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Toluene Elicits a Carbon Starvation Response in *Pseudomonas putida* mt-2 Containing the TOL Plasmid pWW0

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Pseudomonas putida **mt-2(pWWO) exhibited a carbon starvation response in the presence of toluene, a utilizable carbon source. When growth-supporting (4-mg/liter), inhibitory (130-mg/liter), and lethal (267-mg/liter) levels of toluene were provided as the sole carbon source, *P. putida* responded by rapidly inhibiting protein synthesis and by producing 26 new proteins, 22 of which overlapped with those induced by carbon starvation. *P. putida* produced the same proteins when cultures were starved by depleting their carbon source or were downshifted into a carbon-free medium. Carbon supplementation of toluene-exposed cells suppressed the production of the toluene-induced proteins. The level of toluene provided as the sole carbon source influenced the length of time that this response was observed. Following 1.5 to 3 h in a basal salts medium with 4 mg of toluene per liter, protein synthesis increased, the production of the majority of the toluene-induced proteins ceased, and the cells began to grow. In cells provided with 130 mg of toluene per liter, protein synthesis remained inhibited over a 6.5-h experimental period. At this concentration, the production of 15 toluene-induced proteins was prolonged, with nine still detectable in the profiles at 6.5 h. In cells provided with 267 mg of toluene per liter, there was a rapid loss of viability and the toluene-induced proteins were detected prior to death. In cells provided with 4 mg of toluene per liter, the carbon starvation response is transient and likely reflects a period of induction and/or adaptation prior to growth on toluene. At the toluene concentrations which inhibit growth, *P. putida* exhibits a prolonged starvation response despite the presence of an excess of a utilizable carbon source.**

Toluene is widely used as an industrial solvent and in the manufacture of a variety of organic compounds. It is also a component of gasoline. As a consequence of its widespread usage, it has become a ubiquitous environmental pollutant. Microorganisms capable of degrading toluene play a major role in the bioremediation of these contaminated sites. The toxicity of this aromatic hydrocarbon to cells is well documented. Exposure of cells to toluene results in the permeabilization of membranes (6, 18), the leakage of RNA and protein, disaggregation of ribosomes, and inhibition of protein synthesis (18). Its partitioning into cell membranes results in an expansion of the membrane with concomitant loss of membrane function (35). Despite the toxicity of toluene, organisms such as *Pseudomonas putida* mt-2(pWW0) are capable of utilizing it as a sole source of carbon and energy. The TOL plasmid-encoded degradative pathway of this organism is well characterized (5, 10, 41–43). However, the stress response of this organism to the toxicity of toluene has not previously been studied.

Microorganisms respond to a wide variety of environmental stresses by inducing specific stress proteins. The most extensively studied stress response systems include heat shock (27), starvation stress (14), oxidative stress (25), and the SOS response (39). Stress proteins are also produced by microorganisms in response to chemical exposure. Several studies have demonstrated that microorganisms respond to a variety of chemicals by synthesizing unique proteins as well as proteins which overlap with those induced by other stresses (1, 4, 11, 30, 36). At least half of the stress proteins produced by *Escherichia coli* in response to nine different pollutants were unique to each individual chemical, while the remainder overlapped with those induced by carbon starvation or heat shock (4). The unique chemically induced proteins may be useful as toxicity markers because they are synthesized at chemical concentrations that do not inhibit growth (4, 30). In previous chemical stress studies, the organisms were exposed to chemicals that they could not use as carbon and energy sources. Elucidation of the stress responses of microorganisms which can utilize toxic growth substrates such as toluene may provide insight into how microorganisms survive these environmental challenges.

We have studied the stress response of *P. putida* mt-2(pWW0) to toluene when this chemical was provided as the sole carbon source. The goals of this project were to determine (i) if toluene elicits a stress response in *P. putida* mt-2, (ii) whether the stress response of *P. putida* mt-2 is similar in the presence of growth-supporting, inhibitory, and lethal toluene concentrations, and (iii) whether the stress proteins produced are unique to toluene exposure or overlap with those induced by other stresses. This study differs from previous chemical stress studies in that the carbon and energy source of this organism also serves as the chemical stressor.

MATERIALS AND METHODS

Culture conditions. *P. putida* mt-2 ATCC 33015 containing plasmid pWW0 was cultured in a basal salts medium (BSM) (3, 26) supplemented with different carbon sources. To partially induce the TOL catabolic genes prior to any experiment involving toluene, cells were cultured in BSM supplemented with 5 mM *m*-toluate. When toluene was provided as a carbon source, BSM was equilibrated with toluene vapor based on a method described by Evans et al. (7). The toluene (J. T. Baker Chemical Co.) concentration was adjusted by mixing it in various proportions with hexadecane (Fisher Scientific Co.), a hydrocarbon with negligible volatility at 30°C (7). Each toluene mixture (5 ml) was placed into the side arm of a 250-ml biometer flask (32). Preliminary growth studies were performed to determine the levels of toluene which were growth supporting, inhibitory, and lethal to *P. putida* mt-2. Equilibration of BSM with toluene vapor from 1 to 20% (vol/vol) mixtures of toluene in hexadecane supported growth, while 25 to 30%

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mixtures were inhibitory to growth; cells lost viability when BSM was equilibrated with mixtures containing more than 50% (vol/vol) toluene in hexadecane. For this study, 1, 25, and 100% toluene (the latter undiluted with hexadecane) were selected as the growth-supporting, inhibitory, and lethal concentrations, respectively. The toluene concentrations in BSM following equilibration were determined by Strategic Diagnostics, Inc. (Newark, Del.) to be 4, 130, and 267 mg/liter by use of a DTech BTEX test kit. The toluene vapor was equilibrated with 50 ml of BSM at 30°C for at least 2 h prior to each experiment. Each flask stopper was fitted with a needle to allow sampling without disruption of the toluene-equilibrated atmosphere. To ensure that the cells were responding to toluene and that the response was not due to a change in culture conditions, the batch of BSM, the preequilibration times, and the incubation conditions were identical for each experiment. In addition, cells from a single starter culture were used to inoculate each of the toluene-equilibrated and control flasks. The starter culture consisted of early- to mid-log-phase (optical density at 650 nm, 0.2 to 0.35) *m*-toluate-grown cells which were harvested and washed in BSM. Cultures were incubated at 30°C with shaking at 100 rpm. To determine viability, aliquots were removed at selected intervals, diluted in BSM, and plated in triplicate on Trypticase soy agar (Difco Laboratories). The plates were incubated for 24 to 48 h at 30°C. All viability studies were performed a minimum of five times.

Protocols for labeling toluene-exposed *P. putida* cells with L-[^{35}S]methionine. The protein profiles of cells provided with 4, 130, or 267 mg of toluene per liter as the sole carbon source were studied in a series of experiments. To determine the initial response to toluene exposure, mid-log-phase cells (ca. 10^7) were inoculated into toluene-equilibrated BSM containing L-[^{35}S]methionine (20 μCi/ml; >800 Ci/mmol; DuPont-NEN Research Products). Aliquots (8.0 ml) were removed at 1 and 18 min, and the label was chased for 3 min with 10^{-3} M unlabeled L-methionine. To examine the patterns of protein production over a 6.5-h period, 2.0-ml aliquots of toluene-exposed cells were removed at 2.0 min and selected intervals thereafter and labeled with L-[^{35}S]methionine (20 μCi/ml) for 20 min. The label was chased for 3 min with 10^{-3} M unlabeled L-methionine. A second long-term study was performed as just described except 4.0-ml aliquots of cells were labeled for 30 min. The labeling was carried out in the presence of toluene at 30°C with shaking. The control for each labeling study consisted of cells inoculated into BSM supplemented with 5 mM *m*-toluate and equilibrated with hexadecane. The subsequent processing of labeled cells for protein analysis is described below. The protein profiles of toluene-exposed cells were studied in at least six experiments for cells provided with 4 mg of toluene per liter of BSM and in four experiments for cells provided with 130 or 267 mg of toluene per liter.

The effect of carbon supplementation on cells provided with 4, 130, or 267 mg of toluene per liter of BSM was also examined. For these experiments, BSM supplemented with 5 mM *m*-toluate was equilibrated with toluene vapor as previously described. The labeling protocols were performed as described for cells provided with toluene as the sole carbon source. Carbon supplementation experiments were performed twice.

Carbon starvation studies. To determine the initial response of *P. putida* to carbon downshifting, mid-log-phase *m*-toluate-grown cells (ca. 10^7), which were harvested and washed in BSM, were used to inoculate carbon-free BSM containing L-[^{35}S]methionine (20 μCi/ml). Aliquots (8.0 ml) were removed at 1 and 18 min, and the label was chased for 3 min with 10^{-3} M unlabeled L-methionine. To study carbon starvation over a longer period of time, mid-log-phase cells were harvested, washed, and downshifted to carbon-free BSM. Aliquots (8.0 ml) were removed at 2, 60, and 120 min following the downshift from 5 mM *m*-toluate. Cells were labeled with L-[^{35}S]methionine (20 μCi/ml) for 20 min; this was followed by a 3-min chase with 10^{-3} M unlabeled L-methionine. This procedure was also used to label proteins 40 min following the downshift from 5 mM glucose. The subsequent processing of labeled cells for protein analysis is described below.

Starvation following the depletion of 5 mM *m*-toluate from the culture medium was also examined. Aliquots (4.0 ml) were removed 30 min after the cells entered stationary phase. Cells were labeled with L-[^{35}S]methionine (20 μCi/ml) for 30 min; this was followed by a 3-min chase with 10^{-3} M unlabeled L-methionine. The subsequent processing of labeled cells for protein analysis is described below. Carbon starvation by toluate depletion was performed twice.

2-D gel electrophoresis of L-[^{35}S]methionine-labeled proteins. Labeled cells were harvested at 12,000 × *g*, washed in BSM, and suspended in 80 μl of O'Farrell's lysis buffer (31). Cells were broken by 6 cycles of freeze-thawing in a dry ice-ethanol bath. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of the labeled proteins was performed according to the methods of O'Farrell (31) with the Hoefer 250 SE system (Hoefer Scientific Instruments). Isoelectric focusing was run to equilibrium, using a 4% gel with 0.4% Bio-Rad ampholines (pH 3 to 10) and 1.6% Bio-Rad ampholines (pH 5 to 7). A 10% separating gel with a 4% stacking gel was used for the second-dimension sodium dodecyl sulfate-PAGE. Gels were loaded with equivalent amounts of radioactivity (ca. 500,000 cpm), or with 8 to 10 μg of labeled protein if the samples contained less than 500,000 cpm. Gels were dried and proteins were visualized by autoradiography with either Kodak XAR or Kodak Biomax film (Eastman Kodak Co.). Molecular weight determinations were made based on reference gels containing 2-D standards (Bio-Rad Laboratories). The numbers given to the proteins are arbitrary.

Determination of total protein synthesis. Total protein synthesis was determined by measuring the amount of L-[^{35}S]methionine incorporated into cellular proteins for each of the labeling studies described above. Trichloroacetic acid was used to precipitate proteins from 40 μl of cell suspension (2). The radioactivity in precipitates was counted in a Beckman model LS7500 scintillation counter (Beckman Instruments, Inc.). Protein synthesis was expressed as the percentage of L-[^{35}S]methionine incorporated into cellular proteins relative to that of the toluate-grown control (no toluene exposure).

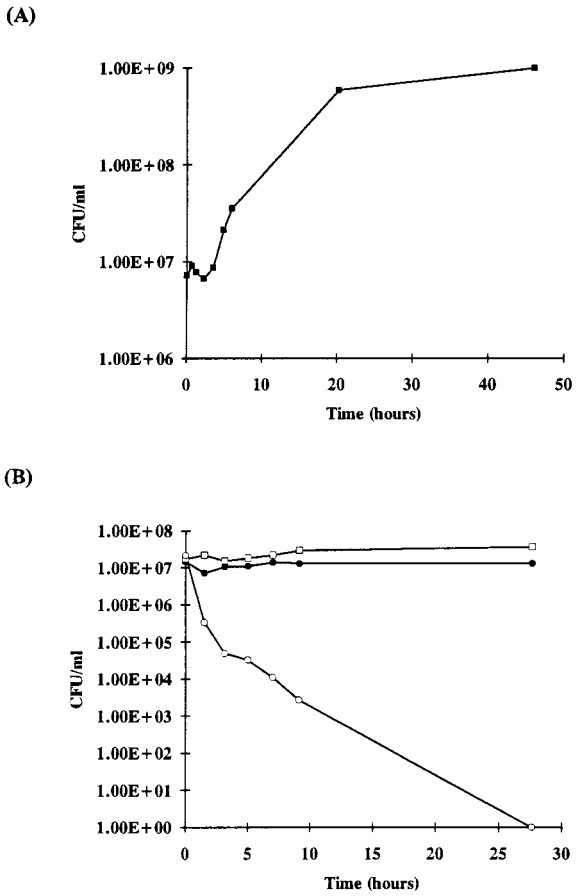

FIG. 1. The growth and survival of *P. putida* provided with toluene as the sole carbon source. (A) Growth of *P. putida* in BSM with 4 mg of toluene per liter. (B) Survival of *P. putida* in BSM with 130 mg (□) and 267 mg (○) of toluene per liter. The control cells were grown on BSM equilibrated with hexadecane (●) to ensure that cells do not grow on hexadecane.

RESULTS

Growth of *P. putida* on toluene. In BSM with 4 mg of toluene per liter, *P. putida* typically had a 2- to 3-h lag, after which cells grew with an average generation time of 1.4 h (Fig. 1A). Cells entered stationary phase in 18 to 24 h, reaching maximum cell densities between 8.5×10^8 and 1.0×10^9 CFU/ml. Growth was inhibited at 130 mg of toluene per liter, yet the cells remained viable (Fig. 1B). Similar growth-supporting and inhibitory concentrations have previously been reported when toluene has been provided in the vapor phase (22, 34, 37). At 267 mg of toluene per liter, there was a rapid loss of cell viability (Fig. 1B). Cells did not grow in BSM equilibrated with hexadecane (Fig. 1B).

Protein synthesis in *P. putida* provided with toluene. Protein synthesis in *P. putida* provided with 4, 130, or 267 mg of toluene per liter as the sole carbon source was inhibited 76 to 89% within the first 20 min of exposure compared to that in the *m*-toluate-grown control cells (Fig. 2A). Protein synthesis in cells provided with 4 mg of toluene per liter began to increase

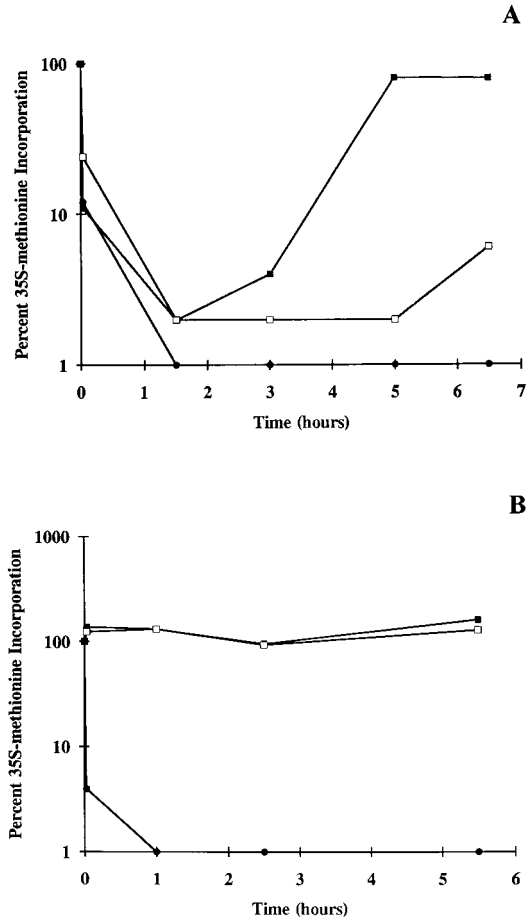

FIG. 2. Protein synthesis in *P. putida* provided with toluene as the sole carbon source (A) or with toluene supplemented with 5 mM *m*-toluate (B). Symbols: ■, 4 mg of toluene per liter; □, 130 mg of toluene per liter; ●, 267 mg of toluene per liter. Protein synthesis was expressed as the percentage of [^{35}S]methionine incorporated into cellular proteins relative to the percentage incorporated in toluate-grown control cells (no toluene exposure).

within 1.5 to 3 h and reached levels comparable to the control within 4 to 6 h. Protein synthesis remained inhibited by over 90% in cells provided with 130 mg of toluene per liter throughout the 6.5-h sampling period. However, the cells continued to incorporate L-[^{35}S]methionine into cellular proteins at low levels. Protein synthesis in cells provided with 267 mg of toluene per liter continued to decline until the levels of L-[^{35}S]methionine incorporation into cellular proteins became negligible. Interestingly, the addition of *m*-toluate as a supplemental carbon source alleviated the inhibition of total protein synthesis in cells exposed to 4 or 130 mg of toluene per liter but not in those exposed to 267 mg/liter (Fig. 2B).

Proteins produced in response to toluene. Twenty-six new proteins were detected in the protein profiles of cells provided with toluene as the sole carbon source that were not detectable in the *m*-toluate-grown control cells. The majority of these proteins appeared during the first 18 min of toluene exposure (Fig. 3). Two toluene-induced proteins (no. 57 and 68) appeared after 1 h of exposure (see Fig. 5). Cells provided with lethal levels of toluene (267 mg/liter) produced a similar subset of proteins during the first 18 min of exposure (38). At this concentration, profiles were rarely obtained beyond the first hour due to the rapid loss of viability, and they will not be discussed further.

The length of time that the toluene-induced proteins were synthesized by cells provided with 4 or 130 mg of toluene per liter is summarized in Fig. 4. Eight of the toluene-induced proteins (no. 4, 10, 12, 24, 28, 42, 45, and 46) were transiently synthesized and were not detected beyond the first 60 min of toluene exposure. The toluene concentration influenced the length of time that the remainder of the subset was synthesized. In cells provided with 4 mg of toluene per liter, the synthesis of all but one of the toluene-induced proteins (no. 68) ceased within 2 to 3 h following exposure, after which new proteins were synthesized which were similar to those in the *m*-toluate-grown control cells. In cells provided with inhibitory levels of toluene (130 mg/liter), the synthesis of 15 proteins was prolonged compared to that of cells growing in 4 mg of toluene per liter. Seven of the 15 (no. 3, 15, 16, 22, 27, 32, and 75) were detected for 3 h, and 8 of them (no. 5 through 8, 11, 57, 64, and 78) were detected throughout a 6.5-h exposure period. In cells provided with either 4 or 130 mg of toluene per liter, protein 68 appeared 1.0 to 1.5 h following exposure and was detected throughout a 6.5-h exposure period.

Overlap of toluene-induced proteins with carbon starvation proteins. We determined that there was substantial overlap of the toluene-induced proteins and those induced by carbon starvation. When *m*-toluate-grown cells were starved by downshifting to carbon-free BSM, 14 new proteins appeared in the profiles within 1 min (Fig. 5). All of these proteins overlapped with those previously identified as the toluene-induced subset of proteins (no. 4 through 8, 10, 11, 15, 16, 27, 28, 32, 42, and 48). These proteins were not detectable in the *m*-toluate-grown control cells. The temporal changes which were observed in the protein profiles of cells starved by carbon downshifting were similarly observed in the profiles of toluene-exposed cells. Eleven of the toluene-induced proteins seen at 1 h were also observed in the 1-h profiles of carbon-starved cells (Fig. 5B and D, no. 5 to 7, 11, 12, 15, 16, 22, 27, 32, and 57). After 2 h, proteins 5, 7, 11, 12, 15, 16, 22, 27, 32, and 57 were still present in both profiles (data not shown).

In all, 22 of the 26 toluene-induced proteins overlapped with those produced by *P. putida* during carbon starvation. Four proteins (no. 64, 68, 75, and 78) were present in the profiles of toluene-exposed cells but not in those of carbon-starved cells. Two of these unique toluene-induced proteins (no. 75 and 78) (shown in Fig. 3B) were detected only during exposure to inhibitory (130-mg/liter) and lethal (267-mg/liter) toluene concentrations. No unique carbon starvation proteins were detected, and none of the 26 toluene-induced proteins was detectable in heat-shocked *P. putida* cells (38).

The arrows in Fig. 5 denote proteins (no. 37 through 41 and 44) which were strongly repressed during the first hour of stress. These proteins were among the first to reappear in the profiles of cells provided with 4 mg of toluene per liter when the production of the toluene-induced proteins ceased and the cells began to grow. These proteins continued to be repressed in carbon-starved cells and were not detectable in the profiles following 2 h of starvation.

To determine whether the proteins observed in response to carbon downshifting were part of a general starvation response, two additional starvation protocols were studied. The protein profiles of cells following exhaustion of *m*-toluate from BSM, as well as those which were starved by downshifting from glucose to carbon-free BSM, were also examined. Starvation proteins (no. 5 to 7, 11, 15, 16, 22, 27, 32, and 57) were detected in the profiles of cells which entered stationary phase as a result of toluate depletion (38). The protein profiles of early-

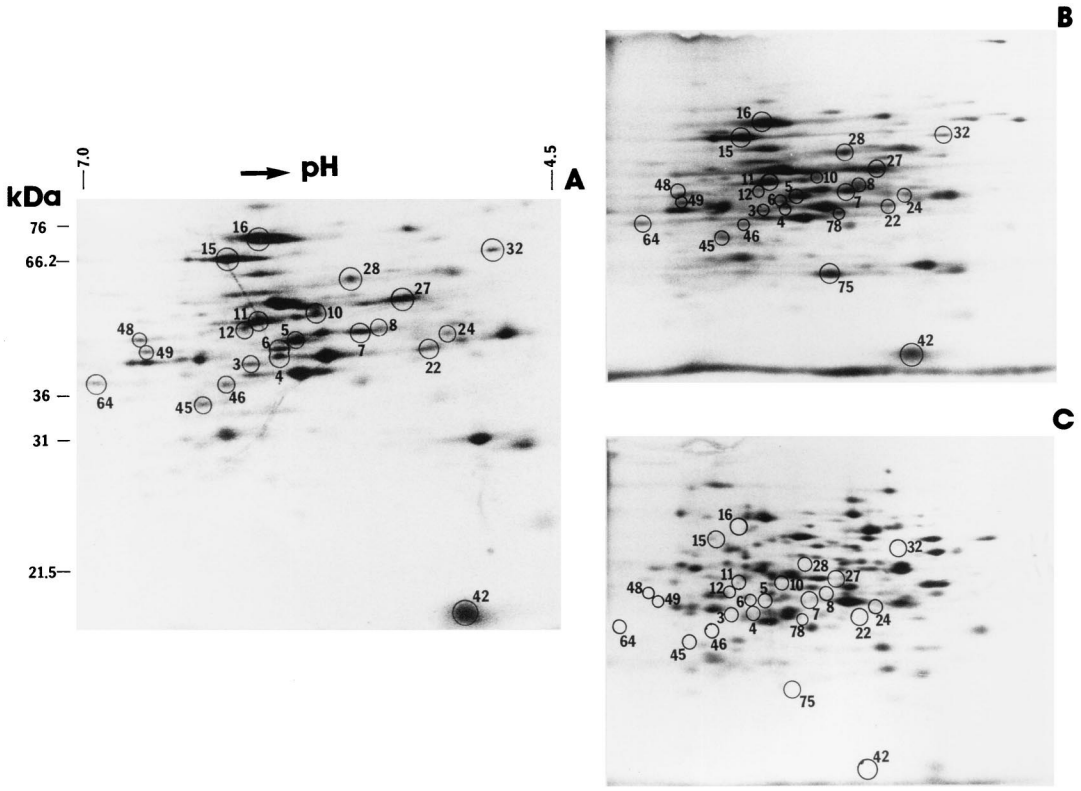

FIG. 3. Protein profiles, following 18 min of exposure, of *P. putida* cells provided with 4 mg of toluene per liter (A), 130 mg of toluene per liter (B), or *m*-toluate (control) (C) as the sole carbon source. The toluene-induced proteins are circled and numbered in panels A and B. The open circles in panel C correspond to the positions of the toluene-induced proteins which were absent from the control profile.

stationary-phase cells were similar to those of toluate-grown cells following 60 min of starvation by downshifting to carbon-free BSM. When mid-log-phase glucose-grown cells were shifted to carbon-free BSM, 11 starvation proteins (no. 3 through 7, 11, 15, 16, 22, 27, and 57) were visible in the protein profiles 40 min after the downshift which were not detectable in the glucose-grown control cells (38). In all cases, the protein profiles of cells starved under different starvation protocols were similar to those observed for cells provided with toluene as the sole carbon source.

To further confirm that the 22 toluene-induced proteins were induced by starvation, the protein profiles of toluene-exposed cells were examined in the presence of a supplemental carbon source. The synthesis of the toluene-induced proteins was suppressed within 1 min of exposure when *m*-toluate was added as a supplemental carbon source (Fig. 6). When 4 or 130 mg of toluene per liter was provided as the sole carbon source (Fig. 6A and C), the profiles were similar to the carbon-starved control (Fig. 6E). The profiles of toluate-supplemented cells exposed to 4 mg (Fig. 6B) or 130 mg (Fig. 6D) of toluene per liter were similar to those of the toluate-grown control cells (Fig. 6F). Similar results were observed for cells grown in BSM with 267 mg of toluene per liter (data not shown).

DISCUSSION

We have determined that when toluene is provided as the sole carbon source it elicits a carbon starvation response in *P. putida*. Cells responded to growth-supporting (4-mg/liter), inhibitory (130-mg/liter), and lethal (267-mg/liter) toluene concentrations with a rapid inhibition of protein synthesis and the production of 26 new proteins, 22 of which overlapped with those induced by carbon starvation. The overlapping proteins were observed in cells which were starved for either *m*-toluate or glucose by downshifting to a carbon-free medium or by carbon depletion. These results suggest that these proteins are synthesized as part of a general starvation response, one that is rapidly induced in response to carbon limitation. Carbon supplementation of toluene-exposed cells rapidly suppressed the production of these proteins. None of the 26 toluene-induced proteins was detectable in heat-shocked *P. putida* cells (38).

Carbon starvation in *P. putida* KT2442 was previously reported to elicit the production of two temporal classes of starvation proteins, one which was transiently produced and the other which was stably synthesized (13). In the current study, eight of the toluene-induced proteins were transiently synthesized in cells provided with 4, 130, or 267 mg of toluene per liter as the sole carbon source and during carbon starvation. These proteins usually were not detectable beyond the first 60 min of exposure. The toluene concentration influenced the length of time that the remainder of the toluene-induced proteins were synthesized. In cells provided with 4 mg of toluene per liter, the synthesis of all but one of the toluene-induced proteins ceased within 2 to 3 h, after which cells synthesized proteins which overlapped with those in the *m*-toluate-grown control cells. In cells provided with 130 mg of toluene per liter, the synthesis of 15 toluene-induced proteins was prolonged, with 9 still detectable in the 2-D profiles at 6.5 h. The production of a similar subset of proteins was observed in carbon-starved cells. In cells provided with 267 mg of toluene per liter, the toluene-induced proteins were observed in the 2-D profiles prior to death.

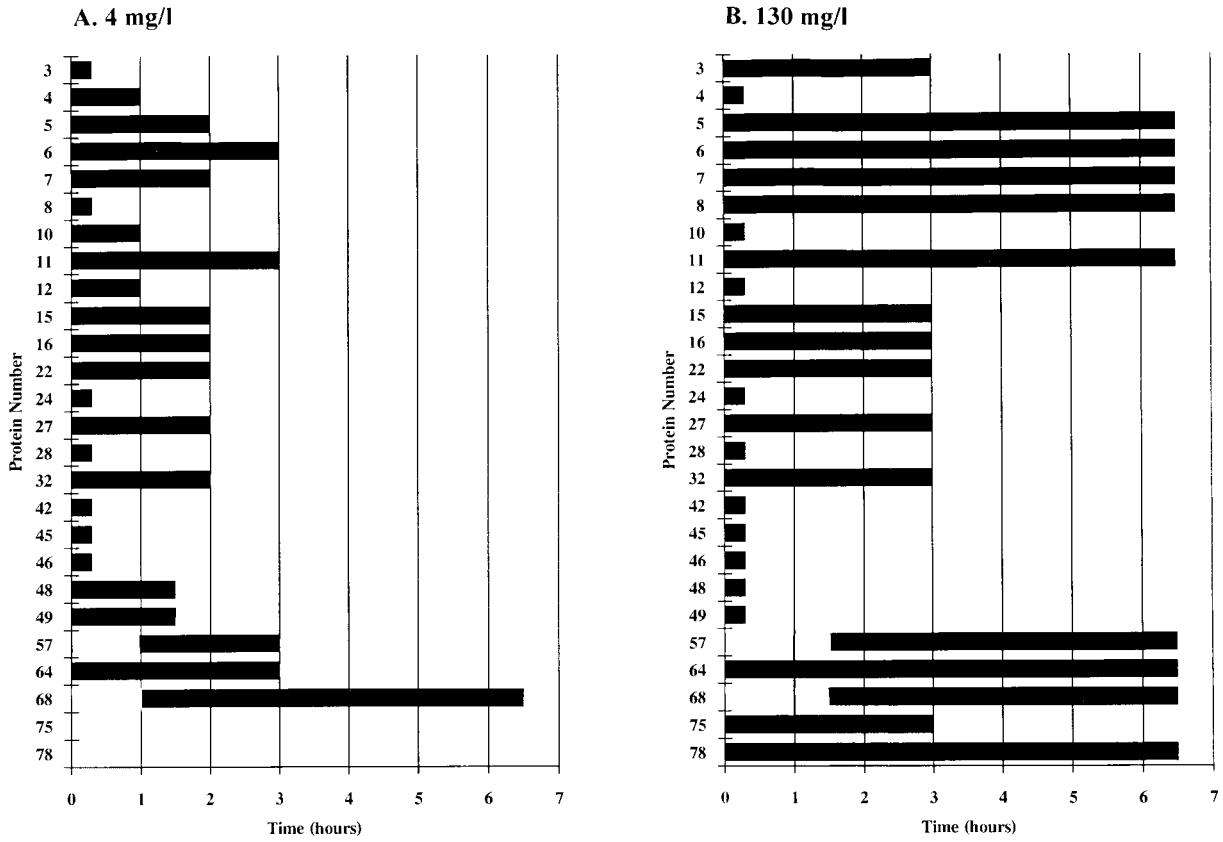

FIG. 4. Summary of the induction patterns for the toluene-induced subset of proteins. The bars indicate the lengths of time that the toluene-induced proteins could be detected on 2-D PAGE gels. Data was compiled from the protein profiles of cells provided with 4 (A) and 130 (B) mg of toluene per liter as the sole carbon source at 2 min, 20 min, 1.0 h, 1.5 h, 1.7 h, 2.0 h, 3.0 h, 4.0 h, 6.0 h, and 6.5 h.

In cells provided with 4 mg of toluene per liter as the sole carbon source, the synthesis of toluene-induced proteins coincided with an inhibition of total protein synthesis and a lag in growth. Total protein synthesis increased within 1.5 to 3 h and reached a level comparable to that of the toluate-grown control within 4 to 6 h. This increase corresponded to the time at which the synthesis of toluene-induced proteins ceased, new proteins were produced which overlapped with those in the *m*-toluate-grown control cells, and cells began to grow. The production of toluene-induced proteins and inhibition of protein synthesis which are observed prior to growth on 4 mg of toluene per liter may reflect the time required for the induction of the upper TOL catabolic operon containing the *xylCAB* genes (5, 43). Although the TOL catabolic genes were partially induced by growing the inoculum in *m*-toluate prior to each experiment, *m*-toluate induces only the genes in the lower catabolic operon (*xylDLEGFJKIH*) (43). Law and Button (21) demonstrated that toluene degradation by *Pseudomonas* sp. strain T2 occurred within minutes to hours depending on the level of induction prior to inoculation. It is also possible that *P. putida* requires a period of adaptation prior to growth on toluene. *P. putida* S12 undergoes a *cis*-to-*trans* isomerization of unsaturated fatty acids following exposure to toluene which is believed to protect the cells against nonspecific membrane permeabilization by increasing membrane ordering (16, 17, 40). Interestingly, the *cis*-to-*trans* isomerization of membrane fatty acids has also been observed in *Vibrio cholerae* during carbon starvation (15). It is possible that *P. putida* undergoes similar changes prior to growth on toluene.

In BSM with 130 mg of toluene per liter, growth is inhibited and the cells exhibit a prolonged carbon starvation response. Several toluene-induced proteins were detected throughout the 6.5-h experimental period. In addition, there was a sustained inhibition of protein synthesis, although the cells continued to incorporate L-[^{35}S]methionine into cellular proteins at low levels. Since the induction of the TOL degradative genes has been shown to proceed more rapidly at higher toluene concentrations (21, 34), the prolonged starvation response should not be due to a longer induction time in the higher level of toluene. The prolonged response may be due to the increased permeability of cells at the higher toluene concentrations. Robertson and Button (34) reported that the leakage and reutilization of toluene metabolic intermediates form a significant metabolic loop in *P. putida* cells. The product leakage increased with toluene concentration, resulting in lower cell yields.

In BSM with 267 mg of toluene per liter, cells synthesized the toluene-induced proteins prior to death and protein synthesis was inhibited in both the presence and absence of supplemental *m*-toluate. The addition of *m*-toluate as a supplemental carbon source suppressed the production of toluene-induced proteins prior to death. Interestingly, carbon-supplemented cells lost viability much more rapidly than non-carbon-supplemented cells (38). Carbon starvation has been shown to provide cross-protection against other stresses, such as heat (12, 19, 28), UV irradiation, (28), $CdCl_2$ (28), H_2O_2 (19), oxidative (12), and osmotic (12, 20). Therefore, the carbon-supplemented cells may have died more rapidly be-

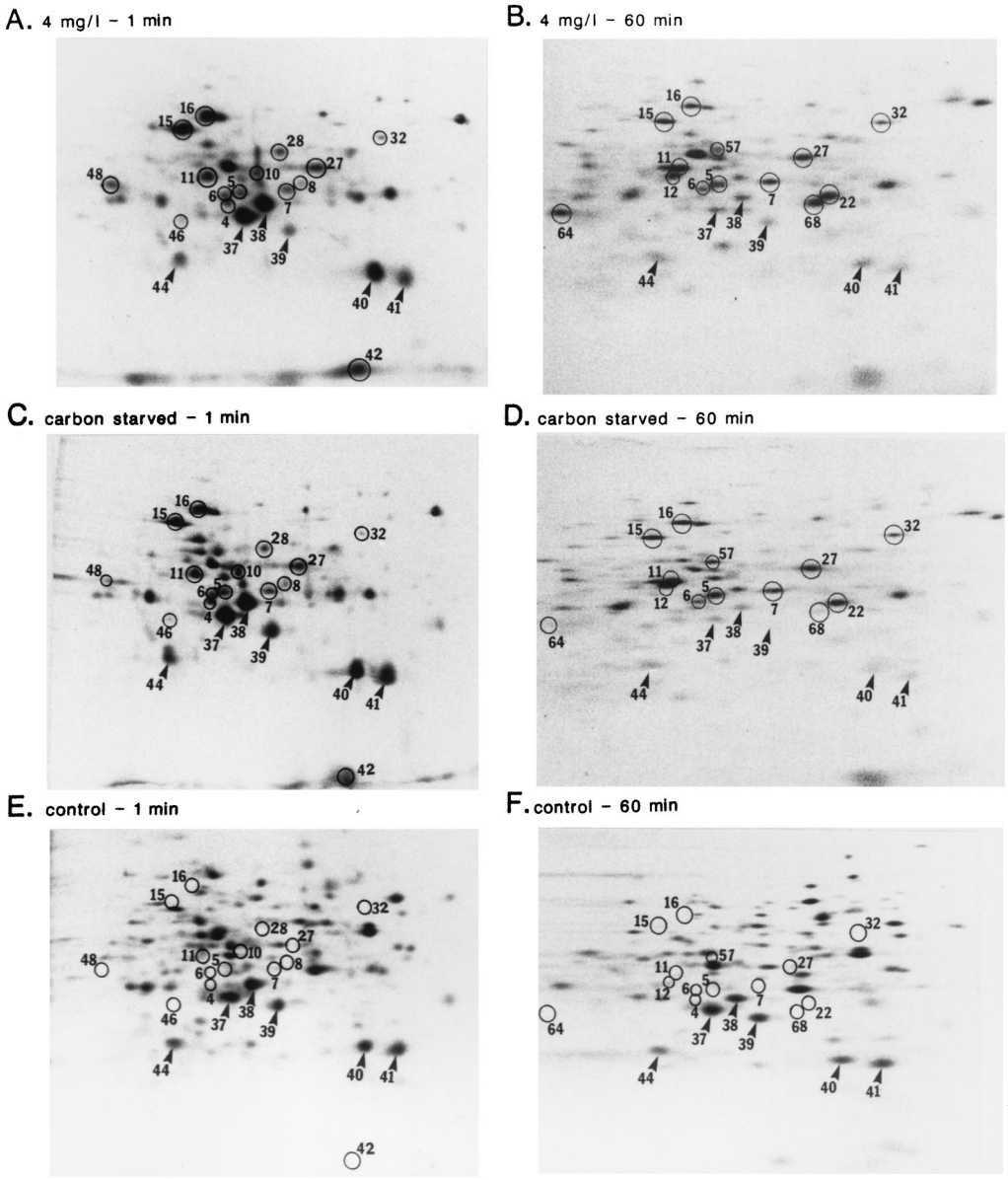

FIG. 5. Overlap of toluene-induced proteins with those induced by carbon starvation following a downshift to carbon-free medium. Shown are the 1- and 60-min protein profiles, respectively, of *P. putida* provided with 4 mg of toluene per liter as the sole carbon source (A and B), carbon-free medium (C and D), or 5 mM *m*-toluate (carbon-containing control) (E and F). The toluene-induced proteins are circled and numbered. Open circles in panels E and F correspond to the positions of the toluene-induced proteins which were absent from the control profiles. The arrows denote proteins which were strongly repressed during the first hour of toluene exposure or carbon starvation; these proteins were among the first to reappear in the profiles as cells began growing in BSM with 4 mg of toluene per liter.

cause they lacked the protection conferred by carbon starvation proteins.

These results suggest that when toluene is provided as the sole carbon source, *P. putida* cells may down-regulate protein synthesis in response to energy depletion. The rapid decrease in total protein synthesis in response to energy depletion at the onset of carbon starvation has been widely reported (9, 12, 28, 29, 33). During the first hour of starvation, the inhibition of protein synthesis is often substantial, with declines of at least 80% reported in *E. coli* (33), *Vibrio* sp. S14 (28), and *P. putida* KT2442 (12). Protein synthesis in carbon-starved *P. putida* mt-2 was similarly inhibited (89 to 92%) within 20 min after cells were suspended in carbon-free BSM (38). Since the addition of *m*-toluate as a supplemental carbon source eliminated the inhibition of protein synthesis in cells exposed to 4 or 130 mg of toluene per liter, it is unlikely that the higher level of toluene directly disrupts protein synthesis.

Previous studies of chemically induced stress protein synthesis have revealed that cells produce stress proteins which overlap with other stress regulons as well as unique stress proteins in response to different chemicals (1, 4, 11, 24, 30, 36). While the majority of the newly synthesized proteins produced by *P. putida* in response to toluene overlapped with those induced by carbon starvation, there were four proteins which were unique to toluene-exposed cells. Two of them were detected only during exposure to inhibitory and lethal levels of toluene, and they may be synthesized in response to more extensive cellular damage.

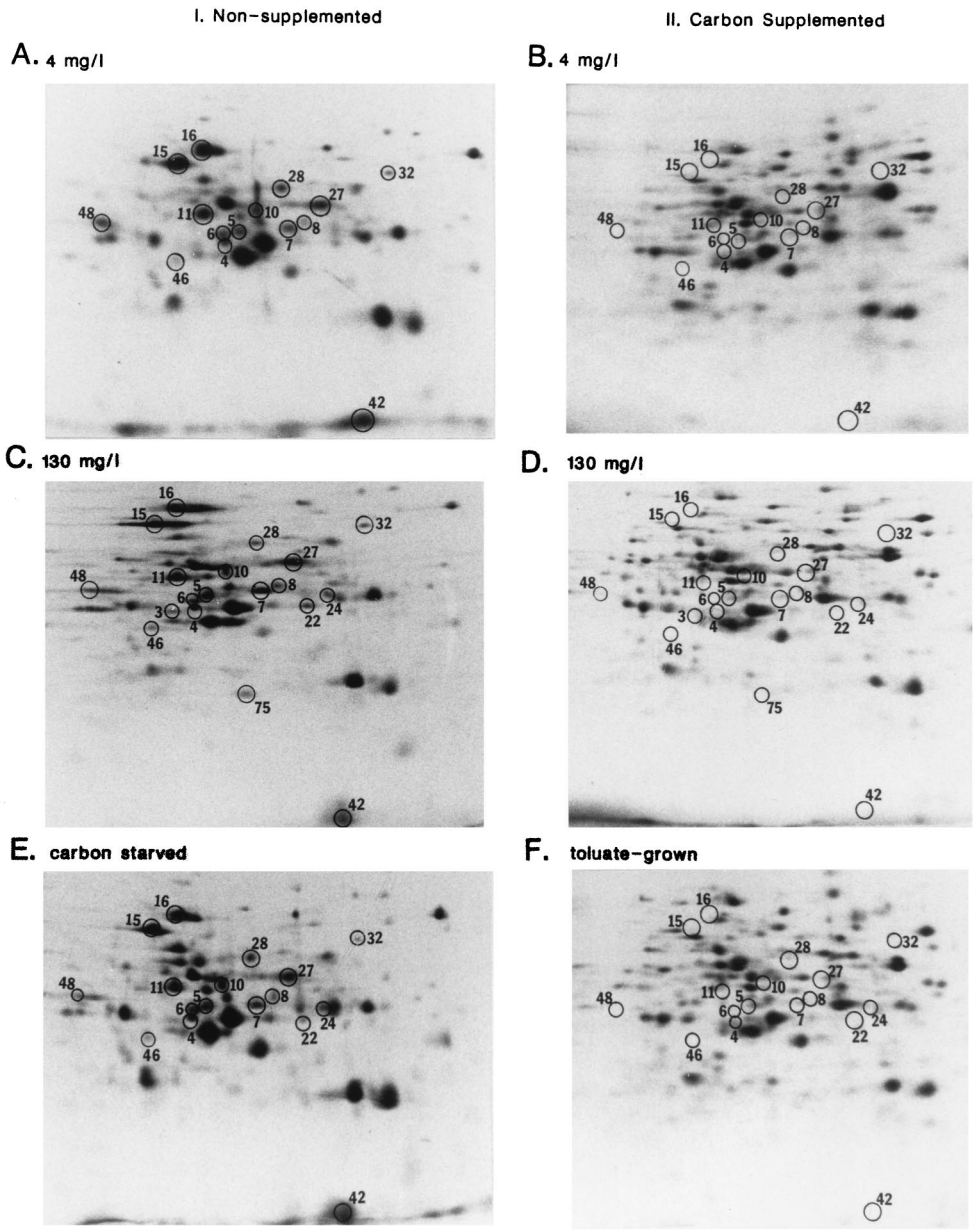

FIG. 6. Protein profiles of *P. putida* following 1 min of toluene exposure in the presence and absence of a supplemental carbon source. Cells were provided with 4 mg of toluene per liter as the sole carbon source (A), 4 mg of toluene per liter and supplemented with 5 mM *m*-toluate (B), 130 mg of toluene per liter as the sole carbon source (C), 130 mg of toluene per liter and supplemented with 5 mM *m*-toluate (D), no carbon source (carbon starved) (E), or *m*-toluate as the sole carbon source (F). The toluene-induced proteins are circled. Open circles in panels B, D, and F correspond to the positions of the toluene-induced proteins which were absent from the profiles of carbon-supplemented or *m*-toluate-grown cells.

It has been suggested that monitoring the transient repression of specific proteins is a sensitive index of pollutant stress and may provide a useful tool for assessing stress conditions in the environment (8, 23). In this study, there were six proteins present in the profiles of *m*-toluate-grown cells that were observed to be transiently repressed during the first few hours of either toluene exposure or carbon starvation. These six proteins were among the first to reappear in the profiles of cells provided with 4 mg of toluene per liter as the total protein synthesis increased and growth resumed. Future studies to determine if the repression of these prominent proteins occurs as a general response to stress may reveal whether they would be useful markers for monitoring stress in *P. putida*.

A carbon starvation response by microorganisms in the presence of a utilizable substrate has not previously been reported. We have observed this response by *P. putida* mt-2 when it is provided with toluene as the sole carbon and energy source. The extent of the response is determined by the concentration of toluene. These results suggest that in cells provided with 4 mg of toluene per liter, *P. putida* mt-2 undergoes a transient period of adaptation and/or induction during which the cells are carbon starved. At 130 mg of toluene per liter, the starva-

tion response is prolonged and growth remains inhibited. At this toluene concentration, cells exhibit a prolonged carbon starvation response despite the presence of an excess of a utilizable carbon source.

Hydrocarbon-degrading microorganisms such as *P. putida* are used in bioremediation to degrade environmental pollutants. At contaminated sites, these organisms will be exposed to a variety of chemicals present in a broad range of concentrations. Further studies of chemically induced stress in these organisms are needed to better understand how they survive in the presence of these chemicals and ultimately utilize them as growth substrates.

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