Responses to Nutrient Starvation in *Pseudomonas putida* KT2442: Two-Dimensional Electrophoretic Analysis of Starvation- and Stress-Induced Proteins

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The responses of *Pseudomonas putida* KT2442 to various forms of nutrient starvation and stress conditions were examined by two-dimensional polyacrylamide electrophoresis. Carbon deprivation resulted in a temporal expression of two classes of starvation-induced proteins: one class was transiently expressed during the initial phase of starvation, and the second class was expressed throughout the entire starvation period. Proteins of the second class could be further subdivided into proteins induced specifically under conditions of carbon starvation, proteins also induced by conditions of stress created by elevated temperature and osmolarity, and finally proteins that were also induced by conditions of nitrogen as well as phosphate starvation. Addition of glucose to a carbon-starved culture led to initiation of a recovery phase. During this phase, repression of starvation-induced proteins as well as induction of a new class of transiently expressed proteins, referred to as maturation proteins, took place.

Studies of the physiology of nondifferentiating bacteria exposed to conditions of environmental stresses and nutrient starvation have during the last decade focused on the analysis of important macromolecular parameters and characteristic phenotypes such as cell shape, stress resistance, and starvation survival. To understand the underlying molecular mechanisms, genetic approaches have been employed in combination with detailed analysis of the pattern of protein synthesis. Important tools are insertional inactivation of starvation-induced genes (6, 13-15, 26, 29, 31) and use of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (O’Farrel gel [22]) analysis of synthesized proteins (6, 11, 12, 15, 18, 19, 25).

In 1986 Groat et al. (6) reported on the induction of 30 proteins in *Escherichia coli* in response to carbon starvation. Furthermore, 13 of these proteins also appeared under conditions of nitrogen and phosphate starvation. Spector et al. (25) and Nystrom et al. (19) concluded from similar 2D-PAGE protein investigations of *Salmonella typhimurium* and *Vibrio* sp. strain S14, respectively, that a group of general starvation- and stress-induced proteins are induced by heat and osmotic shock treatments of nonstarving cultures as well as by nutrient starvation. According to the terminology of Matin (15), such proteins constitute a core set of post-exponential-phase proteins. In *E. coli*, induction of most of these proteins during starvation was found to be independent of cyclic AMP (cAMP)-cAMP receptor protein (CRP) regulation. Such proteins were denoted Pex in order to distinguish them from those specific for carbon starvation, induction of which is dependent on cAMP-CRP (15). In *E. coli*, starvation-induced stress resistance and survival is at least partly dependent on a functional *rpoS* gene, which encodes the stationary-phase sigma factor $\sigma^S$ (28), and independent of cAMP-CRP-regulated gene expression (13, 16). Under conditions of nutrient limitation, induction of many Pex proteins is dependent on expression of $\sigma^S$.

Nutrient-deprived *E. coli* cells synthesize and accumulate trehalose and glycogen and form small, almost spherical cells by reductive divisions. These processes are transient and dependent on $\sigma^S$. Trehalose synthesis accounts, at least in part, for the heat resistance and osmoresistance exhibited by starved *E. coli* cells (3, 7-9, 14). $\sigma^S$-dependent as well as $\sigma^S$-independent mechanisms operate in order to ensure optimal starvation survival. In special cases inactivating mutations in starvation-inducible genes have been shown to cause reduced survival during starvation in *E. coli* (6, 29) and in *S. typhimurium* (26, 27). So far, specific roles for proteins involved in starvation survival have been described in only a few cases, e.g., proteases degrading proteins to free amino acids (24); the ribosome modulation factor Rm, which is part of the stationary-phase ribosomal complex (30, 32); and the product of the *upaA* gene, which is thought to modulate the flow of carbon in the central metabolic pathways (20, 21).

In the process of a detailed description of the physiology of growing, starved, and stressed cells of *Pseudomonas putida* KT2442, we present in this paper a 2D-PAGE analysis of the pattern of protein synthesis. We have identified proteins expressed in relation to growth as well as general and specific stress- and starvation-induced proteins. We have also identified proteins that are expressed during the transition from growth to starvation and vice versa. The presented analysis serves two purposes: (i) a comparison with stress and starvation responses in the well-described but more distantly related bacteria *E. coli*, *S. typhimurium*, and *Vibrio* sp. strain S14 and (ii) identification of condition-specific marker proteins which may become important in further studies of physiological states of *P. putida* KT2442 in more natural environments.

**MATERIALS AND METHODS**

Growth, stress, and starvation conditions for bacteria. The strain *P. putida* KT2442 (4) was used in all studies. Medium used for growth was either the minimal salt medium AB (2) or morpholinepropanesulfonic acid (MOPS) (17) supplemented with 0.2% glucose or 0.3% sodium citrate. The growth temperature was 30°C. The cell mass of the culture was measured.
spectrophotometrically as the optical density at 450 nm (OD₄₅₀). Carbon starvation was accomplished after bacteria of a growing culture (OD₄₅₀ = 0.35) were harvested by centrifugation (preheated rotor and tubes) followed by resuspension in preheated AB medium. Samples were taken for 2D-PAGE analysis 30 min, 60 min, 120 min, 240 min, 24 h, 5 days, 10 days, 30 days, and 4 months after the shift to carbon-free medium. Alternatively, starvation was accomplished by exhaustion of the carbon source in AB medium supplemented with either 0.02% glucose or 0.02% citrate. Starvation for nitrogen was obtained by exhaustion of 0.15 mM NH₄Cl in either AB medium or MOPS supplemented with 0.2% glucose. Starvation for phosphate was obtained by exhaustion of 8 μM Na₂HPO₄ in MOPS supplemented with 0.2% glucose. These conditions resulted in starved cultures with OD₄₅₀ of approximately 0.35. Samples for 2D-PAGE analysis were taken 5 days after inoculation. In addition, growing bacteria present at an OD₄₅₀ of 0.35 were harvested by centrifugation and resuspended in 0.9% NaCl in an attempt to simulate a situation of multiple-nutrient starvation. Samples for 2D-PAGE analysis were taken 5 days later. In all cases the starvation temperature was 30°C, and all incubations were performed aerobically. Recovery of starved bacteria was initiated by adding 0.2% glucose to a culture that was carbon starved for 5 days. Samples were taken for 2D-PAGE analysis 10, 30, 60, and 120 min after addition of glucose.

Cultures exposed to sublethal stress conditions were grown in glucose-supplemented AB minimal medium. At an OD₄₅₀ of 0.3, either solid NaCl to 0.5 or 0.9 M was added in order to increase the medium osmolarity, the culture was incubated at 43°C, or 10 μM H₂O₂ was added in order to increase oxidative stress. Heat shock was performed by placing a small volume of a 25°C-growing culture in a flask preheated at 40°C.

2D-PAGE analysis of [³⁵S]methionine-labeled cellular proteins. Two-milliliter cultures (growing as well as starved) with OD₄₅₀ of approximately 0.35 were labeled with 4 μl of [³⁵S]methionine (15 mCi/ml; Amersham S235) for 15 min during growth, for 15 min at high temperature and high medium osmolarity, and for 20 min during starvation (for up to 10 days). A 30-min labeling time was used for cultures starved for 30 days and 4 months. The cultures were chased for 1 min with 1 μg of unlabeled methionine per ml, chloramphenicol was added to 0.5 mg/ml, and the cells were harvested by centrifugation at 0°C (10,000 × g for 5 min).

Samples for 2D-PAGE analysis were prepared in the following way. The harvested cell pellets were lysed in 240 μl of (preheated to 100°C) buffer I (0.3% sodium dodecyl sulfate [SDS], 0.6 M β-mercaptoethanol, 28 mM Tris-HCl, 22 mM Tris-OH) during a 5-min incubation at 100°C. After cooling to 0°C, 24 μl of buffer II (Tris-OH, 24 mM; Tris-HCl, 476 mM; MgCl₂, 50 mM; and Benzonase 320 U/ml [Merck]) was added. The time of incubation on ice was 10 min. Proteins were precipitated by the addition of 1.2 ml of ice-cold acetone (analytical grade), incubated on ice for 20 min, and then centrifuged at 12,000 × g for 10 min. The precipitated proteins were resuspended in 50 μl of a buffer made up from 1/4 volume of buffer I and 3/4 volume of buffer III (urea, 9.9 M; Nonidet P-40, 4%; ampholytes [pH 3 to 10], 2.2%; dithiothreitol, 100 mM).

The Millipore Investigator 2D-PAGE system was used to resolve the proteins. Five-microliter aliquots were loaded on the isoelectric focusing gels. The Millipore ampholytes with a pH range of 3 to 10 were used. Isoelectric focusing was run to equilibrium. SDS-polyacrylamide gels were fixed in 10% ethanol and 10% acetic acid, dried, and autoradiographed on Kodak XAR5 films (3 to 10 days of exposure except for gels of cells starved for 4 months, for which exposure times were prolonged for up to 3 weeks). All settings and conditions were according to the instructions of Millipore.

All starvation and stress procedures, including labeling of whole-cell proteins followed by 2D-PAGE analysis, were performed at least twice.

Challenge protocol. Exposure of cells to lethal conditions was performed in the following way: cultures were diluted 10² and 10³ times in AB medium and then, at time zero, diluted 10 times in AB medium supplemented with either 20% ethanol or 0.18 mM H₂O₂ or 3 M NaCl or diluted 10 times in 48°C-preheated AB medium. The ethanol challenge was performed at 25°C, and peroxide and high-osmolarity challenges were performed at 30°C. Aliquots (0.1-ml) were taken at time zero and 3, 5, 8, 10, 15, 20, 30, and 40 min and spread on Luria-Bertani plates. After incubation overnight at 30°C, the viable counts were determined for every time point to calculate the fraction of surviving cells.

RESULTS

Synthesis of proteins during carbon, nitrogen, and phosphate starvation. A 2D-PAGE system was used for the separation and analysis of [³⁵S]methionine-labeled proteins from P. putida KT2442. The pattern of specific protein synthesis was measured during growth and after harvesting and resuspension of the cells in carbon-free minimal medium. Autoradiograms of the corresponding gels are shown in Fig. 1, and a summary of the induction pattern is presented in Fig. 2. Figure 1 (panels A and B) shows induction of 49 proteins in response to carbon deprivation. Later after the onset of carbon starvation, additional proteins appeared: 5 after 60 min (Fig. 1C), 12 after 120 min (Fig. 1D), and finally 6 more after 240 min (Fig. 1E and F). After 24 h of starvation (not shown), a stable pattern of protein synthesis which was very similar to the one displayed after 5 days of starvation had developed (Fig. 1F). Except for minor differences, the overall pattern of protein synthesis was found to be relatively stable for at least 4 months of starvation (not shown).

This analysis shows that carbon starvation-induced proteins are induced in a temporal manner during the initial 24 h of starvation. There appear to be two classes of induced proteins with respect to the duration of their synthesis: class 1 constitutes transiently expressed proteins, whereas class 2 proteins, once induced, are stably expressed throughout the entire starvation period. Synthesis of 35 of the 41 identified class 1 proteins was turned on during the initial 30 min of the starvation period, but their expression period varied from less than one to several hours (see Fig. 2B for a summary). Synthesis of the remaining six proteins was initiated at later times. Synthesis of all class 1 proteins was terminated within 24 h of starvation.

In growing cells, approximately 450 proteins were visible on the presented autoradiograms and 420 were visible after 5 days of starvation. We observed 19 proteins (denoted E₁ to E₁₉) which were synthesized in glucose-supplemented minimal medium (Fig. 1A) and switched off during carbon starvation (Fig. 1B and D; Fig. 2E). Apart from the described changes in protein synthesis, there is an almost identical pattern of background proteins when conditions of growth and starvation are compared.

A very similar picture of temporal development in starvation-induced protein synthesis was obtained when the cells had been grown in minimal medium with citrate as the carbon source prior to the shift to carbon-free medium (not shown). Minor differences were the following: four additional class 1
FIG. 1. 2D-PAGE analysis of carbon starvation-induced proteins of *P. putida* KT2442. (A) Exponentially growing cells; (B to F) 30 min (B), 60 min (C), 120 min (D), 240 min (E), and 5 days (F) after removal of the carbon source glucose. Boxed spots indicate the transiently induced class 1 proteins, circled spots indicate the proteins induced throughout the starvation period and denoted class 2 proteins, and “E” with an arrowhead indicates glucose- and growth-induced proteins. The positions where class 2 proteins will appear are marked on panel A. In panel B, positions of induced class 1 and 2 proteins are marked and numbered. In panels C, D, and E, only newly induced class 1 and 2 proteins are numbered. In panel F, all class 2 proteins are marked and numbered. Stars indicate proteins expressed also during nitrogen or phosphate starvation. The positions and sizes (in kilodaltons) of molecular markers are indicated to the right in panels A and F.
proteins were detected in response to citrate deprivation; the glucose-conditioned class 1 proteins 4, 13, and 31 were not expressed; the class 2 proteins 25 and 26 as well as E14 and E16 were difficult to detect, indicating that they were expressed at low levels (not shown); and finally, proteins E4, E13, and E18 were not expressed during growth in citrate-supplemented minimal medium.

When cells entered starvation after exhaustion of a limiting amount of carbon (either glucose or citrate), a final pattern of protein synthesis identical to the one presented in Fig. 1F was observed after 5 days of starvation (not shown).

For the analysis of the pattern of protein synthesis developing during phosphate or nitrogen starvation, we decided to label the cells 5 days after inoculation in growth media with limiting concentrations of the respective nutrient (see Materials and Methods). At this time, the cultures were stable with respect to viable counts and cell mass and small stress-resistant cells had been formed.

Compared with growing cells, cells starved for phosphate showed induction of at least 56 proteins (Fig. 3A). Comparison of the autoradiograms revealed that 14 of these seem to be class 2 carbon starvation-induced proteins as well (see Fig. 2D for a summary). Starvation for nitrogen resulted in induction of at least 43 proteins, of which 12 appeared to be class 2 carbon starvation-induced proteins. Proteins which apparently were induced under all three starvation conditions, e.g., proteins 1, 3, 5, 7a, 12, 15, 23, and 29, are referred to as CNP starvation-induced proteins. On the other hand, proteins 2, 6, 8, 11, 13, 18, 19, 21, 22, 24, 25, and 27 are carbon starvation specific, since they were not induced in response to nitrogen or phosphate starvation (see Fig. 2D).

FIG. 2. Summary of the expression patterns of growth-, starvation-, and stress-induced proteins. (A) Left and right ends of the black lines indicate the time points of induction of class 2 proteins during starvation and their repression during maturation, respectively. Numbering of the proteins is shown on the left and right. (B) Numbers of transiently expressed proteins are boxed in order to show the duration of induction during starvation and maturation. (C) E proteins detected in cells present in glucose-supplemented minimal medium; right and left ends of the black lines indicate the time points of repression during starvation and induction during maturation, respectively. (D) Multiple-induction patterns of class 2 proteins (-C, -P, -N, 4 to 5 days of carbon, nitrogen, and phosphate starvation, respectively; NaCl, 5 days of incubation of cells in 0.9% NaCl; H and Osm, 45 min of incubation at an elevated temperature [43°C] and high medium osmolarity [0.5 M NaCl], respectively). (E) Multiple-induction patterns of E proteins (Glu and Cit, the carbon source in the growth medium was either glucose or citrate, respectively).
FIG. 3. 2D-PAGE analysis of proteins induced under conditions of phosphate (A) and nitrogen (B) starvation. Induced proteins are marked with triangles. An "E" with an arrowhead indicates glucose-induced E proteins. Stars indicate polypeptides expressed during nitrogen, phosphate, or carbon starvation.

FIG. 4. 2D-PAGE analysis of cellular proteins 10 min (A) or 60 min (B) after addition of 0.2% glucose to cells carbon starved for 5 days. An "E" with an arrowhead indicates glucose- and growth-induced proteins, an "M" with an arrowhead indicates transiently expressed maturation proteins, circled spots indicate class 2 starvation proteins, and dotted circles indicate class 2 proteins that are switched off.

similar to the one which developed during carbon starvation (not shown). Only a few nitrogen and phosphate starvation-specific proteins were expressed (Fig. 2D). We have not analyzed the transient phase between growth and this particular situation.

Recovery from starvation. Glucose addition to a carbon-starved culture results in a rapid stimulation of the rate of bulk protein synthesis (5). In order to monitor changes in the pattern of protein synthesis taking place in the transient phase between carbon starvation and growth, cells were labeled with [35S]methionine at different times after glucose addition to a culture starved for 5 days. The 2D-PAGE autoradiograms presented in Fig. 4 show that the pattern of protein synthesis changed rapidly following addition of glucose. Expression of class 2 starvation-induced proteins was switched off in a temporal way: the carbon starvation-specific proteins were switched off first, whereas the CNP starvation-induced proteins were switched off at later times (see Fig. 2A for a summary).

After 60 min most of the class 2 starvation-induced proteins were no longer expressed.

Expression of E proteins followed a temporal pattern of induction. Synthesis of proteins responsive to the presence of glucose in the medium (E1 to E6, E8, E9, E11, and E12) was initiated within 10 min after glucose addition. E proteins (except for E7 and E10) expressed in correlation with bacterial growth were induced later than 2 h after glucose addition (see Fig. 2C for a summary).

During the recovery period, a significant increase in cell mass (measured as the OD450) was detected within the first hour of the poststarvation period (not shown). Microscopic inspection showed that initial cell divisions took place after 2 h, and a significant increase in viable counts was observed approximately 3 h after glucose addition.

A class of transiently expressed proteins, in the following
referred to as maturation proteins, was detected during the recovery phase (Fig. 4). Some were expressed immediately after the glucose addition, either very briefly or for about 1 h, whereas others were expressed somewhat later (+30 to +60 min; Fig. 2B). Interestingly, three of these proteins, labeled M₃, M₄, and M₆, were found to exhibit similar migration patterns with the class 1 starvation proteins 22, 41, and 40, respectively.

**Overlap between stress and starvation responses.** As previously described, the starved *P. putida* KT2442 cells develop a general cross-protection to various physical and chemical stresses (5). However, nonstarved cells express enhanced resistance when exposed to a sublethal treatment prior to a challenge with lethal conditions. Incubation at 43°C for 120 min was found to provoke an increased thermotolerance (Fig. 5A). Further incubation did not lead to any significant increase of resistance. In addition, it was found that resistance to H₂O₂ and ethanol was markedly increased, whereas no significant osmoprotection was obtained. Sublethal exposure to increased medium osmolarity (0.9 M NaCl) resulted in a gradual development of osmoprotection and oxidative protection as well as in increased heat and ethanol tolerance (Fig. 5D). Incubation for 24 h in the presence of 0.9 M NaCl resulted neither in a reduction nor in an increase in the viable counts of the culture. In general, we have found that nutrient starvation induced a higher level of protection than sublethal treatments (Fig. 5B and C). In comparison to the exponential-phase growing culture in which cells are extremely sensitive, a culture carbon starved for 1 day will exhibit almost full survival at the selected time points and chosen conditions.

**A 2D-PAGE analysis** showed induction of 31 proteins as a consequence of exposure to high temperature (43°C) for 45 min (Fig. 6A). As judged from their gel migration behavior, three of these could be identical to already identified CNP starvation-induced proteins, four could be identical to carbon starvation-induced proteins, and one could be identical to a specific nitrogen starvation-induced protein (Fig. 2D). No identity between heat-induced proteins and class 1 carbon
starvation-induced proteins could be detected. Incubation at 43°C caused growth arrest for several hours. In accordance with the growth arrest, synthesis of all growth-related E proteins (E9, E10, and E12 to E15) was switched off. In contrast, expression of E4 and E5 was highly induced. However, the synthesis of several of the glucose-responsive E proteins was found to be switched off.

Proteins such as 1, 22, 28, 141, E6, E12, and 158 were induced immediately after a temperature shift from 25 to 40°C (not shown) and are therefore considered to be heat shock proteins. KT2442 grows at 40°C with a reduced growth rate (5). Further incubation at this temperature did not result in induction of additional proteins (not shown).

Analysis of the pattern of protein synthesis following exposure to 0.5 M NaCl showed induction of 33 proteins (Fig. 6B). Two of these are likely to be identical to already identified CNP starvation-induced proteins, three are likely identical to identified carbon starvation-induced proteins, two are likely identical to identified nitrogen starvation-induced proteins, and four are likely identical to proteins induced under conditions of high temperature (Fig. 2D). E proteins E1 to E5, E8, E9, and E11, were expressed at normal levels, but synthesis of E12 was highly induced. All growth-related E proteins were switched off. Addition of 0.5 M NaCl to the growing culture caused a severe reduction in the growth rate.

Sublethal treatment with H2O2 resulted in enhanced oxidative protection, but no cross-protection against heat, osmotic shock, or ethanol was induced (not shown). 2D-PAGE analysis showed induction of proteins E6 and E12 (not shown), which are also induced by high temperature and/or increased medium osmolarity (Fig. 2D). No starvation-induced proteins were induced.

**DISCUSSION**

Upon carbon deprivation, the strain _P. putida_ KT2442 induces the synthesis of about 72 new proteins not detected in growing cells. Like in _E. coli_ K-12 (6) and _Vibrio_ sp. strain S14 (19), carbon starvation-induced proteins are induced in a temporal way. Deprivation of the exogenous carbon source probably activates specialized control elements which in turn ensure a programmed sequential expression of starvation-specific genes. The final result is the development of a pattern of protein synthesis which is stable throughout long periods of starvation. The 2D-PAGE analysis presented here demonstrates that (i) expression of proteins specifically related to bacterial growth, starvation, and environmental stress conditions produces condition-specific patterns of protein synthesis; (ii) Growth and starvation periods are separated by transition phases exhibiting characteristic patterns of transient protein synthesis; and (iii) starvation for a given macronutrient leads to induction of condition-specific proteins as well as to a set of multiply inducible starvation-induced proteins.

Cells present in liquid cultures growing at high rates are relatively large and cylindrically shaped, with several copies of the genome and with high rRNA content, and they exhibit a low tolerance to environmental stress. In contrast, starved cells have changed cell shape (in many cases, small and spherical), one copy of the genome, a reduced rRNA content, and a reduced rate of protein synthesis, and they are stress resistant and capable of long-term starvation survival (5). Between growth and the stable starvation phase there is a transient phase which seems to be important for the development of the starvation-specific physiological state. If protein synthesis is blocked early in the transient phase, the multiresistant state of starved cells will not develop (5, 19, 23). Temporal induction of most of the identified starvation-induced proteins and repression of growth-related proteins occur during the initial 2 h of the transition phase. This indicates that synthesis of starvation-induced proteins is important for development of the characteristic phenotypes of starved cells. For _P. putida_ KT2442 it was found that a substantially longer period than the initial 2 h is needed for the complete differentiation of the cell physiology (5). Jenkins et al. (12) have suggested that starvation-induced proteins and protective molecules must accumulate to certain cellular levels during the transition phase before exerting their maximal effects.

Although most of the presented experiments involving carbon starvation have been performed with glucose as the pres starvation carbon and energy source, it must be emphasized that a nearly identical differentiation pattern in the cells is observed with citrate as the carbon and energy source (reference 5 and data presented here). We therefore consider the protein synthesis pattern summarized in Fig. 2 to be representative of the species-specific response to starvation for carbon and energy sources. This particular starvation response appears to dominate over other responses, since attempted multiple-nutrient starvation (resuspension and incubation of cells in 0.9% NaCl) induces a pattern of protein synthesis very similar to that observed for carbon-starved cells; also, the survival and general physiological of cells in 0.9% NaCl show great resemblance to those of carbon-starved cells. These results suggest that the carbon starvation response is the most effective in securing starvation survival.

The proteins induced by starvation conditions or during recovery from starvation are at present unidentified. However, it is expected that some proteins are regulatory and others are more directly involved in protection from environmental conditions. We also have no information at present about the stability of the induced proteins; if the transiently expressed proteins (class 1) are not degraded, they will persist in the cells throughout long-term starvation since no dilution by growth occurs. We also have no information at present about the significance of the specific proteins induced by the various stresses, information which is sought at the moment through isolation of mutations in starvation-induced genes. Until such molecular information is available, it makes little sense to speculate about the identities and roles of the various proteins; however, a few indications seem worth mentioning in the context of comparative findings in other bacteria.

Short-term- as well as long-term-starved _KT2442_ bacteria exhibit similar degrees of stress resistance (not shown). Besides the accumulation of protective molecules in the transition phase, active protein protective systems may contribute to the overall stress resistance. Such systems (the chaperones DnaK and GroEL) are weakly induced by starvation in _E. coli_ (6) and by carbon starvation in _Vibrio_ sp. strain S14 (10). _P. putida_ KT2442, like _E. coli_, develops stress-resistant cells in response to carbon, nitrogen, phosphate, and sulfate starvation (5). Therefore, possible candidates for proteins at least partly responsible for stress protection would be expected to be found among the CNP starvation-induced proteins (proteins 1, 3, 5, 7a, 12, 15, 23, and 29).

Another characteristic related to the CNP starvation-induced proteins may be derived from the experiment in which glucose was added to a carbon-starved culture (Fig. 4): switching off of these proteins did not occur immediately after glucose addition; instead, repression correlated with the time at which cell mass started to increase. These data suggest that the inducing signal for this set of proteins is growth arrest caused by nutrient limitation and that the signal responsible for switching off of their synthesis is growth initiation. This group
of proteins may be analogous to the Pex proteins described by Matin (15). On the other hand, synthesis of proteins 2, 8, 18, 19, 21, 24, 25, and 27 is specifically induced by carbon starvation (Fig. 2) and immediately switched off upon glucose addition. They may be similar to the class of cAMP-CRP-induced starvation-induced proteins of E. coli which are believed to be nonessential for starvation survival and development of stress resistance (15).

It has been suggested that protective mechanisms operating during nutrient starvation and during adaptation to various other types of stress (heat, osmotic pressure, and oxygenc conditions) share common features. Jenkins et al. (12) identified in E. coli 11 heat shock proteins, of which 3 were carbon starvation-induced proteins and 8 were general starvation-induced proteins. Jenkins et al. (11) analogously found 22 osmotic shock proteins, of which 5 were identified as general starvation-induced proteins. The overlap of starvation-induced proteins with stress-induced proteins in KT2442 (Fig. 2D) may suggest that some of these (proteins 1, 5, 6, 11, 14, 15, 22, and 28) account for increased stress tolerance during adaptation as well as during starvation. In KT2442 no overlap has been observed between proteins induced by H₂O₂ treatment and starvation, but three proteins induced by the adaptive heat and osmotic pressure treatments were also induced by sublethal treatment with H₂O₂ (not shown). This is in agreement with the increased oxidative tolerance observed with cells preadapted to higher temperature and osmotic pressure.

We could not identify any of the class I carbon starvation-induced proteins among the heat-, osmotic pressure, or H₂O₂-induced proteins. This might explain why the stress protection established under the respective adaptation conditions did not develop the same high degree of multiresistance as found after a period of carbon starvation. Likewise, it has been shown in E. coli that neither rpoS expression nor trehalose synthesis occurs during development of adaptive temperature resistance (8).

Addition of glucose to a carbon-starved culture leads to a transient recovery phase preceding formation of reproductive cells. In the initial part of this phase, which is characterized by a rapid increase in the rate of bulk protein synthesis (5), the expression of most of the carbon starvation-specific proteins is switched off. E proteins responsive to the presence of glucose are induced, and induction of a new class of transiently expressed proteins is detected. We have denoted these maturation proteins as have been suggested by Albertson et al. for proteins synthesized exclusively during the recovery or maturation phase of carbon-starved cells. In the following part of the recovery phase, which is characterized by an increase in cell mass, expression of the CNP starvation-induced proteins ceases. Two of the E proteins expressed in relation to growth are induced together with another temporal class of maturation proteins. After 2 h all starvation-induced proteins as well as the maturation proteins are no longer synthesized. This marks the end of the recovery phase. The cells have now entered the phase of growth and division, and all E proteins are expressed. This course of events is very similar to that described for Vibrio sp. strain S14 (18). Induction of some class 1 starvation-induced proteins during recovery indicates that the transient phases separating growth and starvation might share certain features.

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