Use of Natural mRNAs in the Cell-Free Protein-Synthesizing Systems of the Moderate Halophile *Vibrio costicola*

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In vitro protein synthesis was studied in extracts of the moderate halophile *Vibrio costicola* by using as mRNAs the endogenous mRNA of *V. costicola* and the RNA of the R17 bacteriophage of *Escherichia coli*. Protein synthesis (amino acid incorporation) was dependent on the messenger, ribosomes, soluble cytoplasmic factors, energy source, and tRNA_{Met} (in the R17 RNA system) and was inhibited by certain antibiotics. These properties indicated de novo protein synthesis. In the *V. costicola* system directed by R17 RNA, a protein of the same electrophoretic mobility as the major coat protein of the R17 phage was synthesized. Antibiotic action and the response to added tRNA_{Met} showed that protein synthesis in the R17 RNA system, but not in the endogenous messenger system, absolutely depended on initiation. Optimal activity of both systems was observed in 250 to 300 mM NH_4^+ (as glutamate). Higher salt concentrations, especially those with Cl^- as anion, were generally inhibitory. The R17 RNA-directed system was more sensitive to Cl^- ions than the endogenous system. Glycine betaine stimulated both systems and partly overcame the toxic effects of Cl^- ions. Both systems required Mg^{2+}, but in lower concentrations than the polyuridylic acid-directed system previously studied. Initiation factors were removed from ribosomes by washing with 3.0 to 3.5 M NH_4Cl, concentrations about three times as high as that needed to remove initiation factors from *E. coli* ribosomes. Washing with 4.0 M NH_4Cl damaged *V. costicola* ribosomes, although the initiation factors still functioned. Cl^- ions inhibited the attachment of initiation factors to tRNA_{Met} but had little effect on binding of initiation factors to R17 RNA.

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**MATERIALS AND METHODS**

**Bacterial culture.** *V. costicola* NRC 37001 was grown and harvested as described previously (9).

**Preparation of cellular extracts, ribosomes, and initiation factors.** S-30 extract, that is, the cellular material remaining in the supernatant after centrifuging for 30 min at 30,000 × g_{max} (Beckman type 42.1 rotor), was prepared as described previously (9).

The S-150 extract and the low-salt-washed ribosomes were obtained by centrifuging the S-30 extract at 150,000 × g_{max} for 3 h at 4°C. The soluble S-150 fraction and the ribosomal pellet were then dialyzed or washed and stored according to the procedure of Kamekura and Kushner (9).

A different low-salt extraction buffer than the one described by Kamekura and Kushner (9) was used in the preparation of the cellular extracts and of the ribosomes. It contained 124 mM ammonium glutamate, 8 mM magnesium acetate, 10 mM Tris acetate (pH 7.6), 3 mM spermidine-trihydropclocride, and 6 mM β-mercaptoethanol.

Crude initiation factors were prepared by following the method described for *E. coli* (27), with some modifications to adapt it to *V. costicola*. Ribosomes were homogenized in extraction buffer (15 ml/10 g of wet weight of cells) in which the ammonium glutamate had been replaced by 3.0 to 3.5 M NH_4Cl (high-salt extraction buffer) and left at 4°C overnight. These high-salt-washed ribosomes were spun down (150,000 × g_{max} for 3 h at 4°C), washed once with low-salt extraction buffer, and stored at −70°C. Solid (NH_4)_2SO_4 (0.52 g/ml of supernatant) was added, and the solution was stirred in the cold for 2 h. The precipitated proteins were centrifuged (15,000 × g_{max} for 30 min), dissolved in extraction buffer (0.5 ml/10 g of original wet weight of cells), dialyzed overnight against several liters of this buffer, clarified by centrifugation, and then stored at −70°C.

**Growth of phage R17 and isolation of its RNA.** *E. coli* CSH39 was grown with maximum aeration at 37°C in 2-liter
Erlenmeyer flasks containing 250 ml of 4YT medium (32 g of tryptophane, 20 g of yeast extract, 5 g of NaCl per liter [19]) up to an optical density of 0.4 (550 nm). The bacterial culture was then made 5 mM in CaCl₂ (19), infected with R17 at a multiplicity of 0.15, and incubated for an additional 4 h. With this incubation time, titers of 7 × 10⁻¹² PFU/ml were routinely obtained. For labeling the R17 RNA, 400 μCi of [6⁻³H]Juridine (29 Ci/mmole; NEN Research Products) per liter of medium was added at the time of infection (1).

The following operations were all carried out at 4°C. After incubation, EDTA, MgSO₄, lysozyme, and DNase were added to final concentrations of 50 mM, 200 mM, 100 μg/ml, and 2 μg/ml, respectively (35). The bacteriophage was then collected by the method of Steitz (27). It was precipitated overnight by adding 330 g of (NH₄)₂SO₄ per liter of medium and recovered by centrifugation (25,000 × gmax for 30 min). The pellet was well suspended in 100 ml of standard sodium citrate buffer (SSC (1 SSC = 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.2]) per liter of lysate, and the solution was clarified by centrifugation (25,000 × gmax for 10 min). The phage (20 ml per tube) was then pelleted at 100,000 × gmax (Beckman type 42.1 rotor) for 4 h, and each pellet was resuspended overnight in 2 ml of SSC.

The phage was purified further by two successive sedimentations through CsCl step gradients (39). After each CsCl centrifugation, the visible phage band was removed, diluted to 20 ml with SSC, pelleted at 100,000 × gmax for 4 h and resuspended in SSC (2 to 4 ml). The RNA was then extracted by the method of Steitz (27), which included solubilization of the bacteriophage with 1% sodium dodecylsulfate (SDS), followed by phenol (SSC saturated) extraction and ethanol precipitation.

**In vitro protein synthesis assays.** For the R17 RNA-directed in vitro protein-synthesizing system, the complete reaction mixture (50 μl) contained 15 mM phosphoenolpyruvate, 2 mM ATP, 1 mM GTP, 225 mM ammonium glutamate (including the ammonium glutamate contributed by the cellular extracts and ribosomal preparations), 8 mM Mg²⁺ (as magnesium acetate), 7.5 mM reduced glutathione, 82 mM Tris acetate (pH 7.6). 1.25 μM tRNA from E. coli MRE 600; Boehringer Mannheim Biochemicals, 0.03 mM [¹⁴C]Valine (225 mCi/mmol; ICN Biomedicals Canada Ltd.), 0.05 mM each of the other 19 amino acids, 100 μg of R17 RNA, 0.2 volume of S-30, 0.5 mg of protein) or 10 μl of S-150 (ca. 0.5 mg of protein) and 2 A₁₅₀₅ units of ribosomes or 10 μl of S-150, 4 A₁₅₀₅ units of NH₄Cl-washed ribosomes, and 39 μg of crude initiation factors. The complete reaction mixture (50 μl) for the in vitro translation system directed by endogenous mRNA of V. costicola contained 15 mM phosphoenolpyruvate, 2 mM ATP, 1 mM GTP, 300 mM ammonium glutamate (including the ammonium glutamate contributed by the S-30), 8 mM Mg²⁺ (as magnesium acetate), 7.5 mM reduced glutathione, 82 mM Tris acetate (pH 7.6), [¹⁴C]L-amino acids (1.74 mCi/mg; ICN Biomedicals Canada Ltd.) at a concentration of 0.05 mM each (adjusted with unlabeled amino acids), and a 0.4 volume of S-30 (ca. 1.5 mg of protein).

In both systems, protein synthesis was followed by measuring the amount of radiolabeled amino acid(s) incorporated into high trichloroacetic acid (TCA)-insoluble material. Reaction mixtures were incubated at 30°C for the times indicated. Reactions were stopped by adding 500 μl of 5% TCA; the mixtures were heated at 90°C for 20 min, cooled in ice, and filtered (membrane filters [Millipore Corp.], type HA; pore size, 45 μm). The filters were washed twice with 5 ml of 5% TCA, dried, and mixed with 4 ml of Universal Cocktail (ICN Biomedicals Canada Ltd.). The radioactivity trapped on the filters was then counted.

**Assay of formylmethionyl-tRNA synthetase.** The enzyme reaction was performed in a mixture of the same composition as that for R17 RNA-directed protein synthesis except that R17 RNA was not included and that [¹⁴C]Valine and the other 19 amino acids were replaced by 0.07 mM of [¹⁴C]methionine (285 mCi/mmol; Amersham Corp.). S-150 was used as the source of enzyme. Incubation was at 30°C for 20 min. Synthetase activity was measured by determining the amount of radioactivity rendered insoluble by 500 μl of cold 10% TCA and then retained on Millipore filters (type HA; pore size, 45 μm) (5).

Binding of [¹⁴C]fmet-tRNA or [³H]R17 RNA by initiation factors. Binding of [¹⁴C]fmet-tRNA (29) and [³H]R17 (8) was measured by determining the amount of radioactivity retained on nitrocellulose filters (BA 85; Schleicher & Schuell, Inc.). The reaction mixture contained the following: 225 mM ammonium glutamate, 8 mM Mg²⁺ (as magnesium acetate), 82 mM Tris acetate (pH 7.6), 7.5 mM reduced glutathione, 40 μM of crude initiation factors, and 11 pmol of [¹⁴C]fmet-tRNA (550 dpm/pmol) or 100 μg of [³H]R17 RNA (900 dpm/μg). The reaction mixture (50 μl) was incubated at 30°C for 15 min, cooled on ice, filtered, and washed twice with 3 ml of ice-cold buffer (225 mM ammonium glutamate, 8 mM magnesium acetate, 82 mM Tris acetate (pH 7.6), 6 mM β-mercaptoethanol). The filters were then dried, and the radioactivity was counted.

Synthesis of [¹⁴C]fmet-tRNA. The charging and formylation reactions were done by the method of Voorma et al. (32) with modifications of the reaction mixture to adapt it to V. costicola. The reaction mixture (1 ml) contained the following: 225 mM NH₄ glutamate, 8 mM magnesium acetate, 82 mM Tris acetate (pH 7.6), 6 mM β-mercaptoethanol, 1.5 mM ATP, 10 mM phosphoenolpyruvate, 5 A₂₆₀₅ units of tRNA, 10 μg of folic acid, 3.5 mM [¹⁴C]methionine (225 mCi/mmol; ICN Biomedicals Canada Ltd.), and 250 μl of S-150 of V. costicola. Incubation was at 30°C for 15 min. The initiator tRNA, fmet-tRNA, was then isolated and purified by phenol extraction and ethanol precipitation as described by Voorma et al. (32).

**Gel electrophoresis and autoradiogram.** Following incubation, the reaction mixture of the R17 RNA-directed protein synthesis system was mixed with a 0.125 volume of sample buffer (250 mM Tris hydrochloride [pH 6.8], 5% SDS, 5% 2-mercaptoethanol, 50% glycerol, 0.1% bromophenol blue) and 0.2 A₂₆₀₅ units of R17, as carrier, and heated for 3 min at 90°C (5). Fifty-microliter samples were used for electrophoresis.

Gel slabs for SDS-polyacrylamide gel electrophoresis, with linear gradients of polyacrylamide (10 to 15%) and glycerol (0 to 13%) (5), were prepared by the method of Laemmli (12) and run at 25 mA. Proteins were fixed and stained with Coomassie blue R250 (0.1%) in water-methanol-glacial acetic acid (5:5:2). The gels were destained in a solution of 12.5% isopropanol and 10% acetic acid and then dried in vacuo on Whatman 3 MM paper. The dried gels were autoradiographed at ~80°C with Kodak X-ray film (X-Omat AR) and with an intensifying screen.

**RESULTS AND DISCUSSION**

Characterization of cell-free translation systems directed by natural mRNAs. The RNA of the R17 phage of E. coli was able to act as a messenger for in vitro protein synthesis by extracts of V. costicola. The incorporation of valine, one of

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the predominant amino acids found in the coat protein of this phage (16), was absolutely dependent on the presence of this RNA, as well as on ribosomes and substances found in the S-150 supernatant (Fig. 1). Final incorporation increased with increasing concentrations of ribosomes, mRNA, and S-150 supernatant (Fig. 1). Added tRNAFmet increased, but was not essential for, incorporation (Fig. 1). Incorporation was also absolutely dependent on a mixture of ATP, GTP, and phosphoenolpyruvate (not shown), which presumably served as an energy source in different steps of translation. Effects of individual components of this mixture were not studied.

In an in vitro system containing ribosomes and the S-150 fraction, incorporation was linear for the first 30 min; when S-30 fractions, which contained both ribosomes and the S-150 fraction, were used, valine incorporation reached at least as high a plateau as in the former system by 10 min (not shown). Consequently, in experiments to determine total valine incorporation, the reaction mixture was incubated for 10 min in the presence of S-30 fractions and for 30 min in the presence of S-150 fractions plus ribosomes.

SDS-polyacrylamide gel electrophoresis showed that the newly synthesized proteins made in the presence of R17 RNA comigrated with purified R17 coat protein (Fig. 2).

In vitro protein synthesis directed by endogenous mRNAs of V. coticola was also dependent on the amount of cell extract. Incorporation reached a plateau within 10 min of incubation. This system did not respond to increasing amounts of tRNAFmet (not shown).

Effect of protein synthesis inhibitors. Chloramphenicol and neomycin (10⁻⁴ M), which act on peptide chain formation (10, 20), strongly (93 to 98%) inhibited protein synthesis in both the R17-directed and endogenous systems. Kasugamycin (4 × 10⁻⁴ M) and aurin tricarboxylic acid (4 × 10⁻⁵ M), which inhibit initiation of translation (6, 18), inhibited R17-directed translation by 80% but had less effect (30 and 5% inhibition, respectively) on the endogenous system. Preincubation with RNase completely inhibited translation in both systems.

The above experiments confirm that amino acid incorporation in both systems involves de novo protein synthesis. Both systems are more physiological than the poly(U)-directed system studied previously (3, 9), but there are important differences between them. In the R17-directed system, every ribosome that becomes involved in protein synthesis must first go through normal initiation. In contrast, the translation of endogenous mRNA presumably involves mainly the completion of nascent polypeptides, as in the cell-free translation system directed by endogenous mRNAs of E. coli (6, 10). Preattached ribosomes can carry out elongation immediately without having first to go through initiation.

The differences are reflected by the action of different protein synthesis inhibitors: those (chloramphenicol and neomycin) which inhibit polypeptide elongation act similarly on both systems, while those (kasugamycin and aurin tricarboxylic acid) that primarily affect initiation act much more strongly on the R17-directed system.

Response of translation systems to different ions. The R17-directed protein-synthesizing system required Mg²⁺ and NH₄⁺ ions (ammonium glutamate) at concentrations of 8 and 225 mM, respectively, for optimal activity (Fig. 3A). Potassium glutamate could partly replace ammonium glutamate (Fig. 3A). However, sodium glutamate, NaCl, KCl, or NH₄Cl alone did not support any levels of protein synthesis (not shown).

FIG. 1. Effects of concentrations of different components of the R17 RNA-directed protein-synthesizing system of V. coticola. Symbols: ○, ribosomes; ○, tRNAFmet; □, R17 RNA; ■, S-150.

FIG. 2. Autoradiogram of translated products of R17 RNA-directed in vitro protein synthesis by the S-30 fraction of V. coticola. The time of incubation (in minutes) is indicated above each lane. First lane on the right, SDS-gel electrophoresis of purified A and coat proteins of the R17 bacteriophage stained with Coomassie blue.
The translation of endogenous mRNA also worked best at a Mg^{2+} ion concentration of 8 mM but required slightly more ammonium glutamate (300 mM) than the R17 system for optimal activity (Fig. 3B). Ammonium glutamate could be replaced quite efficiently by potassium glutamate, partly by NH_4Cl and KCl (at lower optimal concentrations), and not at all by sodium glutamate and NaCl (Fig. 3B).

The low Mg^{2+} ion requirement (8 mM) of both systems is typical of translation systems directed by natural mRNAs (15, 17). In contrast, the poly(U)-directed in vitro system of *V. costicola* required more than twice as much Mg^{2+} (38).

Adding NaCl, KCl, or NH_4Cl to either system in the presence of an optimal ammonium glutamate concentration inhibited protein synthesis. For example, addition of 0.1 M of these salts caused approximately 90% inhibition of translation in the R17-directed system. Such an inhibition was also caused by 0.2 M sodium glutamate, 0.4 M ammonium glutamate, or 0.6 M potassium glutamate.

All concentrations of added NaCl, KCl, NH_4Cl, and ammonium glutamate also inhibited translation of endogenous mRNAs, although this system was less sensitive than the R17-directed system. Thus, ca. 0.6 M NaCl was needed to cause 90% inhibition. However, 0.2 M added sodium and potassium glutamate stimulated this system 125 and 100%, respectively; 0.4 M concentrations caused some stimulation, and higher concentrations were inhibitory (not shown).

These results confirm the toxic nature of the Cl^- ions on in vitro protein synthesis by *V. costicola*, effects previously observed with poly(U) as artificial mRNA (3, 9, 38). The greater sensitivity of the R17-directed system than the endogenous system to added Cl^- ions suggests that these ions are more toxic to initiation than to elongation.

The fact that sodium or potassium glutamate stimulated translation directed by endogenous mRNA, in the presence of optimal ammonium glutamate, must be due to the Na^+ and K^+ ions, which are found in high concentrations in these bacteria (25) and which would be expected to play an important role in protein synthesis. This stimulation is probably directed towards elongation rather than initiation.

**mRNA structure.** We considered the possibility that inhibition of the R17 RNA-directed system was due to changes in the secondary structure of the RNA with increasing salt concentrations. Such changes could have resulted in a shielding of the normally available ribosome-binding sites. However, similar results (not shown) were obtained with partially denatured formaldehyde-treated (13) R17 RNA. Thus, salts probably did not act by changing RNA structure. Lodish (13) had previously found that partially denatured viral RNA was more active in stimulating in vitro protein synthesis by *E. coli*, but we did not observe such an increase in our system.

**Effects of added betaine and glutamate.** Glycine betaine (betaine) stimulated protein synthesis in both systems, especially that directed by R17 RNA. Its presence also resulted in higher activity, in both systems, in the presence of NaCl or KCl (Fig. 4A and B). Therefore, it seems that betaine can afford some protection against the toxic action of Cl^- ions, as was previously seen with the poly(U) system (3, 9). Although glutamate also offers protection against Cl^- ions in the poly(U) system (3, 9), further addition of 0.2 M sodium or potassium glutamate was inhibitory in the systems studied now, especially the R17 RNA-directed system (Fig. 4A and B).

**Formylmethionyl-tRNA synthetase.** We investigated this enzyme as a possible site of action of Cl^- ions. However, its activity was only inhibited 20% by as much as 1.0 M NaCl (not shown), and it is clearly not a primary site of Cl^- ion toxicity. It was previously shown (9) that phenylalanyl-tRNA synthetase was not a site of Cl^- action in the poly(U)-directed system.

**Isolation of crude initiation factors of V. costicola.** Initiation factors were isolated from *E. coli* ribosomes by washing with 1 M NH_4Cl (27) but were not isolated from those of *V. costicola*. We found that washing the latter ribosomes with 1.0 and 2.0 M NH_4Cl did not decrease their ability to carry out in vitro protein synthesis. However, those washed with 3.0, 3.5, and 4.0 M NH_4Cl had relative activities of 15, 6, and 2%, respectively. Ribosomes treated with 3.0 and 3.5 M NH_4Cl were active only if protein obtained during the washing procedures (see Materials and Methods) was added.
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FIG. 4. Effects of betaine alone or with sodium (or potassium) glutamate on the R17 RNA-directed protein-synthesizing system (A) and the endogenous translation system of V. costicola (S-30 fraction) (B). Symbols: ○, no addition; ▲, 0.5 M betaine; ■, 0.5 M betaine plus 0.2 M potassium glutamate; ●, 0.5 M betaine plus 0.2 M sodium glutamate. Results are expressed as percentage of control (without added solute). 100% activity represents incorporation of 47 pmol of [14C]valine into hot TCA-insoluble material per 10 μl of S-30 in the R17 RNA system and 14,200 dpm per 20 μl of S-30 in the endogenous system. Higher levels of betaine, up to 1.2 M, caused no further stimulation of activity in either system.

again (Table 1). Presumably, these proteins included the initiation factors. However, a much lower incorporation was observed with 4.0 M NH4Cl-washed ribosomes recombined with the crude initiation factors isolated from them (Table 1). Reconstruction studies (not shown) between 3.5 and 4.0 M NH4Cl-washed ribosomes and their respective initiation factors showed that this lack of incorporation was mainly due to inactive 4.0 M NH4Cl-washed ribosomes rather than inactive extracted initiation factors.

Binding of [14C]fmet-tRNA or [3H]R17 RNA by initiation factors. Retention of [14C]fmet-tRNA or [3H]R17 RNA on nitrocellulose filters was specifically dependent on the addition of crude initiation factors (Table 2). This retention was not due to unspecific binding of the radiolabeled compounds to proteins, since the addition of S-150 from V. costicola, rich in proteins, barely increased the amount of material retained on the filter. Presumably, retention of [14C]fmet-tRNA was caused by the formation of a complex between the tRNA and initiation factor-2 (IF2). This complex is not stable with IF2 isolated from E. coli and is normally stabilized by fixing with glutaraldehyde (29, 30). However, with the initiation factors of V. costicola, fixing with 3% glutaraldehyde did not increase the amount of [14C]fmet-tRNA retained on the filters (not shown). The retention of [3H]R17 RNA was presumably due to the formation of a complex between itself and IF3, as is the case with IF3 of E. coli (7, 22, 33).

In the presence of initiation factors, ribosomes interfered with the retention of both radioactive molecules. In E. coli, IF3, which is required for mRNA binding, has a greater affinity for 30S ribosomal subunits than MS2RNA does (31). There are no data on the association of IF2 with fmet-tRNA, but it is known that the association constant of IF2 with 30S ribosomal subunits, in the presence of IF3 and IF1, is as high as that of IF3 (36). This may also be true for V. costicola, so that the amounts of initiation factors available for the binding of fmet-tRNA to IF2 and R17 RNA to IF3, in the absence of ribosomes, could decrease in the presence of ribosomes due to the higher affinity of the initiation factors for the ribosomes.

These results also show that the retention of [14C]fmet-tRNA and [3H]R17 RNA on nitrocellulose filters was a suitable method to study the effects of salts on the initiation factors of V. costicola. This should, at least, give us some indication of the ability of these factors to recognize their respective ligand, namely fmet-tRNA for IF2 and mRNA for IF3, under different ionic conditions.

Cl− ions had a strong inhibitory effect on the binding of

TABLE 1. R17 RNA-directed incorporation of [14C]valine (dpm) at different concentrations of added crude initiation factors isolated from NH4Cl-washed ribosomes of V. costicola

<table>
<thead>
<tr>
<th>Initiation factor (μg)</th>
<th>Incorporation (dpm) in ribosomes (4 A₅₅₀ units) washed in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.0 M NH₄Cl</td>
</tr>
<tr>
<td>0</td>
<td>4,238</td>
</tr>
<tr>
<td>13</td>
<td>4,238</td>
</tr>
<tr>
<td>26</td>
<td>11,210</td>
</tr>
<tr>
<td>39</td>
<td>12,097</td>
</tr>
</tbody>
</table>

*Crude initiation factors isolated from the ribosomes washed at each NH₄Cl concentration.

TABLE 2. Retention of [14C]fmet-tRNA and [3H]R17 RNA on nitrocellulose filters

<table>
<thead>
<tr>
<th>Components added</th>
<th>[14C]fmet-tRNA (dpm)</th>
<th>[3H]R17 RNA (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>100</td>
<td>152</td>
</tr>
<tr>
<td>Ribosomes (4 A₅₅₀ units)</td>
<td>124</td>
<td>232</td>
</tr>
<tr>
<td>Initiation factors (40 μg)</td>
<td>4,828</td>
<td>6,770</td>
</tr>
<tr>
<td>S-150 (350 μg of proteins)</td>
<td>120</td>
<td>440</td>
</tr>
<tr>
<td>Ribosomes (4 A₅₅₀ units) plus initiation factors (40 μg)</td>
<td>743</td>
<td>2,736</td>
</tr>
</tbody>
</table>

*The reaction mixture contained 225 mM ammonium glutamate, 8 mM Mg²⁺, 82 mM Tris acetate (pH 7.6), 7.5 mM reduced glutathione, and 1 pmol of [14C]fmet-tRNA (550 dpm/μmol) or 100 μg of [3H]R17 RNA (900 dpm/μg).
of those of E. coli.

NH4' ions usually stimulate protein synthesis more than K+ ions (4, 14, 26), but the NH4' requirement in different microorganisms may be quite different. E. coli needs about 60 mM NH4' for maximal activity (5, 28); our present results show that V. costicola needs 250 to 300 mM, while halobacteria may require as much as 3.0 M NH4' (23, 24), although in fact, these organisms contain much lower amounts of this ion (11, 23). The halobacteria specifically require Cl- ions, which are found in high concentrations inside their cells, for protein synthesis (23). As discussed elsewhere (11), this may be one of the major physiological differences between aerobic halophilic eubacteria and archaeabacteria. The former continue to reveal physiological properties somewhat between those of the latter and those of the nonhalophilic eubacteria, to which they are more closely related.

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