

## Formaldehyde Incorporation by a New Methyloph (L3)

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A number of bacterial strains have been isolated and investigated in our search for a promising organism in the production of single-cell protein from methanol. Strain L3 among these isolates was identified as an obligate methyloph which grew only on methanol and formaldehyde as the sole sources of carbon and energy. The organism also grew well in batch and chemostat mixed-substrate cultures containing methanol, formaldehyde, and formate. Although formate was not utilized as a sole carbon and energy source, it was readily taken up and oxidized by either formaldehyde- or methanol-grown cells. The organism incorporated carbon by means of the ribulose monophosphate pathway when growing on either methanol, formaldehyde, or various mixtures of C<sub>1</sub> compounds. Its C<sub>1</sub>-oxidation enzymes included phenazine methosulfate-linked methanol and formaldehyde dehydrogenase and a nicotinamide adenine dinucleotide-linked formate dehydrogenase. Identical inhibition by formaldehyde of the first two dehydrogenases suggested that they are actually the same enzyme. The organism had a rapid growth rate, a high cell yield in the chemostat, a high protein content, and a favorable amino acid distribution for use as a source of single-cell protein. Of special interest was the ability of the organism to utilize formaldehyde via the ribulose monophosphate cycle.

The metabolism of one-carbon compounds by microorganisms is a very active field at present in both theoretical and applied research (25). Much of the recent work with C<sub>1</sub> utilizers has been related to single-cell protein (SCP) research, and a large portion of the work has involved methanol-oxidizing bacteria (9). The advantages of utilizing bacteria in SCP research (20) and of using methanol as a substrate (5) are numerous, and the choice of methanol as the most efficient substrate for growth among the C<sub>1</sub> compounds can hardly be questioned.

From both a theoretical and an applied perspective, however, little is known about the influence of formaldehyde on these methanol-utilizing bacteria. Formaldehyde certainly is accumulated in cells as an intermediate in the oxidation of methanol. It is also certain to be present in varying amounts in various preparations of methanol. Although a fairly toxic substance, it is utilized as a sole carbon and energy source by a variety of methylophs (9, 24). Cell yields of organisms growing on formaldehyde are rarely given in the literature (9), and it is even harder to encounter yields of organisms growing in mixed-substrate cultures containing both formaldehyde and methanol (2). There are few re-

ports which deal with the toxicity of formaldehyde to the methanol-utilizing microorganisms (21-23).

This report describes a number of characteristics of a new methanol-oxidizing bacterium which has many attractive features for SCP use and, in addition, other features pertinent to the relationship of formaldehyde to these bacteria. For example, the strain grows well in mixed-substrate cultures, with reasonably high cell yields. It grows on formaldehyde as a sole carbon and energy source and has a methanol-oxidizing enzyme which is inhibited by formaldehyde. Finally, it is the first organism reported to utilize formaldehyde as a sole carbon source via the ribulose monophosphate (RMP) pathway. Heretofore, it has been tacitly assumed that formaldehyde-grown cells must utilize the serine pathway for incorporation of carbon (9, 11).

### MATERIALS AND METHODS

**Organisms.** Strain L3 was isolated in our laboratory from an enrichment culture containing basal L-salts (19), 4 g of KNO<sub>3</sub> per liter, and a mixture of one-carbon compounds (1% [vol/vol] methanol, 0.1% [wt/vol] sodium formate, and 0.01% formaldehyde). This medium was designed to select for hyphomicrobia and other methylophs.

Cultures were originally inoculated with raw sewage

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liquid obtained from the waste treatment plant in West Lafayette, Ind., and incubated at 30°C for 5 to 7 days. After two sequential transfers in the same medium, the resulting mixed culture was streaked for isolation onto plates of three different media: nutrient agar, nutrient agar supplemented with 0.5% (vol/vol) methanol, and L-salts agar supplemented with 0.5% (vol/vol) methanol. L3 was the only organism which grew on the minimal methanol plates and thus was easily obtained in pure culture.

**Media and chemicals.** The medium eventually used for both batch and chemostat cultivation of L3 was derived from a chemostat medium optimization study similar to that of Mateles and Battat (21). The pre-optimization medium contained 0.1% (vol/vol) CH<sub>3</sub>OH and a modified L-salts base (21) such that the phosphate concentration was five times the L-salts phosphate for pH stability and the nitrate ions were eliminated because of their interference in formaldehyde assays of the culture filtrates (16). The final medium composition included the following salts: Na<sub>2</sub>HPO<sub>4</sub>, 1,050 mg/liter; NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 450 mg/liter; NH<sub>4</sub>Cl, 1,000 mg/liter; MgSO<sub>4</sub>·7H<sub>2</sub>O, 200 mg/liter; KCl, 40 mg/liter; FeSO<sub>4</sub>·7H<sub>2</sub>O, 3 mg/liter; CuSO<sub>4</sub>·5H<sub>2</sub>O, 5 μg/liter; H<sub>3</sub>BO<sub>3</sub>, 10 μg/liter; MnSO<sub>4</sub>·H<sub>2</sub>O, 10 μg/liter; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 70 μg/liter; MoO<sub>3</sub>, 10 μg/liter; and CoCl<sub>2</sub>, 10 μg/liter.

Methanol (99.85% pure) and formate (as the sodium salt) used as substrates were filter sterilized and aseptically added to the autoclaved basal salts medium after it had cooled to either room temperature (liquid medium) or to 50°C (agar media). Formaldehyde was obtained in pure monomeric form and sterilized by adding 0.5 g of powdered paraformaldehyde to 25 ml of distilled water in a Teflon-lined screw-cap tube and heating such tubes for 48 h at 110°C in an oven before use. Cultures normally received 1% (vol/vol) inocula.

Nicotinamide adenine dinucleotide (NAD), reduced NAD, ribose-5-phosphate, lithium hydroxypyruvate, phenazine methosulfate (PMS), 2,6-dichlorophenindophenol, β-mercaptoethanol, and 4,5-dihydroxynaphthalene-2,7-disulfonic acid (chromotropic acid) were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were obtained commercially as reagent-grade materials.

**Chemostat growth studies.** Chemostat cultures were established in a New Brunswick Bio-flo fermentor equipped with automatic pH and temperature controls. The fermentor had continuous monitoring of dissolved oxygen, was aerated by bubbling with filtered room air, and was fed from a 20-liter carboy of presterilized liquid medium. The culture vessel had a working volume of 355 ml. Cultures were routinely kept at pH 7.1 and 30°C, and the agitation and/or aeration rate was manually controlled to keep the dissolved oxygen at 50 to 60%.

**Analyses.** Methanol was assayed with a Varian Aerograph model 3700 gas chromatograph equipped with a flame ionization detector and a 1-m stainless steel column packed with Porapak Q (80 to 100 mesh). The injector, column, and detector temperatures were set at 200, 130, and 200°C, respectively. Helium carrier gas was set at a flow rate of 30 ml/min. Formaldehyde was assayed by a modification (16) of the chromotropic acid method of West and Sen (28). Formate was as-

sayed by the method of Grant (10). The analytical methods for determination of dry weight, protein content, and amino acid analyses have been reported previously (4).

**Enzyme assays.** Cell-free extracts were normally prepared in the following manner. Late-exponential-phase cells were harvested by centrifugation, washed twice in phosphate buffer at pH 7.0, and suspended in 20 mM tris(hydroxymethyl)aminomethane buffer, pH 8.0, containing 5 mM MgCl<sub>2</sub> and 2 mM β-mercaptoethanol. Cells, kept either fresh at 0 to 4°C or frozen, were disrupted by sonic treatment with a Branson sonifier at 20 kc for 3 to 5 min. This was followed by centrifugation at 20,000 × *g* for 20 min to pellet the cell debris. The supernatants represented the crude cell-free extracts which were assayed immediately.

Methanol oxidase was assayed by the method of Janssen and Ruelius (12) at pH 7.5. Methanol dehydrogenase assays were based on the methods of Anthony and Zatman (1) and Goldberg (7) for PMS-linked activity, but they were also examined with other electron acceptors substituting for PMS. The same assay system was also used for formaldehyde dehydrogenase. The NAD- and glutathione-dependent activity of this latter enzyme was also examined (15). Formate dehydrogenase was assayed by the procedures of Kato et al. (14). Our hexosephosphate synthase assay was a variation of that of Lawrence et al. (18) in that no radiolabel was used. The disappearance of formaldehyde as it reacted with ribose-5-phosphate was followed with respect to time. At each time interval, the reaction (in a 2.8-ml volume) was stopped by the addition of 0.2 ml of 4 N hydrochloric acid. A 3-ml quantity of 20% trichloroacetic acid was added to precipitate the protein, and this precipitate was deposited by centrifugation. Supernatants were assayed for remaining formaldehyde. Control samples lacking ribose-5-phosphate or cell extract or both assured the specificity of the reaction. Hydroxypyruvate reductase was assayed by the method of Large and Quayle (17).

**Methanol and formate uptake by whole cells.** Methanol- and formaldehyde-grown cells were washed twice with 0.1 M phosphate buffer (pH 7) and suspended in enough 0.05 M phosphate buffer (pH 7) to yield cell densities of 4 to 8 units of absorbance at 600 nm. Reaction mixtures were prepared in 50-ml Erlenmeyer flasks, each containing 7 ml of L-salts solution with twice the usual phosphates (pH 7), 1 ml of substrate solution which contained either 83 μmol of CH<sub>3</sub>OH or 90 μmol of sodium formate, and 2 ml of cell suspension. Samples were incubated at 30°C on a rotary shaker. After 20 and 40 min of incubation, samples were removed and membrane filtered. Filtrates were assayed for both CH<sub>3</sub>OH and formate. The rate of uptake was thus determined as the rate of loss of each substrate from the extracellular fluid.

## RESULTS

**Characterization of isolate L3.** From the initial enrichment culture containing L3, it was apparent that the strain was a methylotroph capable of growth in a mixed-substrate system of methanol, formaldehyde, and formate. The

organism was subsequently obtained in pure culture on minimal methanol plates. The cells were characterized as motile gram-negative rods with average dimensions of 0.5 by 1.3  $\mu\text{m}$ .

Its colonies were faintly white or gray, small (1 to 2 mm in diameter), and round. They could be easily distinguished from other nonpigmented colonies of methylotrophs by observation of the colonies under low power in the microscope. L3 colonies appeared nearly transparent, with a definite ring of scratchlike marks near the middle of each colony. This inner ring on each colony simplified not only the counting of overlapping or clustered colonies, but also the detection of any contaminating organisms.

Strain L3 did not grow on nutrient agar plates, even if they were supplemented with 0.5% (vol/vol) methanol. In liquid media, the organism grew on either methanol or formaldehyde as a sole carbon and energy source, but not on formate.

Biochemical characterization of the isolate showed that it was oxidase positive, but very weakly catalase positive characteristics similar to those of strain BC3 in our laboratory (4). L3 showed negative tests for nitrate reduction and urease. In carbohydrate fermentation tubes as described previously (4), L3 was found to produce acid, but no gas, and it showed no significant growth in glucose, lactose, sucrose, or maltose. It grew adequately with either nitrate or ammonium as the sole nitrogen source.

The organism showed broad tolerances to the pH, temperature, and phosphate concentration of its medium. Although the optimal growth rate was noted between pH 7.0 and 7.2, the rate dropped only 15% below maximal when grown at either pH 6.0 or 7.7. The optimal temperature range was from 30 to 33°C, but the rate dropped only 20% at 26 and 37.5°C. No growth was observed at 39°C. Phosphate concentration had no influence on the growth rate of L3 except when it was below 3 mM and was apparently limiting. It was examined in concentrations as high as 45 mM.

In addition to the tolerances of the organisms to environmental conditions, a number of other features characterized the strain as promising in SCP-related work. The growth rate of L3 was very rapid for a methanol utilizer, originally yielding a specific growth rate of 0.53  $\text{h}^{-1}$  in batch cultures. With daily transfers of L3, this was increased to a specific growth rate of 0.693  $\text{h}^{-1}$ , which corresponds to a generation time of only 1 h. An amount corresponding to 65 to 70% of the dry weight of the cells was found to be composed of protein, and the amino acid composition of this protein (Table 1) compared well with the United Nations' standards for nutri-

tional protein from microorganisms.

**Growth studies.** L3 was examined in a series of batch cultures to determine both its tolerances to and growth rates on various combinations and concentrations of  $\text{C}_1$  compounds, specifically methanol, formaldehyde, and formate.

Methanol supported growth in concentrations as low as 0.01% and as high as 5% (vol/vol). A profile of specific growth rate versus initial methanol concentration resulted from a series of batch culture experiments (Fig. 1). A plateau of maximal growth rate was found in the range of

TABLE 1. Amino acid profile of cellular protein of strain L3 compared with FAO standards

Amino acid	Composition by wt (%)	
	Strain L3	FAO standard <sup>a</sup>
Alanine	8.4	
Arginine	6.2	
Aspartic acid (plus asparagine)	10.7	
Cysteine	— <sup>b</sup>	3.5 (cysteine + methionine)
Glutamic acid (plus glutamine)	12.3	
Glycine	6.5	
Histidine		
Isoleucine	5.6	4.0
Leucine	9.1	7.0
Lysine	7.3	5.5
Methionine	3.1	3.5 (cysteine + methionine)
Phenylalanine	4.6	6.0 (phenylalanine + tyrosine)
Proline	3.9	
Serine	4.4	
Threonine	5.0	4.0
Tryptophan	— <sup>b</sup>	1.0
Tyrosine	3.8	6.0 (phenylalanine + tyrosine)
Valine	6.7	5.0

<sup>a</sup> Standards set by the Food and Agricultural Organization (FAO) of the United Nations.

<sup>b</sup> —, Cysteine and tryptophan could not be analyzed due to acid hydrolysis in the preparation of the amino acids for analysis.

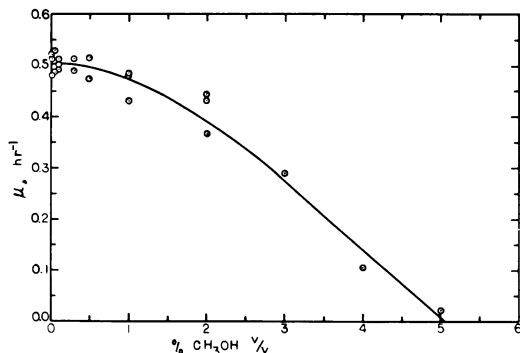


FIG. 1. Effect of initial methanol concentration on the specific growth rate ( $\mu$ ) of strain L3.

0.1 to 0.5% (vol/vol) methanol (24 to 122 mM).

As expected, formaldehyde was a much more toxic substrate for growth and was typically used in the range of 0.01% (wt/vol; 3.3 mM) in growth experiments. Figure 2 shows the profile of specific growth rate of L3 versus initial formaldehyde concentration. Cultures containing 0.05% (wt/vol; 16 mM) or more formaldehyde were unable to grow.

Formate was not utilized by the organism as a sole carbon source, but was completely oxidized by cultures containing methanol and formate. The toxicity of formate was intermediate between the toxicities of methanol and formaldehyde, and it was typically added at a concentration of 0.1% (wt/vol). It was consistently observed that cultures containing methanol, formaldehyde, or both became acidic after growth ensued, whereas any mixed-substrate culture containing formate became alkaline (pH 8.5 to 9.0). It was also observed that pure or mixed-substrate cultures containing formaldehyde had considerably longer lag phases (6 to 10 h longer) than methanol or methanol-formate cultures.

Steady-state chemostat studies were performed with L3 to investigate its potential for continuous cultivation on  $C_1$  compounds. Numerous steady states were achieved with methanol as the sole carbon and energy source in the chemostat, with various dilution rates and substrate concentrations in the feed. As with the batch cultivation, formate was incapable of supporting growth or, thus, of achieving steady-state growth of L3, even if it was supplemented with folic acid, *p*-aminobenzoic acid, and/or serine (2). We have also been frustrated in attempts to reach a steady state with formaldehyde as the sole carbon source. Mixed-substrate chemostat studies have also yielded steady states with relative ease whenever methanol was present. Yields from mixed-substrate cultures were significantly lower than those from methanol cul-

tures, even when the mixed-substrate yield was calculated strictly relative to the methanol consumption. With dilution rates varying from 0.20 to  $0.47 \text{ h}^{-1}$ , the cell-mass yield for methanol-grown chemostat cultures ranged from 40 to 57%, respectively, whereas the highest yield (based on the consumption of methanol alone) from a mixed-substrate culture was 43%. A profile of cell-mass yield versus dilution rate is presented in Fig. 3. Maximal yield was found at a dilution rate of  $0.43 \text{ h}^{-1}$ .

The residual substrate concentrations in the chemostat appeared to be critical to the establishment of steady states and to the regulation of growth to achieve a high cell yield. Formaldehyde, for example, accumulated in the chemostat culture medium as an oxidation product of methanol-grown cells. Many steady-state conditions for methanol-grown cultures reflected a formaldehyde residual of 0.2 to 0.5 mg/liter. Whenever the system was perturbed, however, to increase this formaldehyde production above 2.5 mg/liter, the system was unstable and washed out. Simple addition of as much as 50 mg of exogenous formaldehyde per liter did not result in an instability, because the added material was rapidly oxidized by the cells.

**Enzyme studies.** The contamination problems encountered by other workers with methanol, formaldehyde, and formate oxidizers (2, 8) led us to scrutinize carefully any formaldehyde-grown cultures of L3.

In addition to colonial characteristics and biochemical tests on cultures, the enzymes for the oxidation and incorporation of  $C_1$  compounds were investigated for L3 grown on methanol and on formaldehyde to confirm the identity of all cultures. These methanol-grown and formaldehyde-grown cells will subsequently be referred to as M-cells and F-cells, respectively. A third culture, FM-cells, was also used in the enzyme analyses; the cells were derived from M-cells which were transferred to formaldehyde me-

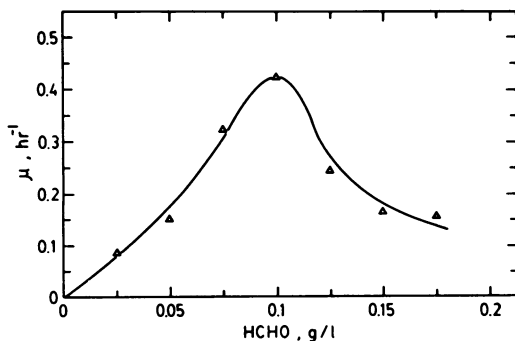


FIG. 2. Effect of initial formaldehyde concentration on the specific growth rate ( $\mu$ ) of strain L3.

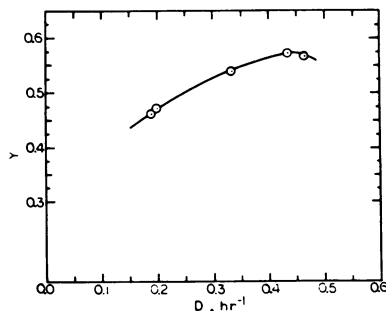


FIG. 3. Cell-mass yields ( $Y$ ) for methanol-grown L3 in the chemostat.  $D$ , Dilution rate.

dium and through 12 successive subcultures (with 1% inocula) in the formaldehyde medium and then inoculated back into methanol medium.

To complement the similarity in colonial characteristics and biochemical tests of the three different cultures of L3, their enzymes appeared to be identical. Variations in relative specific activities were the only distinctions among them. Table 2 lists the activities determined for the various enzymes in each of the three cultures.

The methanol and formaldehyde dehydrogenases of L3 were very similar to those in *Pseudomonas C* reported by Goldberg (7). Each enzyme required PMS, KCN, and ammonium ions for activity in crude cell extracts. Their pH optima were essentially identical, showing maximal activity in the range of 9.0 to 10.5. No activity was demonstrated for formaldehyde dehydrogenase linked to NAD in any of the L3 cultures, nor was there any methanol oxidase activity.

Formate dehydrogenase activity in L3 was linked to NAD and displayed a pH optimum of 7.0. No activity was found linked to PMS for this enzyme. A relatively low activity for this dehydrogenase is reported in Table 2. Similar activity for this enzyme has been reported for *Pseudomonas C* (3). Subsequent experiments, however, suggested to us that the *in vivo* activity for this enzyme in L3 was significantly higher than that reported here.

Because we encountered such low activities of the formate-oxidizing enzyme in L3 despite the fact that formate was oxidized readily by the cells in mixed-substrate cultures, we wanted to demonstrate the *in vivo* function of this enzyme. This was done indirectly by studying the formate uptake rates by whole cells and comparing them with methanol uptake rates. Formate ox-

idation was presumed to result after the formate uptake.

The results of the experiments with whole cells show that uptake of both formate and  $\text{CH}_3\text{OH}$  was greater with F-cells than with M-cells (Table 3). Moreover, for both F- and M-cells, the rates of  $\text{CH}_3\text{OH}$  uptake were only 10 to 25% higher than the formate uptake rates. These findings suggest that the formate dehydrogenase exists at higher concentrations in F-cells than in M-cells. This is in accordance with the finding that in F-cell cultures most of the  $\text{CH}_2\text{O}$  was oxidized to  $\text{CO}_2$  (accompanied by a considerable pH drop) before  $\text{CH}_2\text{O}$  incorporation into cell mass (i.e., actual growth) took place. Furthermore, it is suggested that the formate dehydrogenase activities should be comparable to those of methanol dehydrogenase in L3, but the techniques required to observe this amount of activity have not been found. Johnson and Quayle (13) also found a rather unstable NAD-linked formate dehydrogenase in *Pseudomonas AM-1*. Similar whole-cell experiments with *Pseudomonas C* (using only methanol-grown cells) demonstrated an eightfold-greater rate of methanol oxidation than of formate oxidation (26). This contrast to our findings with L3 may be related to the fact that in *Pseudomonas C* most of the  $\text{CH}_2\text{O}$  is oxidized to  $\text{CO}_2$  through a cyclic scheme through 3-hexulose-5-phosphate rather than directly through formate (3).

Analysis of the  $\text{C}_1$ -incorporation enzymes in L3 demonstrated clearly (Table 2) that the organism utilizes the RMP pathway (27) and hexulosephosphate synthase for growth on either methanol or formaldehyde. Although a high residual reduced NAD oxidase activity was found in L3, similar to that reported by Goldberg (7) for *Pseudomonas C*, no activity beyond this was noted in any assay of hydroxypyruvate reductase. This confirmed that the serine pathway did not operate in L3, even when it was growing on formaldehyde.

Further investigation with the methanol and

TABLE 2. Enzyme activities for methanol- and formaldehyde-grown L3

Enzyme	Sp act <sup>a</sup>		
	M-cells	F-cells	FM-cells
Methanol dehydrogenase	59	27	25
Formaldehyde dehydrogenase	20	23	15
Formate dehydrogenase	4	ND <sup>b</sup>	4
Hexosephosphate synthase	261	277	249
Hydroxypyruvate reductase	0	0	0

<sup>a</sup> Activities are reported as nmol of substrate converted per minute per milligram of protein in crude cell extracts except for hexosephosphate synthase, which has units of micromoles per hour per milligram of protein. See text for a description of cells.

<sup>b</sup> ND, Not determined.

TABLE 3. Methanol and formate uptake rates by strain L3

Cell type <sup>a</sup>	Reaction time (min)	Specific uptake rate ( $\mu\text{mol}/\text{min}$ per mg of protein) of:	
		Methanol	Formate
F-cells	20	1.022	0.961
F-cells	40	1.235	0.967
M-cells	20	0.843	0.630
M-cells	40	0.633	0.330

<sup>a</sup> See text for details.

formaldehyde dehydrogenases in L3 uncovered that each could be exhibited by formaldehyde. It was initially observed that the PMS-linked dehydrogenases from either methanol- or formaldehyde-grown cells could not be distinguished in enzyme assays. Both methanol and formaldehyde reduced PMS and 2,6-dichlorophenolindophenol in the assays with either M-cell or F-cell extracts. Thus, when growing strictly on formaldehyde, the cells retained methanol dehydrogenase activity similar to that in methanol-grown cultures. However, when formaldehyde in the dehydrogenase assays was increased from 13 to 27  $\mu\text{mol}/3\text{ ml}$  in the assay, all of the activity was destroyed. It was determined that, regardless of methanol concentration in the assay, 27  $\mu\text{mol}$  of formaldehyde (or 9 mM) inhibited all PMS-linked activity in M-cell as well as F-cell extracts. This finding indicated that the methanol and formaldehyde dehydrogenases in L3 may actually be the same enzyme, with specificity for either of the two substrates and inhibited from oxidation of either substrate by high concentrations of formaldehyde.

## DISCUSSION

In a search for the ideal methylotroph to be utilized on a large scale for SCP production, one might select for a wide variety of features. It appears that strain L3 as reported here possesses most, if not all, of these features. These include a rapid growth rate, a high protein content, a good amino acid distribution, broad tolerances to pH, temperature, phosphate, and methanol concentrations, and relative ease of achieving steady-state cultures in the chemostat. In addition, L3 grows rapidly in mixed-substrate cultures of methanol, formaldehyde, and formate and utilizes the more energetic RMP cycle (27) for carbon incorporation regardless of the growth substrate(s).

The most intriguing aspect of the metabolism of L3 is its ability to utilize formaldehyde as a sole carbon and energy source via the RMP cycle. Until now, methanol utilization has been associated with either the RMP cycle or the serine pathway in various bacteria (9) and yeasts (6), but formaldehyde utilization was associated solely with the serine pathway in both bacteria (9) and fungi (24). It has also been observed that serine pathway organisms can utilize formate as well as formaldehyde as a carbon source (9). In contrast, L3 cannot use formate as a sole carbon source (although formate is rapidly oxidized in mixed-substrate cultures), but it does utilize formaldehyde. The discovery of hexosephosphate synthase in formaldehyde-grown cells and the corresponding absence of hydroxypyruvate

reductase confirmed the reasoning that the substrate specificity of L3 was due to its strict utilization of the RMP cycle. It seems likely that other methylotrophs will be found similar to L3 in this respect once their substrate specificities and  $\text{C}_1$ -incorporation enzymes are examined.

This utilization of the energetic RMP cycle for growth on methanol and/or formaldehyde is significant with respect to other aspects of SCP as well. Our laboratory in particular is interested in microbial utilization of mixed oxidation products of methane (in natural gas). Because methanol, formaldehyde, and formate would all be present in such preparations of natural gas, it is significant that the organism not only can metabolize formaldehyde and formate in such a mixed-substrate system, but also can utilize two of the three substrates most efficiently via this RMP cycle. It is admitted that the cell yield is lower in such a mixed-substrate system compared with growth on methanol alone, but, nevertheless, yields above 40% are encountered with mixed substrates. It is unfortunate that no steady-state yields for formaldehyde growth of L3 have been obtained as yet. These should also prove interesting in comparison with yields for methanol cultures.

Although *Pseudomonas C* is a facultative methylotroph in contrast to strain L3, the two are similar in many respects, particularly in their methanol and formaldehyde dehydrogenases. These enzymes in both cell types are PMS linked, require KCN and ammonium ions for activity, have extremely high pH optima, and cannot be distinguished from each other by substrate specificity (7). Our discovery that similar properties were associated with the corresponding enzyme(s) in formaldehyde-grown L3 supports the argument that the two dehydrogenases may be a single enzyme which is responsible for both oxidation steps in such organisms.

On the other hand, *Pseudomonas C* apparently differs from L3 in that it is unable to utilize formaldehyde as a sole carbon source. Despite conflicting reports about the activity of this enzyme (7, 26) and the presence of an NAD-linked formaldehyde dehydrogenase (3, 7) in *Pseudomonas C*, it has been reported that these dehydrogenases play a very minor role in formaldehyde oxidation in this organism (3). In *Pseudomonas C* a cyclic oxidation pathway is believed to be the main pathway for the oxidation of formaldehyde (and other  $\text{C}_1$  compounds) to  $\text{CO}_2$ . This regulation of formaldehyde oxidation in the organism may indeed account for its inability to use formaldehyde as a sole carbon source. By the same line of reasoning, because L3 is able to utilize formaldehyde as a sole carbon and energy source, this may be a result of the predominance

of formaldehyde oxidation via formate. Because no NAD-linked formaldehyde dehydrogenase activity was detected in L3, it may be that the PMS-linked activity is responsible for formaldehyde oxidation in these cells.

It is concluded that the characteristics of L3 elucidated here suggest that it is a promising organism in two major respects. It is a valuable strain not only for use in SCP-related work with both pure and mixed one-carbon substrates, but also for the study of the regulation of the C<sub>1</sub>-oxidation and incorporation pathways in RMP cycle methylotrophs.

#### ACKNOWLEDGMENTS

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