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Survival of Denitrifiers in Nitrate-Free, Anaerobic Environments

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Experiments were undertaken to explain the occurrence of a high denitrification capacity in anaerobic, NO_3^--free habitats. Deep layers of freshwater sediments that were buried more than 40 years ago and digested sludge were the habitats studied. The denitrifier populations were 3.1×10^3 and 3.1×10^5 cells cm^{-3} in deep sediments from a river and lake, respectively, and 5.3×10^6 cells cm^{-3} in digested sludge. The denitrification capacities of the samples reflected the population densities. Strict anaerobic procedures were used to obtain the predominant isolates that would grow on anaerobic medium with NO_3^-. All strict anaerobes isolated failed to denitrify. All isolates that denitrified were aerobic, gram-negative bacteria, particularly species of *Pseudomonas* and *Alcaligenes*. No detectable growth was observed when these strains were incubated with electron acceptors other than NO_3^- or O_2. When representative isolates were added to sterile, O_2- and NO_3^--free porewater from their original locations at their natural densities (10^5 cells cm^{-3}), no change in viable population was noted over 3 months of incubation. Metabolic activity was demonstrated in these cells by slow formation of formazan granules when exposed to tetrazolium and by observation of motile cells. When [^{14}C]glucose was added to cell suspensions of the pseudomonads that had been starved for 3 months without electron acceptors (O_2 or NO_3^-), ^{14}C-labeled products, including cell biomass, $^{14}CO_2$, and fermentation products, were produced. The high denitrification capacity of these anaerobic environments appears to be due to conventional respiratory denitrifiers. These organisms have the capacity for long-term survival without O_2 or NO_3^- and appear to be capable of providing for their maintenance by carrying on a low level of fermentation.

To perform microbial denitrification, three major conditions are needed: anaerobiosis, nitrate, and an electron donor. Many reports confirm that denitrifying bacteria are present in environments where these three requirements are not fulfilled. A high denitrification potential, revealing the presence of denitrifiers, is especially prominent in anaerobic, nitrate-free environments such as deep marine sediments (8, 19, 24), freshwater sediments (9), anoxic seawater (3), deep subsurface sediments (5), and digested sludge (11).

It has been proposed that denitrifiers present in these environments might exhibit another kind of metabolism, e.g., the use of alternative electron acceptors such as fumarate or trimethylamine oxide (19), but this has never been proven. Another hypothesis is that bacteria which usually ferment would also be able to denitrify, but very few organisms are known to do both (26). A third hypothesis, which we propose in this article, is that known aerobic denitrifying bacteria use fermentation under anaerobic conditions, at least at a rate sufficient for their survival.

The purpose of this study was to determine which explanation most likely accounts for the presence of denitrifiers in these anaerobic environments. To answer this question, it is important to obtain samples from denitrifying environments where one can be assured that neither oxygen nor nitrate has been present for long periods of time and that recent arrival of organisms from aerobic environments is unlikely. Deep methanogenic sediments are well suited to these criteria. In this article, we report on characterization of denitrifiers from such environments as well as determination of their survival and metabolism under O_2-free and NO_3^--free conditions.

MATERIALS AND METHODS

Sampling of sediment and sludge. Sediment was sampled from two freshwater locations in Michigan: Wintergreen Lake at the W. K. Kellogg Biological Station, and a site near the mouth of the Saginaw River. Wintergreen Lake is a eutrophic lake with extensive methane production in the sediments, and Saginaw River site 9 is 0.5 km below the Saginaw municipal wastewater treatment plant outfall; the plant also treats effluents from manufacturing plants. Samples from Wintergreen Lake were obtained just before the fall turnover of the thermocline.

Intact lake sediment cores were taken in Plexiglas cores (7.6 cm wide and 60 cm long) with a gravity corer. Intact river sediment cores were taken in 7.6-cm-wide and 90-cm-long aluminum cores. Intact subsamples were taken 25 to 28 cm below the sediment surface of Wintergreen Lake sediment cores and 50 to 53 cm below the sediment surface of Saginaw River sediment cores. These subsamples were used for the denitrification potential assay and for isolation of denitrifiers. The organic carbon content, determined by furnace oxidation, was 18.4% ± 0.9% (mean ± standard deviation) for Wintergreen sediments and 8.1% ± 1.5% for Saginaw sediments. Nitrate and nitrite were not detected in samples from either site, as determined by flow injection analysis (Lachat) after extraction with 1 M KCl. The sensitivity of this method was about 3 μM NO_3^-. Further evidence for the lack of NO_3^- was obtained by assaying the sediment anaerobically with C_2H_2 (procedure as for the potential dentrification assay but without NO_3^- addition). No N_2O was detected. The sensitivity of this method, using

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a gas chromatograph with a ^{63}Ni detector, is about 0.02 μM NO_3^-.

Research on Wintergreen Lake sediment for 20 years indicates that these sediments must have been O_2 and NO_3^- free for many years. This conclusion is based on O_2 and NO_3^- profiles, which show that these species only reach the organic ooze layer at the sediment surface briefly during spring and fall water column turnover and that the methane production rates of the sediment are very high (14, 15). Also, the high organic-matter content of the sediment column would immediately scavenge any O_2 and NO_3^- before it penetrated into the consolidated sediments. The time of deposition of the 25- to 28-cm sediment layer is 50 to 54 years earlier, as determined from stratigraphy of sediment, pollen, and diatoms, recorded shifts in watershed land use, and sedimentation rates (12). Because the sampled layer is below more than 20 cm of firmly structured sediment, it is extremely unlikely that any organisms from the aerobic water column could have reached the sampling depth in recent years.

The time of deposition and anaerobiosis of the Saginaw River sediments is less certain because it has not been as well studied and river sediment movement is more dynamic. However, the samples were taken at a depth of 50 to 53 cm, and the entire core was high in organic matter and black from sulfide, indicating that anaerobic sulfate reduction was taking place. The sediment sampled occurred under a layer of polychlorinated biphenyl-contaminated sediment, which was located at a depth of 20 to 30 cm. These polychlorinated biphenyls were deposited at the time of their industrial use in the late 1960s. If the rate of deposition before the occurrence of the polychlorinated biphenyl marker layer was similar to that since, the time of deposition of the sampled layer should be approximately 40 years ago.

Sludge was obtained from the anaerobic digestors of the municipal treatment plants of St. Johns, Coldwater, and Jackson, Mich. The sludges were all anaerobic and intensively producing methane when sampled.

Potential denitrification assay. Subsamples of sediment were taken by the following procedure to minimize exposure to O_2. The sediment was pushed out of the sampling core under air with a plunger until the desired depth was level with the top end of the core. The surface layer was cut off, and disposable syringes (3 ml) with cut-off ends were immediately inserted into the sediment. Subsamples (5 ml) were taken and immediately transferred to 25-ml serum bottles under an O_2-free argon flow. The bottles were capped and flushed with argon. Two milliliters of C_2H_2 was added to inhibit N_2O reduction. The assay was initiated by adding 0.5 ml of O_2-free 7.1 mM KNO_3 solution. Final concentrations were 820 μM NO_3^- and 1,090 μM NO_3^- in the Wintergreen and Saginaw porewaters, respectively.

The denitrification potential of anaerobic sludge was assayed with 2 ml of sludge in Ar-flushed 13-ml serum bottles. One milliliter of C_2H_2 gas was added, and the assay was started by adding 0.15 ml of O_2-free 7.1 mM NO_3^- (final concentration, 500 μM NO_3^-). The differences in porosity of the sediment and sludge samples are the reason for the differences in the final NO_3^- concentrations. Gas samples (0.5 ml) were withdrawn and injected into a Perkin-Elmer 910 gas chromatograph equipped with a ^{63}Ni detector for N_2O analysis. Operational conditions were as described by Parkin et al. (22).

Isolation of denitrifiers. In order to isolate organisms responsible for denitrification from the NO_3^--free and anaerobic zones of the sediment, another set of subsamples were taken in small Plexiglas cores (22 mm wide and 60 mm long) from the same depths but in a parallel set of cores. The subcores were immediately stoppered at both ends (30-mm-long sediment core) and transferred to an anaerobic chamber equipped with an oxygen monitor (Coy, Ann Arbor, Mich.). The first step of enrichment was combined with a most-probable-number (MPN) enumeration procedure for denitrifiers (25). The procedure was modified to be truly anoxic. All dilution bottles (0.85% saline) and tubes of medium (5 mM NO_3^- and 8 g of nutrient broth per liter [Difco]) were prepared under a stream of O_2-free nitrogen (10). The bottles were transferred to the glove box, where all dilutions and inoculations took place. The initial step was performed by taking 10 ml of sediment from the small cores with sterile, cut-off syringes and transferring the sediment subcore to the first dilution bottle containing 90 ml of 0.85% saline plus 1 drop of Tween 80. Samples were shaken by hand for 3 min.

MPN tubes from the highest dilution which were positive for both NO_3^- disappearance and N_2O production after 3 weeks of incubation were selected for further isolation. Samples were streaked onto plates (nutrient broth and nitrate) in the glove box and incubated there. Colonies were picked for purification and verification of denitrification.

To study whether strict anaerobes were responsible for denitrification, we used a complex medium of peptone, glucose, and yeast extract which, as had been established previously, would yield the largest numbers of strict anaerobes from this sediment (13). Five milliliters of sediment was incubated anaerobically in this complex medium with 5 mM nitrate and 10% C_2H_2. Samples from bottles that produced N_2O were enriched in the same liquid medium a second time and then streaked on the same medium containing agar. Colonies were picked to verify denitrification.

All isolates were evaluated for denitrifying ability by measuring N_2O production after growth in nutrient broth with nitrate and C_2H_2.

Characterization and identification. Nine isolates from sediment and three isolates from anaerobic sludge that were confirmed denitrifiers were selected for further characterization. Identification was done with commercially available identification systems: API Rapid NFT, VITEK, Biolog, and fatty acid methyl ester (FAME) analysis. The isolates were all tested for their type of NO_2^- reductase by the immunological technique described by Coyne et al. (4).

Six isolates were selected for testing for the use of alternative electron acceptors; three isolates from sediment (L03, L05, and S04) and three isolates from digested sludge (C07, SJ10, and J04). The tests were performed in anaerobic Hungate tubes containing 10 ml of nutrient broth (8 g/liter) and the following electron acceptors: NO_3^-, 5 mM; fumarate, 7.8 mM; glycine, 16.7 mM; pyruvate, 22.7 mM; and malate, 18.7 mM. Growth was monitored by measuring the optical density at 660 nm.

Growth on fermentable substrates was tested with the following substrates: glucose (1%), Casitone (1%), lactate (1%), and yeast extract (1%). The substrates were added separately to tubes containing nutrient broth prepared anaerobically, and 0.1% yeast extract was also added to the glucose, Casitone, and lactate media.

A 10% inoculum from a culture grown on nutrient broth with nitrate was used. Growth was monitored by measuring the optical density at 660 nm.

Survival experiments. Strains L03 and L05 and two reference strains, *Pseudomonas aeruginosa* PAO1 and *Pseudomonas fluorescens* ATCC 17822, were chosen for survival experiments. Cells were grown anaerobically in 200 ml of

TABLE 1. Capacity for denitrification activity in anaerobic, NO_3^--free environments after addition of NO_3^-

Sample and site	Incubation temp (°C)	Denitrification rate (nmol of N_2O-N cm^{-3} h^{-1})	No. of denitrifiers[a] (cells cm^{-3})
Sediments			
Saginaw River (50–60 cm)	5	0.06	1.3×10^3
Lake Wintergreen (20–30 cm)	5	4.0	3.1×10^5
Digested sludge			
Jackson, Mich.	25	260	5.3×10^6
St. John, Mich.	25	90	7.9×10^5
Coldwater, Mich.	25	50	3.5×10^6

[a] MPN technique.

nutrient broth with 5 mM NO_3^- until the early stationary phase. To remove any potential residual electron acceptors, cells were harvested by centrifugation (10 min, 8,000 rpm). The cells were resuspended in 10 ml of 0.025 mM anaerobic phosphate buffer (pH 7.1).

The resuspended cells were transferred to sterile experimental bottles (160-ml serum bottles) containing 50 ml of 10 mM anaerobic phosphate buffer or 50 ml of anaerobic porewater obtained from Wintergreen Lake sediment by centrifugation of sediment (12,000 rpm). There were two bottles for each strain and for each medium. The final cell density was 10^5 and 10^8 cells per ml in two separate experiments, A and B. Control experiments in which N_2O production in the presence of C_2H_2 was analyzed confirmed that no NO_3^- was carried over. The bottles were incubated at 25°C, and plate counts of recoverable bacteria were performed at approximately 10-day intervals, more frequently at the beginning. Plating and counting were done with a Spiral plater and laser counter (Spiral Systems), respectively.

After 3 months of starvation, a tetrazolium assay (20) was performed to detect metabolic activity of remaining cells. Samples (1 ml) of starving bacteria were transferred to 8-ml, Ar-flushed serum bottles containing 0.1 ml of 0.4% tetrazolium chloride [2,5-diphenyl-3(α-naphthyl)-tetrazolium chloride; Sigma]. After 0, 21, and 42 h, the suspensions were observed by microscopy (Leitz, Diaplan, Germany) on an anaerobic slide prepared in the glove box. The formation of formazan granules was detected by noting refracted light, and at the same time, cell motility was noted.

Metabolic activity measured by conversion of [^{14}C]glucose. After 3 months of starvation, the metabolism of [^{14}C]glucose was determined for strain L03 and *P. fluorescens* ATCC 17822 in the experiment with 10^5 cells per ml. ^{14}C-labeled glucose (ICN; specific activity, 310 μCi μmol^{-1}) in an O_2-free solution was injected into the experimental bottles to a final activity of 0.1 μCi ml^{-1}. This corresponded to 0.32 μM glucose. At this stage, 40 ml of the cell suspension remained in each bottle after sampling for determination of the cell number and for the tetrazolium assay. Replicate bottles were prepared for each strain and for control bottles of phosphate buffer. The turnover of glucose into different pools was monitored for 10 days: incorporation into cells, $^{14}CO_2$ production, and production of ether-extractable acids. A 1-ml liquid sample was withdrawn and transferred to an argon-flushed, stoppered 3-ml vial containing 0.1 ml of 1 N HCl. The sample was filtered through a 0.45-μm filter (Millipore), and the filter was washed with 15 ml of 0.1 N HCl. The filter was transferred to a scintillation vial for liquid scintillation counting.

The filtrate was divided into two portions. To 0.8 ml of the filtrate was added 2 ml of ethyl ether, and the mixture was shaken vigorously to extract fatty acids. The phases were allowed to separate, and 1 ml of the ether fraction was transferred to a scintillation vial containing 10 ml of Safety Solve scintillation liquid. The remaining 0.2 ml of the filtrate was added to an ion exclusion column (Bio-Rad; 300 by 38 mm; Aminex HPX-87H) for analysis of fatty acids. Fractions

TABLE 2. General characteristics and tentative identification of isolates

Isolate[a]	Origin[b]	Dilution used for isolation	Aerobic growth	% gas production from NO_3^-	Motility	Gram reaction	Shape	NO_2^- reductase type	Catalase	Oxidase	Fluorescent pigments on King's medium B	Identification[c]
L01	Sed	10^{-3}	+	>80	+	Neg	Rod	cd_1	+	+	+	*P. fluorescens* B
L03	Sed	10^{-2}	+	>80	+	Neg	Rod	cd_1	+	+	+	*P. fluorescens* B
L04	Sed	10^{-2}	+	>80	+	Neg	Rod	cd_1	+	+	+	*P. fluorescens* B
L05	Sed	10^{-5}	+	>80	+	Neg	Rod	cd_1	+	+	+	*P. fluorescens* B
L06	Sed	10^{-5}	+	>80	+	Neg	Rod	cd_1	+	+	+	*P. fluorescens* B
L09	Sed	10^{-5}	+	>80	+	Neg	Rod	cd_1	+	+	+	*P. fluorescens* B
L10	Sed	10^{-5}	+	>80	+	Neg	Rod	cd_1	+	+	+	*P. fluorescens* B
S02	Sed	10^{-3}	+	>80	+	Neg	Rod	cd_1	+	+	+	*P. fluorescens* B
S04	Sed	10^{-3}	+	>80	+	Neg	Rod	cd_1	+	+	+	*P. fluorescens* B
C07	Slu	10^{-5}	+	>80	+	Neg	Rod	cd_1	+	+	−	Unknown
J04	Slu	10^{-5}	+	>80	+	Neg	Rod	cd_1	+	+	−	*A. faecalis*
SJ10	Slu	10^{-5}	+	>80	+	Neg	Rod	cd_1	+	+	−	*A. faecalis*

[a] First letter indicates source of isolate: L, Wintergreen Lake; S, Saginaw River; C, J, and SJ, Coldwater, Jackson, and St. John sludge reactors, respectively.
[b] Sed, sediment; Slu, sludge.
[c] Based on 125 different tests performed by the API, Biolog, and Vitek systems and FAME analysis.

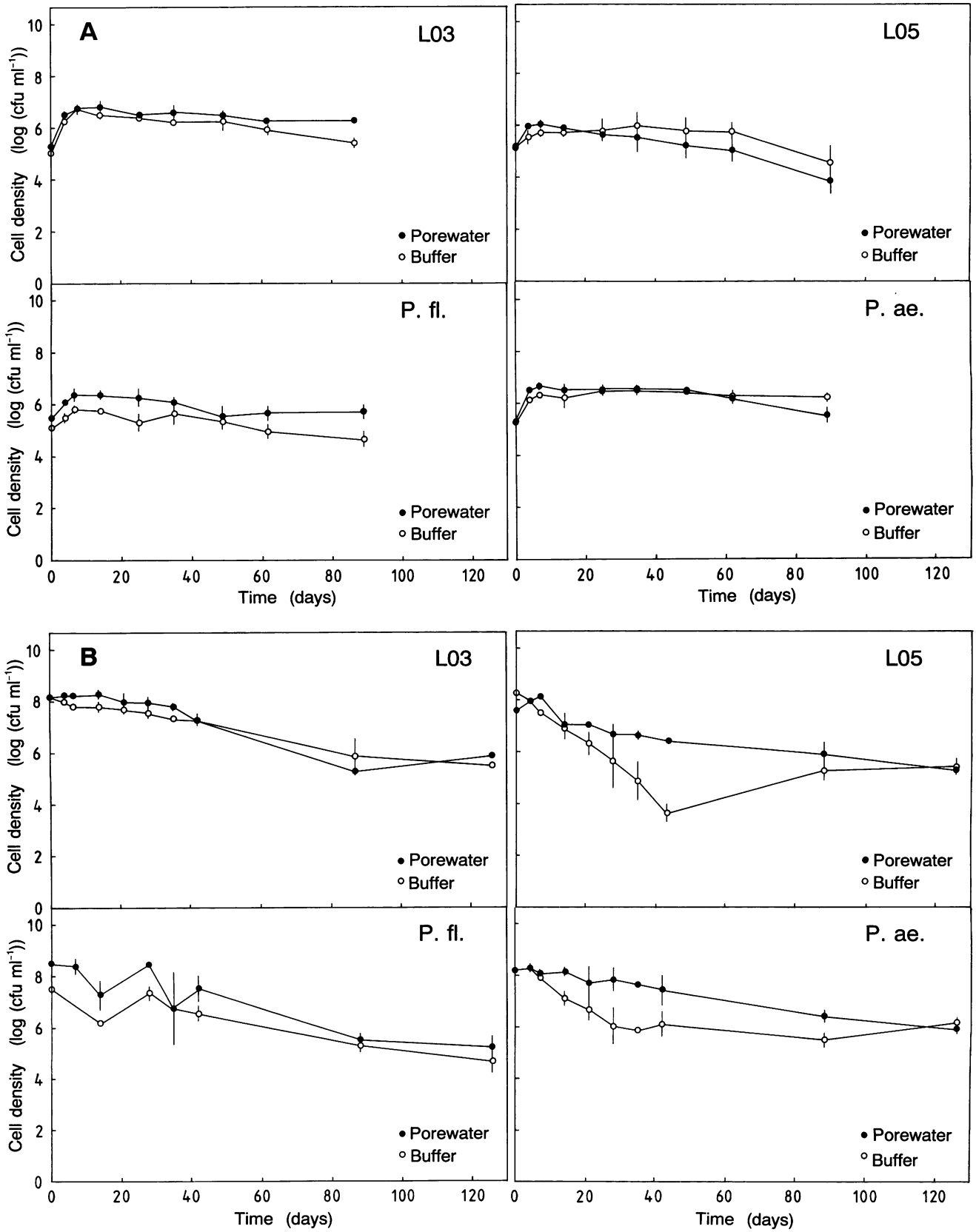

FIG. 1. Survival of isolates L03 and L05, *P. fluorescens* ATCC 17822 (P. fl.), and *P. aeruginosa* PAO1 (P. ae.) in O_2-free phosphate buffer (10 mM, pH 7.1) and sterile porewater from Lake Wintergreen sediments. Cell number is given in CFU. (A) Initial cell density 10^5 cells per ml. (B) Initial cell density 10^8 cells per ml. All experiments were initiated with early-stationary-phase cells. Bars represent standard errors.

(0.6 ml) of the eluate were collected and transferred to scintillation vials containing Safety Solve scintillation liquid.

$^{14}CO_2$ production was monitored by injecting a 1-ml gas sample from the headspace into 10 ml of CO_2 Solve scintillation liquid. All radioactive samples were analyzed on a Packard 1500 Tri-Carb liquid scintillation analyzer.

RESULTS

Denitrification capacity was present in all the samples from anaerobic, NO_3^--free sediments and sludges (Table 1). The cell number (MPN technique) increased with increasing denitrification capacity.

Twelve isolates were selected from among the denitrifiers isolated for further characterization (Table 2). All isolates from the sediment samples were identified as *P. fluorescens* biotype II, a common denitrifier in natural habitats (7). Two isolates from the sludge were identified as *Alcaligenes faecalis*. The third isolate did not match any of the reference data bases. The Biolog, API, and FAME identification systems agreed on the identification of the sediment isolates as fluorescent pseudomonads but did not agree on the correct species and biotype. The classification as biotype II within the fluorescent pseudomonads was based on use of carbon sources and types of pigments produced, as recommended (21). The *A. faecalis* isolates were identified by the API and Biolog identification systems, whereas the FAME system did not give any satisfactory match. No strict anaerobic isolates were found to denitrify. Although some N_2O production was measured in the enrichment cultures, none of the colonies derived from these enrichments denitrified.

No detectable growth was observed for the six isolates tested when an electron acceptor other than NO_3^- was present. None of the strains showed fermentative growth.

When exposed to prolonged anaerobic, NO_3^--free conditions, the isolates from the lake sediment as well as the culture collection strains survived quite well (Fig. 1). In experiment A, when 10^5 cells per ml was the initial cell concentration, the cell number actually increased during the first week of starvation, probably as a result of diminished cell size. After 7 weeks of starvation, the cell number was still higher than the initial number. In experiment B, when the initial cell concentration was 10^8 cells per ml, the cell number decreased gradually, and after 12 weeks of starvation, only about 10^5 cells per ml had survived. The presence of more organic matter due to the higher cell density in experiment B may have prevented the more typical starvation response seen in experiment A. There were no detectable differences in the survival pattern between the fresh sediment and sludge isolates and the culture collection strains, which have been cultivated and stored in the laboratory for years. There was hardly any consistent difference between survival rates in porewater and buffer. A tendency for slightly higher survival in the porewater medium probably reflects the presence of carbon sources or inorganic ions that would support survival.

The addition of tetrazolium to subsamples from the survival experiments led to the formation of formazan granules inside some cells (Fig. 2). Grains were observed only after 42 h of incubation in about 3 of 50 cells (17%), whereas a rapidly growing denitrifying culture would form visible granules within an hour in almost all cells. The cells were also motile.

The formation of radioactive products after the addition of [^{14}C]glucose to suspensions of starving cells is shown in Fig. 3. Most of the labeled C was found in cell material. When the cells were starved in buffer, more C was partitioned to fatty acid production than to $^{14}CO_2$ production. When the cells were starved in porewater, $^{14}CO_2$ production and ^{14}C-fatty acid formation were similar. Ten days after [^{14}C]glucose addition, 25% of the added label was metabolized by cells starved in buffer and about 10% was metabolized by cells starved in porewater.

The samples with the highest content of label in the ether-extractable fraction were analyzed to determine whether fatty acids were produced. Most of the radioactivity was found in an unidentified pool (fraction 7) that eluted before the unused glucose, which eluted in fraction 10 (Fig. 4). Small amounts of radioactivity were found in many fractions in the area where the low-molecular-weight fatty acids eluted. Of these fractions, the most label was found in the fraction where acetate elutes. Label was also found in fractions where succinate, lactate, and formate elute.

DISCUSSION

The quantification of denitrification capacity by the C_2H_2 inhibition technique and the estimation of bacterial numbers by MPN enumeration confirmed that a latent denitrifier population existed in these NO_3^--free, anaerobic environ-

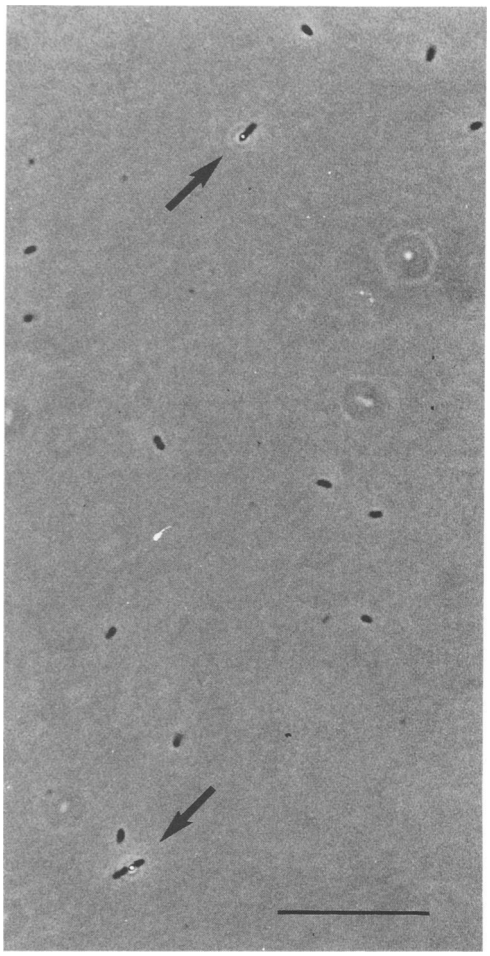

FIG. 2. Photomicrographs of starved cells of isolate L03 42 h after addition of tetrazolium in a dehydrogenase assay. Formazan granules inside the cells (arrows) indicate metabolic activity. Bar, 16 μm.

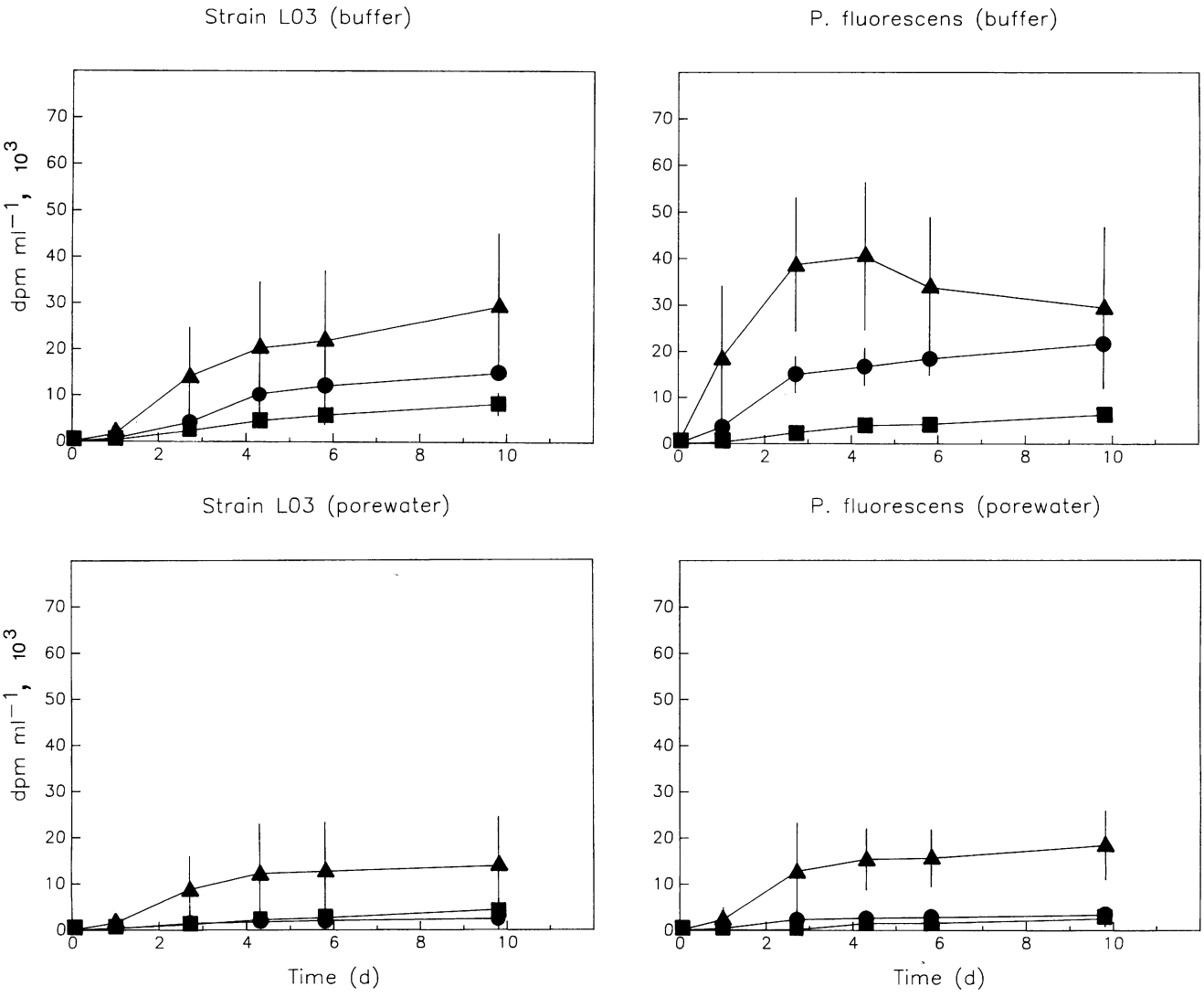

FIG. 3. Product formation after addition of [^{14}C]glucose (0.32 μM) to cell suspensions that had been starved for electron acceptor for 3 months. ▲, incorporated into cell material; ■, CO_2 produced; ●, ether extract after acidification. Bars represent standard errors.

ments. The denitrification rates observed were in accordance with earlier reported rates for similar environments (26). Denitrification capacities have not, however, been reported for deep layers of river sediments, and the low rate at this site is in contrast to that found for the eutrophic lake sediment, which was higher by a factor of almost 100. The organic content in the river sediment was about half that in the lake sediment, which may partially explain the difference.

Because denitrification rates followed the MPN values, isolates from the highest positive dilution should reveal the predominant organisms responsible for the in situ denitrification capacity. However, knowing that enrichment cultures do not always reveal the predominant organisms, we cannot be completely sure that we isolated the most frequent ones. All sediment isolates were common aerobic denitrifiers.

To test whether strict anaerobes could be responsible for the observed denitrification capacity, all sediment handling and isolation procedures were carried out under strict anaerobic conditions. However, no strict denitrifying anaerobes were isolated with this procedure. We cannot exclude the possibility that more unusual anaerobic organisms which we were not able to isolate can denitrify. At least some conversion of NO_3^- to N_2O was measured in the anaerobic enrichments, but it was less than the 80% conversion to gas that is typical of respiratory denitrification (26). Recent isolations and identification of deep subsurface sediment bacteria have also shown that gram-negative aerobes are the major constituents of that community (1, 2), and Francis et al. (5) showed that denitrifiers were present in these layers both with and without NO_3^-.

All the isolates contained heme cd_1 nitrite reductase, which was also shown by Coyne et al. (4) to be the most frequent nitrite reductase type. Also, the two *Alcaligenes* isolates in our study contained the heme cd_1 type. In the study by Coyne et al. (4), both cd_1 and Cu nitrite reductases were found in the genus *Alcaligenes*.

In order to investigate the survival strategies of the

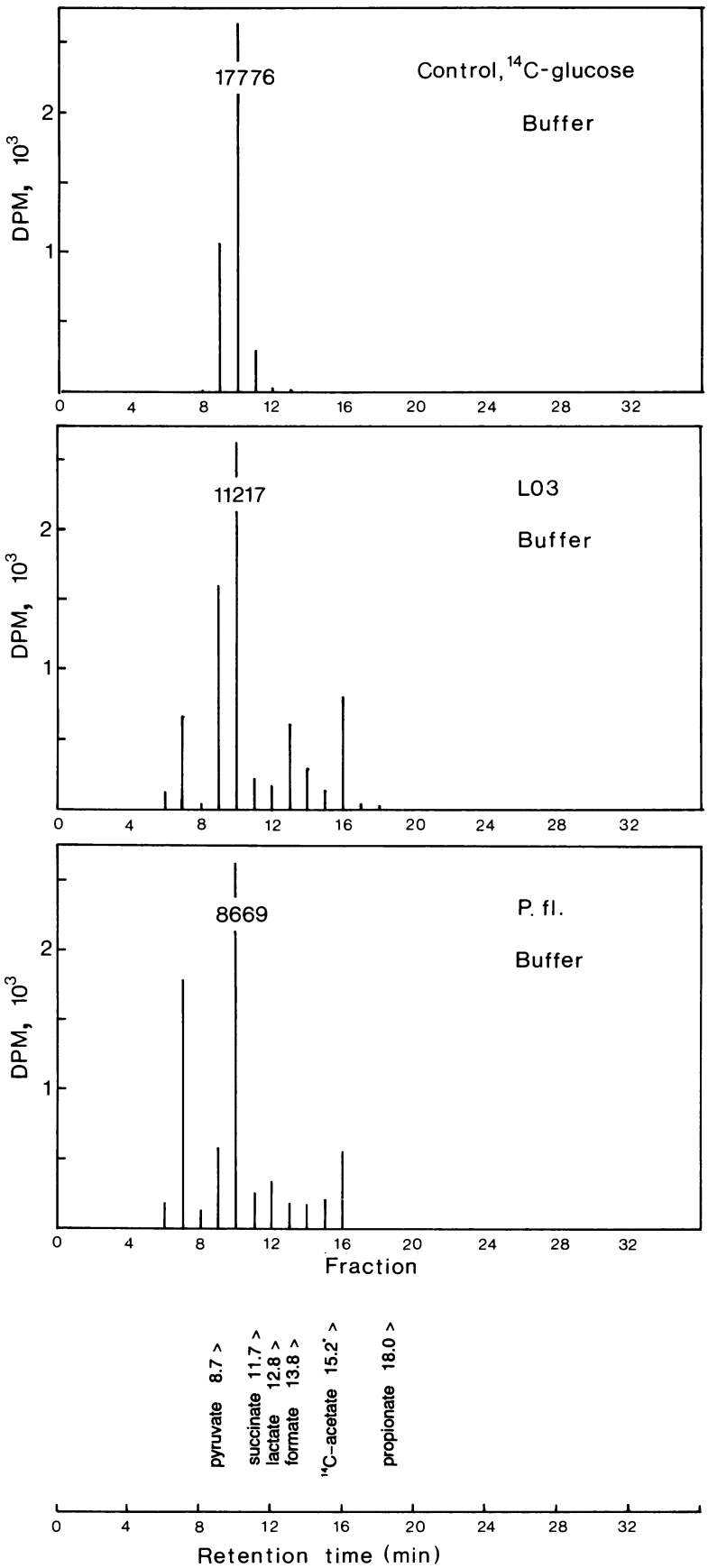

FIG. 4. Fractionation of ^{14}C-labeled components in the starvation solution by chromatography and subsequent scintillation counting. Retention time of standard compounds is shown. Glucose elutes in fraction 10. P. fl., *P. fluorescens* ATCC 17822. *, Detected in fraction 16.

denitrifiers starved for electron acceptors, two sets of survival experiments were set up, with 10^5 and 10^8 cells per ml. The increase in the number of cells in the 10^5 cells per ml experiment (Fig. 3) and the decrease in cell size that was observed suggest the commonly observed survival strategy of producing many but smaller cells (16, 18). The question of whether these microorganisms are carrying out sufficient metabolism to produce maintenance energy has been the subject of discussion in the literature, as reviewed by Morita (16). Morita (17) suggested that starving cells enter a stage of metabolic arrest until favorable conditions again occur. Most of the studies concerning starvation, however, have dealt with starvation for nutrients or electron donors, and very few have dealt with starvation for electron acceptors.

A very low metabolic activity by the starved cells was shown by the slow reduction of tetrazolium. Oren (20) found that this reduction measures not only the "electron transport system activity" in cells possessing an electron transport chain, but also hydrogen production during fermentation processes. The motility of these cells also indicated that energy was being produced in these cells. Thus, these obligate aerobes were capable of at least some electron transport and energy generation after 3 months without either NO_3^- or O_2 as an electron acceptor.

The spike of [^{14}C]glucose was added after 3 months of electron acceptor starvation to see whether these aerobes could metabolize the sugar under these conditions and, if so, to what end products. Label appeared in decreasing amounts in the following fractions: cells > CO_2 > ether extract. Within the ether extract, a significant portion of products fractionated on a fatty acid column as low-molecular-weight fatty acids. Thus, glucose appeared to be fermented by these *Pseudomonas* isolates.

The fact that pseudomonads are known to be unable to ferment glucose or other sugars under anaerobic conditions seems inconsistent with this finding. However, under extreme electron acceptor starvation, it appears that glucose can be fermented. The energy created is probably so minor that it could never support much growth in these organisms, but it may be sufficient to support maintenance. What is clear, however, is that these typically aerobic bacteria can survive in nature for very long periods at high densities without nitrate or oxygen. A low level of fermentation appears to be the only explanation for their existence that is supported by the evidence.

In support of the anaerobic metabolism of pseudomonads, it is now known that this genus contains an anaerobic operator sequence, *anr*, that has operator sequences very similar to those of the *fnr* anaerobic operator in *Escherichia coli* (27). The *Pseudomonas anr* operator controls expression of the operon that carries the ability to ferment arginine (6). Very similar sequences are found for the promoter of the operon that carries denitrifying enzymes and associated cytochrome genes (23). Perhaps this promoter also controls other operons that allow "fermentative" survival of these aerobic organisms.

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