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Isotope Labeling and Microautoradiography of Active Heterotrophic Bacteria on the Basis of Assimilation of $^{14}CO_2$

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Most heterotrophic bacteria assimilate CO_2 in various carboxylation reactions during biosynthesis. In this study, assimilation of $^{14}CO_2$ by heterotrophic bacteria was used for isotope labeling of active microorganisms in pure cultures and environmental samples. Labeled cells were visualized by microautoradiography (MAR) combined with fluorescence in situ hybridization (FISH) to obtain simultaneous information about activity and identity. Cultures of *Escherichia coli* and *Pseudomonas putida* assimilated sufficient $^{14}CO_2$ during growth on various organic substrates to obtain positive MAR signals. The MAR signals were comparable with the traditional MAR approach based on uptake of ^{14}C-labeled organic substrates. Experiments with *E. coli* showed that $^{14}CO_2$ was assimilated during both fermentation and aerobic and anaerobic respiration. The new MAR approach, HetCO_2-MAR, was evaluated by targeting metabolic active filamentous bacteria, including "*Candidatus* Microthrix parvicella" in activated sludge. "*Ca.* Microthrix parvicella" was able to take up oleic acid under anaerobic conditions, as shown by the traditional MAR approach with [^{14}C]oleic acid. However, the new HetCO_2-MAR approach indicated that "*Ca.* Microthrix parvicella," did not significantly grow on oleic acid under anaerobic conditions with or without addition of NO_2^-, whereas the addition of O_2 or NO_3^- initiated growth, as indicated by detectable $^{14}CO_2$ assimilation. This is a metabolic feature that has not been described previously for filamentous bacteria. Such information could not have been derived by using the traditional MAR procedure, whereas the new HetCO_2-MAR approach differentiates better between substrate uptake and substrate metabolism that result in growth. The HetCO_2-MAR results were supported by stable isotope analysis of ^{13}C-labeled phospholipid fatty acids from activated sludge incubated under aerobic and anaerobic conditions in the presence of $^{13}CO_2$. In conclusion, the novel HetCO_2-MAR approach expands the possibility for studies of the ecophysiology of uncultivated microorganisms.

Several isotope-based methods have been introduced in recent years for cultivation-independent characterization of active microorganisms in environmental samples. The novel methodologies include direct isotope analysis of extracted biomarkers, including amino acids, fatty acids, and nucleic acids (9, 10, 24, 32, 36), stable isotope probing (SIP) of DNA or RNA (25, 33), and a new isotope microarray (1). Microautoradiography (MAR) in combination with fluorescence in situ hybridization (FISH) has also been developed for cultivation-independent identification of active bacteria in environmental matrices (3, 21, 29, 30). When targeting heterotrophic bacteria, the traditional MAR approach has been based on the addition of typically ^{14}C- or ^{3}H-labeled organic substrates to environmental samples under defined incubation conditions. Labeled substrate and/or labeled degradation products are then taken up by active heterotrophs and often assimilated into various biomass components. MAR based on inorganic $^{14}CO_2$ as the labeled precursor has been used successfully for years to target autotrophic organisms, e.g., chemolithotrophic nitrifiers from activated sludge (21) and autotrophic *Achromatium* cells from freshwater sediments (18). Combined with FISH, the current MAR approach often provides excellent information about activity and identity at the single-cell level in complex environments.

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Advances in isotope labeling strategies may further expand the potential applications of the MAR approach. For example, isotope labeling of metabolic active heterotrophic bacteria may be improved by using $^{14}CO_2$ as isotope source. This suggestion is based on the old observation that most, if not all, heterotrophic organisms assimilate CO_2 during biosynthesis in various carboxylation reactions induced by enzymes such as pyruvate carboxylase, phosphoenolpyruvate carboxylase, coenzyme A carboxylase, etc. (5, 13, 41). This phenomenon, often described as "heterotrophic CO_2 assimilation," has been used previously for quantification of microbial activity in environmental samples (35, 38, 39), as a measurer of perturbations by xenobiotic compounds (20), and for autoradiographic detection of growing bacteria (34). The majority of these studies on heterotrophic CO_2 assimilation have been inspired by Romanenko (35). However, heterotrophic CO_2 assimilation has received somewhat less attention recently in microbial ecology.

Assimilation of $^{14}CO_2$ in heterotrophic bacteria was visualized by autoradiography as early as 1961, but the scale of autoradiography at that time was reported to range between 0.1 and 1 mm (34). With the tools available today, the resolution of MAR is around a single cell (ca. 1 μm). Hence, $^{14}CO_2$ labeling combined with visualization by MAR-FISH may provide new insights regarding the function and identity of uncultivated heterotrophs.

Some filamentous bacteria in activated sludge, including "*Candidatus* Microthrix parvicella" are extremely difficult to isolate (7, 40). "*Ca.* Microthrix parvicella" is common in activated sludge wastewater treatment systems, where it causes

serious foam problems (4, 7). Unfortunately, the organism is hard to grow and the physiology is poorly understood which has made it difficult to establish efficient control measures. Different in situ techniques including the traditional MAR approach has shown that "*Ca.* Microthrix parvicella" grows mainly (or only) on long-chain fatty acids such as oleic acid (4, 28). Furthermore, "*Ca.* Microthrix parvicella" can accumulate oleic acid and presumably form storage compounds under anaerobic conditions without nitrite and nitrate present. Under subsequent aerobic conditions, these storage compounds may be used to support growth. However, it remains unclear whether "*Ca.* Microthrix parvicella" can use nitrite or nitrate as electron acceptor or to what extent oleic acid is used as a growth substrate under anaerobic conditions in the absence of nitrite or nitrate. These are questions of potential great importance for understanding the competitiveness of the organism in activated sludge systems. However, these questions are difficult to address by using current in situ techniques, including the traditional MAR-method.

In the present study, we examined assimilation of $^{14}CO_2$ by *Escherichia coli* and *Pseudomonas putida* in order to optimize conditions for single-cell detection by FISH combined with MAR (HetCO$_2$-MAR). Subsequently, the HetCO$_2$-MAR approach was used for studying the physiology of "*Ca.* Microthrix parvicella" in activated sludge under different substrate and electron acceptor regimes.

MATERIALS AND METHODS

Isotope labeling of pure cultures. Pure cultures of *E. coli* ATCC 25922 and *P. putida* R1 (26) were grown in liquid mineral medium (LM medium) modified from MacDonald and Spokes (23) with the following composition (per liter): $(NH_4)_2SO_4$, 0.13 g; KH_2PO_4, 0.2 g; $CaCl_2 \cdot 2H_2O$, 20 mg; $MgSO_4 \cdot 7H_2O$, 40 mg; FeNaEDTA, 3.8 mg; HEPES buffer, 4.8 g; and trace element solution (14), 1 ml. The medium was adjusted to pH 7.5 with 10 M NaOH and autoclaved. Various different electron donors and electron acceptors were added from sterile filtered stock solutions before inoculation.

Cells applied for isotope labeling were harvested from fresh cultures grown in LM medium (25°C, 150 rpm in the dark) with the same energy substrate as added during isotope labeling. After being harvested, the cells were resuspended in fresh LM medium to a final optical density measured at 600 nm of 0.5 (equivalent to ca. 2×10^8 cells ml^{-1} derived from microscopically enumeration) and then incubated on the bench for 1 h prior to the simultaneous addition of isotope, electron acceptor, and electron donor.

$NaH^{14}CO_3$ (58 mCi mmol^{-1}; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) was added to 2 ml of cell suspension (described above) in 9.5-ml glass vials (25 μCi ml^{-1}; 0.4 mM $H^{14}CO_3^-$). Immediately after addition of $H^{14}CO_3^-$, the growth was initiated by the addition of electron donor (3 mM glucose, 6 mM pyruvate, 9 mM acetate, or 0.5 g of yeast extract liter^{-1}) and electron acceptor (10 mM NO_3^-). Glass vials were sealed with gas-tight thick rubber stoppers, and finally the oxygen regime was adjusted. Anaerobic conditions were obtained by repeated evacuation of headspace prior to isotope addition and subsequent injection of oxygen free N_2 (99.999% purity). In parallel, cells were labeled with [^{14}C]glucose (310 mCi mmol^{-1}; Amersham Pharmacia Biotech), [^{14}C]pyruvate (20 mCi mmol^{-1}; American Radiolabeled Chemicals, Inc., St. Louis, Mo.), and [^{14}C]acetate (57 mCi mmol^{-1}; Amersham Pharmacia Biotech), with the same total substrate concentration as in the samples with radiolabeled $H^{14}CO_3^-$, except that the isotope concentration was only 5 μCi ml^{-1}. Incorporation of radioactive material during incubation was monitored by using filter count (described below).

Isotope labeling of activated sludge samples. Activated sludge and foam (mainly filamentous bacteria) was collected at the Mou wastewater treatment plant, located 20 km east of Aalborg, Denmark, and stored overnight at 4°C. The activated sludge was diluted and mixed with foam to at final content of suspended solids of 2 g liter^{-1}. The mixture contained many filamentous organisms, and it was almost exclusively "*Ca.* Microthrix parvicella" (>90%) as determined by FISH according to the method of Erhart et al. (16). In some experiments anaerobic conditions were applied, here defined as the absence of oxygen, nitrite, and nitrate. If any nitrite or nitrate was present, the sample was incubated without oxygen until nitrite and nitrate disappeared (monitored with test stickers from Merck, Darmstadt, Germany). MAR experiments were incubated with 0.17 μCi of [1-^{14}C]oleic acid ml^{-1} (57 mCi mmol^{-1}; Amersham-Pharmacia Biotech) or 27.5 μCi of $H^{14}CO_3^-$ ml^{-1} (Amersham-Pharmacia Biotech), which is equivalent to 0.5 mM $H^{14}CO_3^-$. Unlabeled oleic (1 mM) was added to all samples. The background concentration of unlabeled bicarbonate in the activated sludge was ca. 4 mM. All incubations were conducted at 21°C on a rotary table at 150 rpm. All incubations were carried out in 9-ml serum bottles using a final volume of 2.0 ml. Anaerobic conditions were obtained by repeated evacuation of headspace and subsequent injection of N_2 (99.999% purity). In some experiments electron acceptor (2 mM NO_3^-, 1 mM NO_2^-, or atmospheric O_2) was added after 3 h of incubation. Pasteurized sample (70°C, 10 min) was applied as a negative control. Incorporation of radioactive material was monitored by using filter count as described below.

After 6 h of isotope labeling, all samples were fixed in 4% paraformaldehyde and stored at −20°C in 50% ethanol and 50% phosphate-buffered saline for later MAR analysis as described previously (21).

Parallel samples were incubated with $NaH^{13}CO_3$ (99 atom% ^{13}C; Cambridge Isotope Laboratories, Andover, MS) or [18-^{13}C]oleic acid (99 atom% ^{13}C; Sigma-Aldrich, Milwaukee, Wis.) with the same additions of electron acceptors as described above for the radiolabeled compounds. In addition, samples were labeled with $^{13}CO_2$ in the presence of 5 mg of allylthiourea (ATU) liter^{-1}. ATU is an inhibitor of autotrophic ammonia oxidation (6). All samples labeled with stable isotopes were fixed in methanol and dichloromethane for later extraction and analysis of ^{13}C-labeled phospholipid fatty acids (PLFAs; see below).

Quantification of radioactive incorporation (filter count). To avoid loss of radioactive CO_2, all samples were taken through gas-tight rubber stoppers with syringes. Subsamples of suspensions (100 μl) from radioactive incubations (pure culture or activated sludge) were filtered through 0.2-μm-pore-size mixed cellulose filter (Advantec MFS, Inc., Pleasanton, Calif.). Subsequently, 5 ml of 0.1 N HCl was added to the filtration unit. After 3 min of acidification, the acid was washed through the filter, and the filter was immediately transferred to a scintillation vial (20 ml) and dissolved in 10 ml of scintillation fluid (Filter-Count; Packard, Groningen, The Netherlands).

Specific radioactivity in the inorganic carbon pool. For selected samples, an index of specific radioactivity in the inorganic carbon pool was calculated as the ratio between $^{14}CO_2$ and the total CO_2 in the headspace after acidification. The total concentration of inorganic carbon in the samples (LM medium or activated sludge) was determined by headspace gas chromatography after acidification as described previously (11). The radioactive $^{14}CO_2$ was quantified in headspace samples after acidification by trapping CO_2 in ethyleneglycolmonomethylether-ethanolamine (7:1). Trapped CO_2 was transferred to 20-ml polyethylene scintillation vials (Packard). The radioactivity was quantified after the addition of 10 ml of scintillation cocktail (Ultima Gold XR; Packard) by liquid scintillation counting (Packard 1600 TR; Packard).

MAR-FISH. MAR and FISH were carried out as previously described (3, 21) with minor modifications. Briefly, the fixed samples were washed thoroughly in 0.1 N HCl and distilled water. Prior to hybridization, small subsamples were transferred to gelatin-coated coverslips (24 by 60 mm) and immobilized by drying them at 50°C. The samples were briefly rinsed with distilled water to remove precipitates and hybridized with a mixture of fluorescently labeled (Cy3) oligonucleotide probes (Thermo Hybaid, Ulm, Germany) targeting all known *Bacteria* (12) as described previously (2, 12). In some samples the MAR signal from filamentous bacteria was quantified manually by enumeration of silver grains as described by Nielsen et al. (27).

^{13}C-PLFA extraction and analysis. Microbial lipids from activated sludge were analyzed for the abundance of ^{13}C after incubation with $NaH^{13}CO_3$ or [^{13}C]oleic acid. Lipids were extracted by using a mixture of dichloromethane and methanol as described previously (37). Phospholipids (polar lipids) were separated from other extractable lipids by silicic acid column chromatography and then subjected to mild alkaline methanolysis to form fatty acid methyl esters (37). ^{13}C-PLFA methyl esters were then analyzed on a Finnigan Delta Plus XL gas chromatograph combustion isotope ratio mass spectrometer (ThermoQuest, Bremen, Germany). The gas chromatograph (Hewlett-Packard 6890) was equipped with a HP-5MS column (60 m by 0.25 mm [inner diameter]), and a GC/C III combustion interface. He was used as the carrier gas. Fatty acids were identified and named as described previously (19). $\delta^{13}C$ values were determined based on authentic standards certified relative to the international standard PeeDee Belemnite.

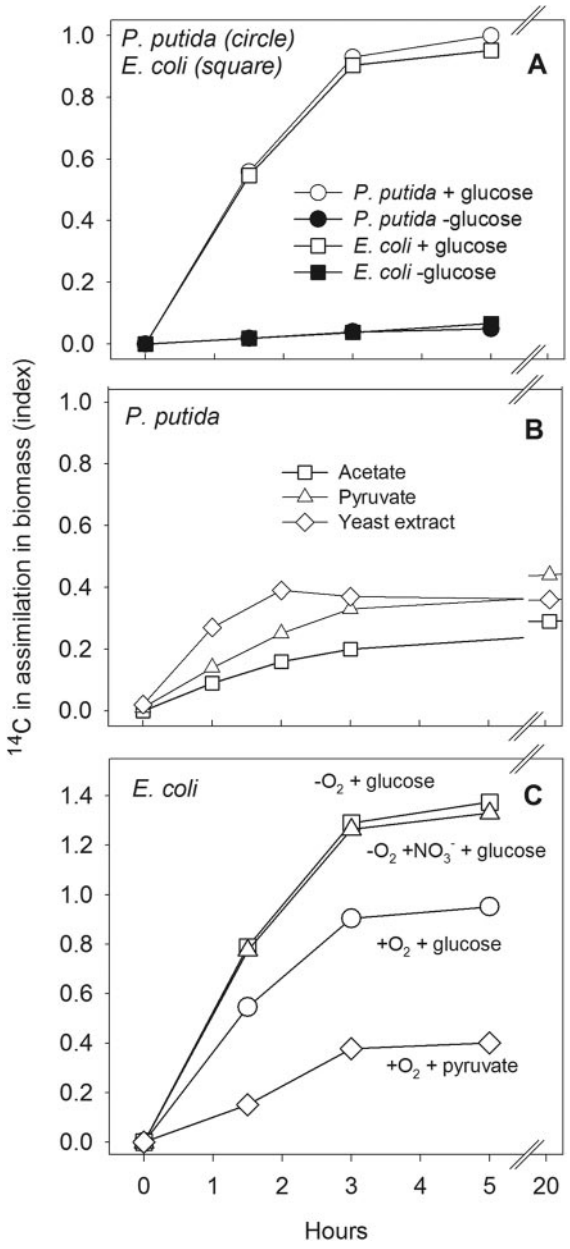

FIG. 1. Isotope labeling of *P. putida* and *E. coli* with $^{14}CO_2$ in the presence of different substrates and oxygen regimes. *P. putida* after 5 h with glucose is defined as index 1.

RESULTS

Isotope labeling of test cultures. Assimilation of $^{14}CO_2$ by *E. coli* and *P. putida* was measured under different growth conditions to examine the effects of selected electron donors and organic substrates on CO_2 assimilation (Fig. 1). To facilitate comparisons of strains, substrates, and electron acceptors, the maximum assimilation observed when growing *P. putida* aerobically on glucose (3 mM) is defined as index value 1. The absolute amount of $^{14}CO_2$ assimilated by *P. putida* at index 1 was equivalent to assimilation of ca. 8% of the added $^{14}CO_2$ in 5 h (2 μCi ml^{-1}). With an initial cell density of 2×10^8 cells ml^{-1}, the average cell specific isotope labeling was $\sim 10^{-14}$ Ci cell^{-1} (considering equal assimilation of isotope among cells). From our experiences this is more than 1 order of magnitude above the experimental detection limit for MAR on single cells with an exposure time of 3 to 4 days.

E. coli and *P. putida* assimilated nearly the same amount of $^{14}CO_2$ when grown under comparable conditions with 3 mM glucose (Fig. 1A). There was hardly any $^{14}CO_2$ assimilation in parallel samples incubated without glucose (less than index 0.06). $^{14}CO_2$ assimilation by *P. putida* varied slightly depending on which organic substrate was used for growth (Fig. 1B). Concentrations of acetate, pyruvate, and yeast extract were adjusted to obtain approximately the same concentration of organic carbon as in the glucose treatments shown in Fig. 1A (see Materials and Methods for details). All organic substrates supported $^{14}CO_2$ assimilation, although the activity was lower than observed for glucose (less than index 0.4). No significant changes in net $^{14}CO_2$ assimilation were observed by extending the incubation period from 3 to 20 h (Fig. 1B). The observed $^{14}CO_2$ assimilation was comparable or greater than in parallel samples incubated without $^{14}CO_2$ in the presence of [^{14}C]glucose, [^{14}C]acetate, or [^{14}C]pyruvate. In these experiments, the maximum isotope labeling was index 0.6 after 9 h of incubation with [^{14}C]glucose (data not shown).

When *E. coli* was grown anaerobically on glucose under fermentative conditions or with NO_3^- as electron acceptor (Fig. 1C), significant amounts of $^{14}CO_2$ was also assimilated. No difference was seen between anaerobic samples grown with or without addition of NO_3^- as electron acceptor. The amount of $^{14}CO_2$ incorporated under anaerobic conditions with glucose as substrate was greater than under aerobic conditions (Fig. 1C). *E. coli* was also grown aerobically with pyruvate (Fig. 1C), and the assimilation of $^{14}CO_2$ after 3 h was close to index 0.4, which is comparable to what was observed for *P. putida* (Fig. 1B).

Differences in growth rates, CO_2 assimilation activity, and isotope dilution resulted in variations in cell-specific $^{14}CO_2$ incorporation under the different incubation conditions. Incubation for 3 h with glucose and $^{14}CO_2$ resulted in a cell-specific radioactivity of ca. 0.6×10^{-14}, 1.0×10^{-14}, or 1.2×10^{-14} Ci cell^{-1} for cells incubated under aerobic conditions, anaerobic conditions with nitrate as electron acceptor, or fermentative conditions, respectively. Nonlinear dilution of $^{14}CO_2$ by $^{12}CO_2$ produced during mineralization of the added organic substrates made it very difficult to quantify exactly the amount of total CO_2 incorporated during the different incubation conditions. For example, the isotope was diluted to 15 and 17% of the initial specific activity during aerobic incubation of *P. putida* and *E. coli*, respectively, for 3 h with glucose as the substrate. Anaerobic incubation of *E. coli* with glucose and NO_3^- resulted in a reduction to 25% of the specific activity, whereas incubation under fermentative conditions diluted the isotope to 38% of the initial specific activity. Regardless, the amounts of $^{14}CO_2$ assimilated into bacterial cells during the experiments (10^{-15} to 10^{-14} Ci cell^{-1}) were sufficient to allow clear visualization by MAR with an exposure time of 3 to 5 days (see below).

MAR of pure cultures. An example of MAR-positive cells is shown in Fig. 2A, where *P. putida* was grown aerobically on glucose in the presence of $^{14}CO_2$. As a relative strong beta-emitter, the incorporated ^{14}C produces silver grains several

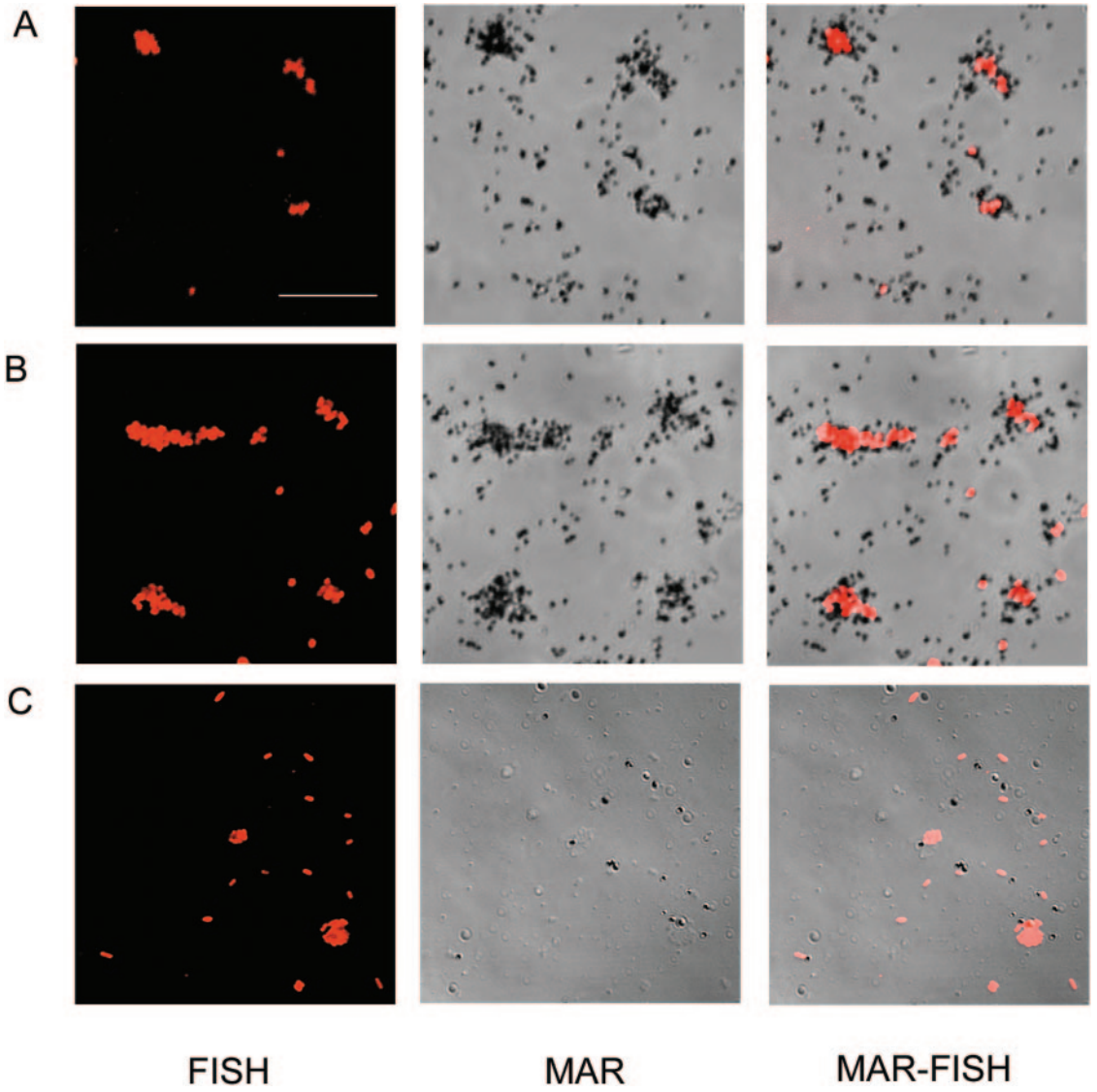

FIG. 2. FISH, MAR, and superimposed MAR-FISH of *P. putida* after incubation with $^{14}CO_2$. Glucose (A) and yeast extract (B) were used as substrates. (C) Negative control without any organic substrate addition. All samples were incubated at aerobic conditions. Scale bar, 10 μm.

microns from the labeled cells, but most silver grains could be detected on top or in the very proximity of individual cells. *P. putida* was also MAR positive when grown on yeast extract (Fig. 2B), pyruvate, or acetate (images not shown). Furthermore, the absence of organic substrate severely reduced $^{14}CO_2$ assimilation, and no MAR-positive cells were observed (Fig. 2C).

E. coli was MAR positive after both aerobic (Fig. 3A) and anaerobic (Fig. 3B) growth on glucose in the presence of $^{14}CO_2$. It is clearly seen that the MAR signal from *E. coli* labeled under anaerobic fermenting conditions was at least as strong as the signal from cells labeled under aerobic conditions. This is in agreement with the biomass labeling results shown in Fig. 1C. Positive MAR signals were also seen for *E. coli* grown with pyruvate and $^{14}CO_2$ (image not shown). *E. coli* was MAR-negative in the absence of added organic substrates (Fig. 3C).

$^{14}CO_2$ assimilation by activated sludge. Activated sludge with a high occurrence of foam-forming filamentous "*Ca.* Microthrix parvicella" was investigated for $^{14}CO_2$ assimilation under different growth conditions to identify factors important for the metabolic activity of the bacterial population. Oleic acid (1 mM) was added to the sludge, together with $^{14}CO_2$. Under aerobic conditions, a significant assimilation was observed, indicating heterotrophic CO_2 assimilation taking place in the activated sludge (evaluated by filter counts). After 7 h, the isotope labeling of biomass in the activated sludge was quantified to 0.43 mCi g of suspended solids^{-1}. This corresponded to an assimilation of 3.1% of the added $^{14}CO_2$ in the presence of 1 mM oleic acid. In contrast, very little $^{14}CO_2$ was assimilated under anaerobic conditions (3 h), indicating insignificant heterotrophic CO_2 assimilation in the presence of oleic acid (0.04 mCi g of suspended solids^{-1}). However, when the anaerobic activated sludge was subsequently exposed to either

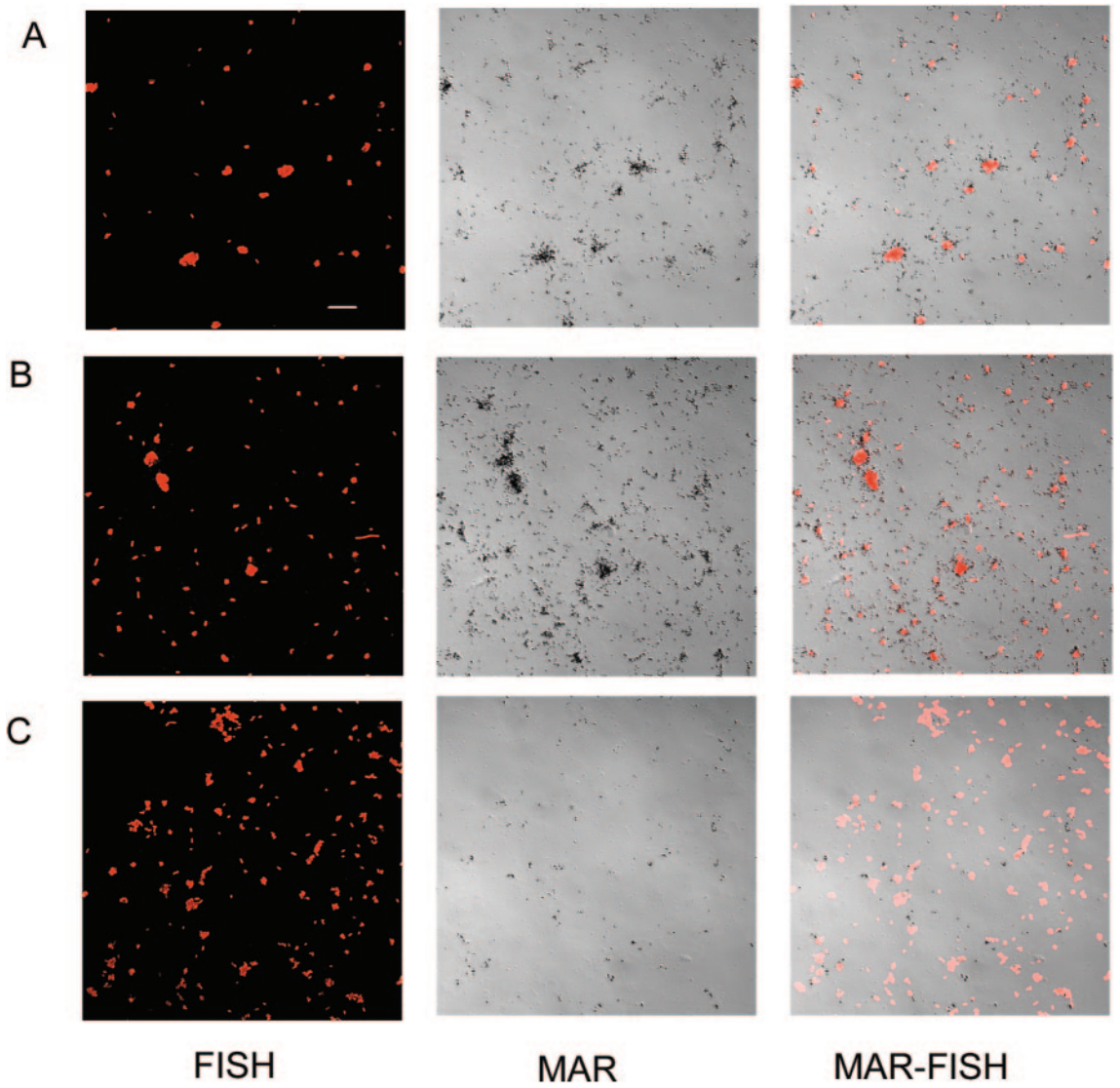

FIG. 3. MAR and FISH and superimposed MAR-FISH of *E. coli* after incubation with $^{14}CO_2$. Glucose was used as energy substrate in the presence (A) or absence (B) of oxygen. (C) Negative control without any organic substrates added. Scale bars, 10 μm.

oxygen or nitrate, substantial amounts of $^{14}CO_2$ were assimilated into biomass (0.36 and 0.42 mCi g of suspended solids^{-1}, respectively). The addition of NO_2^- after 3 h under anaerobic conditions induced assimilation of only 0.11 mCi g of suspended solids^{-1}. Negative control with pasteurized sludge assimilated no detectable $^{14}CO_2$ (<0.001 mCi g of suspended solids^{-1}).

MAR of activated sludge. Two types of MAR approaches were conducted in order to reveal different physiological features of "*Ca.* Microthrix parvicella." In the first experiment, a traditional MAR approach was carried out with [^{14}C]oleic acid as the radiolabeled substrate. [^{14}C]oleic acid was added to activated sludge for 4 h under aerobic and anaerobic conditions. Under both conditions, filamentous "*Ca.* Microthrix parvicella" were MAR positive (data not shown), indicating an active uptake of [^{14}C]oleic acid under both aerobic and anaerobic conditions. However, a different result appeared when the HetCO_2-MAR approach with a combination of $^{14}CO_2$ and unlabeled oleic acid was used. "*Ca.* Microthrix parvicella" filaments were MAR positive under aerobic conditions (Fig. 4A) but MAR negative under anaerobic conditions (Fig. 4B). The MAR signal was quantified by counting the silver grain density along the filaments. In the presence of oxygen, 1.46 silver grains μm^{-1} were observed along the MAR-positive filaments (Table 1), whereas the numbers of silver grains along the filaments incubated in the absence of oxygen (Fig. 4B) were not significantly different from the background (Table 1). In addition to the filamentous "*Ca.* Microthrix parvicella," some nonfilamentous bacteria also assimilated sufficient $^{14}CO_2$ to be MAR positive under aerobic conditions (Fig. 4A).

To examine whether "*Ca.* Microthrix parvicella" take up oleic acid under anaerobic conditions for storage purposes, which may subsequently be used for growth under aerobic conditions, assimilation of $^{14}CO_2$ was investigated after incubation of sludge samples with oleic acid under anaerobic conditions for 3 h, followed by a shift to aerobic conditions for 4 h

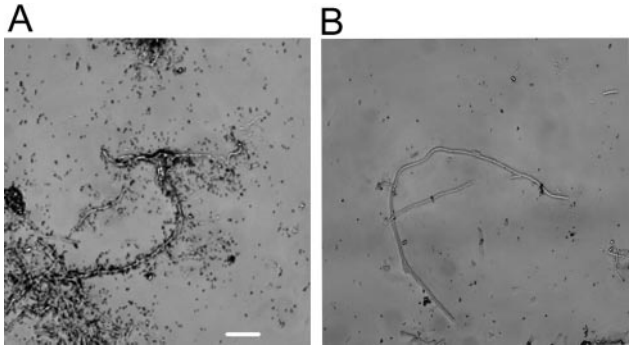

FIG. 4. MAR of filamentous "*Ca.* Microthrix parvicella" in activated sludge after incubation with $^{14}CO_2$ in the presence of unlabeled oleic acid under aerobic (A) and anaerobic (B) conditions. Scale bars, 10 μm.

(Fig. 5A). Parallel experiments with [^{14}C]oleic acid showed that all dissolved [^{14}C]oleic acid (measured as radioactivity in filtrated samples) was assimilated after 0.5 to 1 h (data not shown). With $^{14}CO_2$ as the isotope substrate, MAR-positive filaments were clearly observed after the anaerobic-aerobic transition, suggesting that the oleic acid taken up under anaerobic conditions stimulated metabolic activity and potentially growth under aerobic conditions. In order to see whether $^{14}CO_2$ assimilation could also be induced by nitrite or nitrate addition, samples incubated for 3 h under anaerobic conditions with oleic acid were subsequently exposed to nitrate-reducing (Fig. 5B) or nitrite-reducing conditions (Fig. 5C). MAR-positive filaments were observed with nitrate as electron acceptor but hardly any with nitrite (Fig. 5B and C). Quantification of the MAR signals (Table 1) showed that a comparable amount of $^{14}CO_2$ was assimilated after a transition from anaerobic to aerobic or nitrate-reducing conditions. The amount of $^{14}CO_2$ assimilated after the transition was comparable to what was observed for filaments incubated under aerobic conditions for 7 h without transitions (Student t test, $P > 0.05$ [not significant]). In contrast, transition from anaerobic to nitrite-reducing conditions induced significant less assimilation of $^{14}CO_2$ (Fig. 5C and Table 1), which was nevertheless above the MAR signal observed for samples incubated for 7 h in the absence of oxygen (Student t test, $P < 0.01$). Thus, the results show that oleic acid was most likely taken up and stored under anaerobic conditions without induction of significant growth as indicated by heterotrophic CO_2 fixation. Stored oleic acid or oleic acid derivatives (e.g., lipids) were then able to stimulate metabolic activity and likely growth when oxygen or nitrate was supplied as electron acceptors.

TABLE 1. Quantification of MAR signal from "*Ca.* Microthrix parvicella" in activated sludge after $^{14}CO_2$ labeling in the presence of oleic acid[a]

Treatment	No. of silver grains $\mu m^{-1} \pm$ SD
O_2 (7 h)	1.46 ± 0.36
No O_2 (3 h) → O_2 (4 h)	1.27 ± 0.22
No O_2 (3 h) → No O_2 + NO_3^- (4 h)	1.34 ± 0.46
No O_2 (3 h) → No O_2 + NO_2^- (4 h)	0.23 ± 0.20
No O_2 (7 h)	0.04 ± 0.10

[a] Expressed as described previously by Nielsen et al. (27). SD indicates the standard deviation between enumerated filaments.

^{13}C-PLFA profiles. Activated sludge samples with filamentous "*Ca.* Microthrix parvicella" were incubated with $^{13}CO_2$ and oleic acid to investigate the assimilation of CO_2 into microbial biomass. ^{13}C-PLFA profiles confirmed that $^{13}CO_2$ was incorporated into cell macromolecules such as phospholipids (Fig. 6). Incubation of sludge samples in the presence of $^{13}CO_2$ and ATU (an inhibitor of autotrophic ammonia-oxidizing bacteria), had little effect on the resulting ^{13}C-PLFA profiles. Only three fatty acid groups representing 16:0 and 16:1 species were less labeled in the presence of ATU than without ATU (data not shown). These fatty acids represent PLFAs that are also produced by some autotrophic ammonia-oxidizing bacteria (8). Since the majority of ^{13}C-enriched PLFAs were not affected by ATU inhibition, it may be concluded that heterotrophic rather than chemo-litho-autotrophic CO_2 assimilation appeared to dominate in the sludge samples. This may be due partly to the low initial concentration in the sludge of energy sources for typical autotrophs such as ammonia oxidizers (i.e., an NH_4^+ concentration of <0.01 mM).

Assimilation of $^{13}CO_2$ into microbial PLFAs was stimulated by the addition of oleic acid to sludge samples (Fig. 6A). However, more $^{13}CO_2$ was incorporated into PLFAs under aerobic compared to anaerobic conditions, which is in agreement with the MAR results shown in Fig. 4. Comparison with control samples incubated with $^{13}CO_2$ but without added oleic acid revealed that especially PLFAs representing 16:1ω8/9, 16:0, 18:1ω9/8, and 18:1ω5/7 were labeled in the presence of oleic acid. This was confirmed to a large extent by incubating aerobic sludge samples directly with [^{13}C]oleic acid (Fig. 6B). Comparison of ^{13}C-PLFA profiles from samples incubated with $^{13}CO_2$ and unlabeled oleic acid with profiles from samples incubated with [^{13}C]oleic acid and unlabeled CO_2 revealed differences in the relative enrichments of some PLFAs but relatively few differences among which PLFAs were labeled (Fig. 6B). Thus, the majority of the labeled PLFAs in Fig. 6B likely originated from microorganisms including "*Ca.* Microthrix parvicella" that are associated with oleic acid metabolism in the activated sludge. Some of these microorganisms produced long-chain PLFAs representing unknown C_{20}-C_{22} compounds (Fig. 6). MAR investigations showed that "*Ca.* Microthrix parvicella" was a dominant consumer of oleic acid in aerobic sludge, but also other bacteria consumed oleic acid under aerobic conditions. As a result, it is not known to what extent the unusual microbial fatty acids (C_{20}-C_{22}) can be linked directly to oleic acid consumers such as "*Ca.* Microthrix parvicella."

DISCUSSION

In this study we have shown that CO_2 assimilation occurred during active metabolism of different organic substrates by several heterotrophic bacteria grown under various electron acceptor conditions. This $^{14}CO_2$ incorporation was sufficient for single cell detection by MAR (HetCO$_2$-MAR).

The traditional MAR approach (when targeting heterotrophic organisms) relies on the application of radioactively labeled organic compounds supplied as substrate. In the work presented here, HetCO$_2$-MAR combined with traditional

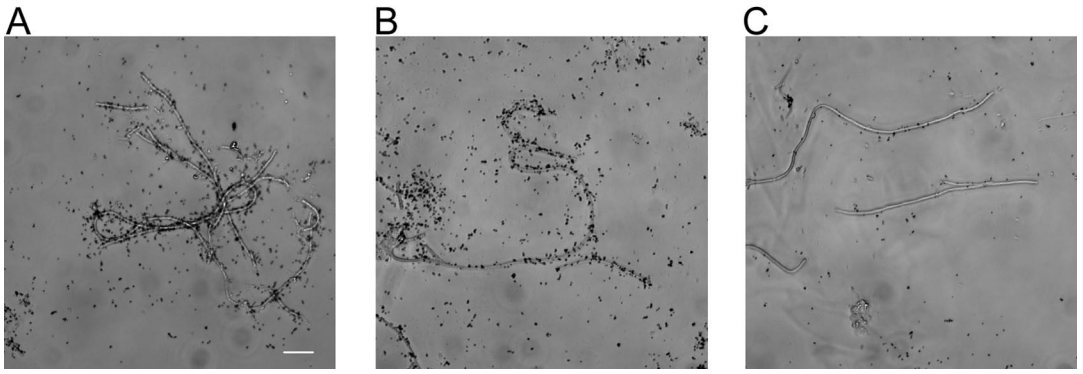

FIG. 5. MAR of filamentous "*Ca.* Microthrix parvicella" in activated sludge labeled with $^{14}CO_2$ in the presence of oleic acid under anaerobic conditions (3 h), followed by the addition of oxygen (A), nitrate (B), or nitrite (C). Scale bar, 10 μm.

MAR enabled differentiation between assimilation of substrate (traditional MAR) and active metabolism ($HetCO_2$-MAR) by the target organism "*Ca.* Microthrix parvicella." Hence, $HetCO_2$-MAR used as supplement to the traditional MAR is proposed as a potential powerful tool for future studies on the ecophysiology of heterotrophic microorganisms. In addition, the $HetCO_2$-MAR approach has a number of potential advantages compared to the traditional MAR. The traditional MAR approach is inherently limited by the availability of isotope-labeled substrates. Homogeneously isotope-labeled complex substrates is normally not commercially available, and it is often difficult and expensive to label active organisms, which only respond to complex organic substrates. In the present study, we showed that complex organic substrates (exemplified by yeast extract) induced significant assimilation of $^{14}CO_2$, sufficient for MAR visualization on a single-cell level (Fig. 2B). In combination with FISH, the $HetCO_2$-MAR approach opens for numerous future applications focusing on $^{14}CO_2$ assimilation by metabolically active heterotrophic microorganisms using a range of nonlabeled organic substrates. The $HetCO_2$-MAR approach also minimizes problems associated with surface adhesive or hydrophobic compounds, which may attach to bacterial surface and induce false MAR-positive cells when the traditional MAR approach is used. In addition, the $HetCO_2$-MAR approach will be very cost-effective when screening the metabolism (but not possible assimilation without further metabolic activity) of many different single compounds in order to elucidate substrate preferences for known or unknown organisms in environmental systems.

A successful MAR experiment relies on sufficient incorporation of the radiotracer into bacterial cells. We have used an exposure time of 3 to 5 days, which requires an incorporation of ca. 10^{-15} Ci cell^{-1}. To get this amount of $^{14}CO_2$ into heterotrophic cells, an experimental design with optimized concentrations of unlabeled and labeled bicarbonate, incubation time, and biomass concentration is required. In experiments with *E. coli* and *P. putida*, we used relatively high biomass concentrations, which caused some isotope dilution due to CO_2 production during respiration (see below). This problem can be eliminated if much lower biomass concentrations are used. In the study of activated sludge with "*Ca.* Microthrix parvicella", we used very high concentrations of $^{14}CO_2$ to overcome the high background concentration of bicarbonate in the activated sludge. Thus, $HetCO_2$-MAR can be made at least as sensitive as the traditional MAR if conditions are optimized for a specific system. $HetCO_2$-MAR may particularly increase the sensitivity (as compared to traditional MAR) in cases where large organic molecules are only available with one or a few ^{14}C atoms.

Our results support previous suggestions that assimilation of $^{14}CO_2$ is a general phenomenon in metabolically active heterotrophic microorganisms (17, 38). Heterotrophic CO_2 assimilation induced by the presence of organic substrates was confirmed by filter count, MAR visualization, and analysis of PLFAs. It has been suggested previously that heterotrophic bacteria assimilate relative constant quantities of inorganic carbon during growth (35), and recently we reported an assimilation of 1.4% ± 0.7% of cell carbon produced by *P. putida* (38). The assimilation of CO_2 has been reported partly to depend on the presence of electron acceptor (15) and to be related to variations in the concentration of external CO_2 (22). In the present study we did not determine the total assimilation of CO_2 relative to the biomass production. However, with an average carbon content of 1.2×10^{-14} mol of C cell^{-1}, the observed assimilation of $^{14}CO_2$ in aerobically grown *P. putida* with glucose (ca. 10^{-14} Ci cell^{-1}) corresponds to a total CO_2 assimilation of at least 3% of biomass C based on the initial specific activity ($^{14}C/^{12}C$) of $^{14}CO_2$. In addition, we have clearly shown that different incorporations of the added $^{14}CO_2$ in our experiments could primarily be explained by different dilutions of the added isotope, dependent on the production of unlabeled CO_2 during the incubation. As an example, after 3 h of incubation of *E. coli*, the specific activity ($^{14}C/^{12}C$) of $^{14}CO_2$ in anaerobic samples was more than twice the specific activity in aerobic samples due to less production of unlabeled CO_2 during fermentative metabolization of glucose. Hence, the observed stimulated assimilation of $^{14}CO_2$ by *E. coli* under anaerobic conditions (Fig. 1C) can be explained mainly by less dilution of $^{14}CO_2$, since fermentative metabolism of glucose produces less CO_2 than aerobic metabolism.

Use of $^{14}CO_2$ for isotope labeling of active heterotrophic microorganisms requires that CO_2 assimilation activity correlate with cell metabolic activity. Our results clearly showed that a significant assimilation of CO_2 only took place when organic substrate was added to cultures of *E. coli* or *P. putida*. A small amount of $^{14}CO_2$ was assimilated without added substrates

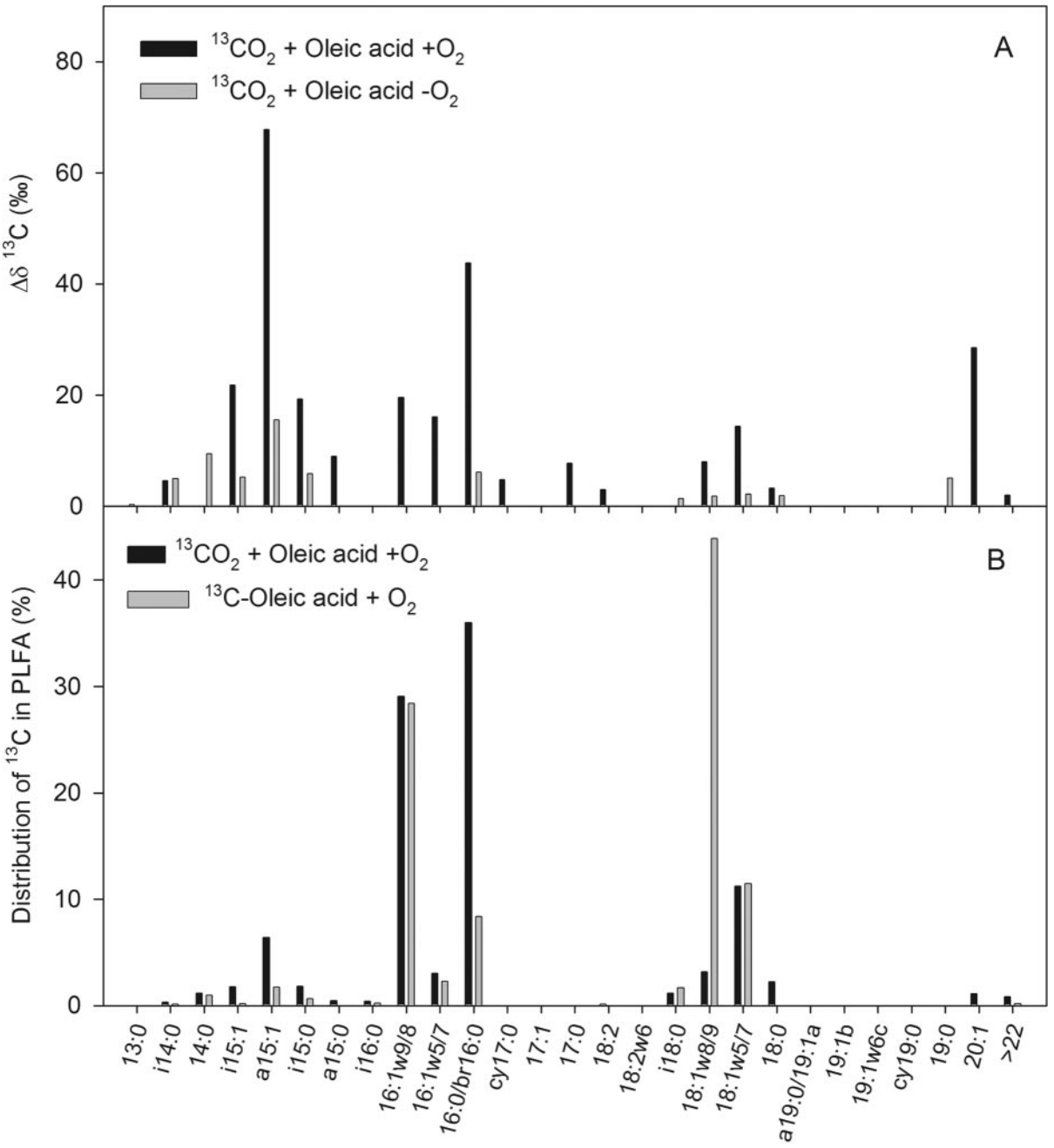

FIG. 6. [^{13}C]PLFA profiles from activated sludge with "*Ca.* Microthrix parvicella" incubated with oleic acid. (A) Comparison of ^{13}C-PLFA profiles after incubation with oleic acid and $^{13}CO_2$ in the presence or absence of oxygen. Enrichment in ^{13}C is expressed as changes in δ^{13}C compared to control samples incubated without $^{13}CO_2$ ($\Delta\delta^{13}$C). (B) Comparison of ^{13}C-PLFA profiles after incubation with either $^{13}CO_2$ and nonlabeled oleic acid or nonlabeled CO_2 and [^{13}C]oleic acid. The relative distribution of ^{13}C incorporated into PLFAs is expressed as a percentage.

(less than index 0.06, Fig. 1), but assimilation was not sufficient to obtain a positive MAR signal with the exposure time applied (Fig. 2C and 3C). A low assimilation of CO_2 in the absence of added organics could be due to use of internal storage compounds or organic exudates released from starved or dead cells. Hence, our observations suggest that heterotrophic CO_2 assimilation is substrate responsive and that starved or metabolic inactive bacteria only assimilate small amounts of CO_2. Nonetheless, we highly recommend including control experiments without the addition of organic substrate in HetCO$_2$-MAR experiments with environmental samples. Background assimilation of CO_2 may take place either due to the metabolism of storage products or organic substrates present in the sample or may be linked to autotrophic activity.

In HetCO$_2$-MAR experiments, $^{14}CO_2$ added to the external medium will diffuse into bacterial cells until isotope equilibrium is approached. Hence, all cells (active and inactive) may contain $^{14}CO_2$ after the incubation. Gray et al. (18) elegantly

showed that thoroughly acidification of the cells before MAR exposure removed dissolved and precipitated CO_2 inside and around the cells. Pearl et al. (31) reported that several species of heterotrophic bacteria precipitated carbonates in marine stromatolites (laminated lithified $CaCO_3$) and that these species contributed to the formation of the stromatolites. Thus, it cannot be ruled out that intracellular precipitation of less-soluble [^{14}C]carbonates may lead to positive MAR signals in rare cases. However, we did not observe MAR-positive cells in a range of control experiments with pasteurized samples or in experiments without the addition of energy sources or electron acceptors. These experiments suggested that thorough acidification prior to MAR exposure did remove all inorganic radiocarbon from the cells (including precipitates). Hence, it is expected that intracellular precipitation of carbonates is a minor problem for the HetCO_2-MAR approach. This is supported by the observation that metabolically active heterotrophs assimilate isotope labeled CO_2 into organic macromolecules such as PLFAs (Fig. 6). Furthermore, this is in agreement with previous studies suggesting that heterotrophs assimilate CO_2 into biomass components such as PLFA (38) and RNA (1). These compounds, and other organic macromolecules, will not be affected by an acidification prior to MAR exposure and will lead to visualization of cells that were de facto metabolically active.

The ecophysiological study of the filamentous bacterium "*Ca.* Microthrix parvicella" in activated sludge showed that this organism could take up oleic acid under anaerobic conditions, but the activity was not associated with a detectable increase in metabolic activity, as indicated by heterotrophic CO_2 assimilation. Heterotrophic CO_2 assimilation depends on both anabolic and catabolic processes and is stimulated during cell growth (38). Hence, our results support previous suggestions indicating that "*Ca.* Microthrix parvicella" is able to take up oleic acid under anaerobic conditions and form storage compounds (e.g., lipids) without initiating balanced growth (4, 28). The stored oleic acid or oleic acid derivatives are then able to support cell growth if oxygen becomes available (4, 28). This hypothesis was supported by the combined results from the traditional MAR and HetCO_2-MAR experiments, and it was further confirmed by the severely attenuated incorporation of $^{13}CO_2$ into PLFAs under an aerobic conditions (Fig. 6A). Quantitative MAR results supported these findings and also suggested that nitrate could be used as an efficient electron acceptor in the absence of oxygen (Table 1). This was further supported by nitrate-stimulated $^{13}CO_2$ incorporation into PLFAs in the absence of oxygen (measured as $\delta^{13}C$ in PLFAs [data not shown]). Under nitrite-reducing conditions, however, only a small amount of $^{14}CO_2$ was incorporated, indicating that "*Ca.* Microthrix parvicella" was not able to oxidize the storage product with nitrite as an electron acceptor. Quantitative results based on heterotrophic assimilation of CO_2 must be interpreted with caution, since changes in metabolism (e.g., electron acceptor) may lead to different cascades of carboxylations in the biosynthesis. However, the observed trends are in agreement with pure culture studies where "*Ca.* Microthrix parvicella" seem to be able to reduce nitrate to nitrite (40). This metabolic feature (anaerobic storage of long-chain fatty acid, followed by growth with oxygen or nitrate) can probably explain why "*Ca.* Microthrix parvicella" grows extremely well in nutrient removal plants under alternating conditions with oxygen and nitrate present as an electron acceptor.

In summary, the novel HetCO_2-MAR approach made it possible for the first time on a single-cell level to distinguish better between uptake and storage of organic compounds and metabolism that initiates true growth. This was clearly illustrated by the unique information on electron acceptors preferences by "*Ca.* Microthrix parvicella" that was obtained with the HetCO_2-MAR approach. To our knowledge, no other methods available would possibly answer this type of question on a single-cell level. We suggest that the HetCO_2-MAR approach will expand the possibilities for studying the ecophysiology of uncultivated heterotrophic microorganisms.

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