Appl Microbiol Biotechnol (1990) 33:596-601

Applied and Microbiology Biotechnology

Cryptic growth within a binary microbial culture

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Received 17 May 1989/Accepted 9 March 1990

Summary. The ability of viable cells of the species *Pseudomonas putida* and *Hyphomicrobium* sp. to metabolize the particulate and soluble cellular organic constituents of both species was studied in a series of batch experiments. Both *P. putida* and *Hyphomicrobium* sp. were grown in individual batch reactors on either the ^{14}C-labelled soluble or the particulate debris of sonicated cells of each species derived from steady-state chemostat cultures. Cell generation times (t_g) observed for *P. putida* cultivated on soluble organic material originating from either sonicated *P. putida* or *Hyphomicrobium* sp. cells, were $t_g = 2.0$ h and $t_g = 6.3$ h, respectively. Corresponding t_g values of *Hyphomicrobium* sp. on soluble organic material originating from sonicated *P. putida* and *Hyphomicrobium* sp. were, respectively, 11.6 h and 4.3 h. While particulate debris originating from either species was solubilized by both *P. putida* and *Hyphomicrobium* sp., no increases in cell numbers were observed for either species. The data indicate that bacteria are capable of scavenging soluble organic material released upon cell lysis at near maximal rates; solubilization of debris also occurred but at much slower overall rates with no observable cell replication. The results reaffirm that cryptic growth and turnover of cellular biomass can be significant under situations of low substrate flux or starvation conditions.

Introduction

Most, if not all, practical and fundamental studies concerning microbial metabolism have employed only *dissolved* limiting substrates (i.e., dissolved organic carbon, inorganic nitrogen, and/or oxygen). All microbial kinetic models describing cell growth employ mathematical rate expressions that are functions only of dissolved electron donors or acceptors. However, 50%–80% of the organic carbon in a natural ecosystem or biological wastewater treatment reactor can be particulate (not passing through a 0.4 μm filter). Biopolymer particulates are defined for this project as intact bacterial cell mass, particulate cellular debris resulting from cell lysis, and extracellular polymeric floc (usually aggregates of lipopolysaccharides and glycoproteins) and thus excludes other forms of colloidal matter (e.g., cellulosic fibers, proteinaeous debris, insoluble hydrocarbons and inorganic precipitates).

Biopolymeric particulates can influence a microbial ecosystem by (1) introducing a new population into the community, (2) competing for substrates required by other bacteria, (3) serving as a source of novel genetic material, and (4) providing a source of nutrients (i.e., organic carbon and nitrogen) especially within an oligotrophic environment. The work reported here is concerned with the latter possibility, of whether there is cycling of organic nutrients within microbial cultures. Regrettably, very little is known about the turnover of cellular biomass in suspended cultures.

A series of articles by Mason et al. (1986a, b), Mason and Hamer (1987), and Bryers and Mason (1987) postulated the following scenario for the net accumulation of microbial cells. A primary soluble carbon energy substrate is metabolized by microbial cells forming more cells, soluble metabolic by-products and respired CO_2. Processes such as maintenance energy requirements detract from cell and by-product formation by intercellular carbon recycling. Energy dissipation can also occur by an external cycle involving the lysis of intact cells followed by (1) scavenging of released soluble organic carbon material by intact cells (i.e., cryptic growth) and (2) solubilization or particulate cellular debris followed by cryptic growth of intact cells on the solubilized material. Mason and Hamer (1987) have described these processes mathematically and experimentally assessed the rate of cryptic growth of the pure culture aerobe *Klebsiella pneumoniae* NC1B 418 on its own soluble intracellular constituents.

This work extends that of Mason and Hamer (1987) to the cycling of cellular carbon within an aerobic binary microbial culture. Unlike Mason and Hamer (1987), we report a protocol to account not only for the

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cryptic growth of each species but also the solubilization of cellular debris.

Materials and methods

Organisms. Pseudomonas putida ATCC 11172 was obtained in freeze-dried form every other month from the American Type Culture Collection (Rockville, MD) and subsequently maintained by monthly subculture on agar slants incubated at 27° C and stored at 4° C. A *Hyphomicrobium ZV620* reported by Gräzer-Lambart et al. (1986), was supplied courtesy of Prof. Dr. G. Hamer, Institute of Aquatic Sciences, Swiss Federal Institutes of Technology, Zürich, Switzerland, and maintained as for *P. putida*.

Cell counts. Total cell counts were determined by acridine orange staining and epifluorescence microscopic counting as described by Hobbie et al. (1977).

Cell cultivation. P. putida was grown in a continuously fed 2-l bioreactor, (Bioengineering, Wald, Switzerland) on a mineral salts medium described by Molin and Nilsson (1983). Glucose (3.0 g l^{-1}), sterilized separately by filtration, was the sole carbon energy substrate, limiting growth. Growth medium was autoclaved, cooled and then delivered continously to the bioreactor by a peristaltic pump. The reaction volume was maintained constant at 1 l. All continuous cultures were carried out at 28° C and pH 7.0. At each culture dilution rate, at least five residence times were allowed to pass to attain steady-state conditions. Dissolved oxygen concentration was measured continuously with an in situ probe and maintained above 30% saturation at all growth rates. Verification of monoculture conditions was done by periodic microscopic observation and by identification of single colonies using an API 20E test system (Analytab Products, Plainview, NY).

Hyphomicrobium sp. was grown in a separate identical 2-l bioreactor (1-l working volume) on a mineral salts medium (Gräzer-Lambart et al. 1986). Methanol (1 mg/l) was the sole carbon energy substrate, limiting growth. Chemostat operation was maintained over increasing dilution rates as described above. The temperature was kept at 28° C and the pH at 7.0. Again this system was maintained completely aerobic as indicated by a measured 35% saturation level.

Repeated chemostat experiments confirmed that *P. putida* did not metabolize methanol and, conversely, *Hyphomicrobium* sp. did not utilize glucose. *P. putida* cells for the experiments described below were taken from continuous culture at a dilution rate (D) of 0.20 h^{-1}; *Hyphomicrobium* sp. cells were withdrawn from cultures at $D = 0.09\ h^{-1}$.

Preparation of cryptic growth and particulate turnover experiment media. After a minimum three residence times at steady-state, exactly 150 ml cell suspension were taken from both the *P. putida* ($D = 0.2\ h^{-1}$) and the *Hyphomicrobium* sp. ($D = 0.09\ h^{-1}$) chemostat cultures. Cell suspensions were centrifuged at 4° C and 11000 *g* for 20 min (McEldowney and Fletcher 1987). Cell pellets were resuspended in 25 ml phosphate buffer (0.03 *M*, pH 6.9). Exactly 0.5 µCi L-[U-^{14}C]glucose (sp. act. 1–10 mCi/mmol) and 0.5 µCi [^{14}C]methanol/ml (sp. act. 30–60 mCi/mmol) (Sigma, St. Louis, MO, USA) were added to the *P. putida* and *Hyphomicrobium* sp. suspensions, respectively. Both suspensions were incubated at 25° C for 1 h to allow assimilation of the labelled substrate. Cells were centrifuged then washed four times in phosphate buffer to remove any non-assimilated labelled substrate. Cells were lysed by sonicating 150 ml-volumes for four 30-s bursts (*P. putida*) and four 3-min bursts (*Hyphomicrobium* sp.) using a Kontes Ultrasonic Cell Disrupter (Kontes Biotechnology, Vineland, NJ) (80 µm amplitude, 40 watts) which resulted in 96% removal of intact *P. putida* and 70% removal of intact *Hyphomicrobium* sp. cells. After sonication, cell suspensions were centrifuged at 2800 *g* for 20 min, then the supernatant was filtered under vacuum through 0.2 µm filter (Nuclespore, Pleasanton, Calif, USA) and added to 175 ml sterile phosphate buffer. In all cases, soluble non-assimilated substrate (glucose or methanol) was < 10% of the total soluble organic carbon measured after cell disruption. After sonication, centrifugation, and filtration, solutions containing the ^{14}C-labelled soluble organic material released from the disrupted cells served as the sole carbon substrate for subsequent cryptic growth experiments.

Biopolymeric particulate cell debris remaining after cell disruption in pellet form was resuspended in 200 ml sterile phosphate buffer and served as the only carbon substrate for subsequent particulate turnover experiments.

Batch cryptic growth experiments. Each experiment was run in duplicate with duplicate uninoculated controls. Further controls consisted of the inoculum described below, introduced into 175 ml carbon-free nutrient solutions, thus providing an estimate of the viable cell lysis rates for each species. Experiments were carried out in 200-ml erlenmeyer flasks, which were presteriized. Either cell-free solution (175 ml) containing soluble organic material released after cell sonication was added to four flasks within a laminar flow hood. Two flasks contained an organic solution originating from *P. putida* cell disruption (cells grown at $D = 0.2\ h^{-1}$) and two contained an organic solution originating from *Hyphomicrobium* sp. cell disruption (cells grown at $D = 0.09\ h^{-1}$). Either steady-state chemostat cell suspension (500 ml) was used as inoculum for the flasks. A *P. putida* inocula was added to two flasks containing, respectively, the soluble organic solution originating from disruption of *P. putida* and *Hyphomicrobium* sp. cells. A *Hyphomicrobium* sp. inocula was added to the remaining two flasks containing, respectively, soluble organic material from *P. putida* and *Hyphomicrobium* sp. disruption.

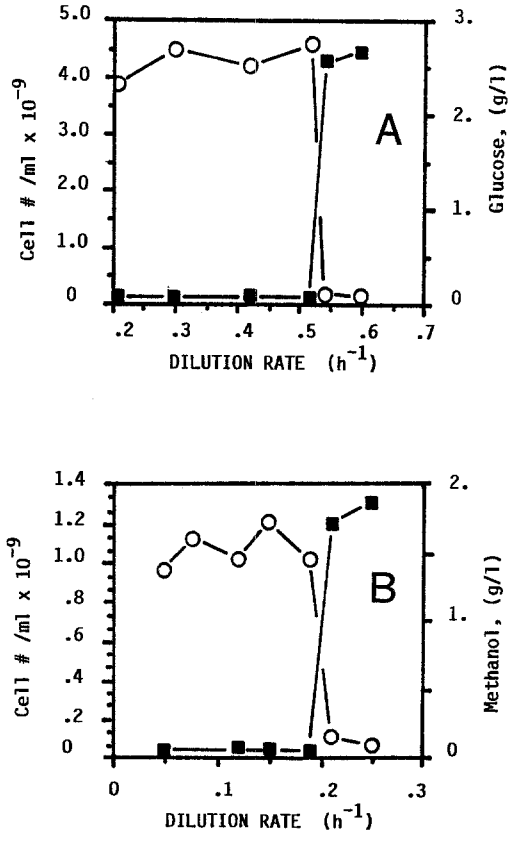

Fig. 1 A, B. Substrate and cell concentrations in continuous culture at 30° C. **A** *Pseudomonas putida* ATCC 11172. **B** *Hyphomicrobium* sp. ZV620

All flasks were mixed well on magnetic stirrers and maintained at 25° C throughout batch cultivation. Samples (2 ml) were taken hourly and analyzed for three different parameters: (1) epifluorescent cell counts (Hobbie et al. 1977), soluble ^{14}C-labelled organic compounds, and particulate ^{14}C-labelled organic material. Soluble material is defined here as that which passes through a 0.22 μm membrane filter (Nuclepore). Scintillation counts (disintegration per minute) were made using a Packard (Meriden, CT) 1900CA Tri-Carb Analyzer.

Particulate hydroylsis experiments. An identical series of experiments were carried out as above but using the resuspended particulate cell debris (see above) of either species as the only carbon substrate. Particulate cell debris of either *P. putida* or *Hyphomicrobium* sp. were resuspended in phosphate buffer and 175 ml of each placed in 200-ml erlenmeyer flasks. Again, 500 μl suspension of viable *P. putida* cells, taken from the chemostat ($D = 0.2\ h^{-1}$) was used to inoculate flasks containing either resuspended *P. putida* or *Hyphomicrobium* sp. cell debris. Similarly, 500 μl of *Hyphomicrobium* sp. viable cell culture ($D = 0.09\ h^{-1}$) was used to inoculate an identical set of flasks containing resuspended debris of *P. putida* and *Hyphomicrobium* spp. cells. Two different controls were also employed in these studies. The first control was particulate cell debris, remaining after sample sonication, resuspended in phosphate buffer as above but not inoculated. The second control was a replicate of those used in the cryptic growth studies, which consisted of inoculating with viable cells in phosphate buffer solution containing no organic carbon. Inoculated solutions were mixed well by magnetic stirrers and maintained at 25° C. Samples (2 ml) were taken every hour after inoculation and analyzed as above for cell count, soluble ^{14}C-labelled material and particulate ^{14}C-labelled material.

Glucose. Aqueous glucose concentrations were determined enzymatically using the hexokinase-based assay (kit no. 16-50) from Sigma.

Methanol. Aqueous methanol concentrations were determined using a Shimadzu GCA-9 gas chromatograph (Columbia, Md, USA) equipped with an 80/100 Carbopack C – 0.1% SP-1000 column and flame ionization detector.

Results

Steady-state chemostat studies

Suspended cell and effluent soluble substrate concentrations for the chemostat cultures of *P. putida* and *Hyphomicrobium* sp. are provided in Fig. 1A and 1B, respectively. Assuming a Monod-like dependency of rate on substrate concentration, the kinetic parameters are:

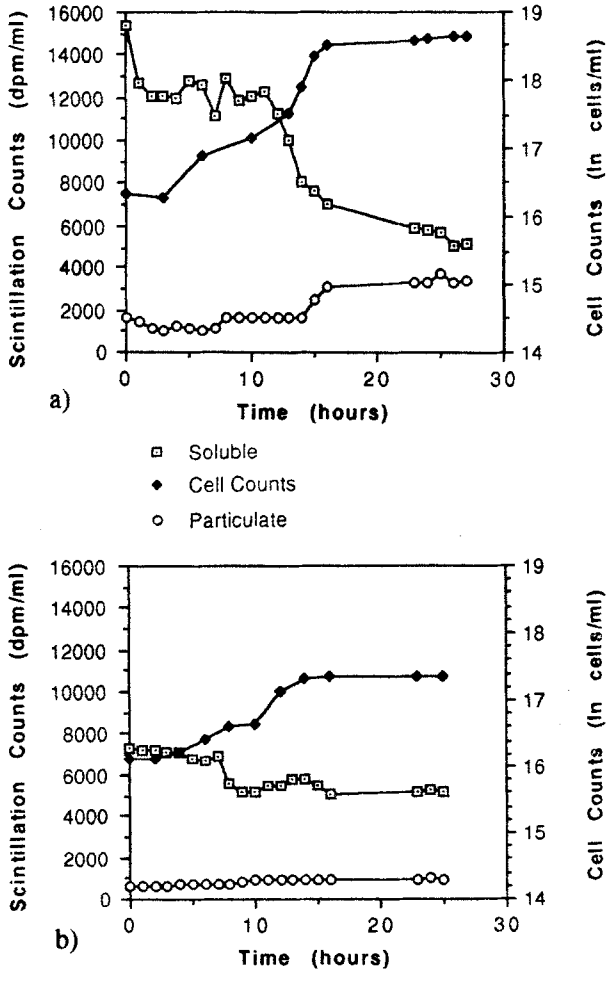

Fig. 2. Cryptic growth of *P. putida* on the soluble contents of **(A)** lysed *P. putida* cells and **(B)** lysed *Hyphomicrobium* sp. cells: dpm = disintegrations per minute

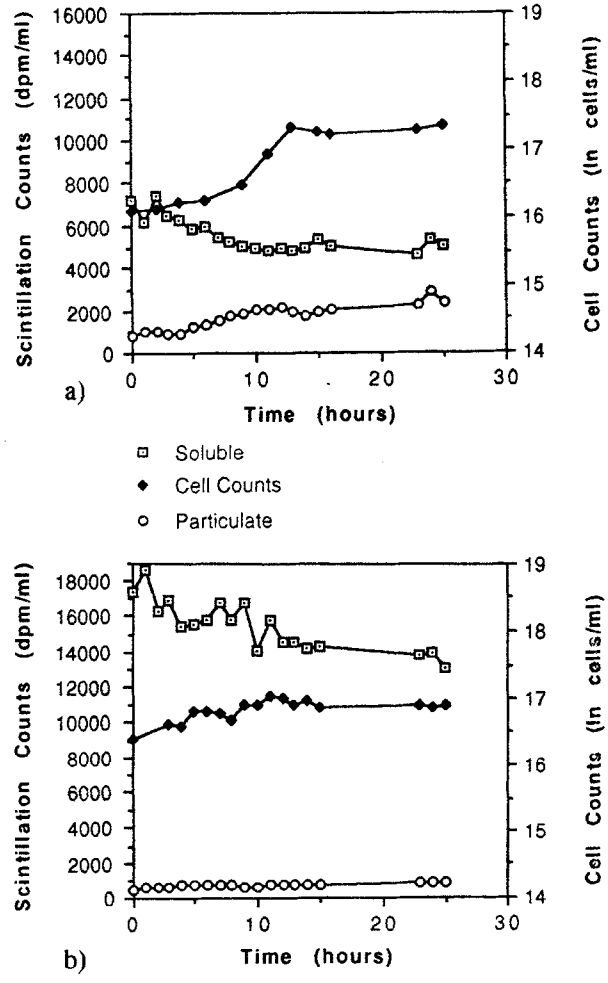

Fig. 3. Cryptic growth of *Hyphomicrobium* sp. on the soluble contents of **(A)** lysed *P. putida* cells and **(B)** lysed *Hyphomicrobium* sp. cells

for *P. putida* $\mu^* = 0.53\ h^{-1}$, ($t_g = 1.3$ h) $K_s = 0.01$ gm glucose l^{-1}, $Y_{x/s} = 0.40$ gm cell gm glucose^{-1}; and for *Hyphomicrobium* sp. $\mu^* = 0.21\ h^{-1}$ ($t_g = 3.3$ h), $K_s = 5 \times 10^{-6}$ gm methanol l^{-1}, $Y_{x/s} = 0.30$ gm cell gm methanol^{-1}, where μ^* = maximum specific growth rate; K_s = saturation coefficient and $Y_{x/s}$ = biomass yield.

Cryptic growth batch studies

All cryptic growth experiments were carried out in duplicate. Experimental results are reported for *P. putida* cultivated on the soluble contents of lysed ^{14}C-labelled *P. putida* (Fig. 2A) and *Hyphomicrobium* sp. (Fig. 2B) and *Hyphomicrobium* sp. cultivated on the soluble contents of lysed ^{14}C-labelled *P. putida* (Fig. 3A) and *Hyphomicrobium* sp. (Fig. 3B). The ^{14}C-labelled soluble organic contents of lysed cells and the resultant concentrations of particulate (cellular) biomass incorporating the ^{14}C label and total intact cell concentrations were determined throughout each experiment.

The results in Fig. 2A and 2B show that viable *P. putida* can metabolize 30%–60% of the soluble organic material released by sonication. Figure 3A and 3B show that viable *Hyphomicrobium* spp. could metabolize only 20%–30% of the soluble organic material released by sonication, irrespective of its origin. Similarly, Mason and Hamer (1987) reported that *K. pneumoniae* was able to metabolize only 50%–60% of the initial organic material released from sonicated *K. pneumoniae* cells. Tacitly, this would imply a portion of the intracellular soluble content of bacteria is not readily metabolized. Another explanation could be that a nutrient other than organic carbon (e.g., nitrogen) may be limiting the cryptic growth process.

Based on total cell concentration, the maximum cell doubling times measured for both species growing on its own soluble lytic products, or that of the other species, are summarized in Table 1.

Particulate hydrolysis batch studies

All particulate growth experiments were carried out in duplicate in batch reactors. Particulate cell debris of either ^{14}C-labelled *P. putida* or *Hyphomicrobium* sp. remaining after sonication and centrifugation was used as the sole carbon source for *P. putida* and *Hyphomicrobium* sp. inoculum. The experimental results are reported for *P. putida* cultivated on particulate cellular debris of *P. putida* (Fig. 4A) and *Hyphomicrobium* sp. (Fig. 4B) and *Hyphomicrobium* sp. grown on particulate debris from *P. putida* (Fig. 5A) and *Hyphomicrobium* sp. (Fig. 5B). Any ^{14}C-labelled particulate concentration, the concentration of any ^{14}C-labelled soluble organics resulting from hydrolysis, and the resultant total cell concentrations (by epifluorescence) (Figs. 4, 5) were determined periodically throughout each experiment.

In all cases, the soluble organic material containing the ^{14}C label remained very low in concentration and essentially constant throughout each experiment. Particulate organic material exhibited no clear trend, appearing essentially constant in all four experimental series. A decrease in particulate concentration is expected if viable cells are solubilizing the particles. However,

Table 1. Summary of kinetic parameters for *Pseudomonas putida* (P.p.) and *Hyphomicrobium* sp. (HM)

Reactor system	Microbial culture	Limiting substrate	Sustrate form	Maximum observed growth rate (h^{-1})	Saturation coefficient	Observed yield
Chemostat	*P. putida*	Glucose	Soluble	0.55	10.0 mg glucose/1	0.42 gm cells/gm glucose
	Hyphomicrobium sp.	Methanol	Soluble	0.21	0.01 mg methanol/1	0.30 gm cells/gm methanol
Cryptic growth studies:				Maximum generation time, t_g (h)		
Batch	*P. putida*	P. p. cell contents	Soluble	2.0	NA	$7.23 \times 10^{+5}$ cells/mg TOC
	P. putida	HM cell contents	Soluble	6.3	NA	$1.8 \times 10^{+6}$ cells/mg TOC
	Hyphomicrobium sp.	P. p. cell contents	Soluble	11.6	NA	$1.8 \times 10^{+4}$ cells/mg TOC
	Hyphomicrobium sp.	HM cell contents	Soluble	4.3	NA	$1.7 \times 10^{+6}$ cells/mg TOC
Particulate turnover studies						
Batch	*P. putida*	P. p. cell debris	Particulate	No growth detected		
	P. putida	HM cell debris	Particulate	No growth detected		
	Hyphomicrobium sp.	P. p. cell debris	Particulate	No growth detected		
	Hyphomicrobium sp.	HM cell debris	Particulate	No growth detected		

NA = not applicable; TOC = total organic carbon

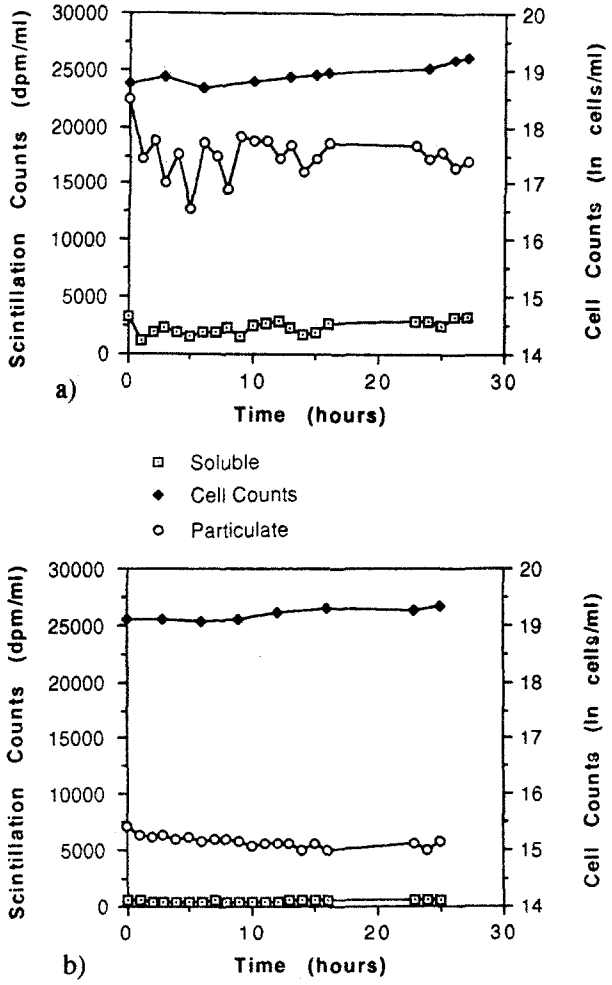

Fig. 4. *Pseudomonas putida* batch cultures utilizing as the only carbon source the particulate debris of lysed **(A)** *P. putida* cells and **(B)** *Hyphomicrobium* sp. cells

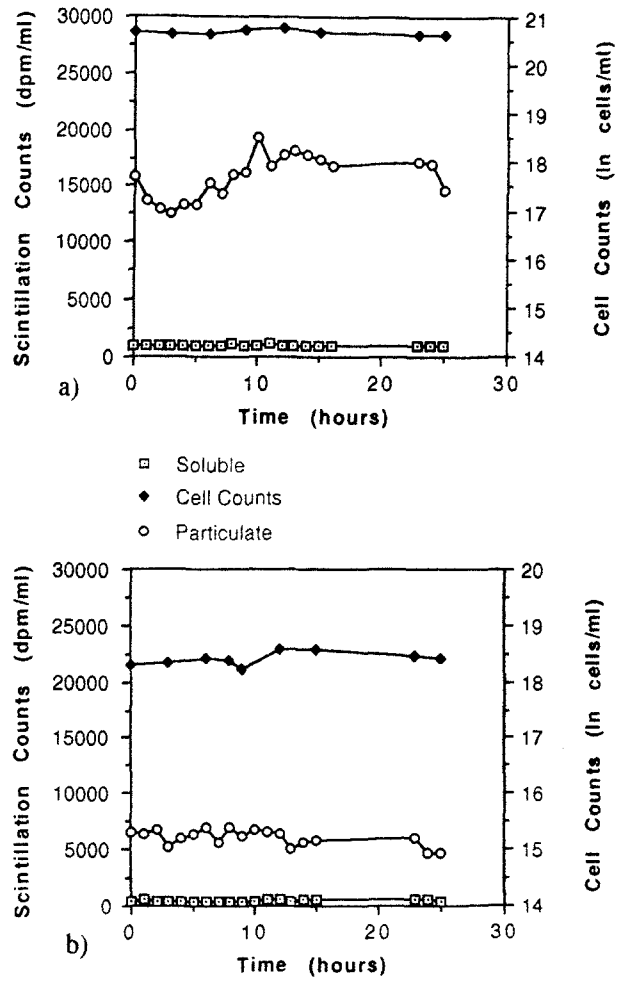

Fig. 5. *Hyphomicrobium* sp. batch cultures utilizing as the only carbon source the particulate debris of lysed **(A)** *P. putida* cells and **(B)** *Hyphomicrobium* sp. cells

the ^{14}C-label method cannot distinguish between the original particulates and resultant intact cells. Thus, an increasing total cell concentration could offset a decreasing particulate concentration, making the measured particulate ^{14}C-label appear roughly constant. Based on epifluorescence counts, cell numbers did not increase during the course of either experiment. No significant increase in soluble organic carbon labelled with ^{14}C was observed, implying that hydrolysis of labelled cell debris did not occur.

Discussion

Death and lysis of microorganisms have been critically reviewed by Mason et al. (1986a) and cryptic growth experimentally studied in pure cultures of *K. pneumoniae* by Mason and Hamer (1987). They reported that *K. pneumoniae* is able to grow on its own soluble lysis products at batch growth rates ranging between 0.69–1.46 h^{-1} at biomass yield values between 0.42–0.52 mg cell C/mg substrate C. Of the dissolved organic carbon released upon sonication, they reported that only 37%–51% was biodegradable by *K. pneumoniae*. Mason and Hamer (1987) suggested cryptic growth is significant in growing microbial cultures, especially for those microbes that exhibit utilization of a broad spectrum of carbon substrates. This argument is reinforced by a study of Drozd et al. (1978) using an obligate methanotrophic bacterium, *Methylococcus* sp.; their claim is that the obligate methane dependence of this bacterium precludes the cryptic metabolism of other complex reduced carbon compounds.

Research here extends the experiments of Mason and Hamer (1987) to the growth of two bacteria, *P. putida* and *Hyphomicrobium* sp., on their own soluble lysis products, and on the soluble lysis products of the other species. Cryptic growth studies indicate that both *P. putida* and *Hyphomicrobium* sp. (an obligate methylotroph) were capable of cryptic growth on their own soluble lysis products. That *P. putida* and *Hyphomicrobium* sp. exhibited near maximum growth rates on the products of their own soluble cell contents ($t_g = 2.0$ h for *P. putida* and $t_g = 4.3$ h for *Hyphomicrobium* sp.) is consistent with the findings of Mason and Hamer (1987). Additionally, the radiolabelling technique de-

scribed here is able to detect *Hyphomicrobium* sp. metabolism of soluble lysis products from either species ($t_g = 11.6$ h on *P. putida* cell contents; $t_g = 4.3$ h on *Hyphomicrobium* sp. contents) despite the strict obligate dependence of *Hyphomicrobium* sp. on soluble methanol. In either series of experiments above, *P. putida* and *Hyphomicrobium* sp. were able to metabolize, respectively, only 50% and 25% of the initial soluble organic concentration. It remains to be seen whether the recalcitrance of this residual organic carbon is due to either (1) a limitation on further growth by insufficient quantities of nutrients other than carbon (i.e., nitrogen) or (2) the chemical nature of the specific residual organic compounds.

Particulate hydroylsis and the subsequent metabolism of soluble organic material were studied in the particulate turnover experiments for both *P. putida* and *Hyphomicrobium* sp. From the data derived here, it is unlikely that the particulate lysis products would contribute significantly to the overall activity in a microbial culture, except in low growth rate systems, such as in aerobic digestion.

The work here reinforces that of Mason and Hamer (1987), suggesting that cryptic growth on soluble lysis products is rapid and need not be restricted by obligate substrate limitation. The literature has modeled death, lysis, cryptic growth and particulate solubilization collectively as "cell decay" or "endogenous metabolism". Results here indicate that "cell decay" can be treated as an initially slow "lysis" step (avoided here using sonication) followed sequentially by two parallel processes, (1) rapid scavenging of soluble lysis prodcuts by the remaining viable cells and (2) the very slow degradation of cellular particulate debris.

Acknowledgements. We would like to thank Dr. C. A. Mason, Institute for Aquatic Sciences, Eidgenössische Technische Hochschule/EAWAG, Zürich, Switzerland, for his comments during the preparation of this paper. This work was supported by the North Carolina Biotechnology Center under grant 86-G-02126.

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