Methanol metabolism in thermotolerant methylotrophic *Bacillus* strains involving a novel catabolic NAD-dependent methanol dehydrogenase as a key enzyme


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**Abstract.** The enzymology of methanol utilization in thermotolerant methylotrophic *Bacillus* strains was investigated. In all strains an immunologically related NAD-dependent methanol dehydrogenase was involved in the initial oxidation of methanol. In cells of *Bacillus* sp. C1 grown under methanol-limiting conditions this enzyme constituted a high percentage of total soluble protein. The methanol dehydrogenase from this organism was purified to homogeneity and characterized. In cell-free extracts the enzyme displayed biphasic kinetics towards methanol, with apparent $K_m$ values of 3.8 and 166 mM. Carbon assimilation was by way of the fructose-1,6-bisphosphate aldolase cleavage and transketolase/transaldolase rearrangement variant of the RuMP cycle of formaldehyde fixation. The key enzymes of the RuMP cycle, hexulose-6-phosphate synthase (HPS) and hexulose-6-phosphate isomerase (HPI), were present at very high levels of activity. Failure of whole cells to oxidize formate, and the absence of formaldehyde- and formate dehydrogenases indicated the operation of a non-linear oxidation sequence for formaldehyde via HPS. A comparison of the levels of methanol dehydrogenase and HPS in cells of *Bacillus* sp. C1 grown on methanol and glucose suggested that the synthesis of these enzymes is not under coordinate control.

**Key words:** *Bacillus* — Methanol — Methylotrophic bacilli — Thermotolerant bacilli — Methylotrophy — Alcohol dehydrogenase — Methanol dehydrogenase — RuMP cycle of formaldehyde fixation — Hexulose-6-phosphate synthase

Over the years it has become clear that Gram-negative methylotrophic bacteria without exception employ a pyrroloquinoline quinone (PQQ) dependent methanol dehydrogenase (MDH; EC 1.1.99.8) for the initial oxidation of methanol (Anthony 1982, 1986; Duine et al. 1978, 1986; de Vries 1986). The situation in Gram-positive methylotrophic bacteria is less clear as relatively few of these organisms have been isolated and studied in detail thus far (Dijkhuizen and Levering 1987). In the methanol-utilizing actinomycete *Nocardia* sp. 239 (Hazeu et al. 1983), the presence of a novel NAD-dependent, PQQ-containing methanol dehydrogenase (nMDH) has been reported (Duine et al. 1984). In this organism nMDH activity resides in a multienzyme complex together with NAD-dependent formaldehyde dehydrogenase and NADH dehydrogenase.

Recently we described the isolation of thermotolerant methylotrophic *Bacillus* strains, employing an NAD-dependent methanol dehydrogenase for methanol oxidation and the RuMP cycle of formaldehyde fixation (Dijkhuizen et al. 1988). NAD-dependent alcohol dehydrogenases (ADH; EC 1.1.1.1) are widely distributed in nature (Sund and Theorell 1963; Brändén et al. 1975) and draw considerable attention, amongst others for phylogenetic reasons (Duester et al. 1986). In general, these enzymes possess a low affinity, if any, for methanol (e.g., Steinbüchel and Schlegel 1984; Rela et al. 1987; Sheehan et al. 1988; Verdun et al. 1988). One example is the NAD-dependent alcohol dehydrogenase from *Bacillus steaerothermophilus* DSM 2334 (Sheehan et al. 1988; Dowds et al. 1988) with apparent $K_m$ values for methanol and ethanol of 20 mM and 82 µM, respectively. This organism, however, is not able to grow on methanol as a carbon source.

In this paper a detailed study of the enzymology of methanol utilization in thermotolerant methylotrophic *Bacillus* strains, and the purification and characterization of the methanol dehydrogenase from *Bacillus* sp. C1 is presented.

**Materials and Methods**

**Organisms**

The organisms used, *Bacillus* sp. C1, PB1, AR2, TS1, TS2 and TS4, and their maintenance have been described before (Dijkhuizen et al. 1988). *Bacillus* sp. S1 and TF are related strains (Al-Awadhi et al. 1988).
For batch cultivation conical flasks filled to 25% of the volume with a 50 mM methanol- or 10 mM glucose-mineral salts medium (Dijkhuizen et al. 1988) of pH 7.5 were incubated in a shaking waterbath. If required the medium was supplemented with 1 ml/l of a vitamin solution, containing (mg/l): biotin, 100; thiamin·HCl, 100; riboflavin, 100; pyridoxal phosphate, 100; panthotenate, 100; nicotinic acid amide, 100; p-aminobenzoic acid, 20; folic acid, 10; vitamin B12, 10; lipoic acid, 10. The methylotrophic Bacillus strains were grown at 50°C and the Gram-negative methylotroph Xanthobacter sp. 25a (Cros et al. 1986) at 30°C. For continuous cultivation of Bacillus sp. C1 the organism was grown in a methanol-limited (50 mM methanol) chemostat on the mineral medium described by Levering et al. (1981). The temperature was controlled at 50°C and the pH was maintained at 7.3 by automatic adjustment with 1 M NaOH.

**Preparation of extracts**

Cells from early to mid-exponential phase of growth were harvested by centrifugation at 3,800 × g for 10 min at 4°C, washed twice and resuspended in 50 mM potassium phosphate buffer pH 7.5, containing 5 mM MgSO₄ (buffer A). Cells were disrupted in the presence of 5 mM dithiothreitol (DTT) by passage through a French pressure cell operating at 1.4 × 10⁵ kN/m². Low-speed cell-free extracts were obtained by centrifugation at 3,800 × g for 10 min at 4°C and used for the determination of NAD(P)H oxidase and NADH dehydrogenase activities. Cell walls and membrane particles were subsequently removed by centrifugation at 25,000 × g for 20 min at 4°C. The supernatants, which contained 5 – 10 mg of protein/ml, were used as crude extracts for enzyme assays and for the purification of methanol dehydrogenase. For enzyme localization studies extracts were centrifuged at 150,000 × g for 60 min at 4°C, yielding a high speed supernatant and a high speed pellet. The pellet was washed twice with buffer A. Extracts and samples obtained in various purifications steps (see below), were stored at –80°C in the presence of 5 mM DTT.

**Enzyme assays**

All assays with Bacillus sp. C1 and Bacillus sp. TS4 were performed at 50°C, using prewarmed buffer solutions. Methanol oxidation rates of whole cells (early exponential growth phase) were determined polarographically, using a Clark-type oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instruments Co., Yellow Springs, OH, USA). Whole cells rapidly lost methanol oxidizing activity, both on ice and at room temperature. The following procedure was therefore adopted which resulted in reproducible activities. Cells were washed once at room temperature in buffer A and assayed immediately. The reaction mixture (3 ml) contained: potassium phosphate buffer pH 7.5, 150 mM; MgSO₄, 15 mM; and cell suspension (0.2 – 0.5 mg cell dry weight). After air saturation and temperature equilibration at 50°C, the reaction was started with 150 μmol of the alcohol to be tested. At 50°C, 100% air saturation corresponds to 0.187 mM O₂/l H₂O.

Spectrophotometric assays were performed with a Perkin-Elmer 124 spectrophotometer. The PQQ-containing methanol dehydrogenase (MDH; EC 1.1.99.8), present in all Gram-negative methylotrophs studied, was assayed according to Ghosh and Quayle (1981), by following DCPPIP reduction at 600 nm (molar absorption coefficient of 22 × 10³ M⁻¹·cm⁻¹). The reaction mixture (1 ml) contained: Tris·HCl pH 9.0, 125 μmol; ammonium chloride, 150 μmol; PMS, 1.25 μmol; DCPPIP, 80 μmol; potassium cyanide, 5 μmol; extract. The reaction was started by the addition of 2 μmol methanol.

The NAD-dependent, PQQ-containing methanol dehydrogenase (nMDH), as observed in Nocardia sp. 239, was assayed by following DCPPIP reduction at 600 nm, according to Duine et al. (1984). The reaction mixture (1 ml) contained tetrasodium pyrophosphate buffer pH 9.0, 100 μmol; ammonium chloride, 120 μmol; NAD, 2.5 μmol; DCPPIP, 40 nmol; extract. The reaction was started by the addition of 2 μmol methanol.

Various methods were tested to measure activity of the NAD-dependent methanol dehydrogenase in the methylotrophic Bacillus strains (see Results section). The following procedure was adopted: Alcohol-dependent NADH production was measured at 50°C, by following the increase in absorbance at 340 nm, in a reaction mixture (1 ml) containing: glycine/KOH buffer pH 9.5, 100 μmol; MgSO₄, 5 μmol; DTT, 1 μmol; NAD, 1 μmol; extract. After temperature equilibration the reaction was started with 500 μmol of the alcohol to be tested. Dilution of extracts resulted in loss of methanol dehydrogenase activity present in Bacillus strains, but could be largely circumvented by using buffer A containing 20% sucrose.

The formaldehyde reductase activity (the activity of NAD-dependent methanol dehydrogenase in the reverse direction) was measured by following the formaldehyde-dependent oxidation of NADH at 340 nm. The reaction mixture (1 ml) contained: potassium phosphate buffer pH 6.7, 50 μmol; DTT, 1 μmol; NADH, 150 nmol; extract. After temperature equilibration the reaction was started with 10 μmol formaldehyde.

NADH dehydrogenase activity was measured by following DCPPIP reduction at 600 nm in a reaction mixture (1 ml) containing: potassium phosphate buffer pH 8.0, 50 μmol; MgCl₂, 5 μmol; DCPPIP, 80 nmol; low-speed cell-free extract. After temperature equilibration at 50°C the reaction was started with 200 nmol NADH.

NAD(P)H oxidase activities were measured at 340 nm in a reaction mixture (1 ml) containing: potassium phosphate buffer pH 7.0, 50 μmol; MgCl₂, 5 μmol; low-speed cell-free extract. The reaction was started with 200 nmol NAD(P)H.

Glucose-6-phosphate isomerase (EC 5.3.1.9) was assayed in a reaction mixture (1 ml) containing: potassium phosphate buffer pH 8.5, 50 μmol; fructose-6-phosphate, 5 μmol; NADP, 0.4 μmol; glucose-6-phosphate dehydrogenase, 1 unit. The reaction was started by the addition of extract.

Hexulose-6-phosphate isomerase activity was determined using a modification of the assay for hexulose-6-phosphate synthase (Levering et al. 1981) in that hexulose-6-phosphate isomerase from Methylphilus methylophilus was omitted.

The following enzymes were assayed according to published methods. NAD-dependent formaldehyde dehydrogenase, EC 1.2.1.1 (van Dijken et al. 1976); NAD-dependent formate dehydrogenase, EC 1.2.1.2, and NAD-independent formate dehydrogenase, EC 1.2.1.3 (Dijkhuizen et al. 1978); hexulose-6-phosphate synthase (HPS; spec-
trophotometric assay of Levering et al. 1981); NADP-dependent glucose-6-phosphate dehydrogenase, EC 1.1.1.49, NADP-dependent 6-phosphogluconate dehydrogenase, EC 1.1.1.44, 6-phosphofructokinase, EC 2.7.1.11, combined activity of 6-phosphogluconate dehydrogenase, EC 4.1.2.12, and KDPG aldolase, EC 4.1.2.14 (van Dijken and Quayle 1977); fructose-1,6-bisphosphate aldolase, EC 4.1.2.13 (van Dijken et al. 1978); phosphoribosylisomerase, EC 5.3.1.6, ribulose phosphate 3-epimerase, EC 5.3.1.1, transketolase, EC 2.2.1.1, transaldolase, EC 2.2.1.2, sedoheptulose-1,7-bisphosphatase, EC 3.1.3.66 (Levering et al. 1982).

Purification of methanol dehydrogenase from Bacillus sp. C1

Methanol dehydrogenase was purified from cells grown at a low dilution rate (D = 0.10 h⁻¹) in a methanol-limited chemostat (S₀ = 50 mM methanol). The viscosity of the crude extract was lowered by adding protamine sulphate (10% solution) to a final concentration of 1 mg per 10 mg protein. The resulting precipitate (nucleic acids) was removed by centrifugation at 25,000 × g for 20 min at 4°C. The supernatant was desalted in buffer B (20 mM Tris-HCl pH 7.5/5 mM MgSO₄/5 mM β-mercaptoethanol) by Sephadex G25 gel filtration (Bio-Rad PD10 column) and subjected to anion-exchange chromatography. The sample was applied onto a Fast Flow Q-Sepharose column (volume 25 ml; washed and equilibrated with buffer B), connected to a Pharmacia FPLC system. Proteins were eluted at a flow rate of 1.0 ml/min by applying a linear 0-1.0 M (NH₄)₂SO₄ gradient in buffer C. Fractions (4 ml) were subjected to anion-exchange chromatography by adding ammonium sulphate (10% solution) to a final concentration of 1 mg per 10 mg protein. The active fractions were pooled and prepared for hydrophobic interaction chromatography by adding ammonium sulphate to a final concentration of 1 M. Samples (13-14 mg of protein) were subsequently applied onto a FPLC Phenyl-Superose HR5/5 column (Pharmacia), equilibrated with 50 mM Tris·HCl pH 7.0/5 mM MgSO₄/5 mM β-mercaptoethanol containing 1.0 M (NH₄)₂SO₄ (buffer C). Bound protein was eluted at a flow rate of 0.4 ml/min with a linear 1.0-0 M (NH₄)₂SO₄ gradient in buffer C. Fractions of 0.5 ml were collected. The active fractions were pooled and stored in small aliquots at -80°C.

Determination of relative molecular mass

The molecular weight of native methanol dehydrogenase was determined on a Superoxer 6 gel filtration column (equilibrated with 100 mM Tris·HCl pH 7.5/5 mM MgSO₄/5 mM β-mercaptoethanol), using gel filtration standards (1,350-670,000 range) of Bio-Rad, Richmond, USA. Samples (200 µl, containing approximately 200 µg of protein) of both crude extract and purified methanol dehydrogenase were used.

Electrophoretic methods

Denaturing gel electrophoresis (SDS-PAGE) was performed as described by Laemmli and Favre (1973) with the following calibration proteins as molecular weight references: phosphorylase A, 94,000; humane transfereine, 80,000; bovine serum albumin, 68,000; catalase, 58,000; fumarase, 50,000; citrate synthase, 46,000; carboanhydrase, 31,000. Gels were stained with Coomassie brilliant blue G-250. Western blotting was performed as described by Towbin et al. (1979) using the alkaline phosphatase-linked-antibody procedure. Following gel electrophoresis under non-denaturing conditions alcohol dehydrogenase from baker’s yeast (Boehringer, Mannheim, FRG), the NAD-dependent methanol dehydrogenase from Bacillus sp. C1 and the PQQ-dependent methanol dehydrogenase from Xanthobacter sp. 25a (Janssen et al. 1987) were visualized by an alcohol-dependent tetrazolium activity staining. The enzyme preparations were loaded on a 6% polyacrylamide gel in 100 mM glycine/Tris pH 9.0. After electrophoresis (100 V, constant current), the gels were stained with incubation with methanol (1%) or ethanol (1%) in staining buffer at 45°C. Staining buffer contained (per 100 ml): glycine·KOH pH 9.5, 10 mmol; MgSO₄, 0.5 mmol; NAD, 0.1 mmol; nitroblue-tetrazolium (NBT), 20 mg; PMS, 8 mg.

Protein determination

Protein was determined by the methods of Lowry et al. (1951; low-speed cell-free extracts only) and Bradford (1976) using bovine serum albumin as a standard.

Formaldehyde and PQQ determination

Formaldehyde was assayed with the method of Avigad (1983). PQQ was determined by measuring the reconstitution of quinoprotein alcohol dehydrogenase from Pseudomonas testosteroni (Groen et al. 1986).

Preparation of antiserum

Antibodies against purified methanol dehydrogenase (Phenyl-Superose pool, Table 1; Fig. 2, lane m) were raised in rabbits by subcutaneous injections (500 µg) in complete Freund’s adjuvant. The specificity of the antibodies were tested by Ouchterlony double diffusion tests and Western blotting.

Electron microscopy and immunocytochemistry

Electron microscopy and immunogold-labeling of NAD-dependent methanol dehydrogenase were performed as described by Douma et al. (1985).

Results

Methanol oxidation

Cells of Bacillus sp. C1 grown on methanol in batch culture oxidized methanol at a high rate (1,100-1,500 nmol min⁻¹ mg⁻¹ of protein). Only short chain (C₁ - C₄) primary alcohols were oxidized, but compared to methanol (100%) at lower relative rates (ethanol, 90%; n-propanol, 57%, n-butanol, 53%). Secondary alcohols and formate were not oxidized. While the organism oxidized various alcohols, it only grew on methanol. Most probably, Bacillus sp. C1 is not able to metabolize aldehydes other than formaldehyde.

Reduced (dithionite) minus oxidized (ferricyanide) cytochrome spectra of low-speed cell-free extracts of methanol- or glucose-grown Bacillus sp. C1 revealed the presence of cytochromes of the c- (552 nm), b- (558 nm) and a-type (603 nm). Potassium cyanide was a potent inhibitor of methanol oxidation (> 90% inhibition by 2 µM in low-speed extracts). Interestingly, also antimycin A acted as an inhibi-
tor of methanol oxidation, both in whole cells (50% inhibition by 60 \text{\mu M antimycin A}) and in low-speed cell-free extracts (> 90% inhibition of both NADH and methanol oxidation by 65 \text{\mu M antimycin A}). These observations indicate that electrons derived from methanol oxidation in Bacillus sp. C1 are donated to the electron transport chain at, or before, the level of cytochrome b. In contrast, the PQQ-dependent methanol dehydrogenase in Gram-negative methylotrophs interacts with the electron transport chain at the level of cytochrome c (Anthony 1982).

All attempts to measure an ammonium-requiring, dye-linked (PMS/DCPIP) methanol dehydrogenase in extracts of the methylotrophic bacilli failed. Also, activity of an NAD-dependent, dye-linked (DCPIP), PQQ-containing methanol dehydrogenase, using the assay as developed for the actinomycete Nocardia sp. 239 (Duine et al. 1984), could not be detected. Incubation of extracts of methanol-grown cells of Bacillus sp. C1 with methanol nevertheless resulted in production of formaldehyde (700 – 800 nmol·min\(^{-1}\)·mg\(^{-1}\) of protein), provided methanol was present at relatively high concentrations (approx. 500 mM). When using these much higher methanol concentrations in the assay as developed for Nocardia sp. 239, we also were able to detect methanol-dependent DCPIP reduction (360 nmol·min\(^{-1}\)·mg\(^{-1}\) of protein) by extracts of Bacillus sp. C1. Omission of NAD from the two assays described above had no effect on the level of activity. Both activities, however, were completely lost when extracts were desalted on a Sephadex G25 column, but could be effectively restored by adding NAD to the reaction mixture. It thus became apparent that NAD in fact was required for methanol oxidation by Bacillus sp. C1 and that this had been masked because of the presence of sufficiently high levels of NAD in the extracts. Extracts of Bacillus sp. C1 subsequently were found to catalyze a methanol-dependent reduction of NAD that could be measured directly at 340 nm. Maximal activities were observed at methanol concentrations of 500 mM. This activity was ammonium-independent, but was strongly stimulated by addition of Mg\(^{2+}\)-ions (5 mM) to the assay buffer. Under optimized conditions (i.e., at 50°C and pH 9.5), extract of cells of Bacillus sp. C1 grown in batch culture were found to possess methanol dehydrogenase activities of 1,000 – 1,200 nmol·min\(^{-1}\)·mg\(^{-1}\) of protein. These high methanol dehydrogenase activities, however, were only observed with undiluted extracts, after preincubation (5 min) of samples and buffers at the assay temperature of 50°C. Starting the reaction with NAD instead of methanol resulted in prolonged acceleration phases and lower final activities. Dilution of samples generally resulted in loss of methanol dehydrogenase activities (only about 10% of the specific activity remained for instance when extract was diluted tenfold in assay buffer) but could be largely overcome by including sucrose in the buffer solutions used. The methanol dehydrogenase activity was not proportional to the amount of extract added to the assay mixtures. Rather, the specific activity increased with increasing protein concentrations. This effect also was observed when sucrose or glycerol were included in the assay buffer. The extracts also catalyzed the reverse reaction, i.e. NADH-dependent formaldehyde reduction (1,200 nmol·min\(^{-1}\)·mg\(^{-1}\) protein). The formaldehyde reductase reaction was not sensitive to dilution inactivation, neither was it affected by Mg\(^{2+}\)-ions. Moreover, the formaldehyde reductase activity was clearly proportional to the amount of protein added. We therefore decided to use this assay during enzyme purification and for localization studies.

Following gel electrophoresis of extracts of methanol-grown cells of Bacillus sp. C1 and Xanthobacter sp. 25a, and Saccharomyces cerevisiae ADH under non-denaturating conditions, the alcohol dehydrogenases present were visualized by staining for activity with methanol and ethanol as substrates. With both substrates the Bacillus sp. C1 activity was found to reside in a single band, the electrophoretic mobility of which was clearly different from that of either yeast ADH or methanol dehydrogenase from Xanthobacter sp. 25a (Fig. 1). The active band from Bacillus sp. C1 extract was excised from the gel and subjected to SDS-PAGE. A single band of approximately 43,000 was observed (results not shown).

**Purification of methanol dehydrogenase from Bacillus sp. C1**

The methanol dehydrogenase was purified to homogeneity from cells of Bacillus sp. C1 grown in chemostat culture under methanol-limitation at \(D = 0.10\) h\(^{-1}\). Extracts of these cells, subjected to SDS-PAGE, possessed a very dominant 43,000 protein band, which suggested that the methanol dehydrogenase constituted already a high percentage of total cell protein. The purification (shown in Table 1 and Fig. 2) was followed with both the formaldehyde reductase and methanol dehydrogenase assays. During ion-exchange chromatography the formaldehyde reductase eluted at 300 mM KCl, resulting in a 2.3-fold increase in specific activity. In this step the NADH dehydrogenase activity eluted at 500 mM KCl. Hydrophobic interaction chromatography turned out to be an effective second purification step, with formaldehyde reductase eluting at relatively low ionic strength (300 mM (NH\(_4\))\(_2\)SO\(_4\)), indicating that the protein is rather hydrophobic in nature. The final preparation, after purification to homogeneity (Fig. 2, lane m), possessed a specific formaldehyde reductase activity of 19.6 \text{\mu mol·min\(^{-1}\)·mg\(^{-1}\)} of protein. The 4.5-fold purification obtained indicates that the enzyme may constitute up to about 22% of total soluble protein at \(D = 0.10\) h\(^{-1}\) in methanol-limited chemostats, assuming that no inactive enzyme is present in the final preparation. The molecular weight of purified native enzyme was estimated to be 300,000 ± 30,000 by gel filtration chromatography. Most likely it is a hexamer or octamer with subunits of 43,000.

The methanol dehydrogenase and formaldehyde reductase activities were stable in cell-free extracts. Storage during 4 days at 4°C, −20°C or −80°C in the absence of protease inhibitors did not result in a significant loss of enzyme activity, provided DTT was present (< 5% loss). However, during purification of the enzyme a remarkable difference was observed between the recoveries of methanol dehydrogenase and formaldehyde reductase activities. After the Q-Sepharose and Phenyl-Superose column steps, 34% of the original formaldehyde reductase activity was recovered whereas the preparation had almost completely lost methanol dehydrogenase activity (> 95% loss). Addition of sucrose, glycerol, iron ions, zinc ions or DTT did not prevent this specific loss of methanol dehydrogenase activity.

**Pyrrroloquinoline quinone analysis**

Nocardia sp. 239 was found to excrete PQQ into the culture medium during growth on methanol (Hazeu et al. 1983).
Fig. 1. Zymogram of extracts of *Xanthobacter* sp. 25a (PQQ-MDH; lane a; *Bacillus* sp. C1, lanes b, d) and purified baker’s yeast ADH (lanes c, e). Samples were loaded on a native polyacrylamide gel (6%) and stained with methanol (lanes a–c) or ethanol (d–e) as the substrate.

Fig. 2A, B. SDS-polyacrylamide (10%) gel electrophoresis of extracts of eight different methanol-grown *Bacillus* strains (A), and samples obtained during purification of methanol dehydrogenase from cells of *Bacillus* sp. CI grown under methanol-limiting conditions (D = 0.10 h⁻¹) in continuous culture (B). A Lanes a–j: *Bacillus* sp. C1, AR2, PBI, TS1, TS2, TS4, marker proteins (see Methods, minus carboanhydrase), *Bacillus* sp. S1, TF, respectively. B Lanes k–o: Crude extract, Q-Sepharose pool, Phenyl-Superose pool, marker proteins (see Methods), baker’s yeast ADH.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein (mg)</th>
<th>Total activity (units)*</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Fold purification</th>
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<tr>
<td>Crude Extract</td>
<td>320</td>
<td>1,408</td>
<td>4.4</td>
<td>100</td>
<td>1.0</td>
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<tr>
<td>Q-Sepharose pool</td>
<td>54.6</td>
<td>541</td>
<td>9.9</td>
<td>38</td>
<td>2.3</td>
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<tr>
<td>Phenyl-Superose pool</td>
<td>24.6</td>
<td>482</td>
<td>19.6</td>
<td>34</td>
<td>4.5</td>
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</table>

*Formaldehyde reductase assay
One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH per min

This organism employs a novel MDH whose activity is both PQQ- and NAD-dependent (Duine et al. 1984). The possible involvement of PQQ in methanol oxidation in *Bacillus* sp. CI was studied by analyzing extracts and purified formaldehyde reductase for the presence of this cofactor. Neither the purified enzyme nor the extract contained PQQ. Only if PQQ is covalently bound to the enzyme can its presence have been overlooked. However, the assay is very sensitive and PQQ is a very stable compound. It thus appears likely that methanol oxidation in *Bacillus* sp. CI is performed by a PQQ-independent, NAD-dependent methanol dehydrogenase.

**Kinetics of methanol oxidation**

Oxidation rates of washed cell suspension of *Bacillus* sp. CI at various methanol concentrations followed normal Michaelis-Menten kinetics. A \( K_m \) value of 2.6 mM methanol was calculated from Eadie-Hofstee plots (Fig. 3A).

Compared to whole cells, the methanol dehydrogenase activity in extracts (and partially purified preparations) displayed a similar substrate specificity (C1–C4 primary alcohols only), although its relative activity towards methanol was considerably lower (methanol, 33%; ethanol, 100%; n-propanol, 71%; n-butanol 87%). The enzyme required NAD(H) as coenzyme, no activity was observed with NADP(H).

Because purified preparations lost methanol dehydrogenase activities, the kinetics of methanol oxidation was further studied in extracts. In extracts the enzyme displayed biphasic kinetics towards methanol (apparent \( K_m \) values of 3.8–166 mM; Fig. 3B) and ethanol (apparent \( K_m \) values 7.5 and 70 mM; not shown). Biphasic kinetics was not observed with whole cells of *Bacillus* sp. CI (see above). Thus, methanol oxidation in vivo becomes saturated at lower methanol concentrations, which results in a relatively low \( K_m \) value. Biphasic kinetics was also observed towards NAD (apparent \( K_m \) values 15 and 190 μM), but not for formaldehyde (apparent \( K_m \) value 2.0 mM) in the reverse reaction.

**Subcellular localization of methanol dehydrogenase**

The subcellular localization of methanol dehydrogenase and NADH dehydrogenase was studied by comparing the levels of these enzymes in cell-free extracts, in high speed supernatants (soluble fractions) and in high speed pellets (membrane fractions). Approximately 70% of the NADH dehydrogenase activity appeared to be membrane associated, while the remaining activity was recovered in the supernatant. Most probably, NADH dehydrogenase has a loose
hexulose-6-phosphate synthase (HPS) and hexulose-6-phosphate isomerase (HPI), were present in all methylotrophic Bacillus strains under investigation. The enzyme data obtained with Bacillus sp. C1 and TS4 are shown in Table 2. A comparison of the levels of methanol dehydrogenase and HPS in glucose and methanol-grown cells of Bacillus sp. C1 suggests that the synthesis of these enzymes is not regulated coordinately. No activities of NAD-(in)dependent formaldehyde or formate dehydrogenases could be detected in Bacillus strains C1 and TS4. This, and the failure of methanol-grown whole cells to oxidize formate, indicated that the organisms do not possess a linear pathway for the oxidation of methanol to CO₂. Energy generation presumably proceeds via the so-called dissimilatory RuMP cycle, involving the enzymes glucose-6-phosphate isomerase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, all of which were present at high activities. The dehydrogenases involved were strictly NAD-dependent, although the high NADPH oxidase and negligible NADPH oxidase activities indicate that NADH is the preferred electron donor for the electron transport chain in these organisms. The enzymes of the Entner-Doudoroff pathway were absent, whereas high activities were detected of 6-phosphofructokinase and FBP aldolase, indicating that the organisms employ the Embden-Meyerhof route for sugar phosphate cleavage. Regeneration of the formaldehyde acceptor molecule ribulose-5-phosphate, from fructose-6-phosphate and glyceraldehyde-3-phosphate, involved the enzymes transketolase, transaldolase, phosphoribosylpyrophosphate isomerase and ribulose phosphate 3-epimerase (RK1A rearrangement variant). Sedoheptulose-1,7-bisphosphatase activity could not be detected. The pathway of methanol metabolism in the Bacillus strains investigated is shown schematically in Fig. 5.

**Discussion**

Although the presence of NAD-dependent alcohol dehydrogenases (ADH; EC 1.1.1.1) has been reported in some methylotrophic bacteria, e.g. in Hyphomicrobiurn X (Brooke and Attwood 1983) and in organism PAR (Bellion and Wu 1978), this enzyme only appeared to function in ethanol catabolism. All Gram-negative methylotrophic bacteria studied employ a PPQ-containing methanol dehydrogenase (MDH, EC 1.1.99.8; Duine et al. 1978) for methanol oxidation. In this group of organisms MDH is located in the periplasmic space. Evidence is emerging that in Gram-positive bacteria, which do not possess a periplasmic space, methanol oxidation is catalyzed by enzymes clearly different from MDH. Previously, Duine et al. (1984), reported the involvement of an NAD-dependent, PPQ-containing methanol dehydrogenase in the actinomycete Nocardia sp. 239. Although this enzyme requires NAD for activity, methanol oxidation did not result in the production of free NADH. In the present study a cytoplasmic, NAD-dependent methanol dehydrogenase was identified as the methanol-oxidizing enzyme in Bacillus strains. No evidence was obtained for the presence of PPQ in Bacillus sp. C1 and methanol oxidation clearly resulted in production of free NADH. The characteristic properties of MDH, nMDH, ADH and the NAD-dependent methanol dehydrogenase as observed in Bacillus sp. C1 are summarized in Table 3. The data indicate that the methylotrophic bacterium Bacillus sp. C1 employs a novel NAD-dependent methanol dehydrogenase for the conver-
methanol/formaldehyde (E° = –182 mV) strongly favours when reaction products are immediately further metabolized. This also explains the relatively high growth yield of the cytochrome b. This becomes especially important when thermotolerant methylotrophic bacteria are grown in extracts of methanol-grown Bacillus sp. Cl and Bacillus sp. TS4.

### Table 2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity in Bacillus sp. Cl</th>
<th>Bacillus sp. TS4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol oxidation rate (whole cells)</td>
<td>1,100 – 1,500 (75)*</td>
<td>1,100 – 1,500</td>
</tr>
<tr>
<td>1. Formaldehyde reductase</td>
<td>1,220 (880)*</td>
<td>1,265</td>
</tr>
<tr>
<td>Formaldehyde dehydrogenase</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2. Hexulose-6-phosphate synthase</td>
<td>17,300 (515)*</td>
<td>19,400</td>
</tr>
<tr>
<td>3. Hexulose-6-phosphate isomerase</td>
<td>5,950</td>
<td>6,700</td>
</tr>
<tr>
<td>4. Glucose-6-phosphate isomerase</td>
<td>1,415</td>
<td>1,480</td>
</tr>
<tr>
<td>5. Glucose-6-phosphate dehydrogenase (NADP)</td>
<td>475 (135)*</td>
<td>930</td>
</tr>
<tr>
<td>6. 6-Phosphogluconate dehydrogenase (NADP)</td>
<td>385</td>
<td>670</td>
</tr>
<tr>
<td>7. 6-Phosphofructokinase</td>
<td>600</td>
<td>670</td>
</tr>
<tr>
<td>8. Fructose-1,6-bisphosphate aldolase</td>
<td>370</td>
<td>1,190</td>
</tr>
<tr>
<td>9. Transketolase</td>
<td>710 (460)*</td>
<td>1,110 (140)*</td>
</tr>
<tr>
<td>10. Transaldolase</td>
<td>60 (9)*</td>
<td>140 (60)*</td>
</tr>
<tr>
<td>11. Phosphoribosyltransferase</td>
<td>80</td>
<td>105</td>
</tr>
<tr>
<td>12. Ribulose phosphate 3-epimerase</td>
<td>775</td>
<td>650</td>
</tr>
<tr>
<td>KDPG aldolase/6-PG dehydratase</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>630</td>
<td>590</td>
</tr>
<tr>
<td>NADPH oxidase</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>NADH dehydrogenase</td>
<td>740</td>
<td>510</td>
</tr>
</tbody>
</table>

* Glucose grown cells
nd not detectable

![Fig. 5. Schematic representation of methanol metabolism in thermotolerant methylotrophic Bacillus strains. The various reactions, indicated by numbers, are catalyzed by the enzymes listed in Table 2. Abbreviations: DHAP, dihydroxyacetone-3-phosphate; GAP, glyceraldehyde-3-phosphate; E4P, erythrose-4-phosphate; Ru5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; H6P, hexulose-6-phosphate; FBP, fructose-1,6-bisphosphate; S7P, sedoheptulose-7-phosphate](image)

sion of methanol into formaldehyde. Inhibition studies revealed that electrons from methanol oxidation are donated to the electron transport chain at, or above, the level of cytochrome b. This also explains the relatively high growth yield of the Bacillus strains on methanol (Dijkhuizen et al. 1988).

In general the rate of an enzymatic reaction is favoured when reaction products are immediately further metabolized. This becomes especially important when thermodynamically unfavourable reactions have to be carried out. Multienzymes, as well as enzyme complexes are then thought to play a role (Sere 1987). The redox potential of the couple methanol/formaldehyde (E° = –182 mV) strongly favours the formaldehyde reductase reaction. It is therefore not surprizing that methanol oxidation in methylotrophic bacteria apparently requires the presence of specially adapted enzymes. In Gram-negative strains, a modifier protein is involved in the regulation of MDH activity by decreasing its affinity for formaldehyde, which is also a substrate for the enzyme in vitro (Page and Anthony 1986). In Nocardia sp. 239, an enzyme complex is formed between the nMDH and NAD-dependent formaldehyde dehydrogenase and NADH dehydrogenase, which further metabolize the reaction products (Duine et al. 1984). The results obtained in the present study show that Bacillus sp. Cl employs a novel methanol dehydrogenase with a relatively low affinity for methanol. The question therefore has to be asked how the organism manages to maintain the high turnover rate of methanol into formaldehyde required for fast growth on the C1 compound methanol (Attwood and Quayle 1984). First of all, the problem of low affinity of the methanol dehydrogenase for methanol is at least partly overcome by synthesizing high levels of the enzyme. In methanol-limited cells, the enzyme may constitute up to 22% of total soluble protein (D = 0.10 h⁻¹). Secondly, the high HPS as well as NADH dehydrogenase activities observed in these bacteria may well ensure that in vivo the methanol dehydrogenase reaction is favoured over the formaldehyde reductase reaction. Maintenance of a relatively high internal pH would also pull the reaction towards formaldehyde and NADH. Various observations indicate that the methanol dehydrogenase present in Bacillus sp. Cl in fact is (part of) a rather complex system. In extracts, but not with whole cells, biphasic kinetics was observed for methanol oxidation. During enzyme purification it was noticed that the enzyme rapidly lost methanol dehydrogenase activity. Moreover, the dehydrogenase activity of the enzyme, but not the reductase activity, appeared to be very sensitive to dilution. Dilution inactivation could be largely overcome via osmotic stabilization, which might be an indication that the methanol dehydrogenase in Bacillus sp. Cl resides in a protein complex. None of the MDH and ADH enzymes described so far require Mg²⁺-ions for activity. Since the reverse reaction does not depend on the presence of Mg²⁺-ions, it appears likely
Table 3. Comparison of characteristic properties of alcohol dehydrogenases from various sources

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Methanol dehydrogenase in methylotrophic bacteria</th>
<th>ADH from Saccharomyces cerevisiae (EC 1.1.1.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram-negative (MDH; EC 1.1.99.8)</td>
<td>Gram-positive Nocardia sp. 239 Bacillus sp. Cl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Assay</th>
<th>PMS/DCPIP (A600)</th>
<th>NAD/DCPIP (A600)</th>
<th>NAD (A660)</th>
<th>NAD (A340)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄⁺-requirement</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg²⁺-stimulation</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NAD-dependence</td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>PQQ-cofactor</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Km [methanol]</td>
<td>5 - 30 µM</td>
<td>nd</td>
<td>3.8 - 166 mM</td>
<td>nu</td>
</tr>
<tr>
<td>Localization</td>
<td>Periplasm</td>
<td>Cytoplasm?</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Structure</td>
<td>1 or 2 subunits of 56,000 - 76,000</td>
<td>nd</td>
<td>6 - 8 subunits of 43,000</td>
<td>4 subunits of 40,000</td>
</tr>
</tbody>
</table>

Table 3 continues...


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