Biochem. J. (1975) **150**, 569–571
Printed in Great Britain

The Autotrophic Growth of *Micrococcus denitrificans* on Methanol

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(*Received* 23 *June* 1975)

Ribulose bisphosphate carboxylase is present at a high specific activity in extracts of methanol-grown *Micrococcus denitrificans*. Enzymic and physiological evidence indicates that, during growth on methanol, the ribulose bisphosphate cycle is the route of carbon assimilation.

Micrococcus denitrificans will grow on methanol as sole source of carbon and energy. It was decided to determine whether this organism assimilates methanol carbon at the oxidation level of formaldehyde via a reduced C_1-fixation pathway such as the ribulose monophosphate cycle or serine pathway (Quayle, 1972), or whether it oxidizes methanol completely to CO_2 and uses its capability to operate the ribulose bisphosphate cycle for carbon assimilation.

Materials and methods

M. denitrificans (N.C.I.B. 8944) was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, U.K. The organism was maintained on glycollate–agar slopes and grown at 30°C in liquid glycollate medium as described by Kornberg & Morris (1965). For growth on methanol or acetate, the glycollate in this medium was replaced by methanol (50mM) or sodium acetate (20mM). Starter cultures (10ml) for growth on methanol were supplemented with Difco yeast extract (0.01%) and $NaHCO_3$ (0.05%) and were used to inoculate larger volumes of medium, which contained $NaHCO_3$ (0.05%) and methanol but no yeast extract. For autotrophic growth on H_2+CO_2, the mineral medium and gas mixture described by Kornberg *et al.* (1960) were used: cultures (500ml) were shaken at 30°C in stoppered 2-litre Buchner flasks and regassed at intervals. The growth of liquid cultures was followed spectrophotometrically at 650nm by using a Unicam SP. 600 spectrophotometer and referring to an E_{650}–dry-weight calibration curve.

Cultures (1 litre) for the preparation of cell-free extracts were harvested in mid-exponential phase and washed once in ice-cold 50mM-Tris–HCl buffer, pH7.5, containing 10mM-$MgCl_2$. Thick cell suspensions in this buffer were disrupted by passing once through a French pressure cell, pre-cooled to 0°C, at a pressure of 15000lb/in^2 (103MPa); whole cells and debris were removed by centrifugation at 35000*g* for 30min and the supernatant was used as the cell-free extract. 3-Hexulose phosphate synthase and phospho-3-hexuloisomerase were assayed in an extract that had been centrifuged for 10min at only 6000*g*. Protein concentrations were determined with the Folin phenol reagent by the method of Kennedy & Fewson (1968), with crystalline bovine plasma albumin as the standard.

Cell-free extracts were assayed at 30°C for the following enzyme activities by the methods quoted: ribulose bisphosphate carboxylase (EC 4.1.1.39), anaerobically by using a continuous spectrophotometric assay as described by Quayle & Pfennig (1975); 3-hexulose phosphate synthase, by the ribose 5-phosphate-dependent disappearance of formaldehyde, Ferenci *et al.* (1974); phospho-3-hexuloisomerase, Cox & Zatman (1974); malyl-CoA lyase (EC 4.1.3.24), Salem *et al.* (1973); ATP malate lyase, Cox & Zatman (1973); hydroxypyruvate reductase (EC 1.1.1.81), measured at pH7.5 with NADPH, Large & Quayle (1963); serine–glyoxylate aminotransferase (EC 2.6.1.45), by using the endogenous NADPH-linked hydroxypyruvate reductase in the linked assay method of Harder & Quayle (1971); glycerate kinase (EC 2.7.1.31), Heptinstall & Quayle (1970); isocitrate lyase (EC 4.1.3.1), Dixon & Kornberg (1959); malate synthase (EC 4.1.3.2), Dixon & Kornberg (1962). Methanol dehydrogenase (EC 1.1.99.8) was assayed by the method of Anthony & Zatman (1964), with 3.3μmol of phenazine methosulphate. Formaldehyde dehydrogenase (NAD^+/GSH linked) (EC 1.2.1.1) and formate dehydrogenase (EC 1.2.1.2) were assayed by using the methods of Johnson & Quayle (1964).

Ribose phosphate isomerase (EC 5.3.1.6), phosphoribulokinase (EC 2.7.1.19), creatine kinase (EC 2.7.3.2) and D-ribose 5-phosphate (disodium salt) were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Lithium hydroxypyruvate was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Other purified enzymes, coenzymes and biochemicals were from Boehringer Corp. (London) Ltd., Lewes, East Sussex BN7 1LG, U.K.

Results and discussion

Micrococcus denitrificans was found to grow aerobically on methanol as sole source of carbon and energy. Addition of $NaHCO_3$ to the liquid growth medium considerably decreased the initial lag phase at low cell concentrations. Cultures in mid-exponential growth phase showed a lag phase of several hours when diluted at least 10-fold into pre-warmed medium containing no $NaHCO_3$, whereas no lag was observed when $NaHCO_3$ was present. Sodium formate and monomethylamine hydrochloride were also found to support growth, though dimethylamine hydrochloride and trimethylamine hydrochloride did not. No significant growth was observed in cultures incubated in the autotrophic growth medium in an atmosphere of methane+air $+CO_2$ (60:30:10). The following mean doubling times for *M. denitrificans* grown in 1-litre shake cultures at 30°C were observed: H_2+CO_2, 5.2h; methanol, 6.1h; acetate, 2.1h; glycollate, 1.7h.

The results of the determinations of enzyme activities in cell-free extracts are given in Table 1.

The induction of ribulose bisphosphate carboxylase in methanol-grown *M. denitrificans* indicates that the route of carbon assimilation is via the ribulose bisphosphate cycle. The finding that the presence of bicarbonate in methanol growth medium decreases the lag phase occurring after inoculation of fresh medium is consistent with this pathway of carbon assimilation. Although some enzymes normally associated with the serine pathway are present (hydroxypyruvate reductase, malyl-CoA lyase, glycerate kinase), they are not present at higher specific activity during growth on methanol. It therefore seems unlikely that the serine pathway is involved in methylotrophic growth. The possibility of the operation of the ribulose monophosphate cycle of formaldehyde fixation is excluded by the absence of 3-hexulose phosphate synthase and phospho-3-hexuloisomerase, key enzymes of this cycle (Kemp, 1972, 1974; Strøm *et al.*, 1974). The growth physiology of *M. denitrificans* on methanol is therefore analogous to that of *Pseudomonas oxalaticus* during growth on formate (Quayle & Keech, 1959) and represents the first case to be reported of autotrophic growth on methanol.

Fixation of carbon from methanol via the ribulose bisphosphate cycle involves a greater energy expenditure than via the serine pathway or the ribulose monophosphate cycle (Strøm *et al.*, 1974). However, in the case of a chemoautotroph, acquisition of the ability to oxidize C_1 compounds to CO_2 would, without elaboration of further biosynthetic enzymes, confer on the organism the capability of growth on such compounds. Hence the gain in nutritional versatility by such a relatively simple step may account for occurrence of this apparently less favourable mode of metabolism of reduced C_1 compounds.

The specific activities of isocitrate lyase and malate synthase are high during the growth of this strain of *M. denitrificans* on acetate, indicating the operation of the glyoxylate cycle (see French *et al.*, 1964). However, the induction of malyl-CoA lyase

Table 1. *Specific activities of various enzymes in cell-free extracts of Micrococcus denitrificans grown on different carbon substrates*

Experimental details are given under 'Materials and Methods'. All values are expressed as nmol/min per mg of protein. NT, not tested.

Enzyme	Growth substrate			
	CO_2+H_2	Methanol	Acetate	Glycollate
Ribulose bisphosphate carboxylase*	65	58	0	0
Hexulose phosphate synthase	NT	0	NT	NT
Phospho-3-hexuloisomerase	NT	0	NT	NT
Hydroxypyruvate reductase (NADPH)†	82	77	88	97
Malyl-CoA lyase	8	8	78	9
ATP malate lyase	<0.2	<0.2	<0.2	<0.2
Serine–glyoxylate aminotransferase	NT	0	NT	NT
Glycerate kinase	18	16	13	NT
Isocitrate lyase	0	0	110	0
Malate synthase	26	15	139	20
Methanol dehydrogenase	0	54	0	0
Formaldehyde dehydrogenase (NAD^+/GSH)	33	734	10	28
Formate dehydrogenase (NAD^+)	0	121	0	0

* Ribulose bisphosphate carboxylase activity expressed as nmol of ribulose bisphosphate carboxylated/min.
† Specific activities when using NADH were approx. 20% of these values.

during growth on this substrate raises the possibility that the malate synthase activity observed might be due, not to malate synthase (EC 4.1.3.2), but to a combination of malyl-CoA lyase and malyl-CoA hydrolase activities, e.g. citrate synthase (EC 4.1.3.7), which is known to hydrolyse (2*S*)-4-malyl-CoA (Eggerer *et al.*, 1964).

We thank the Science Research Council for financial support under Grant no. B/RG/27118.

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