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Regulation of methanol oxidation and carbon dioxide fixation in *Xanthobacter* strain 25a grown in continuous culture

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Abstract. The regulation of C_1-metabolism in *Xanthobacter* strain 25a was studied during growth of the organism on acetate, formate and methanol in chemostat cultures. No activity of methanol dehydrogenase (MDH), formate dehydrogenase (FDS) or ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisC/O) could be detected in cells grown on acetate alone over a range of dilution rates tested. Addition of methanol or formate to the feed resulted in the immediate induction of MDH and FDH and complete utilization ($D = 0.10\ h^{-1}$) of acetate and the C_1-substrates. The activities of these enzymes rapidly dropped at the higher growth rates, which suggests that their synthesis is further controlled via repression by "heterotrophic" substrates such as acetate. Synthesis of RuBisC/O already occurred at low methanol concentrations in the feed, resulting in additive growth yields on acetate/methanol mixtures. The energy generated in the oxidation of formate initially allowed an increased assimilation of acetate (and a decreased dissimilation), resulting in enhanced growth yields on the mixture. RuBisC/O activity could only be detected at the higher formate/acetate ratios in the feed. The data suggest that synthesis of RuBisC/O and CO_2 fixation via the Calvin cycle in *Xanthobacter* strain 25a is controlled via a (de)repression mechanism, as is the case in other facultatively autotrophic bacteria. Autotrophic CO_2 fixation only occurs under conditions with a diminished supply of "heterotrophic" carbon sources and a sufficiently high availability of suitable energy sources. The latter point is further supported by the clearly more pronounced derepressing effect exerted by methanol compared to formate.

Key words: *Xanthobacter* – Methanol – Formate – Methylotrophy – Autotrophy – Calvin cycle – Regulation – Continuous culture – RuBisC/O – Carbon dioxide fixation

All *Xanthobacter* strains (Wiegel et al. 1978; Wiegel and Schlegel 1984) studied are versatile bacteria, able to grow autotrophically on $H_2/O_2/CO_2$, formate and methanol, and heterotrophically on many (multiple-carbon) organic substrates. Three *Xanthobacter* species have been described thus far, namely *X. autotrophicus*, *X. flavus*, and *X. agilis* (Jenni and Aragno 1987; Jenni et al. 1987). Recently we reported the isolation and characterization of *Xanthobacter* strain 25a (Meijer et al. 1990a). This isolate most likely represents a fourth species of the genus *Xanthobacter*, combining characteristic properties of *X. autotrophicus* and *X. flavus* (pleomorphism, slime production, growth on nutrient broth, autotrophic growth at 37° C), *X. flavus* (biotin requirement), as well as *X. agilis* (motility).

Studies on the enzymology of methanol utilization in various *Xanthobacter* strains (Weaver and Lidstrom 1985; Janssen et al. 1987; Meijer et al. 1990a) have shown that this substrate is oxidized to formaldehyde via a PQQ-dependent methanol dehydrogenase (MDH). Oxidation of formate to CO_2 involves an NAD-dependent formate dehydrogenase (FDH). Synthesis of cell material is initiated by assimilation of CO_2 via the Calvin cycle. Three enzymes are specifically involved in the operation of this cycle (Tabita 1988), namely ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisC/O), phosphoribulokinase (PRK) and fructose-1,6-bisphosphatase (FBPase). Synthesis of these enzymes in *Xanthobacter flavus* H4-14 and *Xanthobacter* strain 25a is under strict control (Meijer et al. 1990a–c). The physiological and molecular mechanisms controlling their synthesis, however, still remain to be identified. *Xanthobacter* strains are well-suited for genetic studies (Wilke 1980; Lehmicke and Lidstrom

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Abbreviations: FDH, formate dehydrogenase; FBPase, fructose-1,6-bisphosphatase; ICDH, isocitrate dehydrogenase; MDH, methanol dehydrogenase; PQQ, pyrrolo quinoline quinone; PRK, phosphoribulokinase; RuBisC/O, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuMP, ribulose monophosphate; TCA, tricarboxylic acid cycle

1985; Weaver and Lidstrom 1987) and a genetic analysis of the autotrophic system. No evidence has been obtained thus far for the presence of dual gene clusters for the Calvin cycle enzymes in *Xanthobacter flavus* H4-14 (Meijer et al. 1990b, c), as reported for *Rhodobacter sphaeroides* (Gibson and Tabita 1988) and *Alcaligenes eutrophus* (Husemann et al. 1988).

The aim of the present work was to study the regulation of C_1-metabolism in *Xanthobacter* strain 25a at the physiological level. Further work, focussing on the cloning and characterization of genes encoding Calvin cycle enzymes, will be published elsewhere (Meijer et al. 1990b, c).

Materials and methods

Microorganism and cultivation

Xanthobacter strain 25a, its maintenance, the mineral salts media used for batch and continuous cultures, the procedures followed for cultivation on single and mixed substrates in continuous cultures, harvesting of cells and measurements of growth have been described previously (Dijkhuizen and Harder 1979; Meijer et al. 1990a). Filter-sterilized methanol and heat-sterilized acetate were added at concentrations indicated in the individual experiments. The various measurements were performed after the cultures had reached a steady state, which was assumed to be the case after at least 5 volume displacements.

Preparation of cell-free extracts and enzyme assays

The methods used for preparation of cell-free extracts of *Xanthobacter* strain 25a have been described previously (Meijer et al. 1990a). Enzyme measurements were made on a Hitachi model 100-60 spectrophotometer at 30° C. In all assays the observed rate was linear for at least 3 min and proportional to the amount of extract added. The reaction volumes were 1 ml. The following enzymes were assayed according to published methods; Methanol dehydrogenase (MDH; EC 1.1.9.98), Meijer et al. (1990a); Isocitrate dehydrogenase (ICDH; EC 1.1.1.42), Levering and Dijkhuizen (1985); RuBisC/O (EC 4.1.1.39) and NAD-dependent formate dehydrogenase (FDH; EC 1.2.1.2), Dijkhuizen et al. (1978).

Analytical methods

Methanol and acetate were determined gaschromatographically as described by Heijthuijsen and Hansen (1989) and Laanbrock et al. (1982), respectively. Dry weight values were determined with a total carbon analyzer (Beckman model 915A), connected to an infrared analyzer (Beckman model 865). Protein was determined by the method of Lowry.

Results and discussion

Growth on methanol in chemostat culture

Growth of *Xanthobacter* strain 25a in methanol-limited continuous cultures resulted in complete utilization of the substrate at the various dilution rates tested. The

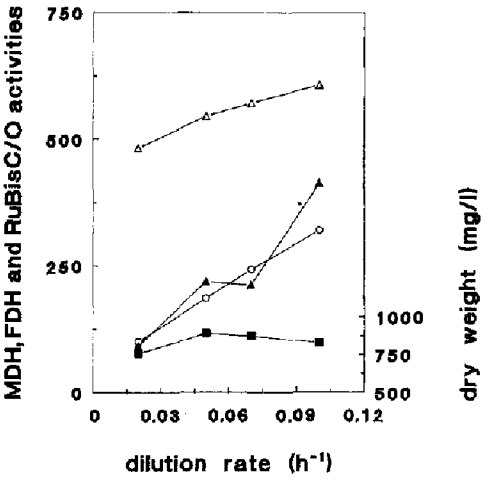

Fig. 1. Effect of increasing dilution rates ($D = 0.02-0.10\ h^{-1}$) of a methanol-limited ($S_R = 100$ mM) continuous culture of *Xanthobacter* strain 25a on a number of steady state culture parameters. ■, Dry weight values; △, MDH; ▲, FDH; ○, RuBisC/O. Enzyme activities are expressed in nmol · min^{-1} · mg^{-1} of protein

relationship between the levels of C_1-enzymes, and the dry weight produced, with dilution rate is shown in Fig. 1. The molar growth yield on methanol was 8.5 g · mol^{-1}, which is considerably lower than the values reported for growth of other gram-negative bacteria on methanol, employing either the ribulose monophosphate (RuMP) cycle (15.7–19.5 g · mol^{-1}), the serine pathway (9.8–14.6 g · mol^{-1}) (Goldberg et al. 1976), or the Calvin cycle (11.0 g · mol^{-1}) (*Paracoccus denitrificans*; van Verseveld and Stouthamer 1978). The specific activities of the dissimilatory enzymes MDH and FDH, and of the Calvin cycle enzyme RuBisC/O, increased with increasing dilution rate (Fig. 1). This response is different from that observed with certain RuMP cycle (Roitsch and Stolp 1985; Greenwood and Jones 1986) or serine pathway (Roitsch and Stolp 1986) methylotrophs, namely a strong increase of MDH activity at the lower dilution rates. The latter organisms, however, synthesize MDH constitutively (although further induction of the enzyme may occur upon addition of methanol to the medium), while in autotrophic bacteria (*Pa. denitrificans* and *Xanthobacter* strains) MDH is induced by methanol (or products derived) and repressed to various degrees by "heterotrophic" substrates (Weaver and Lidstrom 1985; de Vries et al. 1988; this paper).

Growth on acetate and on mixtures of acetate and formate in chemostat culture

No activity of the C_1-enzymes could be detected during growth of *Xanthobacter* strain 25a on acetate alone (molar growth yield 21 g · mol^{-1} at $D = 0.10\ h^{-1}$), whereas the TCA cycle enzyme ICDH was present at a very high specific activity (2.5 µmol · min^{-1} · mg^{-1} protein). Activities of the glyoxylate cycle enzymes could not be detected in cells grown on acetate in batch cultures (Meijer et al. 1990a), or chemostat cultures (this study).

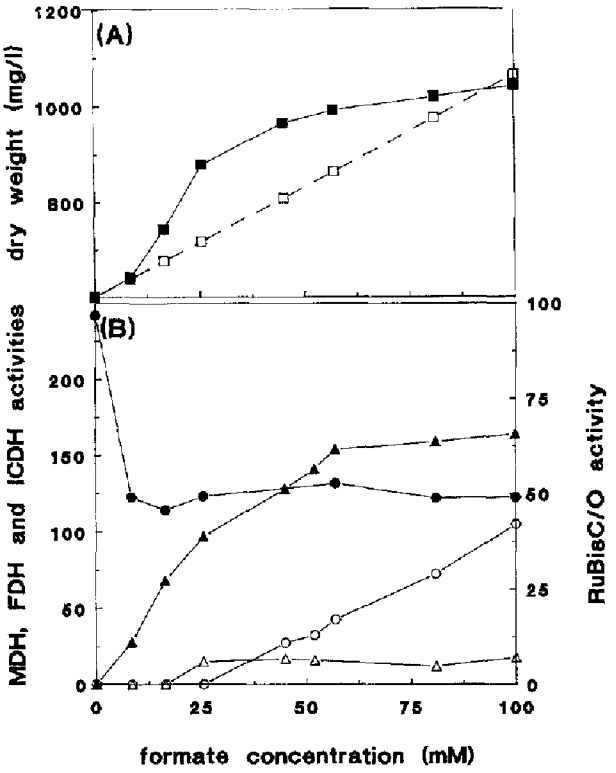

Fig. 2A, B. Effect of stepwise addition of increasing concentrations of formate (0–100 mM) to the feed of an acetate-limited (S_R = 30 mM) continuous culture ($D = 0.10\ h^{-1}$) of *Xanthobacter* strain 25a on a number of steady state culture parameters. A ■, Experimental dry weight values; ∟, sum of the dry weight values obtained during growth on formate and acetate separately. B △, MDH; ▲, FDH; ○, RuBisC/O; ●, ICDH (×0.1). Enzyme activities are expressed in nmol · min^{-1} · mg^{-1} of protein

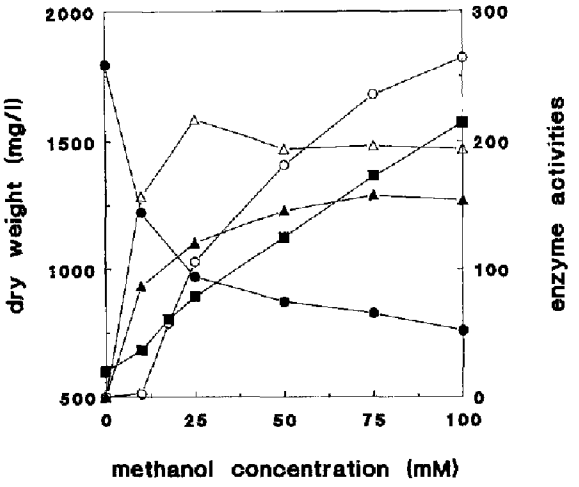

Fig. 3. Effect of stepwise addition of increasing concentrations of methanol (0–100 mM) to the feed of an acetate-limited (S_R = 30 mM) continuous culture ($D = 0.10\ h^{-1}$) of *Xanthobacter* strain 25a on a number of steady state culture parameters. ■, Dry weight values; △, MDH (×0.4); ▲, FDH; ○, RuBisC/O (×2); ●, ICDH (×0.1). Enzyme activities are expressed in nmol · min^{-1} · mg^{-1} of protein

Addition of formate (0–100 mM) to the feed of an acetate-limited continuous culture ($D = 0.10\ h^{-1}$) resulted in simultaneous and complete utilization of the two substrates (Fig. 2). FDH activity appeared immediately, whereas ICDH activity initially decreased rapidly, reflecting a decreased energy generation via the TCA cycle. MDH activities remained very low (< 20 nmol · min^{-1} · mg^{-1} protein), indicating that synthesis of MDH and FDH in *Xanthobacter* strain 25a is not controlled coordinately. Activity of RuBisC/O was detected only at formate concentrations of 45 mM and above. Once these activities appeared, they increased with increasing formate concentrations (Fig. 2B). Thus, at formate concentrations below 45 mM, where RuBisC/O remained repressed, formate only served as an additional energy source. This resulted in a 60% increase of the cultural dry weight, to a 20% higher level than expected on the basis of the molar growth yields on the separate substrates (Fig. 2A; the molar growth yield on formate alone under the same experimental conditions is 4.5 g · mol^{-1}). Since acetate was the only available carbon source, acetate carbon must have been redistributed over the dissimilatory and assimilatory pathways, i.e. more acetate was assimilated and less was dissimilated under these conditions. This redistribution of acetate carbon as the formate concentration was increased is reflected in the strong decrease in specific activity of ICDH. At formate concentrations above 45 mM the increase in dry weight per mmole of formate added dropped, reflecting an increasing contribution of the energetically expensive autotrophic CO_2 fixation to the biosynthesis of cell material.

Growth on mixtures of acetate and methanol in chemostat culture

Addition of methanol (0–100 mM) to the feed of an acetate-limited continuous culture resulted in simultaneous and complete utilization of the two substrates. MDH and FDH activities appeared immediately (Fig. 3), reflecting the inducible nature of these enzymes. A very low activity of RuBisC/O was observed with 10 mM of methanol in the feed. Only when supplying methanol at concentrations of 20 mM, or above, the activity of this enzyme became clearly apparent and gradually increased. Increasing concentrations of methanol in the feed resulted in an almost linear increase in the dry weight produced, reaching values approximately equal to those expected on the basis of the molar growth yields on the single substrates, i.e. growth yields were additive. Carbon assimilation from acetate and methanol (CO_2) apparently proceeds independently from each other during growth in chemostat cultures.

The data (Figs. 2 and 3) thus indicate that the response observed in acetate-limited continuous cultures depends on the energy contents of the second substrate. The additional energy generated by oxidation of methanol, compared to formate oxidation, resulted in derepression of RuBisC/O synthesis at relatively low concentrations of methanol in the feed.

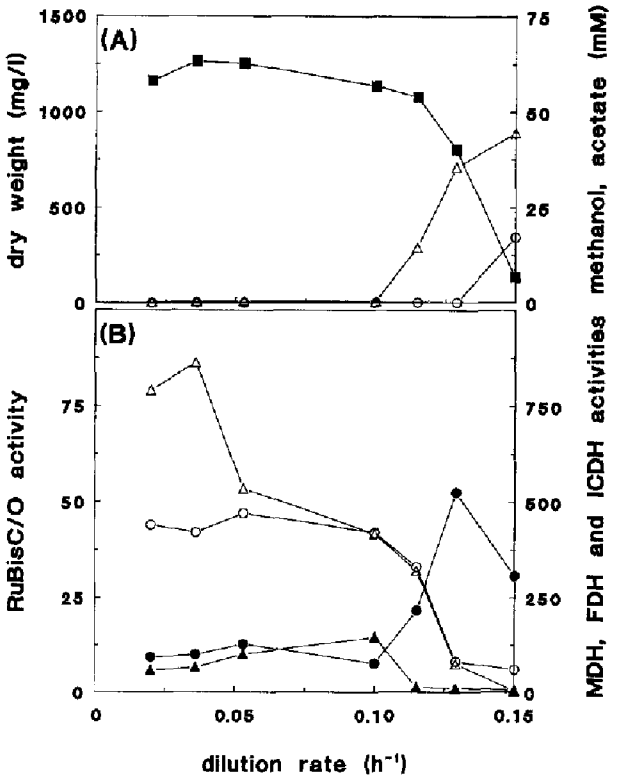

Fig. 4A, B. Effect of increasing dilution rates ($D = 0.02-0.15\ h^{-1}$) of an acetate- ($S_R = 30$ mM) and methanol- ($S_R = 50$ mM) limited continuous culture of *Xanthobacter* strain 25a on a number of steady state culture parameters. **A** ■, Dry weight values; △, ○ residual methanol and acetate concentrations, respectively. **B** △, MDH; ▲, FDH; ○, RuBisC/O; ●, ICDH (×0.1). Enzyme activities are expressed in nmol · min^{-1} · mg^{-1} of protein

Growth on a constant mixture of acetate and methanol in chemostat culture at varying dilution rates

Growth of *Xanthobacter* strain 25a on a constant mixture of acetate ($S_R = 30$ mM) and methanol ($S_R = 100$ mM) in chemostat cultures (Fig. 4A) resulted in complete utilization of both substrates upto a dilution rate of 0.10 h^{-1}. At higher D values residual methanol rapidly accumulated, indicating a switch-off of methanol metabolism at a dilution rate below the μ_{max} on methanol alone (0.15 h^{-1}). Acetate accumulation occurred at a D value of 0.15 h^{-1}, again clearly below the μ_{max} on acetate alone (0.29 h^{-1}), resulting in wash-out of the culture. The relationship between enzyme activity and dilution rate is shown in Fig. 4B. Very high activity levels of MDH were observed under these conditions. RuBisC/O, MDH and FDH activities decreased dramatically above D = 0.10 h^{-1}, rapidly dropping to (almost) zero levels. The switch-off of methanol utilization with increasing dilution rates thus appears to be caused by repressive effects exerted by the "heterotrophic" substrate acetate on these C_1-enzymes (Fig. 4A). Toxic effects of the accumulated methanol probably inhibits growth on acetate, resulting in wash-out of the culture at a D value clearly below the maximal growth rate on acetate.

The results of this study show that the enzymes involved in methanol oxidation and CO_2 fixation in *Xanthobacter* strain 25a are regulated in a different manner. Synthesis of MDH and FDH is controlled via induction by methanol and formate, respectively, and via repression by "heterotrophic" substrates such as acetate. RuBisC/O synthesis appears to be regulated via a (de)repression mechanism and only occurs under conditions with, (a) a diminished supply of alternative carbon sources, and (b) a sufficiently high availability of suitable energy sources. This conclusion is supported by the stronger repression of RuBisC/O synthesis observed in (carbon-excess) batch cultures (Meijer et al. 1990a) compared to (carbon-limited) continuous cultures, and the clearly more pronounced derepressing effect exerted by methanol (compared to formate) addition under the latter growth conditions (this study). As proposed previously for other facultatively autotrophic bacteria (Dijkhuizen and Harder 1984), control of the synthesis of this key enzyme of the Calvin cycle in *Xanthobacter* strain 25a thus appears to be comparable to that observed for other biosynthetic pathways, namely feedback repression by endproduct(s) of the pathway. In order to unravel the molecular details of this control system the genes involved will have to be cloned and regulatory proteins identified.

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