Escherichia coli Proteins Synthesized during Recovery from Starvation

DEBORAH A. SIEGELE* AND LINDA J. GUYNN†

*Department of Biology, Texas A&M University,
College Station, Texas 77843-3258*

Proteins synthesized in *Escherichia coli* during recovery from starvation were resolved by two-dimensional polyacrylamide gel electrophoresis. Nine outgrowth-specific proteins, which appeared in two kinetic groups, that were not detected in either starved or exponential-phase cells were synthesized. Five other proteins whose rate of synthesis during outgrowth was ≥5-fold higher than during exponential growth were observed.

The starvation and exponential phases represent two physiological states that cells must switch between in response to environmental signals. In *Escherichia coli* and other nonsporulating, gram-negative bacteria, starvation results in a morphologically distinct cell that is metabolically less active and more resistant to environmental stresses (8, 9, 13, 17, 21). These changes are accompanied by dramatic changes in the pattern of gene expression.

When nutrients become available and cells resume growth, there is usually a lag before the cells start to divide. During this period, cells may be reversing many of the changes that occurred during starvation. Before exponential growth is achieved, expression of starvation-specific proteins must be turned off and synthesis of proteins needed for growth must be induced.

The ability to respond quickly to the appearance of nutrients will clearly be advantageous in the competition for limited resources. It seems likely that mechanisms have evolved to ensure rapid reentry into the growth cycle. Mutations in several *E. coli* genes cause a defect in reinitiating growth when starved or stationary-phase cells are transferred to fresh medium; such genes include *clpP* (4), *fis* (2), *relA* (5), *relB* (15), *rpoS* (19), *surB* (20), and *cydAB* (20). Proteins expressed only or predominantly during outgrowth from starvation have been observed in *Vibrio* sp. strain S14 (1, 12) and *Pseudomonas putida* KT2442 (7) and during outgrowth of *Bacillus subtilis* spores (22). These outgrowth-specific proteins are likely to be involved in recovery from starvation.

The wealth of genetic, biochemical, and physiological information available for *E. coli* makes it a good system in which to study how cells exit stationary phase and resume growth. To understand how cells control the transition from stationary phase to exponential growth, we examined the pattern of proteins synthesized in *E. coli* after nutrients were added back to starved cells.

Growth of starved cells after the addition of nutrients. *E. coli* K-12 strain ZK126 (W3110 Δ*lacU169 tna-2* [3]) was grown in M63 minimal glucose medium (14) supplemented with 40 μg each of alanine, arginine, glutamine, glycine, histidine, isoleucine, leucine, lysine, proline, serine, threonine, and valine per ml. Exponentially growing cultures were harvested at approximately 1.5×10^8 CFU/ml, washed twice in MBSM buffer (40 mM morpholine propanesulfonic acid [MOPS; pH

7.4], 150 mM NaCl, 1 mM MgSO$_4$), and starved by resuspension in MBSM buffer. Starved cultures were incubated at 37°C with aeration for 24 h. There was no decrease in viability during the starvation period. Growth was initiated by adjusting the medium to final conditions of 1× M63, 0.2% glucose, 2 μg of thiamine per ml, 2 mM MgSO$_4$, and 40 μg of each of the aforementioned 12 amino acids per ml. Growth was monitored by culture density and CFU. Typical results are shown in Fig. 1A. The optical density of the cultures started to increase between 10 and 15 min after nutrients were added and increased exponentially from about 50 min until the onset of stationary phase. The number of cells increased exponentially after 60 min. The increase in optical density at 600 nm before the increase in cell number is presumably due to increases in cell mass and volume that precede cell division (11, 16).

Synthesis of RNA and protein during recovery from starvation. Synthesis of RNA and protein during outgrowth were measured as incorporation of [^{3}H]uridine and [^{3}H]leucine, respectively, into trichloroacetic acid (TCA)-precipitable material as described previously (6). Measurements were done on at least two independent cultures. Representative results are shown in Fig. 1B and C. RNA synthesis began to increase 1 min after nutrients were added and by 2 min was already fivefold higher than in starved cells. Increases in protein synthesis lagged somewhat behind RNA synthesis and were not detected until 3 min. By 4 min, protein synthesis was almost 10-fold higher than in starved cells. These results are comparable to those previously reported for outgrowth of starved *E. coli* cultures (10, 20). The response was slower than that seen for *Vibrio* sp. strain S14, in which synthesis of both RNA and protein increase almost immediately after the addition of nutrients (1, 6).

Specific proteins synthesized during recovery from starvation. To further characterize events occurring during recovery from starvation, we examined the synthesis of individual proteins at different times in the growth cycle. Cultures of ZK126 were grown and starved, and growth was reinitiated by the addition of nutrients as described above. Samples were removed at 2, 5, 15, 30, 45, and 180 min after the addition of nutrients, labeled for 2 min with [^{35}S]methionine (15 μCi/ml, >1,000 Ci/mmol, SJ1515; Amersham, Arlington Heights, Ill.), and then chased for 1 min with 0.2 mM unlabeled methionine. Because of the low rate of protein synthesis after 24 h of starvation, samples from starved cultures were labeled for 2 h.

Proteins synthesized at each time point were separated by two-dimensional polyacrylamide gel electrophoresis as described by O'Farrell (18), with minor modifications. Gels were

_____

* Corresponding author. Phone: (409) 862-4022 Fax: (409) 845-2891. Electronic mail address: d-siegele@tamu.edu.

† Present address: Department of Biochemical Sciences, University of Houston, Houston, TX 77204.
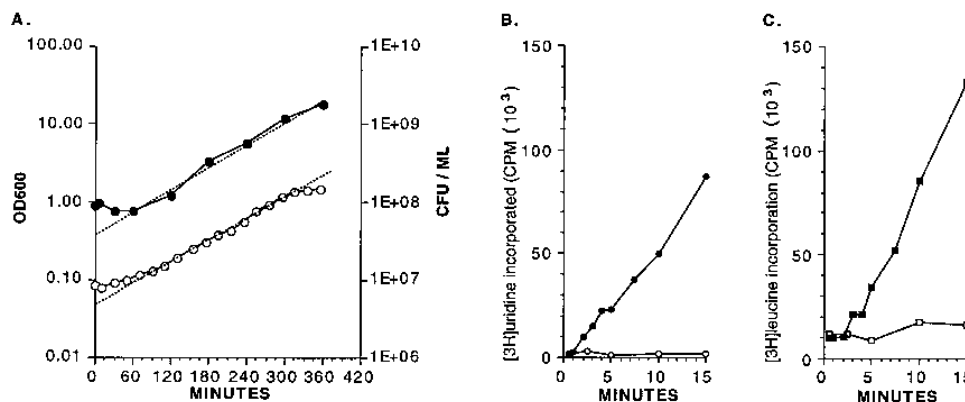
FIG. 1. Growth and synthesis of RNA and protein during recovery from starvation. Growth was initiated by adding nutrients to a 24-h starved culture. (A) Growth was monitored by viable cell count (closed circles) or spectrophotometrically (open circles). The best fit to the linear portion of each growth curve was calculated by linear regression and is shown as a dotted line. OD, optical density at 600 nm. (B) Bulk RNA synthesis was determined by measuring incorporation of [³H]uridine into TCA-precipitable material. (C) Bulk protein synthesis was determined by measuring incorporation of [³H]leucine into TCA-precipitable material. In panels B and C, open symbols indicate incorporation in a 24-h starved culture. [³H]uridine or [³H]leucine was added to a starved culture at time zero, and samples were removed at 0.5, 2.5, 5, 10, and 15 min. Closed symbols indicate incorporation after nutrients were added. Nutrients were added to a starved culture along with [³H]uridine or [³H]leucine at time zero, and samples were removed at 0.5, 1, 2, 3, 4, 5, 7.5, 10, and 15 min.

run on samples from three independent cultures, except for the 2-, 30-, and 45-min time points, which were from two independent cultures. Proteins synthesized at each time point were compared by visual inspection of autoradiograms.

The patterns of protein synthesis during starvation and 5, 15, and 180 min after the addition of nutrients are shown in Fig. 2. Note that because of the long labeling time used for starved cells, we can say whether a particular protein is being synthesized but cannot compare its rate of synthesis during starvation with its rate of synthesis during outgrowth or in exponential phase. The 180-min sample is representative of exponentially growing cells. An identical pattern of protein synthesis was seen in a sample labeled after 10 generations of exponential growth (data not shown).

Starvation-specific proteins were defined as those proteins that were synthesized in starved cells but whose synthesis was not detected in exponential phase cells. Similarly, exponential-phase-specific proteins were defined as those proteins synthesized in exponential-phase cells but whose synthesis was not detected in starved cells. We identified 47 starvation-specific and 33 exponential-phase-specific proteins (Fig. 3).

Five minutes after nutrients were added to starved cells, both starvation-specific and exponential-phase-specific proteins were being made. Most of the exponential-phase-specific proteins were already being synthesized (compare Fig. 2B and 3B), and the majority of starvation-specific proteins were still being synthesized (compare Fig. 2A and 3A). Over the next 40 min, the pattern of protein synthesis changed to more closely resemble the pattern seen in exponential-phase cells. Synthesis of all but seven of the starvation-specific proteins had stopped by 45 min after nutrients were added, and all but five of the exponential phase-specific proteins were being synthesized by this time.

These changes in the pattern of protein synthesis are qualitatively similar to those that occur during recovery from starvation in other gram-negative bacteria (1, 7, 12). Synthesis of different exponential-phase proteins is induced in a specific temporal order, and the synthesis of starvation-induced proteins stops at different times after the addition of nutrients. The starvation-specific proteins were divided into five temporal classes based on how long their synthesis could be detected after the addition of nutrients (Fig. 4A).

Outgrowth-specific proteins. We identified nine proteins that were synthesized only during the initial period of recovery from starvation (proteins 1 through 9 in Fig. 2). Their synthesis was not detected in starved or exponentially growing cells. We call these outgrowth-specific proteins because of their transient synthesis during recovery from starvation. It is possible that synthesis of some or all of these proteins will turn out to also be induced during other growth transitions.

The outgrowth-specific proteins were divided into two classes based on the kinetics of their appearance. Outgrowth-specific proteins 1 through 7 were detected within 2 to 5 min after the addition of nutrients (Fig. 2 and data not shown). There may be other outgrowth-specific proteins synthesized prior to 2 min, but incorporation of [³⁵S]methionine was too low during that time to allow the synthesis of specific proteins to be examined. A second class of proteins appeared after a brief lag. Outgrowth-specific proteins 8 and 9 were first detected in samples pulse-labeled 15 min after the addition of nutrients. The rate of synthesis of the nine outgrowth-specific proteins was induced at least sevenfold over background, except for protein 1, whose rate of synthesis was induced only threefold (Table 1). Expression of the outgrowth-specific proteins was transient. Their synthesis either had stopped or was greatly reduced by 45 min (Fig. 4B).

At least two phases of outgrowth-specific proteins are also observed in other bacteria (1, 7, 12). Transiently expressed immediate upshift (Iup) proteins are detected when *Vibrio* sp. strain S14 cells are pulse-labeled at 0 to 3 min of outgrowth (12). The maturation proteins, seen in *Vibrio* sp. strain S14 (1) and *P. putida* KT2442 (8), comprise a second wave of outgrowth-specific proteins whose synthesis is induced later than the Iup proteins.

The stability of the nine outgrowth-specific proteins was examined with a pulse-chase protocol (data not shown). Outgrowth-specific proteins 1 and 3 were unstable and were not detected after a 60-min chase. The seven other outgrowth-specific proteins were still detected 3 h after labeling. Interestingly, a new protein spot appeared during the 60-min chase of cells pulse-labeled at 5 min. This new protein comigrated with an exponential-phase-specific protein (protein 143 in Fig. 3B). Apparently, there is posttranslational modification of one of
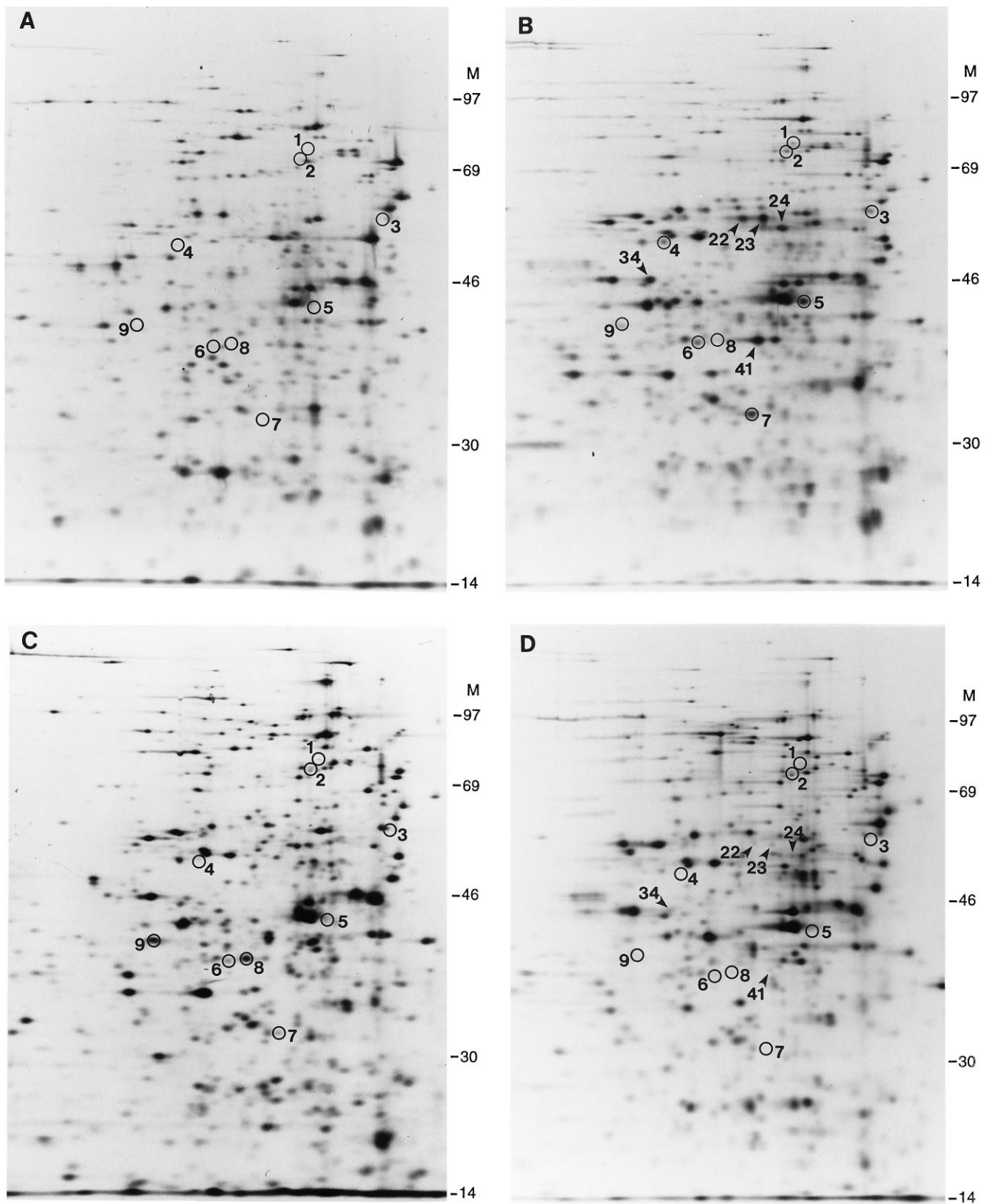
FIG. 2. Two-dimensional polyacrylamide gel electrophoresis of proteins synthesized during recovery from starvation. Cells were labeled with [^{35}S]methionine as described in the text. Labeled samples were precipitated with 5% TCA and resuspended in lysis buffer as described by O'Farrell (18), with minor modifications. Ampholines were from Pharmacia Biotech (Uppsala, Sweden). A total of 10^6 cpm of ^{35}S-labeled protein was loaded on each isoelectric focusing gel. A 10% poly-acrylamide gel was used for the second dimension. The autoradiograms are shown with the basic end of the isoelectric focusing gel at the left. The sizes of the protein molecular weight markers (M) are given in kilodaltons. Proteins synthesized: (A) after 24 h of starvation, (B) 5 min after the addition of nutrients, (C) 15 min after the addition of nutrients, (D) 180 min after the addition of nutrients. Circles indicate the positions of outgrowth-specific proteins, which are designated by arbitrary numbers. Arrows in panels B and D indicate the positions of proteins, designated by arbitrary numbers, whose rate of synthesis at 5 min is greater than during exponential growth.
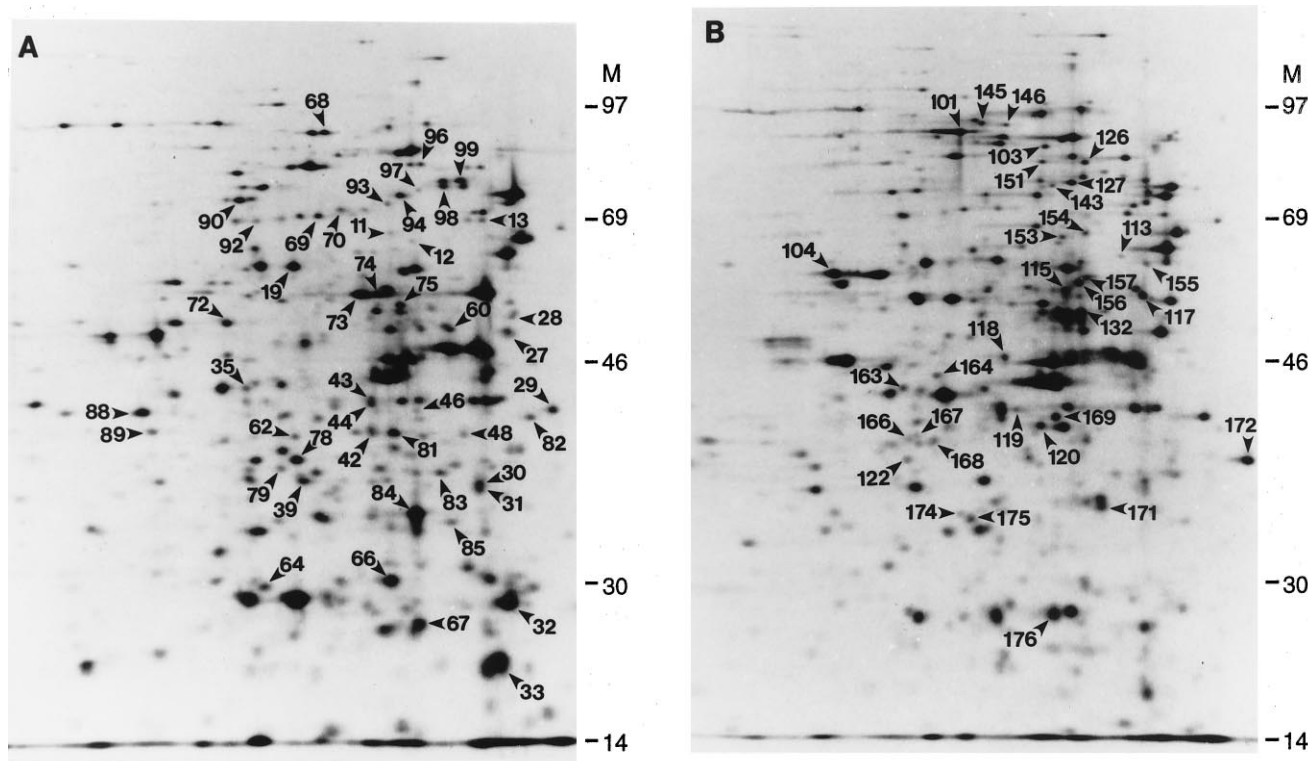
FIG. 3. Two-dimensional polyacrylamide gels showing starvation-specific and exponential phase-specific proteins. Two-dimensional polyacrylamide gel electrophoresis was done as described in the legend to Fig. 2. The autoradiograms are shown with the basic end of the isoelectric focusing gel at the left. The sizes of the protein molecular weight markers (M) are given in kilodaltons. (A) Proteins synthesized after 24 h starvation. (B) Proteins synthesized during exponential growth. Proteins specific for each growth phase are indicated by arrows and designated by arbitrary numbers.

the proteins synthesized at 5 to 7 min that does not occur until sometime later in outgrowth.

Proteins with higher synthesis rates during outgrowth than during exponential growth. In addition to the outgrowth-specific proteins, we detected five proteins whose rate of synthesis at 5 min of outgrowth was at least fivefold greater than it was in exponentially growing cells (Table 1). The positions of these five proteins are indicated with arrows in Fig. 2.

Concluding remarks. Proteins synthesized exclusively or predominantly during recovery from starvation have now been seen in three gram-negative bacteria (references 1, 7, and 12 and this work). Outgrowth-specific proteins in the different organisms may have similar functions. The timing of their expression suggests that they function in reprogramming the cell for exponential growth (1, 7, 12). Outgrowth-specific proteins may be needed to rapidly reverse starvation-induced changes in cell structure, composition, and gene expression.

In this study, we examined the proteins synthesized when glucose plus 12 amino acids were added to *E. coli* cells starved in buffer. It is likely that some of the same outgrowth-specific proteins will be induced when different starvation or outgrowth conditions are used, even though there will be differences in cell physiology and the overall pattern of protein synthesis. In *Vibrio* sp. strain S14, most of the same Iup proteins are induced when growth is initiated by glucose or glucose plus amino acids (12), even though the stringent response is induced when growth is initiated without amino acids (6). Thus, there appears to be a general outgrowth response that may facilitate the transition into the growth phase (12).

Regulation of expression of the outgrowth-specific proteins is likely to be affected by regulators known to be involved in

nutrient shifts. Synthesis of outgrowth-specific protein 7 is not detected when nutrients are added to a starved *rpoS* null strain (19), indicating that its expression is regulated directly or indirectly by the stationary-phase-specific sigma factor σ^S. The
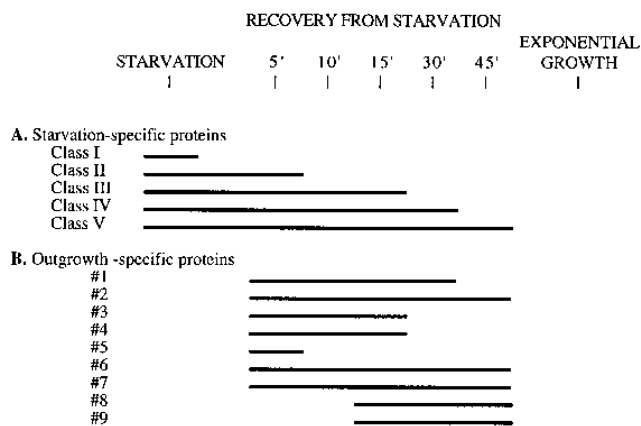

FIG. 4. Times when starvation- and outgrowth-specific proteins were synthesized. (A) Starvation-specific proteins were divided into different temporal classes based on how long after the addition of nutrients their synthesis was detected. The bars indicate when expression of proteins in each temporal class could be detected. Class I contained proteins 69, 70, 72, 73, 74, 75, 78, 79, 81, 82, 83, 84, and 85; class II contained proteins 32, 60, 62, and 88; class III contained proteins 11, 27, 28, 33, 35, 43, 48, 66, 92, and 99; class IV contained proteins 12, 13, 39, 64, 67, 68, 89, 90, 93, 94, 96, 97, and 98; and class V contained proteins 19, 29, 30, 31, 42, 44, and 46. (B) Outgrowth-specific proteins were synthesized transiently. The bars indicate when expression of each outgrowth-specific protein could be detected.

TABLE 1. Increased rates of synthesis of individual proteins
during recovery from starvation[a]

Protein	Fold increase in synthesis rate
1	≥ 3
2	≥ 8
3	≥ 10
4	≥ 10
5	≥ 24
6	≥ 7
7	≥ 12
8	≥ 16
9	≥ 12
22	12
23	27
24	10
34	5
41	38

[a] To determine the relative rate of synthesis of individual proteins, dried gels were exposed to a phosphor imager plate along with a calibration strip spotted with samples of [^{35}S]methionine of known values (counts per minute per square centimeter). The exposed phosphor imager plate was scanned with a Fujix BAS 2000 phosphor imager. MacBAS version 2.1 was used to quantitate the intensity of individual protein spots. In each case, the signals were within the linear range of detection as determined from the calibration strips. Because synthesis of the outgrowth-specific proteins was not detected in exponential-phase cells, the fold increase in synthesis rate was calculated as the rate of synthesis at 5 min (for proteins 1 to 7) or 15 min (for proteins 8 and 9) divided by the background. For proteins that continued to be synthesized during exponential growth, the fold increase in synthesis rate was calculated as the rate of synthesis at 5 min after nutrients were added divided by the rate of synthesis during exponential growth.

observation that the other outgrowth-specific proteins are synthesized in the *rpoS* mutant suggests that there are at least two regulatory pathways controlling expression of these proteins. It remains to be determined whether synthesis of these proteins during recovery from starvation is controlled by other global regulators, such as cyclic AMP or ppGpp.

REFERENCES

1. **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.
2. **Ball, C. A., R. Osuna, K. C. Ferguson, and R. C. Johnson.** 1992. Dramatic changes in Fis levels upon nutrient upshift in *Escherichia coli*. J. Bacteriol. **174:**8043–8056.
3. **Connell, N., Z. Han, F. Moreno, and R. Kolter.** 1987. An *E. coli* promoter induced by the cessation of growth. Mol. Microbiol. **1:**195–201.
4. **Damerau, K., and A. C. St. John.** 1993. Role of Clp protease subunits in degradation of carbon starvation proteins in *Escherichia coli*. J. Bacteriol. **175:**53–63.
5. **Fiil, N., and J. D. Friesen.** 1968. Isolation of relaxed mutants of *Escherichia coli*. J. Bacteriol. **95:**729–731.
6. **Flärdh, K., and S. Kjelleberg.** 1994. Glucose upshift of carbon-starved marine *Vibrio* sp. strain S14 causes amino acid starvation and induction of the stringent response. J. Bacteriol. **176:**5897–5903.
7. **Givskov, M., L. Eberl, and S. Molin.** 1994. Responses to nutrient starvation in *Pseudomonas putida* KT2442: two-dimensional electrophoretic analysis of starvation- and stress-induced proteins. J. Bacteriol. **176:**4816–4824.
8. **Givskov, M., L. Eberl, S. Møller, L. K. Poulsen, and S. Molin.** 1994. Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. J. Bacteriol. **176:**7–14.
9. **Hengge-Aronis, R.** 1996. Regulation of gene expression during entry into stationary phase, p. 1497–1512. *In* F. C. Neidhardt, R. I. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
10. **Jacobson, A., and D. Gillespie.** 1968. Metabolic events occurring during recovery from prolonged glucose starvation in *Escherichia coli*. J. Bacteriol. **95:**1030–1039.
11. **Maaløe, O., and N. O. Kjeldgaard.** 1966. Control of macromolecular synthesis. W. A. Benjamin, New York.
12. **Marouga, R., and S. Kjelleberg.** 1996. Synthesis of immediate upshift (Iup) proteins during recovery of marine *Vibrio* sp. strain S14 subjected to long-term carbon starvation. J. Bacteriol. **178:**817–822.
13. **Matin, A.** 1991. The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. Mol. Microbiol. **5:**3–10.
14. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Mosteller, R. D.** 1978. Evidence that glucose starvation-sensitive mutants are altered in the *relB* locus. J. Bacteriol. **133:**1034–1037.
16. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell: a molecular approach. Sinauer Associates, Inc., Sunderland, Mass.
17. **Nyström, T., R. M. Olsson, and S. Kjelleberg.** 1992. Survival, stress resistance and alterations in protein expression in the marine *Vibrio* sp. S14 during starvation for different individual nutrients. Appl. Environ. Microbiol. **58:**55–65.
18. **O'Farrell, P. H.** 1975. High-resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
19. **Siegele, D. A., and L. J. Guynn.** Unpublished results.
20. **Siegele, D. A., and R. Kolter.** 1993. Isolation and characterization of an *Escherichia coli* mutant defective in resuming growth after starvation. Genes Dev. **7:**2629–2640.
21. **Spector, M. P., and J. W. Foster.** 1993. Starvation-stress response (SSR) of *Salmonella typhimurium*, p. 201–224. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
22. **Wachlin, G., and M. Hecker.** 1982. Activation of protein biosynthesis in outgrowing spores of *Bacillus subtilis*. Z. Allg. Mikrobiol. **22:**495–502.