EFFECT OF TEMPERATURE ON THE CELL-FREE PROTEIN-SYNTHESIZING SYSTEM IN PSYCHROPHILIC AND MESOPHILIC BACTERIA

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Temperature-dependency of poly(U)-directed polyphenylalanine synthesis was examined with different cell-free systems of *Vibrio* sp. strain ABE-1, a psychrophile, and *Pseudomonas aeruginosa*, a mesophile. In S-30 fractions, the activity of *V. ABE-1* was higher than that of *P. aeruginosa* at and below 25°, but the activity of the former decreased rapidly above 25° and was completely lost at 40°. In ribosome-supernatant systems, *V. ABE-1* ribosomes, especially 50S subunits, served a cold-stabilizing function, but *V. ABE-1* supernatant was remarkably thermolabile, which was probably attributable to thermo-inactivation of phenylalanyl-tRNA synthetase present in the supernatant. *V. ABE-1* ribosomes had no activation factor able to be released by washing with 1 M NH₄Cl.

Low-temperature growth of psychrophilic and psychrotrophic bacteria must be maintained by active catabolism and anabolism at low temperature. It has been reported in our laboratory that active ATP-formation was found in *Vibrio* sp. strain ABE-1 (*V. ABE-1*) and *Pseudomonas* sp. strain 351 (*P. 351*) at 7° (1), and that the protein-synthesizing system of *P. 351* was stable by chilling at 0°, but that of *P. aeruginosa* was not (2, 3). Several investigators also reported that some inducing enzymes could be formed in psychrophiles at low temperatures such as 0° (4–7).

We attempted in this study to elucidate the functional differences between the psychrophilic and mesophilic bacteria in protein-synthesizing systems under low temperature. For the purpose of this study, the activity of poly(U)-directed polyphenylalanine synthesis in different cell-free protein-synthesizing systems of *V. ABE-1* and *P. aeruginosa* was measured.

In the present paper, it is reported that the difference between these bacteria in protein-synthesizing activity at 0° seems to be attributable to the functional difference between the bacterial ribosomes, probably 50S subunits, and that the

psychrophile has no activation factor such as the "P-factor" reported by SZER (8).

MATERIALS AND METHODS

Bacterial strain. *Vibrio* sp. strain ABE-1 (9) and a laboratory strain of *Pseudomonas aeruginosa* were used as psychrophilic and mesophilic bacteria, respectively.

Growth conditions. Peptone-broth medium containing 1% of peptone and meat extract respectively was used as the growth and stock media for *P. aeruginosa*. The same medium supplemented with 0.5 M NaCl was used for *V. ABE-1*. *P. aeruginosa* and *V. ABE-1* were cultivated by vigorous shaking at 20° and 10°, respectively.

Preparation of cell-free extracts. Cells in the exponential phase were harvested and washed three times with Buffer I consisting of 20 mM Tris-HCl (pH 7.8), 60 mM NH₄Cl, 10 mM magnesium acetate and 6 mM 2-mercaptoethanol. In the case of *V. ABE-1*, Buffer I containing 0.5 M NaCl was used. Washed cells were ruptured by grinding with sea sand. The paste was diluted with Buffer I, and centrifuged at $9,000 \times g$ for 5 min. The supernatant was re-centrifuged at $30,000 \times g$ for 30 min. The resultant supernatant was incubated for 30 min at the desired temperatures (10° for *V. ABE-1*, and 24° for *P. aeruginosa*), dialyzed against Buffer I for 3 hr, and used as the S-30 fraction.

Ribosomes and supernatant fractions were prepared from the S-30 fraction by centrifugation at $150,000 \times g$ for 150 min. The ribosomes were washed once more with Buffer I.

Preparation of crude aminoacyl-tRNA synthetase and crude tRNA. To obtain crude aminoacyl-tRNA synthetase and crude tRNA, the supernatant fraction prepared from the S-30 fraction was applied to a DEAE-cellulose column (1.5 × 20 cm). After the column was washed with Buffer I, 150 mM and 450 mM of NaCl in Buffer I were successively passed through the column. The crude synthetases were eluted with 150 mM NaCl and the active fractions were combined. Ammonium sulfate to 75% saturation was added to the combined fractions and the precipitate was dissolved in Buffer I and dialyzed against the same buffer overnight. The dialyzed solution was used as crude aminoacyl-tRNA synthetase. The crude tRNA was eluted with 450 mM NaCl. Polynucleotides were precipitated by the addition of two volume of ice-cold ethanol, and the precipitate was dissolved in Buffer I. This solution was used as crude tRNA.

Preparation of ribosomal subunits. The ribosomes were washed with 4.5 ml of 30% (w/v) sucrose by centrifugation at $150,000 \times g$ for 4 hr. The washed ribosomes were suspended in Buffer II consisting of 20 mM Tris-HCl (pH 7.6), 60 mM NH₄Cl, 0.7 mM magnesium acetate and 6 mM 2-mercaptoethanol, and were dialyzed against the same buffer for 5 hr. The ribosomes were layered on 32 ml of 0.3 to 0.9 M linear sucrose density-gradient in Buffer II and centrifuged in a

Beckman SW27 rotor at $39,000 \times g$ for 15 hr at 1° . The gradient was then fractionated and the fractions containing 30S and 50S ribosomal subunits were pooled separately. Each of the fractions was washed with Buffer III (Buffer I containing 50 mM KCl) by centrifugation at $150,000 \times g$ for 15 hr, and resuspended in Buffer III.

Cell-free protein-synthesizing systems. Protein-synthesizing activity was measured by poly(U)-directed polyphenylalanine synthesis. The standard assay system contained in a volume of 0.2 ml: 20 μ mol Tris-HCl (pH 7.8); 2.5 μ mol magnesium acetate; 8 μ mol KCl; 0.2 μ mol adenosine triphosphate (ATP); 5 nmol guanosine triphosphate (GTP); 2.64 μ mol creatine phosphate; 50 μ g creatine kinase; 50 μ g poly(U); 0.8045 nmol [14 C]-L-phenylalanine; and cell-free system. As the cell-free system, either the S-30 fraction (1 mg protein) or supernatant fraction (0.4 mg protein) plus ribosomes (4.5 A_{260} units of 70S or 0.66 unit of 30S plus 0.5 unit of 50S) was used. In the case of the S-30 fraction of *P. aeruginosa*, 5 μ mol of magnesium acetate and 2 μ mol of KCl were used. The reaction was started by the addition of cell-free system, and stopped by the addition of 0.2 ml of ice-cold 10% trichloroacetic acid (TCA). Incorporation of [14 C]-phenylalanine into TCA-insoluble material was determined by the method of BOBIER *et al.* (10) with a minor modification.

Assay of phenylalanyl-tRNA synthetase. The reaction mixture for assay of phenylalanyl-tRNA synthetase contained in a volume of 0.1 ml: 10 μ mol Tris-HCl (pH 7.8); 0.2 μ mol of ATP; 2 μ mol KCl; 1 μ mol magnesium acetate; 0.1 nmol [14 C]-L-phenylalanine; 10 A_{260} unit tRNA; and 60 μ g enzyme. The reaction mixture was incubated at 25° (*V. ABE-1*) or 30° (*P. aeruginosa*) for 30 min, and the radioactivity of phenylalanyl-tRNA was determined by the method of CHERAYIL *et al.* (11).

Chemicals. Poly(U), ATP, GTP, creatine phosphate, and creatine kinase were obtained from Boehringer, Mannheim. L-Phenylalanine[U- 14 C] (531 mCi/mmol) was purchased from the Radiochemical Centre, England.

RESULTS

Effect of temperature on protein synthesis by S-30 fraction

The activity of poly(U)-directed polyphenylalanine synthesis was measured at various temperatures between 0° and 50° . As shown in Fig. 1, the optimum temperatures for protein synthesis by *V. ABE-1* S-30 and *P. aeruginosa* S-30 were 25° and 40° , respectively. In *V. ABE-1* S-30, the incorporation activity markedly decreased above 25° and was completely lost at 40° . However, at 10° , the activity of *V. ABE-1* S-30 was five to six times that of *P. aeruginosa* S-30, and the activities at 0° of *V. ABE-1* and *P. aeruginosa* were 3.1% and 0.6% of the activities at the respective optimum temperatures.

Fig. 1. Effect of temperature of cell-free poly(U)-directed polyphenylalanine synthesis by S-30 fractions from *P. aeruginosa* (●) and *V. ABE-1* (○). The reaction was carried out for 10 min.

Cell-free protein-synthesizing system consisting of ribosomes and S-30 supernatant fractions

As seen in Fig. 1, the protein-synthesizing activity of *V. ABE-1* S-30 was higher at low temperatures and lower at high temperatures than that of *P. aeruginosa* S-30. To elucidate the differences between *V. ABE-1* and *P. aeruginosa* S-30, we attempted to separate each S-30 fraction into ribosomes and supernatant fractions and to measure the protein-synthesizing activities in the homologous and heterologous combination systems of these ribosomes and supernatant fractions. As shown in Table 1, both heterologous systems, *V. ABE-1* ribosomes plus *P. aeruginosa* supernatant and *vice versa*, had protein-synthesizing activity to an extent similar to the homologous systems at 25°. However, the situation was remarkably changed by changing temperature, *i.e.*, the activity was greatly reduced at 35° in the systems containing *V. ABE-1* supernatant, and also reduced at 0° in the systems containing *P. aeruginosa* ribosomes. These results indicated that the essential factors in *V. ABE-1* supernatant and in *P. aeruginosa* ribosomes were thermolabile and cold-labile, respectively.

Thermostability of supernatant fraction

From the above results, it is necessary to elucidate whether *V. ABE-1* supernatant has a thermolabile essential factor and what the factor is. Firstly, we examined the thermostability of the supernatant of *V. ABE-1* and *P. aeruginosa* by preincubation of the supernatant at 35° for 20 min. The results are shown in Table 2. As shown in the table, *V. ABE-1* supernatant lost about 95% of the activity by the preincubation, but the activity of *P. aeruginosa* supernatant lost only about 28%. These results obviously show that some essential component in *V. ABE-1* supernatant is thermolabile.

Secondly, we examined the thermostability of the crude phenylalanyl-tRNA

Table 1. Effect of temperature on poly(U)-directed polyphenylalanine synthesis in the homologous and heterologous systems of *P. aeruginosa* and *V. ABE-1*.

System		[¹⁴ C]-Phenylalanine incorporated (pmol/10 min)		
Ribosomes	Supernatant	0°	25°	35°
<i>P. aeruginosa</i>	<i>P. aeruginosa</i>	0.52	32.4	38.5
<i>P. aeruginosa</i>	<i>V. ABE-1</i>	0.65	48.2	5.7
<i>V. ABE-1</i>	<i>P. aeruginosa</i>	2.00	42.3	40.5
<i>V. ABE-1</i>	<i>V. ABE-1</i>	1.65	52.5	5.2

Table 2. Thermostability of supernatant fractions from *V. ABE-1* and *P. aeruginosa*.

System		Assay temperature (°)	[¹⁴ C]-Phenylalanine incorporated (pmol/10 min)
<i>P. aeruginosa</i>	Control	30	40
	Preincubated ^a	30	29
<i>V. ABE-1</i>	Control	25	39
	Preincubated ^a	25	2

^a Protein-synthesizing system was the same as the respective control except that the supernatant fraction preincubated at 35° for 20 min was used.

synthetases prepared from *V. ABE-1* and *P. aeruginosa*. This was because the enzyme is distributed in the supernatant fractions and some of the aminoacyl-tRNA synthetases have been reported to be thermolabile (12, 13). As was expected, the *V. ABE-1* enzyme was found to be thermolabile and it was inactivated by about 80% by the same preincubation treatment as above, while the *P. aeruginosa* enzyme was inactivated by only about 13% (Table 3). Thus, it may be reasonable to conclude that the main cause of the thermolability of *V. ABE-1* supernatant is attributed to thermo-inactivation of phenylalanyl-tRNA synthetase present in the supernatant.

Table 3. Thermostability of phenylalanyl-tRNA synthetases from *P. aeruginosa* and *V. ABE-1*.

Enzyme		[¹⁴ C]-Phenylalanine incorporated (pmol)	(%)
<i>P. aeruginosa</i>	Control	12.7	100
	Preincubated ^a	11.1	87
<i>V. ABE-1</i>	Control	4.3	100
	Preincubated ^a	0.9	21

^a The enzyme was preincubated at 35° for 20 min. Enzyme activity was measured as described in MATERIALS AND METHODS.

Cold-stability of ribosomal subunits

It is clear, as seen in Table 1, that there is a functional difference between

V. ABE-1 ribosomes and *P. aeruginosa* ribosomes at 0°. Accordingly, we examined the contributions of the individual ribosomal subunits to protein synthesis at 0° by forming hybrid and non-hybrid ribosomes from the large and small ribosomal subunits of *V. ABE-1* and *P. aeruginosa*. The results presented in Table 4 indicate that 50S subunits of *V. ABE-1* may contribute to the high efficiency of protein synthesis in *V. ABE-1* under lower temperatures.

Effect of ribosomal protein released by washing with NH₄Cl

An attempt was made to examine whether the active protein synthesis in psychrophilic bacteria under low temperature was maintained by an activation factor such as the "P-factor," which was reported to be a proteinaceous factor activating protein synthesis at low temperature (8). As shown in Table 5, we could

Table 4. Effect of temperature on the activity of hybrid and non-hybrid ribosomes from the ribosomal subunits of *V. ABE-1* and *P. aeruginosa*.

Ribosomal subunits		[¹⁴ C]-Phenylalanine incorporated (pmol/10 min)	
50S	30S	0°	30°
<i>P. aeruginosa</i>	<i>P. aeruginosa</i>	0.12	4.70
<i>P. aeruginosa</i>	<i>V. ABE-1</i>	0.17	4.50
<i>V. ABE-1</i>	<i>P. aeruginosa</i>	0.32	4.53
<i>V. ABE-1</i>	<i>V. ABE-1</i>	0.35	3.93

Supernatant fraction of *P. aeruginosa* was used in the protein-synthesizing system in this experiment.

Table 5. Effect of released protein on phenylalanine incorporation by NH₄Cl-washed ribosomes.

Ribosomes		Addition	[¹⁴ C]-Phenylalanine incorporated (pmol/10 min)		
			0°	25°	30°
<i>P. aeruginosa</i>	Control	None	0.32	—	40.0
	Washed	None	0.00	—	1.2
	Washed	R. P. ^a	0.00	—	0.5
	Washed	H. R. P. ^b	0.00	—	1.1
<i>V. ABE-1</i>	Control	None	1.00	29.0	—
	Washed	None	0.60	7.5	—
	Washed	R. P. ^a	0.23	4.2	—
	Washed	H. R. P. ^b	0.55	7.4	—

In this experiment, ribosomes were combined homologously with the respective supernatant fraction.

^a R. P. (released protein) was prepared from the washing of *V. ABE-1* ribosomes by the method of SZER (8).

^b H. R. P. (heated released protein) was prepared by heating of R. P. at 60° for 20 min. Almost the same amount (about 50 μg) of R. P. or H. R. P. was added to the reaction mixture.

not find any activation factor in *V. ABE-1* ribosomes. Released protein was more of an inhibitor than an activator, and the inhibition disappeared after heating at 60° for 20 min.

DISCUSSION

Even if the temperature-dependency of the protein-synthesizing system does not always decide the minimum and maximum temperatures for bacterial growth, protein synthesis as well as respiration (phosphorylation) may be one of the most important factors in limiting the growth. As far as we examined the incorporation of [³H]-leucine, protein-synthesizing activities in the psychrotrophic and psychrophilic bacteria were always higher than those in the mesophilic bacteria at low temperature (2, 14). Similar results were obtained in poly(U)-directed polyphenylalanine synthesis by the cell-free systems of *V. ABE-1* and *P. aeruginosa* (Fig. 1). In the present study, we found that *V. ABE-1* ribosomes, especially 50S subunits, contributed to the active protein synthesis in *V. ABE-1* under low temperature (Tables 1 and 4). We have not yet determined what component of the subunits is an essential one and how the component serves an effective function in protein synthesis at low temperature. In this connection, however, SZER (8) isolated an activation factor, "P-factor," from ribosomes of a psychrotrophic bacteria, *Pseudomonas* sp. strain 412, which stimulated poly(U)-directed polyphenylalanine synthesis by washed ribosomes of *P. 412* and *Escherichia coli* at 0°. At first, we presumed that a factor similar to "P-factor" had to be present to participate in *V. ABE-1* systems, but this assumption was disproved in this study (Table 5). At present, we presume that the psychrophilic nature of not only protein-synthesizing systems but also other important metabolic systems in the psychrophiles may be attributed to different characteristics which have been adaptively acquired by them during the process of organic evolution.

An incidental point is that BROEZE *et al.* (15) reported that the initiation step of the translation in *E. coli* system was blocked by chilling, but not the elongation step, and consequently no remarkable difference between a psychrotrophic *P. fluorescens* and *E. coli* in the elongation steps was observed. As stated above, we found the functional difference in 50S subunits of the psychrophile and mesophile at low temperature, but the activity of ribosomal subunits of the psychrophile was only two or three times that of the mesophile subunits. These small differences may be based upon the estimation of the elongation step only, because protein synthesis was measured by poly(U)-directed phenylalanine incorporation in this study.

Further experiments with natural messenger RNA's are in progress.

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