Isolation and Characterization of the Facultative Methylotroph *Mycobacterium* ID-Y

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A facultatively methylotrophic *Mycobacterium* was isolated from Cleveland Harbor, Ohio, USA. The isolate, designated ID-Y, used a wide range of carbon and energy sources including methane and several other hydrocarbons. It displayed a growth cycle from rod-shaped exponential-phase cells, with many cell pairs exhibiting V-formation, to cocco-bacillary stationary-phase cells. A fixation technique involving glutaraldehyde/alcian blue resulted in the observation of a three-layered cell wall. Isolate ID-Y has an ultrastructure similar to that of other mycobacteria, particularly *Mycobacterium phlei* and *Mycobacterium flavum*, which is presently classified as a *Xanthobacter* species.

INTRODUCTION

Two major groups of methane-oxidizing bacteria are recognized, obligate and facultative. Obligate methane-oxidizing bacteria are strictly aerobic, Gram-negative rods, vibrios or cocci which use only C_1 compounds or multiple carbon compounds with no carbon-to-carbon bonds. The majority of the facultative methane-oxidizing bacteria have similar characteristics and also the ability to utilize organic compounds with more than one carbon atom and carbon-to-carbon bonds. The methane-oxidizing mycobacteria have been studied infrequently, possibly due to the difficulty of isolation of these organisms in pure culture; they do not appear to fit the general description of the majority of facultative methylotrophs. This report describes a facultatively methylotrophic *Mycobacterium* that has a three-layered cell wall and displays characteristics similar but not identical to the organism originally described by Nechaeva (1949), and provides an isolate for further study of a facultative methylotroph thought to be no longer in existence.

METHODS

Isolation and maintenance of cultures. Enrichment cultures of methylotrophic bacteria were obtained by inoculating 5 ml Lake Erie water into a rubber-stoppered 74 ml serum bottle containing 35 ml sterile CM mineral salts medium (CM; Weaver & Dugan, 1975). Before inoculation, 10 ml air was withdrawn from and 10 ml sterile CH_4 injected into each of the stoppered serum bottles. After inoculation, the enrichment cultures were incubated statically for 48 h at 30 °C. Samples from the enrichment cultures were then streaked onto CM agar and incubated in a desiccator under CH_4 /air (1:1, v/v) at 30 °C. After several transfers on CM agar under CH_4 /air, one of four isolates, designated ID-Y, was capable of growth on trypticase soy agar (TSA, BBL). ID-Y was repeatedly transferred from TSA plates to CM agar plates under CH_4 /air and from CM plates back to TSA plates. A colony was diluted in liquid CM medium and the greatest dilution demonstrating growth was streaked onto mineral salts agar and incubated under CH_4 /air (1:1, v/v).

Liquid cultures were maintained in rubber-stoppered 125 ml Erlenmeyer flasks containing 25 ml CM medium. Before inoculation the rubber stopper of each flask was swabbed with an acetone-saturated piece of cotton. Culture fluid was injected through the rubber stopper with a sterile syringe. After inoculation, the flask was purged with glasswool-filtered CH_4 /air (1:1, v/v). The bottles were agitated on a rotary shaker at 95 r.p.m. Cultures were also maintained on CM agar plates in a glass desiccator filled with CH_4 /air (1:1, v/v). The desiccators were

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swabbed with acetone before each weekly culture transfer to combat contamination with fungi. The purity of the CH₄ was 99.1%.

Growth studies. The ability of ID-Y to grow on numerous substrates as sole carbon and energy sources was investigated. CM agar plates containing 0.5% (w/v) lignin sulphonate [Maracell E, Marasperse CB, (American Can Co.), Indulin C or Polyfon O, (Westvaco)] or 1% (w/v) glucose or nutrient agar (Difco) were inoculated, incubated for 2 weeks at 21 °C, and observed for growth. Liquid cultures of CM medium containing 1% glucose, 0.2% succinate, 0.1% methanol, 0.1% ethanol, 0.1% hexane or 0.1% hexadecane were incubated at 21 °C on a rotary shaker (160 r.p.m.) and observed for growth after 2 weeks. Flasks containing CM medium and ID-Y were incubated similarly, under an atmosphere of air plus methane, ethane, propane or butane (1:1, v/v) and observed for growth after 2 weeks. Four-day liquid cultures (early stationary) of ID-Y grown on CH₄ were used as inocula for growth studies. The initial cell density for all growth studies in liquid culture was 6×10^7 cells ml⁻¹. Ion Agar no. 2 (Oxoid) at a concentration of 0.9% (w/v) was used in all agar plates. Carbon sources were autoclaved or filter-sterilized separately and added to the growth medium.

Gas analysis. Methane was analysed with a Varian 2740 series gas chromatograph equipped with a 183 × 0.32 cm steel column packed with Porapak Q and a flame ionization detector. The carrier gas was prepurified N₂ with a flow rate of 20 ml min⁻¹. Temperatures of 105 °C for the column and 175 °C for the injector and detector were maintained. Gas samples were collected from the headspace of culture serum bottles with a Pressure-Lok 100 µl gas syringe. Sterile serum bottle controls were analysed to account for adsorption of CH₄ by the bottle or loss via diffusion through the butyl rubber stoppers.

Isolation of DNA and buoyant density determination. Cells were disrupted by liquid nitrogen cryo-impacting (Smucker & Pfister, 1975). The disintegrated cell mass was held at -20 °C until needed. DNA was extracted using a method adapted from Marmur (1961) and Bolivar & Backman (1979). Crude *Escherichia coli* lysates (courtesy of William F. Martin, Ohio State University) were similarly extracted, purified and used for comparison. The DNA was analysed by CsCl density gradient centrifugation with an adaptation of the methods of Schildkraut *et al.* (1968). Solution density was determined from the refractive index (Weast, 1979). The guanine plus cytosine content was calculated from the buoyant density using the relationships published by Schildkraut *et al.* (1962). *E. coli* DNA was used as an internal marker.

Electron microscopy. Specimens were prepared for thin sectioning by fixation for 5 h at 21 °C in a glutaraldehyde/alcan blue solution as employed by Benke & Zelander (1970): 16 ml 25% (v/v) glutaraldehyde and 7 g alcian blue were dissolved in veronal/acetate buffer (Kellenberger *et al.*, 1958) to make 100 ml. The sample was washed twice with distilled H₂O and post-fixed in 2% (w/v) OsO₄ in veronal/acetate buffer overnight at 4 °C. The sample was washed twice in veronal/acetate buffer. Post-fixation in 2% (w/v) uranyl acetate was carried out for 2 h at 21 °C. The cells were washed in veronal/acetate buffer and resuspended in 2% agar. Small blocks of the agar were rinsed with 50% (v/v) acetone and immersed in 2% uranyl acetate in 10% acetone for 15 min. The blocks were dehydrated and embedded in Spurr's low viscosity embedding medium (Spurr, 1969). Thin sections were cut with a diamond knife on an LKB model III ultramicrotome. Sections were post-stained with both uranyl acetate and lead citrate. Cells were prepared for freeze etching by the procedure of Steere (1957). A Philips EM-300 was used to examine all specimens. Exponential-phase cells were used for electron microscopy except where otherwise indicated.

RESULTS AND DISCUSSION

ID-Y cells were non-motile, Gram-positive, acid-fast rods identified as a *Mycobacterium*. Colonies on semi-solid growth media were smooth, raised, entire, and yellow pigmented. Biochemical tests with cells grown on either CM medium under CH₄/air or trypticase soy broth indicated that the cells are catalase and oxidase positive and reduced nitrate to nitrite. Negative results were recorded for phenylalanine deamination, H₂S production, motility, indole production, methyl red/Voges-Proskauer reactions, gelatin hydrolysis, starch hydrolysis and skim milk alteration. No acid or gas was produced from arabinose, dulcitol, galactose, glucose, lactose, maltose, mannose, rhamnose, sorbitol, sucrose, trehalose or xylose.

DNA of the cryoimpacted ID-Y cell preparation yielded about 400 µg per g bacteria (wet weight) and had a density of 1.727 g cm⁻³, which corresponds to a guanine plus cytosine content of 68 mol%. The DNA from ID-Y grown on methane had the same density as that from cells grown on glucose, which supports the conclusion that the isolate is a single strain and not a mixture of two different micro-organisms.

After 14 d ID-Y formed colonies of 1 mm diameter on CM agar under CH₄/air and of 1 to 1.2 mm diameter on 1% glucose/CM agar. The isolate grew poorly on nutrient broth agar, producing pinpoint colonies after 10 d, but grew well in nutrient broth.

ID-Y grew over a temperature range from 15 to 42 °C, with an optimum range from 28 to 35 °C. At temperatures above 31 °C opaque colonies with little or no pigment were produced. However, when plates containing these opaque colonies were transferred to 28 °C the yellow pigment appeared within 2 h. The pigment was completely extractable with ethanol and was the same in the presence or absence of light.

ID-Y was capable of growth on many substrates including compounds as complex as ligninsulphonates (CM agar plates containing 0.5% Maracell E, Marasperse CB, Indulin C or Polyfon O). Growth was also observed on glucose, propane, butane, hexane, hexadecane and methane but not on ethane, ethanol, methanol or succinate. A generation time of 25.7 h on CH₄ and 10.3 h on 0.05% yeast extract was determined for ID-Y at 21 °C.

ID-Y grew as rods of 1.3 to 2.6 µm × 0.2 to 0.3 µm in 24 h nutrient broth cultures during exponential phase but the cells were cocco-bacillary (0.4 to 0.8 µm diameter) during stationary phase. In either glucose or CH₄ cultures, the cells in stationary phase were shorter than those of exponentially growing cells. The organism displayed V-forms in most exponential and some stationary cultures. When grown in hexadecane/CM medium most of the cells partitioned into the oil droplets. Although ID-Y appears to be similar to the methylotrophic *Mycobacterium* spp. originally described by Nechaeva (1949) and Seto *et al.* (1975), which are no longer available, it differs from *Mycobacterium cuneatum* in its ability to grow on hexane. *M. cuneatum* grew on ethane, ethanol and methanol whereas ID-Y did not. ID-Y had a guanine plus cytosine content of 68 mol% as compared to 64.9 mol% and 66.3 mol% for two strains of *M. cuneatum*. ID-Y differs from *M. flavum* var. *methanicum* and *Mycobacterium methanicum* (Nechaeva, 1949) in its ability to reduce nitrate and hydrolyse starch. ID-Y also has traits similar to descriptions of *Mycobacterium gordonae* (Park & DeCiccio, 1974) and to *Xanthobacter flavus*, a species which includes some isolates previously classified as *Mycobacterium flavum* (Wiegel & Schlegel, 1984).

The effect of increasing concentrations of yeast extract on CH₄ consumption is shown in Fig. 1. Supplementation with 0.001% yeast extract resulted in an initial stimulation of CH₄ consumption which probably reflects an increase in cell density due to addition of growth factors. After 2 d, consumption of CH₄ ceased. When the concentration of yeast extract was raised to 0.01%, consumption of CH₄ again ceased after 2 d following a less dramatic period of stimulation, which suggests that the cells switch to yeast extract as their source of carbon and energy.

Although it was difficult to obtain satisfactory negative strains of ID-Y, probably due to the hydrophobic nature of its surface, Fig. 2 shows internal granules which were occasionally observed at each end of cells grown on many different substrates and always in CH₄-grown cells. Also evident was the outer periphery of the cell wall which looked like a light shadow outside the cell. Pairs of cells were frequently arranged in the characteristic V-formation as shown in Fig. 2 and described for other mycobacteria (Edwards, 1970; Hirata, 1979; Imaeda & Ogura, 1963; Petitprez *et al.*, 1967) and *Arthrobacter* species (Kolenbrander & Hohman, 1977).

Fixation of cells using the technique of Kellenberger *et al.* (1958) resulted in poor elucidation of the cell wall with badly condensed DNA. However, when a fixation technique using glutaraldehyde/alcian blue was substituted (Benke & Zelander, 1970), the cells displayed fibrillar DNA, a plasma membrane, and a cell wall (Fig. 3). The cytoplasmic membrane showed three layers with the external layer appearing much more electron dense. A periplasmic space was also observed. The three layers of the cell wall had diameters of: 6 nm, inner wall components; 17 nm, middle wall components; 6 nm, outer wall components. This envelope is very similar to those of other mycobacteria as described by Petitprez *et al.* (1967) and Imaeda *et al.* (1968).

Fig. 4 shows a cell from a glucose exponential-phase culture which has completed septum formation. The septum is composed of four components: a plasma membrane and an inner cell wall component on either side of a transparent zone which had an average diameter of 27 nm. The width of the septum was 21 nm at each end and 30 nm at the centre. A mesosome is in close association with the septum. Throughout division, ID-Y cells were cuneate with a distinct taper from the septal to distal ends. Cells from exponential-phase nutrient broth cultures were morphologically and ultrastructurally similar to glucose- and CH₄-grown cells. However, cells

Fig. 1

Fig. 2

Fig. 1. Consumption of CH_4 by ID-Y grown in CM medium without yeast extract (○) and in CM medium plus 0.01% (□) or 0.001% (▲) yeast extract. ●, Control (sterile CM medium).

Fig. 2. Electron micrograph of two ID-Y cells in a V-formation. The cells were taken from a 1 d culture on CH_4 and negatively stained with 1% uranyl acetate. The internal granules (G) were sometimes observed in cells grown on other substrates and always in CH_4 -grown cells at each end of the cell. Also evident is the outer periphery of the cell (CW) which looks like a capsule. Bar, 0.4 μm .

Fig. 3. Thin sections of ID-Y grown on glucose for 1 d and fixed with glutaraldehyde/alcan blue. iwl, Inner wall layer; M, mesosome; mwl, middle wall layer; N, nuclear material; P, periplasmic space; pm, plasma membrane; owl, outer wall layer; si, septum initiation. Bar, 0.4 μm .

Fig. 4. ID-Y grown in glucose for 1 d and fixed with glutaraldehyde/alcian blue. (a) Cell in which septum formation is complete. (b) Enlargement of septum. iwl, Inner wall layer; M, mesosome; mw, middle wall layer; N, nuclear material; P, periplasmic space; pm, plasma membrane; owl, outer wall layer; s, septum; sp, space. Bars, 0.4 μm (a), 0.1 μm (b).

from late stationary (21 d) nutrient broth cultures had a cocco-bacillary shape and displayed a thicker 8.5 nm inner wall component layer and an electron-dense material which was associated with the plasma membrane and in most cells filled the periplasmic space and extended into vesicular mesosomes which were characteristic of these cells (Fig. 5). Similar vesicular mesosomes have been observed in *Mycobacterium lepraemurium* and a *Mycobacterium* sp. (Imaeda & Ogura, 1963) and in *Bacillus subtilis* (Van Itersen, 1961). The intracytoplasmic membrane system characteristic of obligate (Whittenbury *et al.*, 1970) and some facultative methane-oxidizing bacteria (Patt & Hanson, 1978) were absent in ID-Y. It may be that in ID-Y the mesosomes perform the function of the intracytoplasmic membrane systems of other methane-oxidizing bacteria.

Satisfactory freeze-etch preparations of ID-Y were difficult to obtain. Most of the cells did not cleave and organic material could not be completely removed from replicas. However, surface fibrils and rings were observed in freeze-etch preparations. Imaeda *et al.* (1968) reported that fibrils on the surface of young cells showed an irregular arrangement and sometimes twisted

Fig. 5. ID-Y cells from a 21 d stationary phase culture grown in nutrient broth. cwp, Dense material associated with the outer layer of the plasma membrane; ipm, inner layer of the plasma membrane; iwl, inner cell wall layer; opm, outer layer of the plasma membrane; P, periplasmic space. Bar, 0.1 μ m.

taking a 'spun yarn' form, while older cells displayed few fibrils which frequently ran parallel. Surface rings not previously reported on mycobacteria but similar to those observed on *Arthrobacter crystallopoietes* (Kollenbrander & Hohman, 1977) are present in young and old cells. With each cell division a new ring formed on each sister cell. The presence of surface rings, the growth cycle from rod-shaped exponential-phase cells to coccoid stationary cells, and the V-formation displayed by many cell pairs reflects a morphological similarity between ID-Y and the coryneform bacteria of which arthrobacters are members.

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