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Aerobic and Anaerobic Starvation Metabolism in Methanotrophic Bacteria†

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The capacity for anaerobic metabolism of endogenous and selected exogenous substrates in carbon- and energy-starved methanotrophic bacteria was examined. The methanotrophic isolate strain WP 12 survived extended starvation under anoxic conditions while metabolizing 10-fold less endogenous substrate than did parallel cultures starved under oxic conditions. During aerobic starvation, the cell biomass decreased by 25% and protein and lipids were the preferred endogenous substrates. Aerobic protein degradation (24% of total protein) took place almost exclusively during the initial 24 h of starvation. Metabolized carbon was recovered mainly as CO_2 during aerobic starvation. In contrast, cell biomass decreased by only 2.4% during anaerobic starvation, and metabolized carbon was recovered mainly as organic solutes in the starvation medium. During anaerobic starvation, only the concentration of intracellular low-molecular-weight compounds decreased, whereas no significant changes were measured for cellular protein, lipids, polysaccharides, and nucleic acids. Strain WP 12 was also capable of a limited anaerobic glucose metabolism in the absence of added electron acceptors. Small amounts of CO_2 and organic acids, including acetate, were produced from exogenous glucose under anoxic conditions. Addition of potential anaerobic electron acceptors (fumarate, nitrate, nitrite, or sulfate) to starved cultures of the methanotrophs *Methylobacter albus* BG8, *Methylosinus trichosporium* OB3b, and strain WP 12 did not stimulate anaerobic survival. However, anaerobic starvation of these bacteria generally resulted in better survival than did aerobic starvation. The results suggest that methanotrophic bacteria can enter a state of anaerobic dormancy accompanied by a severe attenuation of endogenous metabolism. In this state, maintenance requirements are presumably provided for by fermentation of certain endogenous substrates. In addition, low-level catabolism of exogenous substrates may support long-term anaerobic survival of some methanotrophic bacteria.

Bacteria often alternate between phases of growth and nongrowth because of fluctuations in substrate availability in their local environment. When growth substrates become limiting, bacteria generally initiate starvation responses that include both morphological and physiological changes (see, e.g., references 16, 21, 23, 25, 29, and 33). These phenotypic changes are integrated parts of survival strategies in which survival time is often optimized by lowering energy metabolism while maintaining some basic cellular processes (7, 31, 35). These basic processes require energy, although the demand can be very small during long-term starvation (7, 23, 26).

Little is known about survival and energy metabolism in methanotrophic bacteria during starvation. However, methanotrophs often live in environments where gradients of nutrients and oxygen can vary significantly over time. In addition, methanotrophic bacteria can utilize only a relatively limited number of electron donors and acceptors. Growth occurs aerobically on reduced C_1 carbon compounds such as methane, methanol, and, occasionally, formaldehyde; aerobic energy transduction proceeds via electron transport systems that include methanol, formaldehyde, and formate dehydrogenases (1, 4, 10). Fermentation or anaerobic respiration has never been demonstrated, and methanotrophs are therefore traditionally considered obligate aerobic respiratory bacteria with restricted catabolic potential (4, 10, 36).

Measurements of oxygen regimes in situ suggest that methanotrophs are frequently exposed to anoxic conditions (20, 28). Furthermore, methanotrophic bacteria have been shown to survive anaerobic starvation relatively well both in situ and in laboratory cultures (see, e.g., references 3, 17, 20, and 27). Bender (3) observed a significant potential for aerobic methane oxidation in sediment that had been anoxic for approximately 90 years, and it was suggested that this capacity was due to the presence of spores or other differentiated resting stages. Other studies have shown that long-term anaerobic survival of methanotrophs can also occur in a nondifferentiated state that facilitates a relatively rapid response to substrate addition (27, 28). For example, nonsporulated cells of *Methylosinus trichosporium* OB3b maintained a capacity for relatively rapid aerobic methane oxidation after 42 days of anoxic incubations (27). In addition, anaerobic starvation of different methanotrophs frequently increased survival and recovery relative to aerobic starvation (27). These studies show that methanotrophic bacteria are capable of starvation survival in both the presence and absence of oxygen. However, the physiology of resting methanotrophs remains uncertain, and it is not known how maintenance energy is generated under starvation conditions.

In the present study, we measured the endogenous metabolism of carbon- and energy-starved methanotrophs during incubations under oxic and anoxic conditions. We examined the effects of potential electron acceptors and donors on survival under anoxic conditions and explored the possibility for an anaerobic metabolism of exogenous glucose.

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MATERIALS AND METHODS

Organisms. The methanotrophic strain WP 12 was isolated from a freshwater wetland as described previously (27); *Methylosinus trichosporium* OB3b and *Methylobacter albus* BG8 were obtained from R. S. Hanson. The cultures were regularly checked for potential heterotrophic contamination. Examination by microscopy showed only one morphotype in each culture, and cells did not grow aerobically or anaerobically when plated onto trypic soy broth agar or nitrate minimal medium (NMM) agar supplemented with 1 mM glucose and 0.01% yeast extract.

Media and starvation conditions. Methanotrophs were grown in NMM in a methane (30%) atmosphere at 30°C as described previously (27). Cells used in the starvation experiments were harvested in late exponential phase by centrifugation, washed twice, and resuspended in NMM or 10 mM phosphate buffer (pH 7.0) as outlined below. The cells were starved for methane in the presence or absence of oxygen in the medium. The two starvation conditions were obtained by flushing the cultures with either filter-sterilized air (oxic conditions) or nitrogen (ultrahigh-purity N_2, 0.6 ppm O_2) (anoxic conditions). The resulting starvation regimes are referred to as aerobic and anaerobic starvation, respectively.

Methane oxidation assay. Aliquots (3 ml) from the starved cultures were centrifuged, washed, and then resuspended in 3 ml of NMM. Methane oxidation rates were calculated from linear decreases in methane concentrations in 16-cm^3 serum bottles after addition of 0.3-cm^3 methane (ultrahigh purity). Gas samples (0.2 cm^3) were taken with a needle and syringe and analyzed on a Shimadzu 14A gas chromatograph equipped with a Porapak Q column and a flame ionization detector.

Plate counts. Culture medium for aerobic plate counts was sampled from methane-starved cultures with a needle and syringe. After serial dilution, 50-μl samples were plated onto NMM agar and incubated at 30°C with an initial atmosphere of 30% methane in air. Colonies were counted after 4 and 6 weeks of incubation.

Endogenous starvation metabolism in strain WP 12. Strain WP 12 was labeled with ^{14}C by adding 0.9 MBq of $^{14}CH_4$ (>99% purity, 2.07 GBq $mmol^{-1}$; Amersham Corp., Amersham, United Kingdom) to the headspace of a 1-liter Erlenmeyer flask containing 200 ml of culture in early exponential phase. The culture was subsequently grown to mid-exponential phase by repeated addition of 1% unlabeled methane to the headspace. The culture was then harvested by centrifugation, washed twice, and resuspended in 10 mM phosphate buffer (pH 7.0) to an A_{600} of 0.2 (density 0.07 mg ml^{-1}). Aliquots (25 ml) were transferred to 160-cm^3 serum bottles, and 5 ml of culture was sampled for analysis of the initial distribution of ^{14}C in bacterial biomass (see below). The bottles were sealed with green neoprene stoppers and then flushed with either filter-sterilized air or N_2. Sealed anoxic bottles were placed in an incubator with an N_2 atmosphere, and oxic and anoxic bottles were subsequently incubated at 30°C on a shaker at 100 rpm.

The distribution of ^{14}C in bacterial biomass and as organic and inorganic carbon in the medium and headspace was determined initially and after 24, 72, and 240 h of methane starvation. Labeled biomass was separated into four fractions by a solvent fractionation scheme partly as described by DiTullio (8) (Fig. 1). We substituted dichloromethane for chloroform in the lipid extraction step (5). Cells were initially filtered onto Whatman GF/F filters, and cell residues plus filter residues were subsequently collected on a new filter after each extraction step (Fig. 1). Cell-free medium collected in the initial filtration step was frozen for later analysis of soluble metabolites. After the medium was thawed, the radioactivity was measured in untreated (pH 7.0) and acidified (pH < 3.0) samples to quantify ^{14}C-labeled metabolites with and without including labeled volatile organic acids. The protein fraction and aliquots of the low-molecular-weight (LMW) fraction and nucleic-acids-plus-polysaccharide (NAP) fraction were radioassayed directly with a Fisher ScintiVerse Bio HP cocktail. The dichloromethane-containing lipid fraction was dried under a heating lamp and then mixed with scintillation cocktail. Production of total $^{14}CO_2$ was calculated from the concentration of $^{14}CO_2$ in 1-cm^3 samples of headspace gas. All samples were analyzed on a model 1218 scintillation counter (LKB Pharmacia, Inc.) with internal standards to correct for quench. Recovery of label in the four fractions (Fig. 1) generally accounted for 94 to 99% of the total activity measured in unfractionated samples.

Effect of exogenous substrates. The effect of potential electron acceptors on the anaerobic survival of strain WP 12, *Methylosinus trichosporium* OB3b, and *Methylobacter albus* BG8 was examined by incubating N_2-flushed cultures with 1 mM nitrate, nitrite, sulfate, or fumarate. Electron acceptors were tested alone or in combination with 1 mM methanol, formate, pyruvate, malate, or glucose.

The effect of exogenous carbon compounds on the survival of methanotrophs was examined by incubating cells under anoxic conditions with 0.1 or 1 mM glucose, 0.01% yeast extract, 0.01% tryptic soy broth, 0.1 mM alanine plus 0.1 mM glycine, 0.1 mM pyruvate, or 1% peat extract. Peat extract was made by homogenizing anoxic peat (from the site where strain WP 12 was isolated) in a blender with an equal volume of autoclaved deionized water. The extract was incubated at 10°C for 5 days, filter sterilized, and added to autoclaved phosphate buffer.

Metabolism of D-[U-^{14}C]glucose. The potential metabolism of glucose under anoxic conditions in cultures of strain WP 12 was examined. Cells were harvested in late exponential phase, washed twice, and resuspended in 10 mM phosphate buffer to a density of 0.3 mg ml^{-1}. Aliquots of 10 ml were transferred to 20-cm^3 bottles, and a mixture of D-[U-^{14}C]glucose (11.19 GBq $mmol^{-1}$; Sigma, St. Louis, Mo.) and unlabeled glucose was added to give a final activity of 40.7 kBq ml^{-1} and a total glucose concentration of 100 μM. The bottles were immediately sealed with neoprene stoppers and then flushed with filter-sterilized N_2 for 60 min. Anoxic bottles were incubated at 30°C in an incubator flushed with N_2 and placed on a shaker at 100 rpm. Production of radiolabeled metabolites in subsamples of headspace gas (1 cm^3) and culture medium (3 ml) was examined. The presence of ^{14}C in bacterial biomass was determined by the fractionation scheme described above (Fig. 1), with the modification that culture samples were initially rinsed with 15 ml rather than 3 ml of phosphate buffer.

D-[U-^{14}C]glucose used in the experiment was initially purified by passing 0.2 ml of an aqueous solution of the compound through an anion-exchange column containing 2 cm^3 of washed Dowex-1 resin (chloride form; Sigma). D-[U-^{14}C]glucose was eluted with autoclaved deionized water, and the fraction with highest activity was filter sterilized and then used in the experiment.

Ion-exchange columns were also used to separate anionic ^{14}C-metabolites from ^{14}C-glucose (19). Cell-free starvation medium from days 0 and 10 was passed through the Dowex columns and then was washed with 15 ml of deionized water. This wash removed excess labeled glucose, which was confirmed by measuring the activity in the eluant (<100 dpm ml^{-1}). Labeled anions were then eluted from the columns with two 4-ml volumes of 1 M NaCl.

The anionic eluants were fractionated by high-pressure liquid chromatography (HPLC) with a reversed-phase column (25 cm by 4.6 mm, Astec VAC C18; Rainin Inc.) and a mobile phase with 150 mM NH_4Cl and 100 mM NaH_2PO_4 adjusted to pH 2.5. The column was operated at 30°C with a flow rate of 0.8 ml min^{-1}. Radiolabeled samples were spiked with unlabeled organic acids prior to injection, and the eluant was subsequently divided into 11 fractions according to the retention times measured for the added organic acids by UV detection at 210 nm. The concentration of label in the different fractions was determined by scintillation counting as described above.

Enzymatic assays. Production of radiolabeled acetate from D-[U-^{14}C]glucose was evaluated by an enzymatic assay to convert acetate to acetyl coenzyme A (acetyl-CoA) (18). Eluants from the anion-exchange columns were incubated for 120 min at 37°C and pH 8 in the presence of 1 U of acetyl-CoA synthetase ml^{-1}, 1 mM CoA, 1 mM ATP, and 10 μM unlabeled acetate. Subsequent HPLC fractionation of samples resulted in a decreased radioactivity in the acetate fraction as a result of a different retention time for acetyl-CoA relative to acetate.

Bulk starvation medium was examined for the presence of radiolabeled ethanol by sequentially incubating samples with alcohol and aldehyde dehydrogenases (1 U ml^{-1}) to obtain radiolabeled acetate. After 120 min of incubation, samples were fractionated by liquid chromatography and the activity in the acetate fraction was determined by scintillation counting.

RESULTS

Endogenous starvation metabolism in strain WP 12. Two patterns of endogenous metabolism were seen in strain WP 12 during methane starvation (Table 1). Aerobic starvation resulted in a 25% loss of labeled biomass over 240 h; protein (12%) and lipid (8%) depletion accounted for most of the decreases. In contrast, only minor changes in the concentration of intracellular label were seen during anaerobic starvation (Table 1). Total labeled biomass decreased by 2.4%, and the fraction containing LMW compounds (including metabolites) was the only pool showing a significant depletion ($P = 0.05$ for $t_{(1),4}$).

During aerobic starvation, most of the carbon lost from labeled cells was recovered as CO_2; a minor fraction (0.5%) was detected as organic carbon in the starvation medium (Table 1). Anaerobic starvation resulted mainly in accumulation of organic carbon in the starvation medium (4.2% of labeled biomass).

Similar linear decreases were seen for LMW compounds during aerobic and anaerobic starvation (Fig. 2A). Lipid transformations took place throughout the aerobic starvation period, whereas no significant changes were seen in anoxic cultures (Fig. 2B). After 240 h, lipid concentrations had decreased by 35% in oxic cultures, which was the greatest relative decrease seen for any of the four pools. Aerobic and anaerobic metabolism of nucleic acids and polysaccharides was apparently confined to the initial 72 h of starvation (Fig. 2C). However, the decrease measured after 240 h of anaerobic starvation was not statistically significant at the 5% level ($P = 0.067$ for

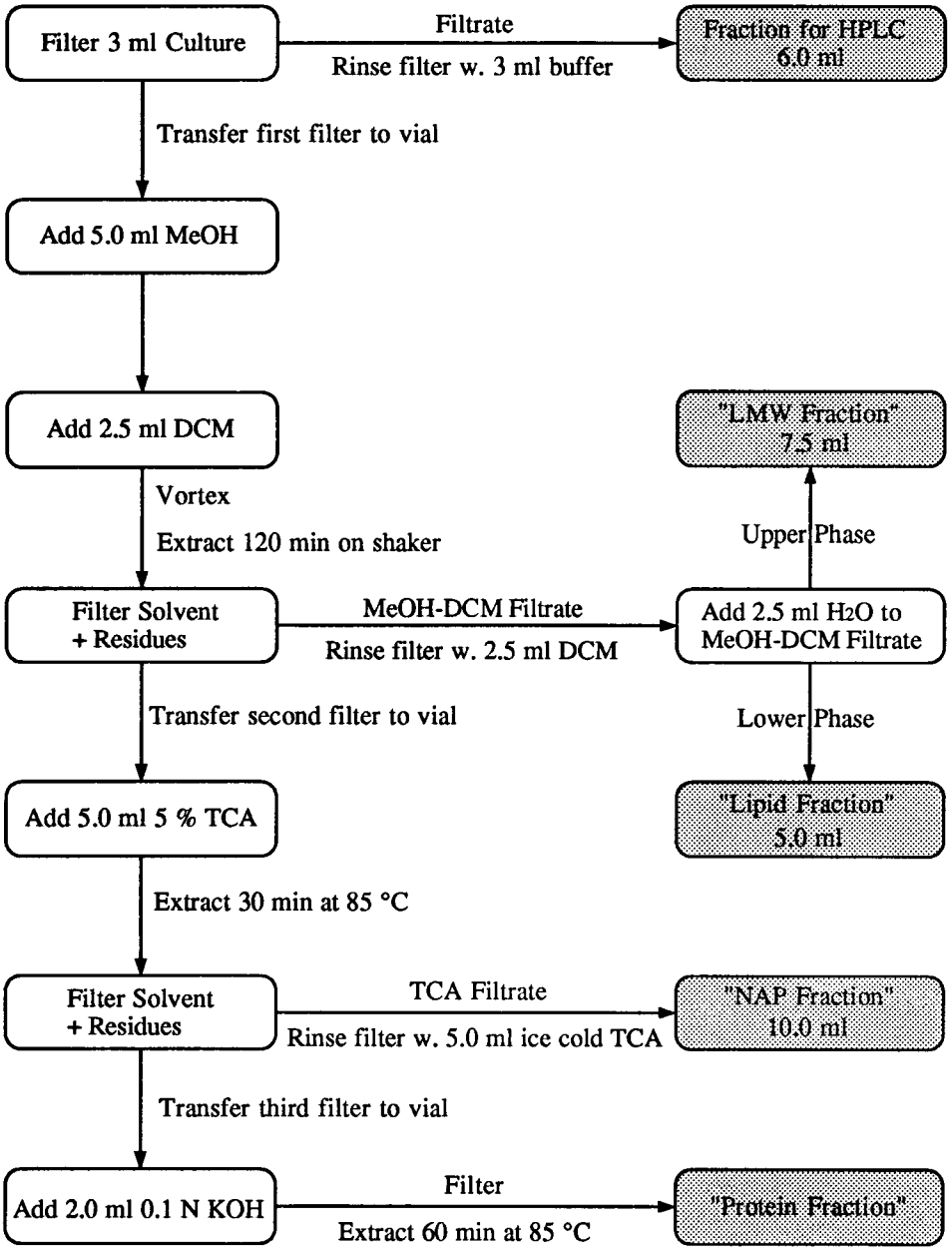

FIG. 1. Outline of the solvent fractionation scheme used for separation of labeled cell material. DCM, dichloromethane; TCA, trichloroacetic acid.

$t_{(1),4}$). Aerobic protein metabolism in strain WP 12 took place almost exclusively during the initial 24 h of starvation (Fig. 2D). During this period, 23% of cellular protein was lost; only 1% was metabolized during the subsequent 216 h of aerobic starvation. No significant decrease in labeled intracellular protein was measured after 240 h of anaerobic starvation.

The large initial decrease in labeled biomass was reflected in a curvilinear production of $^{14}CO_2$ under oxic conditions (Fig. 3A). In contrast, only minor changes in $^{14}CO_2$ production were measured during 240 h of anoxic incubations (Fig. 3A). Instead, a curvilinear accumulation of extracellular nonvolatile organic carbon was observed in the starvation medium (Fig. 3B).

Effect of exogenous substrates on survival. Addition of nitrate, nitrite, sulfate, or fumarate to anoxic starvation medium did not significantly increase the survival of methanotrophic bacteria (data not shown). Small amounts of nitrate and nitrite (<50 nmol ml of culture^{-1}) were reduced to N_2O by anoxic cultures of *Methylosinus trichosporium* OB3b and *Methylobacter albus* BG8, but the cultures did not survive better than parallel cultures incubated in phosphate buffer alone. In these experiments, neither survival nor N_2O production was stimulated by addition of methanol, formate, pyruvate, or glucose.

Addition of potentially fermentable substrates such as trypic soy broth, glucose, peat extract, or a mixture of alanine and glycine to anoxic cultures did not increase poststarvation methane oxidation rates in strain WP 12 (Fig. 4). However, methane oxidation rates were 35% higher after anaerobic starvation than after aerobic starvation (2.7 and 2.0 nmol of CH_4 10^8 cells^{-1} min^{-1}, respectively). Similarly, anoxic incubations of

TABLE 1. Metabolism of endogenous substrates by ^{14}C-labeled strain WP 12 during aerobic and anaerobic starvation[a]

Fraction[b]	Aerobic starvation			Anaerobic starvation		
	Amt (10^3 dpm/ml) on:		Change[c] (% of initial biomass)	Amt (10^3 dpm/ml) on:		Change[c] (% of initial biomass)
	Day 0	Day 10		Day 0	Day 10	
Intracellular						
LMW	21.7	17.6	−3.2	21.6	18.4	−2.4
Lipids	27.9	18.0	−7.6	27.8	26.8	
NAP	13.7	11.2	−1.9	14.4	13.3	
Protein	66.8	50.8	−12.2	65.6	66.7	
Total	130.1	97.6	−25.0	129.4	125.2	−2.4
Extracellular						
CO_2[d]	0.7	20.0	+14.8	0.9	2.4	+1.1
Medium[e]	0.3	1.0	+0.5	0.1	5.5	+4.2

[a] Results are means for triplicate cultures.
[b] See Fig. 1.
[c] Only significant changes are shown ($P \leq 0.05$ for $t_{(1),4}$); changes are calculated as ($count_{day\ 10} - count_{day\ 0}$) × 100/total $count_{day\ 0}$.
[d] CO_2 is calculated as total CO_2 produced per milliliter of culture.
[e] Organic carbon in the starvation medium.

Methylosinus trichosporium OB3b and *Methylobacter albus* BG8 generally resulted in a greater capacity for methane oxidation after starvation than did oxic incubations.

In some starvation experiments, elevated concentrations of yeast extract (≥0.05%) and amino acids (≥1 mM) decreased starvation survival under oxic conditions relative to that of oxic cultures without organics added (data not shown). Under anoxic conditions, methanotrophs at relatively high cell densities (10^8 cells ml^{-1}) were generally unaffected by addition of organic substrates, as indicated by methane oxidation capacity after starvation.

A somewhat different pattern of anaerobic starvation sur-

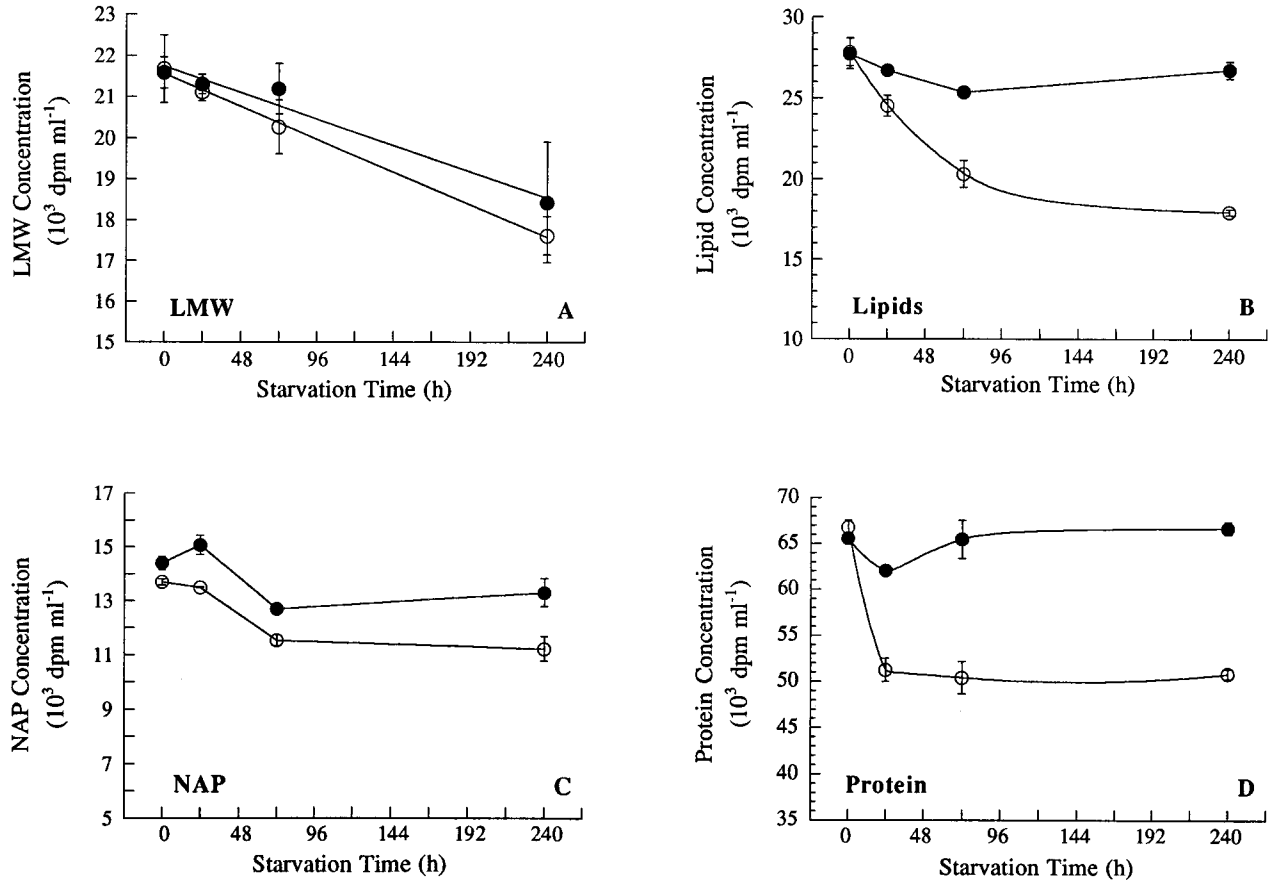

FIG. 2. Time courses of changes in the concentration of cell LMW (A), lipids (B), NAP (C), and protein (D) during aerobic (○) and anaerobic (●) carbon starvation of strain WP 12 (see Fig. 1 for definitions of individual fractions). Data are the means for triplicate cultures ± standard errors.

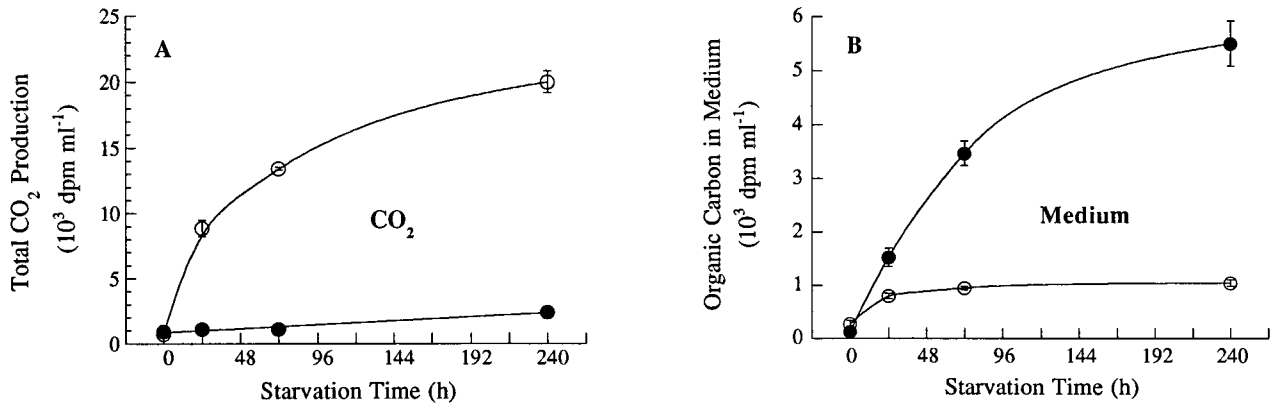

FIG. 3. Time courses of changes in total CO_2 (A) and organic carbon in the starvation medium (B) during aerobic (○) and anaerobic (●) carbon starvation of strain WP 12. Data are the means for triplicate cultures ± standard errors.

vival was seen when strain WP 12 was incubated at lower cell densities (0.9×10^3 cells ml^{-1}) in the presence of 1 mM glucose and 0.01% yeast extract. In these experiments, aerobic plate counts were used to evaluate survival and CFU counts did not decrease after 100 days of anoxic incubation. Recovery on NMM agar in the presence of methane and oxygen was slow, and 3 to 6 weeks of incubation was required before colonies were detectable.

Anaerobic metabolism of D-[U-^{14}C]glucose. Strain WP 12 metabolized small amounts of glucose under anoxic conditions. Relatively high concentrations of label were found in the LMW fraction (e.g., intracellular D-[U-^{14}C]glucose and ^{14}C-metabolites) and as $^{14}CO_2$ and ^{14}C-anions in the anoxic starvation medium (Fig. 5). Small amounts of label were also recovered as bacterial biomass in the protein and NAP fractions (Fig. 5). If labeled and unlabeled glucose were turned over at the same rate, the observed anaerobic metabolism in strain WP 12 corresponded to approximately 0.1 nmol of glucose ml^{-1} day^{-1}.

Some of the anions produced from D-[U-^{14}C]glucose eluted together with acetate when fractionated by liquid chromatography (fraction 5, Fig. 6). The activity in fraction 5 was reduced by 71 to 90% when samples were incubated in the presence of acetyl-CoA synthetase prior to the fractionation. A large unidentified fraction eluted after 3.17 min and was not retained by the column (Fig. 6). Ethanol was not detected in the starvation medium from day 10, as indicated by the absence of increased acetate concentrations after incubation of medium in the presence of alcohol and aldehyde dehydrogenases.

DISCUSSION

In the present study, we examined aspects of aerobic and anaerobic starvation metabolism in methanotrophic bacteria. Metabolism of endogenous and some exogenous substrates was examined primarily with the recently isolated strain WP 12 (27). This bacterium is a non-spore-forming isolate from anoxic peat, and it was tentatively identified as a group I methanotroph (27).

Aerobic and anaerobic endogenous metabolism in carbon- and energy-starved strain WP 12 was evaluated from decreases in the concentration of intracellular macromolecules. The results suggested two types of endogenous metabolism with respect to substrate range and potential energy yield. Under oxic conditions, up to 25% of cell carbon was respired, with proteins and lipids as the preferred substrates (Table 1; Fig. 2).

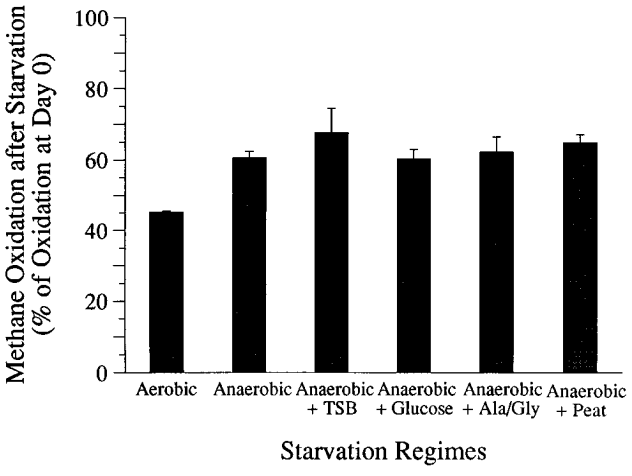

FIG. 4. Effect of different starvation regimes on methane oxidation by strain WP 12. Cells were incubated for 240 h under oxic or anoxic conditions in the absence of methane. Some anoxic cultures were also incubated in the presence of organic substrates (see Materials and Methods for details). Abbreviations: TSB, tryptic soy broth; Ala, alanine; Gly, glycine; Peat, peat extract. Data are the means for triplicate cultures ± standard errors.

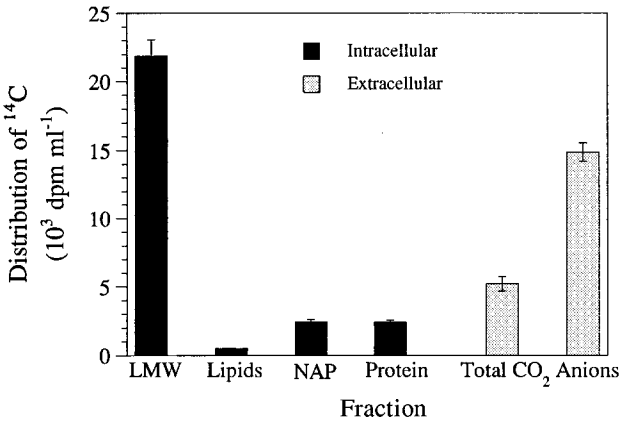

FIG. 5. Formation of ^{14}C-labeled end products from D-[U-^{14}C]glucose in anoxic cultures of strain WP 12. Cultures were incubated for 240 h in the presence of 10 μM labeled and 90 μM unlabeled glucose. Data are the means for triplicate cultures ± standard errors.

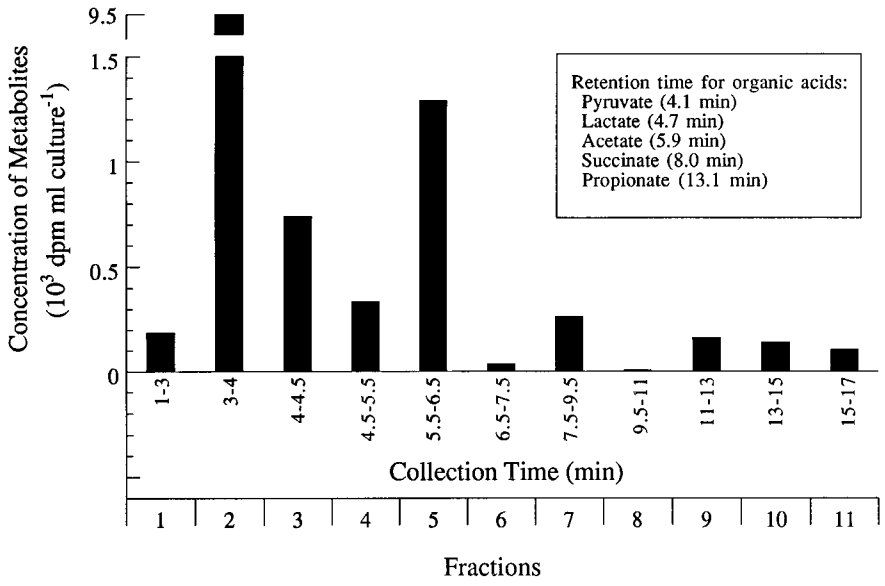

FIG. 6. Fractionation of ^{14}C-labeled anions produced by strain WP 12 under anoxic conditions from D-[U-^{14}C]glucose. Anions were separated by liquid chromatography, and the activity in each fraction was then determined by scintillation counting. The activity in fraction 2 was 9.5×10^3 dpm ml^{-1}. The retention times for known organic acids are also shown.

Under anoxic conditions, a nonrespiratory metabolism based on LMW compounds and perhaps intracellular polysaccharides consumed approximately 10-fold less endogenous substrate (2.4% of cell carbon [Table 1]). Accumulation of organic compounds in the starvation medium during anoxia suggested that endogenous substrates were fermented as described for other bacteria (6, 7). However, the anaerobic metabolism of strain WP 12 was slow and showed a more restricted substrate range than that seen for facultative and obligately anaerobic bacteria (see, e.g., references 6 and 7). Cell leakage may also have contributed to the accumulation of labeled carbon in the anoxic medium, although lysed cells were generally not observed and accumulation of label in parallel oxic culture media was very low (Fig. 3B).

Survival of starved bacteria presumably depends on the ability of the cells to sustain a number of basic processes (7, 35). Maintaining these processes (and thus cell integrity) requires energy, although the demand can be very small during extended starvation (see, e.g., references 7, 23, and 26). Generation of energy during starvation depends on metabolism of endogenous substrates (2, 7) or the ability to gain energy from alternative exogenous substrates (23, 24). However, it remains uncertain how the rate of starvation metabolism per se determines bacterial survival, and it has been suggested that the traditional concept of maintenance energy requirements may not be directly applicable to dormant bacteria (6, 7, 23, 24, 26).

The results of the present study suggested that methanotrophic bacteria consumed significantly more endogenous substrate (10-fold) during the initial 48 h of aerobic starvation than during the same period of anaerobic starvation. Since aerobic respiration generates more energy per mole of substrate oxidized than nonrespiratory anaerobic metabolism does, a simple estimate suggests that at least 10 times more energy could have been produced initially from endogenous substrates in oxic cultures. However, the greater energy production during aerobic starvation did not translate into a better starvation recovery, because poststarvation methane oxidation rates were generally lower for cultures that had been incubated under oxic relative to anoxic conditions. In a previous study, measurements of plate counts, reduction of a tetrazolium salt, and poststarvation protein synthesis similarly suggested better starvation survival for methanotrophic bacteria starved in the absence of oxygen (27). These findings emphasize the complex relationship that apparently exists between measurable endogenous metabolism, maintenance energy requirements, and bacterial survival.

The decreased energy consumption and relatively successful survival of methanotrophic bacteria in the absence of oxygen could have implications for understanding the survival of these bacteria in situ. A survival strategy involving severe depression of metabolic rates and corresponding attenuation of energy-consuming processes under anoxic conditions (anaerobic dormancy) would conserve limited endogenous substrate reserves and subsequently maximize survival time. This strategy is perhaps analogous to the reversible anaerobic hypometabolic response ("metabolic arrest") described in detail for anoxia-tolerant animal cells (see, e.g., references 12, 13, and 34). In these cells, anaerobic dormancy is frequently accompanied by a 5- to 100-fold decrease in fluxes through central pathways such as glycolysis (13, 34). As a result, survival time is extended correspondingly (5 to 100 times) because of a general decrease in rates of biological processes ("slowdown of biological time" [13, 34]). Additional knowledge about anaerobic dormancy in nonsporulating bacteria could thus help explain the survival of certain environmental stresses and may expand current concepts of bacterial dormancy as described by others (see, e.g., references 15, 23, 29, and 33).

Exogenous carbohydrates and other compounds with carbon-carbon bonds are generally not considered growth and energy substrates for methanotrophic bacteria (4, 36). Some authors have speculated that the very limited metabolism of exogenous multicarbon compounds could be due to severe transport limitations rather than inadequate metabolic pathways per se (11, 32). The presence of intracellular label in strain WP 12 incubated with D-[U-^{14}C]glucose suggests a limited capacity for glucose uptake under anoxic conditions by active or passive mechanisms. The high concentration of label

in the LMW pool was presumably due to a combination of labeled glucose and glucose metabolites.

Group I methanotrophs, including *Methylococcus* spp. (4), contain several enzymes involved in carbohydrate metabolism (see, e.g., references 11 and 32). Previous studies have shown that *Methylococcus* strain NCIB 11083 can metabolize stored polyglucose under aerobic starvation conditions, and it was concluded that this process could generate sufficient energy for protein synthesis in the absence of methane (22). Aerobic assimilation of glucose and fructose has also been demonstrated for growing cells of *Methylococcus capsulatus*, although these substrates were not considered important carbon sources (9). The results obtained with strain WP 12 suggest that some methanotrophs are capable of low-level metabolism of exogenous glucose under anoxic conditions. Anaerobic production of $^{14}CO_2$ and [^{14}C]acetate from D-[U-^{14}C]glucose in the absence of exogenous electron acceptors suggests a metabolism that resembles bacterial hexose fermentation in terms of end product formation. Although labeled carbon was also recovered in the bacterial biomass, it is questionable whether this was due to anaerobic assimilation. However, if the observed anaerobic glucose metabolism is coupled to substrate level phosphorylation in group I methanotrophs (e.g., via glyceraldehyde 3-phosphate), this catabolism could potentially play a role in anaerobic starvation survival.

Interestingly, a recent study of starvation survival in denitrifying bacteria showed that some pseudomonads, including *Pseudomonas fluorescens*, were capable of an anaerobic glucose metabolism in the absence of added electron acceptors (14). On the basis of these results, Jørgensen and Tiedje (14) concluded that fermentation of glucose in facultative anaerobic denitrifiers could support survival in oxygen- and nitrate-free environments. The pattern of end product formation by *P. fluorescens* was somewhat similar to that seen for the methanotrophic strain WP 12 and included a low-level production of fatty acids such as acetate (14). However, the two processes differed slightly in that strain WP 12 produced mainly CO_2 and soluble metabolites from added glucose, whereas *P. fluorescens* converted most of the metabolized glucose to bacterial biomass (14).

A potential for a fermentative metabolism has also been suggested for aerobic hydrogen-oxidizing bacteria (30). Lactate, alcohol, and butanediol dehydrogenases and their corresponding metabolites were detected in oxygen-limited cells, whereas the dehydrogenase activity was low in cells grown aerobically. These enzymes were apparently synthesized in the absence of suitable exogenous electron acceptors, and it was suggested that this ability was either an evolutionary relic or associated with advantages during exposure to anoxia (30). Thus, a low-level fermentation of certain exogenous substrates could theoretically extend long-term anaerobic survival in some aerobic bacteria. However, it remains to be shown whether the capacities for anaerobic metabolism of exogenous substrates are indeed coupled to ATP production.

Growth of methanotrophs on methane is a relatively slow process compared with aerobic growth on complex organic substrates by other heterotrophic bacteria. For example, strain WP 12 has a doubling time of 11 to 12 h under seemingly optimal laboratory conditions. In situ, growth is probably even slower, because the availability of substrates such as methane and oxygen is more likely to fluctuate. Although methanotrophs are considered "specialist organisms" with respect to substrates and habitats (4, 10, 32), a relatively successful survival under anoxic conditions would clearly facilitate proliferation under subsequent oxic conditions in situ. A limited capacity for an anaerobic metabolism could thus play an important ecological role in competition with faster-growing aerobic heterotrophs. In support of this, the results of the present study suggest that some methanotrophic bacteria are capable of a low-level anaerobic metabolism of certain exogenous substrates. In addition, these bacteria can survive extended periods of anoxia on energy derived solely from a nonrespiratory metabolism of endogenous substrates. This survival mechanism (anaerobic dormancy) is apparently characterized by very low maintenance energy requirements and corresponding very low rates of endogenous metabolism.

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