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Responses to Multiple-Nutrient Starvation in Marine *Vibrio* sp. Strain CCUG 15956

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The response of marine *Vibrio* sp. strain S14 (CCUG 15956) to long-term (48-h) multiple-nutrient starvation (i.e., starvation for glucose, amino acids, ammonium, and phosphate simultaneously) can be described as a three-phase process. The first phase, defined as the stringent control phase, encompasses an accumulation of guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and decreases in RNA and protein synthesis during the first 40 min. In the second phase, there is a temporary increase in the rates of RNA and protein synthesis between 1 and 3 h paralleling a decrease in the ppGpp pool. The third phase includes a gradual decline in macromolecular synthesis after 3 h. Using two-dimensional gel electrophoresis of pulse-labeled proteins, a total of 66 proteins were identified as starvation inducible (Sti), temporally expressed throughout the three phases of starvation. The inhibition of protein synthesis during the first phase of starvation partly disrupted the subsequent temporally ordered synthesis of starvation proteins and prevented the expression of some late starvation proteins. It was also found that the early temporal class of starvation proteins, which included the majority of the Sti proteins, was the most essential for long-term survival. *Vibrio* sp. strain S14 cultures prestarved (1 h) for glucose, amino acids, ammonium, or phosphate as well as cultures exposed (1 h) to $CdCl_2$ exhibited enhanced survival during the subsequent multiple-nutrient starvation in the presence of chloramphenicol or rifampin, while heat or the addition of cyclic AMP or nalidixic acid prior to starvation had no effect. It was demonstrated that amino acid starvation and $CdCl_2$ exposure, which induced the stringent response, were the most effective in conferring enhanced survival. A few Sti proteins were common to all starvation conditions. In addition, the total number of proteins induced by multiple-nutrient starvation significantly exceeded the sum of those induced by starvation for each of the individual nutrients.

Most natural environments are characterized by their low bioavailability of nutrients (21), and measurements of natural seawater samples from both estuarine and oligotrophic marine waters have revealed that bacterial production is limited not only by grazing and bacteriophages but also by substrate availability (e.g., see references 5, 34, and 36). We and several other groups have been interested in characterizing the physiological adaptation of marine bacterial populations subjected to energy and nutrient starvation (e.g., see references 2, 3, 6, 18, 25, 28, and 39). Long-term survival of marine copiotrophic (35) bacteria in energy- and nutrient-limited microcosms is well documented (20). The *Vibrio* sp. strain S14 used in this study remains 100% viable after 2 weeks in unsupplemented artificial seawater and is culturable after at least 6 months in the starvation regimen (unpublished observations).

The survival of a marine bacterium would be expected to depend on its ability to express specific sets of genes resulting in a phenotype appropriate to a particular circumstance. For example, *Vibrio parahaemolyticus* is capable of producing different cell types adapted to existence in a liquid environment or at a surface (4), and other marine *Vibrio* spp. have been shown to respond to severe lack of energy and nutrients by entering a survival program characterized by major biochemical and morphological changes (1a, 2, 16a, 18, 25). This reorganization and morphogenesis results in a developmental form which is resistant to autolysis, sonic lysis (27), hydrostatic pressure (24), and heat and cold shock (N. Albertson and Å. Jouper-Jaan, unpublished data) and is adhesive to a variety of surfaces (7, 13) and therefore is highly adapted for survival. Most *Vibrio* strains remain metabolically active throughout the starvation-survival process and are primed for substrate availability and rapid recovery (1, 6, 8, 12, 17; N. H. Albertson, T. Nyström, and S. Kjelleberg, J. Gen. Microbiol., in press).

Studies of the survival process in carbon-starved *Escherichia coli* (10) and phosphate-starved *Salmonella typhimurium* (9) have revealed a short-term temporal expression of starvation-specific proteins. In *Vibrio* sp. strain S14, simultaneous starvation for carbon, nitrogen, and phosphorus provokes a temporal induction of membrane and periplasmic proteins and an extensive increase in protein turnover (25). However, little is known concerning the genetic basis and molecular mechanisms of the developmental regulatory processes. For instance, the role of the stringent response has not been thoroughly examined in the starvation-survival process of marine bacteria (12), although stringency has been shown to be involved in the initiation of sporulation in *Bacillus subtilis* (30, 31) and differentiation in both *Streptomyces griseus* (29) and *Myxococcus xanthus* (11). The survival characteristics of a relaxed *relA* strain and its isogenic *relA*$^+$ parent strain of *E. coli* K-12 during carbon starvation conditions were found to be indistinguishable (37). On the other hand, Mosteller and Kwan (23) isolated a number of relaxed control *relB* mutants of *E. coli* K-12 which exhibited a diminished ability to form colonies on solid medium when starved for various nutrients, particularly glucose (22, 23). In addition, Mach et al. (15) demonstrated the importance of the *relA* locus for the survival of *E. coli* cells during prolonged periods of amino acid starvation and also during subsequent multiple-nutrient starvation.

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Here we report that the expression of about 60 polypeptides increases in a temporally ordered way during long-term multiple-energy and -nutrient starvation in marine *Vibrio* sp. strain S14. The importance of protein synthesis at different times of starvation was demonstrated by the inhibitory effect of chloramphenicol and rifampin on long-term survival. We also examined whether a subset of starvation-induced proteins involved in the survival of energy- and nutrient-depleted cells of *Vibrio* sp. strain S14 is induced in response to starvation for different individual nutrients (glucose, amino acid, ammonium or phosphate starvation) and exposure to other stresses such as heat shock, $CdCl_2$, and nalidixic acid. The induction of the stringent response during multiple-nutrient starvation conditions and the possible correlation between the induction of stringency by individual stress conditions and enhanced survival was examined. Two-dimensional gel electrophoresis patterns obtained during the different nutrient limitations and during conditions that induce known stress responses and regulons in *E. coli* were compared.

MATERIALS AND METHODS

Organism, cultivation, starvation, and stress conditions. Marine *Vibrio* sp. strain S14 (CCUG 15956) resembles *Vibrio proteolyticus* (ATCC 15338) but is atypical in four characteristics in that S14 (i) accumulates poly-β-hydroxybutyrate during growth, (ii) is negative for amylase, (iii) does not grow at 40°C, and (iv) does not swarm on solid complex media. Cultures were grown in liquid medium containing 4.0 g of glucose, 2.2 g of $(NH_4)_2SO_4$, 0.54 g of K_2HPO_4, and 4.0 g of Casamino Acids (Difco Laboratories) per liter of nine-salt solution (28) in Erlenmeyer flasks placed in a rotary incubator shaker at 26°C. Addition of $CdCl_2$ (500 μM), nalidixic acid (50, 100, and 200 $\mu g \cdot ml^{-1}$), and cyclic AMP (cAMP) (5 mM) as well as temperature and starvation shifts were initiated when cultures reached an optical density at 420 nm of 0.3 (10^8 cells per ml). Different nutrient limitations were achieved by transferring growing cells, washed in the appropriate medium, to the growth medium lacking glucose, Casamino Acids, ammonium, or phosphate. In the multiple-nutrient starvation regimen, cells were washed and resuspended in unsupplemented nine-salt solution, i.e., simultaneous starvation for glucose, amino acids, ammonium, and phosphate. The starved cell suspensions had an optical density at 420 nm of 0.3 and were incubated statically at 26°C. Shifts from 26 to 35 or 40°C were accomplished by transfer of the culture flask to an incubator shaker at the higher temperature.

Inhibition experiments and viability determinations. The viability of starving cell suspensions was determined after the addition of chloramphenicol (100 $\mu g \cdot ml^{-1}$) and rifampin (300 $\mu g \cdot ml^{-1}$) at appropriate intervals during 48 h of multiple-nutrient starvation. The inhibitory effect of the antibiotics used was determined by comparison of the rate of incorporation of [^{3}H]leucine or [^{3}H]uridine into nontreated cells with the rate of incorporation into antibiotic-treated cells. The concentrations used in this study inhibited target macromolecular synthesis by 96 to 99% throughout the starvation period studied. Viability was determined by spreading serial dilutions on VNSS agar (26), and the number of metabolically active cells was monitored by the method of Zimmerman et al. (42). Separate cultures were allowed to prestarve for the different individual nutrients or were exposed to $CdCl_2$, nalidixic acid, cAMP, or heat for 1 h prior to the multiple-nutrient starvation in the presence of chloramphenicol or rifampin.

Measurement of rate of RNA and protein synthesis. The rate of RNA synthesis was determined by pulse-labeling (15, 30, and 60 s), using 1.2 μg of [5-^{3}H]uridine $\cdot ml^{-1}$ (4.6 TBq $\cdot$ mmol^{-1}; Dupont, NEN Research Products, Boston, Mass.). Total RNA synthesis during each of the starvation and stress conditions was also examined in the presence of the relaxing agent chloramphenicol. The rate of incorporation was determined by assessing the amount of radioactivity precipitable in cold 5% trichloroacetic acid. After incubation for 1 h at 0°C, precipitates were collected on membrane filters as previously described (28). The labeled uridine that was incorporated by the cells was also examined for resistance to alkaline hydrolysis. Protein synthesis was monitored as previously described (28). The relative rates of synthesis were compared with the rate of synthesis of nontreated, exponentially growing cells, which was assigned a value of 1.0.

Assay of nucleotide pools. Cellular guanosine 5′-diphosphate 3′-diphosphate (ppGpp) pools were measured by ion-pair reversed-phase high-performance liquid chromatography. Nucleotide extracts were prepared by the method described by Little and Bremer (14) with slight modifications. Samples (100 ml) of cell suspension were fixed with 10 ml of 5.5% formaldehyde and kept on ice. Cells were harvested (12,000 × *g*, 10 min, 4°C), and the pellets were suspended in 0.5 ml of 0.1 M KOH. After 30 min of incubation on ice, 0.5 ml of 60 mM $NH_4H_2PO_4$ (pH 6.0) was added and the extracts were neutralized with 15 μl of 8.5% H_3PO_4. Cellular debris was removed by centrifugation, and the supernatants were rapidly frozen at −20°C.

A 200-μl sample of the extracts was loaded onto a Lichrosorb RP18 column (5 μm, 4 by 250 mm) with a Spherisorb ODS2 guard column (5 μm, 4 by 10 mm) (LKB Pharmacia). The high-performance liquid chromatography buffer was 60 mM $NH_4H_2PO_4$ (pH 6.0) containing 5 mM tetrabutylammonium dihydrogen phosphate (Fluka Chemie, Buchs, Switzerland). This was mixed with various amounts of methanol with LKB Pharmacia 2156 Solvent conditioner, 2152 LC Controller, and two 2150 HPLC pumps. Isocratic elution with 10% methanol for the initial 5 min was followed by a 5-min linear gradient to 17% methanol and isocratic elution at 17% methanol for 40 min. The flow rate was 0.60 $ml \cdot min^{-1}$. Eluting nucleotides were monitored at 254 nm with a Waters 490 programmable detector. The identity of the cellular ppGpp peak was confirmed by its coelution with ppGpp (Calbiochem) in standard solutions and its UV absorbance spectrum. Peak areas were integrated with Nelson Analytical model 2600 Chromatography Software, and the amount of ppGpp was determined by comparison with the peak areas of the standards. Amounts of extracted ppGpp were expressed as picomoles per milliliter and optical density of the cell suspensions at 610 nm at the time of cell harvest.

Resolution of cell proteins on two-dimensional polyacrylamide gels. At appropriate times of starvation and stress treatment, a portion (5 ml) of the culture was removed and placed in a flask containing [^{35}S]methionine (3.7 MBq $\cdot ml^{-1}$, 46 TBq $\cdot$ mmol^{-1}) for all treatments except Casamino Acids and multiple-nutrient starvation, which received 0.74 MBq $\cdot ml^{-1}$, and the incorporation was allowed to proceed for 10 min. The nontreated control was labeled for 10 min during exponential growth at an optical density at 420 nm of 0.3. Samples for two-dimensional analysis were also prepared at intervals after the inhibition of protein synthesis during the first hour of starvation. Bacterial pellets were dissolved in lysis buffer (9.5% urea, 2%

Nonidet P-40, 5% β-mercaptoethanol, 1.6% Ampholine 5-7 (LKB Instruments, Inc.], 0.4% Ampholine 3-10) to produce extracts for resolution on two-dimensional polyacrylamide gels by the method of O'Farrell (33). The first dimension was an isoelectric focusing gel containing 0.95% each of Ampholine 5-7, Ampholine 3-10, and Servalyt 5-7 (Serva Feinbiochemical). The second dimension was a 12% polyacrylamide gel. Equivalent amounts of radioactivity (approximately 3×10^5 dpm) were loaded for each matched set of gels, i.e., starvation- or stress-treated versus nontreated cells. Autoradiograms (Fuji RX film) were prepared to permit visualization of the ^{35}S-labeled proteins. Proteins which were found to increase in the degree of synthesis relative to the nonstressed control were marked and assigned coordinates based on a standard gel of *Vibrio* sp. strain S14 labeled during exponential growth (1.8 doublings per h). Relative rates of synthesis of individual polypeptides were obtained by computer-assisted microdensitometry at 633 nm with an LKB UltroScan XL 2220 densitometer. The relative rate of synthesis was calculated by dividing the radioactivity of each protein spot by the total radioactivity recovered from the gel.

RESULTS

Synthesis of individual proteins during energy and nutrient starvation. A standard two-dimensional gel of *Vibrio* sp. strain S14 labeled during exponential growth (1.8 doublings per h) is depicted in Fig. 1A. Typical polypeptide patterns obtained after the imposition of multiple-nutrient starvation conditions are presented in Fig. 1B to H. A total of 66 proteins were found to increase in amount relative to the nonstarved control and were thus identified as starvation inducible (Sti). The Sti proteins are listed in Table 1 and marked in the panels of Fig. 1 corresponding to their time of appearance after the onset of starvation. An examination of Fig. 1 and Table 1 reveals that the synthesis of the Sti proteins was time dependent and that these proteins could be grouped in at least six temporal classes (A to F) with respect to their time of appearance during starvation (Table 1). The class A to E Sti proteins could be subdivided into two categories: those transiently synthesized during starvation and those whose synthesis extended throughout the entire starvation period studied. Most Sti proteins (Sti 1 to Sti 38) were induced at the onset of starvation (class A); however, only 58% of these polypeptides were still synthesized after 5 h of starvation. In addition to the sequential induction, the Sti proteins could be categorized based on the kinetics of synthesis as follows: those with a relatively narrow peak of synthesis (e.g., Sti 8, 19, 42, 43, and 56); those with a broad peak of synthesis (e.g., Sti 1, 5, 6, and 28); those with steadily increasing synthesis throughout the starvation period studied (e.g., Sti 27 and 51); and those maximally synthesized at the onset of induction with relatively constant or somewhat decreasing synthesis thereafter (e.g., Sti 33 and 47).

In addition to the induction of new proteins during starvation, several protein spots observed during growth exhibited rapidly diminished intensity after the imposition of starvation conditions, while the synthesis of other polypeptides remained relatively constant during the starvation period studied.

Effect of short-term inhibition of protein synthesis on subsequent induction of Sti proteins. To investigate whether proteins synthesized during the early phase of starvation were necessary for the orderly expression of proteins synthesized at later times, cells were incubated with chloramphenicol during the first hour of starvation, washed free from the inhibitor, and suspended in fresh starvation medium. Subsamples were subsequently removed at 2, 3, 5, and 24 h of starvation, pulse-labeled with [^{35}S]methionine, and processed for two-dimensional gel electrophoretic analysis of Sti proteins. Most of the class A and B proteins were immediately induced when the cells were washed free from chloramphenicol. However, Sti 10 and 33 were significantly delayed in expression and appeared after 5 h of starvation, and Sti 20 was not synthesized at any time during the starvation period studied (Fig. 2A). The same effect was observed for some class C proteins. While most class C proteins had already appeared after 2 h of starvation, Sti 44 was not induced at any time and Sti 47 appeared after a significant lag of 2 to 3 h (Fig. 2B). Furthermore, synthesis of the class D and E proteins Sti 52 and 59, respectively, and the class F proteins Sti 62, 64, and 65 was not elicited after incubation of cells with chloramphenicol during the first hour of starvation (Fig. 2C and D).

Effect of chloramphenicol and rifampin on viability of starved *Vibrio* sp. strain S14. To determine whether protein synthesis during starvation was required for the survival of the cells, we starved suspensions in the presence of chloramphenicol added at different times of starvation and determined the effect on viability at appropriate intervals. We found that inhibition of protein synthesis caused a significant decrease in cell viability during starvation and that the greatest effect on viability was achieved when the inhibitor was added during the first 3 h of starvation (Table 2). These results, which were obtained by the plate count technique, were the same as those given by monitoring the number of respiring cells by the method of Zimmerman et al. (42) (data not shown).

In another set of experiments, chloramphenicol was added for short intervals (2 h) at different times during starvation, after which the cells were washed free from the antibiotic. The addition of chloramphenicol for short intervals also resulted in a pronounced effect on culture viability when added at the onset of starvation, while addition after 4 h and onward had no significant effect (Table 3). Inhibition on the transcriptional level (rifampin) displayed the same effect as did the chloramphenicol treatment (Table 3). It was demonstrated that the inhibitory effect of the antibiotics on the target macromolecular synthesis remained the same throughout the starvation period studied (96 to 99% inhibition). In no case did the loss of viability commence until 6 to 8 h after the cells were washed free from the antibiotics.

Effect of prestress and prestarvation for individual nutrients on starvation resistance. To determine whether survival-related proteins could be induced by starvation for different individual nutrients or other stresses, the treatments' relative effectiveness in affording enhanced survival during the subsequent multiple-nutrient starvation in the presence of chloramphenicol or rifampin was examined. Glucose-, amino acid-, ammonium-, and phosphate-starved (1 h) cells of *Vibrio* sp. strain S14 were strikingly more resistant to multiple starvation in the presence of chloramphenicol or rifampin than their unstarved counterparts (Table 4). Adaptation to $CdCl_2$ also conferred enhanced survival, while heat shock or the addition of cAMP or nalidixic acid prior to starvation had no or limited effect (Table 4). The effect of these different starvation and stress treatments on growth was examined. Three treatments, amino acid starvation, and the addition of $CdCl_2$ and nalidixic acid, led to an immediate and almost complete inhibition of growth (data not shown).

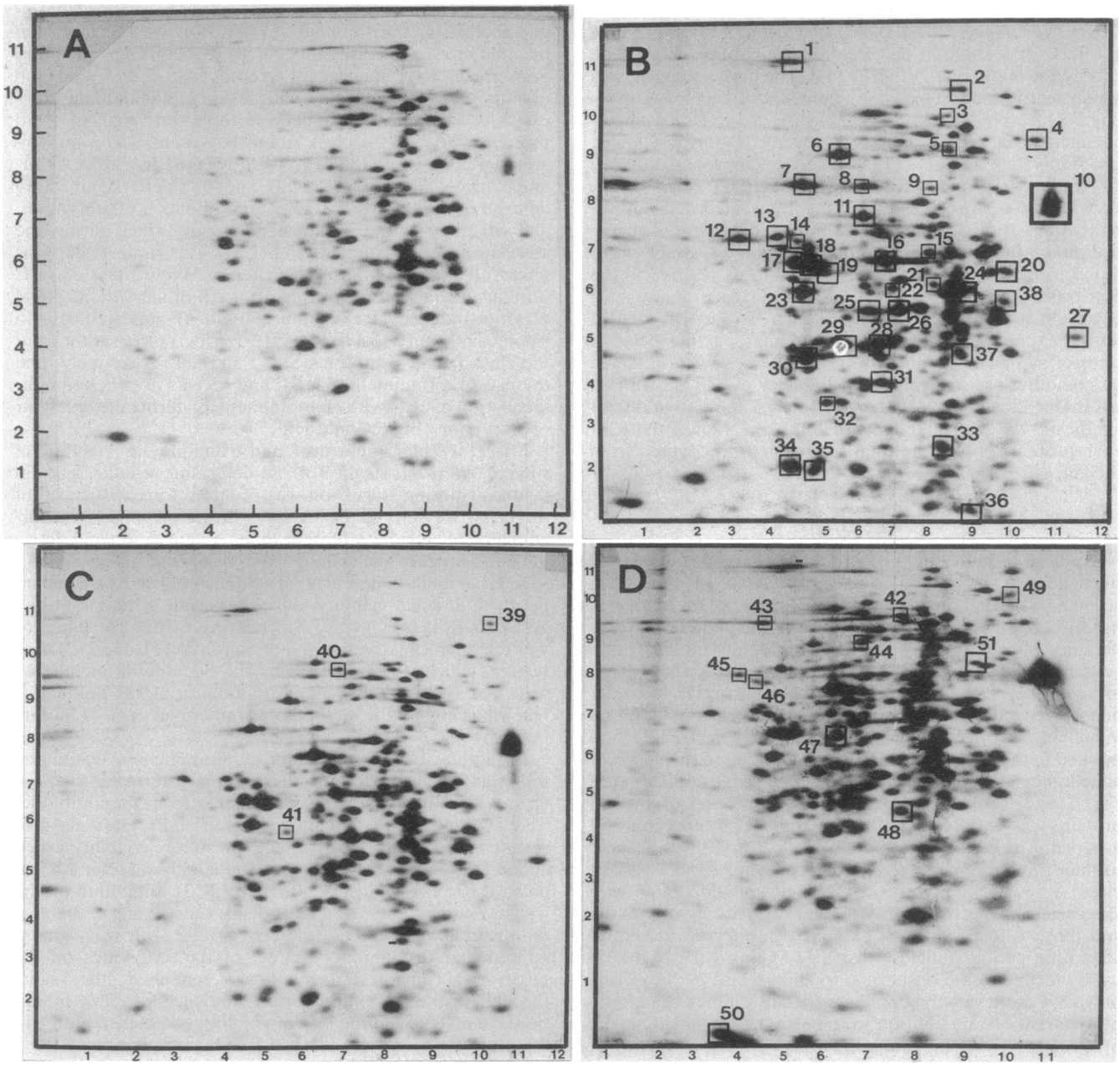

FIG. 1. Two-dimensional gel analysis of proteins induced during multiple-nutrient starvation. (A) Standard two-dimensional polypeptide map of *Vibrio* sp. strain S14 labeled during exponential growth. All proteins identified as inducible on subsequent gels were compared with this standard and assigned coordinates based on that comparison. Cells were labeled for 10 min at 0.3 h (B), 0.8 h (C), 2 h (D), 3 h (E), 5 h (F), 24 h (G), and 48 h (H) of starvation. Proteins marked in each panel were found to increase in amount relative to the nonstarved control and the preceding starvation polypeptide maps. The gel analyses were repeated at least twice to confirm reproducibility.

Ammonium, phosphate, and glucose starvation caused a transient inhibition, while a shift from 26 to 35 or 40°C gradually reduced the growth rate by 60 to 70%. The addition of cAMP had little or no effect on growth. The cells remained 100% viable during all the treatments.

Effect of starvation and stress treatments on total RNA synthesis and ppGpp accumulation. Since multiple-nutrient starvation may cause an internal depletion of amino acids and a buildup of uncharged tRNA in the cell, the occurrence of a stringent controllike response during the initial phase of starvation of *Vibrio* sp. strain S14 cells was examined. A rapid but transient decrease in the rate of total RNA synthesis was demonstrated (Fig. 3). Furthermore, this response could be abolished by the addition of the relaxing agent chloramphenicol (Fig. 3). The total rate of protein synthesis followed essentially the same kinetics as RNA synthesis (Fig. 3).

While starvation for amino acids also effectively reduced RNA synthesis during the first 30 min (Fig. 4A), starvation for glucose, ammonium, or phosphate caused a more intermediate and transient response (Fig. 4B to D). Adaptation to $CdCl_2$ (Fig. 4E) provoked a response similar to that of amino

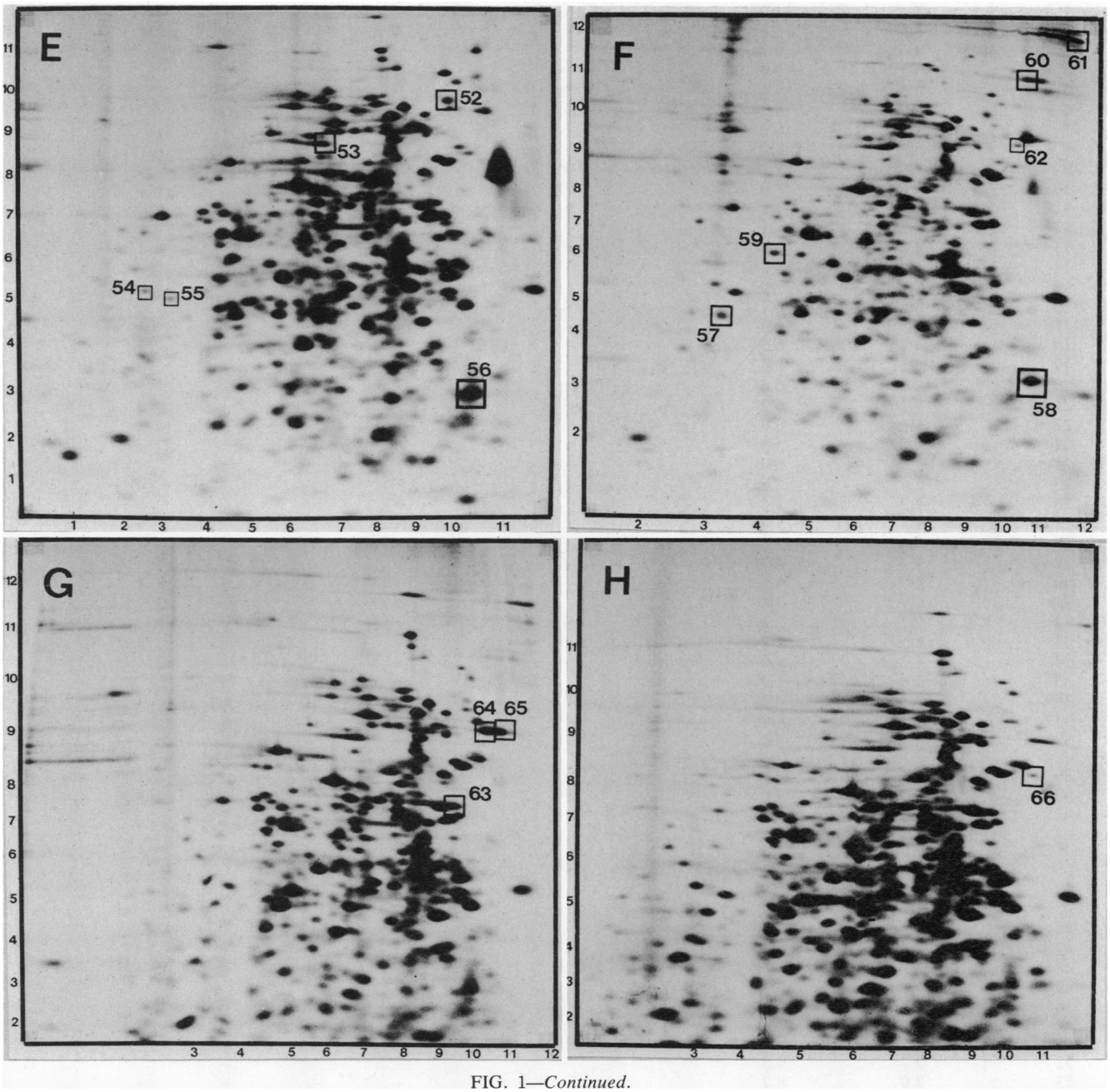

FIG. 1—*Continued.*

acid starvation, judged by the relative decrease in the rate of RNA synthesis. While the total rate of RNA synthesis decreased sharply during amino acid starvation and $CdCl_2$ adaptation, this effect was negated if cells were simultaneously treated with the relaxing agent chloramphenicol (Fig. 4A and E). The transient decrease of RNA synthesis during glucose, ammonium, and phosphate starvation could in no case be relaxed by the addition of chloramphenicol (Fig. 4B to D). Both the addition of nalidixic acid (Fig. 4F) and heat shock (Fig. 4G) caused a gradual decrease in the rate of total RNA synthesis, the kinetics of which were distinct from those obtained during amino acid starvation and $CdCl_2$ exposure. Chloramphenicol counteracted the gradual decrease in RNA synthesis during nalidixic acid treatment but not during heat shock (Fig. 4F and G). The addition of cAMP had no significant effect on RNA synthesis (Fig. 4H).

The alterations in the rate of total RNA synthesis during multiple-nutrient starvation were correlated with pronounced changes in the ppGpp pool size (Fig. 5A). When the ppGpp level rose, RNA synthesis ceased. The subsequent decrease in the ppGpp content appeared to correlate in time with an increase in the rate of RNA synthesis. It should be emphasized that the stringent control phase only proceeded for about 30 to 40 min during the early phase of starvation and that the ppGpp concentrations subsequently leveled off. Amino acid starvation and $CdCl_2$ exposure were the only individual treatments that resulted in an increased pool of

TABLE 1. Numbers, coordinates, and temporal classes of Sti proteins identified in Fig. 1

Starvation protein no.[a]	Polypeptide coordinates[b]		Time of starvation (h)							Temporal class
	x	*y*	0.3	0.8	2	3	5	24	48	
1	4.5	11.0	+	+	+	+				A
2	8.8	10.4	+	+	+	+	+	+	+	
3	8.6	9.9	+	+	+	+	+			
4	10.5	9.4	+	+	+	+	+	+	+	
5	8.6	9.1	+	+	+	+	+	+	+	
6	5.7	8.9	+	+	+	+				
7	4.7	8.3	+	+	+	+	+	+	+	
8	6.2	8.2	+							
9	7.8	8.2	+	+						
10	11.0	8.0	+	+	+	+				
11	6.3	7.5	+	+	+	+	+	+	+	
12	3.2	7.2	+	+	+	+	+	+	+	
13	4.2	7.3	+	+	+	+	+	+	+	
14	4.5	7.2	+	+	+	+	+	+	+	
15	8.2	7.0	+	+	+	+	+	+	+	
16	6.8	6.7	+	+	+	+	+	+	+	
17	4.5	6.6	+	+	+	+	+	+	+	
18	4.9	6.5	+	+	+	+	+	+	+	
19	5.2	6.4	+	+						
20	10.0	6.6	+	+	+	+				
21	8.2	6.2	+	+	+	+	+	+		
22	6.9	6.0	+	+	+	+	+	+	+	
23	4.7	5.8	+	+	+	+	+	+	+	
24	8.8	6.0	+	+	+	+	+	+	+	
25	6.4	5.6	+	+	+	+	+	+	+	
26	7.2	5.6	+	+	+	+	+	+	+	
27	11.5	5.2	+	+	+	+	+	+	+	
28	6.6	4.8	+	+	+	+	+			
29	5.7	4.7	+	+	+	+				
30	4.7	4.5	+	+	+	+	+	+	+	
31	6.7	3.8	+	+	+	+				
32	5.2	3.3	+	+	+	+				
33	8.5	2.7	+	+	+	+	+	+	+	
34	4.2	2.0	+	+	+	+				
35	4.8	1.9	+	+	+	+				
36	9.2	0.7	+	+	+	+	+	+	+	
37	8.8	4.7	+	+	+	+				
38	10.0	5.7	+	+	+	+				
39	10.5	10.7		+	+	+	+			B
40	7.1	9.6		+	+	+	+	+	+	
41	5.5	5.7		+	+	+	+	+	+	
42	7.9	9.5			+					C
43	4.6	9.4			+					
44	6.8	8.9			+	+	+			
45	3.8	8.0			+					
46	4.2	7.8			+	+	+	+	+	
47	6.4	6.3			+	+	+	+	+	
48	7.8	4.5			+	+	+	+	+	
49	9.7	10.3			+	+	+			
50	3.5	0.2			+	+	+	+	+	
51	9.6	8.3			+	+	+	+	+	
52	9.7	9.8				+	+	+		D
53	7.0	8.7				+	+	+	+	
54	2.8	5.1				+	+	+	+	
55	3.2	4.9				+	+	+	+	
56	10.4	3.0				+				
57	3.4	4.3					+	+	+	E
58	10.7	3.4					+	+	+	
59	4.0	5.9					+			
60	11.0	10.7					+			
61	11.8	12.1					+	+	+	
62	10.2	9.2					+	+	+	
63	9.6	7.4						+	+	F
64	10.4	9.1						+	+	
65	10.7	9.1						+	+	
66	10.8	8.2							+	

[a] Sti number.
[b] Coordinates are those shown in Fig. 1.

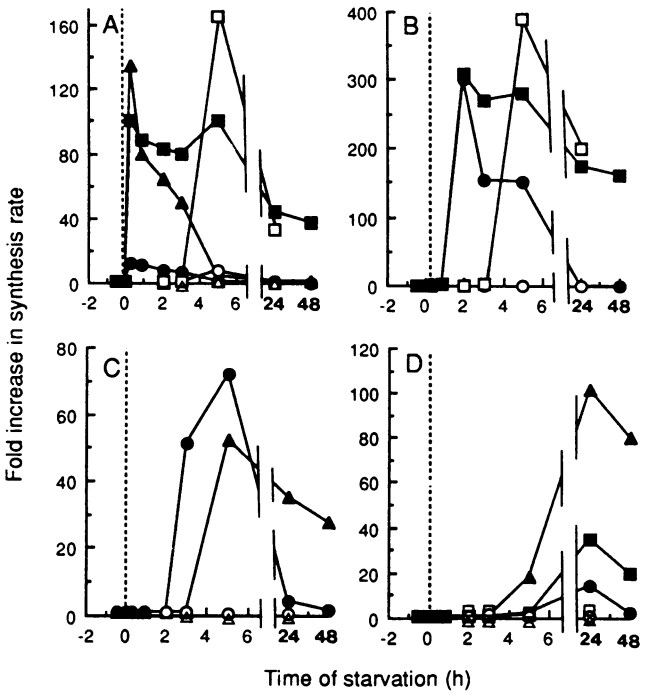

FIG. 2. Fold increase in the rate of synthesis of some Sti proteins during starvation after a short-term (0 to 1 h at the onset of starvation) inhibition of protein synthesis. Symbols: ■, ▲, ●, increase in synthesis of Sti proteins in a nontreated starvation suspension; □, △, ○, synthesis of Sti proteins after incubation with chloramphenicol during the first hour of starvation. (A) Fold increase in the rate of synthesis of class A proteins Sti 10 (●, ○), Sti 20 (▲, △), and Sti 33 (■, □). (B) Synthesis of the class C proteins Sti 44 (●, ○) and Sti 47 (■, □). (C) Synthesis of the class D protein Sti 52 (●, ○) and the class E protein Sti 59 (▲, △). (D) Synthesis of the class F proteins Sti 62 (●, ○), Sti 64 (▲, △), and Sti 65 (■, □).

ppGpp (Fig. 5B and C). The gradual decrease in total RNA synthesis during nalidixic acid exposure, relaxed by the addition of chloramphenicol (Fig. 4F), did not result from a corresponding accumulation of ppGpp.

Two-dimensional gel analysis of proteins induced by stress treatments and starvation for individual nutrients. The experiments described above indicate that the early temporal class of starvation-induced proteins, essential for the longevity of starving S14 cultures, can be induced during starvation for different individual nutrients or during $CdCl_2$ adaptation. To identify subsets of proteins commonly induced regardless of the treatments, we compared the two-dimensional gel patterns of S14 cells subjected to the different stresses.

TABLE 2. Effects of chloramphenicol (Cm) added at different times during starvation

Time of starvation (h) at which Cm was added	% Viability 48 h after Cm addition	% Inhibition of rate of protein synthesis
0	0.2	96
1	2	98
2	5	97
3	15	97
5	62	99
8	67	96
20	70	98

TABLE 3. Effects of short-term (2-h) chloramphenicol (Cm) and rifampin (Rf) addition on subsequent starvation survival

Time of starvation (h) at which 2-h antibiotic incubations were initiated	% Viability 48 h after:	
	Cm addition	Rf addition
0	9	2
2	95	85
4	98	97
6	102	98
10	100	101

A standard two-dimensional gel of *Vibrio* sp. strain S14 labeled during exponential growth is depicted in Fig. 6A. All proteins identified as inducible during subsequent treatments were compared with the standard and assigned coordinates and numbers based on that comparison. Typical polypeptide patterns obtained after 10 min of various starvation and stress conditions are presented in Fig. 6B to I. Proteins marked in each panel were found to increase relative to the nonstressed control pattern and to overlap with one or several of the other treatments. For convenience in describing the different responses, we have listed the results of this visual inspection in Table 5. The same overlap between starvation and stress conditions was seen after 50 min. An examination of Table 5 reveals a considerable overlap among proteins induced by amino acid and glucose starvation. Twelve polypeptides were induced after 10 min of both glucose and amino acid starvation, and three of these were also induced by the addition of cAMP (Table 5). Four of the proteins induced by $CdCl_2$, five induced by nalidixic acid, and one of the heat shock proteins were common to proteins induced by starvation for the different individual nutrients. All but one of the stress-inducible proteins that were found to overlap with the Sti proteins, induced during multiple-energy and -nutrient deprivation, belonged to the early class (class A) of the Sti proteins (Table 5). The main heat shock protein, however (no. 17, Sti 51), also markedly induced during $CdCl_2$ adaptation, was found to belong to class C of the Sti proteins (Table 5). Protein 17 was immediately induced at the onset of heat and $CdCl_2$ adaptation, but the

TABLE 4. Effects of 1-h prestarvation for different individual nutrients, different prestress conditions, and cAMP addition on culture viability during subsequent multiple-nutrient starvation in the presence of chloramphenicol (Cm) or rifampin (Rf)

Prestarvation and prestress conditions[a]	% Viability[b]		
	24 h after Cm addition	48 h after Cm addition	48 h after Rf addition
Log	1.9	0.1	0.03
AA	43	14	6.0
Glc	30	1.3	3.0
N	43	1.3	1.2
P	54	2.0	2.7
Cd	42	10	13
Nx	1.5	0.1	0.01
HS	3.8	0.3	0.02
cAMP	2.0	0.1	0.02

[a] Abbreviations: log, unstarved control; AA, amino acid starvation; Glc, glucose starvation; N, ammonium starvation; P, phosphate starvation; Cd, $CdCl_2$ exposure; Nx, nalidixic acid exposure; HS, heat shock (40°C); cAMP, addition of 5 mM cAMP.

[b] The number of viable cells (100%) at the onset of drug treatment was 1×10^8 to 3.5×10^8 cells per ml.

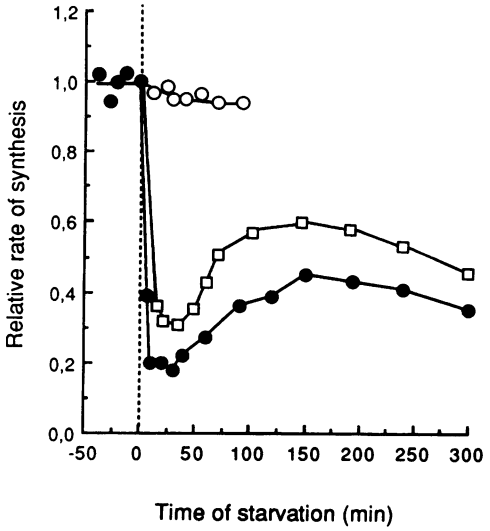

FIG. 3. Effect of multiple-nutrient starvation on total RNA synthesis (●), total RNA synthesis during simultaneous treatment with chloramphenicol (○), and protein synthesis (□) in *Vibrio* sp. strain S14. The rates of synthesis were compared with the rate obtained for exponentially growing cells, which was assigned a value of 1.0. The experiment was repeated three times to confirm reproducibility.

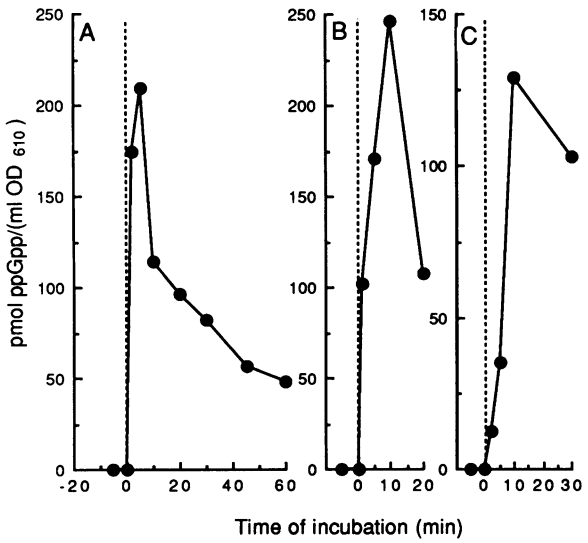

FIG. 5. Accumulation of intracellular ppGpp in *Vibrio* sp. strain S14 at the onset of multiple-nutrient starvation (A), amino acid starvation (B), and $CdCl_2$ exposure (C). Representative results of one of three experiments are shown. Levels of ppGpp during growth were undetectable by the high-performance liquid chromatography method described in Materials and Methods. OD, Optical density.

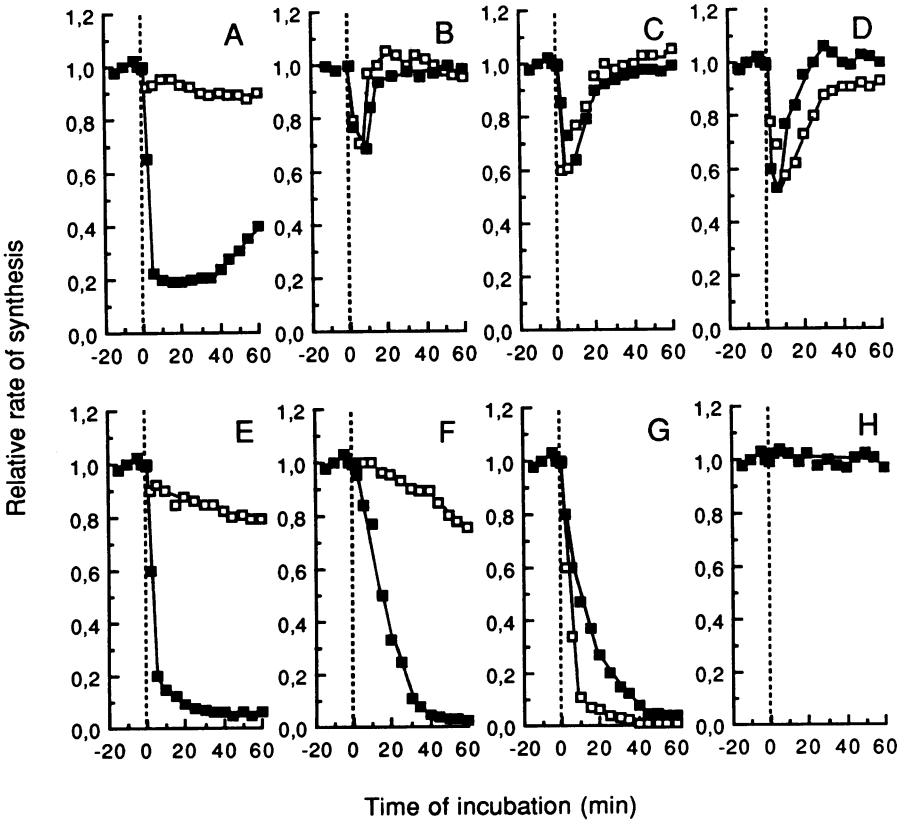

FIG. 4. Levels of total RNA synthesis during different stress and starvation conditions. (A to D) Relative rate of synthesis during amino acid starvation (A), glucose starvation (B), ammonium starvation (C), and phosphate starvation (D). (E to H) Relative rate of synthesis after $CdCl_2$ addition (E), nalidixic acid addition (F), shift to 40°C (G), and addition of cAMP (H). Symbols: ■, rate of synthesis in the control culture; □, rate of synthesis in the presence of chloramphenicol. The relative rates of synthesis were compared with that of nontreated, exponentially growing cells, which was assigned a value of 1.0. Experiments were repeated three times to confirm reproducibility.

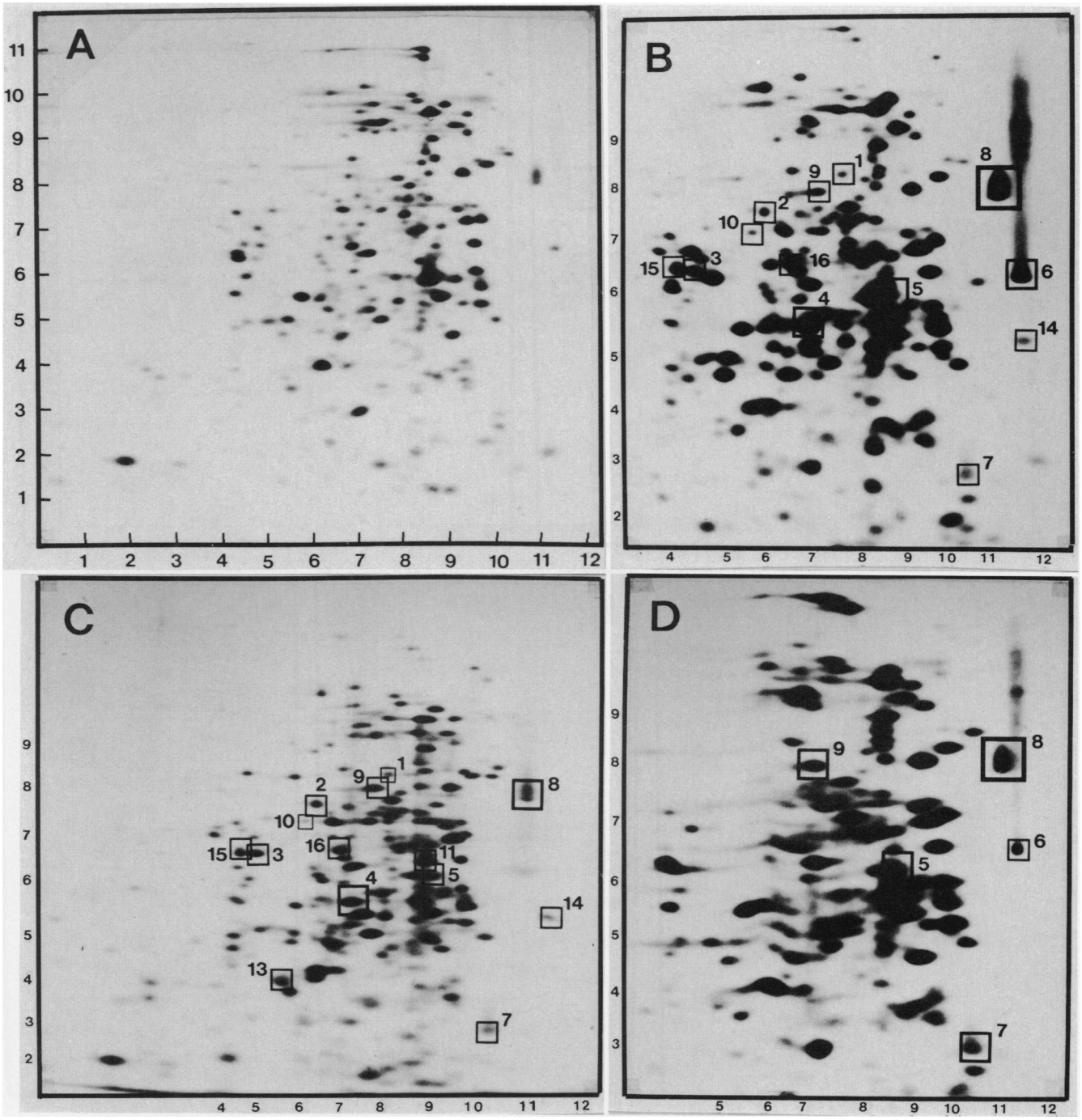

FIG. 6. Stress- and starvation-induced polypeptides of *Vibrio* sp. strain S14. (A) Standard two-dimensional map of an exponentially growing subculture (control); (B) glucose-starved subculture; (C) amino acid-starved subculture; (D) ammonium-starved subculture; (E) phosphate-starved subculture; (F) $CdCl_2$-treated subculture; (G) nalidixic acid-treated subculture; (H) heat-shocked (35°C) subculture; (I) heat-shocked (40°C) subculture. Proteins marked in each panel were found to increase relative to the nonstressed control and to overlap with proteins induced by one or several of the other treatments. Cells were pulse-labeled for 10 min after the imposition of the starvation or stress treatment. The gel analyses were repeated at least twice to confirm reproducibility.

synthesis of this polypeptide was not elicited prior to 2 h of multiple-nutrient starvation. Subsequent to induction, the rate of synthesis of protein 17 increased steadily during multiple-nutrient starvation.

Each starvation and stress treatment included in this study induced a unique group of proteins, and with the exception of the correlation between the amino acid and glucose starvation stimulons, a minority of the polypeptides belonging to one stimulon were found to overlap with proteins induced by the other treatments. The sum of the amino acid and glucose starvation stimulons constituted a major fraction of the early class of proteins responding to multiple-energy

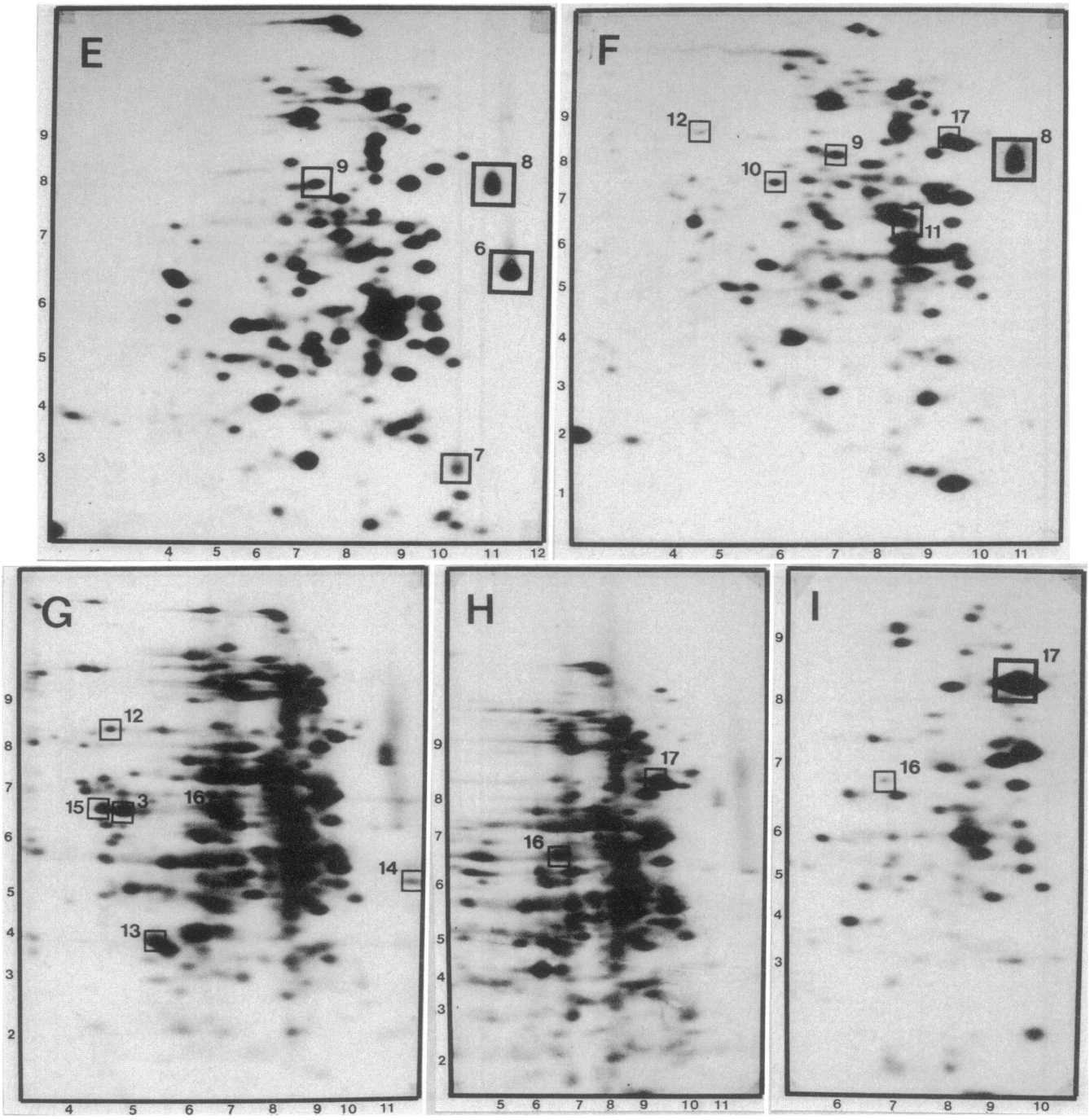

FIG. 6—*Continued.*

and -nutrient starvation, while ammonium and phosphate starvation together induced approximately 18% of the early class of multiple-nutrient starvation proteins (0 to 1 h). An intriguing finding of this study was that the total number of proteins induced during the early phase of multiple starvation significantly exceeded the sum of those induced by the individual-nutrient starvation conditions. A total of 13 of 38 polypeptides belonging to the temporal class A of Sti proteins did not belong to any of the different individual-nutrient starvation stimulons.

DISCUSSION

We identify the response to multiple-nutrient starvation in *Vibrio* sp. strain S14 as a three-phase process based on the kinetics of macromolecular synthesis and the reorganization of macromolecular contents. (i) The first phase encompasses the stringent response with a characteristic accumulation of ppGpp and a rapid decrease in the rate of RNA, protein (this report), and peptidoglycan (27) synthesis. To our knowledge, occurrence of the stringent response during starvation

TABLE 5. Numbers, coordinates, and temporal classes of the proteins identified in Fig. 6 as those that were induced by at least two different starvation or stress treatments

Protein no.	Sti no.[a]	Polypeptide coordinates[b]		Inducing condition[c]									Temporal class[d]
		x	y	AA	Glc	N	P	Cd	Nx	35	40	cAMP	
1	9	7.8	8.2	+	+								A
2	11	6.3	7.5	+	+								A
3	18	4.9	6.5	+	+				+				A
4	26	7.2	5.6	+	+							+	A
5	24	8.8	6.0	+	+	+							A
6		11.4	6.6		+	+	+						
7		10.2	2.8	+	+	+	+						
8	10	11.0	8.0	+	+	+	+	+					A
9		7.4	8.0	+	+	+	+	+				+	
10		6.1	7.2	+	+			+				+	
11		8.8	6.4	+				+					
12		4.7	8.3					+	+				
13		5.4	3.7	+					+				
14	27	11.5	5.2	+	+				+				A
15	17	4.5	6.6	+	+				+				A
16	16	6.8	6.7	+	+				+	+	+		A
17	51	9.6	8.3					+		+	+		C

[a] Proteins identified and designated as Sti during multiple-energy and -nutrient starvation (Fig. 1).

[b] Coordinates are those shown in Fig. 6.

[c] Abbreviations: AA, amino acid starvation; Glc, glucose starvation; N, ammonium starvation; P, phosphate starvation; Cd, $CdCl_2$ exposure; Nx, nalidixic acid exposure; 35, heat shock (35°C); 40, heat shock (40°C); cAMP, addition of 5 mM cAMP.

[d] Previous grouping of Sti proteins in different temporal classes (Table 1). Class A denotes the proteins that appear at the onset of energy and nutrient starvation, and class C denotes the Sti proteins induced after 50 min of starvation.

survival of marine bacteria has so far not been reported. A pronounced increase in the rate of proteolysis during this phase has previously been demonstrated for S14 cells (25). However, since the rate of proteolysis during multiple-nutrient starvation exceeds by five times that of amino acid-starved cells (25), while the accumulation of ppGpp was nearly identical during these different treatments (Fig. 5A and B), the existence of an additional, stringency-independent pathway for protein degradation may be proposed. Nongrowth conditions in *Vibrio* sp. strain S14 apparently provoke a proteolysis rate sufficient to depress the stringent response after approximately 40 min of multiple-nutrient starvation and allow the cells to enter phase 2 of the starvation-survival development program. (ii) The second phase (40 to 180 min) involves a relatively increased respiratory activity (18) and rate of RNA (this report), protein (28, this report), and peptidoglycan (27) synthesis in parallel with the diminishing pool of ppGpp (Fig. 5A). These energy-consuming activities coincide with the degradation of poly-β-hydroxybutyrate (32). The ratio of monounsaturated to saturated fatty acids increases during this phase (16a), as well as the D-alanine content per unit of dry weight (27). (iii) The third phase is characterized by a gradual decrease in endogenous metabolism to low or undetectable levels (18, 28). The ratio of monounsaturated to saturated fatty acids decreases again (16a), and the polar flagellum is lost or shed into the surrounding environment (16).This phase can probably be subdivided into several developmental stages which eventually will lead to recovery and growth or "dormancy" (20). Several Sti proteins, undetectable during growth, were sequentially induced during this phase (Fig. 1), and long-term starvation has previously been shown to provoke the synthesis of high-affinity uptake systems in S14 and other *Vibrio* spp. (1, 6, 8).

The Sti proteins were expressed in a sequential manner, allowing us to identify six temporal classes. The inhibition of protein synthesis during the first hour of starvation partly disrupted the orderly synthesis of late starvation proteins seen for nontreated cells. The results presented in Fig. 2B to D indicate that some Sti proteins induced during phases 2 and 3 depend on protein synthesis during the stringent control phase since the addition of chloramphenicol during this phase (0 to 1 h) abolished the synthesis of some of the class C, D, E, and F Sti proteins. Further work is needed to elucidate these observations or to propose similarities with the temporally ordered expression of genes leading to spore formation in *Bacillus* spp. and *Streptomyces* spp. Such similarities between the starvation survival programs of nondifferentiating and differentiating bacteria have been suggested by Matin et al. (19). It is noteworthy that the stringent response is involved in the initiation of sporulation after amino acid limitation in both *Bacillus* species (30) and *Streptomyces* species (29) and that the kinetics of accumulation and subsequent decline in the ppGpp pool at the onset of nongrowth of *Bacillus* species, *Streptomyces* species, and the *Vibrio* sp. strain S14 used in this study are strikingly similar.

The data presented in this report show that protein synthesis during the initial phase of multiple-nutrient starvation is essential for conferring starvation resistance. This is in accordance with results reported by Reeve and collaborators (37) using *E. coli* K-12. However, it has not previously been shown that inhibitors of transcription or translation present only during the first 2 h of starvation abolish protection against starvation stress. The long-term survival of the marine *Vibrio* sp. strain S14 also allows for an evaluation of the role of protein synthesis subsequent to the initial phase of starvation. It is interesting that an ongoing protein synthesis appears to be essential even for long-term-starved cells since chloramphenicol lowered the culture viability by at least 30% regardless of the time of addition (Table 2). Using two other marine *Vibrio* strains, it was recently demonstrated that the addition of chloramphenicol after 24 h results in a complete loss of viability within an additional 12 h of starvation (Å. Jouper Jaan, personal communication).

In view of the fact that the early phase of multiple-nutrient starvation, which includes the stringent response, promotes starvation resistance and that prestarvation for different individual nutrients enhances survival during subsequent multiple-nutrient starvation in the presence of chloramphenicol or rifampin (Table 4), we investigated the correlation between the induction of stringency and starvation survival. The highest degree of resistance to multiple starvation in the presence of chloramphenicol or rifampin was achieved with cultures prestarved for amino acids or pretreated with $CdCl_2$ (Table 4), and both these treatments provoked a typical stringent response in *Vibrio* sp. strain S14 as judged by the kinetics of RNA synthesis and ppGpp accumulation (Fig. 4A and E, 5B and C). High levels of ppGpp are also rapidly accumulated in *E. coli* K-12 as a consequence of $CdCl_2$ addition (41). Starvation for glucose, ammonium, or phosphate was shown not to induce the stringent response. However, *Vibrio* sp. strain S14 cultures prestarved for any one of these nutrients exhibited enhanced survival during subsequent multiple-nutrient starvation in the presence of inhibitors of protein synthesis (Table 4), but they survived to a lesser extent than cultures prestarved for amino acids or adapted to $CdCl_2$. These results indicate that the stringent

response is of some importance for starvation survival. None of the pretreatments used in this study resulted in a full survival during multiple-nutrient starvation in the presence of inhibitors of protein synthesis.

Three proteins were commonly induced during starvation for the different individual nutrients, and all these treatments promoted enhanced survival during multiple-nutrient starvation in the presence of chloramphenicol. The starvation survival-related subset of proteins in *E. coli* is suggested to be common for carbon, nitrogen, and phosphate starvation and independent of the signal molecule cAMP and its receptor protein (38). By using the technique of Mu d-directed *lac* operon fusion construction, Spector et al. (40) identified a mutation in one locus of *Salmonella typhimurium* (*stiC*) which significantly decreased viability during prolonged periods of nicotinate starvation. This locus was commonly induced under nicotinate, phosphate, ammonium, glucose, isoleucine-valine, adenine, and thiamine limitations and was found to require the RelA regulator for full induction (40). The proteins of unknown identity conferring starvation resistance in *Vibrio* sp. strain S14 were not simply general responders to nongrowth since both $CdCl_2$ exposure and amino acid starvation promoted enhanced survival, while nalidixic acid did not (Table 4). Nalidixic acid primarily induces an SOS response and secondarily induces a heat shock response in *E. coli* K-12 (41). This indicates that preinduction of the SOS and heat shock regulons does not promote a protective effect against starvation stress, provided that nalidixic acid induces a similar response in *Vibrio* sp. strain S14. In addition, an S14 culture shifted from 26 to 35 or 40°C (1 h) did not display an enhanced survival during subsequent multiple-nutrient starvation in the presence of chloramphenicol (Table 4).

Although starvation for individual nutrients and heat shock, nalidixic acid, and $CdCl_2$ treatment provoke unique and individual patterns of protein expression, some proteins were common to different starvation and stress treatments. However, the proteins of one stimulon did not respond coordinately to all starvation and stress treatments, and relatively few of the Sti proteins were found to overlap with the stress-inducible proteins (heat, $CdCl_2$, and nalidixic acid treatment). This suggests, despite an interconnecting regulation for a few specific proteins, major differences in the regulatory pathways controlling the expression of starvation and different stress proteins. In addition, none of the proteins that overlapped between starvation and stress treatments were detected as being universal, nonspecific responders to stress. A most intriguing finding of this study was that 13 of the Sti proteins induced by multiple starvation were unique and not observed when the cells were starved for any of the individual nutrients, indicating that additional sensors and signals may be involved when the bacterial cells are starved for several different nutrients simultaneously.

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