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Lack of ribosomal protein S1 in *Bacillus stearothermophilus*

(agarose–acrylamide composite gel electrophoresis/*Escherichia coli* protein S1/f2 phage RNA)

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ABSTRACT **The 30S ribosomal subunit of *Bacillus stearothermophilus* migrated as a single band when electrophoresed on agarose-acrylamide composite gels. The addition of the ribosomal protein S1 purified from *Escherichia coli* resulted in the appearance of an additional band migrating more slowly; ^{14}C-labeled S1 of *E. coli* was shown to be associated only with this form. Antibody against *E. coli* protein S1 did not crossreact with either the total 30S ribosomal proteins or the postribosomal supernatant from *B. stearothermophilus*. These results indicate that *B. stearothermophilus* lacks a protein equivalent to *E. coli* S1 and may explain our previous finding [*Eur. J. Biochem.* 56, 15–22 (1975)] that *E. coli* S1 greatly stimulated the translation by *B. stearothermophilus* ribosomes of f2 phage RNA.**

In our previous paper (1) we showed that the addition of the 30S ribosomal protein S1 purified from *Escherichia coli* caused a marked stimulation in the *in vitro* synthesis by *Bacillus stearothermophilus* ribosomes of coat protein and replicase in response to f2 phage RNA. Without this protein, *B. stearothermophilus* ribosomes synthesized only A-protein (maturation protein) and a trace amount of coat protein and replicase. Protein S1 is the largest of all the ribosomal proteins of *E. coli* (2) and has been considered to be a fractional protein, that is, a protein present less than one copy per ribosome (3, 4). Recently, this protein was shown to be indispensable for the translation of natural as well as synthetic messengers (5, 6) and for the binding of MS2 phage RNA to ribosomes (7). The 30S ribosomal subunit of *E. coli* was shown (7–9) to exist in at least two forms, a fast migrating form (F-30S) and a slow migrating form (S-30S), which were separable from each other by electrophoresis on agarose–acrylamide composite gels. The protein S1 converted F-30S into S-30S by binding to the 3′-end of 16S RNA (9). The difference between S-30S and F-30S was thus concluded to be only the presence in the former and absence from the latter of the protein S1. MS2 phage RNA was found (7) to bind only to the former.

In this paper, we report that *B. stearothermophilus* 30S ribosomes exist only in F-30S form and *E. coli* S1 converts them into S-30S form. When incubated *in vitro*, ^{14}C-labeled *E. coli* S1 was found to be associated with S-30S form and f2 phage RNA was found to bind preferentially to this form. It thus seems that the 30S ribosomal subunit of *B. stearothermophilus* does not have a protein functionally equivalent to *E. coli* S1.

MATERIALS AND METHODS

Preparation of Ribosomes, f2 Phage and Ribosomal S1. Ribosomes were prepared from *B. stearothermophilus* strain 799 and from *E. coli* A19 and RNA was extracted from f2 phage particles as described (1). The 30S ribosomal protein S1 and S1-depleted 30S subunit from *E. coli* A19 were prepared as described (5, 10). The purity of S1 preparation was examined by dodecylsulfate-gel electrophoresis and found more than 95% pure. Purified protein S1 and *B. stearothermophilus* 30S ribosomes were labeled as reported (11, 12), using [^{14}C]formaldehyde (New England Nuclear, Boston). The labeled materials were extensively dialyzed against buffer I (10 mM Tris·HCl, pH 7.4, containing 10 mM $MgCl_2$, 60 mM NH_4Cl, and 6 mM 2-mercaptoethanol) and kept at −80° until use. The specific radioactivity of the resulting ^{14}C-labeled protein S1 was 1×10^4 cpm/μg of protein and that of *B. stearothermophilus* 30S ribosomes, 2×10^4 cpm/1 A_{260} unit at 88% counting efficiency. (A_{260} unit is that amount of material that gives an absorbance of 1 when dissolved in 1 ml of solvent at a light path of 1 cm.)

Other Methods. Electrophoresis on 0.5% agarose–3% acrylamide composite gels in Tris-boric acid-EDTA buffer, pH 8.3, was performed as described (7, 8). Gels were either stained with 0.2% methylene blue or cut into 1-mm slices, swelled in Soluene 350 (Packard), and their radioactivity determined. Sucrose density gradient analysis of ^{14}C-labeled *E. coli* S1 and *B. stearothermophilus* 30S ribosome complex was performed on 10–30% linear gradient of RNase-free sucrose (Schwarz-Mann) in buffer I. After centrifugation at 45,000 rpm for 3 hr at 4° in a Spinco SW65 rotor, tubes were punctured at the bottom and 7-drop fractions were collected. The absorbance at 260 nm of each fraction was determined spectrophotometrically and the radioactivity was measured with a scintillator cocktail containing Triton X-100. Ouchterlony immunodiffusion was kindly performed by Dr. G. Stöffler as reported (14).

RESULTS AND DISCUSSION

The results reported in our previous paper (1) suggested the possibility that the 30S ribosomal subunit of *B. stearothermophilus* may lack the protein functionally equivalent to *E. coli* S1. If this were the case, it would conceivably migrate as a single band upon electrophoresis on agarose–acrylamide composite gels, since it has been established for *E. coli* 30S ribosomes (8, 9) that they exist in at least two forms and that the slow migrating form (S-30S) has protein S1, whereas the fast migrating form (F-30S) does not. In fact, as shown in Fig. 1, 30S ribosomes of *B. stearothermophilus* (gel 3) gave rise to only a single band upon electrophoresis on 0.5% agarose–3% acrylamide composite gels in EDTA-containing buffer in contrast to *E. coli* 30S ribosomes, which yielded two bands (gel 1 and refs. 8 and 9). Similarly, 70S ribosomes of *B. stearothermophilus* yielded a single band of 30S subunit (gel 2). We noticed that, under the conditions used, the 30S ribosomal subunit of *B. stearothermophilus* always migrated faster than that of *E. coli*. The reason for this is not known.

Abbreviations: F-30S and S-30S, fast and slow migrating form, respectively, of the 30S ribosomal subunit; A_{260} unit, that amount of material that gives an absorbance of 1 when dissolved in 1 ml of solvent at a light path of 1 cm.

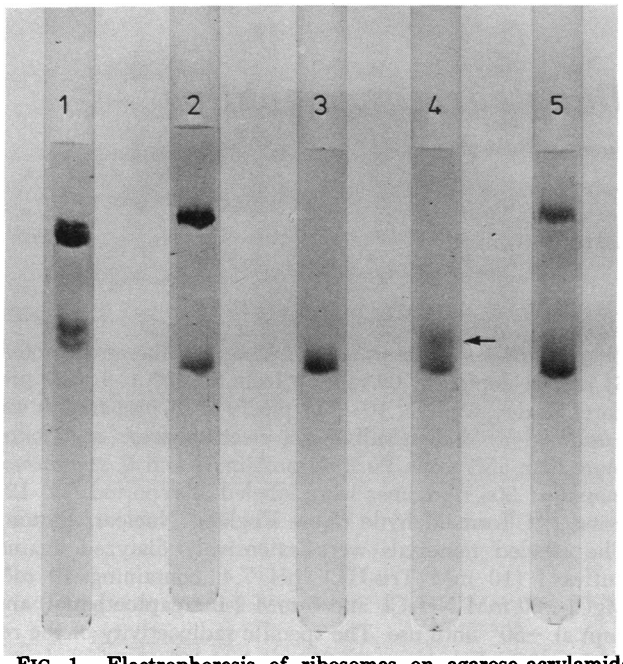

FIG. 1. Electrophoresis of ribosomes on agarose-acrylamide gels. Four-tenths A_{260} unit each of 70S ribosomes of *E. coli* A19 (gel 1) and of *B. stearothermophilus* 799 (gel 2), and 0.25 A_{260} unit of 30S ribosomes of *B. stearothermophilus* (gel 3) were analyzed by electrophoresis on 0.5% agarose–3% acrylamide composite gels (7, 8) prepared in 0.6 × 10 cm glass tubes. In order to see the effect of protein S1, 30S ribosomes of *B. stearothermophilus* were preincubated at 0° for 5 min with 2 μg of protein S1 purified from *E. coli* A19 (the molar ratio of S1 to ribosomes was approximately 6). The mixture was either directly analyzed (gel 4) or incubated for an additional 5 min with 3.3 μg of f2 phage RNA and then subjected to electrophoresis (gel 5). Electrophoresis was at 90 V for 5 hr at 4° and from top to bottom. All gels were stained with 0.2% methylene blue and destained with water. Arrow on gel 4 indicates the position of S-30S. The upper band in gels 1 and 2 corresponds to 50S subunit. The upper band in gel 5 is almost identical with the band of f2 RNA alone (not shown), and is most likely a mixture of f2 RNA and an f2 RNA-protein S1-ribosome complex (see *text* and Fig. 4).

Since we found that the addition of *E. coli* S1 greatly stimulated the synthesis by *B. stearothermophilus* ribosomes of coat protein and replicase of f2 phage (1), we incubated 30S ribosomes of *B. stearothermophilus* with purified *E. coli* S1 and then electrophoresed. This treatment converted a fraction of *B. stearothermophilus* 30S ribosomes into the form migrating more slowly than the main band, as expected (Fig. 1, gel 4), although this new band was not so distinct as that of *E. coli* S-30S. The finding suggests that the 30S ribosomal subunit of *B. stearothermophilus* exists only in F-30S form and that *E. coli* S1 specifically interacts with and converts it into S-30S form, as it does with the 30S ribosomal subunit of *E. coli*.

To prove this further, we incubated 30S ribosomes of *B. stearothermophilus* with *E. coli* S1 labeled *in vitro* with [^{14}C]formaldehyde, as described in *Materials and Methods*, and analyzed the mixture by gel electrophoresis. As illustrated in Fig. 2a, the radioactivity of protein S1 coincided well with the S-30S form of *B. stearothermophilus* ribosomes. Conversely, when ^{14}C-labeled 30S ribosomes of *B. stearothermophilus* were incubated with *E. coli* protein S1 and then electrophoresed, a significant amount of radioactivity migrated more slowly than the main peak of F-30S form (Fig. 2b). The position of S-30S determined independently on gels stained with methylene blue coincided well with this more slowly migrating portion. Results of these two experiments strongly support the conclusion mentioned above.

The same conclusion was also reached from an experiment in which ^{14}C-labeled *E. coli* S1 was mixed with 30S ribosomes of *B. stearothermophilus* and analyzed by sucrose density gradient centrifugation. A considerable amount of *E. coli* S1 was found to be associated with 30S ribosomes of *B. stearothermophilus*, as shown in Fig. 3. The amount of radioactivity associated with *B. stearothermophilus* ribosomes (Fig. 3b) was much smaller than that associated with *E. coli* ribosomes (Fig. 3a), suggesting a weaker binding of *E. coli* S1 to the former than to the latter. To minimize the detachment of bound *E. coli* S1 from 30S ribosomes of *B. stearothermophilus* during centrifugation, glutaraldehyde was added to the mixture before it was placed on the sucrose gradient and then centrifuged. However, this treatment did not significantly enhance the amount of *E. coli* S1 bound to *B. stearothermophilus* 30S ribosomes (data not shown).

From the results shown in Figs. 1, 2, and 3, we concluded that *E. coli* S1 specifically interacts with the 30S ribosomal subunit of *B. stearothermophilus* to form a complex. The next step was to ask if this complex is active in binding of f2 phage RNA, as is the 30S ribosomal subunit of *E. coli* containing S1. When *E. coli* 30S ribosomes were incubated at 0°

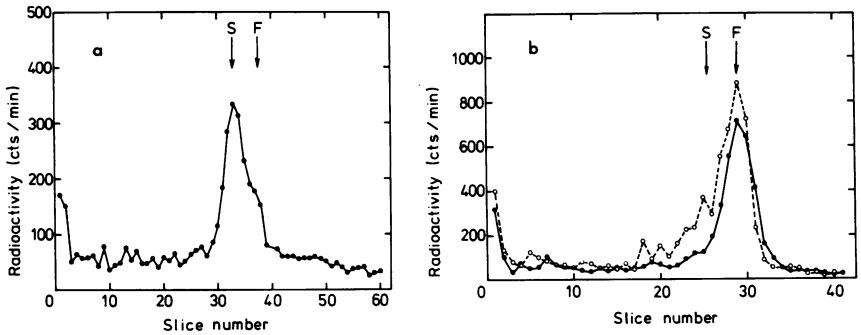

FIG. 2. Binding of *E. coli* protein S1 to 30S ribosomes of *B. stearothermophilus*. (a) 30S ribosomes of *B. stearothermophilus* (0.25 A_{260} unit) were preincubated with 4 μg of *E. coli* protein S1 labeled *in vitro* with [^{14}C]formaldehyde, as described in *Materials and Methods*, and then subjected to electrophoresis as in Fig. 1. Gels were then cut into 1-mm slices, swelled in 0.5 ml of Soluene 350 (Packard) in glass vials at 50° for 3 to 4 hr, and radioactivity was determined. Approximately 10^4 cpm of *E. coli* S1 were used per gel. Arrows S and F indicate the positions of S-30S and F-30S, respectively, which were determined on gels electrophoresed in parallel and stained with methylene blue. (b) 30S ribosomes of *B. stearothermophilus* (0.24 A_{260} unit) labeled *in vitro* with [^{14}C]formaldehyde, as described in *Materials and Methods*, were preincubated with (O- - - -O) or without (●——●) 2 μg of *E. coli* protein S1 and then electrophoresed. Gels were sliced and radioactivity was determined as in (a). Approximately 5 × 10^3 cpm of *B. stearothermophilus* 30S ribosomes were used per gel.

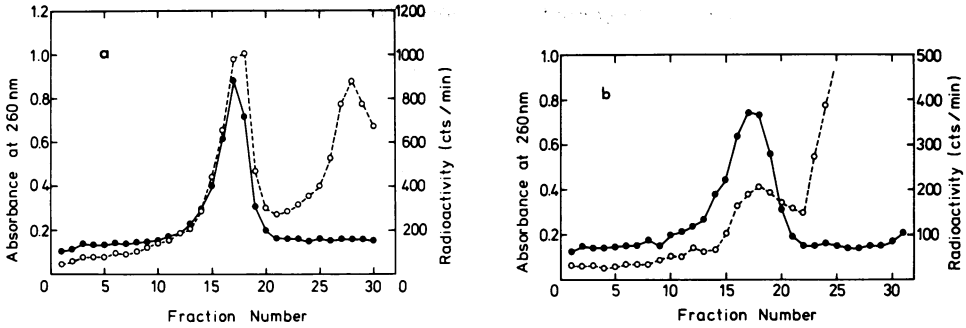

FIG. 3. Sucrose density gradient centrifugation of ribosome-*E. coli* S1 complex. 3 A_{260} units of *E. coli* 30S ribosomes (a) or 3 A_{260} units of *B. stearothermophilus* 30S ribosomes (b) were incubated with 2 μg of *E. coli* S1 (2×10^4 cpm) at 0° for 5 min. Mixtures were analyzed by centrifugation on a 10–30% linear sucrose gradient, as described in *Materials and Methods*. Centrifugation is from right to left. (●——●) Absorbance at 260 nm, (O- - - - -O) radioactivity.

for 5 min with MS2 phage RNA (an RNA coliphage closely related to f2 phage), only the S-30S form was found to bind the RNA (7). We accordingly incubated at 0° ^{14}C-labeled 30S ribosomes of *B. stearothermophilus* with f2 phage RNA in the presence of *E. coli* S1 and applied the mixtures on agarose-acrylamide composite gels. In the absence of f2 RNA, a significant amount of 30S ribosomes migrated more slowly than the main peak, as shown in Fig. 4. Upon the addition of f2 RNA, this more slowly migrating portion almost completely disappeared, and instead a new peak appeared at the position of f2 RNA. This was also noticed on the gels stained with methylene blue (Fig. 1, gels 4 and 5). It is very likely that this peak is a complex consisting of f2 RNA, *E. coli* S1, and *B. stearothermophilus* 30S ribosomes. However, attempts to prove that this was really the case failed to succeed, mainly for the following two reasons. First, when we incubated f2 RNA with ^{14}C-labeled *E. coli* S1 alone and then electrophoresed, a large amount of radioactivity was found to be associated with f2 RNA, suggesting that protein S1 alone bound to f2 RNA. The addition of *B. stearothermophilus* 30S ribosomes did not significantly enhance the binding of radioactive protein S1 to f2 RNA. Second, a considerable amount of ^{14}C-labeled 30S ribosomes of *B. stearothermophilus* comigrated with f2 RNA, even if *E. coli* S1 was omitted. This might be due to the fact that *B. stearothermophilus* ribosomes can bind the region of f2 RNA containing the initiator codon for A-protein (15). It should be noted, however, that incubation of ^{14}C-labeled 30S ribosomes of *B. stearothermophilus* with *E. coli* protein S1 in the presence of f2 RNA decreased the amount of radioactivity migrating more slowly than the main peak to almost negligible level (Fig. 4). This clearly indicates that f2 RNA interacts with S-30S much more efficiently than the native F-30S.

The binding of f2 phage RNA labeled with [^{3}H]uridine to *B. stearothermophilus* ribosomes in the presence or absence of added *E. coli* S1 was also measured, using the Millipore filter method (7). The increase in the ratio of *E. coli* S1 to ribosomes resulted in an increase in the amount of f2 [^{3}H]RNA retained on the filter. Since protein S1 alone bound f2 RNA and the complex was also retained on the filter, we subtracted the value obtained without ribosomes from that obtained with ribosomes. It was found that the values were not quite reproducible for the given amounts of ribosomes and S1, which was likely due to the weak binding of f2 phage RNA to protein S1 alone. Nevertheless, we always observed an increase in the differential values of f2 phage [^{3}H]RNA retained on the filter with increasing amounts of *E. coli* S1.

From all these data described above it seems very likely that the 30S ribosomal subunit of *B. stearothermophilus* does not have a protein functionally equivalent to *E. coli* protein S1 and that *E. coli* S1 specifically interacts with it to make it active with f2 phage RNA. It is not likely that the protein S1 of *B. stearothermophilus*, if present at all, comes off the 30S subunit during the preparation of ribosomes, since the ribosomes of *B. stearothermophilus* were shown (1) to have almost negligible efficiency, and the addition of *E. coli* protein S1 alone rendered them very active, in translating the coat protein cistron of f2 phage RNA when assayed *in vitro* with homologous ribosomal wash and postribosomal supernatant.

It was previously shown (17) that many of the purified 30S ribosomal proteins of *B. stearothermophilus* crossreacted with antibodies against *E. coli* proteins. Thus the data summarized in Fig. 5 further confirm the lack in *B. stearothermophilus* of a protein equivalent to *E. coli* S1. Antibody developed against *E. coli* protein S1 did not show any crossreaction with the total proteins of 30S ribosomal subunit (peripheral well 1), the total proteins of 70S ribosome (well 2), or the postribosomal supernatant (well 3) of *B. stearothermophilus*, whereas it showed a clear precipitin line with their *E. coli* counterparts (wells 7 and 8). We tested eight different concentrations of each of the protein samples and three different batches of antiserum against *E. coli* S1 in order to confirm the negativity of crossreaction, but in no

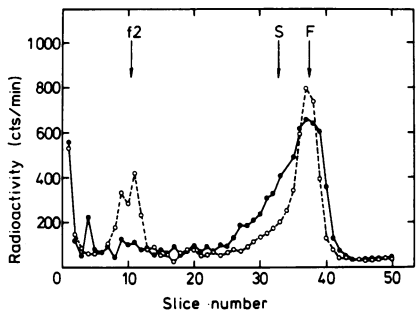

FIG. 4. Binding of f2 phage RNA to S-30S form of *B. stearothermophilus* ribosomes. *B. stearothermophilus* 30S ribosomes [0.48 A_{260} unit (1×10^4 cpm)] labeled *in vitro* with [^{14}C]formaldehyde were preincubated with 4 μg of *E. coli* S1 at 0° for 5 min and then divided into two equal portions. To one of them (O- - -O) was added 8 μg of f2 phage RNA and to the other (●——●) an equal volume of buffer. The mixtures were incubated for a further 5 min and then subjected to electrophoresis. Gels were sliced and radioactivity was determined as in Fig. 2. Arrows f2, S, and F indicate the positions of f2 RNA, S-30S, and F-30S, respectively, which were estimated on gels electrophoresed in parallel and stained with methylene blue as in Fig. 1.

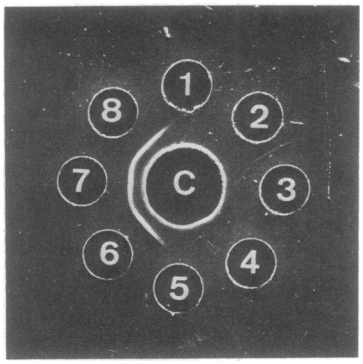

FIG. 5. Ouchterlony double-diffusion of antibody against *E. coli* S1. Ouchterlony double-diffusion tests were carried out as reported (14) with antibody against *E. coli* S1 raised in rabbits. The figure summarizes a series of experiments in which three different lots of antibody and various concentrations of sample proteins were used. The center well contains antibody against *E. coli* S1. The peripheral wells contain: (*1*) 100 μg of total 30S ribosomal proteins (B); (*2*) 300 μg of total 70S ribosomal proteins (B); (*3*) 1000 μg of postribosomal supernatant proteins (B); (*4* and *5*) 4 μg each of two different lots of B-S1; (*6*) 2 μg of purified *E. coli* S1; (*7*) 75 μg of total 30S ribosomal proteins (E); (*8*) 800 μg of postribosomal supernatant proteins (E). B and E stand for *B. stearothermophilus* and *E. coli*, respectively.

case observed any precipitin line. Two different lots of the protein purified from *B. stearothermophilus*, which we designated as B-S1 and correlated with *E. coli* S1 (16, 17), were also tested (wells 4 and 5). They did not crossreact with the antiserum against *E. coli* S1 at all the concentrations (0.5–10 μg per well) tested. Similarly, two other acidic proteins of *B. stearothermophilus*, B-S2 and B-S9 (16, 17), were tested, but neither of them showed any crossreaction (data not shown). Under the same conditions, clear precipitin lines were observed with 0.5–12 μg of protein S1, 12–150 μg of total 30S ribosomal proteins, 15–400 μg of total 70S ribosomal proteins, or with 500–2000 μg of postribosomal supernatant of *E. coli*.

The results reported in this paper may well explain our previous finding (1) that the addition of *E. coli* S1 to an *in vitro* protein-synthesizing system derived from *B. stearothermophilus* greatly stimulated the synthesis of coat protein and replicase of f2 phage, both of which were only negligibly synthesized in its absence. In this connection it is interesting to note that the bulk mRNAs from Gram-negative bacteria were not efficiently translated *in vitro* by ribosomes from Gram-positive bacteria, including two of *Bacillus* bacteria, while those from Gram-positive bacteria were translated by them (13). Our preliminary data suggest that ribosomes of *Bacillus coagulans*, a facultative thermophile, also lack the protein equivalent to *E. coli* S1.

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