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Responses to Nutrient Starvation in *Pseudomonas putida* KT2442: Analysis of General Cross-Protection, Cell Shape, and Macromolecular Content

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The physiology of *Pseudomonas putida* KT2442 with respect to growth and carbon starvation was studied. During the transition from growth to nongrowth, the cell shape changes from cylindrical to spheric, a change which is accompanied by reductions in cell size, DNA and ribosome content, and the rate of total protein synthesis. In addition, a pattern of general cross-protection develops, which enables the cells to survive environmental stresses such as high and low temperatures, elevated osmolarity, solvents, and oxidative agents. Cultures are almost fully viable during 1 month of carbon, nitrogen, and multiple-nutrient starvation and are considered to be in an active nondormant state. In contrast, strain KT2442 does not survive well under conditions of sulfate and phosphate starvation.

In nature, most bacteria are challenged by conditions of widely changing nutrient availability as well as by exposure to various forms of physical stress. Bacteria of the *Pseudomonas* spp. are found in soil, fresh water, sediments, and seawater and are known plant and root colonizers. Especially in the rhizosphere, the nutrient-rich conditions surrounding a growing root stimulate bacterial growth (32). However, in bulk soil as well as in marine environments, nutrient supplies are often scarce (21). Many bacteria have developed mechanisms that allow them to survive starvation for essential nutrients and to reinitiate growth when nutrients again become available. Under severely limited growth conditions, the coordinated expression of protective mechanisms enables the bacteria to tolerate exposure to multiple physical stresses. Because of the extensive analysis of the physiology of starving *Escherichia coli* and marine *Vibrio* cells (see references 23 and 28 for reviews), it is known that there are many qualitative similarities in the carbon starvation responses expressed by these bacteria. During the transition phase between growth and nongrowth, the cells change shape from cylindrical to small spheric and they adjust their metabolic rate to a lower level. The content of ribosomes declines (5); however, it was recently shown that in *Vibrio* sp. strain S14, ribosomes exist in great excess compared with the rate of protein synthesis (8). A general cross-protection against high temperature, oxidative agents such as H_2O_2, high osmolarity, and solvents such as acetone and ethanol develops (see references 10 and 20 for reviews). Although growing cells preadapted by a sublethal treatment can show increased resistance, starved cells in general exhibit a greater resistance than their growing counterparts (12, 13).

It is generally believed that the capability of cells to survive starvation and to tolerate treatments that are lethal during growth is based on the synthesis of a set of proteins commonly referred to as starvation and stress proteins (20). However, there are also differences among the species in the response to starvation for nutrients other than carbon. In contrast to *E. coli* and *Pseudomonas putida* (shown here), the marine *Vibrio* sp. strain S14 does not develop starvation- and stress-resistant small cells in response to nitrogen and phosphorus starvation (11).

In this communication, we report on the *P. putida* nutrient starvation response, especially focusing on carbon starvation, with respect to cell shape, macromolecular content, protein synthesis, and the development of cross-protection against stress.

MATERIALS AND METHODS

Growth and starvation of bacteria. *P. putida* KT2442 (7) was used in all studies. The medium used for growth was either the minimal salt AB medium (4), MOPS (morpholinepropanesulfonic acid) (22) supplemented with 0.4% glucose (wt/vol) or 0.2% citrate (wt/vol), or the rich Luria-Bertani medium (2). The growth temperature was 30°C. Growth was measured spectrophotometrically as optical density at 450 nm (OD_{450}). Carbon starvation was either accomplished after harvesting bacteria of a growing culture (OD_{450} = 0.4) by centrifugation (preheated rotor and tubes) followed by resuspension in preheated AB medium or was established by exhaustion of the carbon source in AB medium supplemented with 0.02% glucose. Starvation for nitrogen was performed by exhaustion of 0.15 mM NH_4Cl in either AB medium or MOPS with 0.4% glucose, starvation for sulfate was performed by exhaustion of 4 μM Na_2SO_4 in AB medium supplemented with 0.4% glucose, and starvation for phosphate was performed by exhaustion of 10 μM Na_2PO_4 in MOPS supplemented with 0.4% glucose. Multiple-nutrient starvation was achieved by resuspending harvested bacteria in 0.9% NaCl. In all cases, the starvation temperature was 30°C. Survival of the starved cultures was monitored by determination of viable counts by plating of 0.1-ml samples of different dilutions on Luria-Bertani plates. Each starvation condition was repeated at least twice with two or three cultures each time.

Flow cytometry. Culture samples of 0.6 ml each were taken, and the cells were harvested by centrifugation and resuspended in 0.1 ml of 10 mM Tris (pH 7.4). One milliliter of 77% ethanol was added, and the cells were fixed overnight. A total of 0.2 ml of the fixed cells was harvested by centrifugation,

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washed with 10 mM Tris (pH 7.4)–10 mM $MgCl_2$, and finally resuspended in 0.1 ml of this buffer. DNA staining solution (0.1 ml [180 μg of mithramycin and 40 μg of ethidium bromide per ml in 10 mM Tris (pH 7.4)–10 mM $MgCl_2$]) was added, and the samples were incubated on ice for at least 2 h prior to flow cytometry.

Forward light scatter, which is a measure of the cell size, and DNA fluorescence of a given cell were measured simultaneously on an Argus fluorescence-based flow cytometer (Skatron, Tranby, Norway). DNA-specific fluorescence was recorded with an Argus B1 filter block, and system performance was monitored with 2-μm-diameter latex spheres. For each sample, 20,000 cells, at a flow rate of 400 to 500 counts/μl, were analyzed. The analysis was accumulated as three-dimensional (DNA, light scatter, cell number) histograms of the culture samples. Analysis of the flow cytometric histograms and transformation into two-dimensional presentations (see Fig. 4A) was performed with the Windows-based software Winflow, developed by F. G. Hansen.

Measurement of protein synthesis. The rate of total protein synthesis of growing as well as carbon-starved cultures was assessed from incorporation of [^{14}C]leucine into trichloroacetic acid-precipitated material. A volume of the bacterial culture was taken and added to 1/10 of a volume of [^{14}C]leucine (diluted to 5 μCi/ml [5.4 Ci/mmol]). Samples (200 μl each) in duplicates were removed at different times after addition of the isotope, and precipitation of macromolecules was performed overnight on ice in 5% trichloroacetic acid supplemented with 1 μg of cold leucine per ml. The precipitates were collected on membrane filters, washed with 5% trichloroacetic acid supplemented with 1 μg of cold leucine per ml, washed with ethanol, and dried. Five milliliters of scintillation liquid was added prior to counting in a scintillation counter.

Ribosome content. The content of ribosomes per cell was estimated by in situ rRNA hybridization (6, 25). Cells were fixed and hybridized as described previously (25). Because of the diminutive cell size of the starved cells and the dim hybridization signals, the cells were counterstained with 4′,6-diamidino-2-phenylindole. This was done by adding 30 μl of 4′,6-diamidino-2-phenylindole (0.3 μg/ml) to each hybridization well subsequent to the hybridization. The cells were stained for 15 min and washed twice in water (15 min each time). A general probe for the bacterial domain, EUB338, was used (31). The probe was labelled directly with rhodamine (Peninsula Laboratories, Belmont, Calif.) during the synthesis of the oligonucleotide.

For video microscopy a Carl Zeiss Axioplan microscope equipped for epifluorescence and phase-contrast microscopy was used. The filter sets 15 and 01 were used to visualize rhodamine and 4′,6-diamidino-2-phenylindole, respectively. For the micrographs, Kodak Ektachrome 400 asa films were used. The charge-coupled device camera (CH250; Photometrics, Tucson, Ariz.) was equipped with a KAF1400 chip cooled to −40°C. Images were captured as 12-bit files with PMIS software (Photometrics) and subsequently were transferred to a Macintosh Quadra 950 computer for image analysis. From each digitalized image, 20 to 30 cells were analyzed. The light intensity from single cells was quantified by dividing the integrated pixel intensity by the cell area, which was defined by circumscribing the cells with a pointing device. This value was considered to reflect the ribosomal content per cell. Image analysis was performed with the software NIH Image (which can be obtained free of charge at the anonymous ftp address zippy.nimh.nih.gov).

Challenge protocol. Growing or starved cultures with a density ranging from 3×10^7 to 8×10^7 cells per ml were diluted 10^2 and 10^3 times in AB medium and then, at time zero, were diluted 10 times in AB medium supplemented with either 20% ethanol, 176 μM H_2O_2, or 3 M NaCl or were diluted at 47°C in preheated AB medium. The ethanol challenge was performed at 25°C, and the peroxide and high-osmolarity challenges were performed at 30°C. Aliquots (0.1 ml each) were spread on Luria-Bertani plates and incubated overnight at 30°C, and the next day the viable counts were determined from the number of CFU. Each challenge experiment was performed at least twice.

When experiments with cultures incubated at 0°C were performed, care was taken to avoid initial heat shock of the cells. Therefore, all cultures incubated at 0°C were diluted in cold medium and allowed to slowly reach room temperature prior to determination of viable counts or exposure to the different challenge conditions.

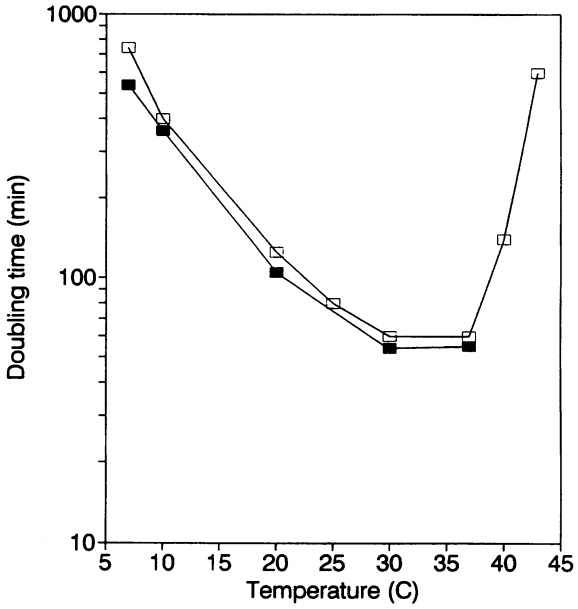

FIG. 1. Doubling times of *P. putida* KT2442 as a function of temperature when grown in AB minimal medium supplemented with either 0.4% glucose (□) or 0.2% citrate (■).

RESULTS

Effect of growth and starvation on cell shape and survival. The generation times of *P. putida* at 30°C in minimal medium with glucose or citrate as the carbon source are 62 and 55 min, respectively (Fig. 1). Slow growth was detected at 43°C, but at 45°C growth was terminated. Cells were found to survive several hours at 45°C before viability started to decline. At the other extreme, we could not detect any growth at 4°C. Microscopic observations revealed that the cells are long and cylindrical during exponential growth (Fig. 2A). This was found to be the case in minimal medium with glucose or citrate as the carbon source as well as in Luria-Bertani medium. However, when the cells were shifted to carbon-free medium by centrifugation or when starvation was initiated by exhaustion of the carbon source, the cells gradually changed their shape to round or coccoid, eventually ending up as very small cells (Fig. 2). Throughout long-term starvation (90 days), the cells maintained this shape. A period of clumping and sticking to surfaces was observed to last from 3 to 24 h after removal of the carbon source (Fig. 2D). The small round cells were also formed in

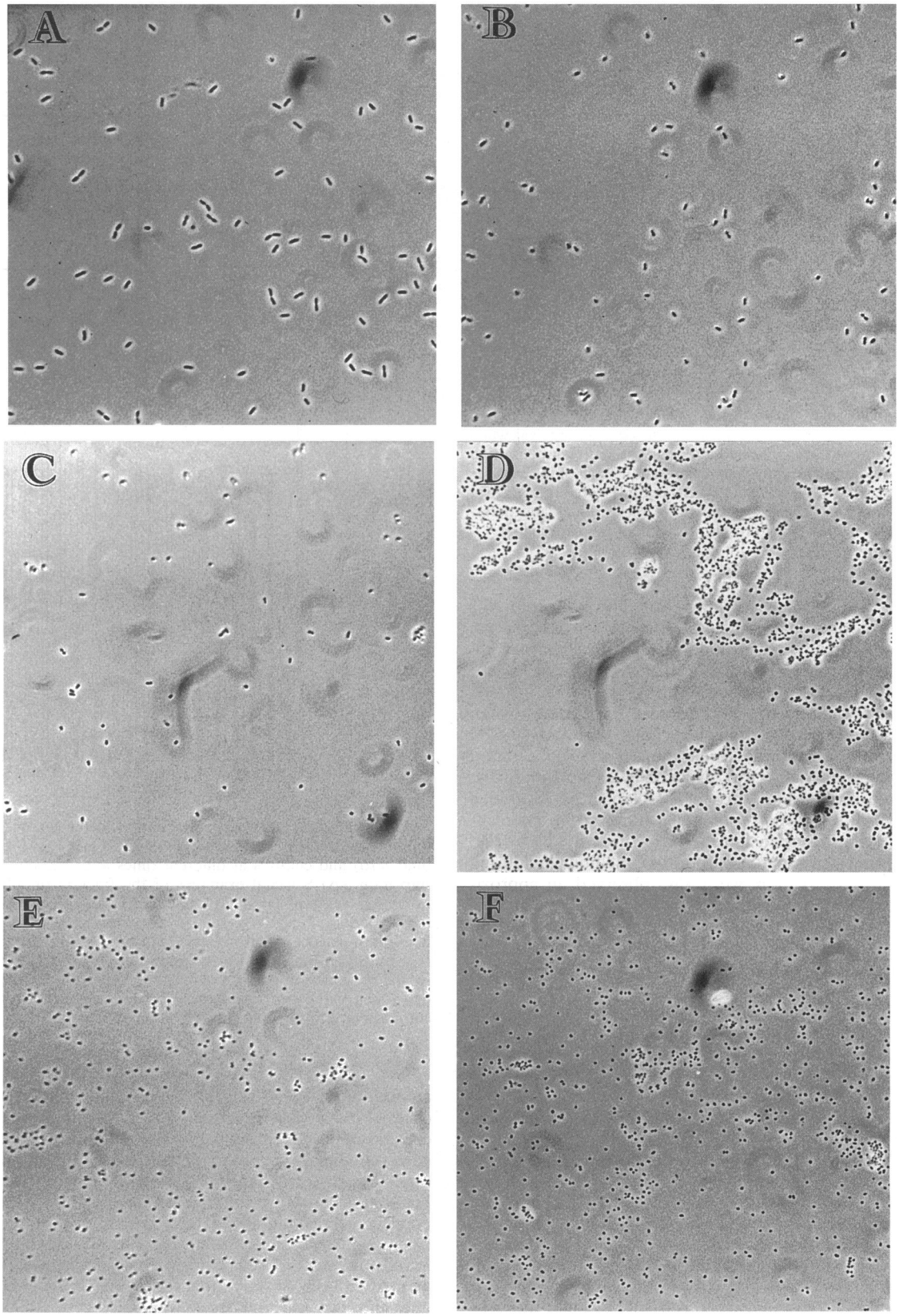

FIG. 2. Microscopic observations of the change in cell shape before and after a shift to a glucose-free medium. (A) Growing cells. (B to F) Cells after 1 h (B), 2 h (C), 5.5 h (D), 28 h (E), and 28 days (F) of starvation.

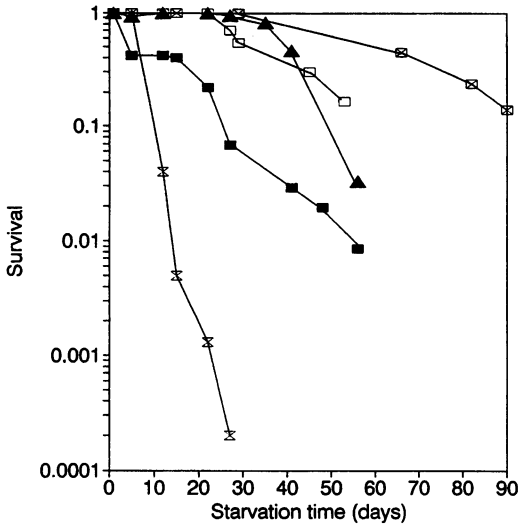

FIG. 3. Survival of *P. putida* KT2442 exhausted for either carbon (⊠), nitrogen (▲), phosphate (▽), or sulfate (■). □, survival when carbon starvation was accomplished after harvest and resuspension in glucose-free medium. The viable counts present in each of the cultures starved for 1 day (1×10^8 to 2×10^8 cells per ml) were arbitrarily set to 1. Survival was expressed as fractions of these values and determined from the viable counts present throughout the experiment.

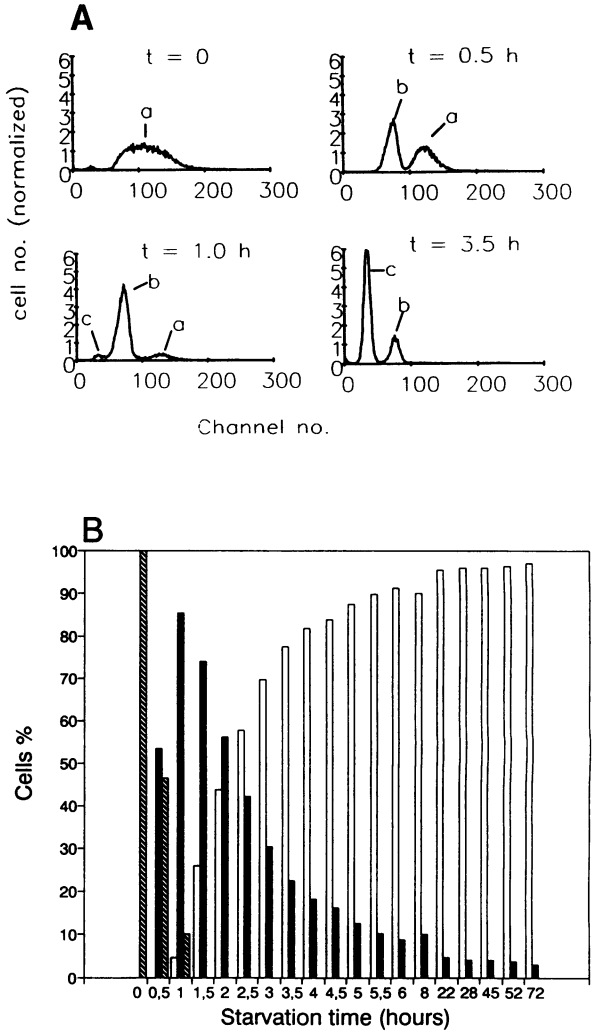

FIG. 4. Flow cytometric determination of the relative DNA content and its distribution in cells obtained from a growing culture (at time zero) and cultures at different times after the onset of carbon starvation. (A) Selected examples of the fraction of cells (cell no.) exhibiting a given amount of recorded DNA-specific fluorescence (channel no.). Peaks c, b, and a are considered to represent the fraction of cells carrying one, two, and three or more chromosomes, respectively. t, time. (B) Bar diagram developed on the basis of the results presented in panel A showing the fraction of cells containing one (open bars), two (solid bars), or three or more (hatched bars) chromosomes throughout the starvation experiment.

response to multiple-nutrient starvation and starvation for either nitrogen, phosphate, or sulfate (not shown). Starvation for sulfate, nitrogen, or phosphate resulted in cells that appeared under the microscope to be highly motile for several days after the onset of starvation (not shown).

The strain was found to be fully viable for 1 month when starved for either glucose or nitrogen (Fig. 3) or when starved for all nutrients (not shown). In addition, it was found that a culture in which starvation was achieved by exhaustion of the carbon source exhibited full survival for a longer period than a culture in which the carbon source was removed by a centrifugation following resuspension in glucose-free medium (Fig. 3). After shifting a culture to a carbon-free medium and during the first 24 h of starvation, we always observed an approximately 10-fold increase in the viable counts of the culture. During this period, the OD of the culture decreased to approximately 80% of the value prior to the shift, indicating that the increase in viable counts reflects the residual cell divisions yielding the minicells.

When either sulfate or phosphate was missing, a reduction in the efficiency of starvation survival was observed (Fig. 3). Ten days after exhaustion of phosphate, the viable counts of the culture started to drop, although the starved cultures had the same OD throughout the experiment.

Effect of growth and carbon starvation on DNA content. The relative DNA content per cell was measured by flow cytometry (Fig. 4A). During exponential growth, the recorded DNA-specific fluorescence showed a broad distribution of genomic content. However, after a shift to glucose-free medium, the fluorescence pattern rapidly changed. Thirty minutes after the onset of starvation, distinct peaks were formed. A rifampin-treated growing *E. coli* K-12 culture produced a distinct peak centered at channel 67.5, which is known to correspond to two fully replicated chromosomes per cell (25a). Since the estimated size of the *Pseudomonas* chromosome exceeds the *E. coli* equivalent by approximately 20% (17) and since the amount of fluorescence is considered to be proportional to the amount of DNA per cell (3), the distinct peak (b) centered at channel 78.5 probably represents a cell population carrying two chromosomes. According to the same consideration, a peak corresponding to cells carrying only one chromosome would be expected close to channel 39. After 1 h of starvation, a cell population carrying what may correspond to one chromosomal equivalent was initially detected (i.e., peak c, centered at channel 42). During the following 4 h, 90% of the culture was found to consist of cells carrying only one chromosome (Fig. 4B). After 1 day of starvation, the distribution of cells carrying one and two chromosomes was stabilized at 95 and 5%, respectively. This distribution was found to be stable throughout a 5-month starvation period (not shown). During the first day of starvation, the light-scattering measurements of the cell

samples indicated an approximately threefold reduction in cell size and it was found that during prolonged starvation a further reduction to one-fifth of the original size was recorded (not shown).

Effect of growth and carbon starvation on protein synthesis and ribosome content. The rate of total protein synthesis, measured by pulse-labelling with radioactive leucine, was reduced during carbon starvation. Exponentially growing cells present at an OD_{450} of 0.4 incorporated the isotope linearly at a rate of approximately 2,700 cpm/min during the 15 min of the labelling experiment (not shown). After harvesting of the culture by centrifugation and resuspension in a glucose-free medium, several pulse-labelling experiments were performed throughout the starvation period. After 6 days of starvation, the incorporation kinetics became slightly curved during the initial 10 min of the labelling experiment (not shown). Assessing the rate of protein synthesis of the starved culture from a single 15-min pulse would therefore lead to an approximately 10% underestimate of the actual rate. We found this minor difference was acceptable, and 15-min labelling times were chosen in order to investigate how rapidly the rate of protein synthesis changed during the first few hours as well as during long-term carbon starvation. As can be seen in Fig. 5A, the rate of protein synthesis declined rapidly during the initial 40 min of starvation, reaching a plateau of one-third of that of the growing culture. During the first day of starvation, the synthesis rate stayed at this level, while the number of cells in the culture reached its maximum value (Fig. 5B). Between 3 and 17 days after the onset of starvation, the protein synthesis rate of the culture was further reduced without a reduction in viable counts, and after 20 days a further reduction in protein synthesis seemed to follow a reduction in viable counts (Fig. 5B).

The ribosome content per cell was measured by intracellular 16S rRNA hybridization of fixed cells as described in Materials and Methods. Upon a shift to a carbon-free medium, the content of ribosomes per cell was found to be constant for 70 min, after which it declined to approximately 50% of the content in growing cells (Fig. 5A). During prolonged starvation, the content of ribosomes slowly declined further, reaching 22% after 30 days of starvation (Fig. 5B) and 15% after 2 months of starvation (not shown).

We also monitored the kinetics of total protein synthesis in carbon-starved cells following addition of glucose (Fig. 6). In a culture starved for 7 days, the rate of protein synthesis increased fivefold within 10 min after the addition of glucose, whereas prolonged starvation seemed to create a lag phase before the rate of protein synthesis started to increase. In the case of the culture starved for 7 days, the OD started to increase approximately 45 min after the addition of glucose, which might indicate a gain in cell mass. In the case of the culture starved for 17 days, the OD started to increase approximately 2 h after the addition of glucose. With the culture starved for 90 days, only a modest increase in density was recorded during the experiment. No increase in viable counts of the three different cultures was detected during the time of the experiment.

Development of a general cross-protection to stress in response to nutrient starvation. A remarkable feature of *E. coli*, *Salmonella typhimurium*, and *Vibrio* sp. is the development of a stress-resistant state when the organism is starved for nutrients. It was therefore obvious that we needed to test whether *P. putida* KT2442 developed a similar stress protection. Growing cells, cells in the transition phase, and carbon-starved cells were challenged with ethanol, H_2O_2, heat shock, and high medium osmolarity. The time-dependent sensitivity

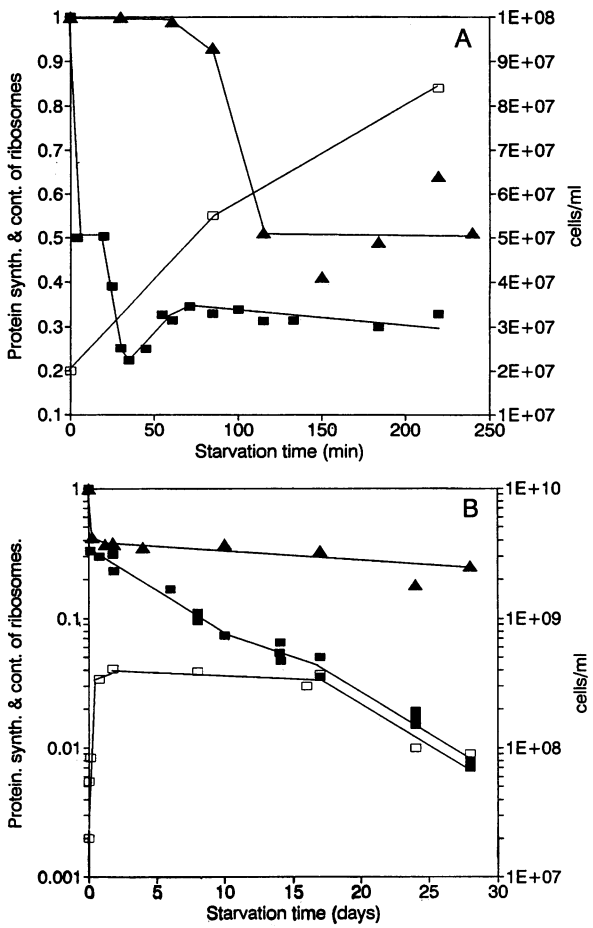

FIG. 5. Protein synthesis (synth.), ribosome content (cont.), and viable counts during growth and starvation. (A) Relative rate of protein synthesis (■), relative ribosome content (▲), and determination of the viable counts (□) during growth and the onset of carbon starvation. The rate of synthesis per 0.2-ml culture was determined after a 15-min labelling period with [^{14}C]leucine. The rate of protein synthesis, as well as the mean light intensity (in situ rRNA hybridization), of a growing culture immediately before the cells were harvested and resuspended in glucose-free medium was arbitrarily set to 1. Standard deviations of the ribosome measurements varied between 10 and 15%. In the scale for viable counts, 1E+07 = 1×10^7, 2E+07 = 2×10^7, etc. (B) Same as panel A but during prolonged starvation.

to the different treatments was determined from the reduction in viable counts of the challenged cultures. Conditions (i.e., concentration, time, and temperature) were chosen such that a rapid decline in the survival of a growing culture was obtained. As demonstrated in Fig. 7, a shift from exponential growth in glucose-supplemented medium to a medium without any carbon source gradually, during the first day of starvation, induced a high degree of resistance to ethanol. Resistance to heat shock and elevated medium osmolarity developed in a similar way (not shown). Full H_2O_2 resistance developed rapidly during the first 90 min of starvation (not shown). A marked increase in tolerance to freezing at −20°C also developed during starvation. Exposure of a growing culture to freezing (−20°C for 24 h) resulted, upon thawing, in a 100-fold reduction in viable counts. In contrast, a culture starved of carbon for 5 days showed a fivefold reduction in the viable counts (not shown).

Development of cross-protection from heat, H_2O_2, ethanol, and high osmolarity was observed following exhaustion of

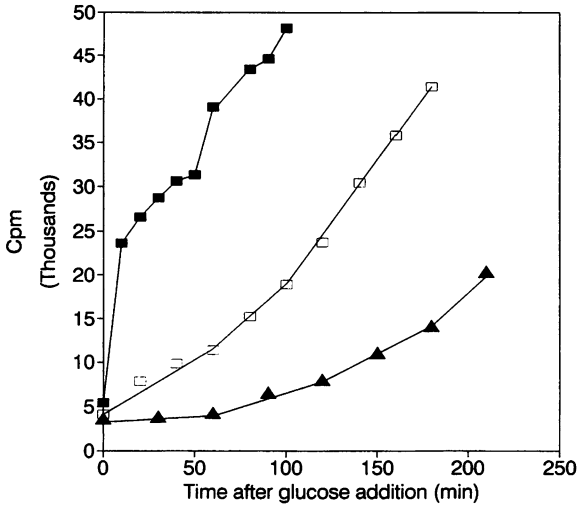

FIG. 6. Reactivation of total protein synthesis in carbon-starved cultures following the addition of 0.2% glucose. Glucose was added at time zero. Culture samples (0.2 ml each) were taken at the time points indicated and labelled with [^{14}C]leucine for 15 min. Results for cultures starved for 7 (■), 17 (□), and 90 (▲) days are shown.

citrate (carbon source) and other nutrients such as nitrogen, phosphate, and sulfate (not shown).

Storage under cold conditions affects the viability of the *P. putida* strain used in this study. An experiment was performed in which aliquots obtained from a carbon-starved culture incubated at 30°C were removed and incubated at 0°C. As a control, a culture of growing cells present in glucose-containing medium was incubated at 0°C. As seen in Fig. 8, viable counts gradually decreased over the 0°C incubation time. However, the longer the prestarvation time at 30°C was, the better the long-term survival at 0°C was. A culture prestarved for 10 days at 30°C exhibited full survival. The culture cooled

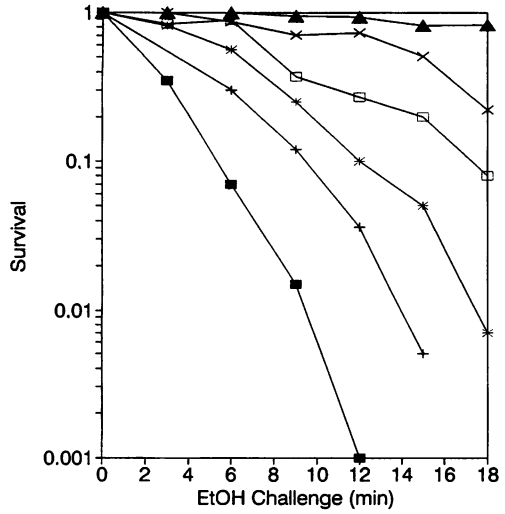

FIG. 7. Challenge of growing and starved cultures with 18% ethanol (EtOH). ■, growing cells. Other symbols represent cells starved for 30 min (+) or 2 (*), 4 (□), 6 (×), or 48 (▲) h. Viable counts present in each of the zero samples (no treatment) were arbitrarily set to 1. Survival was expressed as fractions of these values and was determined from the viable counts present throughout the challenge experiment.

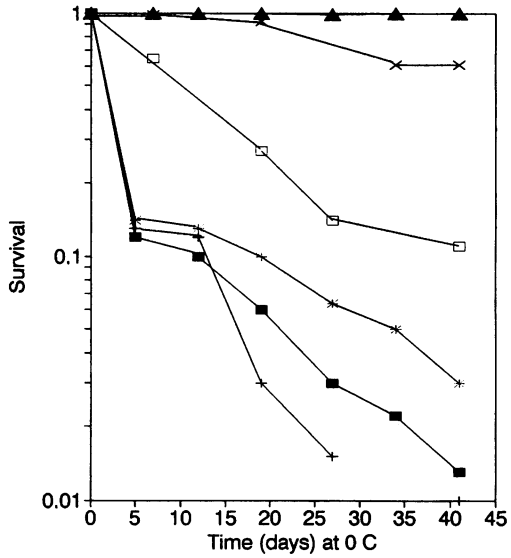

FIG. 8. Survival of cells growing at 30°C and different cultures prestarved at 30°C when exposed to low temperature (0°C). ■, growing cells. Other symbols represent cells prestarved for 10 min (+), 30 min (*), 2 h (□), 1 day (×), and 10 days (▲). The viable counts present in each of the cultures before cooling were arbitrarily set to 1. Survival was expressed as fractions of these values and was determined from the viable counts present throughout the experiment.

in glucose-containing medium also lost viability. In addition, the cultures incubated at 0°C only developed resistance to high osmolarity, heat, H_2O_2, or ethanol if they had been prestarved at 30°C (not shown). Microscopic inspection revealed that the cells did not change shape during the 1½ months of 0°C incubation (not shown). Cells from the cultures prestarved for 24 h and 10 days kept their round shape throughout the time of the experiment and expressed cross-protection against stresses throughout the 0°C incubation period as well (not shown).

DISCUSSION

The *P. putida* strain (KT2442) used in this study is capable of growth within a wide range of temperatures. In addition, it grows almost equally well with glucose or citrate as the carbon source. A common characteristic of the three different *Pseudomonas* isolates so far tested in our laboratory is their capacity to cope with long-term carbon or multiple-nutrient starvation. Whereas a cultured *E. coli* K-12 strain (34), wild-type *E. coli* isolate BJ4 (unpublished results), and an *S. typhimurium* strain (29) lose viability in the range of 1 to 2 orders of magnitude within the first week of starvation, *P. putida* KT2442 was fully viable for 20 days of carbon or multiple-nutrient starvation and was fully viable for even longer if the carbon source was gradually exhausted. After 6 months of carbon starvation, the viability of the culture had dropped by 2 orders of magnitude (not shown). Survival was somewhat reduced under conditions of nitrogen starvation. Under conditions of sulfate and, in particular, phosphate starvation, survival is severely reduced. Cultures of marine *Vibrio* sp. strain S14 are fully viable for 2 weeks in unsupplemented artificial seawater (24) but lose 2 orders of magnitude in viability within 4 days when exposed to nitrogen or phosphate starvation (11). However, simultaneous starvation for carbon and other nutrients seems to enable *Pseudomonas* spp. and *Vibrio* sp. strain S14 to survive deple-

tion of phosphate. Also, *E. coli* has been reported to be more sensitive to phosphate starvation than to carbon or nitrogen starvation (5).

Several macromolecular parameters changed in response to carbon starvation. DNA replication initiation is likely prevented immediately after the onset of starvation. The formation of cells with two chromosomes probably resulted from the running out of DNA replication and cell division, and continuous cell division resulted in the final situation of one chromosome per cell. The rate of total protein synthesis of the culture dropped quickly, cell mass stopped increasing, and the number of cells simultaneously increased 10-fold, reflecting formation of smaller cells by reductive division of the large cells present at the onset of starvation. Both flow cytometric measurements of cell size and microscopic inspections demonstrated this. The cell shape changed from cylindrical to coccoid. This change was easily detectable after 1 h of starvation, and after 5 h all cells in the culture were small. Both *E. coli* K-12 and *Vibrio* sp. strain S14 are known to change cell shape in response to carbon starvation (14, 18, 19). In *E. coli*, the *bolA* gene is responsible for this change and is known to belong to the σ^s regulon. The cultured *Pseudomonas* cells seemed to change shape in a regulated fashion, and like starved *Vibrio* sp. strain S14 cells (15), the newly formed small *Pseudomonas* cells showed increased adhesion properties, resulting in the formation of clumps in the liquid culture.

The rate of total protein synthesis was immediately downregulated in response to the sudden exposure of log-phase cells to the carbon-free medium, reflecting overall reduced translation activity because of energy depletion. A large number of intact ribosomes were still present during the initial 90 min of starvation, as judged from the 16S rRNA hybridization experiments. 16S rRNA liberated from disintegrating ribosomes would be expected to be rapidly degraded, as has been shown to be the case in *E. coli* (5). During the following 17 days of starvation, the rate of protein synthesis was further reduced to approximately 5% of the initial value, whereas the ribosome content slowly declined from 50% to approximately 30%, indicating that rRNA and ribosomes apparently exist in excess of the actual rate of protein synthesis. A similar slow degradation rate of ribosomes was recently reported for *Vibrio* sp. strain S14 (8). The existence of an excess protein-synthesizing capacity in glucose-limited *E. coli* cells was demonstrated as early as 1971 by upshift experiments performed with chemostat cultures (16). The presence of intact ribosomes in starved cells enables a rapid shift from low- to high-level translation activity when metabolizable carbon becomes available, which is reflected by the almost instant reactivation of protein synthesis obtained in a culture starved for 7 days. A similar result was obtained with *Vibrio* sp. strain S14 starved for 200 h (1). During long-term starvation, the low level of protein synthesis, together with the slower reactivation kinetics, may reflect the presence of fewer active ribosomes per culturable cell. We propose that an increasing fraction of the intact ribosomes are being converted to an inactive form, whereas a decreasing active fraction of ribosomes maintain a necessary basal level of translation. It has been proposed that inactive ribosomes are protected from degradation when they are converted into a 100S dimer form present in *E. coli* during starvation (33).

Nutrient starvation resulted in development of a more resistant state that protected the starving nongrowing cells from otherwise lethal exposures to high osmolarity, high and low temperatures, freezing, oxidative stress, and solvents such as ethanol. This has been observed for *E. coli* (12, 13, 20) and *S. typhimurium* (30), whereas *Vibrio* sp. strain S14 develops a stress-resistant state only in response to carbon or multiple-nutrient starvation (11). In contrast to starved *E. coli* K-12 strains, which develop heat resistance up to 57°C (13), the *P. putida* strain only tolerates a temperature elevation of a few degrees above the maximum growth temperature. Exposure of starved cells to 50°C resulted in rapid loss of viability (not shown). Compared with *E. coli*, the full resistance level of strain KT2442 to H_2O_2 was at least 2 orders of magnitude lower (13). On the other hand, resistance to high osmolarity (2.7 M NaCl) was in the same range as that in *E. coli* (12). Prolonged incubation at a temperature in which no growth could be detected (e.g., 0°C) caused loss in viability of log-phase cells and cells in an early stage of carbon starvation. Low temperature apparently constituted a stress condition that the strain was able to survive when prestarved at a higher temperature. Similar observations have been reported for *E. coli* (27). Since no incorporation of labelled leucine could be detected at 0°C (not shown), it is concluded that starvation-induced protein synthesis taking place at the early stage is necessary for the cells to develop the general stress-resistant state and to change shape. The necessity of de novo protein synthesis for starvation survival was initially proposed by Reeve et al. (26). Subsequently, it was shown that *E. coli* and *Vibrio* sp. strain S14 cells show a precise temporal pattern of synthesis of starvation proteins (9, 24). Similar investigations with *P. putida* KT2442 are in progress.

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