with 4 ml of orcinol reagent (mixture of ferric ammonium sulfate 67 mg, orcinol 100 mg and hydrochloric acid 41.5 ml was diluted with water to 50 ml) according to the method of A. H. Brown.\(^{12}\) The rate of color development after 10 min. with the Sample I was 100%, and that with authentic 5'-IMP·2 Na was 100%.

5) Periodate oxidation: The periodate oxidation combined with Schiff’s reaction was carried out on the paper spot detected after paper electrophoresis by the modification of the method of Buchanan, Dekker and Long.\(^{13,14}\) As the Sample I was oxidized by periodate, it is not regarded as 2'- or 3'-nucleotide.

6) Susceptibility to seminal 5'-nucleotidase: The reaction mixture containing 0.5 ml of sample equivalent to 5 mM of nucleotide, 0.5 ml of 0.1 M MgSO$_4$, 0.5 ml of 0.2 M Tris buffer (pH 8.5), and 0.5 ml of the seminal 5'-nucleotidase solution* was incubated at 37°C for 30 min. Inorganic phosphate liberated was estimated by Allen’s method. During incubation the seminal 5’-nucleotidase was prepared by the modification of the method of L. A. Heppel and R. J. Hilmo\(^{15}\) as follows: 3 ml of bull seminal plasma was mixed with 3 ml of 5% protamine sulfate solution. A precipitate was removed by centrifugation and discarded. The supernatant was heated at 50–60°C for 20 min. A precipitate was removed by centrifugation and discarded. The resulting supernatant was filled up to 100 ml with 0.2 M Tris buffer. This solution was used as the seminal 5’-nucleotidase.

\(^{12}\) A. H. Brown, Arch. Biochem., 11, 269 (1946).


### Table III. Accumulation of Ultraviolet-Absorbing Substances by Various Mutants Derived from Strain 1145-2-83

<table>
<thead>
<tr>
<th>Strain</th>
<th>Productivity ($\mu$ mole/ml)</th>
<th>Hp</th>
<th>Is</th>
<th>5'-IMP</th>
<th>5'-GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1145-2-83</td>
<td>4.7</td>
<td>27.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A-1</td>
<td>4.0</td>
<td>34.0</td>
<td>1.0</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td>R-42</td>
<td>4.5</td>
<td>23.3</td>
<td>0.7</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>R-47</td>
<td>5.1</td>
<td>24.8</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>R-218</td>
<td>6.2</td>
<td>14.7</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>R-1125</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-3681</td>
<td>5.6</td>
<td>13.4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>R-5826</td>
<td>6.4</td>
<td>23.0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Cultivation was carried out in S medium for 8 days. In the case of strain R-42 S medium supplemented with 200 mg of adenine per litter was used.

**Fig. 2.** Separation of Ultraviolet-Absorbing Substances Accumulated in S medium.

0.05 ml of the 6-days’ culture fluid was subjected to paper electrophoresis.
Culture broth (cultivated in S medium for 7 days)
boiled for 5 min. at pH 9.0
ppt Sup
PCA added (final 5%)
ppt Sup
adjusted to pH 7.0 with KOH
ppt Sup
Ba(OH)_2 added
ppt Sup
treated with 1% active charcoal at 75~80°C, for 15 min.
concentrated in vacuo
methanol added (final 70%)
ppt Sup
ethanol added (final 70%)
Sup ppt
dissolved in water adjusted to pH 4.0

Fig. 3. Isolation and Purification of 5'-IMP from the Culture Fluid of Strain A-1.

7) Infrared absorption spectrum: Infrared absorption spectrum of the Sample I was identical with that of the authentic 5'-IMP·2Na as shown in Fig. 6.

8) The ratio of the base component to the phosphate component: The ratio of the base component determined ultraviolet spectrophotometrically to the phosphate component of the Sample I was found to be 1.0.

One of the ultraviolet-absorbing substances accumulated in the culture medium of strain R-42 was identified to be 5'-GMP as follows:

1) Paper chromatography: 0.05 ml of the culture fluid was subjected to paper chromatography with Solvent I or Solvent II. Rf values were as follows: with Solvent I the
sample: 0.67, authentic 5'-GMP·2Na 0.66, and with Solvent II the sample: 0.60, authentic 5'-GMP·2Na 0.62.

(2) **Paper electrophoresis**: 0.05 ml of the culture fluid was subjected to paper electrophoresis. The distance migrated to the anode side of the sample and authentic 5'-GMP·2Na was 3.1 cm and 3.2 cm, respectively.

(3) **Ultraviolet absorption spectrum**: The spot on a paper chromatogram was cut out and extracted with 10 ml of 0.1 N HCl. The spectrum of the extract (Fig. 7) was guanine derivative type.

(4) **Periodate oxidation**: The sample was confirmed to be oxidized by periodate.

**Incorporation of adenine derivatives into the cells**

Growth response to adenine, adenosine, 3'-AMP or 5'-AMP in 5'-nucleotidase-lacking mutants was tested. As shown in Fig. 8, the original strain 1143-2-83, which can dephosphorylate both 3'-AMP and 5'-AMP, responded to all adenine derivatives used. Strain R-42, which can dephosphorylate neither 3'-AMP nor 5'-AMP, responded to adenine and adenosine, but did not to 3'-AMP and 5'-AMP. Strain A-1, which can dephosphorylate 3'-AMP but not 5'-AMP, responded to adenine, adenosine and 3'-AMP, but did not

**TABLE IV. REPRESSION OF THE ACCUMULATION OF PURINE DERIVATIVES BY ADENINE DERIVATIVES.**

<table>
<thead>
<tr>
<th>Addition of adenine derivatives (mM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>0</td>
</tr>
<tr>
<td>Adenine 1 mM</td>
<td>36.2</td>
</tr>
<tr>
<td>2 mM</td>
<td>52.2</td>
</tr>
<tr>
<td>4 mM</td>
<td>77.0</td>
</tr>
<tr>
<td>Adenosine 1 mM</td>
<td>48.6</td>
</tr>
<tr>
<td>2 mM</td>
<td>56.4</td>
</tr>
<tr>
<td>4 mM</td>
<td>74.2</td>
</tr>
<tr>
<td>5'-AMP 1 mM</td>
<td>46.0</td>
</tr>
<tr>
<td>2 mM</td>
<td>78.0</td>
</tr>
<tr>
<td>4 mM</td>
<td>80.3</td>
</tr>
</tbody>
</table>

Cultivation was carried out in S medium for 7 days.
not to 5'-AMP. The relationship between the growth response to adenine nucleotide and phosphatase activity in these strains suggests that adenine or adenosine can be incorporated into the bacterial cells, but the adenine nucleotides cannot be incorporated. This possibility may be also supported by the following fact: Feed-back inhibition of purine biosynthesis by 5'-AMP was much lower in the 5'-nucleotidase-lacking strain than in the original strain (Table IV).

Conversion of 5'-GMP to 5'-IMP in the growing culture of strain A-1

As shown in Figs. 9 and 10, strain A-1 accumulated more 5'-IMP in S medium added with 5'-GMP than in S medium alone. As guanosine was not observed during the culture, the reaction 5'-GMP + Inosine → Guanosine + 5'-IMP is not conceivable. Probably 5'-GMP was directly converted to 5'-IMP by GMP-reductase.

Phosphorylation of 5'-AMP to ADP in the growing culture of strain A-1

As shown in Figs. 11 and 12, strain A-1 phosphorylated 5'-AMP to ADP in the growing culture. A small amount of ATP was also formed from 5'-AMP. The strain could phosphorylate not only 5'-AMP but also adenosine.

Separation and identification of ADP formed from 5'-AMP in the culture fluid were carried out by the following methods.

1. Column chromatography: The culture fluid was treated with active charcoal and
fractionated by anion exchange resin as shown in Fig. 13. The ADP-fraction* was used as the Sample II for the following identification tests.

* The ADP-fraction was obtained by concentrating the eluted solution (fractions 100-140) under reduced pressure at 45°C after removing formic acid. This fraction contained about 1 mg of ADP per ml.

(2) Paper chromatography: As shown in Fig. 14, $R_F$ values of the Sample II corresponded to those of authentic ADP.

(3) Paper electrophoresis: The distance mi-
FIG. 11. Formation of ADP during Cultivation of Strain A-1 in S Medium Added with 5'-AMP.

- 5'-AMP, ○○ ADP

FIG. 12. ADP Formed after 6-days' Culture of Strain A-1 at Various 5'-AMP Concentrations.

S medium was employed as the basal medium.

FIG. 13. Fractionation of the Culture fluid by Anion Exchange Resin.

Exchanger: IRA-402-formate, 1.3 cm 7.5 cm.
Sorbed material: The sorbed material was prepared as follows: 500 ml of the culture broth, which was obtained by cultivating strain A-1 in S medium containing 1.5 mg per ml of 5'-AMP for 8 days, was centrifuged in the cold. The supernatant fluid was adjusted to pH 2.0 with 2N HCl and filtered. The clear filtrate was mixed with active charcoal (Shirasagi, Takeda Chemical Industries, Ltd.), and shook. Adsorbed nucleotides, nucleoside and purine-base were then eluted with ammoniacal ethanol (0.4n ammonium hydroxide in 5% ethanol). The effluents concentrated under reduced pressure at 45°C were used for column chromatography.
Eluting solution: Formic acid (Formic) and sodium formate (Formate).
Each 10 ml fraction was collected in a tube,
(1) 0.1 M Formic
(2) 0.1 M Formic, 0.05 M Formate
(3) 0.1 M Formic, 0.1 M Formate
(4) 0.1 M Formic, 0.3 M Formate
(5) 0.1 M Formic, 0.4 M Formate
(6) 0.1 M Formic, 0.5 M Formate
(7) 0.1 M Formic, 1.0 M Formate.

grated to the anode side of the Sample II and authentic ADP was 5.5 cm and 5.6 cm, respectively.

(4) Ultraviolet absorption spectrum: The spot on a paper chromatogram was cut out and extracted with 0.1 n HCl (Fig. 15).

(5) Periodate oxidation: The Sample II was
confirmed to be oxidized by periodate.

6) **Pentose orcinol reaction**: The rate of color development after 10 min. with the Sample II was 100%, and that with authentic ADP was 100%.

7) **The ratio of the base component to the phosphate component**: The ratio was 0.54.

**DISCUSSION**

It was found that the adenine-requiring mutant, *B. subtilis* 1145–2–83, responded to 5'-AMP, while a 5'-nucleotidase-lacking mutant derived by means of total isolation method did not respond to 5'-AMP. From the above fact, it is conceivable that *B. subtilis* 1145–2–83 has permeability barrier to 5'-AMP.

The authors established a technique to get 5'-nucleotidase-lacking mutants from *B. subtilis* 1145–2–83 basing on the facts; (1) the adenine-requiring mutants which do not respond to 5'-AMP are regarded as 5'-nucleotidase-lacking mutants, and (2) such mutants can be easily selected by applying replica plating technique in which adenine-containing medium and 5'-AMP-containing medium are used. This technique may be applied to various kinds of microorganisms having the permeability barrier to nucleotide.

There is a possibility that some of 5'-nucleotidase-lacking mutants may accumulate 5'-nucleotides in their culture medium. Actually mutant R-42 accumulated 5'-GMP and 5'-IMP, and A-1 accumulated 5'-IMP. In both cases, much more amount of inosine was also accumulated. As 5'-IMP was not dephosphorylated in their culture media, the inosine is suggested to have been excreted directly from the cells. Large amounts of 5'-IMP and 5'-GMP may be expected to accumulate by using a mutant whose cellular 5'-nucleotidase is lower than that of A-1 or R-42.

It is also interesting that phosphorylation of 5'-AMP and reduction of 5'-GMP were observed in the culture medium of a 5'-nucleotidase-lacking mutant.
The above properties of adenine-requiring and 5'-nucleotidase-lacking mutants are now being studied in detail.

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