Methanol Dissimilation in *Xanthobacter* H4-14: Activities, Induction and Comparison to *Pseudomonas* AM1 and *Paracoccus denitrificans*

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Methanol dissimilatory enzymes detected in the methanol autotroph *Xanthobacter* H4-14 were a typical phenazine methosulphate-linked methanol dehydrogenase, a NAD⁺-linked formate dehydrogenase, and a dye-linked formaldehyde dehydrogenase that could be assayed only by activity stains of polyacrylamide gels. This same methanol dehydrogenase activity was found in ethanol-grown cells and was apparently utilized for ethanol oxidation. Formaldehyde dehydrogenase activities were investigated in *Paracoccus denitrificans*, *Xanthobacter* H4-14, and *Pseudomonas* AM1. *P. denitrificans* contained a previously reported NAD⁺-linked, GSH-dependent activity, but both *Xanthobacter* H4-14 and *Pseudomonas* AM1 contained numerous activities detected by activity stains of polyacrylamide gels. Induction studies showed that in *Xanthobacter* H4-14, a 10 kDa polypeptide, probably a dehydrogenase-associated cytochrome *c*, was co-induced with methanol dehydrogenase, but the formaldehyde and formate dehydrogenases were not co-regulated. Analogous induction experiments revealed similar patterns in *P. denitrificans*, but no evidence for co-regulation of dissimilatory activities in *Pseudomonas* AM1.

INTRODUCTION

In recent years, methylotrophic bacteria have gained the interest of commercial and industrial concerns as potential sources of single cell protein, chemicals and biochemicals (Anthony, 1982). Successful use of these bacteria will most certainly require a detailed knowledge of their physiology, biochemistry and genetics. One aspect of this requirement is an understanding of the regulation exerted upon the genes responsible for the dissimilation and assimilation of one-carbon compounds. Numerous publications have addressed this issue (Cox & Quayle, 1975; Boulton & Large, 1977; O'Connor & Hanson, 1977; O'Connor, 1981; Marison & Attwood, 1982). Those studies showed that in a variety of methylotrophs the C₁-associated enzyme activities are present at higher levels in cells grown on methylotrophic substrates when compared to cells grown on heterotrophic substrates, suggesting that these activities are regulated by induction or derepression. Dunstan *et al.* (1972*b*), O'Connor & Hanson (1977) and McNerney & O'Connor (1980) followed the induction of several C₁-associated activities in four serine pathway methylotrophs (*Pseudomonas* AM1, *Pseudomonas* 3A2, *Methylobacterium organophilum* and *Hyphomicrobium* X) upon transfer from heterotrophic to methylotrophic growth. The results from these studies suggested that co-ordinate regulation of certain serine pathway enzymes exists in some of these methylotrophs but not in others. Comparison of methanol dissimilatory activities was not made. Little specific attention has been focused on the regulation of the components of the methanol dissimilatory pathway in methylotrophs despite the fact that these are the preliminary reactions in methylotrophic metabolism and include important energy-yielding reactions.

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Abbreviations: ADH, aldehyde dehydrogenase; DCPIP, dichlorophenolindolephenol; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase; MeDH, methanol dehydrogenase; NBT, nitro blue tetrazolium; PMS, phenazine methosulphate.

Fig. 1. The methanol dissimilatory pathway of the methylotrophs used in this study. PQQ, pyrryloquinoline quinone; cyt. c, cytochrome c. For *Xanthobacter* H4-14, the PQQ and cyt. c have not yet been proven to be present. 1, PMS-linked methanol dehydrogenase. 2, Formaldehyde dehydrogenase: (a) *Paracoccus denitrificans* (NAD⁺-linked, GSH-dependent); (b) *Pseudomonas* AM1, *Xanthobacter* H4-14 (identity unknown). 3, Formate dehydrogenase.

Xanthobacter is a recently recognized genus consisting of facultatively chemolithotrophic bacteria, most of which are able to grow on methanol (Wiegel *et al.*, 1978). *Xanthobacter* strain H4-14 has been described by Lidstrom-O'Connor *et al.* (1983) and grows on methanol by oxidizing it to CO_2 (Fig. 1) and incorporating CO_2 via the Calvin-Benson cycle. Thus, this *Xanthobacter* strain, and presumably the others, belongs to a class of methylotrophs termed 'methanol autotrophs'. A number of diverse autotrophic species able to obtain energy from the oxidation of methanol to carbon dioxide have been described (Anthony, 1982; Kelly & Wood, 1982; Loginova & Trotsenko, 1979). Autotrophic growth on methanol is energetically the least efficient of the established methylotrophic pathways (Anthony, 1982). However, it has been suggested that the acquisition by autotrophic bacteria of enzymes capable of converting methanol to carbon dioxide (Fig. 1) is a greater gain in nutritional flexibility than it is a tax on genetics and biochemistry (Sahm *et al.*, 1976).

Methanol dissimilatory activities have been studied in the serine pathway methylotroph *Pseudomonas* AM1 and the autotrophic methylotroph *Paracoccus denitrificans*. *P. denitrificans* contains methanol-inducible activities of a phenazine methosulphate (PMS)-linked methanol dehydrogenase (MeDH), a NAD⁺-linked, GSH-dependent formaldehyde dehydrogenase (FADH) and a NAD⁺-linked formate dehydrogenase (FDH, Fig. 1). In addition, a low level constitutive dye-linked FADH has been detected (Marison & Attwood, 1980). In *Pseudomonas* AM1 the pathway is less clear: it contains methanol-inducible PMS-linked MeDH and NAD⁺-linked FDH activities, but the physiologically significant FADH is uncertain (Fig. 1). A dye-linked FADH is present at low activities (Johnson & Quayle, 1964) but cannot be correlated with methanol growth (Marison & Attwood, 1980). Formaldehyde oxidation in *Pseudomonas* AM1 may be effected via tetrahydrofolate intermediates (Marison & Attwood, 1982; Attwood & Quayle, 1984) but conclusive evidence is still lacking. In addition to the activities noted above, an NAD⁺-linked activity has been reported (M. M. Attwood, 4th International C-1 Symposium, Minneapolis, 1983). It is not yet known which of these activities is the physiologically significant enzyme.

In this communication we report a study of the activities involved in methanol dissimilation in *Xanthobacter* H4-14, a new methanol autotroph, *Pseudomonas* AM1, a serine pathway methylotroph, and *P. denitrificans*, a well studied methanol autotroph. As a prelude to genetic studies, we have examined the pattern of induction of these activities by methanol. Comparison of enzymology and induction patterns has revealed significant differences not only between *Xanthobacter* H4-14 and the serine pathway methylotroph but also between the two methanol autotrophs.

METHODS

Organisms and growth conditions. A streptomycin-resistant, rifampicin-resistant, capsule-less derivative of *Xanthobacter* strain H4-14 (Lidstrom-O'Connor *et al.*, 1983) was used in this study. *Pseudomonas* AM1 (NCIB 9133) and *Paracoccus denitrificans* (NCIB 8944) were obtained from J. R. Quayle, University of Bath, UK.

Bacterial strains were routinely grown on nutrient broth or nutrient agar supplemented with streptomycin sulphate ($20\text{ }\mu\text{g ml}^{-1}$) or rifampicin ($20\text{ }\mu\text{g ml}^{-1}$) when required for *Xanthobacter* H4-14. Cells for biochemical studies were grown in the minimal salts medium of Harder *et al.* (1973). Minimal medium for growth of *Xanthobacter* H4-14 was supplemented with d-biotin ($0.2\text{ }\mu\text{g ml}^{-1}$) and the oxygen tension was lowered by incubation under an atmosphere of 75% (v/v) helium and 25% (v/v) air. Minimal medium for growth of *P. denitrificans* was supplemented with 0.01% (w/v) yeast extract (Cox & Quayle, 1975). For autotrophic growth on methanol, formate or H_2/CO_2 , the medium was further supplemented with 0.02% (w/v) KHCO_3 . For growth on H_2/CO_2 , $8\text{ }\mu\text{M-NiCl}_2$ was added and the cultures were incubated under an atmosphere of 75% (v/v) H_2/CO_2 mixture (6 H_2 :1 CO_2) and 25% air. Sodium formate or heterotrophic carbon sources were added to 0.1% (w/v) and methanol was added to 0.2% (v/v). Small batch cultures were incubated on a gyratory shaker at 200 r.p.m. and $30\text{ }^\circ\text{C}$.

The larger scale induction experiments were done in an 18 l New Brunswick Microferm fermenter containing 12 l of medium at $30\text{ }^\circ\text{C}$. The inocula were concentrates from 1.2 l of cells grown to mid-exponential phase on H_2/CO_2 (*Xanthobacter* H4-14 and *P. denitrificans*) or succinate (*Pseudomonas* AM1) that had been harvested and washed twice with unsupplemented minimal medium. Aeration was maintained at approximately 50 ml min^{-1} for *Xanthobacter* H4-14 or 200 ml min^{-1} for *Pseudomonas* AM1 and *P. denitrificans*. Stirring was maintained at approximately 100 r.p.m. For experiments with *Xanthobacter* H4-14, the initial oxygen tension was lowered by sparging the fermenter with helium (approximately 500 ml min^{-1}) for 10 min with vigorous stirring before inoculation.

Electrophoresis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by minor modifications of the method of Laemmli (1970) as detailed by Weaver *et al.* (1981) except that a uniform acrylamide concentration of 15% (w/v) was used and $45\text{ }\mu\text{g}$ extract protein were applied per lane. Polypeptides were stained with Coomassie Brilliant Blue R-250. Electrophoresis under non-denaturing conditions (PAGE) was as described above with the following exceptions: (1) the gels were 1.5 mm thick; (2) SDS was omitted; (3) the separating gel was 6% (w/v) acrylamide; (4) the stacking gel was 3% (w/v) acrylamide and contained the same buffer system as the separating gel; (5) samples ($200\text{ }\mu\text{g}$ extract protein) were processed by adding a half volume of a loading solution containing 40% (w/v) sucrose plus a trace of bromophenol blue and were not heated; (6) electrophoresis was done at 25 mA at $4\text{ }^\circ\text{C}$.

Densitometric scanning of dried acrylamide gels was done with an LKB densitometer (model 2202 Ultroskan) attached to a recording integrator (model 2220; LKB). Conditions for densitometry were optimized for individual polypeptides or activities so that 'densitometer units' are not comparable between different proteins.

Cell extracts and enzyme assays. Cells were harvested (17000 g , 10 min, $4\text{ }^\circ\text{C}$), washed twice in a half volume of minimal medium, resuspended in a small volume of medium, frozen in a dry ice/ethanol bath and stored at $-70\text{ }^\circ\text{C}$ until needed. After the cells were thawed, deoxyribonuclease I was added to $25\text{ }\mu\text{g ml}^{-1}$ and the cells were disrupted by two passes through a French pressure cell at 20000 lbf in^{-2} (138 MPa). The crude extract was taken as the supernatant fraction after centrifugation at 12000 g for 10 min. Extracts were stored at $-70\text{ }^\circ\text{C}$ after being frozen drop-wise in liquid nitrogen. Protein was determined by the Lowry method with bovine gamma globulin as a standard.

Methanol dehydrogenase (EC 1.1.99.8; MeDH) was assayed by the method of Anthony & Zatman (1964) with the following concentration changes: 125 mM-Tris/HCl (pH 9.0), 50 mM- NH_4Cl , 1 mM-KCN, 2 mM-PMS, 0.1 mM-dichlorophenolindolephenol (DCPIP) and 10 mM-methanol.

Formate dehydrogenase (EC 1.2.1.2; FDH) in extracts of *Xanthobacter* H4-14 and *Pseudomonas* AM1 was assayed by the method of Johnson & Quayle (1964) with the following changes: 50 mM-potassium phosphate (pH 7.0), 0.5 mM- NAD^+ and 10 mM-sodium formate. For extracts of *P. denitrificans*, 40 mM-Tris/HCl (pH 8.4) replaced the phosphate buffer.

Dye-linked formaldehyde dehydrogenase (FADH) in *Pseudomonas* AM1 was measured by the method of Johnson & Quayle (1964) with changes as follows: 50 mM-potassium phosphate (pH 7.0), 0.1 mM-DCPIP, 0.1 mM-KCN (to inhibit FDH activity) and 10 mM-formaldehyde. NAD^+ -linked FADH (EC 1.2.1.1) from *P. denitrificans* was measured as described by Johnson & Quayle (1964) with the following changes: 50 mM-potassium phosphate (pH 8.0), 0.5 mM- NAD^+ , 2.5 mM-reduced glutathione (GSH) and 10 mM-formaldehyde. The assay of FADH in *Xanthobacter* H4-14 is described in Results.

Methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) was determined as described by Marison & Attwood (1982).

The activity of MeDH, FADH, aldehyde dehydrogenase (ADH) and FDH in PAGE gels ('zymograms') was detected as described by Urakami & Komagata (1981) using nitro blue tetrazolium (NBT) except that 50 mM-Tris/HCl (pH 9.0) replaced the glycine buffer for MeDH detection. All substrates (methanol, formaldehyde, acetaldehyde, propionaldehyde, dodecyl aldehyde or formate) were added at 10 mM. For detection of FADH or ADH activities, PMS was not required but was occasionally included in order to enhance stain development. When PMS was included, MeDH could be detected, presumably due to the initial high pH in the gel, remaining traces of ammonium ions and the fact that formaldehyde is a substrate for this enzyme *in vitro*.

Chemicals. Unless otherwise stated, all reagents were standard reagent grade. The following were obtained from Sigma: PMS, DCPIP, NBT, NAD⁺, paraformaldehyde and protein molecular weight standards for some experiments. Bovine gamma globulin, Coomassie Brilliant Blue and protein molecular weight standards for other experiments were obtained from Bio-Rad. Formaldehyde was prepared by autoclaving [2 h, 121 °C, 15 lbf in⁻² (103.5 kPa)] solutions of paraformaldehyde in a sealed vial after sparging of the suspension with helium for 10 min.

RESULTS

Characteristics of methanol dissimilatory activities in Xanthobacter H4-14

Methanol dehydrogenase. In *Xanthobacter* H4-14 extracts, MeDH activity showed an absolute requirement for high pH (optimum pH 9.0), ammonium ions and the dye PMS. Small primary alcohols and formaldehyde were preferred substrates (Table 1). The molecular mass of the native enzyme was approximately 125 kDal, as determined by activity stains of PAGE gels (Table 2). *Xanthobacter* H4-14 grown on ethanol also possessed high levels of MeDH activity (Table 1). In addition, dye-linked 'ethanol dehydrogenase' from these cells co-migrated with MeDH on PAGE gels (data not shown). No NAD⁺ or NADP⁺-linked alcohol dehydrogenase activity could be detected in ethanol-grown *Xanthobacter* H4-14 when assayed over a pH range of 7.0 to 9.0 using phosphate or Tris buffers, 0.5 mM-pyridine nucleotide and 10 mM-ethanol.

Table 1. *Methanol dehydrogenase activity with different alcohols in cell-free extracts of Xanthobacter H4-14 grown on methanol or ethanol*

Substrate	Percentage of activity with methanol*	
	Methanol-grown	Ethanol-grown
Methanol	100	100
Ethanol	91	94
<i>n</i> -Propanol	63	74
<i>i</i> -Propanol	0	0
<i>n</i> -Butanol	47	62
<i>i</i> -Butanol	0	0
Ethylene glycol	0	0
Glycerol	0	0
Formaldehyde	100	ND

* 100% specific activity with methanol was 440 and 200 nmol min⁻¹ mg protein⁻¹ for methanol- and ethanol-grown cells, respectively; ND, not done.

Table 2. *Characteristics of the methanol dissimilatory enzymes in Xanthobacter H4-14*

Activities were measured in cell-free extracts except for formaldehyde dehydrogenase, which could only be detected by activity stains on polyacrylamide gels. Approximate sizes were determined by activity stains on a series of polyacrylamide gels of differing acrylamide concentrations, as compared to molecular weight standards.

Characteristic	Methanol dehydrogenase	Formaldehyde dehydrogenase*	Formate dehydrogenase
Approx. size of native enzyme (kDal)	125	270	105
Requirements for activity	pH 9.0, NH ₄ ⁺	pH 7.0	pH 7.0
Inhibition by KCN and NaN ₃	—	—	+
Electron acceptors	PMS	PMS, NBT	NAD ⁺ , DCPIP, NBT
Specific activity (nmol min ⁻¹ mg protein ⁻¹) in cells grown on:			
methanol	450	+	450
ethanol	250	+	0
formate	45	+	1050
H ₂ /CO ₂	7.5	+	0
succinate or citrate	1	+	0

* Dye-linked, NH₄⁺-independent.

Presumably, *Xanthobacter* H4-14 uses the dye-linked MeDH for the initial oxidation of ethanol when growing on this substrate. This same result has been reported for ethanol-grown *Pseudomonas* AM1 (Dunstan *et al.*, 1972a). MeDH activities were present in significant amounts only in cells grown on methanol or ethanol. Lower levels of activity were found in cells grown on formate, H_2/CO_2 and heterotrophic substrates (Table 2).

Formaldehyde dehydrogenase. Three apparent FADH activities were detected in *Xanthobacter* H4-14. A PMS-linked, NH_4^+ -dependent activity that co-migrated with MeDH activity on zymograms (activity-stained PAGE gels) was detected in cell extracts. In addition, a trace amount of apparent NAD^+ -linked FADH activity was detected in extracts, but this activity could not be clearly distinguished from FDH activity. This low level of activity was found in formate-grown cells as well as methanol-grown cells at a relatively constant fraction of FDH activity. ' NAD^+ -linked FADH' and FDH activities showed nearly identical induction patterns (not shown). These activities responded similarly to enzyme inhibitors and stimulators (see below), showed similar heat inactivation parameters and co-sedimented on sucrose step gradients (not shown). Furthermore, *Xanthobacter* FDH and commercially available *Pseudomonas oxalaticus* FDH showed low levels of reactivity on zymograms with all formaldehyde preparations tested. We concluded that the trace levels of NAD^+ -linked FADH activity in *Xanthobacter* H4-14 were due either to small amounts of formate in the formaldehyde preparations or low levels of formaldehyde utilization by the FDHs.

Except for the activity attributable to FDH, no formaldehyde-dependent, NH_4^+ -independent reduction of PMS, NBT or DCPIP could be convincingly demonstrated in crude extracts of *Xanthobacter* H4-14 by spectrophotometric procedures and with assay pH values ranging from 6.5 to 9.0. Further attempts were made to detect this activity with fractionated extracts. Membrane preparations obtained by centrifugation of crude extracts at 50000 *g* for 1 h contained no detectable activity in either particulate or soluble fractions. In addition, batch-wise DEAE-cellulose fractions of crude extracts contained no detectable activity. However, it was possible to detect a third FADH activity distinct from MeDH and FDH (Table 2) by activity stains of PAGE gels. This activity was stimulated by but did not require PMS, was unaffected by reduced glutathione (5 mM), was approximately 270 kDa in size, and was most reactive towards longer-chain aldehydes (acetaldehyde, propionaldehyde and dodecylaldehyde). This activity was not specifically associated with methanol-grown *Xanthobacter* H4-14, being found at similar levels under all growth regimens examined (Table 2). Its possible role in methanol dissimilation is discussed later. In addition, an 'aldehyde dehydrogenase' (ADH) activity was detected on PAGE gels that reacted with longer-chain aldehydes but not with formaldehyde. This enzyme was approximately 200 kDa in size and was distinct from the dye-linked FADH as well as the other FADH activities mentioned above.

Formate dehydrogenase. Oxidation of formate by *Xanthobacter* H4-14 was accomplished by a NAD^+ -utilizing FDH that was highly specific for formate and tightly regulated. FDH was detected only in extracts from methanol- and formate-grown cells (Table 2). DCPIP and NBT also served as electron acceptors. The activity in crude cell extracts was sensitive to cyanide (7% activity at 0.5 mM-KCN) and azide (25% activity at 0.5 mM- NaN_3) but insensitive to 0.05 mM-dipyridyl, 0.4 mM- $CuSO_4$ and 0.05 mM- $FeSO_4$. Thiol reagents (2-mercaptoethanol, DTT and GSH) were not required for activity or short term stability, but these chemicals typically stimulated FDH activity by 25–50%. Analysis of zymograms revealed a size of approximately 105 kDa for the FDH activity.

Induction of methanol dissimilatory activities in Xanthobacter H4-14

Figs 2 and 3 show the pattern of cell growth, polypeptides and enzyme induction when hydrogen-grown *Xanthobacter* cells were diluted and exposed to methanol. Data for the FADH and ADH activities and two induced polypeptides (Fig. 3*b, c*) were taken from the PAGE zymograms and SDS-PAGE analysis shown in Fig. 2. The levels of MeDH and FDH were initially low or undetectable in hydrogen-grown cells and then were seen to increase dramatically. These activities peaked in the mid-exponential phase of growth, albeit at different times, and then declined precipitously as the culture neared the end of its growth phase. Both the

Fig. 2. Protein and activity stains of PAGE gels of *Xanthobacter* H4-14 samples after transfer from H_2/CO_2 to methanol. (a) Protein stain of SDS-PAGE gel; the positions of the standards (S, kDal) are shown on the left. The positions of the major inducible polypeptides (kDal) are shown on the left. (b) Activity stain of a native PAGE gel using formaldehyde as the substrate (no PMS added). (c) Activity stain of a native PAGE gel using propionaldehyde as the substrate (no PMS added). The positions of ADH and FADH are shown. The time-points of samples are h after transfer to methanol.

Fig. 3. Profiles of growth and C₁-associated activities and polypeptides in *Xanthobacter* H4-14 from the induction experiment shown in Fig. 2. (a) Spectrophotometrically determined activities of methanol dehydrogenase (●) and formate dehydrogenase (■) and absorbance at 600 nm (□). (b) Values derived from densitometer scans of PAGE gels stained for activity of formaldehyde dehydrogenase (▲) and aldehyde dehydrogenase (△). (c) Values derived from densitometer scans of SDS-PAGE protein gels: ▼, 10 kDa polypeptide; ▽, 22 kDa polypeptide.

FADH and ADH activities were initially high in hydrogen-grown cells but decreased significantly upon exposure of the cells to methanol. Both activities subsequently increased as the culture began to grow on methanol, but only the increase in FADH activity approximated the increases in cell density and MeDH and FDH activities.

Marison & Attwood (1982) suggested that oxidation of formaldehyde could involve tetrahydrofolate derivatives. Levels of methylenetetrahydrofolate dehydrogenase, a key enzyme in the proposed pathway, decreased slightly during the course of this induction experiment (data not shown). It is unlikely, therefore, that a tetrahydrofolate-dependent formaldehyde oxidation pathway contributes significantly to methanol dissimilation in *Xanthobacter* H4-14.

SDS-PAGE analysis of the crude extracts revealed three polypeptides (60, 22 and 10 kDa) that clearly increased in amount as the cells adapted to growth on methanol (Fig. 2a). In other methylotrophs, MeDH consists of a dimer of a 60–70 kDa polypeptide, and it is commonly seen as a major protein in methanol-grown cells (Anthony, 1982). Therefore, the prominent 60 kDa band in Fig. 2(a) may represent the subunit of MeDH. The increase in the amount of this polypeptide approximately paralleled MeDH activity for the first 80 h, but the decrease in MeDH activity observed after 80 h was not reflected in a concomitant decrease in the intensity of this band. These results suggest the decrease in MeDH activity was due to post-translational events.

The induction patterns of the 22 and 10 kDa polypeptides (Figs 2a and 3c) were roughly similar to that of the MeDH polypeptide. These two smaller polypeptides are similar in size to the two soluble *c*-type cytochromes associated with MeDH activity in *Pseudomonas* AM1 and *Methylophilus methylotrophus* (O'Keeffe & Anthony, 1980; Beardmore-Gray *et al.*, 1982). In *Pseudomonas* AM1, the larger cytochrome *c* is thought to accept electrons directly from MeDH, but the role of the smaller cytochrome is unclear (Beardmore-Gray *et al.*, 1983). However, the 22 kDa polypeptide of *Xanthobacter* H4-14 was not a prominent component of ethanol-grown cells which would be expected to contain the full complement of MeDH-associated polypeptides (data not shown). The 10 kDa polypeptide was present in these cells. Thus, it was not clear whether these polypeptides were MeDH-associated cytochromes.

Table 3. *Aldehyde dehydrogenase activities detected in three methylotrophs*

Aldehyde dehydrogenase	<i>Xanthobacter</i> H4-14	<i>Pseudomonas</i> AM1	<i>Paracoccus</i> <i>denitrificans</i>
NAD ⁺ -linked, GSH-dependent*	—	—	+
NAD ⁺ -linked, GSH-independent*	—†	—†	—†
Dye-linked, NH ₄ ⁺ -dependent (MeDH)*†	+	+	+
Dye-linked, NH ₄ ⁺ -independent formaldehyde activity (FADH)†	+	FADH-1 + FADH-2 +	NT
Little or no activity with formaldehyde (ADH)	+	ADH-1 + ADH-2 +	NT

NT, Not tested.

* Assayed in cell-free extracts.

† Detected by activity stains of polyacrylamide gels.

‡ Low levels were detected, but were apparently due to traces of formate in the formaldehyde preparations.

Aldehyde dehydrogenases in Pseudomonas AM1

The ADHs detected in *Pseudomonas* AM1 are summarized in Table 3. The dye-linked FADH activity reported by Marison & Attwood (1980) was detected in low levels in cell extracts. We were unable to detect the NAD⁺-linked FADH reported by M. M. Attwood at the 4th C-1 Symposium, Minneapolis, 1983, over a pH range of 6.5–9.0. The reason for our inability to detect this activity is unknown, but may be due to differences in methods of cell breakage or growth conditions. A low level NAD⁺-linked activity was detected but, as was the case with *Xanthobacter* H4-14, this activity could not be distinguished from the FDH and may have been due to traces of formate in our formaldehyde preparations. However, development of zymograms with formaldehyde or propionaldehyde revealed five proteins with strong aldehyde-reducing activities and numerous proteins with minor activities (Fig. 4*b, c*). Characteristics of the ADH activities detected are listed in Table 3. A protein that co-migrated with MeDH activity was clearly the most prominent ADH in crude extracts, but the physiological role of MeDH in formaldehyde oxidation is probably not significant (Marison & Attwood, 1982; Attwood & Quayle, 1984). Two proteins, designated FADH-1 and FADH-2, showed significant activity with formaldehyde. These and ADH-2 showed strong activity with propionaldehyde. An additional protein, ADH-1, showed weaker propionaldehyde dehydrogenase activity. Based on relative staining intensities, FADH-2 was the most specific for formaldehyde.

Induction in Pseudomonas AM1

Analysis of changes in the methanol dissimilatory enzymes and in total polypeptides as *Pseudomonas* AM1 was switched from growth on succinate to growth on methanol is presented in Figs 4 and 5. MeDH activity increased about sixfold during the course of the experiment, and the 60 kDa MeDH polypeptide increased to a high level. Enzymically-measured FADH activity decreased with growth on methanol. This finding is consistent with a previous suggestion that this activity may not be involved in methanol dissimilation (Marison & Attwood, 1982). Fig. 5 includes induction patterns generated from densitometric scanning of the FADH-1 and FADH-2 bands. Except for early time-points, the FADH-2 pattern was similar to the pattern of FADH activity measured spectrophotometrically. The pattern of initial activity increase followed by a slight decrease to a stable level seen with FADH-1 was the type of change expected for an enzyme involved in methanol dissimilation.

FDH activity increased from an undetectable level in succinate-grown *Pseudomonas* AM1 to a broad peak in the early to mid-exponential phase of growth followed by a slow decrease in activity. The initial rate of increase of FDH activity was similar to that for MeDH activity.

Examination of the *Pseudomonas* AM1 extracts by SDS-PAGE clearly showed three inducible polypeptides (Fig. 4*a*). These were probably the MeDH subunits (60 kDa), cytochrome *c*_L (20 kDa) and cytochrome *c*_H (10 kDa), as described by O'Keeffe and Anthony (1980).

Fig. 4. Protein and activity stains of PAGE gels of *Pseudomonas* AM1 after transfer from succinate to methanol. (a) Protein stain of SDS-PAGE gel; the positions of the standards (S, kDa) are shown on the left. The positions of the major inducible polypeptides (kDa) are arrowed. (b) Activity stain of a native 6% PAGE gel using formaldehyde as the substrate (PMS added). (c) Activity stain of a native 6% gel using propionaldehyde as the substrate (PMS added). The positions of the major aldehyde dehydrogenases are shown. The time-points of samples are h after transfer to methanol.

Fig. 5. Profiles of growth and C₁-associated activities and polypeptides in *Pseudomonas* AM1 from the induction experiment shown in Fig. 4. (a) Spectrophotometrically determined activities of methanol dehydrogenase (●), formaldehyde dehydrogenase (X10) (○) and formate dehydrogenase (■) and absorbance at 600 nm (□). (b) Values derived from densitometer scans of PAGE gels stained for activity of formaldehyde dehydrogenases: ▲, FADH-1; △, FADH-2. (c) Values derived from densitometer scans of SDS-PAGE protein gels: ▼, 10 kDa polypeptide; ▽, 20 kDa polypeptide.

Quantification of the cytochrome *c* bands is shown in Fig. 5(c): the amount of cytochrome *c*_H increased steadily with time. The amount of cytochrome *c*_L, which is thought to be most closely associated with MeDH (Beardmore-Gray *et al.*, 1983), increased from a relatively high background level only after a lag of approximately 10 h. This pattern was distinctly different from the pattern of MeDH induction in *Pseudomonas* AM1. Two minor polypeptides of approximately 44 and 30 kDa increased in intensity with growth on methanol, but their identity is unknown.

Induction in *Paracoccus denitrificans*

The induction of the methanol dissimilatory activities and related polypeptides in hydrogen-grown *P. denitrificans* is shown in Figs 6 and 7. Interpretation of these data was made difficult since the initial increase in cell density was apparently due to growth on the trace amount (0.01%) of yeast extract in the medium. Omission of this supplement resulted in long and unpredictable lag times for growth on methanol.

MeDH activity increased upon initial exposure to methanol from an undetectable level in hydrogen-grown *P. denitrificans* to a low level as the cells grew on the yeast extract. After about 30 h, growth on methanol began and MeDH activity increased to a maximal level during late exponential growth and then decreased as growth ceased. FADH activity increased immediately upon exposure of the cells to methanol and continued to increase during growth on methanol, reaching a peak during late exponential growth. This activity also decreased toward the latter stages of the growth cycle. Since *P. denitrificans* possesses a well defined formaldehyde-oxidizing activity, no zymograms were made of the samples from this induction experiment. The pattern of FDH activity was very similar to that of MeDH activity except for the initial period of MeDH increase which was lacking in the FDH pattern.

SDS-PAGE analysis of the *P. denitrificans* extracts clearly revealed the induction of five polypeptides of approximately 64, 44, 22, 20 and 14 kDa (Fig. 6). The strongly induced 64 kDa polypeptide may be the MeDH subunit. However, Bamforth & Quayle (1978) reported a subunit size of 76 kDa for MeDH purified from this same strain of *P. denitrificans*. The reason for this

Fig. 6. Protein SDS-PAGE patterns of *Paracoccus denitrificans* samples after transfer from H_2/CO_2 to methanol. The positions of the standards (S, kDa) are shown on the left and those of the major inducible polypeptides (kDa) are arrowed. The time-points of samples are h after transfer to methanol.

Fig. 7. Profiles of growth and C_1 -associated activities and polypeptides in *Paracoccus denitrificans* from the induction experiment shown in Fig. 6. (a) Spectrophotometrically determined activities of methanol dehydrogenase (●), formaldehyde dehydrogenase (○) and formate dehydrogenase (X10) (■) and absorbance at 600 nm (□). (b) Values derived from densitometer scans of SDS-PAGE protein gels: ▼, 14 kDa polypeptide; ▽, 20 kDa polypeptide.

size discrepancy is unknown, but it may have been due to the difference in the methods used. By analogy to other methylotrophs, two of the three smaller induced polypeptides (22, 20 and 14 kDa) probably represented methanol-specific *c*-type cytochromes. Beardmore-Gray *et al.* (1983) purified two cytochromes *c* from *P. denitrificans* and showed that one reacted specifically with MeDH. Moreover, Van Verseveld & Stouthamer (1978) identified a methanol-inducible cytochrome *c* from spectral data. However, molecular sizes were not determined. The induction pattern of the 20 kDa polypeptide, and to a lesser extent that of the 14 kDa polypeptide, was similar to the pattern of MeDH activity (Fig. 7). The identities of the other methanol-inducible polypeptides are unknown.

DISCUSSION

The facultative chemolithotroph *Xanthobacter* H4-14 was shown to contain three methanol dissimilatory activities: methanol dehydrogenase (MeDH), formaldehyde dehydrogenase (FADH) and formate dehydrogenase (FDH). The MeDH was similar to most other such enzymes in methylotrophs in terms of assay requirements, molecular size, and apparent subunit composition. It seems likely that, as in other MeDHs, this enzyme contains the pyrroloquinoline quinone co-factor and passes electrons to a small, soluble *c*-type cytochrome. MeDH was tightly regulated, being present in significant amounts only when required, i.e. during growth on methanol or ethanol. A change in growth substrates from H_2/CO_2 to methanol resulted in a 65-fold increase in MeDH specific activity. The 60 kDa MeDH subunit was the major polypeptide in methanol-grown cells and represented approximately 10% of the soluble protein.

As is the case for many methylotrophic bacteria, the nature of the formaldehyde-oxidizing component of the methanol-dissimilatory pathway of *Xanthobacter* H4-14 is not absolutely clear. Three possibilities were identified: MeDH, FADH and ADH. It is generally assumed that MeDH plays no significant role in formaldehyde oxidation *in vivo*. Evidence includes the fact that MeDH appears to be a periplasmic enzyme that vectorally translocates methanol-derived formaldehyde to the cytoplasm where, it is hypothesized, it must be acted upon by a distinct FADH (Jones *et al.*, 1982; Kasprzak & Steenkamp, 1983). In addition, the high K_m of MeDH for formaldehyde (Marison & Attwood, 1982) and the ability of MeDH mutants to grow on methylamine (Marison & Attwood, 1980) would suggest that it is not a significant FADH *in vivo*. Of the two ADH activities (other than MeDH) detected in zymograms, only one (FADH) was reactive toward formaldehyde. Both FADH and ADH oxidized aliphatic aldehydes and were not uniquely found in methanol-grown cells. Therefore, they may be required for non- C_1 -related reactions. The inability to detect ADH activity spectrophotometrically in crude extracts is curious. It is possible that the gels stabilized activity, or provided a more sensitive indicator of low activities, in the absence of background reactions. Alternatively, the lack of detectable activity may have been due to the presence of inhibitors that were separated from the enzyme upon electrophoresis. It was not possible to determine whether pyridine nucleotides could serve as electron acceptors for the FADH or ADH.

Two pieces of information suggest that the FADH is indeed involved in methanol dissimilation in *Xanthobacter* H4-14. First, the increase in FADH activity during methanol induction reflected cell growth. Second, a regulatory mutant of *Xanthobacter* H4-14 has been isolated that produces little or no FADH in the presence of methanol, and exhibits a methanol-sensitive phenotype probably due to formaldehyde toxicity (C. A. Weaver & M. E. Lidstrom, unpublished results). Now that a gel assay has been developed, it will be possible to purify both FADH and ADH and characterize them more fully.

If FADH is indeed a part of the methanol dissimilatory pathway, then the initial dramatic decrease in activity upon transfer of hydrogen-grown cells to methanol is an unusual feature. The pattern is reminiscent of the auto-induction mechanism seen in the case of bacterial luciferase (Nealson, 1977). However, preliminary experiments have shown that the pattern of FADH regulation in *Xanthobacter* H4-14 was not simply due to culture dilution and subsequent outgrowth or to a change in energy source (C. A. Weaver & M. E. Lidstrom, unpublished results). It is not known if this phenomenon is a function of pre-growth on hydrogen, dilution into a methanol-containing medium, or both. It is possible that the level of FADH activity is determined by the steady-state level of formaldehyde or another key aldehyde, which would be dependent upon the activity of the aldehyde-producing and aldehyde-consuming reactions. Alternatively, it may be regulated in a general way by the growth rate or nutritional state of the cells, hypotheses that can only be tested using continuous culture.

Anthony (1982) noted that for many methylotrophs FDH is the only readily demonstrable source of reduced pyridine nucleotides. This is apparently the case for *Xanthobacter* H4-14. The FDH from *Xanthobacter* H4-14 was specific for formate and passed electrons to NAD^+ , DCPIP or NBT, but not $NADP^+$. It is distinct from the FDH purified from *Pseudomonas* AM1 in terms of pH preference and sensitivity to cuprous and ferrous salts (Johnson & Quayle, 1964) and is considerably smaller than the FDH obtained from *Pseudomonas oxalaticus* (Muller *et al.*, 1978). The difference in properties among these enzymes that perform the same function is intriguing.

The FDH in *Xanthobacter* H4-14 was tightly regulated, even more so than the MeDH. High levels of activity were found only in methanol- and formate-grown cells, while cells grown on other substrates contained no detectable activity.

For *Xanthobacter* H4-14, induction studies suggested that the MeDH and the 10 kDa polypeptide, which may represent the MeDH-associated cytochrome *c*, were co-regulated. However, the FADH and FDH activities were not. Since formate-grown cells contain only low levels of MeDH and no detectable 10 kDa polypeptide, methanol is probably the inducer for the MeDH and its associated functions, but formate or a derivative is probably the inducer for FDH. FADH regulation has been discussed above.

Dunstan *et al.* (1972*b*) and McNerney & O'Connor (1980) presented evidence for co-ordinate regulation of several serine pathway enzymes in *Pseudomonas* AM1, although MeDH appeared to be regulated separately. We have shown that the methanol dissimilatory functions in *Pseudomonas* AM1 are not co-regulated. MeDH and FDH appeared to be induced at similar rates, but formate-grown *Pseudomonas* AM1 contained only uninduced levels of MeDH (unpublished results), suggesting that this apparent relationship was circumstantial.

It is interesting to note the numerous FDH activities in *Pseudomonas* AM1 detected by activity stains. Since it is unlikely that the spectrophotometrically-determined (dye-linked) FADH is involved in methanol dissimilation due to its low activity and lack of methanol-dependent control, one of the stained activities, perhaps FADH-1, may be the true, physiologically active enzyme. The *in vivo* electron acceptors for these activities may be either NAD⁺, cytochromes or unknown acceptors. Biochemical and mutant analysis of these activities should resolve the question of formaldehyde oxidation in *Pseudomonas* AM1.

In contrast to the induction experiments with *Xanthobacter* H4-14 and *Pseudomonas* AM1, those with *P. denitrificans* revealed several potential examples of co-ordinate regulation. The induction patterns for MeDH and FDH were similar but, as in the cases of *Xanthobacter* H4-14 and *Pseudomonas* AM1, formate-grown *P. denitrificans* had only uninduced levels of MeDH (not shown). The patterns of induction for polypeptides of 14, 20, 24 and 44 kDa were all similar to those for MeDH and FDH. As indicated above, one or two of the smaller polypeptides may represent MeDH-specific *c*-type cytochromes and, thus, may be co-ordinately regulated with MeDH. The other polypeptides may be associated with FDH. Definition of the physical relationships among the genes for these activities and polypeptides will require detailed genetic analysis.

The pattern of induction for the *P. denitrificans* GSH-dependent FADH was especially interesting in that it was apparently induced immediately by methanol in contrast to the delayed induction of MeDH and FDH caused by initial growth on yeast extract. This finding suggests not only independent regulation of FADH by methanol but also the absence of a 'catabolite repression' factor that may control MeDH and possibly FDH gene expression.

Although induction patterns of this type provide important information on regulation of enzyme activities at the whole cell level, they provide little insight into genetic organization or transcriptional regulation. Enzyme activity in cell extracts is a result of transcriptional, translational and effector-mediated regulation. Therefore, measuring enzyme activities without concomitant analyses of the protein and mRNA provides little information concerning these three levels of regulation. In addition, it is known in *E. coli* that different genes present in the *trp* operon are co-transcribed but are expressed in the cell at different levels due to differential termination events (Platt, 1981). Therefore, the presence or absence of co-regulation cannot be taken as evidence for or against the presence of operons. Detailed genetic analysis, which is now available in *Xanthobacter* H4-14 (C. A. Weaver & M. E. Lidstrom, unpublished results) and *Pseudomonas* AM1 (Fulton *et al.*, 1984) will be necessary to determine the molecular details of regulation of these methanol dissimilatory functions.

Three general points of interest can be drawn from the study of these methylotrophs. First, significant differences exist in the enzymology and regulatory patterns for the methanol dissimilatory activities. In particular, the differences between the two methanol autotrophs *Xanthobacter* H4-14 and *Paracoccus denitrificans* are striking. Analogous regulatory differences among other methylotrophs have been noted previously (McNerney & O'Connor, 1980).

Second, the three bacteria have different ways of coping with a common problem,

formaldehyde removal. In the autotrophs, formaldehyde is produced mainly by MeDH and is removed by FADH. In *Pseudomonas* AM1, removal reactions include both FADH and the reactions of the serine pathway. *Xanthobacter* H4-14 apparently does not cope well with formaldehyde and the fact that it grows slowly on methanol after a long lag may be due to formaldehyde toxicity. Formaldehyde-detoxifying (FADH) activity initially decreased in response to methanol, suggesting that formaldehyde could build up due to MeDH activity. This organism was isolated from a low-nutrient lake as a co-culture with a methanotroph in which it apparently grew on low levels of methanol excreted by the methanotroph (Lidstrom-O'Connor *et al.*, 1983). Since this strain was adapted to growth at low methanol concentrations, it is perhaps not surprising that it has not developed efficient means for dealing with high methanol concentrations. It is surprising, however, that no pyridine-nucleotide coupled formaldehyde oxidation could be detected since this deficiency would result in inefficient autotrophic growth. It is possible that the FADH detected on PAGE gels is pyridine-nucleotide linked *in vivo*. *P. denitrificans* not only has an NAD⁺-linked FADH activity but also this activity was induced rapidly in response to methanol, thus apparently solving any potential problems of formaldehyde toxicity during growth at high methanol concentrations. *Pseudomonas* AM1 contains a partially constitutive MeDH so that formaldehyde will be produced from methanol immediately. However, it also has partially constitutive FADH activities and low levels of the serine pathway enzymes which should transform formaldehyde before it reaches toxic levels. The partially constitutive MeDH and FADH activities in *Pseudomonas* AM1 may allow this organism to respond rapidly to methanol in the environment and out-compete less responsive organisms.

Third, the commercial potential of autotrophic methylotrophs should be evaluated, especially in terms of the regulatory properties of the dissimilatory enzymes. The development of genetic techniques in these organisms will allow detailed study and manipulation of these key activities.

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REFERENCES

- ANTHONY, C. (1982). *The Biochemistry of Methylotrophs*. London: Academic Press.
- ANTHONY, C. & ZATMAN, L. (1964). The microbial oxidation of methanol. 2. The methanol-oxidizing enzyme of *Pseudomonas* sp. M27. *Biochemical Journal* **92**, 614–621.
- ATTWOOD, M. & QUAYLE, J. R. (1984). Formaldehyde as a central intermediary metabolite of methylotrophic metabolism. In *Microbial Growth on C₁ Compounds*, pp. 315–323. Edited by R. L. Crawford & R. S. Hanson. Washington: American Society for Microbiology.
- BAMFORTH, C. W. & QUAYLE, J. R. (1978). Aerobic and anaerobic growth of *Paracoccus denitrificans* on methanol. *Archives of Microbiology* **119**, 91–97.
- BEARDMORE-GRAY, M., O'KEEFE, D. T. & ANTHONY, C. (1982). The autoreducible cytochromes *c* of the methylotrophs *Methylophilus methylotrophus* and *Pseudomonas* AM1. *Biochemical Journal* **207**, 161–165.
- BEARDMORE-GRAY, M., O'KEEFE, D. T. & ANTHONY, C. (1983). The methanol:cytochrome *c* oxidoreductase activity of methylotrophs. *Journal of General Microbiology* **129**, 923–933.
- BOULTON, C. A. & LARGE, P. J. (1977). Synthesis of certain assimilatory and dissimilatory enzymes during bacterial adaptation to growth on trimethylamine. *Journal of General Microbiology* **101**, 151–156.
- COX, R. B. & QUAYLE, J. R. (1975). The autotrophic growth of *Micrococcus denitrificans* on methanol. *Biochemical Journal* **150**, 569–571.
- DUNSTAN, P. M., ANTHONY, C. & DRABBLE, W. T. (1972a). Microbial metabolism of C1 and C2 compounds. The involvement of glycollate in the metabolism of ethanol and of acetate by *Pseudomonas* AM1. *Biochemical Journal* **128**, 99–106.
- DUNSTAN, P. M., ANTHONY, C. & DRABBLE, W. T. (1972b). Microbial metabolism of C1 and C2 compounds. The role of glyoxylate, glycollate and acetate in the growth of *Pseudomonas* AM1 on ethanol and C1 compounds. *Biochemical Journal* **128**, 107–115.
- FULTON, G. F., NUNN, D. N. & LIDSTROM, M. E. (1984). Molecular cloning of a methyl coenzyme A lyase gene from *Pseudomonas* sp. strain AM1, a facultative methylotroph. *Journal of Bacteriology* **160**, 718–723.
- HARDER, W., ATTWOOD, M. M. & QUAYLE, J. R. (1973). Methanol assimilation by *Hyphomicrobium* sp. *Journal of General Microbiology* **78**, 155–163.
- JOHNSON, P. A. & QUAYLE, J. R. (1964). Microbial growth on C1 compounds. 6. Oxidation of methanol, formaldehyde and formate by methanol-grown *Pseudomonas* AM1. *Biochemical Journal* **93**, 281–290.
- JONES, C. W., KINGSBURY, S. A. & DAWSON, M. J. (1982). The partial resolution and dye-mediated

- reconstitution of methanol oxidase activity in *Methylophilus methylotrophus*. *FEMS Microbiology Letters* **13**, 195–200.
- KASPRZAK, A. A. & STEENKAMP, D. J. (1983). Localization of the major dehydrogenases in two methylotrophs by radiochemical labeling. *Journal of Bacteriology* **156**, 348–353.
- KELLY, D. P. & WOOD, A. P. (1982). Autotrophic growth of *Thiobacillus* A2 on methanol. *FEMS Microbiology Letters* **15**, 229–233.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LIDSTROM-O'CONNOR, M. E., FULTON, G. L. & WOPAT, A. E. (1983). '*Methylobacterium ethanolicum*': a syntrophic association of two methylotrophic bacteria. *Journal of General Microbiology* **129**, 3139–3148.
- LOGINOVA, N. V. & TROTSSENKO, YU. A. (1979). Autotrophic growth on methanol by bacteria isolated from activated sludge. *FEMS Microbiology Letters* **5**, 239–243.
- MCNERNEY, T. & O'CONNOR, M. L. (1980). Regulation of enzymes associated with C-1 metabolism in three facultative methylotrophs. *Applied and Environmental Microbiology* **40**, 370–375.
- MARISON, I. W. & ATTWOOD, M. M. (1980). Partial purification and characterization of a dye-linked formaldehyde dehydrogenase from *Hyphomicrobium* X. *Journal of General Microbiology* **117**, 305–313.
- MARISON, I. W. & ATTWOOD, M. M. (1982). A possible alternative mechanism for the oxidation of formaldehyde to formate. *Journal of General Microbiology* **128**, 1441–1446.
- MULLER, U., WILLNOW, P., RUSCHIG, U. & HOPFNER, T. (1978). Formate dehydrogenase from *Pseudomonas oxalaticus*. *European Journal of Biochemistry* **83**, 485–498.
- NEALSON, K. H. (1977). Autoinduction of bacterial luciferase: occurrence, mechanism and significance. *Archives of Microbiology* **112**, 73–79.
- O'CONNOR, M. L. (1981). Extension of the model concerning linkage of genes coding for C-1-related functions in *Methylobacterium organophilum*. *Applied and Environmental Microbiology* **41**, 437–441.
- O'CONNOR, M. L. & HANSON, R. S. (1977). Enzyme regulation in *Methylobacterium organophilum*. *Journal of General Microbiology* **101**, 327–332.
- O'KEEFE, D. T. & ANTHONY, C. (1980). The two cytochromes *c* in the facultative methylotroph *Pseudomonas* AM1. *Biochemical Journal* **192**, 411–419.
- PLATT, T. (1981). Termination of transcription and its regulation in the tryptophan operon of *E. coli*. *Cell* **24**, 10–23.
- SAHM, H., COX, R. B. & QUAYLE, J. R. (1976). Metabolism of methanol by *Rhodospseudomonas acidophila*. *Journal of General Microbiology* **94**, 313–322.
- URAKAMI, T. & KOMAGATA, K. (1981). Electrophoretic comparison of enzymes in the Gram negative methanol-utilizing bacteria. *Journal of General and Applied Microbiology* **27**, 381–403.
- VAN VERSEVELD, H. W. & STOUTHAMER, A. H. (1978). Electron-transport chain and coupled oxidative phosphorylation in methanol-grown *Paracoccus denitrificans*. *Archives of Microbiology* **118**, 13–20.
- WEAVER, C. A., REDBORG, A. H. & KONISKY, J. (1981). Plasmid-determined immunity of *Escherichia coli* K-12 to colicin 1a is mediated by a plasmid-encoded membrane protein. *Journal of Bacteriology* **148**, 817–828.
- WIEGEL, J., WILKE, D., BAUMGARTEN, J., OPITZ, R. & SCHLEGEL, H. G. (1978). Transfer of the nitrogen fixing hydrogen bacterium *Corynebacterium autotrophicum* Baumgarten *et al.* to *Xanthobacter* gen. nov. *International Journal of Systematic Bacteriology* **28**, 573–581.