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The Regulation of Exopolysaccharide Production and of Enzymes Involved in C_1 Assimilation in *Methylophilus methylotrophus*

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Methylophilus methylotrophus produced viscous and non-viscous exopolysaccharides (EPS) when grown in batch culture. Both types contained glucose, galactose, mannose and an unidentified 6-desoxyhexose, and were substituted with pyruvate and acetate residues. When the organism was grown in continuous culture only the non-viscous EPS was synthesized; the rate of production was 18·5 mg h^{-1} (g biomass)$^{-1}$ in methanol-limited cultures and increased by approximately 3- and 4-fold when growth was limited by oxygen or nitrogen respectively. The specific activity of methanol dehydrogenase in cell extracts was relatively low when bacteria were grown under conditions of methanol excess and increased 2-fold in carbon-limited cells, reflecting the need to 'scavenge' the small amounts of available methanol. In contrast, the specific activities of several key enzymes of the ribulose monophosphate (RuMP) pathway were greater in cells grown under conditions of nitrogen or oxygen limitation than when growth was limited by the availability of carbon, indicating the potential for increased carbon flux round the cycle when excess methanol was present in the growth medium. When methylotrophs are grown under conditions of methanol excess it is important that there is a mechanism to prevent the overproduction of formaldehyde, and we suggest that these changes in EPS production and in the specific activities of the key enzymes of the RuMP cycle are necessary for the efficient removal of this toxic metabolite of methanol.

INTRODUCTION

Methylophilus methylotrophus can use reduced C_1 compounds as the sole source of carbon and energy. Methanol is oxidized by a quinoprotein, methanol dehydrogenase, to formaldehyde, which then can be either further oxidized to CO_2 or assimilated (see Anthony, 1982). In this organism the KDPG (2-keto-3-deoxy-6-phosphogluconate) aldolase/transaldolase variant of the ribulose monophosphate (RuMP) pathway is responsible for the fixation of C_1 compounds and also has a dissimilatory role. *M. methylotrophus* also contains NAD^+-linked formaldehyde and formate dehydrogenases but usually only a small proportion of formaldehyde is oxidized via these enzymes in RuMP pathway methylotrophs (Anthony, 1982). The first enzyme of the RuMP pathway is hexulose-6-phosphate synthase, which catalyses the condensation of formaldehyde with ribulose monophosphate to form hexulose 6-phosphate. This is then metabolized by hexulose-6-phosphate isomerase, glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase. The resulting 6-phosphogluconate is at a branch point and may enter the assimilatory part of the cycle or be oxidized to ribulose monophosphate and CO_2 in a reaction catalysed by 6-phosphogluconate dehydrogenase; two isoenzymes are present in *M. methylotrophus*, one is specific for NAD^+, while the other uses both NAD^+ and $NADP^+$ (Beardsmore *et al.*, 1982).

Several methylotrophic bacteria produce EPS during growth on reduced C_1 compounds. For

Abbreviations: EPS, exopolysaccharide; KDPG, 2-keto-3-deoxy-6-phosphogluconate; RuMP, ribulose monophosphate.

example, *Methylococcus capsulatus* and bacterium W3A1 synthesize a capsule (Wyss & Moreland, 1968; McIntire & Weyler, 1987), while a viscous slime has been observed in batch cultures of *Methylocystics parvus* OBBP (Hou *et al.*, 1978). Two different acidic polysaccharides are produced by a methane-oxidizing methylotroph, bacterium H-2 (Chida *et al.*, 1983), and Linton *et al.* (1986) reported the presence of a low-viscosity EPS when a *Methylophilus* species was grown in continuous culture. In this paper we demonstrate that *M. methylotrophus* also produces EPS and that the rate of production and yield of polymer vary with growth conditions. The significance of this, and of changes that we have observed in the specific activities of some of the key enzymes of C_1 metabolism is discussed.

METHODS

Growth of bacteria. M. methylotrophus NCIB 10515 (Jenkins *et al.*, 1987) was obtained from the ICI culture collection, Billingham, UK. In batch culture it was grown at 37 °C in Seed 2 medium (Windass *et al.*, 1980) containing methanol (125 mM) as the carbon source and ammonium sulphate (13·6 mM) as the nitrogen source. In continuous culture it was grown in a 1 litre LH Fermentation 500 series chemostat at 37 °C with a culture volume of 740 ml and a dilution rate of 0·13 h^{-1}. The medium contained (l^{-1}): 1·662 g $MgSO_4.7H_2O$; 0·685 g $K_2HPO_4.3H_2O$; 0·205 g K_2SO_4; 1·61 ml H_3PO_4; 39·8 mg $FeSO_4.7H_2O$; 0·72 mg $CuSO_4.5H_2O$; 5·04 mg $MnSO_4$; 3·6 mg $ZnSO_4.7H_2O$; 64·8 mg $CaCO_3$; 144 to 608 mmols methanol and 34 to 102 mmols NH_4^+ [supplied as $(NH_4)_2SO_4$]. It was sterilized by filtration through a 0·2 μm membrane filter with a 0·8 μm prefilter. The vessel was inoculated to 15% (v/v) with an overnight batch culture of cells grown on Seed 2 medium. A pH of 6·8 ± 0·05 was maintained automatically by the addition of 2 M-sulphuric acid or 1·5 M-KOH/0·5 M-NaOH when appropriate. Foaming was controlled by the automatic addition of Silcolapse 5008 (ICI) antifoam when necessary. The dissolved oxygen tension was maintained at 50% saturation, except when cultures were grown under oxygen-limited conditions when the dissolved oxygen tension was below the limit of detection; such cultures were pink, rather than white, since oxygen limitation causes increased production of cytochromes. Cultures were considered to be in the steady state after at least three changes of culture volume and could be maintained in this state for up to 3 weeks. Bacteria were collected on ice, centrifuged at 6000 *g* to separate the cell pellet from the culture supernatant and washed in 20 mM-Tris/HCl buffer, pH 7·5. Pellets could be stored frozen for up to 1 month without loss of enzyme activity.

Investigation of EPS production in batch culture. When *M. methylotrophus* was grown from small inocula in media that initially contained low concentrations of the nitrogen source, low cell densities were obtained, making some of the parameters of interest difficult to measure. To overcome this problem 250 ml flasks containing 100 ml of Seed 2 medium were inoculated with portions of an overnight culture, also grown on Seed 2 medium. Prior to use cells were washed in warm salts medium that did not contain any carbon or nitrogen source and the size of inoculum was adjusted such that the initial OD_{650} of each culture was 0·5. The cultures were incubated at 37 °C with shaking and supplemented with 6·25 mmols methanol after 6 h. After a total of 21 h incubation, during which time the OD_{650} increased by 2- to 3-fold, the cultures were centrifuged at 6000 *g* for 30 min. The supernatant was then removed from the cell pellet. When the viscous EPS was present it formed a distinct layer between the non-viscous material and the cell pellet, and each fraction was carefully removed in turn.

Enzyme assays. Washed cell pellets were suspended in 3 vols of the appropriate sonication buffer and disrupted over ice in a MSE sonicator (150 W) for ten periods of 30 s at an amplitude of 12 μm, interspersed with equivalent cooling periods. The sonication buffer contained 20 mM-KH_2PO_4/NaOH, pH 7·2, 5 mM-$MgCl_2$, except when the $NAD(P)^+$-linked 6-phosphogluconate dehydrogenase was to be assayed, when 20 mM-Tris/HCl pH 7·8, 5 mM-2-mercaptoethanol was used. Prior to assay of the dye-linked enzymes cell debris was removed by centrifugation at 38000 *g* for 20 min at 2 °C; KCN (1 mM) was added to the supernatants to prevent inactivation of methanol dehydrogenase (Beardmore-Gray *et al.*, 1983). When pyridine-dinucleotide-linked enzymes were to be assayed the sonicates were centrifuged at 100000 *g* for 1 h at 2 °C to remove NADH oxidase. Hexulose-6-phosphate synthase and the $NAD(P)^+$-dependent 6-phosphogluconate dehydrogenase were assayed immediately; extracts could be frozen at −20 °C for up to 3 d without loss of activity of the other enzymes.

The following enzymes were assayed by published methods: methanol dehydrogenase EC 1.1.99.8 [Tatra & Goodwin, 1985; modified by the addition of 1 mM-KCN to the assay mixture. The rates of oxygen uptake were therefore divided by two to take into account the inhibition of catalase by KCN (Carver & Jones, 1984).]; NAD^+-linked formaldehyde dehydrogenase EC 1.2.1.1 (Beardsmore *et al.*, 1982); formate dehydrogenase EC 1.2.1.2 (Quayle, 1966); 3-hexulose-6-phosphate synthase plus 3-hexulose-6-phosphate isomerase (Levering *et al.*, 1981; the spectrophotometric assay was used, modified by the omission of exogenous hexulose-6-phosphate isomerase, and by the use of potassium phosphate buffer, pH 7·2); glucose-6-phosphate dehydrogenase EC 1.1.1.49 (Bergmeyer *et al.*, 1974); NAD^+- and $NAD(P)^+$-linked 6-phosphogluconate dehydrogenases EC 1.1.1.44 (Beardsmore *et al.*, 1982; modified by the use of 20 mM-Tris/HCl buffer, pH 7·8, for measurement of the

$NAD(P)^+$-linked enzyme); 6-phosphogluconate dehydratase EC 4.2.1.12 plus KDPG aldolase EC 4.1.2.14 (Wood, 1971). Protein was measured by the method of Lowry. At least two different volumes of each extract were assayed; the variations in the specific activities measured for duplicate assays were less than 10%.

Assay of culture variables. Total carbohydrate was assayed by the method of Dubois, cited by Herbert *et al.* (1971); 6-desoxyhexose by the method of Dische as modified by Osborn (1963); uronic acids by the method of Blumenkrantz & Asboe-Hansen (1973); and ammonia by the method of Di Georgio (1974). The residual methanol concentration was estimated by GLC using a column of 0·2% Carbowax 1500 on 80/100 Carbopak at 70 °C. The reproducibility of the results was determined by setting up triplicate batch cultures and assaying each parameter in duplicate. All results were reproducible to within 10% of the mean. All assays were done at least in duplicate.

Characterization of the EPS. The viscous EPS was diluted with 15 vols 0·9% NaCl, stirred overnight at 3 °C and then centrifuged; this procedure removed cells that had been trapped in the viscous fraction. Before analysis both types of EPS were dialysed against distilled water and lyophilized. The polymers were then hydrolysed by the method of Bryan *et al.* (1986), dried *in vacuo* in the presence of pellets of NaOH and phosphorus pentoxide, and dissolved as a 1% (w/v) solution in 10% (v/v) propan-2-ol. The monomers present were separated by (i) two-dimensional TLC using as solvents butan-2-one/acetic acid/methanol (60:20:20, by vol.) followed by ethyl acetate/pyridine/water (120:50:40, by vol.); after drying the chromatograms were stained with 4-methoxybenzaldehyde reagent (Bryan *et al.*, 1986); (ii) HPLC on a μPorasil column using a mobile phase of aqueous 90% (v/v) acetonitrile containing 2% (v/v) SAMII reagent (Waters).

Freeze-dried EPS was also hydrolysed in 1 M-HCl under nitrogen at 105 °C for 2·5 h. After neutralization with NaOH the organic acid (acetate, pyruvate and succinate) content of the hydrolysate was determined enzymically using commercial kits (Boehringer Mannheim).

Dry weight determinations. Samples (1·5 ml) of culture were centrifuged in an MSE Micro-Centaur for 10 min, washed in distilled water and recentrifuged. Two such pellets were transferred to thin-walled glass vials and dried at 105 °C to constant weight. The dry weight of each culture was determined in triplicate; variations in replicate samples were less than 5% of the mean.

RESULTS

EPS production in batch culture

Preliminary experiments to investigate EPS production by *M. methylotrophus* were done using batch cultures grown in Seed 2 medium; oxygen availability was varied by shaking replicate cultures in either non-baffled or baffled flasks. After 21 h growth all cultures contained residual methanol and NH_4^+ and had produced a non-viscous EPS, the concentration in the supernatant of the culture grown in baffled flasks being $96{\cdot}4 \pm 5{\cdot}0$ μg ml^{-1} ($n = 3$). Approximately 75% of the carbohydrate assayed in the culture supernatant was retained after dialysis, indicating an M_r greater than 12000. The cultures grown in non-baffled flasks, i.e. under conditions of very poor aeration, produced $118{\cdot}4 \pm 5{\cdot}2$ μg ml^{-1} ($n = 3$) of the non-viscous EPS and also contained a viscous EPS, at a concentration of $84{\cdot}3 \pm 3{\cdot}5$ μg ml^{-1} ($n = 3$). On centrifugation this sedimented between the cell pellet and the non-viscous fraction and comprised 10 to 15% of the total culture volume. The exopolymers were hydrolysed and the resulting monomers separated by TLC and HPLC. Both types of EPS contained glucose, galactose and mannose, together with a fourth unknown sugar, which was neither fucose nor rhamnose. Assay of the lyophilized EPS by the method of Dische indicated that about 15% of the total assayable carbohydrate was a 6-desoxyhexose. Both types of EPS also contained acetate (0·4%) and pyruvate (0·1%) residues but succinate and uronic acids were not detected in either.

EPS production and the specific activities of key enzymes of C_1 metabolism in M. methylotrophus provided with various carbon:nitrogen (C:N) ratios in chemostat culture

Production of EPS is often enhanced when bacteria are grown under conditions of carbon excess (see Sutherland, 1982). To investigate whether the presence of excess methanol in the growth medium increased EPS synthesis in *M. methylotrophus* this organism was grown in continuous culture under conditions of oxygen excess and supplied with varying ratios of carbon (as methanol) and nitrogen (as ammonium sulphate) (Table 1). At C:N molar ratios of 10·9 and below methanol was not detected in the culture supernatant, but consideration of the cell yields from methanol indicated that growth was carbon-limited only when the C:N ratio was below

Table 1. *Effect of altering the C : N ratio on biomass and product yields of M. methylotrophus grown in continuous culture at a dilution rate of 0·13 h^{-1}*

ND, Not detectable. *n*, No. of independent determinations. When $n > 1$, mean values are given. Replicates were reproducible to within 10%.

C:N molar ratio		1·4	2·1	5·0	6·2	6·9	7·1	8·8	10·9	11·5	15·2	17·9
[CH_3OH] (mM)	Input	144	207	300	371	414	390	474	478	460	592	608
	Output	ND	ND	ND	ND	ND	ND	ND	ND	36	118	170
[NH_4^+] (mM)	Input	102	100	60	60	60	55	54	44	40	39	34
	Output	62	60	13	5	8	ND	ND	ND	ND	ND	ND
Bacterial dry wt ($g\ l^{-1}$)		1·71	2·4	3·7	3·8	3·9	3·9	4·25	4·4	3·85	3·8	3·37
$Y_{methanol}$ [g biomass (mol methanol)$^{-1}$]		11·9	11·6	12·4	10·3	9·5	9·9	9·0	9·1	9·1	8·0	7·7
[Carbohydrate] ($g\ l^{-1}$)		0·25	0·37	0·47	0·61	1·0	0·89	1·64	2·17	2·58	2·44	2·06
n		1	2	7	3	1	3	2	2	1	1	1

6·2, the $Y_{methanol}$ then being between 11·6 and 12·4 g biomass (mol methanol)$^{-1}$. At C : N ratios above 6·9 residual NH_4^+ was not detected in the culture supernatant but growth was probably not nitrogen-limited until the C : N ratio was above 8·8, when the Y_N for nitrogen-limited growth was 96 to 99 g biomass (mol NH_4^+)$^{-1}$. The precise C : N ratio at which growth switched from carbon to nitrogen limitation cannot be identified; both carbon and nitrogen appear to have been completely metabolized at the intermediate C : N ratios used, but the cell yields suggest that growth was not limited by either of these substrates. The cultures may have been in a transition phase, when growth was 'double-substrate' (i.e. carbon- and nitrogen-) limited. Egli & Quayle (1986) suggested that under such conditions the organism responds to a change in the ratio of the two limiting substrates by altering its cellular composition to match the nutrient limitation.

A non-viscous EPS was present in all the culture supernatants but the viscous polymer observed in batch culture was not produced. The rate of EPS production and the yield of EPS increased about 4-fold and 2·5-fold respectively as the C : N ratio increased from below 6·2 (carbon-limited growth) to above 8·8 (nitrogen-limited growth) (Fig. 1). Thus, as the amount of methanol supplied in the medium rose an increased amount of carbon from formaldehyde was converted to EPS rather than to cell material. The polymer had a similar composition to that produced in batch culture in that it contained glucose, galactose, mannose and an unidentified 6-desoxyhexose and was substituted with pyruvate and acetate residues.

The effect of altering the C : N ratio on the activities of key enzymes of C_1 metabolism was also investigated. The specific activities of formaldehyde and formate dehydrogenases were low and these did not change consistently with growth conditions. However, the activities of other key enzymes of C_1 metabolism did alter (Fig. 2). The specific activity of methanol dehydrogenase was high in extracts of carbon-limited cells and decreased abruptly during the 'transition phase' from carbon- to nitrogen-limited growth to a minimum value at a C : N ratio of 10·9. As the C : N ratio was increased further the steady-state methanol concentration in the medium increased but the specific activity of methanol dehydrogenase remained constant. In contrast, the rate of methanol oxidation *in situ* and the specific activities of hexulose-6-phosphate synthase plus hexulose-6-phosphate isomerase (which are involved in the initial fixation of formaldehyde), glucose-6-phosphate dehydrogenase (which is required for both the assimilatory and dissimilatory RuMP pathway to operate) and both the isoenzymes of 6-phosphogluconate dehydrogenase (which are part of the dissimilatory RuMP pathway) increased in an approximately linear fashion as the C : N ratio was increased.

Fig. 2. Effect of altering the C : N molar ratio on the specific activities of key enzymes of C_1 metabolism in *M. methylotrophus* grown in continuous culture at a dilution rate of 0·13 h^{-1}. Specific activities are expressed as μmol min^{-1} (mg protein)$^{-1}$. (*a*): ●, Methanol dehydrogenase (MDH); ○, *in situ* rate of methanol utilization; △, steady-state methanol concentration; ▲, glucose-6-phosphate dehydrogenase (GPDH). (*b*): ●, Hexulose-6-phosphate synthase plus hexulose-6-phosphate isomerase (HPS); △, NAD^+-linked 6-phosphogluconate dehydrogenase (PGDH-1); ○, $NAD(P)^+$-linked 6-phosphogluconate dehydrogenase (PGDH-2).

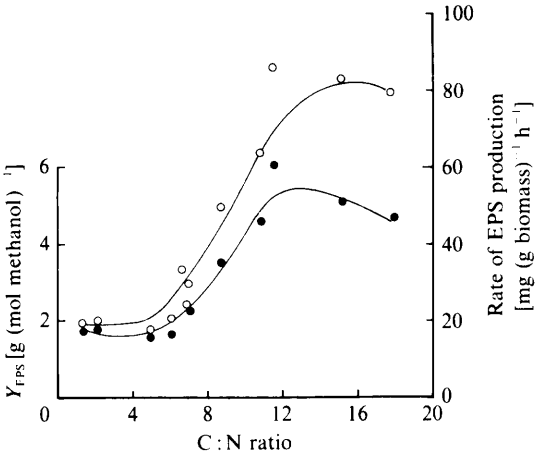
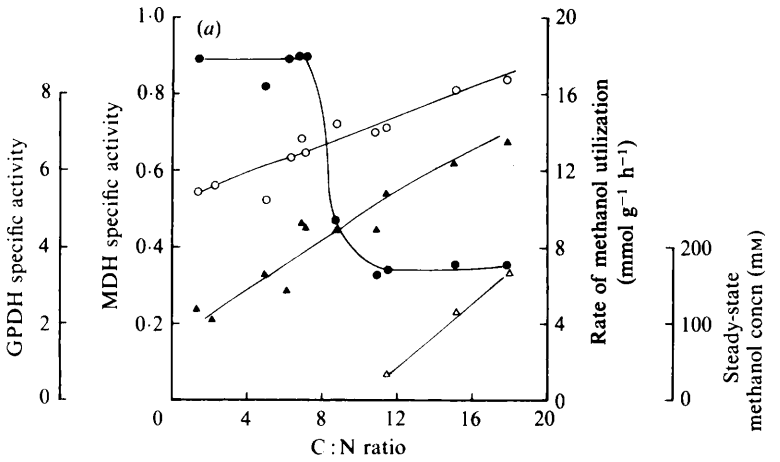


Fig. 1. Effect of altering the C:N molar ratio on the yield and rate of production of EPS by *M. methylotrophus* grown in continuous culture at a dilution rate of 0·13 h^{-1}. ● Y_{EPS}; ○, rate of EPS production.



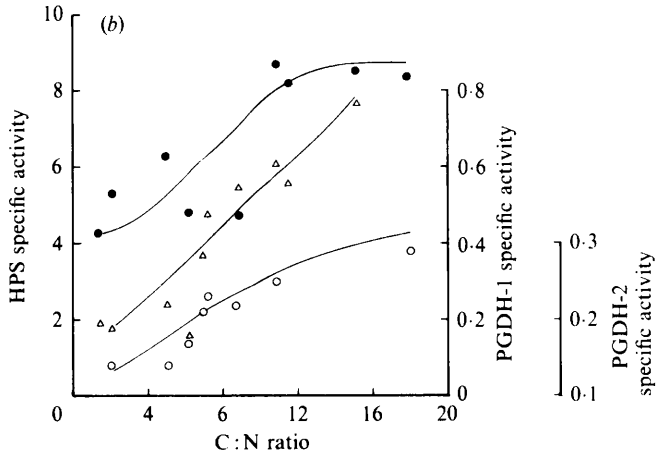


Table 2. *Effect of carbon, oxygen and nitrogen limitation on EPS production and on the specific activities of key enzymes of C_1 metabolism in M. methylotrophus grown in continuous culture at a dilution rate of 0·13 h^{-1}*

Specific activities are expressed as μmol min^{-1} (mg protein)$^{-1}$. Values given are means ± SE obtained from at least three different cultures. ND, Not determined.

	Limiting nutrient		
Specific activity	Carbon	Nitrogen	Oxygen
Methanol dehydrogenase	0·86 ± 0·03	0·36 ± 0·01	0·48 ± 0·03
Hexulose-6-phosphate synthase plus hexulose-6-phosphate isomerase	5·34 ± 0·44	8·43 ± 0·09	4·25 ± 0·27
Glucose-6-phosphate dehydrogenase	2·44 ± 0·14	5·70 ± 0·43	2·41 ± 0·19
6-Phosphogluconate dehydrogenase			
NAD^+-linked	0·17 ± 0·01	0·62 ± 0·05	0·48 ± 0·05
$NAD(P)^+$-linked	0·14 ± 0·01	0·27 ± 0·02	0·18 ± 0·01
6-Phosphogluconate dehydratase plus KDPG aldolase	0·19 ± 0·01	ND	0·48 ± 0·02
Rate of EPS production [mg (g biomass)$^{-1}$ h^{-1}]	18·5 ± 0·59	78·78 ± 4·35	53·30 ± 4·58

EPS production and the specific activities of key enzymes of C_1 metabolism during growth under oxygen limitation in chemostat culture

M. methylotrophus was grown under conditions of oxygen limitation and the steady-state methanol concentration was varied between 42 and 103 mM, depending on the concentration of methanol in the input medium. The rate of EPS production was about 3-fold greater than in carbon-limited cells, and was independent of the C:N ratio (Table 2). The specific activity of glucose-6-phosphate dehydrogenase was similar to that in cells whose growth was limited by carbon. The combined activity of hexulose-6-phosphate synthase plus hexulose-6-phosphate isomerase was slightly lower than that observed in carbon-limited cells while the combined activity of 6-phosphogluconate dehydratase and KDPG aldolase was more than 2-fold greater. The specific activities of methanol dehydrogenase and both the isoenzymes of 6-phosphogluconate dehydrogenase were intermediate between the values measured in carbon- and nitrogen-limited cells.

DISCUSSION

The results presented in this paper demonstrate that *M. methylotrophus* produces two types of EPS when grown in batch culture. Both contained glucose, galactose, mannose and an unidentified 6-desoxyhexose and were substituted with acetate and pyruvate residues, but detailed analyses were not done and they may contain different proportions of the monomers. The difference in the viscosities of the two polymers may be due to a variation in chain length and/or to different degrees of branching. Further work is required to ascertain the significance of the production of these two types of EPS in batch culture.

The non-viscous EPS was also produced when *M. methylotrophus* was grown in continuous culture. Under conditions of carbon limitation the cell yield was about 12 g (mol methanol)$^{-1}$. This is somewhat lower than the 14·7 to 21·6 g (mol methanol)$^{-1}$ predicted by Anthony (1982) for methylotrophs using the KDPG aldolase/transaldolase variant of the RuMP pathway, and it appears that this strain of *M. methylotrophus* converts a considerable amount of carbon to EPS even when methanol is the growth-limiting nutrient. EPS was also produced when another strain of *Methylophilus* was grown in methanol-limited continuous culture, but the polymer had a different composition, consisting of glucose and mannose residues (Linton *et al.*, 1986).

When *M. methylotrophus* was grown in chemostat culture the rate of production of EPS was relatively high if growth was limited by nitrogen or oxygen rather than carbon, but the viscous polymer which was produced in poorly aerated batch cultures was never observed. This may have

been because the high rate of stirring achieved during growth in a chemostat broke up the viscous EPS. Alternatively, it may be that conditions were not suitable for its formation, even when growth was oxygen-limited, since there are many differences between a poorly aerated batch culture and an oxygen-limited continuous culture. Production of EPS was also increased when the *Methylophilus* species studied by Linton *et al.* (1986) was grown under conditions of methanol excess.

Our results also give additional information about the regulation of some of the key enzymes of C_1 metabolism in *M. methylotrophus*. C. W. Jones and co-workers (Greenwood & Jones, 1986; Jones *et al.*, 1987) showed that the specific activity of methanol dehydrogenase in methanol-limited cells decreased as the standing concentration of methanol in the growth medium increased and our results are consistent with this. They also demonstrated that there was no simple linear relationship between either the activity or the concentration of methanol dehydrogenase and the ability of whole cells to oxidize methanol, and their data suggested that although methanol dehydrogenase is the first enzyme involved in methanol metabolism, it is unlikely to catalyse the rate-limiting step in methanol utilization. Our results are consistent with this conclusion since the rate of utilization of methanol *in situ* was highest when the specific activity of methanol dehydrogenase was at a minimum (Fig. 2). Jones *et al.* (1987) also demonstrated that the activities of some key enzymes of the RuMP cycle increased with increasing growth rate when the supply of carbon or oxygen limited growth. We have now shown that the specific activities of hexulose-6-phosphate synthase plus hexulose-6-phosphate isomerase, glucose-6-phosphate dehydrogenase and both isoenzymes of 6-phosphogluconate dehydrogenase increased as the C : N ratio (and hence the steady-state methanol concentration) was raised.

When methylotrophs are grown under conditions of carbon excess there must be a means of preventing accumulation of formaldehyde and maintaining a balance between the production and utilization of this toxic compound (Attwood & Quayle, 1984). In some RuMP pathway organisms, e.g. *Pseudomonas* C, this may be achieved by increasing oxidation via formaldehyde and formate dehydrogenases (Samuelov & Goldberg, 1982), but in *M. methylotrophus* the specific activities of these enzymes were low and did not alter with growth conditions. Although this is not necessarily an indication that there was no change in carbon flux through this oxidative pathway, the fact that there are changes in the specific activities of other key enzymes of C_1 metabolism suggests that the latter are more likely to be significant in the response of *M. methylotrophus* to these altered growth conditions.

Linton *et al.* (1986) suggested that EPS synthesis from methanol is an efficient way of removing formaldehyde when the respiratory capacity of the cell is limited by the availability of oxygen, provided that polymer formation does not lead to the net over-production of reducing equivalents. Our data are consistent with this hypothesis and EPS production may also have a similar function when the biosynthetic capacity of the cell is limited by the availability of nitrogen. The changes we have observed in the specific activities of some of the key enzymes of C_1 metabolism are presumably part of the response of *M. methylotrophus* to the presence of excess methanol in the medium. When carbon is limited the specific activity of methanol dehydrogenase is high so that the small amount of available methanol can be scavenged efficiently; in contrast, when methanol is in excess the specific activity of methanol dehydrogenase is low, minimizing the production of excess formaldehyde (Jones *et al.*, 1987). Most of this formaldehyde is removed by condensation with RuMP to form hexulose-6-phosphate. The rate of fixation of formaldehyde depends not only on the specific activity of hexulose-6-phosphate synthase (which, when measured with hexulose-6-phosphate isomerase increased 2-fold as the C : N ratio increased), but also on the availability of the C_1 acceptor, RuMP. In order to regenerate this compound carbon must flow round either the assimilatory or dissimilatory RuMP cycle; in the latter case this is accompanied by the formation of NAD(P)H which is reoxidized either when it is utilized during biosynthesis or by the electron transport chain, electron transport being coupled to oxidative phosphorylation. When growth was limited by nitrogen availability an increased amount of carbon was converted into EPS rather than cell material. There is a high energy demand associated with the formation of bacterial EPS

(Jarman & Pace, 1984; Linton *et al.*, 1987), and its production by *M. methylotrophus* is therefore likely to assist in the cycling of NAD^+/NADH and ATP/ADP in situations when synthesis of cell material is limited by the supply of nitrogen, or when the respiratory capacity is limited by the availability of oxygen. The rate-limiting steps in the RuMP cycle are not known, but it is likely that the changes observed in the key enzymes of the assimilatory and dissimilatory pathways are necessary to achieve the required carbon flux round the cycle in these particular growth conditions (Jones *et al.*, 1987), and thus regenerate sufficient RuMP to allow efficient removal of formaldehyde.

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