GENETIC AND BIOCHEMICAL STUDIES ON 5'-NUCLEOTIDE FERMENTATION

I. NUCLEOTIDE DEGRADING ABILITY OF *BACILLUS SUBTILIS* AND DERIVATION OF MUTANTS DEVOID OF THIS ABILITY

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The extracellular formation of 5'-nucleotides by micro-organisms has been studied extensively in our country from the industrial point of view (1-4). UCHIDA *et al.* reported the accumulation of 5'-IMP by an adenine requiring mutant derived from a wild strain of *Bacillus subtilis* (5). However, it is well known that the amount of nucleotides accumulated is, in general, far less than that of nucleosides or bases. Therefore, it is believed that the so called "5'-nucleotide fermentation" has a more complicate problem to be solved. Microorganisms prevent, in general, the excess formation and excretion of intermediary metabolites by their own cell regulatory mechanisms which are chiefly connected with the systems of (a) degradations, (b) biosynthesis and (c) transports of metabolites (6).

The authors attempted, in this connection, to analyze and to exclude genetically the unfavorable factors in the phenomenon of 5'-nucleotide fermentation using lines of *B. subtilis* as a model. The present paper deals with the analysis of the ability of nucleotide degradation in *B. subtilis* and the derivation of mutants devoid of such ability.

MATERIALS AND METHODS

Bacterial strains. *Bacillus subtilis* strain K (7) and strain M (Marburg strain) and their auxotrophic mutants were used (see Table 1). Several mutants of strain M lacking alkaline phosphatase (V-11, V-12, AP-1) were also used as the references in an experiment. V-11 and V-12 were kindly supplied by Mr. T. MUKI of the Institute of Applied Microbiology, University of Tokyo. AP-1 was derived by X-ray induced mutation by Mr. K. SANO of our laboratory.

Media. Four media were employed. The CM medium contained 10 g Polypeptone, 10 g meat extract, 1 g yeast extract, and 3 g NaCl in 1,000 ml tap water. The pH was 7.2 after sterilization. The MM medium contained 8.5 g KH_2PO_4 , 2.3 g KOH, 0.5 g Na-citrate $\cdot$ 2H $_2$ O, 1 g $(\text{NH}_4)_2\text{SO}_4$, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 5 g glucose in 1,000 ml distilled water. The pH was 7.2 after sterilization. Appropriate reagents were added to this medium, when

necessary (the supplemented MM medium). The composition of the Tris-MM medium was the same as that of the MM medium except that KH_2PO_4 and KOH were replaced by 2.9 g tris-(hydroxymethyl)-amino-methane and HCl as a buffer system, and appropriate amounts of KH_2PO_4 (2–1,000 $\mu\text{g}/\text{ml}$) was supplemented to adjust the phosphate concentration. The M-1 medium was comprised of 5 g KH_2PO_4 , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6 g urea, 2 g NaNO_3 , 2 ppm Fe^{++} , 2 ppm Mn^{++} , 5 g Polypeptone, 5 g yeast extract, 50 g glucose, 20 g CaCO_3 (sterilized separately) and 1,000 ml distilled water. The pH was 7.0 after sterilization. The CM, MM and M-1 media were chiefly employed as complete medium, minimal medium and fermentation medium respectively.

Genetic techniques. The derivation of mutants was carried out chiefly by X-ray irradiation. For genetic transformation, DNA was prepared from wild strain M or 116 Nia⁻ by the method of LEDERBERG *et al.* (8), and transformant colonies were obtained according to SAITŌ *et al.* (9). For transduction, phage cp-1, a substrain of transducing phage sp-10 which was kindly provided by Prof. IKEDA, was employed. The phage suspensions were prepared by the method of ADAMS (10) using a sensitive wild type strain W-23, and transductant colonies were obtained according to TAKAGI's method (11).

Measurement of 5'-nucleotide degrading activity in intact cells. Cells were obtained by cultivating the test strains for 16–20 hr in CM medium or Tris-MM medium. Cell suspensions were prepared by washing the intact cells with distilled water and resuspending in it. The reaction mixture contained 0.25 ml of the cell suspension (containing 1–1.5 mg cells as dry matter), 5 μmoles of 5'-nucleotide, and 5 μmoles of MgCl_2 in 1 ml (final volume) of 0.25 M buffer solution. Citrate-, acetate-, tris- and glycine buffers were used for the various pH values. The specific activity was expressed as nucleoside or inorganic phosphate formed ($\mu\text{moles}/\text{ml}/\text{mg}$ dry matter/hr). Besides the above method, the "culture test" was also applied to detect the 5'-nucleotide degrading ability: 5'-nucleotide was added to the culture broth of each test strain after 20 hr precultivation at 30° in M-1 medium and the rate of decrease of nucleotide or formation of nucleoside was measured under the same culture conditions. By such method, the activity of degrading enzyme(s) excreted into the culture medium, if any, can also be detected.

Measurement of alkaline phosphatase activity. The method was the same as that in 5'-nucleotide degrading activity except that the 5'-nucleotide was replaced by *p*-nitrophenyl phosphate (NPP) as the substrate and that the pH was fixed at 8.6 in glycine-NaOH buffer solution. The specific activity was defined as the inorganic phosphate formed ($\mu\text{moles}/\text{ml}/\text{mg}$ dry matter/hr). For the experiment reported in Table 5, the method of TORRIANI *et al.* (12) was applied with some modifications: 1 ml of the cell suspension was mixed with 2.5 ml of a solution of 1 M tris buffer, pH 8.0, containing 1.5 mg NPP/ml and the rate of change of optical density at 410 m μ at 25°

was measured. The specific activity was defined as $10^3 \Delta E_{410m\mu}/\text{ml}/\text{unit}$ of cell density (the cell density unit is an optical density of 1.0 at $540 m\mu$ for the original culture).

Fractionation of cell compositions. To investigate the localization of 5'-nucleotide degrading ability of *B. subtilis*, the following fractionation of cell components was carried out. Cells were harvested by cultivating the test strain for 16 hr in 80 ml of CM medium in 500 ml flask at 30° . After washing with distilled water, the cells were resuspended in distilled water (fraction C_1) and 10% sucrose (fraction C_2) at the concentration of 100 mg

Fig. 1. Fractionation of cell compositions.

wet cells/ml. Aliquots were then withdrawn from fraction C_1 and C_2 respectively, and lysozyme was added to give a concentration of $500 \mu\text{g/ml}$, and the suspensions were stirred at 37° for 30–120 min. The viscous lysate prepared from C_1 was designated as fraction L. When protoplast formation was detected by microscopic observation in the lysozyme treated C_2 fraction, the suspension was centrifuged at $3,000 \times g$ for 15 min, and the supernatant was separated (fraction S_1) from the protoplasts pellet. The protoplasts were divided into three portions after washing once with 10% sucrose. The 1st portion was resuspended in fraction S_1 (fraction $P-S_1$) and the 2nd portion was resuspended in 10% sucrose (fraction P). The last portion was resuspended in distilled water, and osmotic shockate (fraction O) was prepared by vigorous shaking at 37° followed by homogenizer treatment. Fraction S_1 was further centrifuged at $10,000 \times g$ for 20 min, and the 2nd supernatant (S_2) and debris (D) obtained. Each fraction was prepared from an equal volume of intact cells. 5'-Nucleotide degrading activities in these fractions were measured by the same method as in intact cells.

Measurement of growth in liquid medium. Cells were harvested from 16 hr cultures of the test strain in CM medium and, after washing once, inoculated into 7 ml of the supplemented MM medium at the concentration of ca. 5×10^7 cells/ml, and cultivation was carried out in MONOD tubes on a seesaw-type shaking machine at 37°. Growth was determined periodically by measuring the cell turbidity using a SHIMADZU-KOTAKI photoelectric colorimeter.

Accumulation of nucleotides and their derivatives. Cells of the test strain were inoculated into 20 ml of M-1 medium at the concentration of ca. 5×10^7 cells/ml and cultivation was carried out at 30° using a 500 ml flask on a reciprocal shaker. Aliquots were withdrawn periodically from the culture and centrifuged. Nucleotides, nucleosides and bases in the supernatants were subjected to analysis.

Identification and determination. Nucleotides, nucleosides and bases were identified with authentic samples by means of paper chromatography (7), column chromatography (14), paper electrophoresis and U.V. absorption spectra. Determination of these substances was carried out by paper chromatography and column chromatography. Inorganic phosphate was assayed by ALLEN's method (15).

RESULTS

5'-Nucleotide degrading abilities in various strains of B. subtilis K and M

Some mutants derived from *B. subtilis* K have the ability of producing a large amount of purine derivatives such as inosine and/or hypoxanthine (7) instead of 5'-IMP. Some mutants of strain M also produce hypoxanthine (see Table 1). These facts suggest that both strains K and M have the potential of degrading 5'-nucleotides to nucleosides and/or bases. This was confirmed by a preliminary experiment in which 0.4% of 5'-nucleotides,

Table 1. Difference in 5'-IMP degrading activity between *Bacillus subtilis* strains K and M.

Strain	Genotype	Specific activity ^a	Accumulation ^b
K	K wild type	1.1	—
	No. 2 ade-	1.2	Hypoxanthine
	B-4 ade- his-	1.0	Inosine
	C-30 ade- his- tyr-	0.7	Inosine
M	M wild type	<0.1	—
	116 nia-	0.2	—
	160 Sr try-	<0.1	—
	MX-59 try- ade-	0.1	Hypoxanthine

^a Inosine plus hypoxanthine formed (μ mole/ml/mg dry matter/hr). Intact cells harvested from 16 hr culture in 50 ml of CM medium were used.

^b After 3 days cultivation at 30° in M-1 medium.

5'-AMP, 5'-GMP, 5'-IMP, 5'-CMP and 5'-UMP, added to the 20 hr culture of various mutants derived from K and M, were found to be completely degraded to their nucleosides and bases at 30° within 24 hr (see *Materials and methods*). Table 1 shows such activities in intact cells harvested from

Fig. 2. Repressive effect of inorganic phosphate on formation of 5'-IMP degrading enzyme(s) in *Bacillus subtilis* M.

Intact cells harvested from 20 hr culture in 50 ml of Tris-MM containing 25 μ g/ml L-tryptophan and 2 μ g/ml KH_2PO_4 (low phosphate) or 1,000 μ g/ml KH_2PO_4 (high phosphate) were used.

Fig. 3. Repressive effect of inorganic phosphate on formation of alkaline phosphatase in *Bacillus subtilis* M.

Intact cells harvested from 20 hr culture in 50 ml of Tris-MM containing 25 μ g/ml L-tryptophan and 2 μ g/ml KH_2PO_4 (low phosphate) or 1,000 μ g/ml KH_2PO_4 (high phosphate) were used.

16 hr cultures in the CM medium using 5'-IMP as the substrate. It was found that all strains belonging to M had lower activities (one tenth or less) compared with the strains belonging to K, although strain M had enough ability to degrade 0.4% 5'-nucleotide in the cultures within 24 hr. The difference in activity between M and K was analyzed in connection with the repressive effect of inorganic phosphate on the formation of 5'-nucleotide-cleaving enzymes, some of which seemed to split 5'-nucleotide to nucleoside and phosphate. In the experiment shown in Fig. 2, formation of the cleaving enzyme(s) in strain M had largely induced to the levels of strain K in response to low level of inorganic phosphate (2 μ g/ml) in the growth medium. 1,000 μ g/ml of phosphate, which was the same amount as in the CM medium, repressed the formation. Strain 160 S^r (tryptophan-less mutant) had half the activity of the wild strain M which was also repressed by 1,000 μ g/ml phosphate as in the case of strain M. Such a repressive effect of inorganic phosphate on 5'-nucleotide degradation in the lines of strain M resembles that on alkaline phosphatase of the same lines (16). Therefore, the comparison between the 5'-nucleotide degrading activity and alkaline phosphatase activity was attempted using strain M and 160 S^r. The result confirmed that both activities were simultaneously repressed by phosphate. An apparent difference was found, however, in that alkaline phosphatase was almost completely repressed by 1,000 μ g/ml phosphate, while on the other hand, a little activity was still remained in the 5'-nucleotide degradation under the same condition (Fig. 2 and 3). Thus, it might be considered that strain M possessed the other 5'-nucleotide-cleaving enzyme(s) as well as alkaline phosphatase. This assumption will be supported in the later section.

Localization of 5'-nucleotide degrading activity in cell compositions

To investigate the localization of 5'-nucleotide degrading activity of *B. subtilis*, activities were compared in several cell fractions prepared from the intact cells of strain C-30, which was one of the inosine producing mutants of K (7). The methods were illustrated in detail in "Materials and methods" as well as in Fig. 1. Figs. 4a and 4b show the results with the intact cells. Strain C-30 degraded 5'-IMP to inosine rapidly (Fig. 4a). The optimum pH of this reaction was in the range of 8-9 (Fig. 4b). This pattern is similar to that of alkaline phosphatase (17). Fig. 5 shows the activities at pH 8.6 in every cell fractions. The activity of intact cells (C₁ or C₂) was comparable to that of the total lysate (L). An interesting result was that protoplasts (P) prepared from intact cells by lysozyme treatment were almost devoid of the activity, all of which appeared in the supernatant (S₁ or S₂). The activity which appeared by preparing osmotic shockate (O) from protoplasts was comparatively low in contrast to that in the supernatant. From these results, it may be concluded that almost all of the activity of 5'-nucleotide degradation is localized at least outside the cell membrane of C-30 cells.

Fig. 4. Degradation of 5'-IMP by C-30 intact cells (a, b).

(a) Reaction mixture (1ml) contained 5 μmoles 5'-IMP, 5 μmoles MgCl_2 , 250 μmoles tris buffer, pH 8.6, and 1.3mg intact cells (as dry matter). ● 5'-IMP; ○ inosine plus hypoxanthine.

(b) ○ Acetate buffer; ● tris buffer; ▲ glycine buffer.

Fig. 5. Loss of 5'-IMP degrading activity by treatment of intact cells with lysozyme.

1 ml of reaction mixture (C₁ or C₂) contained 4.5mg of C-30 intact cells (as dry matter) prepared from 16hr culture in CM medium. L, S₁, S₂, P-S₁, O, P and D are cell fractions indicated in Fig. 1.

Isolation of mutants devoid of 5'-nucleotide degrading ability

It seems almost impossible to accumulate 5'-nucleotide in the culture medium by strains which possess such degrading activity as shown in previous sections, even if these strains possess the potential of 5'-nucleotide synthesis.

Attempts were made to isolate the mutants devoid of such activity from the lines of strains K and M, and to determine whether the genetic change brings about a characteristic favorable to 5'-nucleotide accumulation. The authors succeeded in obtaining such mutants from one of the purine auxotrophs by isolating secondary mutants which showed growth response to nucleoside but not to nucleotide using replica plating technique. The principle of such mutant derivation is based on the following two assumptions: (A) nucleotide degrading activity is located at the surface of the cell, and (B) the rate of uptake of nucleotides into the cell is lower than that of nucleosides or bases. Assumption (A) is strongly supported by the experiment reported in the previous section. Assumption (B) is also supported indirectly by the different growth response pattern to 5'-IMP between MX-9 and MX-129, both of which are purine auxotrophs (Figs. 6a and 6b). MX-129 derived from 160Sr had about half the activity of MX-9 derived from strain M in 5'-nucleotide degradation, and this difference was well reflected in the growth rate in the liquid medium containing 0.2 μ moles/ml

Fig. 6. Growth response of mutant strains to 5'-IMP and inosine.

Additions to MM medium: T, L-try 25 γ /ml; I, L-try+inosine 0.2 μ mole/ml; J-0.2, J-0.6 and J-2, L-try+5'-IMP 0.2 μ moles/ml, 0.6 μ moles/ml and 2 μ moles/ml respectively.

Lineage of mutants used: Strain M $\rightarrow$ MX-9
 $\rightarrow$ 160Sr $\rightarrow$ MX-129 $\rightarrow$ MX-46 $\rightarrow$ MX-97

of 5'-IMP. It should be expected, therefore, that the mutants, if isolated, devoid of activity to degrade 5'-IMP, showed no growth response to 5'-IMP of the above concentration but showed normal growth response to inosine of the same molar concentration.

Sixtyseven such mutants were isolated from strain MX-129 by X-ray irradiation. Among them, four strains were regarded as the mutants almost devoid of 5'-IMP degrading activity by the "culture test", 18 strains partially lost such activity. The other 45 strains as well as many mutants which showed slight response to 5'-IMP could not be distinguished from the parent strain MX-129 because 0.4% 5'-IMP added to the culture was completely degraded within 24 hr. The growth response of the typical mutant MX-46 was indicated in Fig. 6c. MX-46 showed no growth response to 0.2 μ moles/ml of 5'-IMP although it showed slight response to 0.6 and 2 μ moles/ml of 5'-IMP respectively. The other three mutants showed the same growth pattern as MX-46. Fig. 6d shows the case in a secondary mutant MX-97

Table 2. Induction of mutants lacking 5'-IMP degrading activity.

Genetic derivation of mutants	Genotype ^a	Growth response ^b		
		Try	Try+inosine	Try+5'-IMP
160 Sr	try-	+	+	+
↓ X-rays irradiation				
MX-129	try- pur-	-	+	+
↓ X-rays irradiation				
MX-46	try- pur- J ⁿ	-	+	-
↓ X-rays irradiation				
MX-97	try- pur- J ⁿ J ^u	-	+	+
↓ Transformation by wild type-DNA				
MT-16	try- J ⁿ J ^u	+	+	+

^a Abbreviations: Jⁿ, cannot degrade 5'-IMP; J^u, can utilize 5'-IMP for growth.

^b The amounts of nutrients added to MM plates: Try, 25 μ g/ml; inosine, 0.2 μ mole/ml; 5'-IMP, 0.2 μ mole/ml.

derived from MX-46, which showed slight response even to 0.2 μ moles/ml of 5'-IMP. MX-97 seems to be a mutant altering the permeability of 5'-IMP into the cell, because MX-97 also possessed no 5'-IMP degrading activity as MX-46. The genetic derivation of mutants described above were summarized in Table 2.

Characterization of strain MX-46

Fig. 7 shows the pH *versus* 5'-IMP degrading activity curves obtained with MX-46 and wild strain M. It was observed that MX-46 had lost the activity through all the pH range, although about 10% of the activity of wild strain still remained. The defect of the activity was non-specific to all purine and pyrimidine nucleotides tested (Table 3). Various interpretations are possible as to the mutational event occurring in MX-46. One

possibility is that a non-specific phosphatase, such as alkaline phosphatase, was lost in activity. To test the possibility, MX-46 was compared with the original strains MX-129, 160 S^r and M in the formation of 5'-IMP cleaving

Fig. 7. Defect of 5'-IMP degrading activity in MX-46.

Intact cells harvested from 20 hr culture in 50 ml of Tris-MM containing 25 μ g/ml L-tryptophan, 50 μ g/ml inosine and 2 μ g/ml KH_2PO_4 (low phosphate) were used.

Table 3. Non-specific loss of nucleotide degrading activity in MX-46.

Substrate	Specific activity (% of control)
5'-IMP	8.3
5'-GMP	2.9
5'-AMP	10
3'(2')-AMP	8.3
5'-UMP	2.5
5'-CMP	1.7

The specific activity of strain M is indicated as 100%.

enzyme(s) as well as alkaline phosphatase. As shown in Table 4, MX-46 was devoid of both activities coordinately (one fifth of MX-129, one tenth of wild strain). Moreover, both residual activities in MX-46 were found simultaneously repressed by phosphate. These facts indicate an intimate relationship between the two activities. However, it was also found that three alkaline phosphatase negative mutants, V-11, V-12 and AP-1, possessed about 15-20% activity of the wild strain in 5'-nucleotide degradation in spite of complete loss of alkaline phosphatase activity (Table 5). From these results, it seems probable

that MX-46 had lost 5'-nucleotide degrading activity not by a single defect of alkaline phosphatase activity, but by simultaneous defects of at least two or more kinds of enzymatic activities caused by one mutational event.

Accumulation of nucleotides by mutants

The mutants described in previous sections seem to have acquired the favorable characteristic for 5'-nucleotide accumulation in the culture medium. To test this point, the following two experiments were carried out.

In the first experiment, a pur⁺-transformant, MT-16, was derived from

Table 4. Activities of nucleotide degradation and alkaline phosphatase in mutants of strain M.

Strain	Genotype	Specific activity ^a			
		Nucleotide degradation ^b		Alkaline phosphatase	
		Low Pi	High Pi	Low Pi	High Pi
M	Wild type	2.39	0.41	1.57	<0.1
160 S ^r	try ⁻	1.11	0.15	0.87	<0.1
MX-129	try ⁻ pur ⁻	1.12	0.20	1.02	<0.1
MX-46	try ⁻ pur ⁻ J ⁿ	0.20	<0.10	0.16	<0.1

^a Inorganic phosphate formed (μ moles/ml/mg dry matter/hr). Intact cells harvested from 20 hr culture in 50 ml of Tris-MM containing 25 μ g/ml L-try and 2 μ g/ml KH_2PO_4 (low Pi) or 1,000 μ g/ml KH_2PO_4 (high Pi) were used.

^b 5'-IMP was used as the substrate.

Table 5. Degradation of 5'-IMP by mutants lacking alkaline phosphatase.

Strain			Specific activity (% of wild type)	
			alkaline phosphatase ^a	5'-IMP degradation ^b
M	Wild	type	100	100
160 S ^r	try ⁻		55	42
AP-1	AP ⁻		0.6	22
V-11	arg ⁻	AP ⁻	0.4	14
V-12	arg ⁻	AP ⁻	0.3	17
MX-46	try ⁻	pur ⁻ J ⁿ	7.2	6

^a Assayed by the method of Torriani *et al.* with some modifications (see text). Intact cells used were those from 20 hr culture in 50 ml of Tris-MM containing 25 μ g/ml L-try and 2 μ g/ml KH_2PO_4 (low phosphate).

^b The rate of 5'-IMP degradation per 10^{10} cells under shaking condition at pH 8.6 was determined after the addition of 0.4% 5'-IMP to the 20 hr culture at 30° in 3 ml M-1 medium (KH_2PO_4 , 5,000 γ /ml).

MX-97 by wild type-DNA (see Table 2), and nucleotide accumulation was checked in a 3 day-old culture. Fig. 8 indicates one of the paper chromatographic results, in which MT-16 showed several U.V. absorbing spots at the R_f region corresponding to nucleotides. In contrast to MT-16, strains MX-129, 160 S^r and M showed no spots at the same R_f region, but rather showed several spots at the R_f region corresponding to nucleosides and bases. The main substances accumulated in the culture of MT-16 were identified as AMP, GMP, CMP and UMP (0.37, 0.30, 0.08 and 0.14g/liter respectively) by means of column chromatography using Dowex-1 $\times$ 4 formate type (Fig. 9) and other optical observations (see *Materials and methods*).

From this experiment, it is concluded that MT-16 had excreted the four kinds of mononucleotides into the culture and preserved them from degradation at least for three days. The formation of such mononucleotides described above might be due to the *de novo* synthesis of purine and pyrimidine and/or the decomposition of the preformed RNA (18).

In the second experiment, adenine requiring mutants were further

Fig. 8. Paper chromatogram of the accumulated substances.

Cultivation was carried out for 3 days in M-1 medium at 30°. 0.05 ml of the supernatant of each mutant culture was used as a sample. Solvent system used: n-propanol, ammonia and water (20:15:3). Authentic samples indicated: 1, 5'-AMP; 2, 5'-GMP; 3, 5'-CMP; 4, 5'-UMP; 5, 5'-IMP; 6, adenine; 7, hypoxanthine; 8, cytosine; 9, uracil; 10, inosine.

Fig. 9. Gradient elution of nucleotides in the 4 ml culture fluid of strain MT-16.

MT-16 is a pur⁺-transformant derived from MX-97 by wild type-DNA. Column: Dowex 1×4 formate type, 200—400 mesh, 1×10 cm.

derived from MT-16 and the possibility of IMP accumulation was checked. The results of this experiment are reproduced in Table 6. MX-59, an adenine requiring mutant derived from 160 S^r as the reference of this experiment, accumulated hypoxanthine instead of IMP owing to its nucleo-

Table 6. Accumulation of IMP derivatives by mutants of strain M.

Genetic derivation of mutants	Genotype	Accumulation (g/liter)	
		Hypoxanthine	IMP
X-rays — 160 S ^r — X-rays	try ⁻	<0.1	<0.1
MX-59	try-ade ⁻	2.2	<0.1
→ MX-129	try-pur ⁻	—	—
↓ X-rays	try-pur-J ⁿ	—	—
MX-46	try-pur-J ⁿ	—	—
↓ X-rays	try-pur-J ⁿ	—	—
MX-97	try-J ⁿ	<0.1	<0.1
↓ Transformation ^{a)}	try-J ⁿ ade ⁻	<0.1	<0.1
MT-16	J ⁿ ade ⁻	1.8	0.25
↓ X-rays			
MX-7			
↓ Transduction ^{b)}			
MT-11			

a) Transformed by pur⁺nia-DNA extracted from strain 116.

b) Transduced by phage cp-1, a substrain of sp-10.

tide degrading ability. MX-7, which was derived from MT-16 and also one of adenine requiring mutants, accumulated no hypoxanthine or inosine as well as no IMP. However, MT-11, a try⁺-transductant derived from MX-7, accumulated small amounts of IMP together with hypoxanthine. It was found later that MT-11 had acquired one tenth of the degrading activity of the wild type by the transduction of tryptophan prototrophy from strain W-23. From this experiment, it might be considered that some inhibitory action against IMP accumulation was brought about by the defect of nucleotide degrading activity and the partial restoration of activity rather made it possible to accumulate IMP although it was gradually degraded to hypoxanthine.

DISCUSSION

A good deal of work has been done on the bacterial phosphatases which cleave organic phosphate esters such as 5'-nucleotides (19, 20). MISAWA *et al.* (21) have studied the acid and alkaline phosphatase activities in intact cells using 14 different species and have concluded that there are considerable differences between these species in the levels of activity and repression by inorganic phosphate.

In the species of *B. subtilis*, too, our experiments showed remarkable differences in the levels of repression by inorganic phosphate on the formation of nucleotide cleaving enzyme(s) between strain K and strain M and even between the mutants belonging to M. The occurrence of mutants such as MX-46 enables us to postulate that some mutational event(s) can bring about higher levels of repression by phosphate which simultaneously acts on the formations of alkaline phosphatase and the other one or more nucleotide cleaving enzymes. In this connection, the presence of a common regulatory mechanism on the formation of phosphatases in strain M reported by HIROTA *et al.* (22) and GORINI's idea of "super repressed mutation" (23) are very suggestive to us. Although further enzymatic and genetic analysis would be necessary for investigation, mutants such as MX-46 may become a good tool for the investigation of the physiological role of bacterial phosphatases in connection with the problem of enzyme localization. Nucleotide degrading activity was suggested to be localized outside the cell membrane in the experiment using strain C-30. A similar finding was reported in the alkaline phosphatase of *E. coli* (24). These facts were interesting in connection with the general problem of the difficulty of organic phosphate ester uptake to the cell.

Finally, the accumulation phenomena observed in several mutants isolated constituted the most important problem. Although MT-16 has the ability of accumulating small amount of mononucleotides in the culture, an adenine requiring mutant, MX-7, derived from MT-16, has no ability of IMP accumulation caused by the *de novo* synthesis of IMP, and MT-11, a try⁺-transductant from MX-7, which has partially acquired the degrading activity, accumulates IMP together with hypoxanthine. These conflicting observations suggest that the defect of degrading activity is favorable for the reservation of the preformed nucleotides in the culture, but unfavorable for the *de novo* synthesis or excretion of nucleotides. A possible interpretation is that the defect of activity directly causes the high levels of nucleotide pools in the cytoplasm which strongly repress the formation of enzymes belonging to purine and pyrimidine biosynthesis provided that, as in the case of feed-back inhibitor (25), the active forms of the co-repressors are nucleotides (26). The presence of a strong regulatory mechanism of purine biosynthesis was found in strain MT-16 which will be reported in a following paper.

SUMMARY

Both strains K and M, belonging to *B. subtilis*, were found to possess 5'-nucleotide degrading activity. However, strain M was characterized by the fact that the formation of degrading enzyme(s) was well repressed by inorganic phosphate in contrast to strain K. The strong activity in strain C-30, an inosine producing mutant belonging to K, was suggested to be localized outside the cell membrane.

A mutant MX-46, which was derived from a purine auxotroph belonging to M, showed no growth response to $0.2 \mu\text{moles/ml}$ of 5'-IMP and has non-specifically lost 90% of the nucleotide degrading activity as well as alkaline phosphatase activity of the wild strain. On the other hand, alkaline phosphatase negative mutants derived independently from strain M still possessed 15-20% of nucleotide degrading activity of the wild strain. Therefore, it was concluded that MX-46 was different from normal alkaline phosphatase negative mutants.

A pur^+ -transformant MT-16, derived from MX-46 via MX-97, accumulated four mononucleotides in the culture medium. An adenine requiring mutant, MX-7, derived from MT-16, did not accumulate IMP, and a try^+ -transductant MT-11 derived from MX-7, which had partially acquired the degrading activity, accumulated IMP together with hypoxanthine. The relationship between the bacterial degrading activity and the accumulation of nucleotide was discussed in view of these observations.

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