Hexose Phosphate Synthase and Tricarboxylic Acid-Cycle Enzymes in Bacterium 4B6, an Obligate Methylophile

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Eleven pure cultures of bacteria that grow with trimethylamine as sole source of carbon and energy have been isolated from river- and ditch-water, soil, air and fish. Of 49 compounds tested, only methylamine, dimethylamine or trimethylamine supports the growth of one isolate, bacterium 4B6, indicating that this organism is a new type of obligate methylophile. Results of experiments with extracts of bacterium 4B6 reveal (i) the presence of hexose phosphate synthase (Kemp & Quayle, 1966), (ii) the absence of 2-oxoglutarate dehydrogenase and malate dehydrogenase, (iii) and low activities of other tricarboxylic acid-cycle enzymes.

Materials and methods

The mineral medium used was mineral base E of Owens & Keddle (1969). Filter-sterilized trimethylammonium chloride or sodium acetate solutions were added to the autoclaved mineral medium to give a final concentration of 20 mM. Bacterium 4B6 was grown in media fortified with vitamin B₁₂ (2.5 µg/l) and bacterium 5B1 in media fortified with thiamin hydrochloride (0.5 mg/l). Solidified media were prepared by the addition of Oxoid Ionagar no. 2 (10 g/l) to the liquid media. Stock cultures were maintained on slopes of trimethylamine agar medium at 4°C and subcultured every 3 months.

Three of the cultures isolated with trimethylamine as sole source of carbon and energy were used in the present work. Bacteria 4B6 and 5B1 are non-pigmented, Gram-negative, obligately aerobic, non-motile, rod-shaped bacteria; *Pseudomonas* 3A2 is a pink-pigmented bacterium similar in its properties to *Pseudomonas* AM1 (Peel & Quayle, 1961). Both bacterium 5B1 and *Pseudomonas* 3A2 are typical facultative methylophiles that grow on a variety of C₁ and other substrates but not on methane.

The ability of bacterium 4B6 to grow on each of a variety of potential growth substrates was tested in vitamin B₁₂-supplemented mineral medium containing 0.1% of filter-sterilized growth substrate. For most substrates the tests were done both on solidified and in shaken liquid media. Liquid media (100 ml) were inoculated with 1 ml of an exponential-phase trimethylamine culture; solidified media were inoculated by streaking. Growth on methane was tested in liquid medium as described by Whittenbury

et al. (1970). All cultures were incubated at 30°C and examined for growth periodically up to 14 days.

Organisms for the preparation of extracts were grown in conical flasks containing 1 litre of trimethylamine- or acetate-containing medium incubated on an orbital shaker at 30°C. Cells were harvested in mid-exponential phase after 16–24 h growth and washed twice with ice-cold 100 mM-sodium phosphate buffer, pH 7.5. Extracts were prepared by sonication; washed suspensions (12 ml) containing 100–200 mg wet wt./ml in 100 mM-sodium phosphate buffer, pH 7.5, were sonicated at 20 kHz for 5 min at 0°C with an MSE 100 W ultrasonic disintegrator. Whole cells and debris were removed by centrifuging the crude sonicated suspension at 35 000 g for 30 min; the supernatant was then spun at 100 000 g for 1 h, and this final supernatant was used for the enzyme assays. Extracts of bacterium 4B6 for the determination of hexose phosphate synthase activity were prepared by sonication of cells suspended in sodium phosphate buffer containing 1 mM-MgCl₂ (Lawrence *et al.*, 1970), and the 35 000 g supernatant was used for the assays. Extracts for assays of particulate malate dehydrogenase (FAD) activity were prepared by sonication of organisms suspended in 50 mM-tris-HCl buffer, pH 7.5; the crude sonicated suspension was spun at 10 000 g for 10 min, and the supernatant was used. Protein concentrations were determined with the Folin phenol reagent (Kennedy & Fewson, 1968).

Isocitrate dehydrogenase (NADP) (EC 1.1.1.42), malate dehydrogenase (EC 1.1.1.37) and glutamate dehydrogenase (EC 1.4.1.2–4) activities were assayed as described by Sottocasa *et al.* (1967), isocitrate dehydrogenase (NAD) (EC 1.1.1.41) activity by the method of Kornberg (1955), citrate synthase (EC 4.1.3.7) activity by the method of Srere *et al.* (1963), succinyl-CoA synthetase (EC 6.2.1.5) activity by the method of Kaufman *et al.* (1953) and fumarate hydratase (EC 4.2.1.2) activity by following the disappearance of fumarate at 240 nm (Racker, 1950). Aconitate hydratase (EC 4.2.1.3) activity was determined with *cis*-aconitate as substrate as described by Pearce *et al.* (1969). Succinate dehydrogenase (EC 1.3.99.1) activity was assayed by following the reduction of 2,6-dichlorophenol-indophenol in the presence of 1 mM-phenazine methosulphate and 1 mM-KCN by the method of Veeger *et al.* (1969). 2-Oxo-

glutarate dehydrogenase (EC 1.2.4.2) activity was assayed by the methods of Amarasingham & Davis (1965) with the NAD analogue 3-acetylpyridine-adenine dinucleotide and of Mukherjee *et al.* (1965) with NAD; the values quoted in Table 1 were obtained with the NAD analogue. The activity of particulate malate dehydrogenase (FAD) was assayed by the method of Benziman (1969), NAD- and NADP-dependent malate dehydrogenase (decarboxylating) (EC 1.1.1.38 and 1.1.1.40) activities by the methods of Scrutton (1971) and isocitrate lyase (EC 4.1.3.1) activity by the continuous method of Dixon & Kornberg (1959). Hydroxypyruvate reductase and hexose phosphate synthase activities were assayed by using the methods of Lawrence *et al.* (1970) and of Dahl *et al.* (1972) respectively

Results

The following substrates failed to support the growth of bacterium 4B6: tetramethylammonium chloride, trimethylsulphonium chloride, trimethylamine *N*-oxide, triethylamine, diethylamine, ethylamine, ethanolamine, putrescine, tyramine, benzylamine, choline, betaine, carnitine, methane, methanol, formate, formamide, ethanol, acetate, glyoxylate, glycollate, oxalate, glycine, sarcosine, citrate, succinate, fumarate, malate, pyruvate, lactate, serine,

aspartate, glutamate, lysine, arginine, glycerate, malonate, glutarate, glycerol, sorbitol, glucose, ribose, xylose, benzoate, 3,4-dihydroxybenzoate and mandelate. Bacterium 4B6 grew well in shaken liquid culture and on solidified media containing trimethylamine, dimethylamine or methylamine; mean generation times in shaken liquid culture were 2.5 h on trimethylamine, 4 h on dimethylamine and 5 h on methylamine. As methylamines are the obligatory growth substrates of bacterium 4B6 this organism can be considered to be an obligate methylotroph.

Extracts of bacterium 4B6 were examined for the enzymes of the tricarboxylic acid cycle, and their specific activities were compared with those found in extracts of the facultative methylotrophs bacterium 5B1 and *Pseudomonas* 3A2. The results are shown in Table 1. For trimethylamine-grown organisms it is evident that, with the exception of citrate synthase and NAD-dependent isocitrate dehydrogenase, the activities in extracts of bacterium 4B6 are much lower than those found in extracts of the facultative methylotrophs. In particular, no 2-oxoglutarate dehydrogenase or malate dehydrogenase (EC 1.1.1.37) activities were observed in extracts of bacterium 4B6; several attempts were made to demonstrate 2-oxoglutarate dehydrogenase activity by using the assay methods of Amarasingham & Davis (1965) and Mukherjee *et al.* (1965), but with

Table 1. *Specific activities of enzymes in cell extracts of bacterium 4B6 and two typical facultative methylotrophs*

All values are expressed as nmol/min per mg of protein, and were determined as described under 'Materials and methods'. N.D., Not determined.

Organism	Bacterium 4B6	<i>Pseudomonas</i> 3A2		Bacterium 5B1	
Growth substrate ...	Trimethylamine	Trimethylamine	Acetate	Trimethylamine	Acetate
Citrate synthase	29	25	270	41	194
Aconitate hydratase	10	132	940	400	1540
Isocitrate dehydrogenase (NADP)	13	136	970	220	1900
Isocitrate dehydrogenase (NAD)	75	0	0	0	0
2-Oxoglutarate dehydrogenase	0	1.6	32	7	59
Succinyl-CoA synthetase	2	11	59	14	84
Succinate dehydrogenase	0.6	20	95	24	110
Fumarate hydratase	9	122	510	210	760
Malate dehydrogenase	0	660	3800	675	10300
Glutamate dehydrogenase (NADP)	144	0	0	109	250
Glutamate dehydrogenase (NAD)	0	0	0	0	0
Isocitrate lyase	1.1	1.4	1.4	8	143
Hydroxypyruvate reductase	0	624	172	385	99
Hexose phosphate synthase	975	0	N.D.	0	N.D.

negative results. Extracts of bacterium 4B6 also lacked particulate malate dehydrogenase (FAD) as well as NAD- and NADP-dependent malate dehydrogenases (decarboxylating).

The activities of the tricarboxylic acid-cycle enzymes in the facultative methylotrophs when grown on trimethylamine are much lower than those in acetate-grown organisms. Presumably during growth on trimethylamine the cycle functions principally for the provision of intermediates for biosynthesis. The low isocitrate lyase activity in extracts of acetate-grown *Pseudomonas* 3A2 implies that this organism assimilates C_2 units (and perhaps C_1 units during growth on trimethylamine) by the pathway suggested by Dunstan *et al.* (1972*a,b*) for ethanol and C_1 -unit assimilation in the similar pink-pigmented facultative methylotroph *Pseudomonas* AM1.

Extracts of trimethylamine-grown *Pseudomonas* 3A2 and bacterium 5B1 contain high hydroxypyruvate reductase activity, consistent with the operation of the serine pathway for the assimilation of C_1 units (Heptinstall & Quayle, 1970). This enzyme is absent from extracts of bacterium 4B6, which contain instead a high activity of hexose phosphate synthase, a key enzyme in the ribose phosphate pathway of formaldehyde assimilation (Kemp & Quayle, 1966).

Discussion

As discussed by Dahl *et al.* (1972), it is currently considered that there are two categories of organism able to grow on reduced C_1 compounds as their sole source of carbon and energy (cf. Foster & Davis, 1966). These are: (i) the obligate methylotrophs, whose growth is strictly dependent on the presence of methane or methanol [or, according to Wilkinson (1971), dimethyl ether]; (ii) the facultative methylotrophs, which are unable to grow on methane, but are capable of growth on reduced C_1 compounds such as methanol, formate or methylamine as well as on a variety of other organic compounds. With the isolation of organism W1, which grows only on methanol or methylamine (Dahl *et al.*, 1972), and of bacterium 4B6, which grows only on methylamines, a problem is emerging concerning the definition of an obligate methylotroph, and indeed of methylotrophy. There is an obvious sense in which these two organisms can be considered to be obligate methylotrophs, namely that their growth is strictly dependent on ' C_1 ' substrates. It seems to us that the common property of all methylotrophs (obligates and facultatives) can best be regarded as their ability to grow non-autotrophically at the expense of carbon compounds containing one or more carbon atoms but containing no carbon-carbon bonds. With the obligate methylotrophs such compounds are their unique growth substrates, upon which their growth is absolutely dependent, whereas the facultative methylotrophs have the added property of being able to grow on a variety of other organic compounds. A further advantage of this concept is that the apparent anomaly of considering trimethylamine, trimethylamine *N*-oxide, trimethylsulphonium ion, dimethylamine, dimethyl ether etc. as ' C_1 compounds' is also removed.

Those obligate methylotrophs that can grow on methane use either the serine pathway or the ribose phosphate pathway for the assimilation of C_1 units (Lawrence & Quayle, 1970); most, if not all, of the facultative methylotrophs appear to use the serine pathway (Quayle, 1972). Until very recently the ribose phosphate pathway had been observed only in those obligate methylotrophs able to grow on methane; now, however, organism W1 (Dahl *et al.*, 1972) and bacterium 4B6, neither of which can grow on methane, have both been shown to possess hexose phosphate synthase, a key enzyme of that pathway. These observations would seem to eliminate the possibility that this enzyme is uniquely associated with the assimilation of methane carbon. The absence of hydroxypyruvate reductase, a key enzyme in the serine pathway (Heptinstall & Quayle, 1970), indicates the absence of the latter in bacterium 4B6.

The absence of 2-oxoglutarate dehydrogenase, associated with low activities of other tricarboxylic acid-cycle enzymes, has been suggested as an explanation of the failure of the obligate lithotrophs and obligate phototrophs to grow heterotrophically (for a recent review see Kelly, 1971). The absence of 2-oxoglutarate dehydrogenase has also been reported for some obligate methylotrophs (Patel *et al.*, 1969; Wilkinson, 1971; Dahl *et al.*, 1972), suggesting a common biochemical basis for obligate lithotrophy, obligate phototrophy and obligate methylotrophy. All the tricarboxylic acid-cycle enzymes in bacterium 4B6 are present at significantly lower specific activities than those found in acetate-grown facultative methylotrophs, and 2-oxoglutarate dehydrogenase was not detected. These results provide further support for the above concept of the biochemical basis of obligate methylotrophy.

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