The linear oxidation of formaldehyde to CO₂ as the proper energy generating sequence for the assimilation of methanol in *Acetobacter methanolicus MB58*<sup>※</sup>

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Abstract. *Acetobacter methanolicus MB58* can grow on methanol. Since this substrate exhibits to be energy deficient there must be a chance to oxidize methanol to CO₂ merely for purpose of energy generation. For the assimilation of methanol the FBP variant of the RuMP pathway is used. Hence methanol can be oxidized cyclically via 6-phosphogluconate. Since *Acetobacter methanolicus MB58* possesses all enzymes for a linear oxidation via formate the question arises which of both sequences is responsible for generation of the energy required. In order to clarify this the linear sequence was blocked by inhibiting the formate dehydrogenase with hypophosphite and by mutagenesis inducing mutants defective in formaldelyde or formate dehydrogenase. It has been shown that the linear dissimilatory sequence is indispensable for methylo trophic growth. Although the cyclic oxidation of formaldehyde to CO₂ has not been influenced by hypophosphite and with mutants both the wild type and the formaldehyde dehydrogenase defect mutants cannot grow on methanol. The cyclic oxidation of formaldehyde does not seem to be coupled to a sufficient energy generation, probably it operates only detoxifying and provides reducing equivalents for syntheses. The regulation between assimilation and dissimilation of formaldehyde in *Acetobacter methanolicus MB58* is discussed.

Key words: *Acetobacter methanolicus MB58* — Methylo trophicity — Linear and cyclic dissimilatory sequence of formaldehyde — Energy generation — Mutants — Regulation

Methanol is an energy deficit substrate, this means that ATP and reducing equivalents generated enroute to a central precursor, e.g. glyceraldehyde-3-phosphate or phosphoglycerate do not suffice to assimilate the substrate carbon by microorganisms (Babel 1980; Babel and Müller 1985). For this purpose energy must be produced by oxidation of methanol to CO₂. *Acetobacter methanolicus MB58* assimilates methanol via the FBP-variant of the RuMP-pathway (Babel and Müller 1977; Babel 1984). Since this strain can also grow on glucose or glycerol as sole carbon and energy source reducing equivalents required for the assimilation of the precursor carbon ought to be released from the TCA cycle. However, during methylotrophic growth of *Acetobacter methanolicus MB58* the TCA cycle does not seem operate energetically (Babel and Hofmann 1977; Müller-Kraft and Babel 1987) as in other methylotrophic bacteria (Davey et al. 1972). Thus the RuMP-cycle (Strøm et al. 1974; Babel and Hoffmann 1975) and the linear dissimilatory sequence for the oxidation of formaldehyde become favourite for energy production. The RuMP-cycle also seems to be ruled out for the following reasons: Firstly, both dehydrogenases of the cyclic formaldehyde oxidation are NAD⁺- and NADP⁺-dependent, however, the specific activity of NAD⁺-linked 6-phosphogluconate dehydrogenase is less by a half in methanol grown cells than in glucose grown ones (Steudel et al. 1980). Thus the cyclic oxidation of formaldehyde is only relevant to the energy generation if *Acetobacter methanolicus MB58* possesses NADPH-NAD⁺: transhydrogenase activity (EC 1.6.1.1). But this enzyme could not be found. Secondly, the cyclic oxidation of formaldehyde to CO₂ provides 2 mol NAD(P)H. On a growth yield of 0.4 g dry weight per g methanol, which is experimentally obtained, it can be predicted using the modified Y<sub>ATP</sub> concept (Anthony 1978; Babel and Müller 1985) that at least 1 mol ATP must be generated per mol formaldehyde oxidized. Because of the extremely low P/O quotient for NADH (about 0.1 in acetate acid bacteria, cf. Stouthamer 1962; Babel 1984) such an ATP yield is unlikely hence the linear dissimilatory sequence for formaldehyde seems to be responsible for the energy budget with methylotrophic growth of *Acetobacter methanolicus MB58*.

The linear dissimilatory sequence of formaldehyde is catalyzed by a dye-mediated formaldehyde dehydrogenase (Gründig and Babel 1987) and by a membrane-bound apparently cytochrome-coupled formate dehydrogenase (Babel and Mothes 1978; Gründig and Doronina 1984). If this sequence is really physiologically relevant to *Acetobacter methanolicus MB58* the organism should not be able to grow on methanol if the linear oxidation of formaldehyde to CO₂ is blocked. This was tested by the inhibition of formate dehydrogenase activity in the wild type and by means of mutants.

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Abbreviations: ATP, Adenosine-5'-triphosphate; DCPIP, 2,6-dichlorophenolindophenol; DW, dry weight; ETP, electron transport phosphorylation; FBP, fructose-1,6-bisphosphate; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; PMS, phenazine methosulfate; RuMP, ribulose monophosphate; Ru5P, ribulose-5-phosphate; SDS, sodiumdodecylsulphate; TCA, tricarboxylic acid; TYB, toluylene blue
Materials and methods

Growth conditions

*Acetobacter methanolicus* MB58 was grown with agitation in 500 ml flasks with 100 ml medium (Steudel et al. 1980; Uhlig et al. 1986) supplemented with 0.5% (m/v) of the appropriate substrate as the sole carbon and energy source and 50 mg/l pantothenic acid. The cells were harvested in the mid-logarithmic growth phase, washed twice with phosphate buffer, 50 mM, pH 7.4 for the assay of methanol dehydrogenase and cytoplasmic enzymes or with phosphate buffer, 50 mM, pH 5.0 for the assay of dye-mediated formaldehyde or formate dehydrogenase and for measurements on whole cells.

Preparation of cell-free extracts

Cell disruption was performed in the above mentioned buffers with a French pressure cell press (Aminco Instruments Company, Silverspring, USA) using a twofold passage at 90 MPa. Clear supernatant and pellet fractions were obtained after centrifugation (40 min, 16,000 × g, 4°C). The pellet fraction was washed once with phosphate buffer, 50 mM, pH 5.0 and resuspended in the twofold original volume.

Assay of enzyme activity

All enzymes were assayed at 30°C with a Specord UV/VIS spectrophotometer. The following enzymes were measured spectrophotometrically by methods described or quoted by Gründig and Doronina (1984): Methanol dehydrogenase (EC 1.1.1.99.8), DCPIP-mediated formate dehydrogenase, hexulose-6-phosphate synthase and isomerase, NAD(P)^+/-linked glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and NAD(P)^+/-linked 6-phosphogluconate dehydrogenase (EC 1.1.1.44). Phosphoglucoisomerase (EC 5.3.1.9) was assayed according to Bergmeyer (1974). TYB-mediated formaldehyde dehydrogenase was measured in the pellet fraction with phosphate buffer, 100 mM, pH 5.0, TYB, 0.15 mM and formaldehyde, 20 mM under anaerobic conditions as described for formate dehydrogenase (Gründig and Doronina 1984). For calculating specific activities ε was determined to be 1.3 × 10^3 I · M⁻¹ · cm⁻¹. Cytochrome c was determined using redox difference spectra as described by Babel and Steudel (1977).

Protein and dry weight determinations

Protein was measured by the method of Bradford (1976) using 1 h incubation of pellet samples with the reaction mixture. Dry weight of bacterial suspensions was determined measuring the extinction at 700 nm and by using a calibration curve.

Gas turnover

The oxygen consumption of whole cells was measured with an oxygen electrode according to Steudel et al. (1980). Manometric determinations were performed by the Warburg technique at 30°C (shaking frequency 100/min). We used 2.5 ml washed cell suspension (0.85 g/l DW final concentration). The reaction was started with 0.5 ml substrate. In these experiments methanol, 150 mM, formaldehyde, 20 mM or formate, 30 mM were used. All experiments were performed in duplo.

Substrate consumption with resting cells

30 ml of washed cell suspensions (0.85 g/l DW) were placed in 100 ml Erlenmeyer flasks, which were closed with plastic stoppers with a small hole to prevent evaporation. Incubations were performed in a shaking water bath (30°C, 75 rpm). In these experiments the same substrate concentrations were used as in manometric measurements. Samples were taken with syringes and biomass was separated immediately by centrifugation (2 min, 16,000 × g, 4°C) in closed tubes. The remaining supernatant was stored at −20°C until analytical procedures were performed. For detection of intracellular formate concentrations biomass probes were resuspended in 100 μl Tris-HCl buffer, 50 mM, pH 8.5 supplemented with SDS, 0.2% and incubated at 37°C for 1 h in closed tubes. The clear solutions obtained by centrifugation (2 min, 16,000 × g) were used for measurement of formic acid.

Determination of substrate concentrations

Formaldehyde was determined according to Chrastil and Wilson (1973) and formic acid according to a modified method (Sleat and Mah 1984) of Lang and Lang (1972). Methanol was determined gaschromatographically.

Isolation of mutants defective in the linear dissimilatory sequence of formaldehyde

Methanol grown cells (10^16–10^20 cells per ml) were incubated in Sörensen buffer, pH 5.4 with 0.05 mg/ml MNNG at 30°C for 1 h. Then the cells were washed three times with Sörensen buffer and plated on glucose or glycerol agar. Mutants, which were not able to grow on methanol as the sole carbon and energy source were identified replicating the obtained colonies on methanol agar. Methanol^- mutants were checked for the absence of formaldehyde and formate dehydrogenase in colonies. For this purpose the colonies were grown on filter paper, which was placed on a solid medium. The filter with the colonies was washed on a Büchner funnel with the buffer, which was used in the test agar. Then the filter was placed on test agar with the colonies up and incubated at 30°C in the dark.

TYB-mediated formaldehyde dehydrogenase was assayed in colonies by two methods. The first method is based on the fact that *Acetobacter methanolicus* MB58 produces formic acid from formaldehyde in the presence of hypophosphate. The formation of this acid was detected with a pH-indicator. The test agar contained in phosphate buffer, 25 mM, pH 5.2; agar-agar, 0.8%, Congo red, 0.05%, NaH₂PO₄, 20 mM and formaldehyde, 20 mM. After about 2 h in the red agar a blue corona was formed only around those colonies which contain TYB-mediated formaldehyde dehydrogenase.

In a second method TYB was used as an electron acceptor for the formaldehyde dehydrogenase. The test agar contained in phosphate buffer, 25 mM, pH 5.0; toluylen blue, 0.05% and formaldehyde, 20 mM. After some minutes