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Ribonucleic Acid and Ribosomes of *Bacillus stearothermophilus*[1]

GRADY F. SAUNDERS AND L. LEON CAMPBELL
Department of Microbiology, University of Illinois, Urbana, Illinois

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ABSTRACT

SAUNDERS, GRADY F. (University of Illinois, Urbana), AND L. LEON CAMPBELL. Ribonucleic acid and ribosomes of *Bacillus stearothermophilus*. J. Bacteriol. **91**:332–339. 1966.—The ability of some thermophilic bacteria to grow at temperatures as high as 76 C emphasizes the remarkable thermal stability of their crucial macromolecules. An investigation of the ribonucleic acid (RNA) and ribosomes of *Bacillus stearothermophilus* was conducted. Washed log-phase cells were disrupted either by sonic treatment or by alumina grinding in 10^{-2} M $MgCl_2$–10^{-2} M tris-(hydroxymethyl)aminomethane buffer, *p*H 7.4 (TM buffer). Ultracentrifugal analysis revealed peaks at 72.5*S*, 101*S*, and 135*S*, with the 101*S* peak being the most prominent. By lowering the Mg^{++} concentration to 10^{-3} M, the ribosome preparation was dissociated to give 40*S*, 31*S*, and 54*S* peaks. These in turn were reassociated in the presence of 10^{-2} M Mg^{++} to give the larger 73*S* and 135*S* particles. When heated in TM buffer, *Escherichia coli* ribosomes began a gradual dissociation at 58 C, and at 70 C underwent a large hyperchromic shift with a T_m at 72.8 C. In contrast, *B. stearothermophilus* ribosomes did not show a hyperchromic shift below 70 C; they had a T_m of 77.9 C. The thermal denaturation curves of the 4*S*, 16*S*, and 23*S* RNA from both organisms were virtually identical. The gross amino acid composition of *B. stearothermophilus* ribosomes showed no marked differences from that reported for *E. coli* ribosomes. These data suggest that the unusual thermal stability of *B. stearothermophilus* ribosomes may reflect either an unusual packing arrangement of the protein to the RNA or differences in the primary structure of the ribosomal proteins.

Much information concerning the nature of thermophily has accumulated during the past 20 years. The early literature has been covered in the reviews of Gaughran (8) and Allen (1). The literature to 1956 concerned with the relative thermal stability of a number of enzymes and structural proteins has been reviewed by Koffler (13). Since 1957 other papers have appeared on this subject (3–5, 17, 18, 21–24, 29). However, other than the α-amylase of *Bacillus stearothermophilus* (3, 4, 17, 18), none of the enzymes studied to date has been isolated in a high state of purity. Thus, it is not possible to determine whether the heat stability of the enzymes reported is due to a unique structure or to the presence of protective materials in the enzyme preparations. The ability of some thermophilic bacteria to grow at temperatures as high as 76 C, however, emphasizes the remarkable thermal stability of their crucial macromolecules.

Studies on the deoxyribonucleic acid (DNA) base composition of thermophilic bacteria have shown that there is no apparent relationship between the thermal stability of DNA and the ability of cells to proliferate at high temperatures, since the DNA melts at temperatures comparable to that of mesophilic organisms (19, 31).

Friedman and Weinstein (7) reported that a subcellular amino acid-incorporating system from *B. stearothermophilus* is active at 65 C. Studies on the ribosomes and ribonucleic acid (RNA) of a thermophilic bacterium (2, 16, 28) suggest that RNA has a higher thermal stability within the ribosome than in its isolated state.

In this paper we describe some properties of the ribosomes and RNA of *B. stearothermophilus* and compare them with similar components of

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Escherichia coli. Coincidentally, the DNA base composition of both organisms is 50% guanine + cytosine.

MATERIALS AND METHODS

Organisms and growth media. B. stearothermophilus strain 10 was grown at 65 C in a medium containing (per liter of distilled water): Trypticase, 20 g; yeast extract, 3 g; NaCl, 8.5 g; $CaCl_2 \cdot 2H_2O$, 1.47 g; $FeCl_3 \cdot 6H_2O$, 7 mg; $MgSO_4 \cdot 7H_2O$, 15 mg; and $MnCl_2 \cdot 4H_2O$, 1 mg. (The *p*H was adjusted to 7.3 to 7.4.) *E. coli* strain B was grown at 37 C in the same medium. For the isotope-labeling experiments, yeast extract was omitted from the medium.

Isolation of ribosomes. Cells were grown in baffle-bottom DeLong flasks (Bellco Glass, Inc., Vineland, N. J.) with shaking at 133 rev/min, describing an 0.5-inch (1.27-cm) diameter circle, and were harvested in the logarithmic phase of growth. The cells were harvested in the cold, and were washed three times with 10^{-2} M $MgCl_2$–10^{-2} M tris(hydroxymethyl)-aminomethane (Tris) hydrochloride, *p*H 7.4 (TM buffer). All subsequent isolation steps were carried out at 4 C. The cells were broken either by grinding with alumina or by two 30-sec pulses of sonic oscillation in a Branson Sonifier (model S110). The cell debris was removed by two 15-min centrifugations at 25,000 × *g* in a Sorvall centrifuge (rotor SS34). Ribosomes were pelleted by centrifugation at 105,000 × *g* for 3 hr (40 rotor) in a Spinco (model L) preparative ultracentrifuge. The pellet was suspended in TM buffer and dialyzed overnight at 4 C against the desired buffer.

Sedimentation velocities. Sedimentation velocity studies were performed in a Spinco (model E) analytical ultracentrifuge at 20 C. The sample was always equilibrated against the solvent by dialysis prior to ultracentrifugation. All runs were made in a 12-mm standard cell fitted with a Kel F centerpiece. The sedimenting boundaries of the ribosomes were followed with the Schlieren optical system.

Extraction and purification of RNA. To washed cells in 3 × 10^{-2} M Tris buffer (*p*H 7.7), lysozyme (400 μg/ml) and deoxyribonuclease (50 μg/ml) were added. The mixture was incubated at 37 C for 5 min, and then $MgCl_2$ was added to a concentration of 10^{-2} M. The mixture was incubated at 37 C for 3 min, Duponol (to a final concentration of 0.4%) was added, and the mixture was shaken for 5 to 10 sec. An equal volume of phenol buffered with 0.01 M KH_2PO_4 (*p*H 6.7) was added immediately to minimize activity of the cell ribonuclease.

RNA was purified by the method of Gierer and Schramm (10). RNA was precipitated from the aqueous phase after the removal of ether, with three volumes of cold ethyl alcohol, and the precipitate was dissolved in $\frac{1}{10}$× SSC (0.015 M NaCl–0.0015 M trisodium citrate, *p*H 7). The RNA solution was dialyzed against 2,000 volumes of 2× SSC (0.30 M NaCl–0.03 M trisodium citrate, *p*H 7) in the cold for 18 hr.

Base composition analysis of RNA. The RNA solution was hydrolyzed by adding an equal volume of 0.6 N KOH and incubating the mixture at 37 C for 18 hr. The resulting 2′–3′ nucleotides were separated on a Dowex-1-8X formate column with a resin bed 9 mm in diameter by 50 mm high. The method of elution was described by Hayashi and Spiegelman (11). The eluents used were: 0.005 N HCOOH, to remove weakly adsorbed material (nucleosides and free bases); 0.025 N HCOOH, to elute cytidylic acid; 0.1 N HCOOH, for adenylic acid; 0.05 N HCOOH plus 0.05 N $HCOONH_4$, for uridylic acid; and 0.1 N HCOOH plus 0.2 N $HCOONH_4$, for guanylic acid. Unlabeled *E. coli* carrier RNA of known base composition was added prior to hydrolysis to give an absorbancy marker in the nucleotide regions and to provide internal controls for the preferential loss of individual nucleotides. The eluate was collected in 5-ml fractions, and the absorbancy at 260 mμ was determined for each fraction. The radioactivity of each fraction was determined by liquid scintillation counting in a Packard Tri Carb.

RNA fractionation on methylated albumin-kieselguhr (MAK) columns. MAK columns were prepared as described by Mandell and Hershey (15). The RNA solution was adjusted to 20 μg/ml in 0.1 M NaCl–0.05 M phosphate buffer (*p*H 6.7) and charged onto the column. RNA was eluted with a linear gradient of NaCl, and the RNA fractions were identified by absorbancy at 260 mμ.

Melting temperature. Thermal denaturation of RNA and ribosomes was followed in a Gilford (model 2000) spectrophotometer equipped with a heated cell compartment and a temperature readout assembly. All samples had initial absorbancies at 260 mμ of 0.40 to 0.60.

Amino acid analysis. For these analyses large quantities of ribosomes were prepared by a modification of the method of Staehelin et al. (25). Washed cells were broken by brief sonic treatment with a Branson Sonifier, and the cell debris was removed by centrifugation. The time of sonic treatment varied with the quantity of cells used (generally 2 min for 40 ml of a 50% cell suspension). The crude extract was carefully layered onto sucrose layers. The contents of the tube then consisted of a bottom layer of 7.7 ml of 80% sucrose, a middle layer of 14 ml of 1.0 M sucrose, and a top sample layer of 14 ml. All solutions were adjusted to 10^{-2} M $MgCl_2$–10^{-2} M Tris-HCl (*p*H 7.4). The tubes were centrifuged at 2 C for 4 hr in a Spinco model L preparative ultracentrifuge in a no. 30 fixed-angle rotor. The centrifuge was allowed to decelerate without braking, and three fractions were removed from each tube with the aid of a syringe. The upper 20-ml fraction (fraction A) contained little ultraviolet-absorbing material. The 6-ml fraction below this (fraction B) showed peaks at 45*S*, 57*S*, and 70*S* in the analytical ultracentrifuge. Fraction C was the 9 ml immediately above the pellet. After dialyzing out the sucrose and passing fraction C through a second cycle of centrifugation through the sucrose layers, the main ribosome layer appeared in the fraction B position. This fraction showed peaks at 56*S*, 73*S*, and 100*S*. This material (primarily 73*S*) was exhaustively dialyzed against demineralized water and concentrated by

lyophilization. Approximately 2 mg of protein, estimated by the method of Lowry et al. (14), was dried and hydrolyzed for various times in 1.0 ml of glass-distilled constant-boiling HCl at 110 C under vacuum.

The amino acid composition of the hydrolysates was determined on a Spinco (model 120 B) automatic amino acid analyzer equipped for accelerated analysis and employing the Beckman custom spherical resins. Cystine was determined as cysteic acid after performic acid oxidation (12) of the ribosomal protein followed by hydrolysis and analysis on the amino acid anayzer. Tryptophan was not determined.

RESULTS AND DISCUSSION

Figure 1 shows the Schlieren diagram obtained in the analytical ultracentrifuge of the partially purified *B. stearothermophilus* ribosomes in TM buffer. The large peaks are at 4*S*, 72.5*S*, 101*S*, and 135*S* (corrected for solvent viscosity), with the 101*S* being the most prominent. Dialysis of

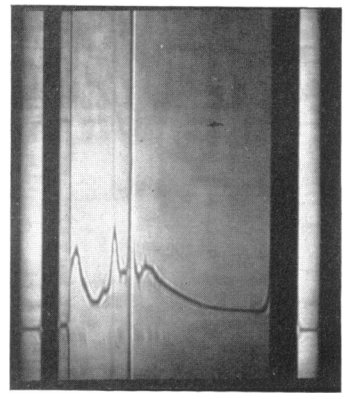

FIG. 1. *Sedimentation pattern of Bacillus stearothermophilus ribosomes in TM buffer. Centrifugation was carried out at 35,600 rev/min at a concentration of 10.7 mg of RNA/ml. The direction of sedimentation is from left to right.*

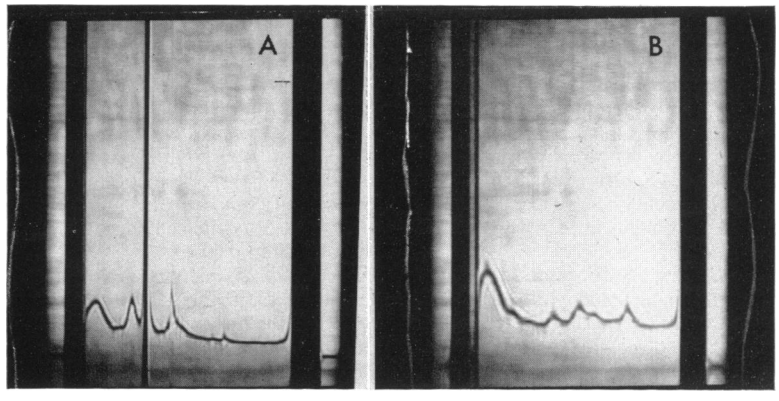

FIG. 2. *Effect of Mg^{++} concentration on sedimentation pattern of Bacillus stearothermophilus ribosomes. (A) Ribosomes in 10^{-3} M $MgCl_2$–10^{-2} M Tris buffer (pH 7.4). (B) Reconstituted ribosomes in TM buffer. Conditions of centrifugation and RNA concentration were the same as in Fig. 1. Photographs taken 16 min after reaching speed.*

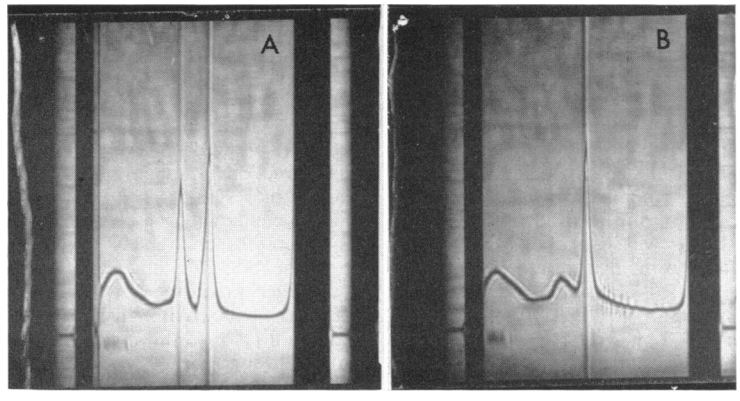

FIG. 3. *Breakdown of Bacillus stearothermophilus ribosomes in low Mg^{++} buffer. (A) Sedimentation pattern of ribosomes in 10^{-4} M $MgCl_2$–10^{-2} M Tris buffer (pH 7.4). (B) Sedimentation pattern of ribosomes in A after treatment with ribonuclease (50 μg/ml for 15 min at room temperature). Conditions for centrifugation and RNA concentration were the same as in Fig. 1. Photographs taken 32 min after reaching speed.*

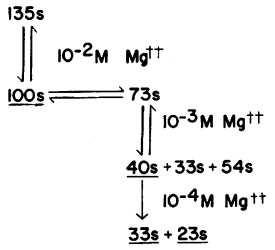

FIG. 4. *Postulated interconversion scheme for Bacillus stearothermophilus ribosomes. The major peaks at each Mg^{++} concentration are underlined. The 24S peak is ribonuclease-sensitive.*

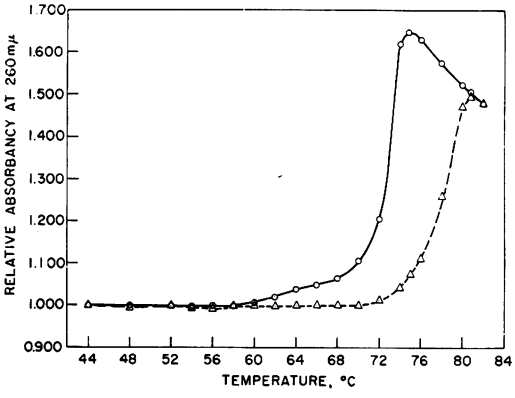

FIG. 5. *Thermal denaturation profile of Bacillus stearothermophilus and Escherichia coli ribosomes in TM buffer.* ○, *E. coli ribosomes;* △, *B. stearothermophilus ribosomes.*

the ribosome preparation from TM buffer into 10^{-3} M $MgCl_2$–10^{-2} M Tris (*p*H 7.4) buffer overnight at 4 C resulted in partial degradation. Figure 2A shows a prominent hypersharp peak at 40*S* and two other large peaks at 31*S* and 54*S*. After dialysis from the 10^{-3} M $MgCl_2$ back into TM buffer, the pattern obtained is shown in Fig. 2B. Here the two larger peaks were at 73*S* and 135*S*. Small peaks were also found at 54*S* and approximately 100*S*. This shows that the breakdown products can be reassociated in the presence of 10^{-2} M $MgCl_2$ to give the larger 73*S* and 135*S* particles.

Dialysis of the thermophile ribosome preparation into 10^{-4} M $MgCl_2$–10^{-2} M Tris (*p*H 7.4) buffer resulted in further breakdown (Fig. 3A). The major peaks were at 33*S* and 24*S*, with no larger peaks observable. Treatment of this preparation with 50 μg of ribonuclease/ml for 15 min at room temperature resulted in the loss of the 23*S* peak (Fig. 3B). The 33*S* peak remained hypersharp.

Tissieres et al. (30) have shown that in 10^{-2} M Mg^{++} the predominant particle is 100*S* in *E. coli* ribosomes. Lowering the Mg^{++} concentration to 10^{-3} M results in conversion of a majority of the particles to 70*S*. A further decrease in Mg^{++} to 2.5×10^{-4} M leads to the appearance of 30*S* and 50*S* ribosomes from *E. coli*.

Figure 4 summarizes the possible interconversion scheme for *B. stearothermophilus* ribosomes. These results suggest that *B. stearothermophilus* ribosomes are a little more sensitive to Mg^{++} concentration than are *E. coli* ribosomes.

To compare the thermal stability of the ribosomes, the purified preparation was centrifuged through a sucrose density gradient in TM buffer, and the major peak was isolated and dialyzed against TM buffer. A similar preparation of *E.*

TABLE 1. *Base composition of Bacillus stearothermophilus DNA and RNA*

Sample	Buoyant density	Guanine + cytosine*	T_m	Guanine + cytosine†
	g/cc	%	*C*	%
DNA.....	1.713	50.5	90.8	52.4
	Mole per cent‡				
	Guanine	Adenine	Cytosine	Uracil	Guanine + cytosine
					%
mRNA.....	29.3	26.8	21.1	22.8	50.4
Bulk RNA....	34.0	23.0	23.5	18.2	57.5

* Base composition was calculated from the buoyant density value according to the equations of Sueoka (26).

† Base composition from T_m measurements was calculated by the equation of Marmur and Doty (20): per cent guanine + cytosine = $(T_m - 69.3)$ 2.439.

‡ Base composition determined after hydrolysis of the RNA with 0.6 N KOH and chromatography on a Dowex-1-8X formate column.

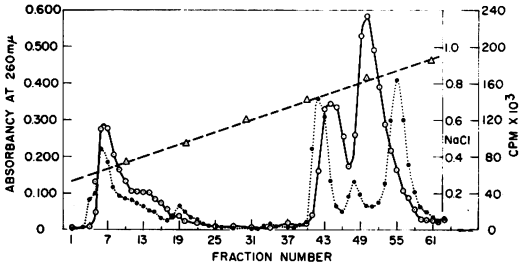

FIG. 6. *Co-chromatography of Bacillus stearothermophilus RNA and Escherichia coli RNA on a methylated albumin-kieselguhr column.* ○, *E. coli RNA, identified by absorbancy at 260 mμ;* ●, *B. stearothermophilus RNA identified by P^{32} radioactivity;* △, *salt molarity in the fractions collected.*

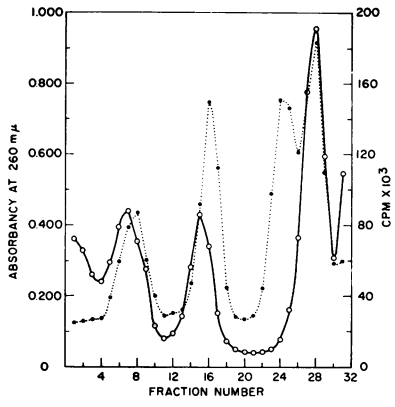

FIG. 7. *Sucrose density gradient co-centrifugation profile of Bacillus stearothermophilus and Escherichia coli RNA. A 0.5-ml sample containing 600 μg of RNA was layered onto 28 ml of a 5 to 20% linear sucrose gradient in TM buffer. The tubes were centrifuged in an SW 25 rotor in a model L preparative ultracentrifuge at 25,000 rev/min for 12 hr at 2 C. The tubes were pierced, and fractions were collected and assayed for radioactivity and absorbancy at 260 mμ. ○, E. coli RNA (absorbancy); ●, B. stearothermophilus RNA (radioactivity, count/min).*

TABLE 2. *Base composition of Bacillus stearothermophilus RNA fractions*

Fraction	Mole per cent				Guanine + cytosine
	Guanine	Adenine	Cytosine	Uracil	
					%
4S	31.8	18.7	30.5	18.9	62.3
16S	32.0	21.0	29.4	17.7	61.4
23S	33.1	25.0	23.1	18.8	56.2

coli ribosomes was given the same treatment. Figure 5 shows the melting curves obtained from the two preparations. The large hyperchromic shift (ca. 60%) noted is probably partially due to protein dissociation from the RNA. The *E. coli* particles began gradual dissociation at about 58 C, and then at 70 C underwent a large hyperchromic shift with a T_m at 72.8 C. On the other hand, the ribosomes of *B. stearothermophilus* did not show any rise in absorbancy until about 72 C; they had a T_m of 77.9 C. The temperature at which the ribosomes of *B. stearothermophilus* begin to melt (72 C) is very close to the temperature above which the organism will not grow (76 C).

To investigate the possibility that the greater heat stability of *B. stearothermophilus* ribosomes was due to the presence of an unusual ribosomal RNA species, we undertook a study of the RNA classes of this organism. Table 1 shows the nucleotide composition of the DNA and cellular RNA. The messenger RNA (mRNA) was labeled with a 30-sec P^{32} pulse and isolated by the cold phenol method. The generation time of *B. stearothermophilus* strain 10 under the growth conditions used here is 31 min. The base composition of the mRNA agrees well with the DNA com-

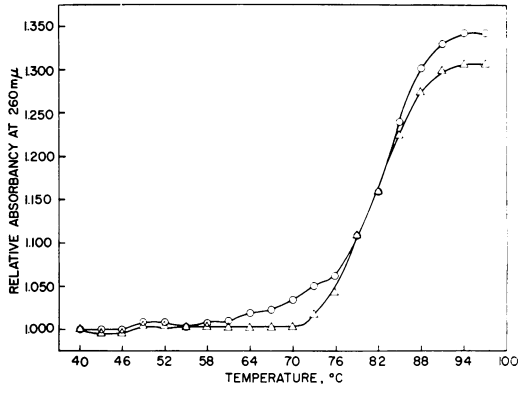

FIG. 8. *Melting curve of the 4S RNA of Bacillus stearothermophilus and Escherichia coli. The purified RNA was fractionated on a MAK column and dialyzed against TM buffer. △, E. coli; ○, B. stearothermophilus.*

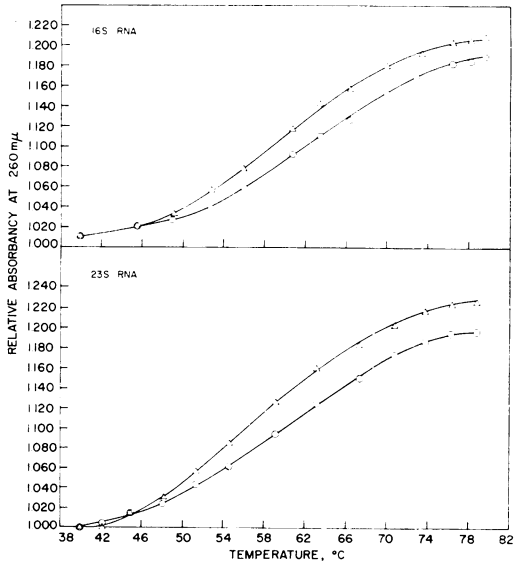

FIG. 9. *Melting curve of 16S and 23S RNA from Bacillus stearothermophilus and Escherichia coli. Purified RNA was fractionated on a MAK column, and the contents of the peak tubes were dialyzed into 0.15 M NaCl–0.015 M trisodium citrate (pH 7). △, E. coli; ○, B. stearothermophilus.*

TABLE 3. *Amino acid composition (mole per cent) of Bacillus stearothermophilus ribosomes*

Amino acid	Hydrolysis time (hr)			Avg
	22	49	72	
Lysine	6.33	6.39	6.18	6.30
Histidine	1.80	1.86	2.14	1.93
Arginine	5.10	4.80	5.12	5.01
Aspartic acid	8.81	9.20	9.48	9.16
Threonine	5.25	4.88	4.78	5.53*
Serine	3.89	3.30	2.78	4.44*
Glutamic acid	13.64	13.25	13.10	13.33
Proline	4.31	4.59	4.44	4.44
Glycine	8.96	9.47	9.44	9.29
Alanine	10.29	10.61	10.64	10.51
Valine	8.40	8.82	9.07	9.07†
Methionine	2.33	2.25	2.43	2.33
Isoleucine	6.36	6.85	6.71	6.64
Leucine	8.14	8.47	8.41	8.34
Tyrosine	2.65	1.82	2.01	2.16
Phenylalanine	3.76	3.46	3.40	3.55
½ Cystine	0.82	—	—	0.82

* Extrapolated to zero hydrolysis time.
† The 72-hr value.

position. The nucleotide composition of the bulk RNA (which is usually largely ribosomal in origin) was 57.5% guanine + cytosine (GC), which was slightly higher than that found by Hayashi and Spiegelman (11) for *E. coli* (54.3% GC), *B. megaterium* (53.9% GC), and *Pseudomonas aeruginosa* (53.3% GC).

To establish the size classes of the RNA present in *B. stearothermophilus*, steady-state labeled RNA was prepared. Cells were grown for six generations in a P^{32} medium, washed twice with unlabeled medium, and then chased for 1.5 generations in the same medium. RNA was isolated from the Duponol-lysozyme treated cells by the cold phenol method. Purified RNA from *B. stearothermophilus* and *E. coli* were co-chromatographed on a MAK column. The RNA was eluted with a linear gradient of NaCl, and the *E. coli* RNA was identified by absorbancy at 260 mμ (Fig. 6).

The 4*S* material in both preparations elutes at approximately the same salt molarity. However, the two larger peaks, presumably ribosomal RNA, are split further in the thermophile than are the corresponding *E. coli* RNA peaks. The MAK column has been shown by Sueoka and Cheng (27) to select for size and composition of the RNA species; the larger the molecule the later it elutes, and the higher the GC content the earlier the elution.

To check for size differences in the two high molecular weight species, the RNA preparations were centrifuged through a sucrose gradient. P^{32} (steady state)-labeled *B. stearothermophilus* RNA and cold *E. coli* RNA were layered onto a 3 to 15% sucrose gradient in TM buffer. The centrifugation was carried out in an SW-25 rotor at 25,000 rev/min at 0 C for 5 hr. The 16*S* and 23*S* peaks from both organisms were at approximately the same positions (Fig. 7), so no gross size differences exist. These results have been confirmed in the analytical ultracentrifuge at a concentration of 20 μg/ml.

The nucleotide compositions of the peak tubes obtained from the MAK column (Fig. 6) are given in Table 2. The 4*S* RNA peak is 62% in GC, with adenine approximately equal to uracil and guanine approximately equal to cytosine. Dunn, Smith, and Spahr (6) and Giacomoni and Spiegelman (9) have shown the soluble RNA of *E. coli* to be 61% in GC.

The 16*S* RNA of *B. stearothermophilus* is 61% GC, which is consistent with its elution from a MAK column before the 16*S* RNA of *E. coli* (54% GC). The 23*S* peak of the thermophile is approximately twice the size of the 16*S* peak, and is slightly higher in GC than *E. coli* 23*S* RNA (56% versus 53%). The elution pattern from a MAK column may reflect a subtle change in secondary structure from the *E. coli* counterpart.

The thermal denaturation curves of the 4*S*, 16*S*, and 23*S* fractions from both organisms are virtually identical (Fig. 8 and 9).

The amino acid composition of *B. stearothermophilus* strain 10 ribosomes is presented in Table 3. The data are expressed in terms of mole percentages in each analysis.

The threonine and serine values were extrapolated to zero hydrolysis time by the method of least squares. The amount of valine was observed to increase with time of hydrolysis; the 72-hr value was used in the composition calculations.

A comparison of the amino acid composition of *B. stearothermophilus* ribosomes with that reported by Spahr (24) for *E. coli* ribosomes shows no marked differences. The basic amino acid content of *E. coli* ribosomes is 17.78%, whereas in *B. stearothermophilus* ribosomes it is 13.24%; the half cystine content is approximately three times higher in the thermophile.

In agreement with the work of Teece and his coworkers (2, 16, 28) with another thermophilic bacterium, the differences, if any, in the gross properties of the RNA species present in *B. stearothermophilus* and *E. coli* are slight and probably could not account for the increased stability of the protein-synthesizing system. Therefore, the more stable ribosomes of the thermophile may reflect a difference in the ribosomal protein

moiety. However, the amino acid composition of the ribosomal proteins of the two species shows no significant differences. The slightly higher cystine content in the ribosomal protein of *B. stearothermophilus* may reflect the presence of more disulfide bonds, which could lead to greater thermostability; however, the quantity of cystine is still very low. A different packing arrangement in the ribosomal protein or a difference in the primary structure of the protein could conceivably result in a greater thermostability of the ribosomes of *B. stearothermophilus*. These possibilities are currently being investigated.

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