APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1996, p. 133–140 Vol. 62, No. 1
0099-2240/96/$04.00+0

Dynamics of Substrate Consumption and Enzyme Synthesis in *Chelatobacter heintzii* during Growth in Carbon-Limited Continuous Culture with Different Mixtures of Glucose and Nitrilotriacetate

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Received 29 August 1995/Accepted 5 November 1995

Regulation of nitrilotriacetate (NTA) degradation and expression of NTA monooxygenase (NTA-MO) in the NTA-degrading strain *Chelatobacter heintzii* ATCC 29600 in continuous culture at a dilution rate of 0.06 h^{-1} under transient growth conditions when the feed was switched between media containing NTA, glucose, or different mixtures thereof as the sole carbon and energy sources was investigated. A transition from NTA to glucose was accompanied by a rapid loss of NTA-MO. A transition from glucose to NTA resulted in a lag phase of some 25 h until NTA-MO expression started, and approximately 100 h was needed before a steady state for NTA-MO specific activity was reached. This transient lag phase was markedly shortened when mixtures of NTA plus glucose were supplied instead of NTA only; for example, when a mixture of 90% glucose and 10% NTA was used, induction of NTA-MO was detected after 30 min. This suggests a strong positive influence of alternative carbon substrates on the expression of other enzymes under natural environmental conditions. Regulation of NTA-MO expression and the fate of NTA-MO were also studied during starvation of both glucose-grown and NTA-grown cultures. Starvation of NTA-grown cells led to a loss of NTA-MO protein. No synthesis of NTA-MO (derepression) was observed when glucose-grown cells were starved.

The synthetic organic complexing agent nitrilotriacetic acid (NTA) is used in large quantities for a number of different applications, for example as a substitute for polyphosphates in washing powders or as a metal-trapping compound in the galvanizing industry and in environmental or nuclear metal decontamination processes (11, 31, 32, 34, 35, 47). Because NTA is potentially able to remobilize heavy metals deposited in river sediments or soils (1, 31, 48), the degradation of this agent has attracted considerable interest. Several obligately aerobic and denitrifying NTA-degrading strains have been isolated and characterized (10, 13–16, 26, 49, 54), and the biochemistry of an oxygen-dependent degradation pathway as well as an oxygen-independent degradation pathway has been elucidated (25, 27, 50, 51). Although the metabolic pathway for NTA degradation is known, there is little information concerning the regulation of NTA-degrading enzymes. Studies of the dependence of enzyme expression on the substrate mixture and growth rate have been restricted to steady-state conditions (3). However, in wastewater treatment plants as well as in natural aquatic systems microorganisms are confronted with a constantly changing environment and rarely experience steady-state conditions. Because of shock loading, diurnal and weekly cycles, heavy rainfalls, etc., they encounter periods in which substrates are plentiful followed by times in which they are scarce. It is well known that changing environmental conditions strongly influence the physiological behavior of microbial cells and that adaptation to different environmental conditions can result in lag phases. Such adaptation processes can severely affect the efficiency of biodegradation over long periods in both engineered and natural systems (6). Especially when degradation efficiency in wastewater treatment plants is considered, it is of interest to know the time scale of such adaptation processes; however, also with respect to natural environments it is useful to know how fast microbes are able to adapt to the sudden appearance of a chemical compound that can be used as a new substrate.

In order to obtain information concerning the dynamic behavior of microbial NTA degradation under transient conditions, *Chelatobacter heintzii* ATCC 29600 was cultivated in carbon-limited continuous culture and the influence of changes in the composition of the inflowing medium and the time required for the induction of NTA metabolism were studied. The results suggest that the use of mixtures of carbon substrates has a strong influence on the dynamics of expression of degradative enzymes, and they contribute to a better understanding of the principles of microbial metabolic regulation under complex environmental growth conditions.

MATERIALS AND METHODS

Microbial strain and cultivation. *C. heintzii* ATCC 29600 (2) was used in all experiments. The organism was precultured in synthetic medium as described previously (13) with 2 g of NTA ($C_6H_6NO_6Na_3 \cdot H_2O$) $liter^{-1}$ as the only source of carbon and nitrogen. For continuous cultivation this medium was modified in the following way: 1.9 ml of H_3PO_4 $liter^{-1}$ (85% [wt/wt]) was used instead of Na phosphate-K phosphate buffer; vitamins were omitted; the concentrations of $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ were lowered to 0.3 and 0.02 g $liter^{-1}$, respectively; and 10 mg of silicone anti-foam (Fluka, Buchs, Switzerland) $liter^{-1}$ was added. The concentrations of carbon and nitrogen sources used varied between 0 and 2.75 g of NTA $liter^{-1}$, 0 and 2 g of glucose monohydrate $liter^{-1}$, and 0 and 1.38 g of NH_4Cl $liter^{-1}$. The compositions of the media employed in the different transient experiments are given below. While the feed was shifted from one medium type to another, special care was taken to avoid mixing of the two media in the tubing by separating them with an air gap. In transient experiments the dilution rate was kept at 0.06 h^{-1}. For starvation experiments the feed was stopped. The bioreactor (2-liter working volume; MBR, Wetzikon, Switzerland) was aerated at a rate of 0.2 volume of air per volume of culture liquid per min, and the pH was maintained at 6.8 with H_3PO_4 (1 M) and KOH-NaOH (0.5M,

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each). The temperature was maintained at 30°C. Steady-state conditions were defined as constancy in both biomass concentration and NTA monooxygenase (NTA-MO) specific activity. Steady-state datum points represent the averages for three independent samples.

Immunoquantification of components A and B of NTA-MO. Samples (2 ml) of culture suspension of a known biomass concentration were collected from the bioreactor, and cells were immediately harvested by centrifugation at 20,000 × *g* for 5 min. The pellet was resuspended in 0.2 ml of sample buffer (30), boiled for 3 min, immediately frozen, and stored at −80°C. For quantification, lysed cells in sample buffer were thawed and mildly sonified for 30 s and 10.0-μl portions of this suspension were loaded onto five different sodium dodecyl sulfate-polyacrylamide gels (29). For calibration, four standard concentrations of both purified component A (cA) and component B (cB) of NTA-MO were loaded onto each gel. For detection of NTA-MO components A and B the procedure described by Uetz et al. (51) was used. Gels were blotted onto a nitrocellulose sheet. The nitrocellulose sheet was incubated in 20 ml of phosphate-buffered saline (PBS) containing 4% polyvinylpyrrolidone for 2 h. Subsequently, it was incubated in 20 ml of PBS containing 2% polyvinylpyrrolidone, 10 μl of antiserum against cA, and 10 μl of antiserum against cB for 1 h. This was followed by extensive washing with PBS containing 2% polyvinylpyrrolidone and incubation in the same buffer including 1 μCi of ^{125}I-labelled protein A for 45 min. After the sheet was washed with PBS and dried, it was exposed for 60 h to Fuji RX medical X-ray film. For quantification of cA and cB the developed film was scanned with a computing densitometer model 300A gel scanner (Molecular Dynamics, Sunnyvale, Calif.). The detection limits for cA and cB were in the range of 0.3 and 0.2 ng of protein, respectively.

Determination of NTA-stimulated oxygen uptake rate of cells. A portion (4 ml) of culture liquid was taken directly from the bioreactor, and cells were collected by centrifugation, washed once with Tris-HCl buffer (50 mM; pH 7.5), and resuspended in the same volume of buffer. The NTA-stimulated oxygen uptake rate was recorded at 25°C in a Clark-type oxygen cell (Rank Brothers). The total volume of the assay was 3.0 ml, consisting of 2.88 ml of cell suspension with a known optical density plus 120 μl of NTA-$MgCl_2$ (0.1 M each). Sampling and assay times were standardized.

Analytical methods. The biomass concentration in the culture was measured by filtration through a 0.2-μm-pore-size polycarbonate membrane filter (Nuclepore, Cambridge, Mass.). Cells collected on filters were washed once with distilled water, and filters were dried at 100°C to constant weight. Optical density was determined in a 1-cm cuvette at 546 nm with a Uvikon 860 spectrophotometer (Kontron, Zürich, Switzerland). Biomass measurements were carried out in triplicate.

The concentration of NTA was measured by high-pressure ion-exclusion chromatography as described by Schneider et al. (45). The detection limit was 1 mg liter^{-1}.

Concentrations of protein in standard NTA-MO solutions used for quantification of components A and B were determined by the test of Bradford (8). Bovine serum albumin (Fluka) was used as a standard.

RESULTS

Transition from NTA as a single substrate to glucose as a single substrate. In natural environments microorganisms rarely encounter stable growth conditions; rather, they have to adapt to fluctuating substrate availability. Also, they encounter mixtures of substrates for growth rather than single substrates (12, 23). In order to investigate how long cells of *C. heintzii* ATCC 29600 retain their NTA-degrading activity when NTA is suddenly replaced by another carbon and energy source, cells were initially cultivated in carbon-limited chemostat culture at a constant dilution rate of 0.06 h^{-1} with NTA supplied in the feed. After establishment of steady-state conditions, the feed was switched to a medium containing glucose. During the shift, biomass concentration was monitored and the presence of NTA-degrading enzymes was assessed by monitoring of the NTA-stimulated oxygen uptake rate as well as by immunoquantification of NTA-MO components (Fig. 1).

The dry weight of the culture increased substantially after the substrate shift, although a lag of 2 to 3 h was observed before growth accelerated (Fig. 1A). This increase reflects the higher yield coefficient of *C. heintzii* with glucose [$Y_{X/\text{glucose}}$ = 0.38 g of dry biomass (g of glucose)$^{-1}$] compared with that for NTA [$Y_{X/\text{NTA}}$ = 0.21 g of dry biomass (g of NTA · H_3)$^{-1}$] during growth at the dilution rate of 0.06 h^{-1} (3). From the biomass curve it can be calculated that the specific growth rate increased transiently during the first 10 h to approximately 0.08 h^{-1} and then slowly approached the dilution rate again. The NTA-stimulated specific oxygen uptake rate of the cells started to decrease exponentially after about 1 h until it became undetectable after about 40 h (Fig. 1B).

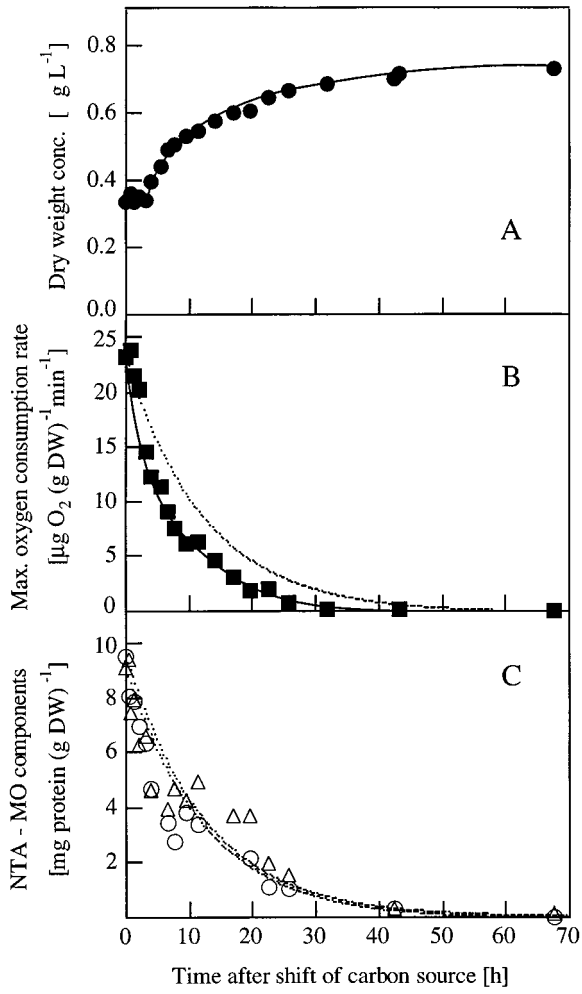

FIG. 1. Transient behavior of *C. heintzii* grown in carbon-limited continuous culture after switching of the feed from a medium containing NTA as the only carbon and energy source (S_0 = 1.87 g of NTA · H_3 liter^{-1}) to one containing only glucose (S_0 = 2 g of glucose · H_2O liter^{-1}). The total carbon concentration in the feed (S_0) before and after the switch was always 60.6 mM. Throughout the experiment the dilution rate was kept constant at D = 0.06 h^{-1}. (A) Dry weight concentration (conc.) (●); (B) maximum NTA-stimulated specific oxygen consumption rate (■) after the medium shift and theoretical time course of this rate, including both hydraulic dilution and a transient maximum growth rate of the culture (calculated in total as washout at a rate of 0.08 h^{-1}) (dotted line); (C) immunological quantification of NTA-MO components cA (△) and cB (○) and theoretical time courses for washout of cA and cB (dotted lines). DW, dry weight.

The decrease of the specific oxygen uptake rate proceeded faster than the theoretical washout curve (the dotted line in Fig. 1B represents the total of both washout at the hydraulic dilution rate and dilution of activity among newly formed cells, assuming for the calculation washout at a rate of 0.08 h^{-1}). This suggests that synthesis of enzymes responsible for NTA degradation ceased and that NTA-MO present not only was diluted among newly formed cells but was in addition subjected to degradation. Assuming washout at a rate of 0.08 h^{-1}, the time needed to reach an NTA-stimulated specific oxygen uptake rate of 1 μg of O_2 [g (dry weight)]$^{-1}$ min^{-1} would be 42

h, whereas the data in Fig. 1B demonstrate that this oxygen uptake rate was already reached after 26 h. Figure 1C shows the immunoquantification of monooxygenase components A and B after the medium was switched from NTA to glucose. Taking into account the limited accuracy of NTA-MO quantification, the pattern closely resembled that obtained for the NTA-stimulated specific oxygen uptake rate. A decrease of the concentration of enzyme from 9 mg of protein [g (dry weight)]$^{-1}$ to the background level of approximately 0.03 mg of protein [g (dry weight)]$^{-1}$ for each component (3) was observed within 40 h. The rate at which these components were lost also suggests that intracellular degradation of NTA-MO protein took place.

Transition from glucose to NTA. To investigate the time required for induction of NTA-metabolizing enzymes, *C. heintzii* was first cultured in continuous culture on glucose as the only carbon and energy source and after steady state was reached the inlet medium was switched to one containing NTA only. Throughout this experiment the NTA-stimulated specific oxygen uptake rate, the levels of monooxygenase components A and B, the biomass concentration, and the NTA concentration in the culture were monitored as a function of time (Fig. 2). During steady-state growth with glucose, the cells exhibited no NTA-stimulated oxygen uptake and the levels of NTA-MO components A and B were always close to the detection limit. The changes in dry weight and NTA concentration in the culture following the shift are plotted in Fig. 2A. Not unexpectedly, dry weight initially decreased after the substrate shift because glucose-grown cells were unable to metabolize the newly introduced substrate NTA. Dry weight started to increase again as soon as the level of induction allowed growth rates higher than the dilution rate. After approximately 80 h a steady-state biomass concentration which corresponded to that expected from the yield during growth of *C. heintzii* with NTA was reached. Correspondingly, the concentration of NTA in the culture increased after the shift from glucose to NTA (substrate concentration in the feed [S_0] = 1.91 g of NTA · H_3 liter^{-1}), an initial rate corresponding to the theoretical wash-in curve, and it subsequently increased to about 1.2 g of NTA · H_3 liter^{-1}. After some 10 to 15 h the concentration of NTA in the culture started to deviate from the wash-in curve, indicating that induction of NTA-metabolizing enzymes in *C. heintzii* had started. After this, growth resumed and NTA was completely consumed.

After the shift the NTA-stimulated specific oxygen uptake rate of the cells remained close to zero until after some 20 h a continuous increase was observed, indicating the start of induction of NTA-metabolizing enzymes. The maximum level of the NTA-stimulated specific oxygen uptake rate was reached some 100 h after replacement of glucose in the feed by NTA. The induction pattern for monooxygenase components A and B was slightly different (Fig. 2C). The enzyme content started to increase approximately 20 h after the shift, but the maximum level of induction was reached within approximately 50 h. The slightly higher background content of component B compared with component A before induction started cannot be considered significant.

Transitions from glucose to different mixtures of NTA plus glucose. Both under carbon-sufficient growth conditions in batch culture and in carbon-limited chemostat culture *C. heintzii* and other NTA-utilizing bacterial strains were shown to utilize NTA together with other suitable carbon sources (3, 13). Since in wastewater treatment plants and surface waters NTA-degrading microorganisms would utilize NTA in conjunction with a range of other available carbon sources, the influence of the availability of a second easily degradable substrate, such as glucose, on the process of adaptation to NTA of *C. heintzii* was investigated in continuous culture by subjecting the cells to a shift from glucose to different mixtures of NTA and glucose. Each transient experiment was started with a culture at steady state on a medium containing glucose as the only carbon and energy source, and the culture was then shifted to medium which contained 10, 50, 90 or 99% NTA carbon (NTA-C). The results obtained for shifts to mixtures containing 50 and 10% NTA are shown in Fig. 3. The start of induction was arbitrarily defined as the point at which the specific NTA-stimulated oxygen uptake rate reached 0.4 μg of O_2 [g (dry weight)]$^{-1}$ min^{-1}. This value was some two to three times higher than the average background oxygen uptake rates of cells grown on glucose as the only carbon substrate when exposed to NTA. In the different experiments the time needed for *C. heintzii* culture until induction started was graphically determined by linear extrapolation of the maximum slope of the curve back to 0.4 μg of O_2 [g (dry weight)]$^{-1}$ min^{-1}. The

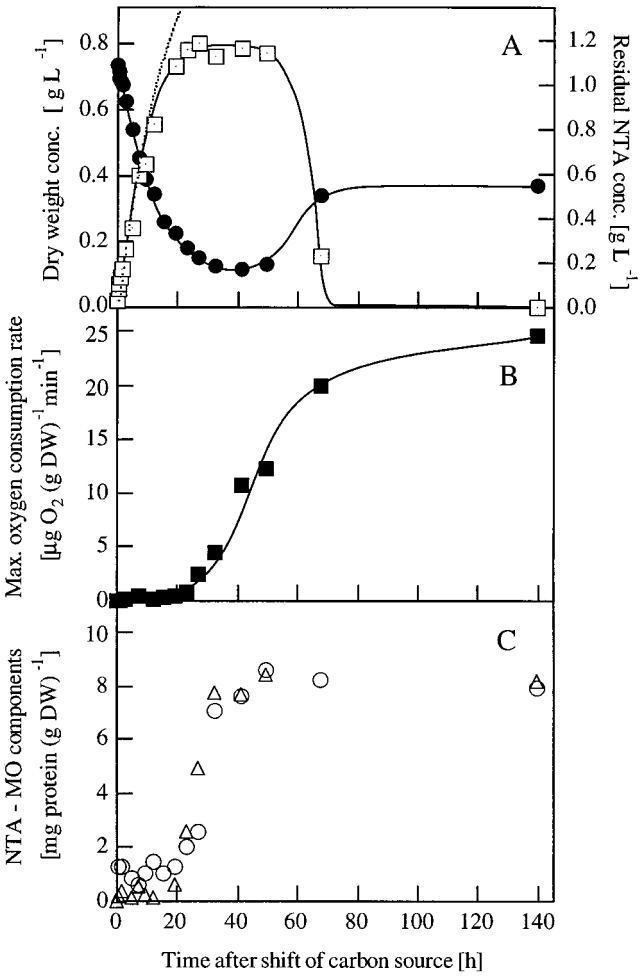

FIG. 2. Transient behavior of *C. heintzii* grown in carbon-limited continuous culture after switching of the feed from a medium containing glucose as the only carbon and energy source (S_0 = 2 g of glucose · H_2O liter^{-1}) to one containing only NTA (S_0 = 1.87 g of NTA · H_3 liter^{-1}). The total carbon concentration in the feed (S_0) before and after the switch was always 60.6 mM. Throughout the experiment the dilution rate was kept constant at D = 0.06 h^{-1}. (A) Dry weight concentration (conc.) (●) and residual NTA concentration (□) in the culture vessel; (B) maximum NTA-stimulated specific oxygen consumption rate; (C) immunological quantification of NTA-MO components cA (△) and cB (○). DW, dry weight.

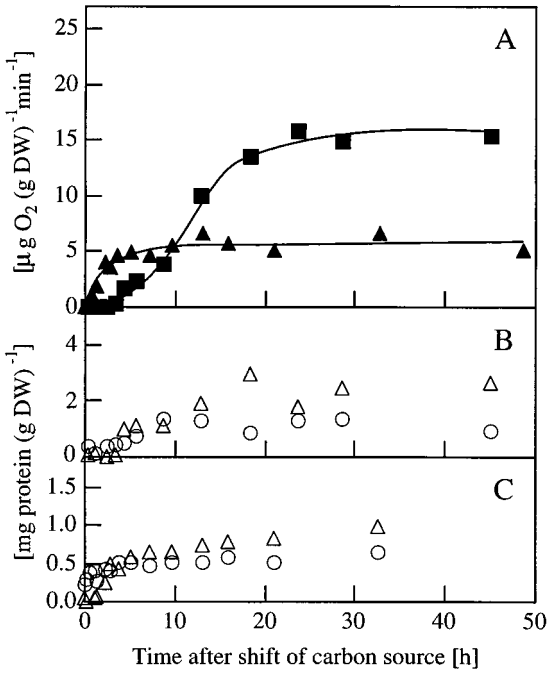

FIG. 3. Transient behavior of *C. heintzii* grown in carbon-limited continuous culture after switching of the feed from a medium containing glucose as the only carbon and energy source to one containing a mixture of glucose and NTA with a fraction of NTA-C of 10 or 50%. The total carbon concentration in the feed (S_0) before and after the switch was always 60.6 mM, and the dilution rate was kept constant at $D = 0.06\ h^{-1}$. (A) Maximum NTA-stimulated specific oxygen consumption rate as a function of time. ■, 50% of NTA-C; ▲, 10% NTA-C. DW, dry weight. (B and C) Immunological quantification of NTA-MO components cA (△) and cB (○) as a function of time when mixtures containing 50% (B) and 10% (C) NTA-C were supplied.

time required before induction started in the different experiments is plotted versus the fraction of NTA-C in the medium feed (Fig. 4).

The data clearly indicate that induction of NTA-degrading enzymes proceeded faster when the culture was supplied with mixtures of glucose and NTA rather than with NTA alone. When mixtures containing up to 80% NTA-C were supplied, the time needed before detectable induction of NTA metabolism started was linearly related to the proportion of NTA in the substrate mixture. Addition of only 1% glucose carbon (glucose-C) to the inflowing medium reduced this period from 24 h to 9 h. A substrate mixture with 90% glucose-C and 10% NTA-C reduced the time until the NTA-stimulated oxygen uptake rate reached 0.4 μg of O_2 [g (dry weight)]$^{-1}$ min^{-1} to 30 min. Furthermore, maximum slopes of the curves for the increase in the NTA-stimulated oxygen uptake rate (Fig. 2A and 3A) were markedly steeper when the feed was shifted to media with a low proportion of NTA-C, i.e., {1.2 and 0.9 μg of O_2 [g (dry weight)]$^{-1}$ min^{-1}} h^{-1} for 10 and 50% NTA-C, respectively, than when it was shifted to media with a proportion of NTA-C equal to or higher than 90%, i.e., {approximately 0.4 μg of O_2 [g (dry weight)]$^{-1}$ min^{-1}} h^{-1}.

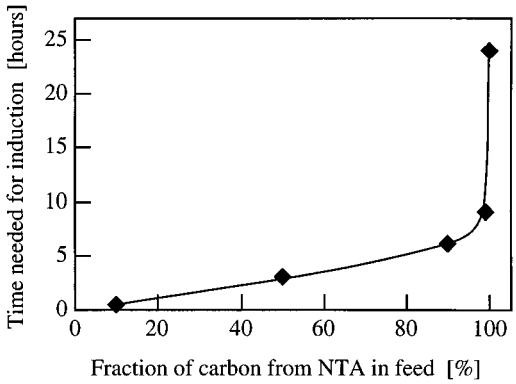

FIG. 4. Time needed to initiate induction of NTA-degrading metabolism in *C. heintzii* grown in carbon-limited continuous culture when the substrate in the feed was switched from glucose as the only carbon and energy source to glucose-NTA mixtures containing different proportions of NTA-C. The total carbon concentration in the feed (S_0) was always 60.6 mM. The dilution rate was kept constant at $D = 0.06\ h^{-1}$.

Additionally, it should be mentioned that the maximum specific NTA-stimulated oxygen uptake rates as well as the levels of NTA-MO components A and B that were reached after establishment of steady-state conditions were proportional (although not linearly) to the fractions of NTA-C in the different media used (compare Fig. 1A and B; Fig. 2A and B; and Fig. 3A, B, and C). The final levels of expression of NTA-degrading enzymes corresponded to the steady-state levels previously reported by Bally et al. (3).

Loss of NTA-degrading capacity during starvation. Under environmental conditions microorganisms frequently experience extended periods of very slow growth or even starvation. We therefore determined whether cells of *C. heintzii* induced for NTA degradation lose or retain NTA-MO under such conditions and, vice versa, whether starvation of uninduced cells previously grown on an easily degradable substrate such as glucose will lead to derepression of NTA-MO. For this investigation, cells were grown in steady-state chemostat culture with either NTA or glucose as the only substrate and starvation conditions were initiated by switching off the medium feed. The cellular NTA-MO protein content and the specific maximum NTA-stimulated oxygen uptake rate were monitored as a function of time. Within 200 h of starvation glucose-grown cells did not show any detectable specific NTA-stimulated oxygen uptake. Consistent with this, no increased expression of NTA-MO above the background level for glucose-grown cells was observed (data not shown).

When grown under conditions of NTA limitation and subsequently exposed to starvation, cells of *C. heintzii* exhibited a rapid decrease of their NTA-stimulated specific oxygen uptake rate. The decrease started within 30 min after the feed was interrupted; a rate equal to half the initial rate was reached after about 18 h, and after 190 h of starvation cells had completely lost their ability to oxidize NTA (Fig. 5A). In contrast to the specific NTA-stimulated oxygen uptake rate a rapid initial decrease of the cellular content of NTA-MO components cA and cB was observed. Half of the maximum amount of NTA-MO protein had already disappeared within the first 5 h after the feed was stopped, and the concentrations of the NTA-MO components cA and cB had decreased to background levels of about 0.03 to 0.05 mg of protein [g (dry weight)]$^{-1}$ after 100 h of starvation. NTA-MO component B was always present in lower concentrations than component A (Fig. 5B).

DISCUSSION

Microbial growth rarely takes place in a state of balanced growth—as defined by Campbell (9)—and especially in nature cells have to continuously adapt their metabolic apparatus to constantly changing environmental conditions, such as the spectrum of nutrients and nutrient concentrations, tempera-

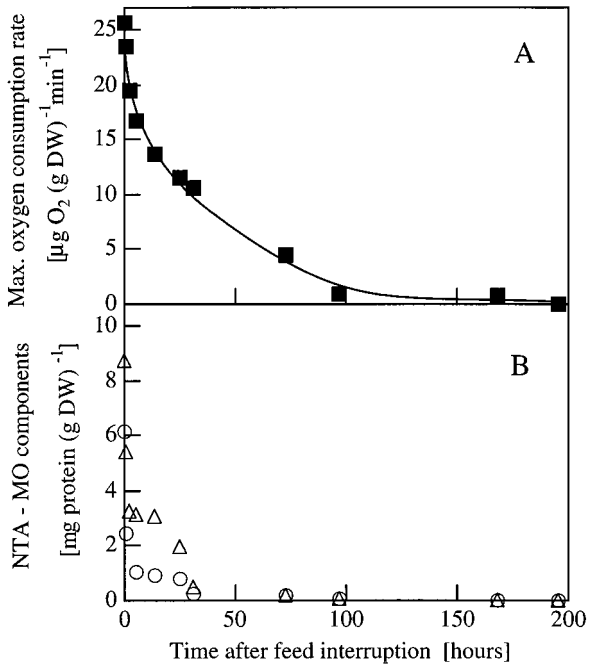

FIG. 5. NTA utilization capacity of *C. heintzii* grown in carbon-limited continuous culture on NTA as the only carbon and energy source when cells were subjected to starvation by switching off of the feed pump. (A) Maximum (Max.) NTA-stimulated specific oxygen consumption rate [■]; (B) immunological quantification of NTA-MO components cA (△) and cB (○). DW, dry weight.

ture, redox potential, etc. Hence, adequate metabolic flexibility is important for successful competition of a genospecies within a mixed population (21). Such transient environmental conditions also affect expression of enzymes involved in biodegradation. The efficacy of the degradation of organic pollutants—especially those which are only transiently available and are degraded via inducible enzyme systems—is therefore highly dependent on the periods within which expression of specific enzymes can be achieved.

Several studies concerning transient responses have been published for *Saccharomyces cerevisiae* (7, 52) and bacteria (17, 22, 28, 39, 41, 46). Nevertheless, Barford et al. (6) concluded that insufficient experimental data are available to establish a mechanistic model for mathematical description of transient growth conditions, and today this still applies because of the fact that only a few additional studies have since been published (21, 42). Hence, principally empirical models (4, 5) which can be used for the description of transient behavior during growth of cultures with both single and mixed substrates have been developed. The work presented here gives information in particular with respect to the times involved in the regulation of expression of the specific enzyme NTA-MO when a continuous culture of *C. heintzii* was subjected to nutrient shifts. The findings could be useful for the development of models as tools for predicting the behavior of environmental systems affected by changes in substrate spectrum and availability.

Regulation of enzyme synthesis by induction or by repression and derepression? When cultures are growing with different substrates, the question arises as to whether expression of enzymes is regulated by induction or by repression and derepression, or even by a combination of the two. There are a number of observations that suggest that repression of the synthesis of NTA-utilizing enzymes by glucose is not very likely. For example, in batch culture all NTA-utilizing bacterial strains tested so far, including *C. heintzii* ATCC 29600, have been able to utilize NTA in combination with glucose or other suitable carbon sources (13). This demonstrates that the synthesis of NTA-utilizing enzymes is not affected by the presence of high extracellular glucose concentrations or that such an effect is easily overruled by NTA.

The data shown in Fig. 1 and those given in a previous study (3) do not allow final elucidation of the dominating factor in the regulation of NTA-MO during growth in continuous culture. The reduction of NTA degradation-associated metabolic activities after a switch of the feed from NTA to 100% glucose-C (Fig. 1) might be due not only to the absence of NTA and its inducing effect but also to the presence of glucose exerting repression. Any possible repression by glucose can be excluded in the experiment in which the feed to a chemostat culture growing on NTA was interrupted. Here also a slow decrease of the concentration of NTA-MO was observed (Fig. 5). This indicates that the lack of expression of this enzyme—or at least the reduction of expression to a certain low constitutive level—was probably due to the absence of NTA and the absence of its inducing effect rather than to repression by glucose. Furthermore, the observation that no expression above the low constitutive level of NTA-MO occurred when the feed to a chemostat culture growing on glucose was switched off indicates that repression by glucose cannot be the reason for reduced expression of NTA-MO. The same behavior was observed with cells grown on complex media (data not shown).

Rate of NTA-MO turnover. Under conditions of carbon starvation and during transient growth, turnover of many proteins in living organisms is increased. This allows rearrangement of the cellular protein composition and adaptation of the set of enzymes to the new conditions (40–42, 53). Assuming that the rate of synthesis of NTA-MO in *C. heintzii* in the experiment described in the legend to Fig. 5 was insignificant after the feed of NTA was interrupted and that NTA-MO was intracellularly degraded, the turnover rates of both components of the NTA-MO can be estimated from the initial degradation rates given by the slope of the curve at time zero in Fig. 5B. For NTA-MO components A and B degradation rates of 28 and 94% per hour, respectively, can be calculated for the initial phase (note that on the basis of the maximum NTA-stimulated oxygen consumption rate shown in Fig. 5A this degradation does not appear to be so extensive; the reason for this discrepancy is presently not known). These NTA-MO degradation rates estimated from Fig. 5B are rather high compared with the turnover rates of 4 to 7% for total cellular protein or ribosomal protein typically observed for bacteria such as *Escherichia coli* or *Bacillus* species either experiencing starvation or during lag phases (20, 32, 33, 43). In spite of the fact that estimation of the NTA-MO component turnover rate is based on the degradation to a nonimmunogenic state only and not necessarily to single amino acids, the two NTA-MO components seem to be proteins exhibiting a very rapid rate of degradation under starvation conditions. This contrasts with the behavior observed when cells were exposed to a shift from NTA to glucose as the only substrate for growth (Fig. 1). If the initial degradation rate during the first 4 h, during which the biomass concentration remained approximately constant, is estimated from Fig. 1C, a degradation rate of approximately 5% per hour can be calculated for both components. A similar rate can be estimated from Fig. 1B for the NTA-stimulated maximum specific oxygen uptake, which was always approximately proportional to the NTA-MO content of cells. This suggests that in *C. heintzii* the (virtually) immediate availability of glucose as a

carbon and energy source after the substrate shift reduces the degradation rate of NTA-MO protein during the adaptation phase.

Segregation of the population versus adaptation of cellular composition. It is generally accepted that large enough microbial monocultures can be considered homogeneous rather than segregated (23, 24). Nevertheless, one may argue that during the simultaneous utilization of mixtures of substrates variations in enzyme activities in a culture can be explained either by different levels of expression or by segregation of the culture into metabolically or even genetically different subpopulations (e.g., by heterogeneous distribution of plasmids).

A strong argument against segregation of the culture is the discontinuous increase of steady-state NTA-MO content in a culture of *C. heintzii* observed during growth at a constant dilution rate in the chemostat with NTA-glucose mixtures with increasing fractions of NTA-C (3). Regulation of the NTA-MO content in *C. heintzii* at the level of subpopulations, i.e., one subpopulation degrading only NTA and a second one growing exclusively on glucose, should result in a linear increase of the measured NTA-MO specific activity with increasing proportions of NTA in the substrate mixture. Furthermore, the high degradation rate estimated above for NTA-MO during starvation strongly suggests that the decrease in the cellular NTA-MO content was mainly due to degradation of the two subunits of the enzyme and that, therefore, the culture was adjusting the enzyme content within single cells.

Influence of inducible and constitutive enzyme systems on transient growth behavior. In the few reports concerning the transient behavior of microbial cultures that have been published (22, 28, 38, 46, 55), information is usually restricted to the concentrations of biomass and substrates in the culture. In most of these studies a change in dilution rate rather than in nutrient composition has been investigated. In a study similar to the one presented here, Standing et al. (46) examined the effect produced when the medium fed was switched from glucose to xylose (which is metabolized by inducible enzymes). The reported response pattern with respect to biomass and substrate concentration was analogous to that observed in this study. Unfortunately, specific activities of enzymes involved in xylose metabolism were not monitored by these authors. The results of Standing et al. (46) have subsequently been discussed by Harrison and Topiwala (24) and Baloo and Ramkrishna (4, 5). The latter simulated the substrate transition with the help of a structured cybernetic model. They incorporated the constitutive level of an inducible metabolic system used for utilization of a new substrate into their model and found a very strong dependence on the level of constitutivity. The lower this level was, the longer was the time needed to reach the next steady state. Both Standing et al.'s and our experimental results support this dependence. In our case the high constitutive level of glucose-metabolizing enzymes in *C. heintzii* led to a smooth and fast transition when the medium was switched from NTA to glucose, whereas in the opposite direction—starting out from a very low constitutive level—a long lag period was observed before the content of NTA-MO increased. Therefore, we can assume that in the experiment for which the results are shown in Fig. 2A, initially after the medium shift, biomass and NTA concentration were only dilution rate dependent and cells unable to metabolize NTA were washed out and NTA was washed in. Further, it seems that duration of the mid-phase of the curve was influenced by the time needed for induction of NTA-metabolizing enzymes and that the subsequent recovery phase was determined mainly by the difference of the maximum specific growth rate with the new substrate and the dilution rate used in our experiment.

Effect of an additional C source on the time needed for induction of NTA-MO. The data obtained here clearly suggest that the availability of an additional carbon and energy source has a strong influence on the ability of a bacterial cell to react when it is suddenly confronted with a new substrate which requires induction of enzymes involved in its transport and catabolism. A glucose proportion of 1% of the total amount of carbon in the medium supplied was sufficient to exhibit a marked accelerating effect. In fact, glucose did not inhibit induction, but rather it supported induction of NTA-MO expression. When mixtures containing less than 90% NTA-C were supplied, the time needed before synthesis of NTA-MO started, and to a lesser extent also the subsequent rate of enzyme synthesis, was directly proportional to the fraction of glucose-C in the feed. This suggests that the greater the fraction of glucose that remained for the culture to use to maintain the metabolic activity established before the nutrient switch, the more energy was available for the rearrangement of cellular metabolism and particularly for synthesis of the NTA-degrading enzymes. The overproportionally large accelerating effect of as little as 1% glucose-C, reducing the time needed for induction from 23 h to 10 h, suggests a triggering level for a utilizable carbon source in the range of 1%. This fraction of carbon may be the substrate needed to deliver the energy and carbon for maintenance of the cell without forcing the organism to sacrifice its own constituents as an energy source. One percent of the specific glucose consumption rate of *C. heintzii* cultivated at $D = 0.06\ h^{-1}$ corresponds to a value of about 0.004 g of glucose [g (dry weight)]$^{-1}$ h^{-1}. Interestingly, this consumption rate is approximately 10 times lower than the values for maintenance coefficients given by Pirt (44).

It may be argued that the extracellular NTA concentration itself is the important factor influencing the time needed to start induction of NTA-MO. Although the data presented in this study cannot give a definite answer, this seems rather unlikely. Considering that the resulting concentrations of NTA must have been almost identical for the two experiments in which cells were shifted either from glucose to NTA alone or from glucose to a mixture of 99% NTA and 1% glucose, it seems unlikely that the small differences in NTA concentrations in the initial phase right after the shift can lead to a change in the time needed to induce NTA-MO expression after the shift as large as the one observed.

In a similar study Gottschal et al. (22) investigated the response of *Thiobacillus* strain A2 during a transition from heterotrophic growth with acetate to autotrophic growth with thiosulfate and CO_2. They observed that addition of a second substrate as an energy source (acetate or formate) reduced the time needed to start induction of enzymes involved in the use of thiosulfate and CO_2. The fact that the "autotrophic" carbon and energy source formate also reduced the induction time indicates that the role of such compounds as an energy source, rather than as a carbon source, is crucial during such transient conditions. Whereas for *C. heintzii* and NTA-glucose expression of NTA-degrading enzymes was not affected by glucose, the autotrophic metabolism of *Thiobacillus* strain A2, i.e., its use of thiosulfate and CO_2, was repressed by acetate. These differences might be due to the fact that the two systems are metabolically quite different.

Ecological and practical implications of time required for induction. Because glucose contributes a significant fraction to the pool of utilizable carbon substrates in both treatment plants and the natural environment (36, 37), *C. heintzii* and most likely also other microorganisms with constitutive capacities to use glucose should be able to respond quite quickly to changes in the substrate spectrum in their environment: This

suggests that extended induction times as observed in the experiment involving the transition from pure glucose to NTA as a single carbon substrate are not to be expected in nature (except when other environmental factors do not allow induction). With respect to NTA this seems to be the case, considering its relatively fast disappearance upon pulse addition in various environments (e.g., 8 h in an activated sludge tank and 3 h during infiltration in a river bank [18, 19]). This suggests that time courses of induction as reported for experiments using the single-substrate strategy are probably of little relevance for the situation in nature.

The acceleration of induction observed when the medium was shifted from glucose to mixtures thereof with NTA compared with shifts to pure NTA might have an implication of general practical importance: for example, for industrial wastewater treatment plants which are frequently exposed to shock loading of quite persistent pollutants degraded via inducible metabolic pathways the addition of a certain amount of an easily degradable substrate to the wastewater stream during an appropriate period might provide a method to ensure rapid induction and improve removal efficiency.

ACKNOWLEDGMENTS

We are indebted to C. A. Mason for correcting the English, G. Hamer for continuous support, T. Uetz for his help with the immunological enzyme quantification, and U. Lendenmann for adaptation of growth models. M.B. acknowledges the hospitality of the groups of E. Kellenberger and T. Bickle at the Biocenter of the University of Basel.

This research was supported by the Research Commission of ETH Zürich (grant no. 0.330.089.86/3) and by a grant from Unilever Merseyside GB/Unilever Switzerland.

REFERENCES

1. **Anderson, R. L., W. E. Bishop, and R. L. Campbell.** 1985. A review of the environmental and mammalian toxicology of nitrilotriacetic acid. Crit. Rev. Toxicol. **15:**1–102.
2. **Auling, G., H. J. Busse, T. Egli, T. El-Banna, and E. Stackebrandt.** 1993. Description of the gram-negative, obligately aerobic, nitrilotriacetate (NTA)-utilising bacteria as *Chelatobacter heintzii*, gen. nov., sp. nov., and *Chelatococcus asaccharovorans*, gen. nov., sp. nov. Syst. Appl. Microbiol. **16:**104–112.
3. **Bally, M., E. Wilberg, M. Kühni, and T. Egli.** 1994. Growth and regulation of enzyme synthesis in the nitrilotriacetic acid (NTA) degrading *Chelatobacter heintzii* ATCC 29600. Microbiology **140:**1927–1936.
4. **Baloo, S., and D. Ramkrishna.** 1991. Metabolic regulation in bacterial continuous cultures. I. Biotechnol. Bioeng. **38:**1337–1352.
5. **Baloo, S., and D. Ramkrishna.** 1991. Metabolic regulation in bacterial continuous cultures. II. Biotechnol. Bioeng. **38:**1353–1363.
6. **Barford, J. P., N. B. Pamment, and R. J. Hall.** 1982. Lag phases and transients, p. 55–89. *In* M. J. Bazin (ed.), Microbial population dynamics—1982. CRC Press, Boca Raton, Fla.
7. **Beck, C., and H. K. von Meyenburg.** 1968. Enzyme pattern and aerobic growth of *Saccharomyces cerevisiae* under various degrees of glucose limitation. J. Bacteriol. **96:**479–486.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
9. **Campbell, A.** 1957. Synchronization of cell division. Bacteriol. Rev. **21:**263–272.
10. **Cripps, R. E., and A. S. Noble.** 1973. The metabolism of nitrilotriacetate by a pseudomonad. Biochem. J. **136:**1059–1068.
11. **Egli, T.** 1988. (An)aerobic breakdown of chelating agents used in household detergents. Microbiol. Sci. **5:**36–41.
12. **Egli, T., U. Lendenmann, and M. Snozzi.** 1993. Kinetics of microbial growth with mixtures of carbon sources. Antonie Leeuwenhoek **63:**243–274.
13. **Egli, T., H. U. Weilenmann, T. El-Banna, and G. Auling.** 1988. Gram-negative, aerobic, nitrilotriacetate-utilizing bacteria from wastewater and soil. Syst. Appl. Microbiol. **10:**97–305.
14. **Enfors, S. O., and N. Molin.** 1973. Biodegradation of nitrilotriacetate (NTA) by bacteria. I. Isolation of bacteria able to grow anaerobically with NTA as sole carbon source. Water Res. **7:**881–888.
15. **Enfors, S. O., and N. Molin.** 1973. Biodegradation of nitrilotriacetate (NTA) by bacteria. II. Cultivation of an NTA-degrading bacterium in anaerobic medium. Water Res. **7:**889–893.
16. **Focht, D. D., and H. A. Joseph.** 1971. Bacterial degradation of nitrilotriacetic acid. Can. J. Microbiol. **17:**1553–1556.
17. **Franks, P. A., R. J. Hall, and P. M. Linklater.** 1980. Mechanistic model of the growth of *Streptococcus cremoris* HP at superoptimal temperatures. Biotechnol. Bioeng. **22:**1465–1487.
18. **Giger, W., M. Ahel, M. Koch, H. U. Laubscher, C. Schaffner, and J. Schneider.** 1987. Behaviour of alkylphenol polyethoxylate surfactants and of nitrilotriacetate in sewage treatment. Water Sci. Technol. **19:**449–460.
19. **Giger, W., H. Ponusz, A. Alder, D. Baschnagel, D. Renggli, and C. Schaffner.** 1987. Auftreten und Verhalten von NTA und EDTA in schweizerischen Gewässern und im Trinkwasser, p. 9–12. *In* EAWAG Jahresbericht 1987, Dübendorf, Switzerland.
20. **Goldberg, A. L., and A. C. St. John.** 1976. Intracellular protein degradation in mammalian and bacterial cells: part 2. Annu. Rev. Biochem. **45:**747–803.
21. **Gottschal, A. L.** 1993. Growth kinetics and competition—some contemporary comments. Antonie Leeuwenhoek **63:**299–313.
22. **Gottschal, J. C., A. Pol, and J. G. Kuenen.** 1981. Metabolic flexibility of *Thiobacillus* A2 during substrate transitions in the chemostat. Arch. Microbiol. **129:**23–28.
23. **Harder, W., and L. Dijkhuizen.** 1982. Strategies of mixed substrate utilisation in microorganisms. Philos. Trans. R. Soc. Lond. B **297:**459–480.
24. **Harrison, D. E. F., and H. H. Topiwala.** 1974. Transient and oscillatory states of continuous culture. Adv. Biochem. Eng. **3:**167–219.
25. **Jenal-Wanner, U., and T. Egli.** 1993. Anaerobic degradation of nitrilotriacetate (NTA) in a denitrifying bacterium: purification and characterization of the NTA dehydrogenase-nitrate reductase enzyme complex. Appl. Environ. Microbiol. **59:**3350–3359.
26. **Kakii, K., H. Yamaguchi, Y. Iguchi, M. Teshima, T. Shirakashi, and M. Kuriyama.** 1986. Isolation and growth characteristics of nitrilotriacetate-degrading bacteria. J. Ferment. Technol. **64:**103–108.
27. **Kemmler, J.** 1993. Biochemistry of nitrilotriacetate degradation in the facultatively denitrifying bacterium TE 11. Ph.D. thesis no. 9983. Swiss Federal Institute of Technology, Zürich.
28. **Kjeldgaard, N. O., O. Maaløe, and M. Schaechter.** 1958. The transition between different physiological states during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. **19:**607–616.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
30. **Lendenmann, U. (Swiss Federal Institute of Water Resources and Water Pollution Control, Dübendorf).** 1994. Personal communication.
31. **Macaskie, L. E.** 1991. The application of biotechnology to the treatment of wastes produced from the nuclear fuel cycle: biodegradation and bioaccumulation as a means of treating radionuclide-containing streams. Crit. Rev. Biotechnol. **11:**41–112.
32. **Mandelstam, J.** 1960. The intracellular turnover of protein and nucleic acids and its role in biochemical differentiation. **24:**289–308.
33. **Mandelstam, J., and H. Halvorson.** 1960. Turnover of protein and nucleic acid in soluble and ribosome fractions of non-growing *Escherichia coli*. Biochim. Biophys. Acta **40:**43–49.
34. **McCrary, A. L., and W. L. Howard.** 1979. Chelating agents, p. 339–368. *In* M. Grayson and D. Eckroth (ed.), Kirk-Othmer encyclopaedia of chemical technology, 3rd ed., vol. 5. Wiley, New York.
35. **Mottola, H. A.** 1974. Nitrilotriacetic acid as a chelating agent: applications, toxicology and bioenvironmental impact. Toxicol. Environ. Chem. Rev. **2:**99–161.
36. **Münster, U.** 1993. Concentration and fluxes of organic carbon substrates in the environment. Antonie Leeuwenhoek **63:**243–274.
37. **Münster, U., and R. J. Chróst.** 1990. Origin, composition and microbial utilisation of dissolved organic matter, p. 8–46. *In* J. Overbeck and R. J. Chróst (ed.), Aquatic microbial ecology: biochemical and molecular approaches. Springer, New York.
38. **Nagai, S., Y. Nishizawa, E. Isao, and S. Aiba.** 1968. Response of a chemostatic culture of *Azotobacter vinelandii* to a delta type of pulse in glucose. J. Gen. Appl. Microbiol. **14:**121–134.
39. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell, a molecular approach. Sinauer Associates, Inc. Publishers, Sunderland, Mass.
40. **Nyström, T.** 1993. Global systems approach to the physiology of the starved cell, p. 129–150. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
41. **Omlin, B. D.** 1990. Dentrifikation und Assimilation von Nitrat bei *Paracoccus denitrificans:* Untersuchungen zum Wechsel zwischen oxischen und anoxischen Bedingungen in Kontinuierlicher Kultur. Ph.D. thesis, ETH No. 9348. Swiss Federal Institute of Technology, Zürich.
42. **Östling, J., L. Holmquist, K. Flärdh, B. Svenblad, Å. Jouper-Jaan, and S. Kjelleberg.** 1993. Starvation recovery of *Vibrio*, p. 103–127. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York.
43. **Pine, M. J.** 1972. Turnover of intracellular proteins. Annu. Rev. Microbiol. **26:**103–126.
44. **Pirt, S. J.** 1975. Principles of microbe and cell cultivation. Blackwell Scientific Publications, Oxford.
45. **Schneider, R. P., F. Zürcher, T. Egli, and G. Hamer.** 1988. Determination of

nitrilotriacetate in biological matrices using ion exclusion chromatography. Anal. Biochem. **173:**278–284.
46. **Standing, C. N., A. G. Frederickson, and H. M. Tsuchiya.** 1972. Batch- and continuous-culture transients for two substrate systems. Appl. Microbiol. **23:**354–359.
47. **Tack, F., M. Verloo, and J. De Brabander.** 1993. Heavy metal extraction from a contaminated dredged material using NTA in combination with acid treatment, p. 628–629. *In* H. J. P. Eijsackers and T. Hamers (ed.), Integrated soil and sediment research: a basis for proper protection. Kluwer Academic Publishers, Dordrecht, The Netherlands.
48. **Tiedje, J. M.** 1980. Nitrilotriacetate: hindsights and gunsights, p. 114–119. *In* A. W. Maki, K. L. Dixon, and J. Cairns, Jr. (ed.), Biotransformation and fate of chemicals in the aquatic environment. American Society for Microbiology, Washington, D.C.
49. **Tiedje, J. M., B. B. Mason, C. B. Warren, and E. J. Malec.** 1973. Metabolism of nitrilotriacetate by cells of *Pseudomonas* species. Appl. Microbiol. **25:**811–818.
50. **Uetz, T., and T. Egli.** 1993. Characterization of an inducible, membrane-bound iminodiacetate dehydrogenase from *Chelatobacter heintzii* ATCC 29600. Biodegradation **3:**423–434.
51. **Uetz, T., R. Schneider, M. Snozzi, and T. Egli.** 1992. Purification and characterization of a two-component monooxygenase that hydroxylates nitrilotriacetate from "*Chelatobacter*" strain ATCC 29600. J. Bacteriol. **174:**1179–1188.
52. **von Meyenburg, H. K.** 1969. Energetics of the budding cycle of *Saccharomyces cerevisiae* during glucose limited aerobic growth. Arch. Microbiol. **66:**289–303.
53. **Wanner, U., and T. Egli.** 1990. Dynamics of microbial growth and cell composition in batch culture. FEMS Microbiol. Rev. **75:**19–44.
54. **Wanner, U., J. Kemmler, H. U. Weilenmann, T. Egli, T. El-Banna, and G. Auling.** 1990. Isolation and growth of a bacterium able to degrade nitrilotriacetic acid under denitrifying conditions. Biodegradation **1:**31–42.
55. **Young, T. B., III, and H. R. Bungay III.** 1973. Dynamic analysis of a microbial process: a systems engineering approach. Biotechnol. Bioeng. **15:**377–393.