Incorporation of [^{14}C]Methanol and [^{14}C]Bicarbonate
by *Hyphomicrobium* sp. 53-49 under
Various Growth Conditions: Aerobic,
Denitrifying and Autotrophic

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Received June 22, 1983

A study was made of the incorporation of methanol and bicarbonate into the cell constituents of denitrifying or aerobic methanol grown and autotrophic $\text{H}_2\text{-O}_2\text{-CO}_2$ grown *Hyphomicrobium* sp. 53-49. Cells were incubated with [^{14}C]methanol or [^{14}C]bicarbonate, and the distribution of the radioactivity in the nonvolatile constituents of ethanol extracts of cells was examined. When denitrifying grown cells were incubated with [^{14}C]methanol, the major part of the radioactivity was fixed to serine as the first stable compound. Aerobic methanol grown cells also fixed [^{14}C]methanol mainly to serine. These results suggest that methanol grown cells assimilate methanol by the serine pathway. When denitrifying or aerobic methanol grown cells were incubated with [^{14}C]bicarbonate, malate was mainly observed as a nonvolatile compound in the initial period of the incubation. Autotrophic grown cells also fixed the major part of [^{14}C]bicarbonate to malate. In this case, phosphoglyceric acid was found in the phosphorylated compounds area.

A number of studies on the assimilation of C_1 compounds by microorganisms have been reported, and there were reviews by Ribbons¹⁾ and Quayle.^{2~4)}

These studies revealed the following pathways for C_1 -compound assimilation⁵⁾: the ribulose biphosphate cycle, the two serine pathways, the ribulose monophosphate cycle, mixing of the ribulose biphosphate and ribulose monophosphate cycles, a pentose phosphate cycle, and one, not yet completely understood.

In the case of *Hyphomicrobia*, C_1 -compound assimilative pathways were reviewed, e.g. by Hirsch⁶⁾ and Harder.⁷⁾ *Hyphomicrobium* strains are the only bacteria that are able to denitrify on growth on C_1 compounds.⁸⁾ However, there were few reports of

C_1 -compound assimilative pathways of *Hyphomicrobium* which was grown on C_1 compounds with denitrification, except methylamine.⁹⁾

Hydrogenomonas eutropha strain H16 had been the only bacterium known that was able to denitrify on growth on H_2 and CO_2 .¹⁰⁾ In the preceding papers, we reported that *Hyphomicrobium* sp. 53-49 was a denitrifying bacterium capable of utilizing methanol and of autotrophic growth in a $\text{H}_2\text{-CO}_2$ gas phase as a sole energy and carbon source.^{11,12)} This strain was characterized as a C_1 and C_2 -methylotroph which is able to utilize only C_1 and C_2 compounds aerobically and to denitrify or grow autotrophically as hydrogen bacteria do.

There has been no report of comparison of

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C₁-compound assimilative pathways of methylotrophs performed using an organism which can assimilate methanol under aerobic or denitrifying conditions, and also assimilate CO₂ under H₂-utilizing conditions.

The present paper describes the investigation and comparison of C₁-assimilation pathways of *Hyphomicrobium* sp. 53-49 grown under various conditions, that is, aerobic methanol, denitrifying methanol, and autotrophic grown. The investigation was carried out using [¹⁴C]methanol or [¹⁴C]bicarbonate, and the intermediate metabolites were detected by two-dimensional paper chromatography.

MATERIALS AND METHODS

Organism. *Hyphomicrobium* sp. 53-49 used was isolated from soil as a denitrifying bacterium capable of utilizing methanol.¹¹⁾

Aerobic methanol grown cells were cultivated on medium #9 which contained Na₂HPO₄·12H₂O 2.85 g, KH₂PO₄ 1.36 g, MgSO₄·7H₂O 0.2 g, CaCl₂·2H₂O 10 mg, FeSO₄·7H₂O 5 mg, MnSO₄·nH₂O (*n*=4~6) 2.5 mg, NaMoO₄·2H₂O 2.5 mg, (NH₄)₂HPO₄ 3 g and CH₃OH 5 g in one liter of distilled water. The culture was incubated aerobically at 30°C in a 1-liter Erlenmeyer-flask with 400 ml of the medium shaken on a rotary shaker.

Denitrifying methanol grown cells were cultivated on medium #13 of which the composition was the same as

that of medium #9 except that KNO₃ (5 g/liter) was used as a nitrogen source instead of ammonium phosphate. Cells were incubated at 30°C in a 2.5-liter Woulff's bottle filled with the medium stirred slowly with a magnetic-stirrer under a nitrogen gas phase.

Autotrophic grown cells were cultivated on medium #16 in a 5-liter mini-jar-fermentor as described in the previous paper.¹²⁾

Each culture was harvested at the logarithmic growth phase by centrifugation, and the cells were washed twice with 10 mM phosphate buffer, pH 7.0.

FIG. 1. Reaction Flask.

¹⁴C Incorporation was carried out in the flask. The gases below were blown into the reaction mixture stirred by the magnetic stirrer, vigorously. The reaction mixture was pre-incubated for 10~20 min. Gases such as air, Ar and H₂-O₂ were used for aerobic, denitrifying and autotrophic grown cells, respectively.

TABLE I. REACTION CONDITIONS

Growth conditions of cells	Addition to reaction mixture per 1 ml					Reaction conditions	Calculated incorporated carbon after 1 min ^a (nmol/mg dry cells)
	Cell (mg of dry cells)	¹⁴ CH ₃ OH (μmol)	NaH ¹⁴ CO ₃ (μmol)	KNO ₃ (μmol)	CH ₃ OH (μmol)		
CH ₃ OH Aerobic	15.8	0.17 (10 μCi)	—	—	—	Aerobic	0.72
	7.8	—	0.75 (20 μCi)	—	5.7	Aerobic	0.055
CH ₃ OH Anaerobic with denitrification	35.6	0.17 (10 μCi)	—	2.5	—	Anaerobic	0.19
	4.3	—	0.75 (20 μCi)	5.7	5.7	Anaerobic	0.16
Autotrophic	27.9	—	0.35 (20 μCi)	—	—	H ₂ :O ₂ , 2:1	1.02

^a These values were calculated from the total radioactivity of spots on the paper chromatograms.

Incorporation of [^{14}C]methanol and [^{14}C]bicarbonate into cell constituents. Cells were suspended in 10 mM phosphate buffer, pH 7.0 for [^{14}C]methanol incorporation or pH 8.0 for [^{14}C]bicarbonate. The incubation was carried out in a flask containing a 7~8 ml reaction mixture as shown in Fig. 1. One ml of the reaction mixture consisted of 4.3~35.6 mg (dry weight) of cells, [^{14}C]methanol or sodium [^{14}C]bicarbonate and 10 μmol of phosphate buffer, pH 7.0 or 8.0. Details of the reaction conditions are given in Table I. Incubation was performed with stirring at 30°C and the gas flowed through the side tube of the incubation flask. At suitable intervals, 1 ml of the reaction mixture was removed and transferred immediately into 4 ml of hot ethanol.

Extraction of ^{14}C -labeled compounds. The reaction mixture transferred to the hot ethanol as described above was boiled for 20 min under a small reflux condenser, with a few drops of 10N formic acid when sodium [^{14}C]bicarbonate was used. After centrifugation, the resultant precipitate was boiled with 5 ml of 20% ethanol and centrifuged again. The combined supernatant was evaporated to dryness under reduced pressure at 45°C. The residue was dissolved in 100 μl of 20% ethanol, and 3, 10, 20 or 50 μl of the solution was applied to filter paper (Toyo No. 50, 40 $\times$ 40 cm) for two-dimensional descending paper chromatography.

Chromatography and radioautography. The filter paper was developed at 30°C using the following solvent systems¹³: phenol–water (100:40, v/v) as the first solvent and *n*-butanol–propionic acid–water (142:71:100, v/v) as the second solvent.

After chromatography, the paper was exposed to X-ray film for a week to detect the radioactive areas. Ketose, aldose and amino acid were detected by spraying with the following reagents: For ketose, 0.2% naphthoresorcinol in ethanol–phosphoric acid (10:1, v/v); for aldose and ketose, 2 g of diphenylamine, 2 ml of aniline and 10 ml of 80% phosphoric acid in 110 ml of ethanol; and for amino acid, 0.02% ninhydrin in *n*-butanol. Extraction and identification of each spot on the chromatogram were done according to the methods in the literature.^{13~21)}

Measurement of radioactivity. The radioactive areas on the paper chromatograms were cut out and placed in vials containing 20 ml of liquid scintillator consisting of 0.4% of 2,5-diphenyloxazole in toluene. The radioactivity was measured with a liquid scintillation spectrometer (Packard 'Tricab' Model 3320). Total carbon incorporated after 1 min was calculated from the activity (Table I).

The serine and glycine fraction was cut out and eluted from the paper with water and dried under reduced pressure at 45°C. The residue was dissolved in 10 μl of water, followed by the addition of 10 ng of glycine and 10 ng of serine (and 10 ng of glucose, fructose and galactose as standard compounds, if necessary). A sample of

5 μl was applied to an Avicel SF TLC plate (5 $\times$ 10 cm) and electrophorased at 600 V in 0.75N formate for 20 min. After serine and glycine were detected with ninhydrin reagent, these and other areas on the plate were scraped off and collected, and then the radioactivity was measured.

RESULTS

Incorporation of [^{14}C]methanol into cell constituents

When denitrifying methanol grown cells were incubated with [^{14}C]methanol anaerobically for 5 sec, the radioactivity appeared mostly in the center area of the paper chromatogram (Fig. 2a). No radioactivity was found near the starting point.

When aerobic methanol grown cells were incubated with [^{14}C]methanol aerobically for 15 sec, the radioactivity appeared in more spots than when denitrifying grown cells were used (Fig. 2b).

The time courses of total radioactivity incorporated into denitrifying grown and aerobic grown cells are shown in Figs. 3a and b, respectively. As can be seen in these figures, the pattern looks similar to the pulse-labelling one. The distribution of the radioactivity in the nonvolatile constituents over a period of 20 min is shown in Fig. 3a (denitrifying grown cells) and Fig. 3b (aerobic grown cells).

In the case of denitrifying grown cells, over 50% of the radioactivity fixed was found in serine and glycine as the major products in the initial period, and the percentage of these amino acids decreased rapidly with time. Aspartate also showed a negative slope. Phosphorylated compounds (spot Nos. 7 and 8 in Fig. 2a) and other radioactive spots were the minor products. The ratio of serine to glycine is shown in Table IIa. Most of the radioactivity was found in serine in the initial period, then the ratio changed with time, and, in the end, most of the activity was found in glycine.

In the case of aerobic methanol grown cells, serine and glycine were also the major products in the initial period (Fig. 3b). The percentages of serine + glycine and aspartate decreased with time. Phosphorylated compounds (spot Nos. 8 and 11 in Fig. 2b) were recognized

a

b

FIG. 2. Radioautograms of Metabolites Produced from $[^{14}\text{C}]$ Methanol by *Hyphomicrobium* sp. 53-49.

Cells were incubated with $[^{14}\text{C}]$ methanol ($10\ \mu\text{Ci}$, $0.17\ \mu\text{mol}$ per ml of reaction mixture). 1 ml of the reaction mixture was withdrawn, and extracted with ethanol-water. After drying, the extract was dissolved in $100\ \mu\text{l}$ of 20% ethanol, and $20\ \mu\text{l}$ of this solution was used for paper chromatography.

a: Denitrifying grown cells were incubated for 5 sec under denitrifying conditions.

b: Aerobic grown cells were incubated for 15 sec under aerobic conditions.

FIG. 3. Time Course of Total Radioactivity and Its Distribution Incorporated from $[^{14}\text{C}]$ Methanol into Cell Constituents of Methanol Grown *Hyphomicrobium* sp. 53-49.

Cells were incubated with $[^{14}\text{C}]$ methanol ($10\ \mu\text{Ci}$, $0.17\ \mu\text{mol}$ per ml of reaction mixture). At the time indicated, a 1 ml aliquot was removed and poured into 4 ml of hot ethanol. The analytical method is described under MATERIALS AND METHODS.

a: Denitrifying grown cells were reacted under denitrifying conditions.

b: Aerobic grown cells were reacted under aerobic conditions.

○—○, serine+glycine; □—□, phosphorylated compounds No. 8; △—△, aspartate; ●—●, phosphorylated compounds No. 7; ■—■, alanine; ■—■, glutamate; ●—●, spot No. 14; ▲—▲, phosphorylated compounds No. 11; ○—○, total radioactivity incorporated.

TABLE II. DISTRIBUTION OF RADIOACTIVITY IN SERINE AND GLYCINE IN THE SERINE+GLYCINE FRACTION INCORPORATED FROM $[^{14}\text{C}]$ METHANOL

a		
Time	Serine (%)	Glycine (%)
5 sec	99.9	0.13
15 sec	92.6	7.4
30 sec	86.2	13.8
1 min	75.6	24.4
2 min	—	—
5 min	17.3	82.7
10 min	14.3	85.7
20 min	—	—

b

Time	Serine (%)	Glycine (%)
15 sec	92.9	7.1
30 sec	88.3	11.7
1 min	58.5	41.5
2 min	—	—
5 min	29.8	70.2
10 min	26.8	73.2
20 min	30.3	69.7

a: Cells grown on medium # 13 with denitrification were incubated with $[^{14}\text{C}]$ methanol under denitrifying conditions.

b: Cells grown on medium # 9 aerobically were incubated with $[^{14}\text{C}]$ methanol under aerobic conditions.

as mixtures, which could neither be separated clearly nor measured quantitatively. Other radioactive spots were the minor products in the initial period or did not show negative

slopes. The ratio of serine to glycine is shown in Table IIb. Most of the radioactivity was found in serine in the initial period, then the ratio changed with time, the same as for the

FIG. 4. Radioautogram of Metabolites Produced from [^{14}C]Bicarbonate by *Hyphomicrobium* sp. 53-49. Autotrophic grown cells were incubated with [^{14}C]bicarbonate (20 μCi , 0.35 μmol per ml of reaction mixture) for 5 sec under $\text{H}_2\text{-O}_2$ gas phase conditions. 1 ml of the reaction mixture was withdrawn, and extracted with ethanol-water. After drying, the extract was dissolved in 100 μl of 20% ethanol, and 20 μl of this solution was used for paper chromatography.

denitrifying grown cells.

Incorporation of [^{14}C]bicarbonate into cell constituents

When denitrifying methanol grown cells were anaerobically incubated with [^{14}C]bicarbonate, methanol and KNO_3 , a few radioactive spots were faintly detected on the paper chromatogram in the initial period. Conversely, when aerobic methanol grown cells were aerobically incubated with [^{14}C]bicarbonate and methanol, radioactive spots were observed in several areas in the early period except around the starting point of the paper chromatogram.

On the other hand, in the case of autotrophic grown cells which were incubated with [^{14}C]bicarbonate under a $\text{H}_2\text{-O}_2$ (2:1) gas flow for only 5 sec, more and higher radioactive spots appeared on the paper chromatogram than when methanol utilizing cells were used (Fig. 4). High radioactivity was also found in the area near the starting point of the paper chromatogram.

The time courses of total radioactivity incorporated into anaerobic, aerobic and autotrophic grown cells are shown in Figs. 5a, b and c, respectively. The distribution of radioactivity in the cell constituents over a period of 20 min for anaerobic and aerobic grown cells and 320 sec for autotrophic grown cells is also shown in Figs. 5a, b and c, respectively.

In the case of denitrifying grown cells, malate and serine+glycine were the major products in the initial period, and the percentages of these radioactivities decreased with time. Over 30% of the radioactivity fixed was found in malate in the initial period. The ratio of serine to glycine is shown in Table IIIa. The percentage of serine was lower and the change in the ratio was slower than in the case of [^{14}C]methanol.

When aerobic grown cells were used, malate was also the major product for the period from 15 sec to 1 min. Compared with denitrifying grown cells, the percentage of serine+glycine increased with time and they became the major

FIG. 5. Time Course of Total Radioactivity and Its Distribution Incorporated from $[^{14}\text{C}]$ Bicarbonate into Cell Constituents of Methanol Grown and Autotrophic Grown *Hyphomicrobium* sp. 53-49.

Cells were incubated with $[^{14}\text{C}]$ bicarbonate ($20 \mu\text{Ci}$, $0.75 \mu\text{mol}$ per ml of reaction mixture). At the time indicated, a 1 ml aliquot was removed and poured into 4 ml of hot ethanol. The analytical method is described under MATERIALS AND METHODS.

a: Denitrifying grown cells were reacted under denitrifying conditions.

b: Aerobic grown cells were reacted under aerobic conditions.

c: Autotrophic grown cells were incubated with $[^{14}\text{C}]$ bicarbonate ($20 \mu\text{Ci}$, $0.35 \mu\text{mol}$ per ml of reaction mixture) under H_2 - O_2 gas phase conditions.

○---○, malate; ○—○, serine + glycine; ■---■, glutamate; △—△, aspartate; ▲---▲, pyruvate; □---□, citrate; ▽—▽, spot No. 39; □—□, phosphorylated compounds (phosphorylated compounds 1 in Fig. 5c); ▲—▲, phosphorylated compounds 2; ○—○, total radioactivity incorporated.

products finally (see Figs. 5a and b). The radioactive spot near the starting point (P-compounds) was shown to be a mixture of compounds which could not be separated quantitatively. Other radioactivities were distributed in many spots including those of

TCA-cycle members. As shown in Table IIIb, the ratio of serine to glycine was very low. Most of the radioactivity was found in glycine (98% initially) and the ratio was constant during the whole period. These phenomena were in contrast with the $[^{14}\text{C}]$ methanol in-

TABLE III. DISTRIBUTION OF RADIOACTIVITY IN SERINE AND GLYCINE IN THE SERINE + GLYCINE FRACTION INCORPORATED FROM [^{14}C]BICARBONATE

a		
Time	Serine (%)	Glycine (%)
15 sec	68.8	31.2
30 sec	53.0	47.0
1 min	50.1	49.9
2 min	56.5	43.5
5 min	49.0	51.0
10 min	41.4	58.6
20 min	40.8	59.2

b		
Time	Serine (%)	Glycine (%)
15 sec	2.0	98.0
30 sec	3.7	96.3
1 min	2.2	97.8
2 min	2.6	97.4
5 min	2.6	97.4
10 min	2.5	97.5
20 min	2.3	97.7

c		
Time	Serine (%)	Glycine (%)
2 sec	24.2	75.8
5 sec	17.4	82.6
10 sec	16.9	83.1
20 sec	17.2	82.8
40 sec	22.6	77.4
80 sec	23.4	76.6
160 sec	26.4	73.6
320 sec	17.7	82.3

a: Cells grown on medium #13 with denitrification were incubated with [^{14}C]bicarbonate under denitrifying conditions.

b: Cells grown on medium #9 aerobically were incubated with [^{14}C]bicarbonate under aerobic conditions.

c: Cells grown on medium #16 autotrophically were incubated with [^{14}C]bicarbonate under $\text{H}_2\text{-O}_2$ gas phase conditions.

corporation.

In the case of autotrophic grown cells, malate, serine + glycine and phosphorylated compounds 1 (P-compounds 1 in Fig. 4) were recognized as the main products. Phosphoglycerate was detected in the phosphorylat-

ed compounds 1 fraction. The percentage of these spots decreased with time, glutamate, on the other hand, increased with time. Table IIIc shows that the percentage of serine was lower than that of glycine and their distribution did not change with time.

DISCUSSION

Methylotrophs oxidize methanol to carbon dioxide through the following pathway.

And under aerobic conditions, one of the assimilative pathways of carbon is the serine pathway in which carbon is assimilated through the serine-glycine cycle. *Hyphomicrobia* have the same pathway.

But there has been no study of C_1 - compound assimilative pathways of *Hyphomicrobium* spp. grown under denitrifying or autotrophic conditions. The authors investigated the assimilation of C_1 compounds by *Hyphomicrobium* sp. 53-49 which can grow on methanol aerobically or anaerobically with denitrification and on CO_2 autotrophically under a $\text{H}_2\text{-O}_2\text{-CO}_2$ atmosphere. *Hyphomicrobium* sp. 53-49 requires carbon dioxide for both autotrophic and heterotrophic growth.¹¹⁾

When [^{14}C]methanol was incorporated into the cell constituents of denitrifying and aerobic methanol grown cells, the radioactivity was found in serine + glycine, especially in serine, as the first stable compounds. The percentage of serine decreased rapidly with time. These results suggest that *Hyphomicrobium* sp. 53-49 which is grown with denitrification or grown aerobically using methanol has the serine pathway.

When [^{14}C]bicarbonate was incorporated into the constituents of denitrifying grown cells, radioactivity was found in malate as the first stable compound. TCA-cycle members (not shown in the figures) and aspartate were detected, and showed negative slopes. These results suggest that this organism has some carboxylase(s) which form(s) malate or oxalo-

acetate and other TCA-cycle members. In addition, serine+glycine were recognized in the earlier period. They were not very high in percentage but high enough for a carbon assimilative pathway. Considering the distribution of the radioactivity between serine and glycine, a part of CO_2 may be fixed to serine and glycine by the reverse action of formate oxidation or alternative routes. Because, in this experiment, the reaction mixture contained methanol as an energy source, there is the possibility of CO_2 reduction to formate.^{22,23)}

When aerobically grown cells incorporated [^{14}C]bicarbonate into cell constituents, most of the radioactivity was found in malate which is similar to the case of denitrifying grown cells. This also suggests that this organism has some carboxylase(s). The percentage of serine+glycine was not high at first but increased with time. And further, serine was negligible in amount compared to glycine throughout the incubation period. This shows carbon dioxide is not incorporated through the serine pathway by aerobically grown cells.

When cells autotrophically grown under a $\text{H}_2\text{-O}_2\text{-CO}_2$ atmosphere were used, [^{14}C]bicarbonate was fixed strongly compared to in the case of methanol grown cells. The radioactivity was found in malate and serine+glycine. Glycine was dominant in the serine+glycine fraction. The same results were obtained for aerobic grown cells except that the percentage of serine+glycine decreased with time. These results suggest that carbon dioxide is fixed through a similar pathway as in the case of aerobic grown cells. Furthermore, in the case of autotrophic grown cells, the radioactivity detected in the phosphorylated compound 1 was strong. A large amount of the compound 1 fraction was phosphoglycerate. This phenomenon was not observed clearly when methanol grown cells were used. This may suggest that the ribulose biphosphate cycle or a similar cycle is present in *Hyphomicrobium* sp. 53-49 grown autotrophically. This idea is supported by the fact that

hydrogen bacteria grown autotrophically on $\text{H}_2\text{-O}_2\text{-CO}_2$ have the ribulose biphosphate cycle.^{24,25)}

These results suggest that *Hyphomicrobium* sp. 53-49 assimilates methanol and carbon dioxide as in the following scheme:

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