Isolation and Characterization of Methane-utilizing Yeasts

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Five yeast strains capable of utilizing CH_4 as the sole energy source for growth have been isolated using selective enrichments for methanotrophic micro-organisms. One of the pure cultures was selected for single-cell isolations and antibiotic screening to verify more rigorously the purity of the methanotrophic yeast cultures. Examination of the ultra-structure of this isolate confirmed its eukaryotic nature. The rates of CH_4 oxidation by the isolates were determined by assaying whole cell suspensions for CH_4 -dependent oxygen consumption in an oxygen electrode and by measuring the conversion of $^{14}\text{CH}_4$ to $^{14}\text{CO}_2$ and incorporation of ^{14}C into cellular material by whole cells. Characterization of the isolates suggests that four different species of yeast have been obtained that are capable of utilizing CH_4 .

INTRODUCTION

The ability to utilize hydrocarbons as the sole carbon and energy source for growth has been reported for many eukaryotic and prokaryotic micro-organisms. The compounds utilized most frequently are *n*-alkanes that contain more than 10 carbon atoms. The use of gaseous hydrocarbons as energy sources for microbial growth is much more limited, especially among eukaryotic organisms (Whittenbury, 1971; Shennan & Levi, 1974). An isolate of *Graphium* has been reported to grow on ethane (Zajic *et al.*, 1969; Volesky & Zajic, 1971). The use of CH_4 as a substrate for growth has so far been restricted to prokaryotic organisms (Whittenbury, 1971; Quayle, 1972; Shennan & Levi, 1974). Both eukaryotic and prokaryotic isolates have been obtained that are capable of growing on methanol, an intermediate in CH_4 metabolism (Cooney & Levine, 1972).

This paper reports the enrichment and isolation of five yeast strains capable of growth on CH_4 . Evidence for their CH_4 -oxidizing capabilities and some general characteristics of these eukaryotic organisms are described.

METHODS

Organisms. The five yeast strains described in this paper were isolated from the following sources: isolates Y and NY, MY, and CY from lake water samples from the thermoclines of Lake Mendota (Madison, Wis., U.S.A.), Lake Monona (Madison, Wis., U.S.A.) and Lake 227 (Experimental Lakes Area, Northwestern Ontario, Canada), respectively; isolate WY from a soil sample of the Oal horizon of a fen, Lake Wingra (Madison, Wis., U.S.A.).

Enrichment and isolation. Isolate Y was obtained during an enrichment for facultative CH_4 -oxidizing bacteria using the procedure of Patt *et al.* (1974). A lake water sample from the thermocline of Lake Mendota was used as the inoculum. The enrichment was carried out at 25 °C.

In order to selectively enrich for and isolate other strains of CH_4 -oxidizing yeasts, water or soil samples obtained from locations where CH_4 oxidation was known to occur were used to inoculate flasks containing YM broth [1% (w/v) glucose, 0.3% (w/v) malt extract, 0.3% (w/v) yeast extract, 0.5% (w/v) peptone broth; pH 3.5]. These were incubated at 20 °C without shaking until turbidity developed. The suspension was

then dilution plated on to a basal medium agar with a vitamin and amino acid supplement, or filtered through 0.45 μm Millipore filters which were placed on filter pads wetted with the same medium. The medium contained (per litre, in distilled water): KH_2PO_4 , 0.85 g; K_2HPO_4 , 0.15 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; NaCl , 0.1 g; CaCl_2 , 0.1 g; H_3BO_3 , 500 μg ; $\text{CaSO}_4 \cdot 5\text{H}_2\text{O}$, 40 μg ; KI , 100 μg ; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 200 μg ; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 400 μg ; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 200 μg ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 400 μg . Either $(\text{NH}_4)_2\text{SO}_4$ (0.5 g l^{-1}) or KNO_3 (2 g l^{-1}) was added as the nitrogen source. When required, a vitamin supplement and/or an amino acid supplement was added to the medium. The final concentrations of the supplements were (per litre): biotin, 2 μg ; calcium pantothenate, 400 μg ; folic acid, 2 μg ; inositol, 2 mg; niacin, 400 μg ; *p*-aminobenzoic acid, 200 μg ; pyridoxine.HCl, 400 μg ; riboflavin, 200 μg ; thiamin.HCl, 400 μg ; and L-histidine.HCl, 10 μg ; DL-methionine, 20 μg ; DL-tryptophan, 20 μg . The final pH of the medium was about 5.5. The plates and filters were incubated in BBL anaerobic jars at 20 °C under an atmosphere of 70% CH_4 /20% air/10% CO_2 (all %, v/v). The colonies that formed were examined microscopically. Those containing large budding cells were transferred and grown as clones on YM plates, then transferred to filters on the basal medium and incubated in an atmosphere of CH_4 /air/ CO_2 to establish pure colonies.

Growth and culture maintenance. All isolates were grown and maintained on filters or in liquid culture in the supplemented basal medium under a 70% CH_4 /20% air/10% CO_2 atmosphere (except isolate CY which was grown in the same medium without the amino acid and vitamin supplements). All were incubated at 20 °C without shaking.

Criteria for purity. Individual cells from a culture of isolate Y grown in the basal salts medium under the CH_4 /air/ CO_2 atmosphere described above were isolated by the method of DeVay & Schnarhorst (1963). Individual colonies were grown on YM agar then tested for growth in YM broth in the presence of lincomycin (100 $\mu\text{g ml}^{-1}$), penicillin (25 $\mu\text{g ml}^{-1}$), chloramphenicol (40 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$) and gramicidin (50 $\mu\text{g ml}^{-1}$).

Characterization of isolates. The morphological, nutritional and sexual characteristics of the isolates were examined as suggested by van der Walt (1970). Tests used to determine the carbon assimilation patterns were carried out in liquid cultures. The liquid cultures used to detect fermentative ability were overlaid with sterile mineral oil after inoculation. Temperature optima and maxima for growth were determined by growing the isolates in YM broth without shaking.

Microscopy. Cells of isolate Y grown on YM plates were fixed in 4% (w/v) glutaraldehyde for 2 h on ice, washed and suspended in 2% (w/v) ionagar, post-fixed in 1.5% (w/v) potassium permanganate for 2 h and stained with 1.5% (w/v) uranyl acetate for 1.5 h. After dehydration, all preparations were embedded in Spurr epoxy resin (Spurr, 1969) using a 12 h incubation at each infiltration step. Thin sections were cut with a diamond knife on a MT-2 Sorvall microtome, placed on carbon-coated grids and stained for 30 min with 2% (w/v) uranyl acetate, then for 15 min with Reynolds lead citrate (Reynolds, 1963). Specimens were examined and photographed in a Hitachi HU-11E-1 electron microscope.

Assays for methane oxidation by resting cell suspensions. Methane oxidation by whole cells was determined in two ways. For the radioisotopic assay, cells grown on CH_4 were harvested, washed by centrifugation with 0.05 M-potassium phosphate buffer pH 7.0, and resuspended in the basal medium to give a final cell concentration of 0.2 to 0.4 mg dry wt ml^{-1} . This suspension (2.5 ml) was placed in a 5 ml serum vial sealed with a rubber septum. Vials were evacuated, flushed with CH_4 and filled with an atmosphere containing CH_4 , N_2 , air and CO_2 in the proportions indicated. Approximately 1 μCi of $^{14}\text{CH}_4$ was then injected and vials were incubated on a rotary shaker at 20 °C for the time specified. Cold 0.5 M-NaOH (2 ml) was then injected into each vial slowly with shaking to trap any CO_2 present, and the vials were flushed with N_2 to eliminate any remaining $^{14}\text{CH}_4$. The vials were connected to a CO_2 trap containing phenethylamine/methanol/Packard NCS tissue solubilizer (2:2:1, by vol.), acidified by injection of 0.5 ml 2 M- H_2SO_4 and 0.6 ml of ice-cold 60% (w/v) trichloroacetic acid, placed in an ice bath and gassed with N_2 for 7 min (flow rate 10 ml min^{-1}). A sample of the trapping solution was counted in Liquifluor (New England Nuclear). The cell material precipitated by the cold trichloroacetic acid was filtered through Gelman GF/A filters, dried and counted in Liquifluor to measure incorporation of $^{14}\text{CH}_4$ into cellular material.

In order to measure induction of CH_4 -oxidizing activity, cells grown on glucose were harvested, washed by centrifugation three times in 0.05 M-phosphate buffer pH 7.0, and resuspended in the basal medium. The cultures were incubated under an atmosphere of 70% CH_4 /20% air/10% CO_2 at 20 °C without shaking. Samples were taken at various times and assayed for CH_4 oxidation as described above. Induction of the CH_4 -oxidizing system was determined by comparing the amounts of $^{14}\text{CO}_2$ produced and of radioactivity incorporated into cellular material with controls using cells grown on CH_4 .

The rate of CH_4 oxidation was also determined using a Rank oxygen electrode (Rank Bros, Bottisham, Cambs.). The assay mixture contained, in 1.0 ml: 0.8 ml of 0.05 M-phosphate buffer pH 7.0, 0.10 ml of a whole cell suspension that contained 5 mg dry wt ml^{-1} and 0.1 ml of phosphate buffer saturated with CH_4 . The buffer and cells were added to the electrode chamber first to determine the endogenous activity of the cells. The assay was started by injection of the CH_4 -saturated buffer. Oxygen uptake was measured at 25 °C.

Analytical methods. Lactic acid concentration was determined by the method of Nanni and Baldini (Dawes *et al.*, 1971), using lithium lactate as a standard. Absorbance was measured at 568 nm on a Gilford 2000 spectrophotometer.

For dry weight determinations, cells were harvested from a measured volume of culture by centrifugation and washed with phosphate buffer. The pellet was resuspended in 1.0 ml distilled water and placed on a pre-dried, pre-weighed metal planchet. The planchet was dried to constant weight in an oven at 90 °C.

Materials. Methane (C.P. grade, 99 %) was purchased from the Matheson Co., Joliet, Ill., U.S.A. No trace contaminants were detectable by gas chromatography using the technique of Patt *et al.* (1974).

Radioactive $^{14}\text{CH}_4$ was purchased from the Amersham Corp., Arlington Heights, Ill., U.S.A. The radiochemical purity of the preparation was determined by the supplier to be 99 % using gas radiochromatography on silica gel.

RESULTS

Isolation and enrichment

The first isolate (Y) was obtained during an enrichment for CH_4 -oxidizing bacteria. Some of the pale-pink pigmented colonies that grew when dilution plates were incubated under CH_4 contained large rod-shaped cells ($3 \times 7 \mu\text{m}$) that reproduced by budding.

Single-cell isolations ensured that this isolate did not contain any prokaryotic contaminants that enabled survival on CH_4 . The 30 resulting clones grew on glucose in the presence of all the antibiotics tested; however, better growth was observed in the presence of penicillin and kanamycin than in the presence of chloramphenicol, gramicidin or lincomycin. No bacterial contaminants were observed in these cultures. Every clone retained the ability to grow with CH_4 as an energy source when incubated in basal salts medium under an atmosphere containing CH_4 . All oxidized $^{14}\text{CH}_4$ to $^{14}\text{CO}_2$.

The development of a selective enrichment procedure resulted in the isolation of four additional yeast strains capable of growth on CH_4 . An isolate was successfully obtained from every source. However, the incubation of the inoculum in medium at a low pH (3.5 to 4.0) was necessary at the initial step of the enrichment to allow the yeast strains to proliferate faster than methanotrophic bacteria present in the samples.

Morphological description

All the isolates formed bright-pink colonies when grown chemoheterotrophically on YM agar, although the production of capsular material and the shade of pink varied from isolate to isolate. When grown on CH_4 , the organisms showed only slight pigment production.

The cells of the different isolates ranged from rod-shaped ($3 \times 7 \mu\text{m}$) to more oval-shaped ($3 \times 5 \mu\text{m}$) (Fig. 1). All reproduced by budding. The fine structure of isolate Y was examined to determine whether the organisms were eukaryotes. An electron micrograph of a cell from a clone obtained from single-cell isolation is shown in Fig. 2. The intracytoplasmic organization of the cell into membrane-bounded organelles confirms the eukaryotic nature of this organism.

Growth on methane

The isolates grew in liquid culture in stoppered test tubes with 5 to 15 ml medium. Little or no growth occurred if N_2 was added to the atmosphere instead of CH_4 . The organisms could also be grown on filters. No clones grew on inoculated filters incubated in the presence of air. Nitrate served as a better nitrogen source during growth on CH_4 than did ammonia. The highest cell yields were obtained when the atmosphere contained a small amount of O_2 (4 to 5 %) and additional CO_2 (10 %); a cell yield of $2.1 \text{ g dry wt l}^{-1}$ was obtained under these conditions. The fastest generation time observed was approximately 2 d for isolate MY. The generation times of the other isolates grown in liquid culture were much slower, usually being 7 d or more. Attempts to grow the organisms in larger volumes of liquid media on CH_4 consistently resulted in lower growth yields. Cell lysis occurred when attempts were made to increase gas transfer by stirring or shaking the cultures during incubation.

Fig. 1. Phase contrast micrographs of methane-utilizing yeasts: (a) isolate Y showing several stages of budding; (b) isolate MY; (c) isolate CY. Bar markers represent 5 μm .

Assays for methane oxidation by resting cell suspensions

Table 1 shows the results of a typical radioisotopic experiment using resting cells of the five isolates grown on CH_4 . All oxidized CH_4 to CO_2 . Oxidation rates varied from 0.02 to 0.18 $\mu\text{mol CH}_4$ oxidized h^{-1} (mg dry wt) $^{-1}$. When the atmosphere in the vials contained 10% CO_2 , little or no labelled carbon from CH_4 appeared in cellular material. However, if no CO_2 was added other than that present in the air, large amounts of radioactivity were incorporated by the cells (Table 1).

Similar experiments using cells that had been grown on glucose and transferred to CH_4 showed induction of the CH_4 -oxidizing system within 24 to 48 h of transfer. Glucose-grown cells transferred to the basal medium incubated under N_2 did not produce any $^{14}\text{CO}_2$ from $^{14}\text{CH}_4$ (Table 1).

A Rank oxygen electrode was used to measure O_2 utilization by washed cell suspensions from CH_4 -grown yeast cultures. Oxygen consumption was CH_4 dependent. Rates of 0.20 to 0.30 $\mu\text{mol O}_2$ consumed h^{-1} (mg dry wt) $^{-1}$ were obtained for resting cell suspensions of all the isolates.

Nutritional characteristics

A number of C_1 compounds, alcohols and hydrocarbons were tested as carbon and energy sources for the isolates (Table 2). Methanol, formaldehyde or formate did not support growth even when added at extremely low levels or as vapours. All the isolates tested grew on methylamine, ethane and butane. Only isolate CY grew on the higher hydrocarbons. Ethanol and (some) other alcohols did support growth. All grew well with acetate as the energy source.

The isolates grew aerobically with metabolically diverse compounds as substrates,

Fig. 2. Electron micrograph of isolate Y grown on YM agar: N, nucleus; NM, nuclear membrane; M, mitochondrion; L, lipid globule; CM, cytoplasmic membrane; CW, cell wall. Bar marker represents 1 μm .

Table 1. Oxidation of $^{14}\text{CH}_4$ by methane-grown cultures of the yeast isolates

Unless otherwise indicated, cell suspensions were incubated under an atmosphere of 40% N_2 /25% CH_4 /25% air/10% CO_2 for 15 h, and the specific activity of $^{14}\text{CH}_4$ was 2.8×10^6 d.p.m. μmol^{-1} .

Isolate	$^{14}\text{CO}_2$ trapped (d.p.m.)	CH_4 oxidized [$\mu\text{mol h}^{-1}$ (mg dry wt) $^{-1}$]	^{14}C incorporated into cellular material (d.p.m.)
CY	94 232	0.017	245
NY	351 847	0.064	10
Y	392 719	—	99
MY	54 901	0.032	310
WY	250 964	0.097	52
CY (1)*	114 070	0.18	14 786
CY (2)*	172	—	95

* Suspensions of methane-grown cells (1) or glucose-grown cells (2) were incubated under an atmosphere of 50% N_2 /25% air/25% CH_4 for 22 h, and the specific activity of $^{14}\text{CH}_4$ was 2.4×10^6 d.p.m. μmol^{-1} .

Table 2. *Utilization of C₁ compounds, alcohols and hydrocarbons by methanotrophic yeasts*

For each isolate, the results indicate the growth on the test compound compared with growth on CH₄: ++, better growth than on CH₄; +, growth equal to that on CH₄; ±, slight growth; —, no growth. (The isolates did not all grow equally well on CH₄.)

Carbon and energy source	Isolate				
	Y	CY	NY	MY	WY
Methane	+	+	+	+	+
Methanol	—	—	—	—	—
Formaldehyde	—	—	—	—	—
Formate	—	—	—	—	—
Dimethyl ether	±	±	—	±	ND
Methylamine	+	+	+	±	+
Trimethylamine	±	±	—	+	—
Ethane	±	±	+	+	±
Butane	+	±	±	+	ND
Nonane	—	+	—	—	—
Hexadecane	—	++	—	—	—
Ethanol	++	++	—	++	++
Propanol	—	++	—	++	ND
Isobutanol	±	—	—	+	ND
Octanol	—	—	—	—	ND
Glycollate	—	—	—	—	—
Glyoxylate	—	±	+	+	—
Acetate	++	++	++	++	++

ND, Not determined.

Table 3. *Growth characteristics of isolates capable of growth on methane*

Growth in basal medium supplemented with the test compound is indicated as follows: +, growth; ±, slight growth; —, no growth.

Characteristic	Isolate				
	Y	CY	NY	MY	WY
Aerobic growth on:					
Glucose	+	+	+	+	+
Sucrose	+	+	—	+	+
Raffinose	+	+	+	+	+
Inulin	+	+	—	—	—
Soluble starch	+	—	+	—	—
L-Rhamnose	—	+	—	+	+
Glycerol	—	+	+	+	+
Inositol	—	—	—	—	—
Succinate	+	+	+	+	+
Citrate	—	+	—	+	+
Hexadecane	—	+	—	—	—
Sugar fermentation*	—	+	—	+	+
Utilize nitrate as N source	+	+	±	—	—
Vitamin requirement	—	—	+	+	+
Optimum growth temp. (°C)	15	15–20	15	25–30	25–30
Maximum growth temp. (°C)	32	32	30	42	42

* Not an alcoholic fermentation. Gas was not produced; fermentation was determined by acid production. Sugars fermented included glucose, sucrose and raffinose.

including complex and simple sugars, dicarboxylic acids and alcohols, but each isolate varied in its ability to use certain compounds. Table 3 summarizes some of the growth and nutritional characteristics of each isolate. Three of the isolates were able to ferment sugars slowly, though the fermentation was not of the typical alcoholic type. Growth was slow with no gas production. Use of an indicator in the medium suggested that acid was produced; an assay for end-products indicated a small amount of lactic acid ($8 \mu\text{g ml}^{-1}$) was produced by the cultures after 4 weeks incubation.

All of the isolates grew well with ammonia as a nitrogen source, including when CH_4 was used as the energy source. Three of the isolates also used nitrate although it was not as good a nitrogen source as ammonia except when the organisms were grown with CH_4 as the energy source. Nitrate was then the preferred nitrogen source.

The isolates varied in their requirements for supplements to the basal medium. Isolate CY required no additions to a mineral salts base, isolate Y required the amino acid supplement for growth and the remaining three all required the addition of vitamin supplement for growth when glucose served as the carbon source.

Isolates Y, CY and NY exhibited lower growth temperature optima (15 to 20 °C) and lower maximum growth temperatures (30 to 32 °C) than the other isolates. Isolates MY and WY, which came from warmer sources, grew well at 30 °C and their maximum growth temperature was 42 °C.

Sexual characteristics

Ascospore production was not observed during culture of any of the isolates on several different sporulation media. All the isolates except WY exhibited pseudomycelial formation. No true mycelium was formed.

DISCUSSION

This paper describes the first isolation of eukaryotic micro-organisms that are capable of growth on CH_4 as the sole energy source. These pink-pigmented yeasts exhibit most characteristics common to other yeasts including similar morphology, ultrastructure, nutritional requirements and means of asexual reproduction. Rudd *et al.* (1976) have reported that enrichment cultures of CH_4 -oxidizing organisms from shield lakes were dominated by a 'large yeast-like' micro-organism in doublets, but they did not characterize these organisms further. Our successful enrichment and isolation of CH_4 -oxidizing yeasts from five different sources where CH_4 oxidation was known to occur suggest that these eukaryotic micro-organisms may play a part in the natural oxidation of this substrate even though the yeasts were not the predominant members of CH_4 -oxidizing populations observed in this study.

Methanotrophic bacteria oxidize CH_4 aerobically to CO_2 ; methanol, formaldehyde and formate are intermediates in the pathway. Formaldehyde serves as the carbon source and is directly assimilated into cellular material by the organisms via either the ribulose 5-phosphate pathway or the serine pathway of formaldehyde fixation (Quayle, 1972). Nutritional studies to determine the ability of the yeasts to use various C_1 compounds as substrates for growth have provided little information as to the possible intermediates of CH_4 metabolism in these organisms. Methanol, formaldehyde and formate did not support growth of the organisms. However, the failure of methanotrophic bacteria to grow on methanol is common (Whittenbury *et al.*, 1970; Patt *et al.*, 1974). The results of radioisotopic studies indicate that CO_2 is the final product of CH_4 oxidation by the yeasts. These studies also suggest that CH_4 or one of the intermediates does not serve as a carbon source for the organisms. Little or no radioactivity appeared in trichloroacetic acid-precipitable material when the assay was run under conditions most suitable for growth, i.e. when the cultures were incubated under an atmosphere supplemented with 10% CO_2 . When the CO_2 content was limited to the amount present in the 25% air in the atmosphere of the assay vial,

labelled carbon appeared in cellular material in much higher quantities. This suggests that the yeasts may assimilate CO_2 as a carbon source, since omission of the additional CO_2 would permit the $^{14}\text{CO}_2$ produced from $^{14}\text{CH}_4$ to become a significant portion of this gas present in the atmosphere.

Examination of the morphological and physiological characteristics of the organisms suggests that at least four different species of yeast were isolated that are capable of growth on CH_4 . Isolates MY and WY may be the same organism. These two isolates utilized the same organic compounds as energy sources, grew poorly on nitrate, required vitamins for growth and fermented the same sugars. The other three isolates were more psychrophilic and grew well on nitrate, but differed from each other in several other characteristics. Isolate CY grew in a minimal medium without supplements, degraded higher hydrocarbons and could grow fermentatively on some sugars. Isolates Y and NY did not grow anaerobically; they differed from each other in the supplements they required for growth and in the compounds that served as carbon and energy sources for growth. Further studies are currently being undertaken to determine the taxonomic positions of these new methanotrophic yeasts.

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