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Stability of Ribosomes and Ribosomal Ribonucleic Acid from *Bacillus stearothermophilus*[1]

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After heating at 65 C, ribosomes isolated from *Bacillus stearothermophilus* were strikingly more heat-stable than comparable preparations from *Escherichia coli* when tested for ability to support polyuridylic acid-directed phenylalanine incorporation at 37 C. The stability of ribosomes was also determined by measurements of hyperchromicity at 259 mμ while heating them from 25 to 90 C. In standard buffer containing 0.01 M Mg^{++}, the T_m (temperature at the midpoint of total hyperchromicity) of *E. coli* and *B. stearothermophilus* ribosomes was 71 and 81 C, respectively. In a magnesium-free buffer, the T_m of *E. coli* and *B. stearothermophilus* ribosomes was 44 and 64 C, respectively. Putrescine (0.01 M) was more effective in stabilizing ribosomes from *B. stearothermophilus* than those from *E. coli*. Spermidine (0.001 M), on the other hand, was more effective in stabilizing ribosomes from *E. coli* than those from *B. stearothermophilus*. Melting curves of total ribosomal ribonucleic acid (rRNA) from *E. coli* and *B. stearothermophilus* revealed T_m values of 50 and 60 C, respectively. Putrescine stabilized thermophile rRNA, but had no effect on *E. coli* rRNA. Sucrose density gradients demonstrated that thermophile 23*S* ribonucleic acid was degraded during storage at −20 C; the 23*S* component from *E. coli* was stable under these conditions. The results are discussed in terms of the mechanism of ribosome heat stability and the role of the ribosome in governing the temperature limits for bacterial growth.

The intriguing topic of microbial thermophily has attracted extensive investigation in the past decade. The earlier literature has been reviewed by Gaughran (9), Allen (2), and Koffler (10). Whereas initial investigations were concerned primarily with enzymes and structural proteins, the emphasis in the past 5 years has been on the nucleic acids and ribosomes of thermophiles.

Previous reports by Friedman and Weinstein (6-8) demonstrated that a subcellular system derived from *Bacillus stearothermophilus* has a high temperature optimum for amino acid incorporation, and that it is therefore useful in studying the effects of temperature on the coding properties of nucleic acids. Heat-denaturation profiles of soluble ribonucleic acid (sRNA) isolated from *B. stearothermophilus* and *Escherichia coli* did not reveal significant differences between the two nucleic acid preparations (3, 8, 13). Nevertheless, the proper secondary structure of sRNA, essential for amino acid acceptor capacity (3), could be maintained at high temperatures in vivo by regulation of the polycation concentration (3, 8). Aminoacyl sRNA synthetases of *B. stearothermophilus* were shown to be more heat-stable than the comparable enzymes from *E. coli* (3, 8). The present study compares the ribosomes and ribosomal ribonucleic acid (rRNA) from *B. stearothermophilus* to those from *E. coli* and the effects of polyamines on their thermostability.

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MATERIALS AND METHODS

Organisms, media, and growth conditions. B. stearothermophilus NCA 2184 was grown at 65 C in a medium of the following composition (grams per liter): tryptone (Difco), 10.0; yeast extract (Difco), 5.0; NaCl, 5.0; glucose, 20; *p*H adjusted to 7.0 with 1 N NaOH. Cells (mid- to late-log phase) were harvested at an optical density of 1.0, when read against a medium blank at 550 mμ. The bacteria were washed once in "standard buffer" (0.01 M tris(hydroxymethyl) aminomethane (Tris) chloride, *p*H 7.8; 0.01 M magnesium acetate; 0.06 M KCl; and 0.006 M β-mercaptoethanol); and frozen until used. *E. coli* B was grown at

37 C in the same medium and processed in the same manner.

Preparation of ribosomes and rRNA. The frozen bacteria were disrupted by grinding with twice their wet weight of alumina. This and all subsequent preparative steps were performed at 5 C. The cell paste was suspended in an amount of standard buffer equal to three times the wet weight of cells. Alumina and intact cells were removed by centrifugation at 20,000 × *g* for 20 min. The supernatant fluid was decanted, and deoxyribonuclease (3 μg/ml) was added. After 5 min, the suspension was centrifuged at 20,000 × *g* for 20 min. The supernatant fluid was aspirated and centrifuged at 30,000 × *g* for 30 min to obtain the S-30 fraction. The S-30 was centrifuged at 45,000 rev/min (122,000 × *g*) for 90 min in the 50S rotor of a Spinco (model L) centrifuge to obtain the ribosomal supernatant fluid (S-122) and ribosomal pellet (P-122). The surface of the ribosomal pellets was rinsed with standard buffer, and then the pellet was resuspended in standard buffer with the aid of a Teflon pestle. The suspension was centrifuged at 10,000 × *g* for 10 min. The supernatant fluid was used to test the heat stability of ribosomes or was extracted with phenol to obtain rRNA. In the latter case, one volume of the ribosome suspension was shaken vigorously with an equal volume of water-saturated phenol. The emulsion was clarified by centrifugation at 3,500 × *g* for 5 min at room temperature. The aqueous layer was removed and extracted twice with 0.5 volume of water-saturated phenol. Finally, the aqueous layer was chilled at 5 C and sufficient 5% NaCl was added to bring the final concentration to 0.1%. The rRNA was precipitated with two volumes of absolute ethyl alcohol and the suspension was placed at −20 C overnight. After centrifugation at 10,000 × *g* for 10 min, the pellet was resuspended in a small volume of water and dialyzed against distilled water overnight.

Heat stability of ribosomes in protein synthesis. Ribosomes from *B. stearothermophilus* or *E. coli* were suspended in standard buffer at a concentration of 1,050 optical density (OD) units (determined at 260 mμ) per ml and heated at 65 C. Samples were removed at various times, placed in test tubes on ice, and then tested for their ability to function in protein synthesis at 37 C. The incubation mixture for protein synthesis contained, in a 0.4-ml volume, the following components (in micromoles unless otherwise specified): Tris chloride buffer (*p*H 7.8), 4.0; magnesium acetate, 4.0; KCl, 24.0; β-mercaptoethanol, 1.4; adenosine triphosphate, 0.25; guanosine triphosphate, 0.01; phosphoenolpyruvate, 1.25; phosphoenolpyruvate kinase, 12 μg; a mixture of ^{12}C-L-amino acids (8), excluding the radioactive amino acids, 0.0125 of each; ^{14}C-phenylalanine (165 mc/mmole), 0.76 mμmoles; ribosomes, 10 OD units; 0.1 ml of the homologous S-122 fraction; and polyuridylic acid (poly U; *S* = 8.5), 100 μg. Samples were incubated at 37 C for 20 min, after which they were processed; the radioactivity was measured as previously described (8). The results are expressed as "net polymer stimulation," i.e., the total ^{14}C-phenylalanine incorporated in the presence of poly U minus that incorporated in the absence of poly U.

Melting curves. Thermal denaturation profiles of ribosomes and rRNA preparations were obtained with a Beckman (model DUR) spectrophotometer attached to a Gilford (model 2000) multiple-sample absorbance recorder. Both ribosomes and rRNA were tested at initial OD values of 0.4 to 0.6 (at 259 mμ).

Sucrose density gradients. Twelve OD (259 mμ) units of rRNA was layered on a 26-ml linear gradient of 5 to 20% sucrose in acetate buffer (0.01 M acetic acid-0.1 M NaCl; *p*H 5.1). After centrifugation in the SW 25 rotor of the Spinco model L preparative ultracentrifuge at 24,000 rev/min at 4 C for 15 hr, the bottom of the gradient tube was punctured and the effluent was assayed for OD at 260 mμ with a Gilford model 2000 recorder.

Materials. Poly U was purchased from Miles Laboratories, Inc. ^{14}C-phenylalanine was obtained from Schwarz Bio Research Inc., Orangeburg, N.Y. Deoxyribonuclease (electrophoretically purified) was obtained from Worthington Biochemical Corp., Freehold, N.J., and rabbit muscle pyruvate kinase (Boehringer) from Calbiochem. Phenol was purchased from Allied Chemical Corp., Morristown, N.J., and was redistilled just prior to use. Levigated alumina was a product of Norton Abrasives, Worcester, Mass. Putrescine dihydrochloride was purchased from California Foundation for Biochemical Research, and spermidine trihydrochloride from Nutritional Biohemicals Corp., Cleveland, Ohio.

RESULTS

Figure 1 presents the data obtained when samples taken from heated ribosomal suspensions of *B. stearothermophilus* and *E. coli* were tested for their ability to support poly U-directed phenylalanine incorporation at 37 C. After 5 min at 65 C, 94% of the initial activity was retained by the thermophile ribosomes, whereas only 21% was retained by *E. coli* ribosomes. When the time interval at 65 C was extended to 15 min, incorporation values were 80 and 5% for thermophile and *E. coli* ribosomes, respectively. At the end of 30 min, the corresponding values were 74 and 4%, respectively. Similar results on the heat stability of mesophile and thermophile ribosomes were recently reported by Algranati and Lengyel (1).

Further confirmation of the heat stability of ribosomes from *B. stearothermophilus* was obtained by studying the heat denaturation profiles of ribosomes. When assayed in standard buffer (0.01 M Mg), the T_m for *E. coli* ribosomes was 71 C, whereas the T_m for thermophile ribosomes was 81 C (Fig. 2). When tested in standard buffer minus magnesium (Fig. 3A), the T_m values obtained for *E. coli* and *B. stearothermophilus* ribosomes were 44 and 64 C, respectively. Similar observations on the greater heat stability of thermophile ribosomes as measured by thermal denaturation profiles have been reported by others (12, 13). When the ribosomes were suspended in standard buffer minus magnesium and supplemented with 0.01 M putrescine (Fig. 3B), the T_m

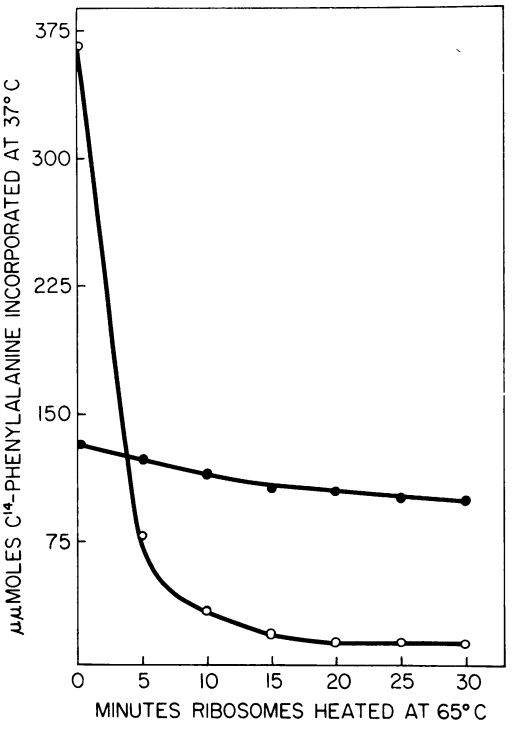

FIG. 1. *Heat stability of ribosomes from Escherichia coli* (○) *and Bacillus stearothermophilus* (●).

values obtained for *E. coli* and *B. stearothermophilus* ribosomes were 47 and 71 C, respectively. Under these conditions, therefore, putrescine was more effective in stabilizing thermophile ribosomes than *E. coli* ribosomes. As we shall describe shortly, a similar difference in response to putrescine was obtained with rRNA. When ribosomes were assayed in standard buffer minus magnesium and supplemented with 0.001 M spermidine (Fig. 3C), the T_m values obtained for *E. coli* and *B. stearothermophilus* were 51 and 67 C, respectively. In this case, the results are the opposite of those obtained with putrescine, since spermidine is somewhat more effective in stabilizing *E. coli* ribosomes than *B. stearothermophilus* ribosomes.

Comparable studies were performed with purified rRNA. In standard buffer minus magnesium (Fig. 4A), the T_m values for *E. coli* and *B. stearothermophilus* rRNA were 50 and 60 C, respectively. The greater thermostability of thermophile rRNA has been observed by others (12, 13), and is in agreement with the base composition data which reveal a higher guanine plus cytosine content in *B. stearothermophilus* rRNA than in *E. coli* rRNA (12, 13). When the same materials were tested in standard buffer minus magnesium and supplemented with 0.01 M putrescine, T_m values for *E. coli* rRNA and *B. stearothermophilus* rRNA were 50 and 65 C, respectively (Fig. 4B). Therefore, whereas this polyamine had no effect on *E. coli* rRNA, it increased the T_m of thermophile rRNA by 5 C. When heat denaturation curves were done in standard buffer minus magnesium and supplemented with 0.001 M spermidine, T_m values for *E. coli* rRNA and *B. stearothermophilus* rRNA were 59 and 71 C, respectively (Fig. 4C.) Spermidine, therefore, stabilized both types of nucleic acid. Mangiantini et al. (12) reported that spermidine did not stabilize *B. stearothermophilus* rRNA, but their studies were carried out in buffer containing magnesium.

rRNA preparations from *E. coli* and *B. stearothermophilus* were analyzed by means of the sucrose density gradient technique (Fig. 5). *E. coli* rRNA was fractionated into 16*S* and 23*S* components in the approximate ratio of 1:2 (OD, 259

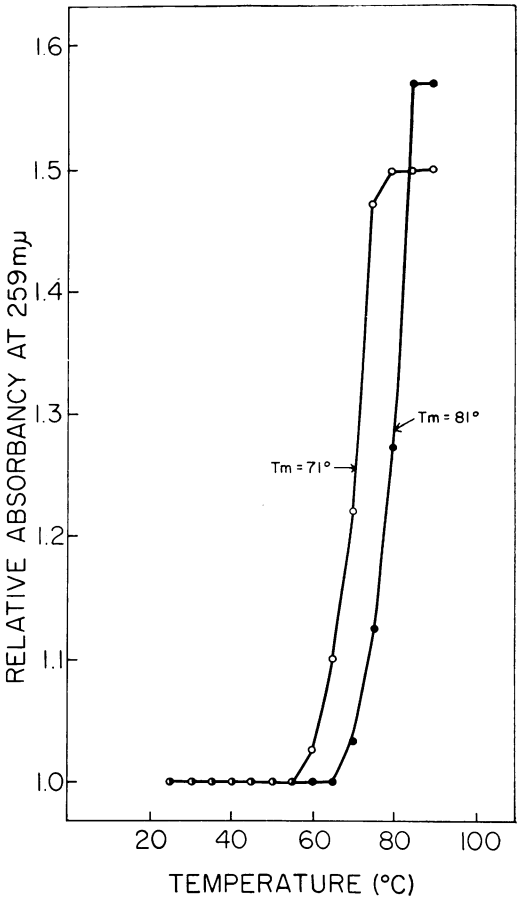

FIG. 2. *Thermal denaturation profiles of ribosomes from Escherichia coli* (○) *and Bacillus stearothermophilus* (●) *in standard buffer.*

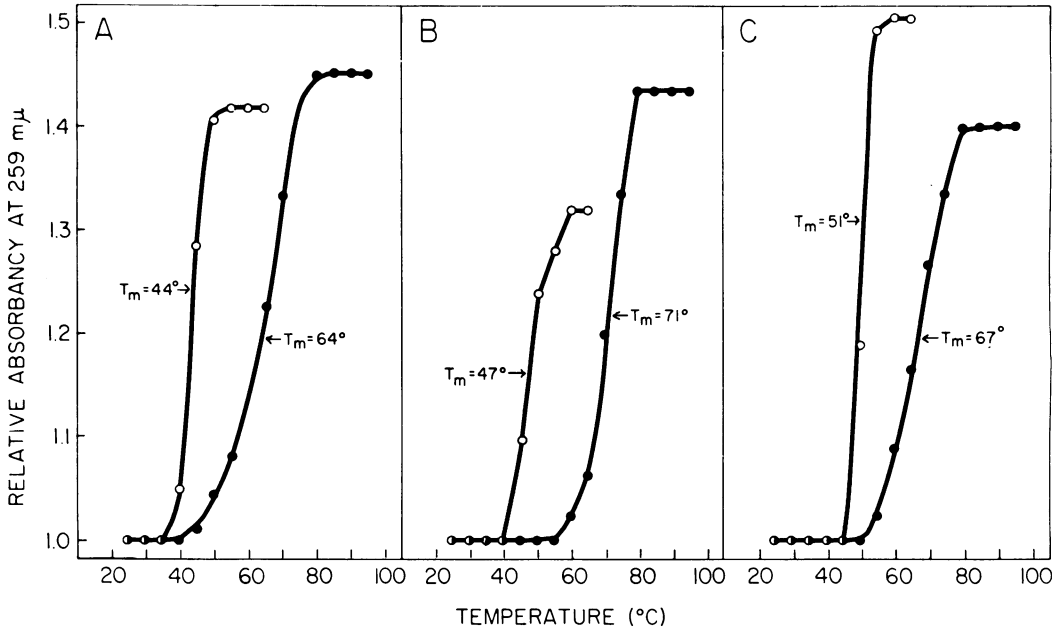

FIG. 3. *Thermal denaturation profiles of ribosomes from Escherichia coli* (○) *and Bacillus stearothermophilus* (●). (*A*) *Standard buffer minus magnesium.* (*B*) *Standard buffer minus magnesium plus 0.01* M *putrescine.* (*C*) *Standard buffer minus magnesium plus 0.001* M *spermidine.*

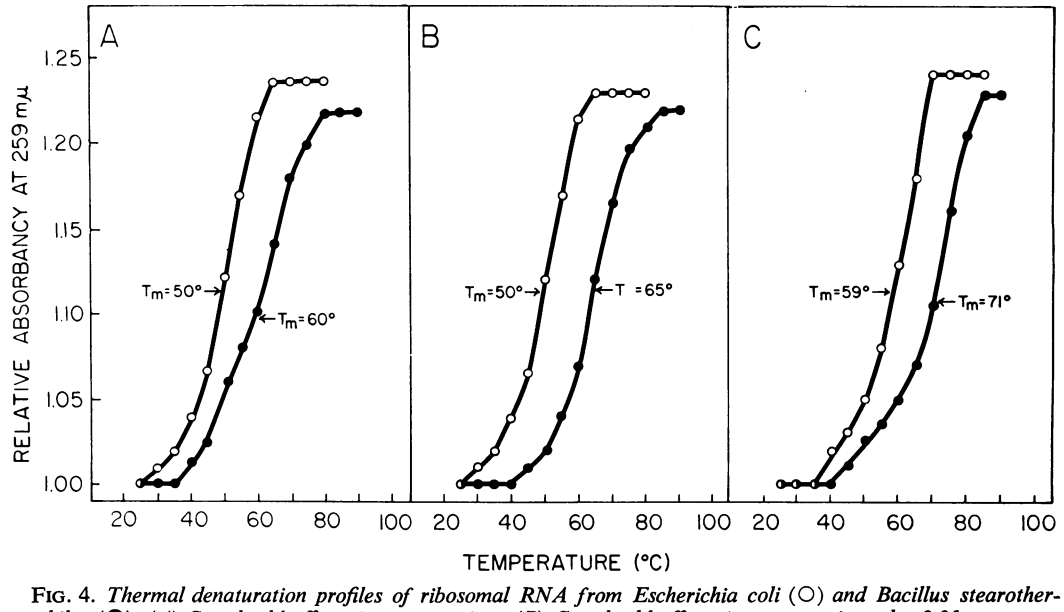

FIG. 4. *Thermal denaturation profiles of ribosomal RNA from Escherichia coli* (○) *and Bacillus stearothermophilus* (●). (*A*) *Standard buffer minus magnesium.* (*B*) *Standard buffer minus magnesium plus 0.01* M *putrescine.* (*C*) *Standard buffer minus magnesium plus 0.001* M *spermidine.*

mμ), as has been reported previously by Kurland (11). Two peaks of RNA, having sedimentation coefficients of approximately 16*S* and 23*S*, were also obtained with *B. stearothermophilus* rRNA. The amount of the 16*S* component exceeded that of the 23*S* component. When sucrose gradients were run immediately after the isolation of *B. stearothermophilus* rRNA, the 23*S* to 16*S* ratio was 1.6:1. The change in ratio, which occurs after brief storage at −20 C, suggests preferential deg-

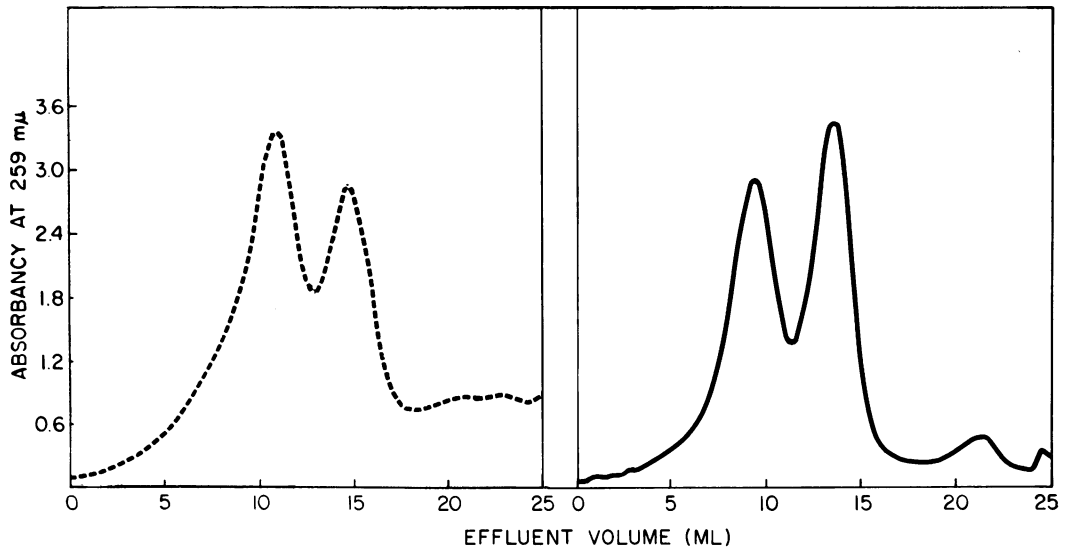

FIG. 5. *Sucrose density gradient centrifugation profile of ribosomal RNA from Escherichia coli (left) and Bacillus stearothermophilus (right).*

radation of the 23*S* fraction and its conversion to 16*S* RNA. Mangiantini et al. (12) found that, when rRNA from *B. stearothermophilus* strain B was studied in an analytical ultracentrifuge, the major component was 16*S*. There was a small but variable amount of a 21*S* component, as well as a 10*S*-11*S* RNA. These authors suggested that the 21*S* material may be particularly labile and split into subunits, giving rise to the 10*S*-11*S* peak. Further evidence for the instability of thermophile 23*S* RNA can be seen in the methylated albumin-kieselguhr column and sucrose density gradient profiles reported by Saunders and Campbell (13). Results similar to those of Mangiantini et al. (12) were obtained with rRNA from *B. cereus* in studies described by Takai and Kondo (14). Further work is needed to establish whether instability of 23*S* rRNA is a general characteristic of gram-positive bacilli. In addition, it will be of interest to determine whether the instability observed with *B. stearothermophilus* 23*S* RNA is an intrinsic characteristic of the macromolecule (perhaps due to the presence of subunits) or the result of a specific ribonuclease which remains bound to the RNA after phenol extraction.

DISCUSSION

The present studies provide further evidence for the heat stability of thermophile ribosomes. At the present time it is not clear whether this heat stability is inherent in the structure of thermophile rRNA, or ribosomal protein, or rather a consequence of an additional component which is tightly bound to these ribosomes. The present studies, as well as previous evidence (12, 13), indicate that *B. stearothermophilus* ribosomal RNA has a higher T_m than *E. coli* rRNA. This may account, at least in part, for the heat stability of the ribosomes. A comparison of the data presented in Fig. 3 and 4 reveals that, in the absence of polyamines, thermophile ribosomes have a higher T_m than their corresponding rRNA. This effect was not observed with *E. coli* preparations. In addition, the slope of the melting curve was less steep with thermophile ribosomes than with *E. coli* ribosomes. These results suggest that the protein moiety of thermophile ribosomes may also play a role in stabilizing the ribosomes against heat denaturation. However, starch-gel electrophoresis (12), as well as polyacrylamide disc electrophoresis in urea (J. G. Flaks, *personal communication*), of ribosomal proteins has revealed only minor differences between preparations obtained from *B. stearothermophilus* and *E. coli*. In addition, the amino acid composition of thermophile ribosomal protein, when compared to a similar preparation obtained from *E. coli*, showed no marked difference, with the possible exception of a slightly higher cystine content in the ribosomal protein of *B. stearothermophilus* (13). Since the ribosomes employed for studies on thermostability have not been extensively purified, thermostability may be due to a stabilizing factor which is tightly bound to the ribosome. The ability of putrescine to stabilize both thermophile

rRNA and thermophile ribosomes against heat denaturation suggests the possibility that the stabilizing factor may be a polyamine. In preliminary studies, we analyzed the polyamine content of *B. stearothermophilus* grown at 65 C and found that the total polyamine concentration was actually lower than values obtained by Dubin and Rosenthal (5) for *E. coli*. The possible presence of a low concentration of a specific polyamine which is responsible for heat stability has not been excluded, but our preliminary results indicate that in *B. stearothermophilus* (as in *E. coli*) the major components are putrescine and spermidine.

The present studies may be relevant to the general problem of thermobiosis, since the heat stability of ribosomes may play an important role in governing the maximal growth temperature of bacteria. Indeed, a recent study demonstrates a correlation between the T_m of bacterial ribosomes and the maximal growth temperature of the organisms from which they were derived (B. Pace and L. L. Campbell, Bacteriol. Proc., p. 98, 1966). Results obtained by Arcà et al. (4), indicating that at high temperatures wrong amino acids may be attached to sRNA, suggest that coding errors may also be instrumental in fixing the maximal temperature for growth. The relative importance of these factors in limiting the temperature range for the growth of various organisms remains to be determined.

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