

Ribosomes Exist in Large Excess over the Apparent Demand for Protein Synthesis during Carbon Starvation in Marine *Vibrio* sp. Strain CCUG 15956

KLAS FLÄRDH,¹ PAUL S. COHEN,² AND STAFFAN KJELLEBERG^{1*}

Department of General and Marine Microbiology, University of Göteborg, Carl Skottsbergs gata 22, S-413 19 Göteborg, Sweden,¹ and Department of Microbiology, University of Rhode Island, Kingston, Rhode Island 02881²

Received 29 April 1992/Accepted 18 August 1992

Carbon starvation induces the development of a starvation- and stress-resistant cell state in marine *Vibrio* sp. strain S14 (CCUG 15956). The starved cells remain highly responsive to nutrients during prolonged starvation and exhibit instantaneous severalfold increases in the rates of protein synthesis and RNA synthesis when substrate is added. In order to elucidate the physiological basis for the survival of cells that are starved for a long time, as well as the capacity of these cells for rapid and efficient recovery, we analyzed the ribosome content of carbon-starved *Vibrio* sp. strain S14 cells. By using direct chemical measurements of the amounts of ribosomal particles in carbon-starved cultures, we demonstrated that ribosomes were lost relatively slowly (half life, 79 h) and that they existed in large excess over the apparent demand for protein synthesis. After 24 h of starvation the total rate of protein synthesis was 2.3% of the rate during growth, and after 3 days this rate was 0.7% of the rate during growth; the relative amounts of ribosomal particles at these times were 81 and 52%, respectively. The ribosome population consisted of 90% 70S monoribosomes, and no polyribosomes were detected in the starved cells. The 70S monoribosomes were responsible for the bulk of the protein synthesis during carbon starvation; some activity was also detected in the polyribosome size region on sucrose density gradients. We suggest that nongrowing carbon-starved *Vibrio* sp. strain S14 cells possess an excess protein synthesis capacity, which may be essential for their ability to immediately initiate an upshift program when substrate is added.

It is well established that bacterial growth in natural environments is restricted by energy and/or nutrient shortages (40, 57), and there is a growing interest in the adaptations and responses of bacteria to nutrient limitation (see references 15 and 25). In particular, many marine waters are oligotrophic and are characterized by extremely low concentrations of bioavailable organic carbon (39, 40). Growth of the heterotrophic bacterial populations that inhabit these waters is frequently limited because of energy and nutrient depletion (21, 22, 60), and the life cycles of the bacteria may include intermittent periods of growth and starvation-induced non-growth, as well as unbalanced growth (7, 23). In order to better understand the autecology of marine bacteria and to evaluate the participation of these organisms in nutrient cycling in marine environments, an improved knowledge of the physiological state induced by energy and nutrient starvation is needed. Bacteria such as *Vibrio* species, which are commonly isolated from a variety of marine ecosystems (5, 8), should have evolved adequate responses to nutrient limitation and are suitable model organisms for such studies.

The starvation-induced responses of marine *Vibrio* spp. have been extensively studied and described (for reviews, see references 22, 23, and 38). *Vibrio* spp. respond to energy and nutrient deprivation by a time-dependent series of adaptive physiological and molecular realignments (46). These responses are similar to those of other nondifferentiating bacteria, such as *Escherichia coli* and *Salmonella typhimurium* (35, 56). While the morphological manifesta-

tions of the starvation response in *E. coli* and *Vibrio* spp. are minor, these organisms have some characteristics in common with the developmental processes of the true differentiating bacteria (e.g., the sporulation of *Bacillus* spp.); in particular, energy and nutrient starvation induces a sequential and temporally ordered synthesis of starvation proteins (16, 47). A resistant cell state develops, and this cell state is able to survive starvation and to withstand multiple environmental stresses (18, 19, 44, 48, 50).

The regulatory mechanisms that are involved in starvation-induced development and the molecular basis for the stress-resistant state are still largely unknown, but these mechanisms are now slowly being revealed. For example, *E. coli* mutants that are not capable of stationary-phase survival have been isolated (58), and tentative regulators of starvation protein synthesis have been identified (17, 28). A specific set of starvation-induced proteins (Pex) have been identified as the proteins that are essential for starvation survival and development of cross-protection in *E. coli* (19, 55). Most of the Pex proteins are among the proteins that *E. coli* synthesizes regardless of whether it is starved for carbon, nitrogen, or phosphorus (35). In contrast, we recently found in *Vibrio* sp. strain S14 that carbon starvation alone is the determinant for the development of starvation and stress resistance, as well as for the induction of stringent control (50). In fact, nitrogen and phosphorus starvation in the presence of carbon did not evoke any of these responses (50).

In contrast to bacterial spores, even ultramicrocells of *Vibrio* sp. strain S14 that are starved for a long time (120 h) are not dormant. These cells possess highly efficient transport systems (4, 31) and synthesize protein at low, but easily

* Corresponding author.

detectable rates (45, 49). The metabolically active ultramicrocells are capable of rapidly recovering from starvation and respond instantaneously to the addition of nutrients by increasing the rates of RNA synthesis and protein synthesis severalfold (3). The upshift initiates a temporally ordered program of protein synthesis, including repression of starvation-induced (Sti) proteins (47), transient expression of some maturation specific (Mat) proteins, and induction of growth-related proteins. After a lag period or maturation phase, the length of which depends on starvation time, DNA synthesis and cell division resume, and culture growth resumes (3).

Unfortunately, detailed information about the physiological status of cells that are starved for a long time and how these cells are prepared for rapid recovery is lacking. However, it may be that the nature and the functionality of the protein synthesis system in itself are of utmost importance for maintaining viability and substrate responsiveness. Albertson et al. (3) suggested that starved *Vibrio* sp. strain S14 possesses an excess of stable RNA and ribosomes that can be used in the rapid recovery process. It has also been proposed that extremely slowly growing *E. coli* cells contain extra RNA that is not used for protein synthesis, but can be put into action in response to increased nutrient supplies (24). Davis et al. (10) observed a correlation between loss of ribosomes and loss of viability during phosphate deprivation and suggested that ribosome degradation is a major cause of cell death during starvation. Initially, it would be beneficial to starved cells to degrade ribosomes because such degradation would provide the cells with an endogenous source of the essential nutrients. However, if too much of the protein synthesis capacity was lost, the cells would have difficulty in recovering from starvation and would eventually die (10). Extensive and rapid degradation of ribosomes in *E. coli* has been observed repeatedly during stationary phase and during starvation for various nutrients (see references 10, 20, 30, 33, 34, 41, and 51). In addition, correlations among starvation survival, maximal growth rate, and functional characteristics of the ribosomes have been described for a series of natural isolates of *E. coli* (37).

In this paper we describe a study of the protein synthesis machinery during carbon starvation of *Vibrio* sp. strain S14. By directly measuring the amounts of ribosomal particles in carbon-deprived cells, we found that ribosomes were very slowly degraded and existed in large excess in relation to the actual rate of protein synthesis in the starved cells. Polyribosomes were not detected in the starved cells, and ribosomes appeared to exist mainly as monomeric 70S particles. The role of ribosome stability in the ability of the organism to survive and to recover from starvation is discussed below.

MATERIALS AND METHODS

Bacterium, growth, and starvation conditions. Marine *Vibrio* sp. strain S14 (CCUG 15956) has been described previously (1, 31). On the basis of its 16S rRNA sequence, this strain resembles species that reside in the core of the genus *Vibrio* (11, 14). The cells were grown in batches of marine minimal medium (MMM) (52) at 26°C with rotary shaking. For each experiment, cultures were grown overnight in liquid MMM supplemented with glucose (1 g liter⁻¹) and were diluted 100-fold into fresh MMM supplemented with 0.15 g of glucose per liter. The cells grew exponentially (generation time, 75 min) in this medium until glucose deprivation caused cessation of growth. The resulting starvation cultures were incubated for 9 days at 26°C with slow rotary shaking. Viability was assessed by plating serial

dilutions in a nine-salt solution (artificial seawater) onto VNSS agar (32).

Measurement of the rates of total protein synthesis and RNA synthesis. The rate of total protein synthesis was determined as the rate of radioactive leucine incorporation into hot trichloroacetic acid (TCA)-insoluble material. Subvolumes of an experimental culture were given L-[4,5-³H]leucine (Amersham) and unlabeled carrier leucine (1.2 μM; 98 GBq mmol⁻¹). At appropriate intervals, 200 μl was removed and added to 2 ml of ice-cold 5% TCA, and the resulting preparations were heated at 90°C for 15 min and kept on ice for 1 h prior to collection of the precipitated material on polycarbonate membrane filters (pore size, 0.2 μm; Poretics Corp., Livermore, Calif.). The precipitates were washed three times with ice-cold 5% TCA and then transferred to scintillation vials. Aquassure (Dupont, NEN Research Products, Boston, Mass.) was used as the scintillation cocktail, and the radioactivity was determined with a Beckman model 6000LL liquid scintillation counter. The rates of leucine incorporation were determined from the slopes of the linear incorporation graphs, as described by Nyström et al. (49), and were expressed as picomoles per minute per microgram of cellular protein. The rate of total RNA synthesis was determined by pulse-labeling preparations for 30 s with 0.13 mM [5,6-³H]uridine (1.4 TBq mmol⁻¹; Dupont, NEN Research Products). The rate of incorporation was determined by measuring the amount of radioactivity that was precipitated in ice-cold 5% TCA, as described above for leucine incorporation, except that the samples were not heat treated.

Chemical analyses of RNA and protein concentrations. RNA concentrations were determined by using the orcinol reaction, as described by Cooper (9), and yeast RNA as the standard. Samples were placed into equal volumes of 10% TCA on ice, and precipitates were collected by centrifugation prior to analysis. Protein concentrations were determined by using the Peterson modification of the method of Lowry et al. (53) and a protein assay kit (procedure no. P5656; Sigma Diagnostics, St. Louis, Mo.), including the optional TCA-deoxycholate precipitation. Bovine serum albumin was used as the standard.

Preparation and quantitation of ribosomal particles. In order to determine the ribosome content in *Vibrio* sp. strain S14 during long-term carbon starvation, growing and starved cells were harvested, lysed, and fractionated by differential centrifugations. Subvolumes (250 ml) of the experimental cultures were withdrawn at the onset of starvation and after different times of carbon starvation. The cells were harvested by centrifugation at 10,000 × *g*_{max} and 4°C for 10 min and were stored frozen at -70°C. The pellets were thawed, resuspended in 1 ml of buffer A (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 6 mM 2-mercaptoethanol, 1.25 mM dithiothreitol), frozen, and thawed again, and 1 ml of buffer B (10 mM Tris-HCl [pH 7.5], 21 mM magnesium acetate, 60 mM KCl, 6 mM 2-mercaptoethanol, 1.25 mM dithiothreitol) was added together with lysozyme (300 μg ml⁻¹). After 15 min of incubation, 80 μl of 10% Triton X-100 was added. The lysates were kept on ice for another 15 min, and then 6 ml of buffer C (10 mM Tris-HCl [pH 7.5], 10.5 mM magnesium acetate, 0.5 mM EDTA, 30 mM KCl, 6 mM 2-mercaptoethanol, 1.25 mM dithiothreitol) was added together with DNase I (5 μg ml⁻¹). The lysates were cleared by pelleting unlysed cells by centrifugation at 7,000 × *g*_{max} and 4°C for 10 min. The supernatants were centrifuged twice at 30,000 × *g*_{max} and 4°C for 30 min, and the resulting extracts were designated the S-30 extracts. The ribosomal particles in the S-30 extracts were pelleted by centrifuging the extracts at

150,000 $\times g_{\max}$ and 4°C for 60 min in a Beckman type 80Ti rotor. The crude ribosome pellets (designated P-150) were resuspended in buffer C and, along with the supernatants (designated S-150) and subsamples of the lysates, cleared lysates, and S-30 extracts, were stored frozen at -70°C until the protein and RNA concentrations were determined chemically. The terms particulate RNA and soluble RNA are used below for the pelleted RNA (P-150) and the RNA that stayed in the supernatant (S-150), respectively. The amounts of ribosomal particles after the different lengths of starvation were expressed as the amounts of particulate RNA per volume of cell suspension. The efficiency of the lysis procedure was determined for each sample as the percentage of RNA that was retained in the cleared lysates when unlysed cells had been removed. The chemically determined RNA and protein concentrations were corrected for the efficiency of lysis in each individual sample. This efficiency varied from 90 to 100% at the onset of starvation to about 40% for cells that were starved for a long time.

Analysis of ribosomal particles by density gradient centrifugation. Resuspended ribosomal pellets (P-150) were dialyzed against buffer D (same as buffer C, except that the magnesium acetate concentration was 0.105 mM and the EDTA concentration was 0.005 mM) to dissociate the ribosomes into 30S and 50S subunits. Samples were loaded onto 17-ml sucrose density gradients (5 to 20% sucrose in 10 mM Tris-HCl [pH 7.5]-0.10 mM magnesium acetate-30 mM KCl) and spun at 26,000 rpm in a Beckman type SW28.1 rotor for 8 h at 4°C. Prior to centrifugation, a small portion of radioactively labeled reference ribosomes was added to each sample to indicate the positions of intact 30S and 50S subunits. Reference ribosomes were obtained by labeling exponentially growing cells in MMM for two generations with [5,6-³H]uridine (0.3 μ M; 1.4 TBq \cdot mmol⁻¹; Dupont, NEN Research Products) and preparing an S-30 extract in buffer D as described above. The bottoms of the gradient tubes were punctured, and the contents of the tubes were pumped through the flow cuvette of a Beckman model DU-65 spectrophotometer and into a fraction collector, which divided each gradient into 34 fractions. The absorbance profiles were read at 254 nm. The positions of the reference ribosomal subunits were located by determining the radioactivity in the fractions.

Extraction and analysis of polyribosomes. The method which we used for polysome extraction was adopted from the freeze-thaw-lysozyme lysis procedure described by Ron et al. (54). Chloramphenicol (final concentration, 200 μ g ml⁻¹) was added to 50 ml of log-phase cells that were grown to an optical density at 610 nm (OD₆₁₀) of 0.2 in MMM supplemented with 1 g of glucose per liter, to 50 ml of cells that had been starved for 24 h, or to 75 ml of cells that had been starved for 144 h. After 30 s, the cells were poured over crushed ice and centrifuged at 10,000 $\times g_{\max}$ and 2°C for 10 min. The resulting pellets were resuspended in 0.5 ml of a solution containing 10 mM Tris-HCl [pH 7.5], 30 mM KCl, 5.25 mM magnesium acetate, 0.25 mM EDTA, 6 mM 2-mercaptoethanol, 100 μ g of chloramphenicol per ml, and 1.0 mg of lysozyme per ml and were twice frozen in liquid nitrogen and thawed in a 4°C water bath. Deoxycholate (final concentration, 0.3%) was added to the suspensions, as were DNase I (final concentration, 5 μ g ml⁻¹) and dithiothreitol (final concentration, 1.25 mM), before the preparations were incubated on ice for 10 min. The extracts were cleared by centrifugation for 12 min in a Beckman type TLA100.2 rotor at 30,000 $\times g_{\max}$ and then were loaded onto 17-ml 10 to 30% sucrose density gradients (10 mM Tris-HCl [pH 7.5], 30 mM

KCl, 5.0 mM magnesium acetate). The gradients were centrifuged in a Beckman type SW 28.1 rotor at 26,000 rpm and 4°C for 3 h. The gradients were analyzed as described above. In order to compare the protein synthesis activities on the different classes of mono- and polyribosomes after different starvation times, the cells were pulsed with L-[4,5-³H]leucine (final concentration, 70 nM; Amersham) for 10 s before quenching with chloramphenicol, harvesting of the cells, and polysome extraction. The specific activities were 0.16 TBq mmol⁻¹ for log-phase cells and cells that were starved for 24 h and 1.6 TBq mmol⁻¹ for cells that were starved for 144 h. The levels of hot TCA-insoluble radioactivity in the fractions were determined as described above after 0.1 mg of bovine serum albumin was added to each fraction.

We tested the possibility that starved cells contained RNases that degraded polysomes during the extraction procedure. Radioactive log-phase polyribosomes were used as probes for RNase activity in starved cells. Such polysomes were obtained by labeling exponentially growing cells for two generations with [2-¹⁴C]uridine (final concentration, 5 μ M; 0.13 GBq mol⁻¹; Amersham). The labeled cells were extracted separately or after they were mixed with equal amounts of unlabeled cells that had been starved for 24 or 144 h, and the polysomes were analyzed as described above.

RESULTS

Carbon starvation conditions, survival, and endogenous metabolism. Since carbon starvation is the determinant for the development of starvation and stress resistance (50), a defined glucose starvation condition was chosen for these experiments. The way in which starvation was imposed on the cells, as well as the minimal medium (MMM), has not been used in previous studies of *Vibrio* sp. strain S14, and therefore, the carbon starvation response under these conditions was investigated. The *Vibrio* sp. strain S14 cultures that were grown in MMM supplemented with 0.15 g of glucose per liter stopped growing at an OD₆₁₀ of 0.2. The growth arrest, which defined the onset of starvation, occurred immediately, and no deceleration phase could be distinguished (Fig. 1). No biomass increase was observed after the onset of starvation. Concomitantly, we observed abrupt decreases in the rates of total RNA synthesis and total protein synthesis (Fig. 1). As has been described previously for multiple-nutrient-starved *Vibrio* sp. strain S14 (47), the cells entered the second phase of starvation after 30 min, as demonstrated by the relative increases in the rates of macromolecular syntheses at that time. After 1.5 h of carbon deprivation, we observed a slow decrease in the total rate of protein synthesis that was continuous throughout the experimental period (Fig. 1). After 2 days the rate of leucine incorporation was less than 1% of the rate during growth (Table 1).

As Fig. 2A shows, the optical density decreased gradually during the starvation time which we studied. The level of viability was 100% for at least 5 days and thereafter decreased slowly (Fig. 2A). The time of onset and the rate of loss of viability varied slightly between the individual experiments, but the decrease in viability did not exceed 1 log after 9 days of starvation.

The decrease in the total cellular protein content was greatest during the first days, but the rate of decrease decelerated markedly after 4 days of starvation (Fig. 2B). The rate of degradation of total RNA, as determined by the orcinol method, was very low during the first 24 h and increased to about 1% h⁻¹ after 48 h of carbon starvation

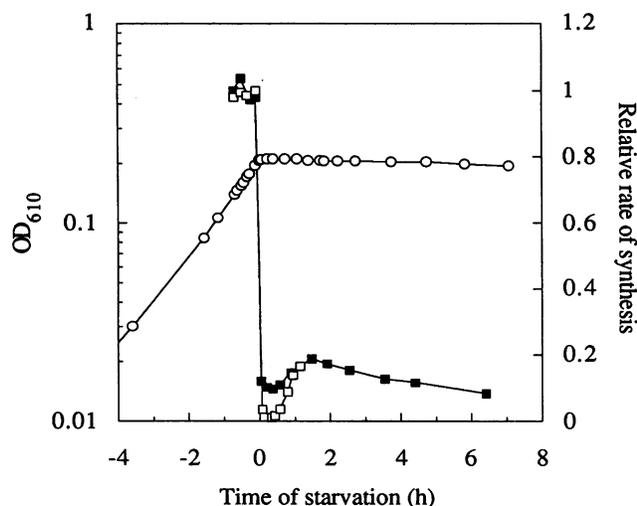


FIG. 1. Effects of glucose deprivation on biomass (○) and the total rates of protein synthesis (■) and RNA synthesis (□). Cells were grown exponentially in minimal medium containing 0.15 g of glucose per liter until the carbon source was consumed and growth stopped. Biomass was determined by measuring OD_{610} . The rates of protein synthesis and RNA synthesis were determined as the rates of incorporation of [3H]leucine and [3H]uridine, respectively, and were compared with the rates prior to growth arrest, which were assigned values of 1.0. Representative data from one of two replicate experiments are shown.

(Fig. 2B). After 9 days, 50% of the total cellular protein and 15% of the total RNA remained (Fig. 2B).

Amount of ribosomes in carbon-deprived cells. We found that the loss of ribosomes during carbon starvation was a relatively slow process. The ribosome contents of the starving cell suspensions decreased during the first 3 days, with an apparent half-life of 79 h (Fig. 3). Subsequently, the rate of ribosome loss accelerated slightly (half-life, 40 to 50 h). The numbers of ribosomes per cell could be estimated by using the amounts of particulate RNA (Fig. 3), the molecular weight of the RNA in one *E. coli* ribosome (42), and the viability data (Fig. 2A). According to such calculations, the cells contained 20,000 to 35,000 ribosomes at the onset, 16,000 ribosomes after 24 h of starvation, and 8,000 ribosomes after 4 days of starvation.

The crude ribosome pellets used for these analyses contained only particulate RNA and insignificant amounts of potentially interfering substances, such as tRNA, polysaccharides, and DNA fragments. Thus, we expected that the

TABLE 1. Rate of protein synthesis during carbon starvation

Length of starvation (h)	Rate of protein synthesis (pmol of leucine \cdot min $^{-1}$ \cdot mg of protein $^{-1}$) ^a	Relative rate of synthesis (% of rate during growth)
0	4.34	100
24	0.098	2.3
48	0.035	0.80
72	0.029	0.68
96	0.026	0.59
144	0.0085	0.20
216	0.0071	0.16

^a Determined by measuring the rate of [3H]leucine incorporation into hot TCA-insoluble material.

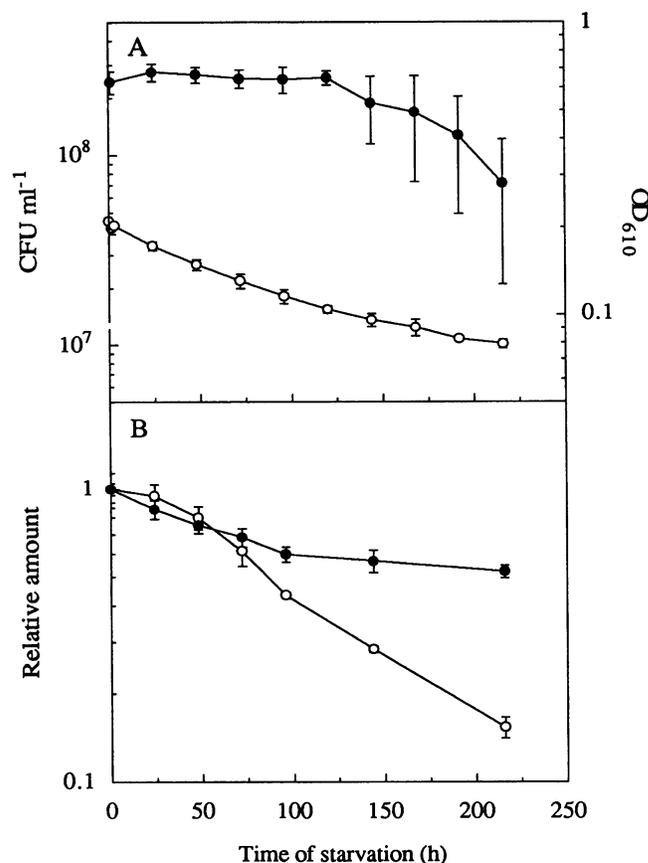


FIG. 2. Viability, biomass, protein content, and RNA content during 9 days of carbon starvation. (A) Number of viable cells, as determined by plating onto complex solid medium (●). Biomass was determined by measuring the optical density of the starved cell suspension (○). (B) Amount of protein per unit volume of starved cell suspension relative to the amount of protein at the onset of starvation (●). The amount of RNA per unit volume of starved cell suspension relative to the amount of RNA at the onset of starvation (○) was determined by using the orcinol reaction. The means and standard deviations from at least six determinations are shown.

orcinol method would give accurate measurements of the amounts of ribosomal particles. The validity of the data obtained from the chemical analysis of sedimentable RNA was further confirmed by the results of a density gradient centrifugation analysis of the P-150 preparations from cells that had been starved for various times. The amounts of particulate RNA that were loaded onto identical gradients were 109 μ g for log-phase cells and cells that had been starved for 24, 48, and 72 h and 54.5 μ g for cells that had been starved for 96 h. The absorbance profiles of a typical set of gradients are shown in Fig. 4. We detected no significant UV-absorbing material except the 30S and 50S particles. Integration of the 30S and 50S peaks demonstrated that the ratios of 30S particles to 50S particles ranged from 1:2.0 to 1:2.2 for all of the samples and that the sum of the areas in each gradient was directly proportional to the amount of RNA loaded (data not shown). Also evident from this analysis was the similarity in the shapes of the subunit peaks for all of the starvation times, which, in addition to the absence of particles other than 30S and 50S particles, indicated that abnormal ribosomal particles were not present

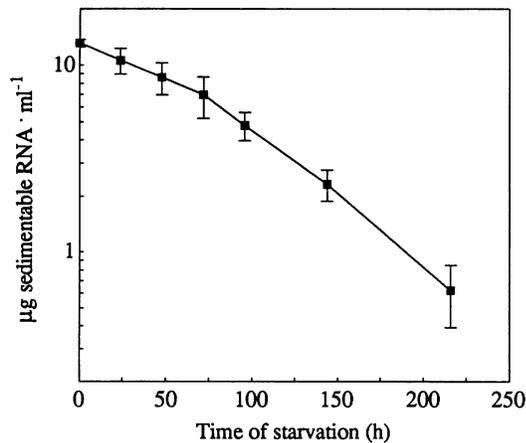


FIG. 3. Stability of ribosomes during carbon starvation. The amount of ribosomal particles per unit volume of starved cell suspension was determined by measuring the amount of particulate RNA that sedimented at $150,000 \times g_{\max}$. Means and standard deviations from at least six determinations are shown.

in detectable amounts during the process of degradation (Fig. 4). In addition, the ratio of soluble RNA to particulate RNA (Table 2) indicated that only a minor fraction of the RNA from degraded ribosomal particles could have been present as soluble RNA. Throughout the starvation period, more than 70% of the total RNA was particulate, and there was only a minor, temporal increase in the amount of soluble RNA with greater starvation times (Table 2).

Polyribosomes during carbon starvation. Polyribosomes were not detected in cells that were starved for either 24 or 144 h (Fig. 5B and C). The 70S monosomes accounted for 88% of the ribosome population in the starved cells, while 12% of the ribosomes were 30S or 50S subunits: 65% of the ribosomes in exponentially growing cells sedimented as polyribosomes, 23% sedimented as 70S monomers, and 12% dissociated into subunits (Fig. 5A).

The protein synthesis activities in the different classes of poly-, di-, and monosomes were analyzed by adding a short

pulse of [³H]leucine prior to inhibition of peptide chain elongation with chloramphenicol and polyribosome extraction. The short [³H]leucine pulse revealed that protein synthesis in the cells that were starved for a long time took place mainly on 70S monosomes (Fig. 5B and C). Although no polyribosomes were detected by absorbance measurements, we observed a low rate of protein synthesis in the size region of the small polysomes in the density gradients (Fig. 5B and C). The specific leucine incorporation activity (i.e., amount of hot TCA-precipitable radioactivity per absorbance unit) of the 70S ribosomes in the cells that were starved for 24 h was about 200-fold lower than the activity in the growing cells. During growth, the specific activities of the poly- and monosome fractions were approximately equal (data not shown).

The absence of polyribosomes in the starved cells was not due to the presence of RNase activity in the cell extracts. This was demonstrated by the results of control experiments in which radioactively labeled polysomes from exponentially growing cells were used as probes for such enzyme activity. The polysome profiles of growing cells were identical, whether the labeled cells had been extracted and analyzed separately or after mixing with unlabeled cells that had been starved for 24 or 144 h (Fig. 6).

DISCUSSION

In this study we found that *Vibrio* sp. strain S14 cells that have been deprived of exogenous carbon for prolonged periods of time retain ribosomes in a large excess over the apparent demand for translation. The rate of total protein synthesis decreased immediately at the onset of starvation (Fig. 1) and after 48 h was less than 1% of the rate of synthesis during growth (Table 1). The content of ribosomal particles decreased slowly, and after 72 h of starvation 52% of the ribosomes were still intact (Fig. 3). This is a significantly lower degradation rate than that reported previously for *E. coli* strains, which lose ribosomes and rRNA relatively rapidly during stationary phase and starvation (see references 10, 20, 30, 33, 34, 41, and 51). Nath and Koch (41) reported that rRNA is degraded at a rate of about $10\% \text{ h}^{-1}$ for at least 6 h during glucose, nitrogen, or phosphorus

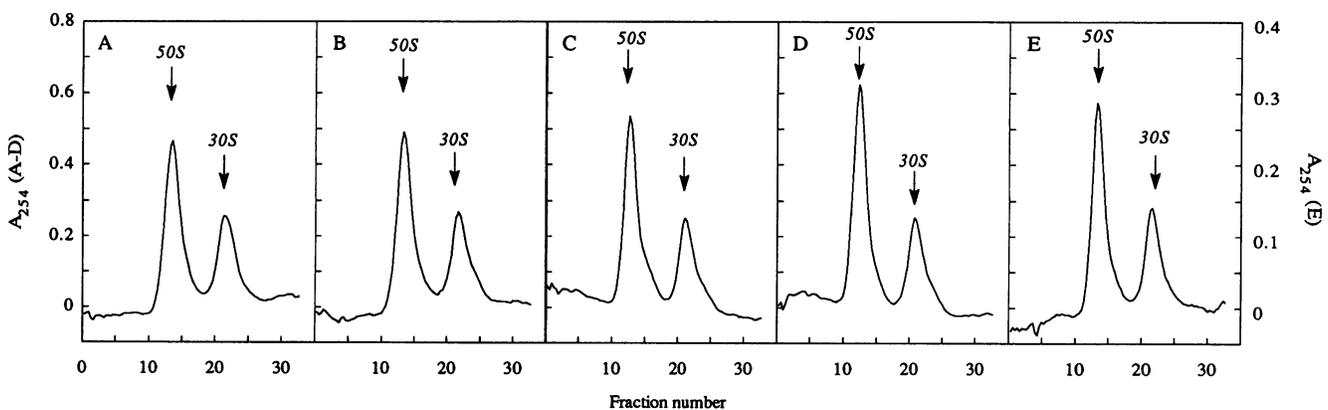


FIG. 4. Density gradient analysis of ribosomal subunits from cells that were carbon starved for 0 h (A), 24 h (B), 48 h (C), 72 h (D), and 96 h (E). Crude ribosomal particles were dialyzed against buffer D (0.1 mM Mg^{2+}) and were sedimented through 5 to 20% sucrose density gradients. Either $109 \mu\text{g}$ (A through D) or $54.5 \mu\text{g}$ (E) of particulate RNA was loaded onto each gradient. The A_{254} profiles were determined by pumping the contents of each gradient through a spectrophotometer. Fractions were collected starting from the bottom of the tubes. A small amount of radioactively labeled ribosomes from growing cells was applied to each gradient; these labeled 30S and 50S subunits did not affect the A_{254} profiles and were detected by liquid scintillation, and their positions in the gradients are indicated.

TABLE 2. Distribution of RNA and protein in soluble and particulate fractions at different times during carbon starvation

Length of starvation (h)	Soluble RNA ^a		Particulate RNA ^b		Soluble protein ^a		Particulate protein ^b	
	Concn ($\mu\text{g ml}^{-1}$) ^c	% of total RNA ^d	Concn ($\mu\text{g ml}^{-1}$)	% of total RNA	Concn ($\mu\text{g ml}^{-1}$)	% of total protein ^e	Concn ($\mu\text{g ml}^{-1}$)	% of total protein
0	1.2	8.1	14	92	27	78	7.8	22
24	2.4	16	13	84	21	78	6.0	22
48	2.6	21	9.9	79	20	80	5.0	20
72	2.2	23	7.5	77	17	81	4.0	19
96	1.7	27	4.6	73	16	86	2.8	15
144	1.0	26	2.8	74	17	89	2.2	11
216	0.3	22	1.0	78	13	91	1.4	9.4

^a Soluble RNA or protein was the RNA or protein that was not sedimented by centrifugation at $150,000 \times g_{\text{max}}$ (S-150).

^b Particulate RNA or protein was the RNA or protein that was sedimented by centrifugation at $150,000 \times g_{\text{max}}$ (P-150).

^c Micrograms per milliliter of starved cell suspension.

^d The total RNA was the sum of the soluble and particulate RNA fractions.

^e The total protein was the sum of the soluble and particulate protein fractions.

starvation. Okamura et al. (51) reported that more than 90% of labeled sedimentable ribosomes were lost after 6 h of carbon deprivation. However, two marine *Vibrio* spp. were recently reported to retain 10 to 26% of their original 16S rRNA for 15 days in stationary phase or during multiple-nutrient starvation (26).

Davis and coworkers (10) observed a correlation between the loss of ribosomes and the relatively rapid decrease in viability displayed by *E. coli* cells that had been deprived of phosphate. When 20 to 50% of the cells were viable, one-half or more of the total nucleic acid, including the majority of the ribosomes, had been lost. Similar results were reported to have been obtained for carbon and nitrogen starvation as well. It was proposed that ribosome degradation is a major cause of cell death during starvation (10). The slow and continuous loss of ribosomal particles in carbon-deprived *Vibrio* sp. strain S14 is not paralleled by a decrease in viability, at least not for the first 5 days of starvation (this study).

Abnormal ribosomal particles were not observed during starvation of *Vibrio* sp. strain S14; such particles have been reported to be formed during magnesium starvation (36) and inhibition of protein synthesis (27) in *E. coli*. Ribosome degradation in carbon-starved *Vibrio* sp. strain S14 seems to occur in accordance with the model presented by Kaplan and Apirion (20), who suggested that after the initial disintegration of the ribosome subunits, further degradation of the rRNA proceeds rapidly to nucleotides. Wada et al. (59) have reported that during the transition of *E. coli* to stationary phase, a decrease in the overall rate of protein synthesis is accompanied by the dimerization of 70S ribosomes into 100S particles. These authors proposed that the 100S particles may be a storage form of unoccupied ribosomes and found that they constituted 40% of the total ribosomal particles during stationary phase (59). Such ribosome dimers were not detected in the ribosome population after *Vibrio* sp. strain S14 cells were deprived of carbon (Fig. 5).

Polyribosomes were not detected by absorbance measurements of density gradients from carbon-starved cultures (Fig. 5). This finding is in accordance with observations of *E. coli* during short-term glucose starvation (12). Some active polysomes appeared to be present in the starved cells, however. Low protein synthesis activity was found in the polysome size region in the sucrose gradients (Fig. 5). The difference in the total rates of protein synthesis between growing and starved cells was reflected in the specific protein synthesis activity observed on the 70S ribosomes,

which was approximately 200-fold lower after 24 h of starvation. This observation may be interpreted such that a small fraction (1/200) of the ribosomes were translating with the same rate as during growth, or that all ribosomes were elongating at a 200-fold-reduced rate. Naturally, an intermediate situation may exist. If nontranslating ribosomes are present, it is possible that they are bound to mRNA, and if they are, they may have paused in the process of initiation, elongation, or termination. The absence of polysomes in carbon-deprived cells indicates that either the availability of mRNA or the rate of translation initiation limits protein synthesis. The size of the mRNA pool during starvation is not known, but a starvation-induced increase in the mean mRNA stability and the presence of extremely long-lived starvation-specific messages have been reported previously for *Vibrio* sp. strain S14 (2). The initiation step or some step after initiation complex formation is believed to be the rate-limiting step in translation in exponentially growing cells (43). A similar situation may exist in carbon-starved *Vibrio* sp. strain S14, and a severe limitation at this step may explain the dearth of polyribosomes in such cells. By using a *Vibrio* sp. strain S14 cell-free protein-synthesizing system, the functional characteristics of the ribosomes in starved cells will be analyzed further in our laboratory.

The discussion about rate-limiting factors for protein synthesis in carbon-deprived cells is also relevant for understanding the recovery process. As mentioned above, multiple-nutrient-starved *Vibrio* sp. strain S14 cells respond instantaneously to nutrients by increasing the rate of protein synthesis. The length of the subsequent lag phase or maturation phase before DNA synthesis, cell division, and re-growth commence is dependent on the length of prestarvation (3). An ordered and sequential pattern of protein synthesis, as revealed by two-dimensional gel electrophoresis of pulse-labeled cells, was observed during the recovery process. This pattern included a set of maturation-specific (Mat) proteins (3). An understanding of the very fast initial response of the translation apparatus and how it is initiated may contribute to the elucidation of the sensing and signaling pathways involved in the induction of the Mat proteins.

The connection between the capacity for starvation survival and the characteristics of the translation machinery has recently been emphasized by Mikkola (37). Natural isolates of *E. coli* generally do not exhibit optimized ribosome performance characteristics as do commonly used laboratory strains and thus deviate from the optimization principle for rapid bacterial growth regarding ribosome efficiency and

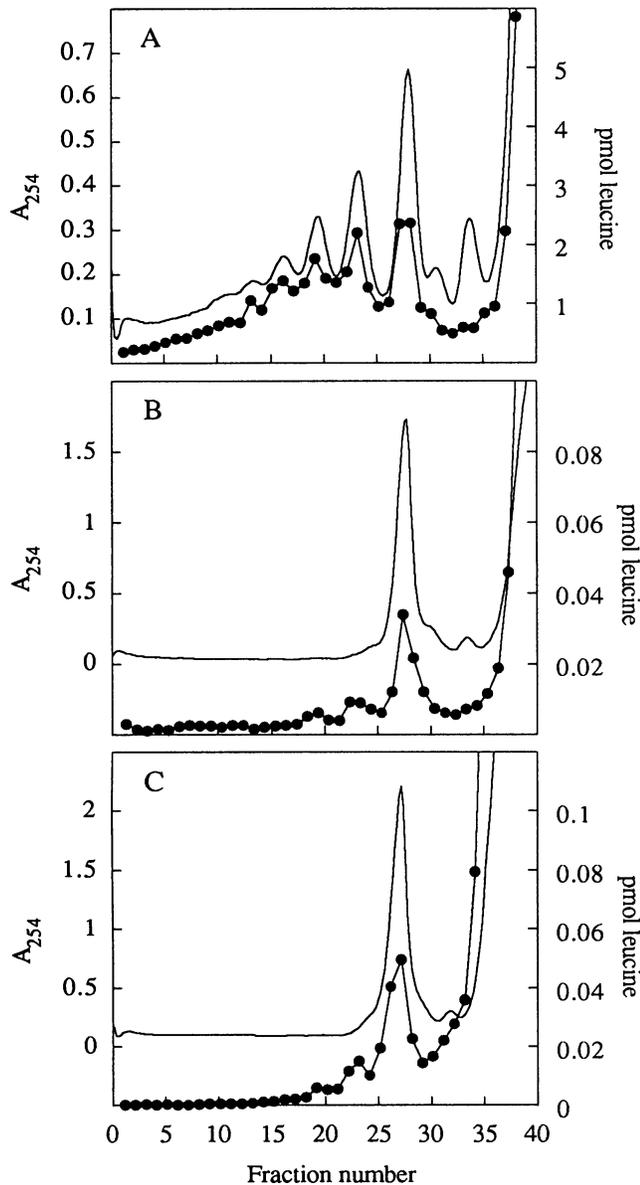


FIG. 5. Polyribosome profiles and protein synthesis in growing cells (A), as well as in cells that were starved for carbon for 24 h (B) and 144 h (C). Polyribosomes were prepared by using the freeze-thaw-lysozyme lysis procedure (54), sedimented through 10 to 30% sucrose density gradients, and detected by determining A_{254} (solid line), as in the experiment shown in Fig. 4. Fractions were collected starting from the bottom of the tubes. The radioactive leucine that was incorporated into nascent polypeptides during a 10-s pulse prior to cell harvest was detected by hot TCA precipitation and liquid scintillation. Results are expressed as picomoles of leucine in individual fractions (●).

mass investment in the translation apparatus (13, 29). It has been concluded that maximal bacterial growth rate and ribosome efficiency are not selected for in many natural bacterial populations. Natural isolates are also superior to laboratory strains in starvation survival, and a clear correlation has been demonstrated among slow loss of viability, slow growth, and slow ribosome phenotype. Interestingly, all of the natural isolates tested evolved the same character-

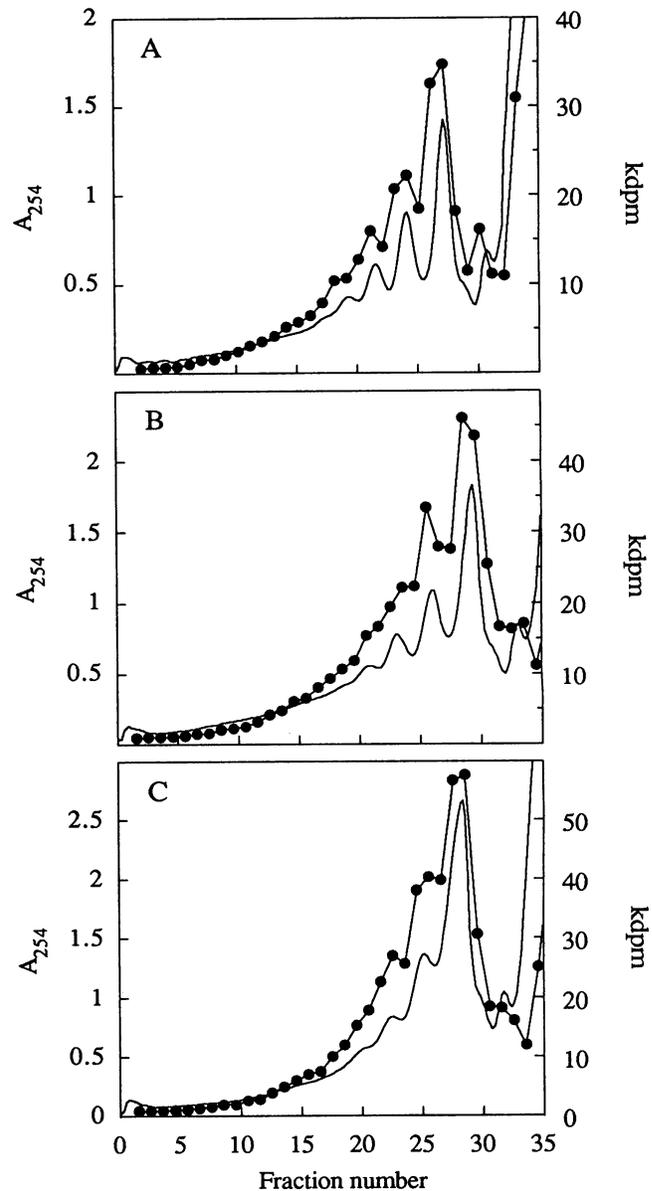


FIG. 6. Stability of polyribosomes obtained from growing cells during extraction in the presence of starved cells. Exponentially growing cells were labeled for two generations with [14 C]uridine. The labeled cells were extracted separately (A) or after they were mixed with an equal amount of unlabeled cells that had been starved for carbon for 24 h (B) or 144 h (C). The total polyribosome content in each gradient was analyzed by determining the A_{254} (solid line), as in the experiment shown in Fig. 4. The labeled polysomes were detected by TCA precipitation and liquid scintillation (●). Fractions were collected starting from the bottom of the tubes.

istics as laboratory strains, including curtailed starvation resistance, after a few hundred generations in glucose-limited chemostats (37). As indicated by these data, the demands and selection pressures that operate on the translation apparatus may be very different in nongrowing starved cells compared with cells growing rapidly in laboratory media.

It has been suggested that the proportionality between bacterial growth rate and ribosome concentration can be exploited for autecological studies of the physiological status

of individual cells in environmental samples (see reference 42). The use of labeled probes against specific rRNA sequences and fluorescence microscopy should allow not only the detection of specific species or other taxa, but also estimates of growth rates in situ. Our data, as well as the data of Kramer and Singleton (26), have important implications for such analyses. Nongrowing carbon-starved bacterial cells contain significant amounts of ribosomal particles; in fact, they may harbor as many as 8,000 ribosomes per cell after 96 h of nongrowth (this study). This should be compared with the 6,700 ribosomes per cell in exponentially growing *E. coli* B/r with a generation time of 100 min at 37°C (6). The natural *E. coli* isolates that had slow ribosomal performance characteristics and displayed a slow loss of viability during starvation could compensate for their lower ribosome efficiency by increasing ribosome concentrations during growth at low rates (37). Clearly, the relationships among physiological status, RNA and ribosome contents, ribosome stability, and starvation survival in natural bacterial isolates need further attention.

ACKNOWLEDGMENT

This study was supported by a grant from the Swedish Natural Science Research Council.

REFERENCES

- Albertson, N. H., T. Nyström, and S. Kjelleberg. 1990. Exoprotease activity of two marine bacteria during starvation. *Appl. Environ. Microbiol.* **56**:218–223.
- Albertson, N. H., T. Nyström, and S. Kjelleberg. 1990. Functional mRNA half-lives in the marine *Vibrio* sp. S14 during starvation and recovery. *J. Gen. Microbiol.* **136**:2195–2199.
- Albertson, N. H., T. Nyström, and S. Kjelleberg. 1990. Macromolecular synthesis during recovery of the marine *Vibrio* sp. S14 from starvation. *J. Gen. Microbiol.* **136**:2201–2207.
- Albertson, N. H., T. Nyström, and S. Kjelleberg. 1990. Starvation-induced modulations in binding protein-dependent glucose transport by the marine *Vibrio* sp. S14. *FEMS Microbiol. Lett.* **70**:205–210.
- Austin, B. 1988. *Marine microbiology*. Cambridge University Press, Cambridge.
- Bremer, H., and P. P. Dennis. 1987. Modulation of chemical composition and other parameters of the cell by growth rate, p. 1527–1542. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
- Chin-Leo, G., and D. L. Kirchman. 1990. Unbalanced growth in natural assemblages of marine bacterioplankton. *Mar. Ecol. Prog. Ser.* **63**:1–8.
- Colwell, R. R. 1984. *Vibrios in the environment*. John Wiley & Sons, New York.
- Cooper, T. G. 1977. *The tools of biochemistry*. John Wiley & Sons, New York.
- Davis, B. D., S. M. Luger, and P. C. Tai. 1986. Role of ribosome degradation in the death of starved *Escherichia coli* cells. *J. Bacteriol.* **166**:439–445.
- Dorsch, M., D. Lane, and E. Stackebrandt. 1992. Towards a phylogeny of the genus *Vibrio* based on 16S rRNA sequences. *Int. J. Syst. Bacteriol.* **42**:58–63.
- Dresden, M. H., and M. B. Hoagland. 1967. Polyribosomes of *Escherichia coli*. Breakdown during glucose starvation. *J. Biol. Chem.* **242**:1065–1068.
- Ehrenberg, M., and C. G. Kurland. 1984. Costs of accuracy determined by a maximal growth rate constraint. *Q. Rev. Biophys.* **17**:45–82.
- Goodman, A. (University of New South Wales, Sydney, Australia). 1992. Personal communication.
- Gottschal, J. C. 1992. Substrate capturing and growth in various ecosystems. *J. Appl. Bacteriol. Symp. Suppl.* **73**:39s–48s.
- Groat, R. G., J. E. Schultz, E. Zychlinsky, A. Bockman, and A. Matin. 1986. Starvation proteins in *Escherichia coli*: kinetics of synthesis and role in starvation survival. *J. Bacteriol.* **168**:486–493.
- Jenkins, D. E., E. A. Auger, and A. Matin. 1991. Role of RpoH, a heat shock regulator protein, in *Escherichia coli* carbon starvation protein synthesis and survival. *J. Bacteriol.* **173**:1992–1996.
- Jenkins, D. E., S. A. Chaisson, and A. Matin. 1990. Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. *J. Bacteriol.* **172**:2779–2781.
- Jenkins, D. E., J. E. Schultz, and A. Matin. 1988. Starvation-induced cross protection against heat or H₂O₂ challenge in *Escherichia coli*. *J. Bacteriol.* **170**:3910–3914.
- Kaplan, R., and D. Apirion. 1975. The fate of ribosomes in *Escherichia coli* cells starved for a carbon source. *J. Biol. Chem.* **250**:1854–1863.
- Kirchman, D. L. 1990. Limitation of bacterial growth by dissolved organic matter in the subarctic Pacific. *Mar. Ecol. Prog. Ser.* **62**:47–54.
- Kjelleberg, S., K. Flårdh, T. Nyström, and D. J. W. Moriarty. *In* T. Ford (ed.), *Aquatic microbiology: an ecological approach*, in press. Blackwell Scientific Publications, Inc., Boston.
- Kjelleberg, S., M. Hermansson, P. Mårdén, and G. W. Jones. 1987. The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. *Annu. Rev. Microbiol.* **41**:25–49.
- Koch, A. L. 1971. The adaptive responses of *Escherichia coli* to a feast and famine existence. *Adv. Microb. Physiol.* **6**:147–217.
- Kolter, R. 1992. Life and death in stationary phase. *ASM News* **58**:75–79.
- Kramer, J. G., and F. L. Singleton. 1992. Variations in rRNA content of marine *Vibrio* spp. during starvation survival and recovery. *Appl. Environ. Microbiol.* **58**:201–207.
- Kurland, C. G., M. Nomura, and J. D. Watson. 1962. The physical properties of the chloromycetin particles. *J. Mol. Biol.* **4**:388–394.
- Lange, R., and R. Hengge-Aronis. 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol. Microbiol.* **5**:49–59.
- Maaløe, O. 1979. Regulation of the protein-synthesizing machinery—ribosomes, tRNA, factors, and so on, p. 487–542. *In* R. F. Goldberg (ed.), *Biological regulation and development*, vol. 1. Plenum Publishing Corp., New York.
- Mandelstam, J. 1960. The intracellular turnover of protein and nucleic acids and its role in biochemical differentiation. *Bacteriol. Rev.* **24**:289–308.
- Mårdén, P., T. Nyström, and S. Kjelleberg. 1987. Uptake of leucine by a marine Gram-negative heterotrophic bacterium during exposure to starvation conditions. *FEMS Microbiol. Ecol.* **45**:233–241.
- Mårdén, P., A. Tunlid, K. Malmcrona-Friberg, G. Odham, and S. Kjelleberg. 1985. Physiological and morphological changes during short term starvation of marine bacterial isolates. *Arch. Microbiol.* **142**:326–332.
- Maruyama, H., and D. Mizuno. 1970. Ribosome degradation and the degradation products in starved *Escherichia coli*. I. Comparison of the degradation rate and of the nucleotide pool between *Escherichia coli* B and Q-13 strains in phosphate deficiency. *Biochim. Biophys. Acta* **199**:159–165.
- Maruyama, H., M. Ono, and D. Mizuno. 1970. Ribosome degradation and the degradation products in starved *Escherichia coli*. III. Ribosomal RNA degradation during the complete deprivation of nutrients. *Biochim. Biophys. Acta* **199**:176–183.
- Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz. 1989. Genetic basis of starvation survival in nondifferentiating bacteria. *Annu. Rev. Microbiol.* **43**:293–316.
- McCarthy, B. J. 1962. The effect of magnesium starvation on the ribosome content of *Escherichia coli*. *Biochim. Biophys. Acta* **55**:880–888.

37. Mikkola, R. 1991. Ph.D. thesis. Uppsala University, Uppsala, Sweden.
38. Morita, R. Y. 1985. Starvation and miniaturisation of heterotrophs, with special emphasis on the maintenance of the starved viable state, p. 111–130. *In* M. Fletcher and G. D. Floodgate (ed.), *Bacteria in their natural environments*. Academic Press, London.
39. Morita, R. Y. 1986. Starvation-survival: the normal mode of most bacteria in the ocean, p. 243–248. *In* F. Megusar and M. Gantar (ed.), *Perspectives in microbial ecology*. Slovene Society for Microbiology, Ljubljana, Yugoslavia.
40. Morita, R. Y. 1988. Bioavailability of energy and its relationship to growth and starvation survival in nature. *Can. J. Microbiol.* **34**:436–441.
41. Nath, K., and A. L. Koch. 1971. Protein degradation in *Escherichia coli*. II. Strain differences in the degradation of protein and nucleic acid resulting from starvation. *J. Biol. Chem.* **246**:6956–6967.
42. Neidhardt, F. C., J. I. Ingraham, and M. Schaechter. 1990. *Physiology of the bacterial cell. A molecular approach*. Sinauer Associates, Inc., Sunderland, Mass.
43. Nomura, M., D. M. Bedwell, M. Yamagishi, J. R. Cole, and J. M. Kolb. 1987. RNA polymerase and regulation of RNA synthesis in *Escherichia coli*: RNA polymerase concentration, stringent control, and ribosome feedback regulation, p. 137–149. *In* W. S. Reznikoff, R. R. Burgess, J. E. Dahlberg, C. A. Gross, M. T. Record, and M. P. Wickens (ed.), *RNA polymerase and the regulation of transcription*. Elsevier Science Publishing, Inc., New York.
44. Novitsky, J. A., and R. Y. Morita. 1978. Starvation induced barotolerance as a survival mechanism of a psychrophilic marine vibrio in the waters of the Antarctic Convergence. *Mar. Biol.* **49**:7–10.
45. Nyström, T., N. Albertson, and S. Kjelleberg. 1988. Synthesis of membrane and periplasmic proteins during starvation of a marine *Vibrio* sp. *J. Gen. Microbiol.* **134**:1645–1651.
46. Nyström, T., N. H. Albertson, K. Flärdh, and S. Kjelleberg. 1990. Physiological and molecular adaptation to starvation and recovery from starvation by the marine *Vibrio* sp. S14. *FEMS Microbiol. Ecol.* **74**:129–140.
47. Nyström, T., K. Flärdh, and S. Kjelleberg. 1990. Responses to multiple-nutrient starvation in a marine *Vibrio* sp. strain CCUG 15956. *J. Bacteriol.* **172**:7085–7097.
48. Nyström, T., and S. Kjelleberg. 1989. Role of protein synthesis in the cell division and starvation induced resistance to autolysis of a marine *Vibrio* during the initial phase of starvation. *J. Gen. Microbiol.* **135**:1599–1606.
49. Nyström, T., P. Mårdén, and S. Kjelleberg. 1986. Relative changes in incorporation rates of leucine and methionine during starvation survival of two bacteria isolated from marine waters. *FEMS Microbiol. Ecol.* **38**:285–292.
50. Nyström, T., R. M. Olsson, and S. Kjelleberg. 1992. Survival, stress resistance, and alterations in protein expression in the marine *Vibrio* sp. strain S14 during starvation for different individual nutrients. *Appl. Environ. Microbiol.* **58**:55–65.
51. Okamura, S., H. B. Maruyama, and T. Yanagita. 1973. Ribosome degradation and degradation products in starved *Escherichia coli*. VI. Prolonged culture during glucose starvation. *J. Biochem.* **73**:915–922.
52. Östling, J., A. Goodman, and S. Kjelleberg. 1991. Behaviour of IncP-1 plasmids and a miniMu transposon in a marine *Vibrio* sp.: isolation of starvation inducible *lac* operon fusions. *FEMS Microbiol. Ecol.* **86**:83–94.
53. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83**:346–356.
54. Ron, E. Z., R. E. Kohler, and B. D. Davis. 1966. Polysomes extracted from *Escherichia coli* by freeze-thaw-lysozyme lysis. *Science* **153**:1119–1120.
55. Schultz, J. E., G. I. Latter, and A. Matin. 1988. Differential regulation by cyclic AMP of starvation protein synthesis in *Escherichia coli*. *J. Bacteriol.* **170**:3903–3909.
56. Siegele, D. A., and R. Kolter. 1992. Life after log. *J. Bacteriol.* **174**:345–348.
57. Tempest, D. W., and O. M. Neijssel. 1978. Eco-physiological aspects of microbial growth in aerobic nutrient-limited environments. *Adv. Microb. Ecol.* **2**:105–153.
58. Tormo, A., M. Almirón, and R. Kolter. 1990. *surA*, an *Escherichia coli* gene essential for survival in stationary phase. *J. Bacteriol.* **172**:4339–4347.
59. Wada, A., Y. Yamazaki, N. Fujita, and A. Ishihama. 1990. Structure and probable genetic location of a “ribosome modulation factor” associated with 100S ribosomes in stationary-phase *Escherichia coli* cells. *Proc. Natl. Acad. Sci. USA* **87**:2657–2661.
60. Wheeler, P. A., and D. L. Kirchmann. 1986. Utilization of inorganic and organic nitrogen by bacteria in marine systems. *Limnol. Oceanogr.* **31**:998–1009.