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

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Received 19 October 1989/Accepted 2 April 1990

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 was. 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\(_4\)Cl, concentrations about three times as high as that needed to remove initiation factors from *E. coli* ribosomes. Washing with 4.0 M NH\(_4\)Cl 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.

Polyuridylic acid [poly(U)]-directed incorporation of phenylalanine by cell extracts of the moderate halophile *Vibrio costicola* is inhibited by Cl\(^-\) ions (9), which prevent ribosome binding to the artificial mRNA (3).

There are a number of differences between a translation system directed by poly(U) and that directed by a natural mRNA. Poly(U) has no Shine-Dalgarno sequence, which normally plays an important role in the initial attachment of natural mRNAs to ribosomes. At high Mg\(^{2+}\) concentrations (18 mM), the initiation factors are not required in the poly(U)-directed system of *Escherichia coli* (15). Finally, the initiator tRNA is not involved, since poly(U) has no AUG initiator codon. Therefore, the initiation process of protein synthesis is not as well represented in an in vitro translation system directed by poly(U) as in one directed by a natural messenger.

We wanted to study the effects of Cl\(^-\) ions on protein synthesis in *V. costicola* directed by a natural mRNA. Unfortunately, none have been isolated from this or any other moderate halophile, although some studies have been done with a mixture of unidentified endogenous mRNAs (38). We have now found that a natural mRNA which functions well in the cell-free translation system of *E. coli*, that of bacteriophage R17 (2), can function in vitro with *V. costicola*. This study describes some properties of both *V. costicola* systems and studies the site of action of different ions.

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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\(_{\text{max}}\) (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\(_{\text{max}}\) 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-trihydrochloride, 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\(_4\)Cl (high-salt extraction buffer) and left at 4°C overnight. These high-salt-washed ribosomes were spun down (150,000 × g\(_{\text{max}}\) for 3 h at 4°C), washed once with low-salt extraction buffer, and stored at −70°C. Solid (NH\(_4\))\(_2\)SO\(_4\) (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\(_{\text{max}}\) 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 trypoptone, 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 × 10⁴ PFU/ml were routinely obtained. For labeling the R17 RNA, 400 µCi of [6-³H]uridine (29 Ci/mmol; 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 centrifuged 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 × g max for 30 min). The pellet was well suspended in 100 ml of standard sodium citrate buffer (SSC) (1× SSC is 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 × g max for 10 min). The phage (20 ml per tube) was then pelleted at 100,000 × g max (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 × g max 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 dodecyl sulfate (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 tRNA⁷⁵⁴ (from E. coli MRE 600; Boehringer Mannheim Biochemicals), 0.03 mM [³H]valine (225 cpm/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 (ca. 0.5 mg of protein) or 10 µl of S-150 (ca. 0.5 mg of protein) and 2 A₂₆₀ units of ribosomes or 10 µl of S-150, 4 A₂₆₀ 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]amino acids (1.74 mM/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 RNA (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 µg of crude initiation factors, and 11 pmol of [¹⁴C]fmet-tRNA (550 dpm/pmol) or 100 µg of [³H]R17 RNA (900 dpm/pµg). 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 A₂₆₀ units of tRNA⁷⁵⁴, 10 µg of folinic acid, 3.5 mM [¹⁴C]methionine (225 cpm/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% bromphenol blue) and 0.2 A₂₆₀ 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 3MM 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
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 tRNA\textsuperscript{Met} 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 \textit{V. cisticola} 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 tRNA\textsuperscript{Met} (not shown).

\textbf{Effect of protein synthesis inhibitors.} Chloramphenicol and neomycin (10\textsuperscript{-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 \times 10\textsuperscript{-4} M) and aurin tricarboxylic acid (4 \times 10\textsuperscript{-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 \textit{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.

\textbf{Response of translation systems to different ions.} The R17-directed protein-synthesizing system required Mg\textsuperscript{2+} and NH\textsubscript{4}\textsuperscript{+} 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 NH\textsubscript{4}Cl alone did not support any levels of protein synthesis (not shown).
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\(_4\)Cl 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\(_4\)Cl 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\(_4\)Cl, 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 affords 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\(_4\)Cl (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\(_4\)Cl 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\(_4\)Cl had relative activities of 15, 6, and 2%, respectively. Ribosomes treated with 3.0 and 3.5 M NH\(_4\)Cl 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: O, 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 pmoles 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.

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 a (μg)</th>
<th>Incorporation (dpm)/in ribosomes (4 A260 units) washed in:</th>
<th>3.0 M NH4Cl</th>
<th>3.5 M NH4Cl</th>
<th>4.0 M NH4Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4,238</td>
<td>1,746</td>
<td>721</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>8,210</td>
<td>5,855</td>
<td>953</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>11,210</td>
<td>9,775</td>
<td>1,004</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>12,097</td>
<td>10,531</td>
<td>1,253</td>
<td></td>
</tr>
</tbody>
</table>

a 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 a</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 A260 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 A260 units) plus initiation factors (40 μg)</td>
<td>743</td>
<td>2,736</td>
</tr>
</tbody>
</table>

a The reaction mixture contained 225 mM ammonium glutamate, 8 mM Mg2+, 82 mM Tris acetate (pH 7.6), 7.5 mM reduced glutathione, and 11 pmoles of [14C]fmet-tRNA (550 dpm/pmol) or 100 μg of [3H]R17 RNA (900 dpm/μg).
Halophile *E. coli* and of the extremely halophilic archaeabacterium *Halobacterium cutirubrum*, the most studied species of this type.

Ribosomes of the extreme halophiles require high salt (mainly high KCl) concentrations for stability. Those of *V. costicola* are stable in both high and low salt concentrations, while those of *E. coli* are stable only in low salt concentrations (37). Higher concentrations of NH₄Cl are needed to remove initiation factors from the ribosomes of *V. costicola* than from 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.

**ACKNOWLEDGMENT**

This work was supported by a grant to D.J.K. from the Natural Sciences and Engineering Research Council of Canada.

**LITERATURE CITED**