Alternative Carbon Assimilation Pathways in Methane-utilizing Bacteria

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(Accepted for publication 31 August 1970)

An examination of three species of methane-utilizing bacteria has shown (Lawrence, Kemp & Quayle, 1970) that the bacteria use one of two pathways of carbon assimilation, the serine pathway (Heptinstall & Quayle, 1970) or the ribose phosphate cycle of formaldehyde fixation (Kemp & Quayle, 1967). The isolation of many new types of methane-utilizing bacteria by Whittenbury, Phillips & Wilkinson (1970) has made possible an extension of these studies, and this paper reports the results of an examination of eight of the new isolates. The examination is based on the distribution of two key enzymes, each of which appears to be specifically involved in one of the assimilation pathways, namely hydroxypyruvate reductase (serine pathway) and hexose phosphate synthetase (ribose phosphate cycle).

METHODS

Growth of the organisms. The following organisms were kindly provided by Dr R. Whittenbury, Department of General Microbiology, University of Edinburgh (the symbols in parentheses denote the particular strain): '*Methylosinus sporium*' (5), '*Methylosinus trichosporium*' (OB 3 b), '*Methylocystis parvus*' (OB BP), '*Methylomonas agile*' (Y1), '*Methylomonas rosaceus*' (BG 2), '*Methylomonas methanica*' (25), '*Methylococcus minimus*' (TMC) and '*Methylobacter capsulatus*' (1521). The bacteria were grown in liquid salts medium (Foster & Davis, 1966) under an atmosphere of methane-air (1:1) in conical flasks shaken at 30°. '*Methylobacter capsulatus*' would grow in liquid medium only if the starter culture was supplemented with an aqueous extract of agar. The extract was prepared from Difco agar by the method of Dworkin & Foster (1956) and 0.5 ml. (containing 22.5 mg. dry wt) was added to 10 ml. of the starter culture. *Pseudomonas methanica* (Dworkin & Foster, 1956), *Methylococcus capsulatus* (Foster & Davis, 1966) and *Methanomonas methanooxidans* (Stocks & McCleskey, 1964) were grown up in a fermentor on methane as carbon source using the growth technique described by Lawrence *et al.* (1970). The bacterial paste harvested from the fermentor was freeze-dried and stored at -15° until used.

Preparation of bacteria-free extracts. Bacteria, 1 g. (wet wt) or 0.1 g. (dry wt), were suspended in 5 ml. of 20 mM-sodium phosphate buffer, pH 7.0, supplemented with 5 mM-magnesium chloride, sonicated until maximum breakage had occurred (< 8 min.) in an MSE sonicator (type 100 W) and the resulting suspensions centrifuged at 90,000 g for 30 min. The supernatant fraction was poured off and the pellet resuspended in 5 ml. of the same buffer used to suspend the bacteria.

Enzyme assays. The activities of hexose phosphate synthetase and hydroxypyruvate reductase in the supernatant and pellet fractions were measured by the procedures of Lawrence *et al.* (1970). The specific activity of hydroxypyruvate reductase is expressed as μ moles of NADH oxidized/h./mg. protein. The specific activity of hexose phosphate synthetase is expressed as μ moles of [14 C]formaldehyde fixed/h./mg. protein. In the assays for hexose phosphate synthetase, control assays were performed by incubating [14 C]formaldehyde with extract in the absence of ribose 5-phosphate. The control values were subtracted from the test values obtained with ribose 5-phosphate present in the assay mixture.

Protein determinations. Protein was determined by the method of Folin and Ciocalteu as described by Layne (1957), with bovine serum albumin as standard.

RESULTS AND DISCUSSION

The enzyme distribution in the eight new isolates and in *Pseudomonas methanica* (Dworkin & Foster), *Methylococcus capsulatus* (Foster & Davis) and *Methanomonas methanooxidans* (Stocks & McCleskey) is shown in Table 1. It can be seen that the clear division of organisms into those which contain hydroxypyruvate reductase and those which contain hexose phosphate synthetase, initially seen in the case of the

Table 1. *Specific activities of hydroxypyruvate reductase and hexose phosphate synthetase in extracts of methane-grown bacteria*

Organism	Test of membrane system	Hydroxypyruvate reductase activity (μ moles/mg. protein/h.)		Hexose phosphate synthetase activity (μ moles HCHO fixed/mg. protein/h.)	
		Supernatant	Pellet	Supernatant	Pellet
' <i>Methylosinus sporium</i> ' (5)	II	80	n.d.	n.d.	n.d.
' <i>Methylosinus trichosporium</i> ' (OB 3 b)	II	46.3	n.d.	n.d.	n.d.
' <i>Methylocystis parvus</i> ' (OB BP)	II	91	n.d.	n.d.	n.d.
<i>Methanomonas methanooxidans</i> (Stocks & McCleskey)	II	72.3	n.d.	1.11	0.54*
' <i>Methylomonas agile</i> ' (Y 1)	I	n.d.	n.d.	18.7	8.70
' <i>Methylomonas rosaceus</i> ' (BG 2)	I	n.d.	n.d.	108.8	15.5
' <i>Methylomonas methanica</i> ' (25)	I	n.d.	n.d.	78.1	31.4
' <i>Methylobacter capsulatus</i> ' (1521)	I	n.d.	n.d.	35.7	18.2
' <i>Methylococcus minimus</i> ' (TMC)	I	n.d.	n.d.	132.2	17.8
<i>Pseudomonas methanica</i> (Dworkin & Foster)	I	0.62	n.d.	54.8	10.6*
<i>Methylococcus capsulatus</i> (Foster & Davis)	I	n.d.	n.d.	18.2	22.5*

n.d. = None detectable.

* The pellet fraction was washed thrice with 20 mM-phosphate buffer, pH 7.0, + 5 mM-MgCl₂ before assay.

last-named three organisms by Lawrence *et al.* (1970), precisely holds over the wider range that have now been tested. Whittenbury *et al.* (1970) have tentatively classified the methane-utilizers on the basis of morphology, fine structure and type of resting stage into two vibrioid groups, '*Methylosinus*' and '*Methylocystis*', and three rod/coccoid groups, '*Methylomonas*', '*Methylobacter*' and '*Methylococcus*'. On this new

classification, *Mtn. methanooxidans* (Stocks & McCleskey) appears to be a strain of 'Methylosinus' (R. Whittenbury, personal communication), *Ps. methanica* (Dworkin & Foster) is a strain of 'Methylomonas methanica', and *Mtc. capsulatus* (Foster & Davis) remains similarly named. The 11 organisms in Table 1 which have now been screened, although they only constitute a small sample of the total number of strains isolated, are nevertheless representative of each of the different groups proposed by Whittenbury *et al.* (1970). It thus seems likely that the dichotomy of assimilation pathway observed in this sample of organisms will be a general property amongst methane-utilizing bacteria.

All the methane-utilizing bacteria which have been examined so far possess complex internal membrane systems (Proctor, Norris & Ribbons, 1969; Whittenbury, 1969; Davies & Whittenbury, 1970; Whittenbury *et al.* 1970) which have been divided into two basic arrangements (Davies & Whittenbury, 1970): a series of bundles of disc-shaped vesicles distributed throughout the organism (type I) and a system of paired membranes running throughout the organism or aggregated at the periphery (type II).

It is most interesting to note that the subdivision of the organisms into two classes based on assimilation pathway corresponds exactly with their subdivision based on membrane system. Thus an organism using the serine pathway possesses a type II membrane system, whereas an organism using the ribose phosphate cycle possesses a type I membrane system. It therefore appears likely that the methane-utilizing bacteria, far from being a closely similar group of organisms as might be anticipated from their striking nutritional similarity of being able to grow only on methane or methanol, may in fact be divided into at least two major groups possessing fundamental differences in their mode of carbon assimilation and system of internal membranes. These differences are wide enough to suggest that the two groups of organisms may have evolved quite separately as methane utilizers. If this were so, it raises the question as to why both groups have convergently evolved as obligate methylootrophs.

We acknowledge generous financial support from the Agricultural Division, Imperial Chemical Industries Ltd, Billingham, Co. Durham, and the Science Research Council for a Studentship held by A. J. L.

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