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A Methylotrophic Thermotolerant Actinomycete With a NAD^+-Dependent Methanol Dehydrogenase

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ABSTRACT

The methylotrophic thermo tolerant actinomycete strain 381, was 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 suspensions 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. The organism can also utilizes pyruvate, casamino acid, glucose, glycerol and many other carbon sources as described before. Preliminary enzyme studies suggested that the cell extracts contained phosphoribulokinase and (RUBP) ribulose bisphosphate carboxylase (the two key enzymes for Calvin cycle) which–compounds indicating that actinomycete strain 381, assimilates C1by the means of Serine pathway.

INTRODUCTION

Most methylotrophic bacteria so far described oxidize methanol by means of a NAD $(P)^+$–independent dehydrogenase, the activity of which is measured at high pH in the presence of $NH4^+$ salts via dichlorophenol-indophenol reduction (1,4,7). The enzyme oxidizes a wide range of primary alcohols in vitro, with phenazine methosulphate as the electron acceptor. This classical methanol dehydrogenase (MDH) is a quinoprotein, since pyrroloquinoline quinone (PQQ) is its prosthetic group (3, 4,5,7).

The specific activity of MDH, in crude extracts from different bacteria, varies over a wide range between 4 and 1300 nmol per minute per mg protein, but it is usually between 60 and 600 nmol per minute per mg protein. This reflects to some extent, the variety of growth conditions and methods of cell breakage and enzyme assay (8,9,10). However, this type of methanol dehydrogenase may not be universally involved in the bacterial oxidation of methanol. For example, in some gram-positive methylotrophic bacteria, classical methanol dehydrogenase could not be detected (3,12,13).

Cell free extracts of Nocardia sp. 239 were reported to oxidize methanol (although at a low rate) via a dye-linked dehydrogenase at pH 7.0 in the absence of $NH4^+$ salts (28). Other investigations confirmed that in this organism, which has recently been classified as Amycolatopsis methanolica (1,15, 16,17,18), the presence of the classical methanol dehydrogenase could not be demonstrated (19). 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 (4,9, 20).

MATERIALS AND METHODS

Chemicals

2,6-Dichlorophenolindophenol (sodium salt), flavin mononucleotide (FMN) (disodium salt), flavin adenine dinucleotide (FAD) (sodium salt), phenazine methosulphate, and Brilliant blue G were supplied by Sigma Chemical Company Ltd., other chemicals for general purposes, were supplied by BDH Ltd.

Actinomycetes strain 381

In the course of an investigation of methanol utilization by microorganisms, an actinomycete able to grow on methanol was isolated from compost and temporarily named Actinomycete strain 381. The aerial mycelium of the organism developed well at 45 ºC 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 as well as CMM, but the aerial mycelium produced on rich media was sparse or non-existent. The colonies which appeared on Bennett's agar plates were cream to yellowish, neither changing in color when becoming older nor producing pigments.

Actinomycete 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-butanol, n-propanol, propandi-ol, n-pentanol and n-octanol. Although the actinomycete strain 381 grew well on medium containing only

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methanol, inorganic salts and vitamins, the growth rate was stimulated by the addition of an organic substance, such as glucose (1% w/v), yeast extract (0.5% w/v), or casamino acids 0.01% (w/v) to the medium. The organism also metabolizes other substances as its sole source of carbon and energy. These include glycerol, adonitol, meso-erythritol meso-inositol, mannitol, L-arabinose, D-arabinose, dextrin, D-fructose, D-mannose, D-fucose, L-fucose, D-cellobiose, lactose, maltose, melibiose, sucrose, L-proline salicin, L-flueonine (at a concentration of 1% w/v) and quinic acid (0.01% w/v). The organism could use ammonium chloride as a nitrogen source.

RESULTS

Substrate Oxidation by the Cell Suspension

Methanol, pyruvate, dimethyl-sulphide and trimethylamine oxidase activities, and potential respiration rates were assayed polarographically at 45 ºC using a Clark-type oxygen electrode. proposed percentage of substrate (Table 1). The rate of oxygen A 3 ml reaction mixture contained broth culture medium plus an appropriate amount of fresh cells (1.4 mg wet weight) and 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.

Methanol Dehydrogenase (MDH)

Methanol dehydrogenase activity was measured spectrophotometrically at 45 ºC with NAD^+ and 2,6 dichlorophenolindophenol (DCPIP) at pH 9.0, in the presence of ammonium chloride, cyanide and 20 mM methanol. Freshly prepared cell extracts disrupted by X-Press was used and the rate of change in absorbance was recorded. A trace of activity could be detected in the absence of NAD^+, either in the presence or absence of phenazine methosulphate, or flavin coenzymes. The addition of NAD^+ stimulated activity at least ten fold (Table 2). The activity was also stimulated by $NH4^+$, but was inhibited by addition of KCN.

NAD^+-dependent methanol dehydrogenase 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.

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

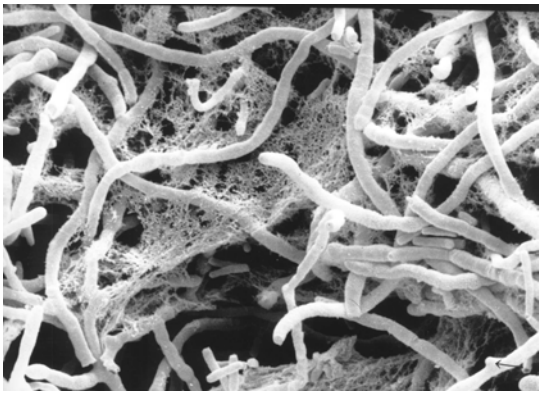

Fig. 2. **Septation at stage and some mature spores showing spore surface**

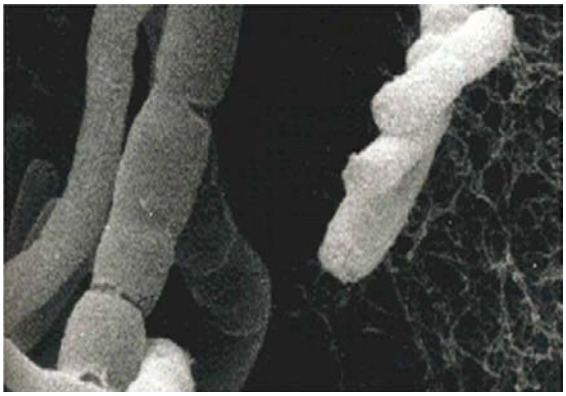

Table 1. **Oxygen uptake by actinomycete stain 381in CMM medium With various substrates at 45 ºC**

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
Dimethyl-amine (0.1% v/v)	0.03
Dimethyl-amine (0.4% v/v)	0.00
Trimethyl-amine (0.1% v/v)	1.50
Trimethyl-amine 0.4%(v/v)	0.37
Dimethyl-sulphide 0.1%(v/v)	0.24
Dimethyl-sulphide 0.4%(v/v)	0.08
Formaldehyde (0.005% v/v)	0.00
Formaldehyde (0.01% v/v)	0.00

*Reduction of dissolved oxygen concentration (as % of saturation) per minute.
Each incubation contained approximately 0.4 gram wet weight of the bacterium

Table 2. **The effect of KCN, NAD^+ and $NH4^+$ addition on the dehydrogenase activity of actinomycete strain 381**

Substrate	Addition of:			Dichlorophenol-indophenol (nmol min^{-1} mg^{-1} protein)
Methanol	KCN	NAD^+	NH_4^+	
+	+	+	+	2.21
+	–	+	+	2.76
+	–	–	+	0.28
+	–	+	–	1.38
Boiled cell extract or no methanol	+	+	+	0.23

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. The initial rate of change of absorbance followed at 600 nm. The reaction mixture contained in a total volume of 3 ml: 0.3 mmole of tetrasodiun pyrophosphate (TSPP) pH 9.0; 0.12 µmole of 2,6 dichlorophenolindophenol; 0.36 µmole of NH_4Cl; 6.0 µmoles of CH_3OH; 7.5 µmoles of NAD^+ and 3 µmoles of KCN.

Table 3. **The methanol dehydrogenase activities in act actinomycete 381**

Substrate (Methanol)	Addition of cell extract	DCPIP reduction (nmol min^{-1} mg^{-1} protein)
+	No extract	0.11
+	cell supernatant	3.04
+	broken cell suspension	19.61

Bacteria were broken in the X-Press. After centrifugation at 48,000 X g for 20 min at 2 ºC, the supernatants were assayed for MDH activity.
The initial rate of change of absorbance followed at 600 nm. The reaction mixture contained in a total volume of 3 ml: 0.3 mmole of tetrasodiun pyrophosphate (TSPP) pH 9.0; 0.12 µmole of 2,6 dichlorophenolindophenol; 0.36 µmole of NH4Cl; 6.0 µmoles of CH3OH;7.5 µmoles of NAD+ and 3 µmoles of KCN.

DISCUSION

Methanol-utilizing *actinomycete* strain 381 is a facultative methylotroph, capable of growing under autotrophic and heterotrophic conditions. NAD^+-dependent methanol dehydrogenase has previously been described in Nocardia species 239 (13,14,21,22). Preliminary chemotaxonomic studies have been carried out on actinomycete strain 381 to examine its relationship to the Nocardia species 239.
Chemical analyses of whole cell hydrolysates demonstrated the presence of meso-DAP, arabinose and galactose in major quantities. This chemical profile is characteristic of the so-called wall type IV actinomycetes (6,23,24,25). Mycolic acids were not present and the membrane fatty acid pattern was complex including saturated, monounsaturated and branched iso, anteiso and 10-methyl fatty acids. Isoprenoid quinones were present exclusively as menaquinones having eight isoprene units and containing two, four or six points of saturation. The major polar lipids present in chloroform /methanol extracts, were phosphatidylglycerol, diphosphatidyl-glycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidyl-choline. This additional chemical information confirms the taxonomic position of strain 381 as an amycolate wall type IV *actinomycete* (14,24,25,27,28).
Following the enzymatic investigation of the organism it was examined for the presence and activity of key enzymes operative in the ribulose monophosphate, ribulose bisphosphate and serine assimilatory pathways. The discovery that the two key enzymes, hydroxypyruvate and glyoxylate reductase of the serine pathway were present in actinomycete strain 381, was not in good agreement with Amycolatopsis methanolica which assimilates the methanol via ribulose monophosphate (19).
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 (1, 25). 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.

The detectable activities of ribulose bisphosphate carboxylase and phosphor-ribulokinase, the two key enzymes of the ribulose bisphosphate pathway (RUBP; Calvin cycle), were much lower than the activity of methanol dehydrogenase. Therefore 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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