The linear oxidation of formaldehyde to CO$_2$
as the proper energy generating sequence for
the assimilation of methanol in Acetobacter methanolicus MB58*

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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 CO$_2$ 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-phos-
phogluconate. 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 hypophosphate and by mutations in-
ducing mutants defective in formaldehyde or formate dehy-
drogenase. It has been shown that the linear dissimilatory
sequence is indispensable for methylotrophic growth.

Although the cyclic oxidation of formaldehyde to CO$_2$ has
not been influenced by hypophosphate and with mutants
both the wild type and the formaldehyde dehydrogenase
defect mutants cannot grow on methanol. The cyclic oxida-
tion 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 dis-
cussed.

Key words: Acetobacter methanolicus MB58 —
Methylophagy — Linear and cyclic dissimilatory sequence
of formaldehyde — Energy generation — Mutants — Regu-
lation

Methanol is an energy deficient substrate, this means that
ATP and reducing equivalents generated enroute to a central
precursor, e.g. glyceraldehyde-3-phosphate or phospho-
glycerate do not suffice to assimilate the substrate carbon
by microorganisms (Babel 1980; Babel and Müller 1985).

* Dedicated to Prof. Dr. Dr. S. M. Rapoport on occasion of his
75th birthday

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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 methosul-
fate; RuMP, ribulose monophosphate; Ru5P, ribulose-5-
phosphate; SDS, sodiumdodecylsulfate; TCA, tricarboxylic acid;
TYB, toluylene blue

For this purpose energy must be produced by oxidation of
methanol to CO$_2$. 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 MB 58 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 favourable for energy production. The
RuMP-cycle also seems to be ruled out for the following
reasons: Firstly, both dehydrogenases of the cyclic formalde-
hyde oxidation are NAD$^+$- and NADP$^+$-dependent,
however, the specific activity of NAD$^+$-linked 6-phosphogluco-
nate 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 CO$_2$ 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 pre-
dicted using the modified $Y_{ATP}$ 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 acet
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
(Grünzig and Babel 1987) and by a membrane-bound appar-
etly 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 CO$_2$
is blocked. This was tested by the inhibition of formate
dehydrogenase activity in the wild type and by means of
mutants.
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 pantethonic 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 cytoplasmatic 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 (Amino Instruments Company, Silver Springs, 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 l · 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 (10¹⁸ - 10²⁰ 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 wiped over 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; tolulylene blue, 0.05% and formaldehyde, 20 mM. After some minutes