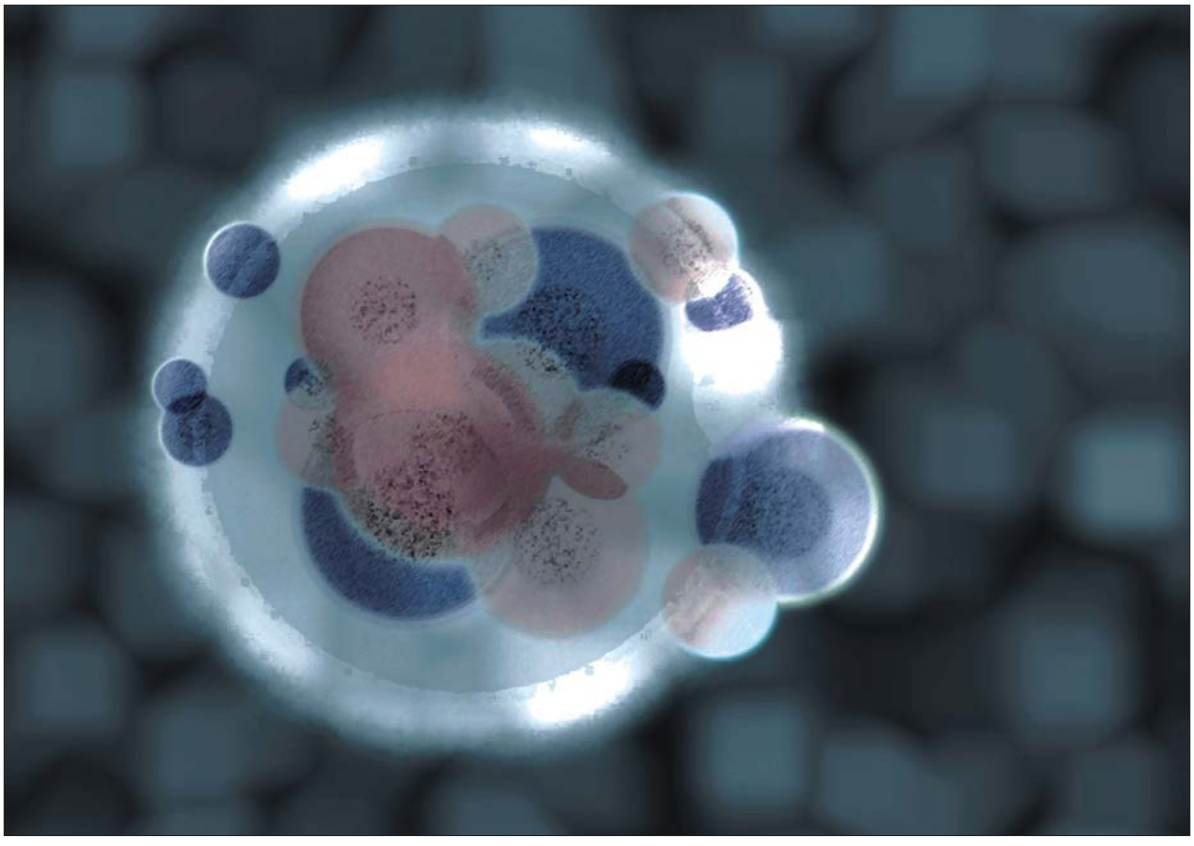

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NAD^+-Dependent Methanol Dehydrogenase from One-Carbon Compound Utilizer Thermoactinomycete

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Abstract: The methylotrophic thermotolerant actinomycete strain 381, isolated from a compost sample, assimilated methanol during growth. Methanol dehydrogenase could not be detected in cell extracts using dye-linked assays in the absence of NAD^+. Dichlorophenol-indophenol-linked methanol oxidation was detected in broken cell suspension in the presence of NAD^+ and the absence of cyanide. Taxonomic studies showed that actinomycete strain 381 was distinct from *Nocardia* species 239, which has also been reported to contain an NAD^+-dependent methanol dehydrogenase.

Key words: Methylotrophic, thermotolerant, actinomycetes, NAD^+-dependent, methanol dehydrogenase (MDH)

INTRODUCTION

Most methylotrophic bacteria so far described oxidize methanol by means of an NAD $(P)^+$-independent dehydrogenase (EC 1.1.99.8), the activity of which is measured at high pH in the presence of NH_4^+ salts via dichlorophenol-indophenol reduction. The enzyme oxidizes a wide range of primary alcohols *in vitro*, with 5-methylphenazinium methyl sulfate (PMS; 'phenazine methosulphate' as the electron acceptor (Amaratunga *et al*., 1997; Anthony, 1998, 1999; Anthony and Ghosh, 1998; Bishop *et al*., 1998). This classical methanol dehydrogenase (EC 1.1.99.8; MDH) is a quinoprotein, having pyrroloquinoline quinone (PQQ) is its prosthetic group (De Boer *et al*., 1990; Cozier *et al*., 1995; Euverink *et al*., 1996; Bystrykh *et al*., 1997; Duine, 1999).

The specific activity of MDH, in crude extracts from different bacteria varies over a wide range 70-22000 pmol/sec/mg protein, being usually 1-10 nmol/sec/mg protein. This reflects to some extent the variety of growth conditions and methods of cell breakage and enzyme assay (Frank *et al*., 1989; Frébortová *et al*., 1997; Ferry, 1999; Felder *et al*., 2000). However, this type of MDH may not be universally involved in the bacterial oxidation of methanol. For example, in some Gram-positive methylotrophic bacteria classical MDH could not be detected (Hazeu *et al*., 1983; Goodfellow, 1996; Goodwin and Anthony, 1998).

The main objective of our study was to characterize the isolated microorganism by physiological and biochemical way (assimilation and dissimilation of a variety of one carbon compounds). Cell free extracts of *Nocardia* sp. 239 was reported to oxidize methanol via a dye-linked dehydrogenase (although at a low rate) in the absence of NH_4^+ salts at pH 7.0 (Hektor and Dijkhuizen, 1996; Hektor *et al*., 2000). Other investigations also confirmed this enzyme in the above orgamism (recently classified as *Amycolatopsis methanolica* (Itoh *et al*., 2000); the presence of the classical MDH has not been demonstrated (Luykx *et al*., 1998; Kim *et al*., 1999; Malashenko *et al*., 2000). Since it was observed that the methanol-grown organism excreted PQQ (just like Gram-negative methanol utilizers), the question arose whether an unusual quinoprotein, methanol oxidizing enzyme might be present in this organism (Kato *et al*., 1975; Felder *et al*., 2000).

MATERIALS AND METHODS

Chemicals: 2, 6-dichlorophenolindophenol (sodium salt; DCPIP), flavin mononucleotide (FMN); disodium salt), flavin adenine dinucleotide (FAD; sodium salt), 5-methylphenazinium methyl sulfate and Brilliant blue G were from Sigma, other chemicals for general purposes were supplied by *Becton Dickinson*.

***Pseudonocardia* sp.** (called actinomycete strain 381) able to grow on methanol was isolated from compost. The aerial mycelium of the orgamism developed well at 45°C on the solid Chelate Mineral Medium (CMM) containing 0.5% (V/V) methanol. Under such conditions, the aerial mycelium was white, straight and only rarely branched and did not form whorls or spirals (Fig. 1). The aerial

mycelium fragmented to produce oval shaped spores with relatively smooth-surfaces (Fig. 2). Substrate mycelium had a characteristic zig-zag appearance. The organism is a facultative methylotroph, capable of growing on rich media such as Bennett's and glucose-yeast extract agar and CMM, however, the aerial mycelium produced on rich media was sparse or it did developed. The colonies which appeared on Bennett's agar plates were cream to yellowish and did not change color or produce any pigment when becoming older.

Substrate oxidation by the cell suspension: Methanol, pyruvate, dimethyl sulfide and trim-ethylamine oxidation and potential respiration rates were assayed polarographically at 45°C using a Clark-type oxygen electrode. A 3 mL reaction mixture contained broth culture medium plus an appropriate amount of fresh cells (1.4 mg wet mass) and substrate (Table 1). The rate of oxygen consumption was corrected as necessary for endogenous respiration and autoxidation. For the measurement of potential respiration rates, cells were used immediately after removal from the incubator. Oxygen consumption rates were measured on addition of different substrates and the rate of change in dissolved oxygen content was recorded, over a period of 10 min.

Spectrophotometric and kinetic determinations: Enzymatic activities of the 381 strain were assayed spectrophotometrically. The protein content was determined according to Bradford (1976). The assays were done on *LKB* Ultrospec II spectrophotometer; the rate of absorbance change was recorded. Dehydrogenase activity was assayed at 45°C with and without NAD^+, in two different systems. Freshly prepared cell extract of strain 381 were disrupted using X-press and used for further measurements.

- NAD^+-independent methanol dehydrogenase. This enzyme was assayed at 45°C as a dye-linked dehydrogenase according to Anthony and Zatman (1976) and by the addition of potassium cyanide. The re action mixture contained in a total volume of 3 mL (in µmol): Tris-HCl (pH 9.0) 300, NH_4Cl 45.0, CH_3OH 20.0, PMS 3.3, KCN 3.0, DCPIP 0.13 and cell extract as required. The reaction was started by the addition of freshly prepared cell extract and the initial rat of reduction of DCPIP was measured at 600 nm in the presence of PMS as an electron carrier. Endogenous levels of activity for each of the assays were determined by repeating the assay without substrate. Controls were also carried out using boiled cell extract.

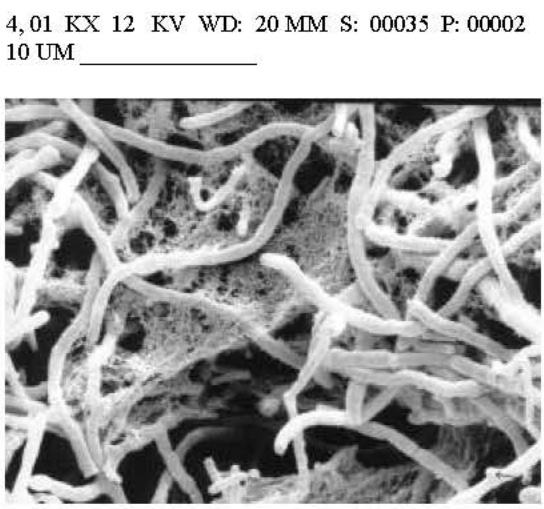

Fig. 1: The start of the sporulation process: septation and swelling can be seen

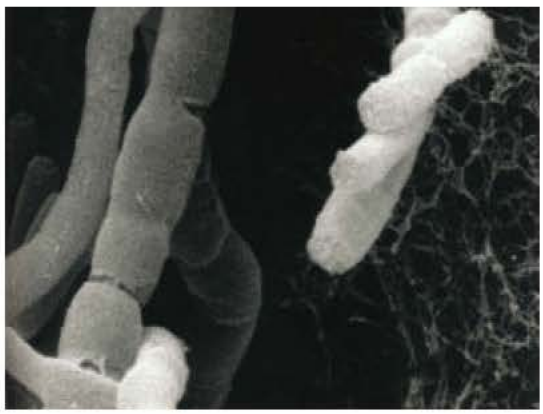

Fig. 2: Septation at advanced stage and some mature spores showing spore surface

Table 1: Oxygen uptake by *actinomycete* stain 381 in CMM medium with various substrate at 45°C

Substrates	Oxygen uptake rate*
Methanol (0.5% V/V)	8.00
Pyruvate (0.05% W/V)	1.40
Pyruvate (0.1% W/V)	5.31
Methanol (0.5% V/V) + pyruvate (0.05% W/V)	6.67
Methanol (0.5% V/V) + pyruvate (0.1% W/V)	9.13
Dimethylamine (0.1% V/V)	0.03
Dimethylamine (0.4% V/V)	0.00
Trimethylamine (0.1% V/V)	1.50
Trimethylamine (0.4% V/V)	0.37
Dimethylsulphide (0.1% V/V)	0.24
Dimethylsulphide (0.4% V/V)	0.08
Formaldehyde (0.005% V/V)	0.00
Formaldehyde (0.01% V/V)	0.00

*Decrease in dissolved oxygen concentration (as % of saturation) per min; each incubation vessel contained approximately 0.4 g wet mass bacterium

- NAD^+- dependent methanol dehydrogenase. An enzyme assay system (total volume of 3 mL) was prepared with the following composition (in μmol): NH_4Cl 360 tetrasodium diphosphate (pH 9.0) 300, NAD^+ 7.5, CH_3OH 6.0, KCN 3.0, DCPIP 0.13 and cell extract as required. The reaction was done as in the case of (*a*).

One unit of enzyme activity was defined as the amount of enzyme which catalyses the reduction of 1 μmol of DCPIP per min (Bamforth and Quayle, 1977) the molar absorption coefficient of DCPIP AT 600 nm and pH 9.0 is 21.99×10^3 L $mol^{-1}cm^{-1}$ (Armstrong, 1964).

RESULTS AND DISCUSSION

The strain 381 was characterized as a wall type IV actinomycete facultative methylotroph capable of growth on CMM broth medium containing methanol (0.1, 0.5 or 1.0% V/V). The organism was not able to grow on solid medium containing 0.1% (V/V) of ethanol, n-propanol, n-butanol, n-pentanol, n-octanol and propandiol. Although the strain 381 grew well on medium containing only methanol, inorganic salts and vitamins, the growth rate was stimulated by the addition of an organic substance, such as 1% glucose, 0.5% yeast extract, or 0.01% casamino acids (all three W/V) to the medium. The organism also metabolized other substances as its sole source of carbon and energy. These include glycerol, adonitol, *meso*-erythritol *meso*-inositol, mannitol, D-fucose, L-fucose, D-arabinose, L-arabinose, D- mannose, D- fructose, cellobiose, lactose, maltose, melibiose, sucrose, dextrin, L-proline, salicin, L-threonine (all at concentration of 1% W/V) and (1R,3R, 4R, 5R)-1, 3, 4, 5-tetrahydroxycyclohexanecarboxylic acid (quinic acid; 0.01% W/V); it used ammonium chloride as a nitrogen source. Oxygen consumption rates were measured according to the method described. The results recorded in Table 1 demonstrated the oxidation of methanol and, to a lesser extent, of trimethylamine and pyruvate. Consistent with the growth experiments, dimethylamine was not oxidized.

Methanol dehydrogenase: A trace of MDH activity could be detected in the absence of NAD^+, either in the presence or absence of PMS or flavin coenzymes. The addition of NAD^+ stimulated activity at least ten-fold (Table 2). The activity was also stimulated by NH_4^+, but it was inhibited by addition of KCN. NAD^+-dependent MDH activity could also be detected in extracts from the Braun-cell homogenizing (Table 3). Maximum activity required the presence of the particulate fraction of the cell, presumably maintenance fragments.

Table 2: The effect of KCN, NAD^+ and NH_4^+ addition on the methanol dehydrogenase activity of actinomycete strain 381

Substrate (Methanol)	KCN	NAD^+	NH_4^+	DCPIP (L mol^{-1} cm^{-1})
+	+	+	+	2.21
+	-	+	+	2.76
+	-	-	+	0.28
+	-	+	-	1.38
Control 1	+	+	+	0.23
Control 2	+	+	+	0.20

Bacteria were broken in the X-Press. After centrifugation at 48,000 x g for 20 min at 2°C, the supernatants were assayed for MDH activity

Table 3: The methanol dehydrogenase activities in actinomycete 381

Addition of cell extract	Substrate (Methanol)	DCPIP (L mol^{-1} cm^{-1})
No extract	+	0.11
cell supernatant	+	3.04
broken cell suspension	+	19.61

As previously mentioned most methylotrophic bacteria so far described oxidize methanol by means of an NAD $(P)^+$-independent dehydrogenase. The enzyme oxidizes a wide range of primary alcohols *in vitro*, with phenazine methosulphate as the electron acceptor (Amaratunga *et al*., 1997; Anthony and Ghosh, 1998; Anthony, 1999).

NAD^+-dependent methanol-utilizing *actinomycete* strain 381 was found a facultative methylotroph, capable of growing under autotrophic and heterotrophic conditions. Dichlorophenol-indophenol-Linked methanol oxidation was detected in broken cell suspensions of the bacteria in the presence of NAD^+ and the absence of cyanide. Preliminary chemotaxonomic studies have been carried out on *actinomycete* strain 381 to examine its relationship to the *Nocardia* species 239. NAD^+-dependent methanol dehydrogenase has also been described in *Nocardia* species 239 (Hektor *et al*., 2000; Piersma *et al*., 1998).

In the strain 381, the enzymes, hydroxypyruvate and glyoxylate reductase were much more active in comparison to all other enzymes including methanol dehydrogenase. The activity was confirmed using the control organism *Methylobacterium organophilum* (Schuppler *et al*., 1995; Springer *et al*., 1998; Turlin *et al*., 1996; Van Spanning *et al*., 2000). The results of the activity of methanol dehydrogenase showed that even under the best assay conditions, the activity was much lower than the activities of the two assimilatory enzymes of the serine cycle. This may reflect tight control of methanol utilization at the level of methanol

dehydrogenase, but alternatively could indicate severe disorganization of the complex methanol oxidizing machinery during either the preparation of the cell free extract and or during the enzyme system preparation (Ferry, 1999; Hazeu *et al*., 1983).

The results suggest that the major assimilation route is the serine pathway. The negligible activity of hexulose phosphate synthase and phosphoribuloisomerase, the two key enzymes of the ribulose monophosphate (RuMP) pathway, suggest that neither the RuMP nor RuBP pathways are operational. This conclusion provides further evidence that the serine cycle was responsible for the assimilation of the carbon substrates in *actinomycete* strain 381.

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