Enzymatic Production of 5-Methyluridine from Purine Nucleosides and Thymine by *Erwinia carotovora* AJ-2992

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Microorganisms that produce 5-methyluridine (ribothymidine) directly from purine nucleosides and thymine were screened from our stock cultures. Of the 400 strains tested, *Erwinia carotovora* AJ-2992 was found to possess the most potent ability as to production of 5-methyluridine from guanosine and thymine. In the presence of intact cells of *Er. carotovora* AJ-2992 as the enzyme source, 222 mM 5-methyluridine was produced from 300 mM guanosine and 300 mM thymine at 60°C on 48 hr incubation. The enzymatic production of 5-methyluridine by *Er. carotovora* AJ-2992 was found to involve the following two successive reactions *via* ribose-1-phosphate as an intermediate: phosphorolysis of purine nucleosides to ribose-1-phosphate and purine bases by purine nucleoside phosphorylase, followed by condensation of ribose-1-phosphate and thymine into 5-methyluridine by pyrimidine nucleoside phosphorylase.

It is known that enzymes catalyzing the transglycosylation of nucleosides are widely distributed in eukaryotes and prokaryotes. Through the application of the corresponding reactions, several natural nucleosides and their analogues, such as adenine arabinoside,^{1,2)} ribavirin,^{3~7)} 2',3'-dideoxyadenosine⁸⁾ *etc.*, have been synthesized. Recently, 3'-azido-3'-deoxythymidine (AZT) was found to be effective as a remedy for AIDS⁹⁾ and is now widely used in the world as the sole drug for AIDS. Due to the great demand for AZT, the development of an industrial production system for thymidine, an important intermediate for AZT, has been expected. Thymidine can be obtained by extraction from a hydrolysate of deoxynucleic acids derived from salmon and herring sperm, but this method suffers from the unstable supply of raw materials, the troublesome purification of the product and the intractable waste. With this background, the development of a simple and efficient method for the production of thymidine has been required. On the other hand, thymidine can be obtained through the chemical reduction of 5-methyluridine. Several chemical

methods^{10~12)} were studied as to the synthesis of 5-methyluridine, a precursor of thymidine. However, there remain some practical limitations to these approaches, which are laborious and which give low yields. Recently, Hori *et al.*¹³⁾ reported the enzymatic synthesis of 5-methyluridine from inosine and thymine by a thermophile, *Bacillus stearothermophilus*, but the production yield was rather low. In addition, the mechanism underlying the enzymatic synthesis of 5-methyluridine has not been elucidated yet. With this background, we newly screened more effective microorganisms producing 5-methyluridine from purine nucleosides and thymine at high temperature, and found that *Er. carotovora* AJ-2992 is a potent 5-methyluridine-producer, from guanosine and thymine. Through investigation of the reaction conditions, we constructed an enzymatic system for the effective production of 5-methyluridine.

This paper describes the screening of 5-methyluridine-producing microorganisms, the reaction conditions for 5-methyluridine production and the mechanism underlying 5-methyluridine synthesis by *Er. carotovora* AJ-

2992.

Materials and Methods

Chemicals. Inosine and guanosine were products of Ajinomoto Co., Inc. α -D-Ribose-1-phosphate (R-1-P) dicyclohexyl ammonium salt was purchased from Sigma Chemicals (St. Louis, U.S.A.). All other chemicals used in this study were commercially available and of analytical grade.

Microorganisms and cultivation. Microorganisms from stock cultures kept in our laboratory were subjected to screening. A loopful of cells of each microorganism was inoculated into 5 ml of medium I (pH 7.0), comprising 1% polypeptone (Nihon Pharmaceuticals, Tokyo, Japan), 1% meat extract (Wako Pure Chemical Industries, Osaka, Japan), 0.5% yeast extract (Nihon Pharmaceuticals, Tokyo, Japan) and 0.5% NaCl, and then aerobically cultivated at 30°C for 24 hr. The cells were then harvested by centrifugation at $12,000 \times g$ at 4°C for 10 min and used as the enzyme source. Cells, 50 mg/ml on a wet weight basis, were added to a reaction mixture, comprising 50 mM guanosine, 50 mM thymine and 100 mM potassium phosphate buffer (pH 7.0), followed by incubation at 60°C for 24 hr.

The cultivation of *Erwinia carotovora* AJ-2992, which was selected as the best strain for 5-methyluridine production, was carried out as follows: a loopful of cells of *Er. carotovora* AJ-2992 subcultured on a bouillon agar slant was inoculated into 5 ml of medium I in a test tube. After aerobic cultivation at 30°C for 24 hr, 1 ml of the culture was transferred to 50 ml of medium I in a 500 ml flask, followed by aerobic cultivation at 30°C for 16 hr.

Reaction methods. Cells of *Er. carotovora* AJ-2992 thus obtained were harvested by centrifugation at $10,000 \times g$ at 4°C for 10 min and then rinsed with 50 mM Tris-HCl buffer (pH 8.5). The wet cells thus obtained were used as the enzyme source. The standard reaction mixture, comprising 50 mM Tris-HCl buffer (pH 8.5), 20 mM K_2HPO_4 , 100 mM guanosine, 100 mM thymine and intact cells (50 mg/ml on a wet weight basis), was incubated at 60°C for 24 hr or 48 hr with standing.

Analytical methods. The reacted mixture was centrifuged at $10,000 \times g$ at 4°C for 10 min and then 1.5 μ l of the supernatant was spotted onto a silica gel thin layer chromatography (TLC) plate (Kieselgel 60 F₂₅₄; Merck Japan, Tokyo, Japan), developed with a solvent system of chloroform-methanol (5:1, by volume), and detected qualitatively under UV light. Quantitative determination of nucleosides and bases was carried out by high-performance liquid chromatography (HPLC) under the following conditions: resin, Cosmosil 5C₁₈ (Nakarai Tesque, Kyoto, Japan); column size, 4.6 by 250 mm;

solvent, 5% methanol, 0.14% acetic acid; flow rate, 1.0 ml/min; temperature, 40°C; and detector, UV monitor (254 nm).

R-1-P was detected by TLC under the following conditions: solid phase, Kieselgel 60 F₂₅₄; developing solution, *n*-butanol-acetic acid-water (2:1:1, by volume); color-developing agent, ethanol-*p*-anisaldehyde-acetic acid-sulfuric acid (90:5:1:5, by volume).

Enzyme purification. *Er. carotovora* AJ-2992 was cultivated as described above at 30°C for 16 hr in medium I. All subsequent procedures were performed at 7°C.

(Step 1) Harvested cells from 3 liters of culture were suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 0.2 mM dithiothreitol (DTT) and then disrupted with a sonic oscillator for 60 min in an ice bath. The debris was removed by centrifugation at $15,000 \times g$ at 4°C for 20 min.

(Step 2) The supernatant obtained was kept at 60°C for 60 min in a bath and then centrifuged at $15,000 \times g$ at 4°C for 20 min.

(Step 3) The supernatant obtained was fractionated with solid ammonium sulfate (30~60% saturation), followed by dialysis against the same buffer at 4°C for overnight.

(Step 4) The dialysate was applied to a 3×50 cm DEAE-Toyopearl 650M (Tosoh, Tokyo, Japan) column equilibrated with the same buffer. After washing with the buffer, the enzymes were eluted with a NaCl linear gradient, from 0 to 0.6 M, in the same buffer. Active fractions were collected and the enzyme was concentrated by the addition of solid ammonium sulfate to 60% saturation. The precipitated proteins were collected by centrifugation, dissolved in the same buffer and then applied to a HPLC column, under the following conditions: resin, Butyl-TOYOPEARLPAK 650S (Tosoh, Tokyo, Japan); column size, 2.2 by 20 cm; solvent A, 50 mM potassium phosphate buffer (pH 7.0) containing 0.2 mM DTT and 1.8 M ammonium sulfate; solvent B, solvent A without ammonium sulfate; gradient, 0 to 100% solvent B, 180 min; flow rate, 2.5 ml/min. Active fractions were collected and concentrated as described above. The 5-methyluridine-producing activity was divided into two fractions, A (phosphorolysis of purine nucleosides) and B (phosphorolysis of pyrimidine nucleosides), with these treatments.

Enzyme assays. (i) Assay for the ribosyl transfer reaction. The standard reaction mixture, comprising 10 mM inosine, 10 mM thymine, 20 mM K_2HPO_4 , 50 mM Tris-HCl buffer (pH 8.5) and enzyme in a total volume of 1.0 ml, was incubated at 60°C for 40 min. The reaction was stopped by the addition of 4 ml of 0.1 N HCl. 5-Methyluridine formed was quantitatively determined by HPLC.

(ii) Assay for phosphorolysis of inosine and 5-methyluridine. The reaction mixture, comprising 10 mM inosine or 5-methyluridine, 100 mM potassium phosphate buffer

(pH 7.0) and enzyme in a total volume of 1.0 ml, was incubated at 30°C for 10 min. The reaction was stopped by the addition of 4 ml of 0.1 N HCl and the hypoxanthine or thymine formed was determined by HPLC.

The protein concentrations were determined with a Protein Assay Kit (Japan Bio-Rad Laboratories, Tokyo, Japan), with bovine serum albumin as a standard.

Results

Screening of 5-methyluridine-producing microorganisms

A total of 400 strains belonging to 40 genera, selected from our stock cultures, were tested as to their ability to produce 5-methyluridine from guanosine and thymine. In the first screening, 268 strains were found to possess 5-methyluridine-producing ability by means of the TLC method (Table I). In particular, strains belonging to genera *Erwinia*, *Flavobacterium* and *Micrococcus* produced 5-methyluridine in high yields. The amounts of 5-methyluridine produced by the selected strains belonging to genera *Erwinia*, *Flavobacterium* and *Micrococcus* are presented in Table II (the second screening).

Optimal reaction conditions

The optimal reaction conditions were investigated using intact cells of *Er. carotovora* AJ-2992. The optimal pH for 5-methyluridine production from guanosine and thymine was found to be about 8.5, the activity being observed in a wide pH range, especially in the alkaline region (Fig. 1). The optimal temperature was around 60°C. The optimal phosphate concentration for 5-methyluridine production was 20 mM, but the change in the activity level was only slight in the phosphate concentration range tested (Fig. 2). The effect of the substrate concentrations on 5-methyluridine production was investigated in the presence of various concentrations of guanosine and thymine under the optimal conditions mentioned above. The amount of 5-methyluridine formed in the reaction mixture increased in proportion to the amounts of guanosine and thymine added. The best conversion yield was obtained when 300 mM guanosine and 300 mM thymine were

Table I. DISTRIBUTION OF 5-METHYLURIDINE-PRODUCERS

Reaction mixtures, comprising 50 mM guanosine, 50 mM thymine, 100 mM potassium phosphate buffer (pH 7.0) and 50 mg/ml cells, on a wet weight basis, were incubated at 60°C for 24 hr.

Genus	No. of strains tested	No. of 5-methyluridine producers		
		++	+	±
<i>Achromobacter</i>	32	5	6	6
<i>Acinetobacter</i>	2	0	0	1
<i>Aeromonas</i>	2	1	0	0
<i>Agrobacterium</i>	4	3	1	0
<i>Alcaligenes</i>	13	0	0	4
<i>Arthrobacter</i>	18	6	4	2
<i>Azotobacter</i>	2	0	0	0
<i>Bacillus</i>	31	19	6	0
<i>Beijerinckia</i>	1	0	0	0
<i>Brevibacterium</i>	27	7	2	8
<i>Cellulomonas</i>	4	4	0	0
<i>Citrobacter</i>	6	1	1	4
<i>Corynebacterium</i>	21	0	0	1
<i>Escherichia</i>	30	2	11	14
<i>Enterobacter</i>	3	1	1	0
<i>Erwinia</i>	41	37	1	0
<i>Flavobacterium</i>	14	5	4	5
<i>Hafnia</i>	1	0	0	1
<i>Klebsiella</i>	4	1	0	3
<i>Kluyvera</i>	5	0	2	3
<i>Kurthia</i>	1	0	0	1
<i>Microbacterium</i>	4	2	0	2
<i>Micrococcus</i>	16	10	0	4
<i>Mycoplana</i>	4	0	0	3
<i>Nocardia</i>	11	0	0	6
<i>Planococcus</i>	2	2	0	0
<i>Protaminobacter</i>	3	2	0	0
<i>Proteus</i>	11	6	0	2
<i>Propionibacterium</i>	1	0	0	1
<i>Pseudomonas</i>	25	2	0	0
<i>Rhizobium</i>	4	0	0	2
<i>Rhodococcus</i>	13	6	0	2
<i>Salmonella</i>	4	0	0	4
<i>Sarcina</i>	7	6	0	1
<i>Serratia</i>	12	2	2	6
<i>Sporosarcina</i>	1	0	0	1
<i>Staphylococcus</i>	1	1	0	0
<i>Streptomyces</i>	11	1	0	3
<i>Vibrio</i>	4	1	1	0
<i>Xanthomonas</i>	4	2	0	1

++, more than 15 mM; +, 5 to 15 mM; ±, less than 5 mM.

added to the reaction mixture. When 500 mM guanosine and thymine were used, the amount of 5-methyluridine formed increased, but the

Table II. SCREENING OF MICROORGANISMS
HAVING POTENT ABILITY AS TO 5-
METHYLURIDINE PRODUCTION

Reaction mixtures, comprising 100 mM guanosine, 100 mM thymine, 100 mM potassium phosphate buffer (pH 7.0) and 50 mg/ml cells, on a wet weight basis, were incubated at 60°C for 3 hr or 24 hr.

Strains		5-Methyluridine formed (mM)	
		3 hr	24 hr
<i>Erwinia carotovora</i>	AJ-2948	38.0	53.0
	AJ-2956	25.3	56.8
	AJ-2958	31.1	55.7
	AJ-2960	19.7	54.5
	AJ-2964	25.1	51.1
	AJ-2965	29.7	57.7
	AJ-2990	27.8	58.2
	AJ-2992	47.1	61.6
<i>Flavobacterium arborescens</i>	AJ-2450	10.6	45.1
<i>Flavobacterium rhenanum</i>	AJ-2468	28.6	35.6
<i>Flavobacterium esteroaromaticum</i>	AJ-2471	14.4	32.7
<i>Micrococcus luteus</i>	AJ-1001	16.2	53.7

Table III. EFFECT OF THE SUBSTRATE CONCENTRATIONS
ON 5-METHYLURIDINE PRODUCTION BY
Erwinia carotovora AJ-2992

Reaction mixtures, comprising guanosine and thymine, as indicated in the the table, 20 mM K_2HPO_4 , 50 mM Tris-HCl buffer (pH 8.5) and 50 mg/ml cells, on a wet weight basis, in a total volume of 4 ml, were incubated at 60°C for 48 hr with standing.

Substrate concentration (mM)		5-Methyluridine formed (mM)	Conversion yield (mol%)
Guanosine	Thymine		
50	50	23.9	47.8
100	100	52.0	52.0
200	200	107.7	53.9
300	300	181.0	60.3
500	500	275.7	55.1

conversion yield decreased (Table III).

Time course of 5-methyluridine production

Figure 3 shows the time courses of 5-methyluridine production from guanosine and thymine with shaking (100 ml-scale). The amount of 5-methyluridine produced increased with

Fig. 1. Effect of pH on the Production of 5-Methyluridine by *Erwinia carotovora* AJ-2992.

Reaction mixtures, comprising 100 mM guanosine, 100 mM thymine, 50 mg/ml cells on a wet weight basis and the buffer indicated in the figure, were incubated at 60°C for 24 hr. (Δ) 100 mM potassium phosphate buffer; ($\circ$) 50 mM Tris-HCl buffer + 100 mM K_2HPO_4 ; ($\square$) 50 mM glycine-NaOH buffer + 100 mM K_2HPO_4 ; (∇) 50 mM $NaHCO_3$ -NaOH buffer + 100 mM K_2HPO_4 .

Fig. 2. Effect of the Phosphate Concentration on 5-Methyluridine Production by *Erwinia carotovora* AJ-2992.

Reaction mixtures, comprising 100 mM guanosine, 100 mM thymine, 50 mg/ml cells on a wet weight basis and phosphate at the concentration indicated in the figure, were incubated at 60°C for 24 hr.

the reaction time and also increased in proportion to the amount of added intact cells. Under the conditions with 50 mg/ml of intact

cells (wet weight basis), the amounts of 5-methyluridine produced in the presence of 300 mM and 500 mM guanosine and thymine were 222 mM (yield, 74%) and 265 mM (yield, 53%), respectively. With 100 mg/ml of intact cells (wet weight basis), 314 mM (yield, 63%) 5-methyluridine was produced from 500 mM guanosine and thymine.

Fig. 3. Time Course of 5-Methyluridine Production by *Erwinia carotovora* AJ-2992.

Reaction mixtures, comprising guanosine and thymine, 20 mM K_2HPO_4 , 50 mM Tris-HCl buffer (pH 8.5) and intact cells in a total volume of 100 ml, were incubated at 60°C with shaking. The concentrations of guanosine, thymine and intact cells were varied as indicated in the figure. (A) 300 mM guanosine and 300 mM thymine, (B) 500 mM guanosine and 500 mM thymine. (○) 50 mg/ml and (●) 100 mg/ml cells, on a wet weight basis.

Production of 5-methyluridine from various nucleosides

Table IV shows the production of 5-methyluridine from various nucleosides, as ribose donors, and thymine in the presence of intact cells of *Er. carotovora* AJ-2992. Guanosine was found to be the best ribose donor, while the other purine nucleosides, such as adenosine and inosine, were relatively poor ribose donor, compared to guanosine. In addition, 5-methyluridine was effectively produced from pyrimidine nucleosides such as uridine and cytidine, but was not produced from xanthosine or 5-aminoimidazole-4-carboxamide riboside.

Enzyme purification and properties of the enzymes

The 5-methyluridine-producing enzymes

Table IV. PRODUCTION OF 5-METHYLURIDINE FROM VARIOUS NUCLEOSIDES AND THYMINE BY *Erwinia carotovora* AJ-2992

Reaction mixtures, comprising 100 mM nucleoside, as indicated in the table, 100 mM thymine, 20 mM K_2HPO_4 , 50 mM Tris-HCl buffer (pH 8.5) and 50 mg/ml cells, on a wet weight basis, were incubated at 60°C for 48 hr.

Nucleosides	5-Methyluridine formed (mM)
Adenosine	23.8
Guanosine	63.6
Inosine	29.5
Xanthosine	0.0
Uridine	48.7
Cytidine	51.6
AICAR*	0.0

* AICAR, 5-aminoimidazole-4-carboxamide riboside.

Table V. TWO ENZYMES INVOLVED IN 5-METHYLURIDINE SYNTHESIS

The two fractions obtained on Butyl-Toyopearl column chromatography were used as enzyme sources. Determination of nucleosides and bases formed in the reaction mixtures was carried out by HPLC. The reaction systems were described under Materials and Methods.

Fractions	Phosphorolysis of inosine	Phosphorolysis of 5-methyluridine	Transribosylation to thymine
	Hypoxanthine formed (mM)	Thymine formed (mM)	5-Methyluridine formed (mM)
A	1.07	0	0
B	trace	3.20	0
A + B	ND*	ND	1.75

* ND, not determined.

present in *Er. carotovora* AJ-2992 were partially purified by heat treatment, ammonium sulfate fractionation, DEAE-Toyopearl column chromatography and Butyl-Toyopearl column chromatography. With these treatments, the 5-methyluridine-producing activity was divided into two fractions, A and B. As shown in Table V, the enzyme in fraction A could split the ribosyl linkage of inosine in the presence of inorganic phosphate. On the other hand the enzyme in fraction B could split the ribosyl linkage of 5-methyluridine in the presence of inorganic phosphate. Only a mixture of fractions A and B could transfer the ribosyl group of inosine to thymine in the presence of inorganic phosphate, no splitting activity being observed in its absence. These results indicated that 5-methyluridine was produced through the successive reactions of the two enzymes mentioned above and that inorganic phosphate was indispensable for the reaction. The optimal pH of the former reaction with fraction A and the latter reaction with fraction

B were 9.0 and 7.5, respectively. The optimal temperatures for these reactions were both 70°C. The substrate specificities of these enzymes are shown in Table VI. The enzyme in fraction A phosphorolysed purine ribonucleosides and purine deoxyribonucleosides, but not xanthosine, 5-aminoimidazole-4-carboxamide riboside or pyrimidine nucleosides. The enzyme in fraction B phosphorolysed uridine, 5-methyluridine, deoxyuridine and thymidine, but not cytidine, orotidine or purine nucleosides. Based on these observations, the enzymes in fractions A and B were identified as "purine nucleoside phosphorylase (PNPase) (EC 2.4.2.1)" and "pyrimidine nucleoside phosphorylase (PyNPase) (EC 2.4.2.2.)", respectively.

Isolation of an intermediate of the reaction

To identify an intermediate of the reaction, the product of the phosphorolysis of inosine by PNPase was isolated from the reaction mixture. Three mg (as protein) of PNPase was added to the reaction mixture, comprising 50 mM potassium phosphate buffer (pH 8.0) and 40 mM inosine in a total volume of 100 ml. After 18 hr-incubation at 60°C, the reaction mixture was applied to a column of SP207, which has been previously equilibrated with distilled water, and then eluted with distilled water. The fractions containing the phosphorolysed product were pooled and lyophilized. To obtain an authentic R-1-P sodium salt, a proposed intermediate, R-1-P dicyclohexyl ammonium salt, dissolved in distilled water was applied to a column of Diaion SK-1B (Na⁺ form), which had been previously equilibrated with distilled water, and then eluted with distilled water. The sodium salt of R-1-P was obtained as the effluent, and the fractions were pooled, lyophilized and used as the authentic R-1-P sample. The isolate was decomposed to ribose under acidic conditions. In addition, the ¹H-NMR spectrum of the isolate showed the same pattern as that of authentic R-1-P, as shown in Fig. 4. These results confirmed that the intermediate of the reaction producing 5-methyluridine from gua-

Table VI. SUBSTRATE SPECIFICITIES OF THE TWO ACTIVE FRACTIONS

Reaction mixtures, comprising 1 mM nucleoside, 100 mM K₂HPO₄, 100 mM glycine-NaOH buffer (pH 9.0) and fraction A (10 µg as protein), or 1 mM nucleoside, 100 mM potassium phosphate buffer (pH 7.5) and fraction B (10 µg as protein), in a total volume of 1.0 ml, were incubated at 30°C for 5 min. The bases formed were determined by HPLC.

Substrates	Relative activity	
	Fraction A	Fraction B
Adenosine	57	0
Guanosine	64	0
Inosine	100	0
Xanthosine	0	0
Uridine	0	108
5-Methyluridine	0	100
Cytidine	0	0
Orotidine	0	0
AICAR	0	0
2'-Deoxyadenosine	41	0
2'-Deoxyguanosine	47	0
2'-Deoxyinosine	92	0
2'-Deoxyuridine	0	27
Thymidine	0	14

Fig. 4. ^1H -NMR Spectra of (A) Authentic and (B) Isolated R-1-P.

Fig. 5. Mechanism of 5-Methyluridine Production.

nosine and thymine was R-1-P. In fact, 5-methyluridine could be produced from the isolated R-1-P and thymine by PyNPase (fraction B).

Mechanism of 5-methyluridine production

Judging from that two enzymes, PNPase and PyNPase, were involved in the 5-methyluridine production and an intermediate of the reaction was R-1-P, the reaction mechanism of 5-methyluridine production from guanosine and thymine by *Er. carotovora* AJ-2992 was proposed to be as follows: guanosine is phosphorylated to R-1-P and guanine through the forward reaction of PNPase in the presence of inorganic phosphate, and then the ribose moiety of R-1-P formed through the former reaction is transferred to thymine to form 5-methyluridine through the reverse reaction of PyNPase (Fig. 5).

Discussion

We screened bacterial strains which can directly produce 5-methyluridine from guanosine and thymine. Among them, we newly selected a bacterium, *Er. carotovora* AJ-2992, with potent ability as to the production of 5-methyluridine. Sakai *et al.*¹⁴⁾ also reported that *Er. carotovora* possessed extremely high potency as to the transfer of the N-ribosyl group between purine and pyrimidine nucleosides.

As to the enzymatic production of 5-methyluridine, Hori *et al.*¹³⁾ reported the production from inosine and thymine by *Bacillus stearothermophilus*, but the production yield

was rather low and the reaction mechanism has not been elucidated yet.

In contrast to their results, our present investigation showed that a newly selected bacterium, *Er. carotovora* AJ-2992, has strong ability, producing 222 mM 5-methyluridine, with a molar yield of 74%, from 300 mM guanosine and thymine. In addition, the reaction mechanism for the production of 5-methyluridine from guanosine and thymine by *Er. carotovora* AJ-2992 has been elucidated by investigating the properties of the enzymes concerned in 5-methyluridine synthesis.

Many bacteria have the ability to transfer the N-ribosyl group between purine and pyrimidine nucleosides, and to synthesize nucleosides from bases and R-1-P. R-1-P is the intermediate in the transribosylation reaction between purine and pyrimidine nucleosides.¹⁵⁾ Also in the reaction in our study, two enzymes are successively involved, inorganic phosphate is indispensable and an intermediate of the reaction is R-1-P. In the system involving AJ-2992, the 5-methyluridine-producing reaction could proceed well even with a low concentration of phosphate. This suggests that phosphate might be recycled efficiently in this reaction system.

The mechanism underlying this reaction consists of the following two successive reactions: 1) R-1-P formation from purine nucleosides and inorganic phosphate through the forward reaction of PNPase and 2) 5-methyluridine formation from R-1-P and thymine through the reverse reaction of PyNPase. In fact, an intermediate of the reaction is R-1-P,

and 5-methyluridine is only synthesized from guanosine and thymine with the co-existence of PNPase and PyNPase.

The first enzyme catalyzing R-1-P formation from guanosine and inorganic phosphate is considered to be PNPase (EC 2.4.2.1) from its substrate specificity, that is, it can phosphoryse various purine ribonucleosides and purine deoxyribonucleosides. On the other hand, the second enzyme catalyzing 5-methyluridine formation from R-1-P and thymine is considered to be PyNPase (EC 2.4.2.2) from its substrate specificity, that is, it can phosphoryse uridine, 5-methyluridine, 2'-deoxyuridine and thymidine, but not cytidine or orotidine.

Among the purine nucleosides tested, guanosine was the best ribose donor for 5-methyluridine production. This may be due to the low solubility of guanine which was formed through phosphorysis of guanosine. Among the purine bases tested, *i.e.*, guanine, adenine and hypoxanthine, guanine showed the lowest solubility in the reaction mixture and was removed from the reaction system. Therefore, equilibrium of the reaction catalyzed by PNPase may shift to R-1-P synthesis.

PNPases purified from rabbit liver¹⁶⁾ and yeast¹⁷⁾ cannot phosphoryse adenosine, but the PNPases purified from *Er. carotovora* AJ-2992 and *Enterobacter aerogenes* AJ-11125¹⁸⁾ can phosphoryse adenosine and 2'-deoxyadenosine. This indicated that PNPases in bacteria might be enzymes with wide substrate specificities for various purine nucleosides. PNPase and PyNPase purified from *Er. carotovora* were reported by Sakai *et al.*^{19,20)} They showed that the optimum pHs of the PNPase and PyNPase from *Er. carotovora* were 7.8 and 7.0, respectively, and that these enzymes could not phosphoryse deoxyribonucleosides. In contrast, the optimum pHs of the PNPase and PyNPase purified from *Er. carotovora* AJ-2992 were 9.0 and 7.5, respectively, and PNPase can even phosphoryse purine deoxyribonucleosides, such as 2'-deoxyadenosine, 2'-deoxyguanosine and 2'-deoxyinosine, and PyNPase can phosphoryse pyrimidine de-

oxyribonucleosides, such as thymidine and 2'-deoxyuridine. These results suggest that the enzymes isolated by us may be different from the nucleoside phosphorylases isolated by Sakai *et al.*

In our recent work,⁶⁾ ribavirin was produced from orotidine and 1,2,4-triazole-3-carboxamide by the same strain, *Er. carotovora* AJ-2992, which presumably has a orotidine-phosphorolyzing enzyme. But the PyNPase purified from AJ-2992 could not phosphoryse orotidine. Judging from the results, the orotidine-phosphorolyzing enzyme in AJ-2992 may be different from the PyNPase purified in this study.

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