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Maintenance and Induction of Naphthalene Degradation Activity in *Pseudomonas putida* and an *Alcaligenes* sp. under Different Culture Conditions

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The expression of xenobiotic-degradative genes in indigenous bacteria or in bacteria introduced into an ecosystem is essential for the successful bioremediation of contaminated environments. The maintenance of naphthalene utilization activity is studied in *Pseudomonas putida* (ATCC 17484) and an *Alcaligenes* sp. (strain NP-Alk) under different batch culture conditions. Levels of activity decreased exponentially in stationary phase with half-lives of 43 and 13 h for strains ATCC 17484 and NP-Alk, respectively. Activity half-lives were 2.7 and 5.3 times longer, respectively, in starved cultures than in stationary-phase cultures following growth on naphthalene. The treatment of starved cultures with chloramphenicol caused a loss of activity more rapid than that measured in untreated starved cultures, suggesting a continued enzyme synthesis in starved cultures in the absence of a substrate. Following growth in nutrient medium, activity decreased to undetectable levels in the *Alcaligenes* sp. but remained at measurable levels in the pseudomonad even after 9 months. The induction of naphthalene degradation activities in these cultures, when followed by radiorespirometry with ^{14}C-labeled naphthalene as the substrate, was consistent with activity maintenance data. In the pseudomonad, naphthalene degradation activity was present constitutively at low levels under all growth conditions and was rapidly (in approximately 15 min) induced to high levels upon exposure to naphthalene. Adaptation in the uninduced *Alcaligenes* sp. occurred after many hours of exposure to naphthalene. In vivo labeling with ^{35}S, to monitor the extent of de novo enzyme synthesis by naphthalene-challenged cells, provided an independent confirmation of the results.

The time required for degradative bacteria to adapt upon exposure to xenobiotic substrates may be an important determinant of contaminant fate in the environment (2, 39, 41). In fact, predictions of contaminant persistence based upon degradation rate constants for fully induced bacteria may seriously underestimate the ability of contaminants to persist if adaptation periods are not considered (22). Adaptation may involve the de novo synthesis of enzymes, the growth and proliferation of degradative populations, intra- and interspecific transfers of genetic information, or molecular alterations of existing enzyme systems, and minutes to years may pass before active degradation occurs (38). The duration of the acclimation period preceding measurable xenobiotic degradation by bacteria in environmental samples has been considered evidence to support one or another of these adaptive mechanisms (1, 40). It may be possible to accelerate the adaptation process by providing specific inducers of degradative genes (28), nutrient elements to promote cell growth (21, 27, 35), or solid supports for the conjugal transfer of plasmids (23). The directed evolution of novel enzymatic pathways may be accomplished by the addition of several organisms, each carrying different catabolic genes, to promote recombination (38).

The physiological state or culture history of bacteria may dictate the subsequent course of degradation. Most xenobiotic-degradative enzymes are inducible, often by metabolites of the target compounds. Inducer formation depends on the level of constitutive degradative enzyme expression by the cell, which may be quite variable among different microorganisms degrading different compounds (6, 27). Comeau et al. (7) found that at equal cell densities, 2,4-dichlorophenooxyacetic acid mineralization by a noninduced *Pseudomonas cepacia* strain lagged 0.6 days behind mineralization by fully induced bacteria in soil. Likewise, *p*-nitrophenol mineralization rates and related levels of enzyme activity declined significantly in *Pseudomonas putida* following short-term growth under nonselective conditions (27). Conversely, prolonged culture (2 or more years) under nonselective conditions was required to obtain cells of a toluene-degrading pseudomonad with low or undetectable levels of enzyme activity for use in induction studies (30).

Recent results with nongrowing cells suggest that degradative enzymes are relatively stable and are maintained under starvation conditions following growth on xenobiotic compounds. Cells of an actinomycete held under starvation conditions for 8 weeks following growth on *p*-nitrophenol remained capable of degrading the compound despite starvation-induced filament fragmentation and cell dwarfing (14). Similar results were obtained by Truex et al. (36) in a study in which cells of a *P. cepacia* strain that had been starved for a long period (60 to 80 days) outperformed cells that had been starved for a short period (2 days) in degrading quinoline in soil columns. In neither case did starvation result in the production of cells uninduced for the degradation of their previous growth substrate. A more complete knowledge of the physiology and regulation of degradative-enzyme systems in bacteria may provide useful insights into an effective means of stimulating or preparing cells for in situ bioremediation applications.

We recently reported that soil-sorbed naphthalene was differentially available to two bacterial species when they were fully induced for naphthalene metabolism (11). As a prelude to studying the bioavailability of soil-sorbed naphthalene to bacteria under enzyme-limited, as opposed to sorption-limited

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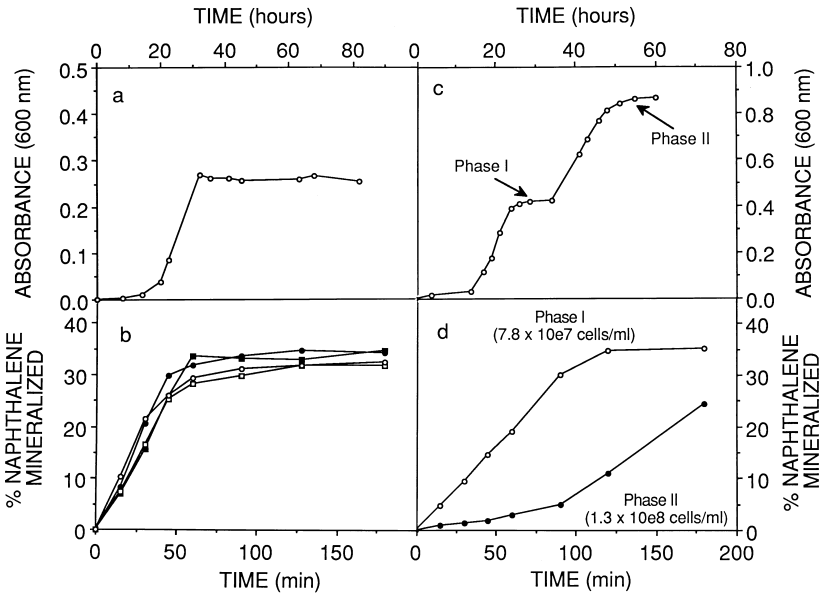

FIG. 1. Growth curves for strains ATCC 17484 (a) and NP-Alk (c) in minimal medium with naphthalene (200 μg ml^{-1}) as the sole carbon and energy source. [^{14}C]naphthalene mineralization curves for strain ATCC 17484 (1.0×10^7 cells ml^{-1}) in stationary-phase cultures 22 (open circles), 47 (filled circles), 72 (open squares), and 122 (filled squares) h old (b) and for strain NP-Alk during phase I and phase II (d) at the indicated cell densities are represented.

conditions, we undertook this study to elucidate enzyme maintenance and induction characteristics of the same organisms.

MATERIALS AND METHODS

Organisms and growth conditions. The organisms used in this study included *P. putida* (ATCC 17484) and a gram-negative isolate designated NP-Alk from petroleum-contaminated soil (11). Attempts to identify NP-Alk have tentatively placed it in the genus *Alcaligenes* on the basis of its peritrichous flagellation, its inability to utilize glucose as a carbon and energy source, its fatty acid methyl ester profile (Microbial Identification System, Newark, Del.), its carbon source utilization profile (Biolog, Inc., Hayward, Calif.), and its positive reaction to a fluorescent oligonucleotide probe specific for the β subgroup of the *Proteobacteria.*

Growth of organisms in minimal medium with naphthalene as the sole carbon source (11) was monitored by measuring the A_{600} of cultures over time. In studies on the maintenance of naphthalene degradation activity, cultures were either allowed to enter stationary phase, harvested by centrifugation (12,100 × *g*, 20 min), and resuspended in phosphate-buffered saline (PBS; 8.5 g of NaCl, 0.6 g of KH_2PO_4, 0.3 g of Na_2HPO_4 per liter of distilled water, pH 7.0) in late-log phase (starvation regimen) or grown under nonselective conditions in one-half-strength nutrient broth with periodic transfers to fresh medium. Cells were also starved after being grown in nutrient medium, washed, and resuspended in PBS.

The survival of cells during stationary phase or under starvation conditions was estimated by plating the cells onto nutrient agar (4 g of nutrient broth, 15 g of agar per liter of distilled water). The maintenance of the degradative phenotype was assessed by plating cells onto minimal agar (11) with naphthalene supplied in the covers of inverted plates as the sole carbon and energy source. Colonies grown on nutrient agar were also replica plated onto minimal agar with vapors from solid naphthalene as the carbon source to check for the degradative phenotype.

Measurement of naphthalene utilization activity. A whole-cell, spectrophotometric assay for naphthalene oxygenase activity (33) was used in this study. The disappearance of naphthalene (20 μl of a 10-mM solution in 100% ethanol) added to 3 ml of a washed-cell suspension in a quartz cuvet (with a target A_{600} of 0.15) was monitored as the decrease in A_{277} as a function of time in a Perkin-Elmer model 320 spectrophotometer. Analysis of culture supernatants during growth on naphthalene showed that salicylate did not accumulate, and corrections to absorbance data were therefore not required. Naphthalene consumption rates were normalized to the protein contents of cell suspensions determined by the method of Lowry et al. (24). Activity half-lives were estimated from first-order decay constants obtained by fitting activity data as a function of culture age to a first-order decay model by nonlinear regression analysis (SYSTAT). Since the oxygenase assay monitors the combined uptake and conversion of naphthalene to non-UV-absorbing products, we refer to the assay's measurements as naphthalene utilization activities in this report.

^{14}C induction studies. The induction of naphthalene degradation activities in uninduced cultures was assessed by monitoring the production of $^{14}CO_2$ by washed-cell suspensions incubated in PBS solutions containing [1-^{14}C]naphthalene (Sigma) at initial levels of activity of about 5×10^3 dpm ml^{-1}. Total naphthalene concentrations were varied by adding various amounts of a stock naphthalene-PBS solution of known concentration (by A_{277}). This solution was prepared by heating PBS containing solid naphthalene (at approximately two times the solubility limit of 31.69 mg per liter [26]) to 95°C with stirring. The contents were cooled to room temperature with stirring and were sterilized by filtration through a 0.2-μm-pore-size filter (Nuclepore). Details of the radiorespirometric assay were published previously (11).

^{35}S induction studies. The induction of naphthalene-degradative enzymes was also assessed in labeling experiments with $^{35}SNa_2SO_4$ (ICN Biochemicals, Inc.). Cells with different culture histories were washed in PBS and added to PBS solutions containing variable concentrations of unlabeled naphthalene and 0.4 μCi of $^{35}SNa_2SO_4$ ml^{-1}. Control cells were suspended in PBS with ^{35}S but without naphthalene. After appropriate incubation periods, cells were pelleted by centrifugation, washed in PBS, and resuspended in 50 μl of distilled water. Aliquots (10 μl) of cell suspensions were removed for protein analysis (24), and the remaining suspensions were diluted with distilled water to a constant protein concentration and then diluted twofold in a lysis solution consisting of 125 mM Tris-HCl (pH 6.8), 10% mercaptoethanol, 4% sodium dodecyl sulfate (SDS), and 20% glycerol. Samples were boiled for 5 min and frozen at −20°C until analysis by SDS-polyacrylamide gel electrophoresis (PAGE) following the method of Laemmli (20). Proteins and molecular weight standards (Bio-Rad) were separated by electrophoresis in 10% polyacrylamide gels run at specified conditions. Gels were stained with Coomassie blue and dried under vacuum. Autoradiograms (Kodak X-Omat AR film) of labeled proteins were exposed for periods of 0.1 to 10 days before development. Spots of $^{35}SO_4$-spiked India ink applied to the edges of the dried gels allowed the alignment of the autoradiograms with gels in which unlabeled molecular weight markers were used. This method permits the identification of proteins expressed specifically in response to naphthalene in previously uninduced cells (12).

RESULTS

Growth of organisms. When grown in minimal medium with naphthalene (200 μg ml^{-1}) as the sole carbon and energy source, strain ATCC 17484 showed normal batch culture kinetics and attained a cell density of approximately 5×10^8 cells ml^{-1} (Fig. 1a). Cultures maintained to a high degree the capacity to degrade naphthalene for several days following entry into stationary phase (Fig. 1b). In contrast, strain NP-Alk showed an unusual biphasic growth pattern in batch culture (Fig. 1c). Following the mineralization of naphthalene during the first phase of growth, the culture entered a stationary phase

lasting from 15 to 20 h. A second growth phase then ensued, during which the cell density was roughly doubled. Analysis of filtered (0.2-μm-pore-size filter) culture supernatant fluids collected after phase I growth by high-performance liquid chromatography (HPLC) UV detection showed the absence of known intermediates of naphthalene degradation (18) or of low-molecular-weight fatty acids. The reinoculation of filter-sterilized (0.2-μm-pore-size Nuclepore filter) phase I supernatant fluid supported the growth of NP-Alk to a density of approximately 5×10^8 cells ml^{-1}; however, no other naphthalene degraders in our culture collection, including strain ATCC 17484, would grow in this naphthalene-depleted culture medium. Further attempts to identify the soluble product(s) of naphthalene degradation by strain NP-Alk, including gas chromatography-mass spectrometry analysis of ethyl acetate extracts of acidified culture fluids have been unsuccessful. A comparison of the naphthalene degradation capacities of phase I and phase II cells (Fig. 1d) indicated that this second growth phase was unrelated to the utilization of naphthalene or its metabolites per se, since cells in phase II were poorly induced for the utilization of naphthalene.

The redirection of cellular metabolism in phase II growth toward utilization of the soluble constituent(s) produced during naphthalene mineralization was accompanied by major changes in protein profiles of crude cell lysates (data not shown). The appearance of proteins with molecular masses of 85.3, 76.7, 31, and 22 kDa accompanied the disappearance of proteins with molecular masses of 77.5, 52.8, and 40.8 kDa as cells progressed from phase I to phase II. The latter proteins corresponded to naphthalene (salicylate)-specific proteins identified in induction experiments with ^{35}S labeling (see below and reference 12). Interestingly, electron transparent storage granules were abundant in phase II but not in phase I cells as shown by transmission electron microscopy of thin sections (data not shown).

Maintenance of naphthalene utilization activity. Naphthalene utilization activity was monitored as a function of age in stationary-phase cultures of the two organisms. In accordance with the mineralization data above, levels of activity declined more slowly in strain ATCC 17484 than in strain NP-Alk (Fig. 2a; Table 1). Measurable activity (>0.2 ng naphthalene [μg of protein]$^{-1}$ min^{-1}) persisted in cultures of strain ATCC 17484 for several weeks into stationary phase. When cells were harvested after growth on naphthalene (following phase I growth in NP-Alk) and resuspended in the same volume of PBS (starvation regimen), the apparent half-life of naphthalene utilization activity increased significantly for both organisms (Fig. 2b; Table 1).

To determine if the enhanced levels of activity in starved cultures were due to the maintenance of existing enzymes or continued enzyme synthesis, early-stationary-phase (phase I for NP-Alk) cultures were harvested and resuspended in PBS to their original densities. The suspensions were divided equally into two flasks, and after overnight (approximately 20 h) incubation to deplete residual carbon and allow the completion of cell division cycles, chloramphenicol (50 μg ml^{-1}) was added to one flask from each set. Over the following 4 weeks, cell densities remained fairly constant and approximately equal in the chloramphenicol-treated and untreated cultures of both organisms (Fig. 3), with no loss of degradative phenotype. However, levels of naphthalene utilization activity, normalized to the protein content of the cultures, declined three and eight times more rapidly in the chloramphenicol-treated cultures than in the untreated cultures for strains ATCC 17484 and NP-Alk, respectively (Fig. 3a and b). These results suggest that the continued synthesis of naphthalene-degradative enzymes by starved cells was responsible for their enhanced maintenance of utilization activity. Activity half-lives are therefore functional descriptions of the competing processes of enzyme synthesis and decay rather than zero-order measurements of enzyme decay alone.

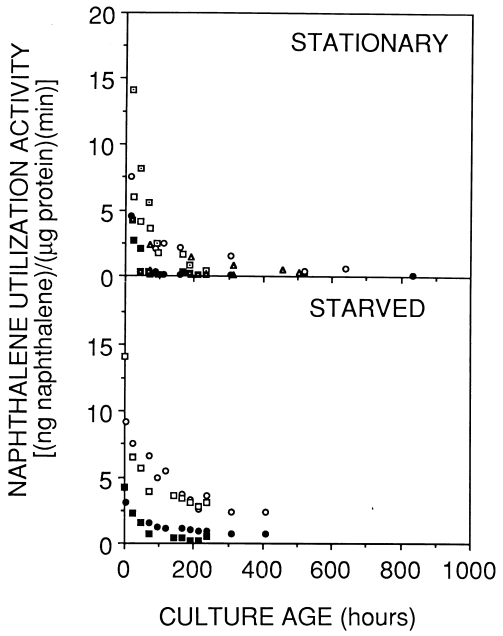

FIG. 2. Maintenance of naphthalene utilization activities in stationary-phase (upper panel) and starved (lower panel) cultures of strains ATCC 17484 (open symbols; different symbols represent different experiments) and NP-Alk (filled symbols) as a function of culture age following growth on naphthalene.

Over longer periods, cell densities in both starved and stationary-phase cultures of strain NP-Alk declined by less than 1 order of magnitude over a 180-day culture period as indicated by plate counts on nutrient agar (Fig. 4). Over the same period, cell densities of strain ATCC 17484 declined by over 5 orders of magnitude with somewhat better survival rates for stationary as opposed to starved cells. Strain NP-Alk was not readily recovered on minimal agar medium with naphthalene as the carbon source, but replica-plating experiments showed that 100% of the cells retained the ability to degrade naphthalene. Strain ATCC 17484, on the other hand, grew equally well on nutrient and minimal agar (with naphthalene) media.

The results showing levels of activity for starved and stationary-phase cultures were confirmed in naphthalene mineralization assays. At approximately equal cell densities, the initial rate of naphthalene mineralization in starved cultures ex-

TABLE 1. Maintenance of naphthalene utilization activities in starved and stationary-phase cultures of strains ATCC 17484 and NP-Alk

Organism	Naphthalene utilization activity half-life (h)[a] in culture		Ratio of naphthalene use in starved/stationary-phase cultures
	Stationary phase	Starved	
ATCC 17484	43.3	115.5	2.7
NP-Alk	13.1	69.3	5.3

[a] Functional half-lives of utilization activities (see the text for an explanation) were determined with first-order decay constants obtained from nonlinear model fits of plots of activity versus time.

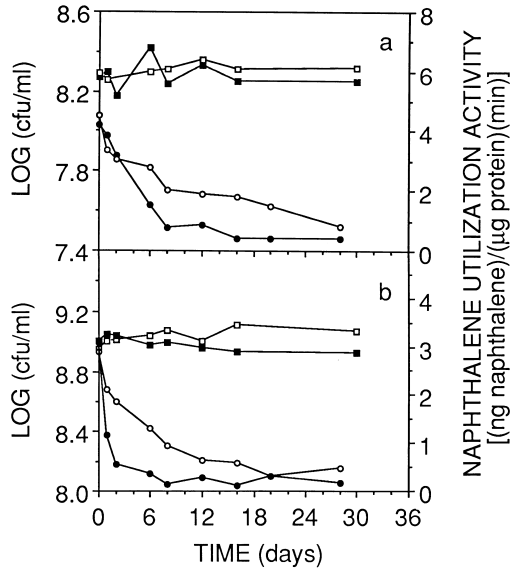

FIG. 3. Maintenance of viability (squares) and naphthalene utilization activities (circles) in starved cultures of strains ATCC 17484 (a) and NP-Alk (b) treated (filled symbols) or untreated (open symbols) with chloramphenicol (50 μg ml^{-1}) as a function of culture age. Viability data represent means of quadruplicate plate counts on nutrient agar.

ceeded those of cultures held in stationary phase for the same period (11 days) (Fig. 5). In strain ATCC 17484, the extent of naphthalene mineralization by stationary-phase cultures exceeded that by starved cultures (Fig. 5a), while the opposite was true for strain NP-Alk (Fig. 5b). With stationary-phase cells of strain NP-Alk, a lag in the onset of rapid naphthalene mineralization indicative of enzyme induction was evident at expanded time scales, consistent with the curve for phase II cells in Fig. 1d.

Induction of enzyme activity. Organisms grown previously in nutrient medium had very low levels of enzyme activity and produced distinctly S-shaped mineralization curves when naphthalene was present at concentrations insufficient to support growth (Fig. 5). The lengths of adaptation periods for the two organisms differed by more than a factor of 50, being very short for strain ATCC 17484 and quite prolonged for strain NP-Alk. Figure 6 shows that the rapid induction of naphthalene degradation activity in strain ATCC 17484 was due to fairly high constitutive levels of degradative activity in this

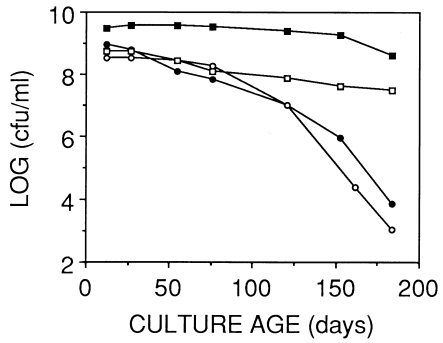

FIG. 4. Survival of strains ATCC 17484 (circles) and NP-Alk (squares) in stationary-phase (filled symbols) and starved (open symbols) cultures as a function of culture age. Points represent means of quadruplicate plate counts on nutrient agar.

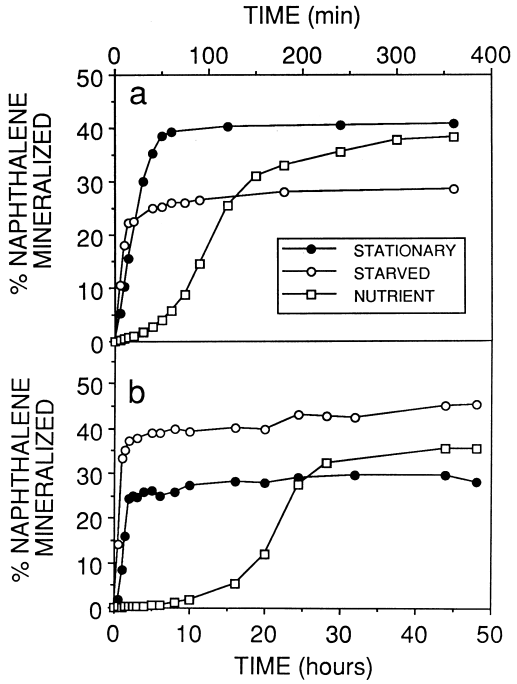

FIG. 5. Comparison of the naphthalene (101.3 ng ml^{-1}) mineralization kinetics of starved and stationary-phase (at 11 days) cells and in long-term uninduced cells (growth in nutrient medium for approximately 11 months) of strains ATCC 17484 (a) and NP-Alk (b).

organism. Cells grown under nonselective conditions for 248 days mineralized naphthalene immediately upon exposure to the compound at a rate which remained linear for 12 to 15 min. After this time, upward curvature of the mineralization data was indicative of enzyme induction. At the inflection at 50 min, the culture became substrate limited and the rest of the mineralization curve was first order. Conversely, for strain NP-Alk, levels of mineralization activity remained undetectable for approximately 10 h following exposure to naphthalene (Fig. 5b). The induction period itself was also protracted, requiring another 10 h before substrate limitation slowed the rate of mineralization.

With both organisms, the acclimation periods preceding

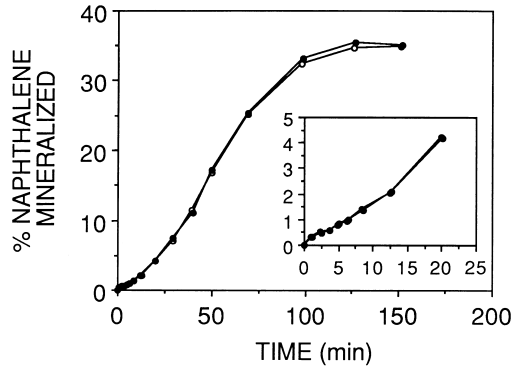

FIG. 6. Mineralization of naphthalene by uninduced cells of strain ATCC 17484 (after 248 days of culture in nutrient broth) in duplicate vials containing 63.4 ± 1.0 ng of naphthalene ml^{-1} and at a cell density of 0.9×10^7 ml^{-1}. The initial mineralization datum points in the inset show the linearity of activity over the first 15 min of the experiment.

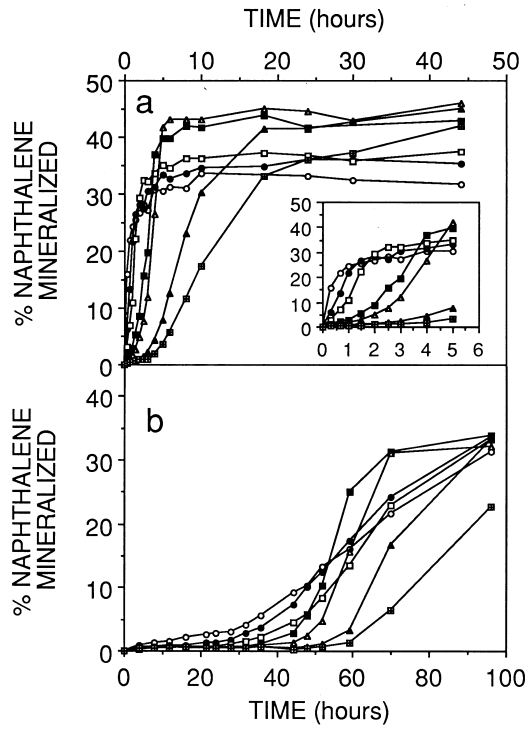

FIG. 7. Influence of naphthalene concentration on the kinetics of degradation by uninduced cells (after 7 days with a daily transfer to fresh minimal medium with succinate) of strain ATCC 17484 (a) and by strain NP-Alk following 182 days of culture in nutrient broth (b). For both organisms, cell densities were $\sim 10^7$ cells ml^{-1} and naphthalene concentrations were 0.020 (open circles), 0.058 (filled circles), 0.115 (open squares), 0.515 (filled squares), 1.01 (open triangles), 5.02 (filled triangles), or 10.04 (squares with crosses) μg of naphthalene ml^{-1}. The inset in panel a shows the early portions of the mineralization curves.

measurable degradation and the shapes of the subsequent mineralization curves in induction experiments were concentration dependent (Fig. 7). At naphthalene concentrations of less than 100 ng ml^{-1} and cell densities of approximately 10^7 ml^{-1}, increases in the sizes of biodegradative populations were negligible and upward curvature in the mineralization data was construed to represent simple induction of degradative enzyme synthesis. This rate of induction was relatively rapid, occurring within the first hour in experiments with strain ATCC 17484 and within the first 30 h (depending on culture history) in experiments with strain NP-Alk. At higher naphthalene concentrations (0.5 to 10 μg ml^{-1}), mineralization curves showed longer lag periods and assumed a more distinct S shape. Here, the lag periods preceding rapid mineralization were about 2 to 4 h for strain ATCC 17484 and approximately 20 to 50 h for strain NP-Alk, depending on naphthalene concentration and culture history. At the highest naphthalene concentration, the number of CFU increased more than twofold, indicating that anabolic processes in addition to degradative-enzyme synthesis had occurred.

Figure 8 shows the labeling pattern by $^{35}SO_4^{2-}$ of total cellular proteins in induction experiments with strain ATCC 17484. In the absence of the substrate (PBS control) (Fig. 8, lane 0), no detectable ^{35}S was incorporated by the cells. At low (0.1 μg of naphthalene ml^{-1}) (Fig. 8, lane 0.1) and intermediate (1.0 μg of naphthalene ml^{-1}) (Fig. 8, lane 1.0) concentrations, preferential labeling of a few proteins occurred. The majority of these labeled proteins was specific to naphthalene-challenged cells (12) and did not generally correspond to the prominent bands in the Coomassie blue-stained gel. In contrast, cells induced with 10 μg of naphthalene ml^{-1} (Fig. 8, lane 10) showed general labeling of all cellular proteins and relative labeling intensities which more closely corresponded to staining intensities with Coomassie blue. These results support the notion that concentration-dependent variations in the lengths of the adaptation periods are related to the extent of growth and anabolic protein synthesis which accompanies induction and naphthalene dissimilation.

A similar naphthalene concentration-dependent incorporation of $^{35}SO_4^{2-}$ into cellular proteins was observed with strain NP-Alk (data not shown). Whereas the naphthalene-specific proteins of strain ATCC 17484 had molecular weights similar to those detected in induction experiments with other *P. putida* strains (PpG7 and 9816; data not shown), naphthalene-specific proteins in strain NP-Alk varied significantly in their molecular weights from those of the pseudomonads (Fig. 9) (12).

In vivo labeling with $^{35}SO_4^{2-}$ was used in a quantitative comparison of the extent of de novo naphthalene-specific protein synthesis required for the dissimilation of a constant mass of naphthalene by cells (a constant biomass on a protein basis) with different culture histories. In accordance with the data shown in Fig. 5, the labeling intensities of naphthalene-specific proteins increased in the following order: starved cells < stationary-phase cells < nutrient broth-grown cells for strain NP-Alk. Low and approximately equal labeling intensities of proteins in starved and stationary-phase cells of strain ATCC 17484 were consistent with the prolonged maintenance of naphthalene utilization activity in this organism under both culture conditions. Starvation (for 12 days) of cells following growth in nutrient broth resulted in a lower level of naphthalene-specific protein synthesis in comparison with cells which were freshly grown in nutrient broth prior to induction.

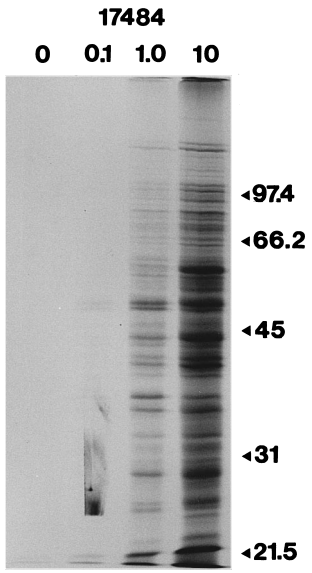

FIG. 8. Autoradiogram (8-day exposure) of crude cell lysates of *P. putida* ATCC 17484 separated by SDS-PAGE following 44 h of induction with 0 (PBS negative control), 0.1, 1.0, and 10 μg of naphthalene ml^{-1} in the presence of 0.4 μCi of $^{35}SNa_2SO_4$ ml^{-1}. Cells (250 μg of protein in 75 ml) were starved for 6 h following growth in nutrient broth prior to induction. Molecular weight markers (in thousands) are indicated by arrowheads.

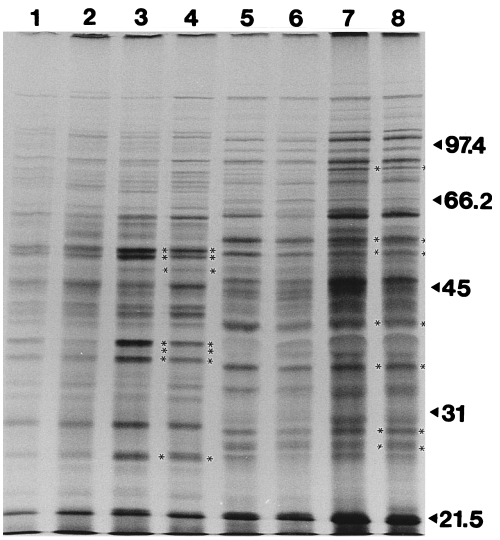

FIG. 9. Autoradiogram (6-day exposure) of crude cell lysates of *P. putida* ATCC 17484 (lanes 1 to 4) and *Alcaligenes* sp. NP-Alk (lanes 5 to 8) separated by SDS-PAGE following 24 (strain ATCC 17484) or 48 (strain NP-Alk) h of induction with naphthalene (approximately 4 μg ml^{-1}) in the presence of 0.4 μCi of $^{35}SNa_2SO_4$ ml^{-1}. Cells (500 μg of protein in 60 ml) were held in stationary phase (lanes 1 and 5) or under starvation conditions in PBS (lanes 2 and 6) for 12 days following growth on naphthalene prior to induction. The incorporation of ^{35}S in response to naphthalene by early-stationary-phase cells (lanes 3 and 7) and cells starved for 12 days in PBS (lanes 4 and 8) following growth in nutrient broth are also shown. Naphthalene-specific proteins are indicated by asterisks in lanes 4 and 8 for the two organisms.

DISCUSSION

Microbial degradation processes in nature result from the combined enzyme-, cell-, and community-based activities of microorganisms whose regulation and kinetics are likely to be highly individualized and variable in space and time. These activities are governed by a multitude of environmental factors, including nutrient status, the availability of alternative substrates, predation, population density, and contaminant concentration, pre-exposure, and bioavailability. These factors make it difficult to predict contaminant fates or to interpret biodegradability data from natural samples. The demonstration here, that two organisms carrying out the same biochemical process differ dramatically in their regulation of that process, indicates that environmental extrapolations based on laboratory experience with a single organism can be misleading. It cannot be inferred from biodegradability studies in which acclimation periods preceding degradation vary that a particular adaptation mechanism (enzyme induction, cell proliferation, etc.) is at work.

Results from biodegradability tests are also sensitive to the concentration of the test compounds (2, 10, 16, 22, 29, 37). When the combination of degradative population size and test compound concentration is insufficient to support growth, a fairly rapid rate of adaptation may be observed because of simple enzyme induction in previously unadapted cells. When population size and test compound concentration combinations are capable of supporting growth (34), apparent lengths of adaptation periods may increase because of the additional anabolic requirements of the organisms. As shown here, the time required for one organism (strain NP-Alk) to adapt by simple enzyme induction is longer than the time required for cell growth at high substrate concentrations in another organism (strain ATCC 17484). Therefore, the kinetics of degradation by bacteria in environmental samples may reveal little about the molecular and cellular details of the process.

The discrepancies in the regulation and kinetics of degradation observed here are likely manifestations of the contrasting lifestyles of the copiotrophic pseudomonad and the oligotrophic *Alcaligenes* sp. The biochemistry and genetics of naphthalene degradation have been studied in a variety of *P. putida* strains (18, 31, 33, 42). The plasmid-encoded naphthalene catabolic genes, which are expressed constitutively at low levels (5), have a high degree of homology even among degradative plasmids of different sizes (43). In most strains, the coordinate transcription of both the upper and lower *nah* operons is activated 24- to 30-fold (4, 5, 42) by the association of the *nahR* gene product with the inducer, salicylate, provided exogenously or produced from naphthalene at basal rates. Such activation occurs in a matter of minutes following exposure to inducing substrates (17) and is not subject to catabolite repression (32). Here, we observed that naphthalene utilization activities in cultures that were starved for a short period and in stationary-phase cultures of *P. putida* ATCC 17484 lasted a relatively long time. We also confirm that a significant constitutive level of enzyme expression in the absence of inducers keeps cells poised for rapid enzyme induction (within 12 to 15 min) upon the introduction of naphthalene. Previously uninduced cells begin logarithmic growth at the expense of naphthalene only a few hours after initial exposure to the compound. Cells grow rapidly but not very efficiently (approximately 0.3 μg of protein per μg of naphthalene) and are not well suited to long-term survival under nutrient-limited conditions (Fig. 4). This lifestyle has been described as copiotrophic.

Despite the similar biochemistry of dissimilation by naphthalene *Alcaligenes* sp. strain NP-Alk, its regulation of naphthalene degradation differs dramatically from that of *P. putida* ATCC 17484. The biphasic growth pattern of this organism with naphthalene as the substrate is similar to diauxie. A redirection of cellular metabolism to the utilization of phase I growth products results in a virtual shutdown of naphthalene degradation machinery as cells double their number and elaborate new proteins. Extremely low basal levels of enzyme synthesis make the detection of naphthalene degradation by uninduced cells difficult for many hours after the addition of the substrate.

Strain NP-Alk shows many characteristics of an oligotrophic organism. Although the induction rate of naphthalene degradation enzymes is relatively low, fully induced, nongrowing cells of this organism show a lower K_m and a lower maximum rate of metabolism for the substrate than strain ATCC 17484 (11). In degrading naphthalene, strain NP-Alk elaborates a soluble product which is unusable as a substrate by other organisms but which supports the doubling of the population in batch culture with an ultimate yield of about 0.6 μg of protein per μg of naphthalene. At the completion of growth, cells contain abundant storage polymers and are able to survive for over 6 months with little change in viability (Fig. 4).

Alcaligenes sp. NP-Alk is a novel organism. In addition to its unusual physiology, the naphthalene-specific proteins synthesized by this organism are completely different from those synthesized by the *Pseudomonas* spp. tested (12). In addition to its apparently different regulatory control of naphthalene-degradative enzyme synthesis, it is likely that this organism has a different genotype than the one elucidated for pseudomonads on the basis of the NAH7 plasmid. Since cells fully uninduced for naphthalene degradation are readily generated, this organism may provide an interesting model for future studies of regulation of xenobiotic-degradative genes.

A common response of bacteria to nutrient depletion is to increase the synthesis of catabolic enzymes (13) and/or substrate scavenging proteins (25). The synthesis of both upper and meta pathway enzymes encoded by the TOL plasmid (pWW0) was strongly induced only as cells entered the late-exponential phase of growth (15). The growth-phase-dependent expression of TOL plasmid genes, as well as of other genes related to bacterial survival in nutrient-poor environments, is related to the genes' common dependence on RpoN-like sigma factors for transcription (3, 8, 9, 19). Although the transcription of the (NAH7 plasmid-encoded) *nah* genes occurs with the normal sigma factor (σ^{70}), studies with a *P. putida* strain showed the strongest expression of the *nah* genes in late-log phase (in nutrient medium with naphthalene), even though there was no evidence for catabolite repression of the *nah* genes (5). Spikes in light output following the discontinuation of naphthalene additions to continuous cultures of an engineered *P. fluorescens* strain containing a *lux-nah* fusion plasmid (17) are also suggestive of some form of growth-phase-dependent regulation of the expression of naphthalene-degradative genes.

In this context, our observation that levels of naphthalene utilization activity remained higher in starved than in stationary-phase cultures may indicate a continued synthesis of enzymes during starvation. Indeed, levels of activity decreased much more rapidly in starved cultures treated with chloramphenicol than in untreated, starved cultures. The more rapid rate of activity and the lower level of de novo protein synthesis required for the mineralization of naphthalene by starved cells may be advantageous in certain environmental applications. Coupled with reductions in cell volumes, which would alleviate delivery problems due to clogging, the enhanced retention and expression of degradative functions in starved cultures may offer an ideal means of cell preparation for bioremediation applications involving the introduction of organisms to subsurface environments.

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