Metabolic characteristics of an aerobe isolated from a methylotrophic methanogenic enrichment culture

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An anaerobic methylotrophic methanogenic enrichment culture, with sustained metabolic characteristics, including that of methanation for over a decade, was the choice of the present study on interspecies interactions. Growth and methanation by the enrichment were suppressed in the presence of antibiotics, and no methanogen grown on methanol could be isolated using stringent techniques. The present study confirmed syntrophic metabolic interactions in this enrichment with the isolation of a strain of *Pseudomonas* sp. The organism had characteristic metabolic versatility in metabolizing a variety of substrates including alcohols, aliphatic acids, amino acids, and sugars. Anaerobic growth was favoured with nitrate in the growth medium. Cells grown anaerobically with methanol, revealed maximal nitrate reductase activity. Constitutive oxidative activity of the membrane system emerged from the high-specific oxygen uptake and nitrate reductase activities of the aerobically and anerobically grown cells respectively. Cells grown anaerobically on various alcohols effectively oxidized methanol in the presence of flavins, cofactor FAD and the methanogenic cofactor F_{420}, suggesting a constitutive alcohol oxidizing capacity. In cells grown anaerobically on methanol, the rate of methanol oxidation with F_{420} was three times that of FAD. Efficient utilization of alcohols in the presence of F_{420} is a novel feature of the present study. The results suggest that utilization of methanol by the mixed culture would involve metabolic interactions between the *Pseudomonas* sp. and the methanogen(s). Methylotrophic, methanogenic partnership involving an aerobe is a novel feature hitherto unreported among anaerobic syntrophic associations and is of ecological significance.

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1. Introduction

Mixed microbial populations endowed with characteristic and specific metabolic capabilities occur widely in niches of natural habitats. Their coexistence is sustained by metabolic interactions that allow the flow of carbon, energy and other intermediates toward their mutual benefit (Wolin 1982). The process of anaerobic digestion – or biomethanation of organic matter – has gained credence as a renewable alternative technology towards energy generation by effecting the degradation of complex organic substrates to methane. The process also contributes simultaneously to abatement of environmental pollution. The process involving mutually interdependent, but diverse microbial populations, is an ideal choice for the study of syntrophic association of several anaerobic-fermentative bacteria with methanogens (McInerney 1988; Zehnder *et al* 1982; Schink 1997). The fermentative bacteria supply the growth substrates especially acetate, H_2, CO_2 and other one-carbon compounds that fuel the methanogenic conversions, while the methanogens ably remove the inhibitory products like H_2, and CO_2. Methanogenic archeae, a diverse group of obligate anaerobes, occurring in most anaerobic habitats, form the terminal electron

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sink during anaerobic digestion of organic matter to methane (Archer 1984; Boone *et al* 1993). Methanogens derive the energy for growth only by methanogenesis and are the only organisms known to produce methane as a catabolic end product (Thauer 1990, 1998; Blaut 1994). The metabolic interactions amongst the microbes during methanogenesis involve 'inter-species hydrogen-transfer mechanisms' (Sparling and Gottschalk 1990; Stams 1994) and the earliest reported study of such symbiotic association involving a methanogen was on *Methanobacillus omelianskii* (Bryant *et al* 1967). In addition, research from our laboratory provided evidence for an efficient interactive metabolic control prevailing during active biomethanation of polymeric carbohydrates (Krishnan and Lalitha 1990) or proteins (Swaminathan 1995). This metabolic control was characterized by maintenance of steady-state levels of many of the key intermediates involved. These studies provided an experimental approach toward understanding the catalytic efficiency of microbial metabolic interactions. Attempting further exploration on this aspect of metabolic control, a mixed methanogenic culture growing on methanol was studied. This culture was enriched from the slurry derived from a high-rate anaerobic digester. The reactor was operated on the leaves of *Leucaena leucocephala* with sustained methane yields up to 85–90 l/kg wet wt./day (Lalitha *et al* 1994a). Observations on consistent biomethanation effected by this enriched mixed methanogenic culture over a decade, were suggestive of metabolic partnerships among methylotrophs. Initial studies on this mixed culture revealed the presence of a phototroph of the *Rhodopseudomonad* type (Lalitha *et al* 1994b). Methanation by the enrichment culture was effective even after the elimination of the phototroph by maintaining this enrichment in the dark for several generations. Further, attempts were made to address if any other metabolic partner(s) were present and the results described in this paper establish the co-existence of a facultative bacterium with the methanogen(s). Details on the isolation and characterization of the facultative organism are described in this paper. It is interesting that oxidation of methanol under anaerobic conditions, thermodynamically an uphill task, was mediated by the methylotrophic methanogenic enrichment, especially when methanol itself was apparently not the growth substrate for any of the methanogen(s) present. This microbial consortium thus assumes considerable fundamental and ecological significance related to the process of biomethanation.

2. Materials and methods

2.1 *Materials*

Methyl viologen, sulphanilamide, sodium dithionate, 2-bromoethanesulphonate, N-(1-napthyl)-ethylenediamine dihydrochloride, dithiothretiol, were purchased from Sigma Chemical Company (St. Louis, MO, USA). All the growth substrates used in the study were analytical reagent grade from E. Merk (Mumbai, India). Yeast extract, tryptone, agar, and all antibiotics were from Hi Media (Mumbai, India). All other chemicals used were analytical reagent grade from E. Merk (Mumbai, India). Serum vials (35 ml) with butyl rubber stoppers were from Nathany Industries (Kolkata, India). Roll tubes were purchased from Bellco Glass, Inc (NJ, USA).

2.2 *Enrichment of the methanogenic culture*

The source of inoculum used for enrichment was the digested slurry from laboratory anaerobic reactors of 2 l capacity operated semicontinuously, using the leaves of *Leucaena leucocephala* as input feed (4 g) that generated sustained output gas at 0·8–1·0 l/l/day with 85% methane content (Vasanthy *et al* 1986). Methanogenic enrichment was carried out using a minimal medium as described earlier (Lalitha *et al* 1994b) using the roll tube technique (Hungate 1969).

2.3 *Growth studies*

2.3a *Studies on the methanogenic enrichment*: Further experiments with the isolate of methanogenic enrichment were carried out anaerobically in the dark in 35 ml serum vials, sealed with butyl rubber stoppers, containing 20 ml of sterile medium. Anaerobic growth media were prepared under an atmosphere of oxygen-free nitrogen (Hungate 1969; Bryant *et al* 1968). Stock solutions of antibiotics were filter-sterilized and added at a concentration of 50 mg/l. The methanogenic inhibitor, 2-bromoethanesulphonate, was used at 0·28 mM. Methanogeneis was routinely monitored during growth in minimal medium as described earlier (Lalitha *et al* 1994b). Coenzyme F_{420} was partially purified from a pure culture of *Methanosarcina barkeri* (Deppenmeier *et al* 1990). All growth experiments were carried out at 37ºC.

2.3b *Studies on the facultative isolate*: After comparing the composition of a wide variety of media described for the growth of facultative bacteria (Atlas 1997), four specific media, A, B, C, D were formulated with glycerol as the added growth substrate. Various additions to the media were as follows in g/l (unless specified otherwise) with pH adjusted to 7·4: medium A: K_2HPO_4, 1·15; KH_2PO_4, 0·625; $MgSO_4{\cdot}7H_2O$, 0·02; NH_4NO_3, 1·0; glycerol, 3 ml, and yeast extract, 0·5 g; medium B: K_2HPO_4, 2·28; NaH_2PO_4, 1·38; $(NH_4)_2SO_4$, 0·5; $MgSO_4{\cdot}7H_2O$, 0·02; and glycerol, 3 ml; medium C: NaH_2PO_4, 3·0; K_2HPO_4, 4·1; $FeSO_4{\cdot}5H_2O$, 5 ppm; yeast extract, 1·0;

tryptone, 1·0; $MgSO_4 \cdot 7H_2O$, 0·5; and glycerol, 3 ml; and medium D: KNO_3, 10; NH_4Cl, 10; K_2HPO_4, 1·5; $MgSO_4 \cdot 7H_2O$, 0·2; NaCl, 0·2; and glycerol, 3 ml. Growth was monitored by the levels of turbidity, dry weight and total cell protein. The production of pyoverdine and pyocyanin was monitored using King's medium (King *et al* 1954). Gas analyses were done using a dual injector Tracor model 540, gas chromatograph (Austin, USA) as described earlier (Lalitha *et al* 1994b). For light microscopy, Leitz Wetzler (Gottingen, Germany) model Ortholux microscope, equipped with an Orthomat camera attachment. Scanning electron microscopy of the samples fixed with 2% glutaraldehyde and dehydrated as described by Albrecht *et al* (1976) was done using Jeol electron microscope operated at under standard conditions at 80 kV.

2.4 *Nitrate reductase assay*

Nitrate reductase activity was determined as described previously with dithionite and methyl viologen as the electron donor (Fernandez-Lopez *et al* 1994). Nitrite was determined by the diazotization procedure (Nicholas and Nason 1957).

2.5 *Oxygen uptake experiments*

Pseudomonas MC cells from 100 ml culture, grown aerobically and anaerobically in medium C for 48 h, were collected by centrifugation at 8,000 *g* using Beckman Model J2-21 centrifuge (Beckman Instruments, Inc., UK) and the cells pellet resuspended in 10 ml of 0·1 M phosphate buffer, pH 7·4. The rate of oxygen uptake during the oxidation of various alcohols by the cells was followed at 25ºC in a Gilson 5/6 H model oxygraph (Gilson medical electronics, Middleton, WI, USA) and the reaction volume of 1·2 ml contained: 10 mM alcohol; 0·15 mM FAD or 0·1 ml of partially purified F_{420} (of absorbance at 420 nm not less than 1·0 O.D./ml); 0·02 ml of 1% N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD), 0·1 ml cells.

3. Results

3.1 *General characteristics of the methylotrophic methanogenic enrichment*

Methanogenic enrichment grown on methanol was obtained originally from the active slurry of a semi-continuously operated anaerobic digester degrading leafy biomass of *Leucaena leucocephala* (Vasanthy *et al* 1986). This selective enrichment culture, with a high rate biomethanation efficiency, was successfully maintained for over a decade, in a minimal medium with resultant sustained methane yield of 16–20 mmol/l during a 40 to 45 day cycle. Phototrophic growth initially observed was eliminated by growing the cultures in the dark for several generations. This methylotrophic methanogenic enrichment thus devoid of the phototroph, was the parent culture used for the isolation procedure. Several attempts to isolate a methanol utilizing methanogen from this enrichment culture, by repeated colony picking using the roll-tube technique (Hungate 1969) followed by serial dilutions, were futile. Colony picking always revealed more than one morphological form, while serial dilutions of liquid culture of the colonies resulted in the cessation of methanation. Other methanogenic substrates tested neither resolved nor supported the growth of methanogen(s). The following possibilities were therefore considered: (i) that the methanogen(s) existed in a syntrophic relationship with one or more of the non-methanogens in the culture and/or (ii) methanol was not the preferred carbon source for the growth of the methanogen(s) as a result of which an obligate dependence of the methanogen(s) on the non-methanogen(s) existed.

Both the possibilities were explored by altering the culture conditions for the growth of the enrichment. Various antibiotics individually added to the culture medium inhibited methanation considerably (table 1). In the presence of chloramphenicol and cycloserine, the inhibition was complete while the other antibiotics inhibited methanation by 70–90%. Ampicillin, amoxycillin

Table 1. Effect of antibiotics on methanation by mixed methanogenic enrichment.

Antibiotics added*	Methane yield (mmol/l)** (%)
Streptomycin	60 ± 2·0
Gentamycin	4 ± 0·5
Kanamycin	4 ± 0·5
Chloramphenicol	ND
Penicillin G	15 ± 1·2
Erythromycin	30 ± 1·8
Bacitracin	30 ± 1·4
Ampicillin	72 ± 3·0
Ammoxicillin	59 ± 2·2
Cycloserine	ND
Vancomycin	11 ± 1·5
Tetracycline	ND

Culture grown anaerobically at 37ºC in the minimal medium for 15 days.
Values are averages ± SEM of three independent experiments.
*Antibiotics added at the level of 50 mg/l culture.
**Methane yield represented as percent of that from the control cultures grown in the absence of added antibiotic. Control values were 17·2 ± mmol/l of the culture and taken as 100%.
ND, not detectable.

and streptomycin were the least effective in inhibiting growth and methanation. Antibiotics do not inhibit the growth of methanogens owing to the novel biochemistry of methanogens and to the presence of structural components of the cell wall material and cell membrane in archeae that are very different from those of eubacteria (Lang and Ahring 2001). The suppression of growth and methanation by the enrichment culture notable in the presence of antibiotics, were suggestive of syntrophic associations between methanogen(s) and non-methanogen(s). Addition of 2-bromoethane sulphonate, a potent methanogenic inhibitor, to the growth medium, completely inhibited methanation as expected.

In addition, the methanogenic cultures, anaerobically grown in the presence of various antibiotics, showed little or no aerobic growth. It is tantamount to suggest that the presence of non-methanogen(s) was obligatory to the growth of the methanogen(s) that failed to get resolved in pure culture, but always co-existed with the facultative non-methanogen(s) even after repeated roll-tube experiments and serial dilution studies.

3.2 *The nature of aerobic growth and isolation of the non-methanogen*

Further attempts were made to isolate the non-methanogenic facultative organism(s) by plating on tryptone-yeast agar medium by repeated enumeration after selective colony picking. The isolate, thus purified, was maintained in the minimal medium. Plating on nutrient-agar resulted in the formation of creamy-yellowish oval colonies after overnight incubation at 37ºC. Diffusible yellowish-green pigment(s) surrounding the colonies were identified as pyoverdine using spectral and fluorescence characteristics of the pigments as described before (Meyer and Abdallah 1978). Based on the characteristics listed in table 2, the facultative bacterium was confirmed as a strain of fluorescent *Pseudomonas*. The isolate was deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Germany (DSM 9370) and the Microbial Type Culture Collection (MTCC) in India (MTCC 2450). The isolate is hereafter referred to as *Pseudomonas MC*.

3.3 *Growth of* Pseudomonas MC *on alcohols*

Since *Pseudomonas MC* was isolated from a methanol-grown enrichment culture, the aerobic growth of the isolate on different alcohols was followed (table 3). Growth was reasonably of a high level on all alcohols except 2-propanol. The growth parameters for glycerol grown cells were the highest among the alcohols tested, with the dry weight levels reaching about 800 mg/l in 60 h, while cells grown on other alcohols had dry weight levels of 60–80% of that of glycerol grown cells.

Anaerobic growth of the organism was insignificant when grown devoid of nitrate in the growth media. Preliminary experiments in the presence of varying concentrations of added nitrate (required as an electron-acceptor during anaerobic growth), indicated 0·4% nitrate concentration to be optimal. Anaerobic growth of the isolate also revealed better utilization of glycerol among various

Table 2. Taxanomical properties of the isolate *Pseudomonas MC*.

Morphological features:	
Form	Rods
Size	1–5 μM
Motility	Highly motile
Flagellation	Polar
Gram stain	Negative
Physiological properties:	
pH for growth	6·0–7·5
Nitrate reduction	Positive
H_2S production	Negative
Oxidative/fermentative metabolism of carbohydrates	Oxidative
Hydrolysis of gelatin	Positive
Hydrolysis of starch	Positive
Hydrolysis of amide	Positive
Growth on medium containing thioglycollate	Growth throughout the medium
Catalase activity	Positive
Cytochrome c oxidase activity	Positive

Table 3. Growth of *Pseudomonas MC* on alcohols.

	Growth period		
	24 h	48 h	60 h
Growth substrate	Dry weight (mg/l)		
Aerobic growth			
Methanol	339 ± 38	418 ± 28	519 ± 42
Ethanol	328 ± 29	549 ± 40	697 ± 31
1-proponal	213 ± 46	411 ± 28	540 ± 22
2-propanol	184 ± 26	299 ± 28	396 ± 31
Glycerol	317 ± 20	648 ± 30	873 ± 43
Anaerobic growth			
Methanol	50 ± 18	120 ± 21	150 ± 21
Ethanol	167 ± 30	279 ± 18	339 ± 29
1-proponal	117 ± 18	209 ± 28	261 ± 21
2-propanol	46 ± 12	117 ± 22	151 ± 10
Glycerol	247 ± 28	414 ± 20	525 ± 21

Cells grown aerobically or anaerobically at 37ºC in minimal medium for 24 h; 48 h; 60 h.
Values are averages ± SEM of three independent experiments done in duplicates.

alcohols tested but with other alcohols, growth was about 40–60% with notably the least growth on methanol. Thus glycerol was found to be a good growth substrate for *Pseudomonas MC* under both aerobic and anaerobic conditions, justifying its choice as growth substrate for further studies.

3.4 *Choice of the growth medium*

Aerobic growth of the cells grown for 48 h with glycerol, in terms of dry weight and total protein, was best in medium C and was three- to five-fold higher than that in the other media. Hence medium C was used for further studies. Growth of the isolate was poor in simple media, but could be markedly enhanced by adding yeast extract and tryptone. Aerobic growth monitored over a period of 60 h in medium C, using glycerol as the growth substrate, was characterized by a lag period of about 6 h followed by a linear increase up to 50 h, beyond which it remained steady till the end of the period of observation.

3.5 *Growth of* Pseudomonas MC

Among the aliphatic acids tested, dry weight for aerobically grown cells on acetate and aspartate were significantly high (table 4) but relatively much less on sugars, a trait that was in accordance with earlier reports on other *Pseudomonas* species (Palleroni 1984). In general, anaerobic growth of cells on aliphatic acids was only about 50–60% of that of aerobic growth.

Cells grown aerobically on glycerol for 48 h in medium C, readily utilized methanol and ethanol with a high rate of oxygen uptake (about 125 natom oxygen/min/mg protein) and the rates with 2-propanol and glycerol were also high with 80–90% efficiency (table 5). These results are suggestive of a constitutive alcohol oxidative capacity in the cells. Nitrate reductase activity of *Pseudomonas MC* cells anaerobically grown on methanol, was of a high level when compared to that of cells grown on glucose (table 6).

3.6 *Methanol utilization by* Pseudomonas MC

Oxidation of methanol by the *Pseudomonas MC* grown on glycerol, was assessed by exploring requirement of various cofactors. $NADP^+$-dependant methanol dehydrogenase, flavin-linked alcohol oxidase, and H_2O_2 dependant methanol peroxidase activities were found to be either at a very low level or undetectable in both the membranous and soluble fractions of the anaerobically grown cell extracts. The activity of pyrroloquinone (PQQ) dependant methanol dehydrogenase was carefully estimated after blocking the electron flow toward the respiratory chain and also at various pH in the presence of methyl amine or ammonium ions as described earlier (Anthony and Zatman 1967). But the activity levels were negligible. Studies on the effect of various electron acceptors during oxidation of methanol indicated that neither NAD^+, $NADP^+$ nor DCPIP could be used as an electron acceptor (table 7). Addition of ferricyanide (30 mM) resulted in the lowering of specific oxygen uptake by 30%. In contrast, added FAD enhanced specific oxygen uptake rate by 100%. Owing to the flavin-linked methanol oxidation in the isolate observed in the

Table 4. Growth of *Pseudomonas MC* on aliphatic acids and sugars.

	Dry weight (mg/l)	
Growth substrate	Aerobic	Anaerobic
Aliphatic acids		
Formate	349 ± 46	213 ± 31
Acetate	522 ± 45	301 ± 33
Succinate	400 ± 36	183 ± 23
Citrate	348 ± 24	349 ± 64
Aspartate	500 ± 35	371 ± 79
Glutamate	400 ± 30	112 ± 79
Sugars		
Glucose	301 ± 92	621 ± 74
Fructose	199 ± 36	551 ± 73
Sorbitol	242 ± 58	580 ± 54
Mannitol	391 ± 112	658 ± 39

Aerobic and anaerobic growth of *Pseudomonas MC* cells in medium C at 37ºC monitored for 60 h, in the presence of different growth substrates as indicated.
Values are averages ± SEM of three independent experiments done in duplicates.

Table 5. Oxidation of alcohols by *Pseudomonas MC* cells grown on glycerol.

Substrate	Cellular oxygen uptake (natoms oxygen/min/mg protein)
Methanol	123 ± 20
Ethanol	125 ± 28
1-propanol	97 ± 34
2-propanol	100 ± 16
Glycerol	118 ± 30

Cells grown aerobically on glycerol in medium C at 37ºC for 48 h, harvested and resuspended in 0·1 M phosphate buffer pH 7·4.
Oxygen uptake by the cell suspension measured in the presence of various alcohols (80 mM) added as indicated.
Values are averages ± SEM of three independent experiments.

Table 6. Nitrate reductase activity in anaerobically grown *Pseudomonas MC*.

	Nitrate reductase activity (units)			
	Specific activity*		Total activity**	
Growth substrate	Membrane fraction	Soluble fraction	Membrane fraction	Soluble fraction
Glucose	42 ± 2	266 ± 11	2442 ± 20	2356 ± 31
Methanol	281 ± 9	294 ± 13	2337 ± 21	1941 ± 19
Glucose + methanol	47 ± 5	140 ± 9	2418 ± 25	1285 ± 16

Cells grown anaerobically in medium C at 37ºC for 60 h in the presence of growth substrate at 90 mM.
Values are averages ± SEM of three independent experiments done in duplicates.
*Unit of enzyme activity given as nmol nitrate reduced/min/mg protein.
**Unit of enzyme activity given as nmol nitrate reduced/min/l culture.

Table 7. Methanol oxidation in the presence of exogenously added electron acceptors.

Electron acceptor	Cellular oxygen uptake (natoms oxygen/min/mg protein)
Control	71 ± 5
NAD^+	84 ± 8
$NADP^+$	89 ± 12
Ferricyanide	61 ± 5
DCPIP	62 ± 11
FAD	140 ± 12

Cells grown anaerobically on glycerol in medium C at 37ºC for 48 h, harvested and resuspended in 0·1 M phosphate buffer pH 7·4.
Oxygen uptake by the cell suspension measured in the presence of methanol with added electron acceptors as indicated.
Electron acceptors added and tested individually in concentrations: NAD^+, 0·2 mM; $NADP^+$, 0·2 mM; pottasium ferricyanide, 30 mM; 2,6-dichlorophenol-indophenol, 35 mM; and, FAD, 0·15 mM.
Values are averages ± SEM of three independent experiments done in duplicates.

present study, the effect of the flavin analogue, 8-hydroxy 5-deazaflavin designated as F_{420}, produced by methanogens, was also tested. Results presented in table 8, reveal that though both F_{420} and FAD were effective as cofactors during methanol oxidation, the former was more effective with anaerobically grown cells and the latter with those grown aerobically. Methanol oxidation rates, in the presence of FAD as the cofactor, were reasonably high and elicited no differences between those of the cells grown either aerobically or anaerobically on different alcohols. An exception was noted in the cells grown on glycerol; for anaerobic growth on glycerol, the oxygen uptake rates in the presence of FAD was two-fold higher than that for aerobic growth. Interestingly, F_{420} proved to be as effective as FAD with glycerol grown cells. Also, it was only with F_{420} as cofactor, anaerobic cells, irrespective of the growth substrate, revealed two- to three-fold higher rates of methanol oxidation than that of aerobic cells; this enhancement was singularly high in cells grown anaerobically on methanol with six-fold enhancement in the presence of F_{420}. These results suggest that FAD and F_{420} linked methanol dehydrogenation could be constitutive in alcohol grown cells of *Pseudomonas MC*. Though aerobic oxidation of methanol in methanol grown cells appears to be preferentially FAD linked, F_{420}-linked methanol oxidation in *Pseudomonas MC* has emerged to be a very interesting feature revealed by the present study. These results strongly suggest that flavin linked methanol oxidation could be constitutive in *Pseudomonas MC*.

4. Discussion

Mixed methanogenic populations are mutually interdependent for their survival through syntrophic associations. Reports on hydrogen and formate transfer between species in syntrophic cultures utilizing ethanol and lactate (Stams 1994) and the activities of formate lyase and formate dehydrogenase in complex anaerobic communities (Thiele and Zeikus 1988), confirm such interactions.

Studies with mixed cultures broadly fall into two categories: (i) the use of culture-enrichment techniques to adapt suitable organisms from the natural inoculum towards utilization and degradation of specific compounds, and identify the partners later, and (ii) the use of defined pure microorganisms in cocultures to effect desired biotransformation reactions.

The former procedure was in fact adopted in the present study to enrich a methanol-utilizing methanogenic consortium with the desirable trait of methanogenic activity. This consortium conserved its metabolic characteristics, including that of methane generation, during maintenance with methanol as the growth substrate for over a decade (Lalitha *et al* 1994b). This methodology

Table 8. Oxidation of methanol by *Pseudomonas MC* cells grown on different alcohols.

	Cellular oxygen uptake (natoms oxygen/min/mg protein)	
Growth substrate	Aerobic growth	Anaerobic growth
FAD dependent		
Methanol	134 ± 60	141 ± 52
Ethanol	120 ± 50	107 ± 48
1-propanol	94 ± 34	86 ± 66
Glycerol	96 ± 47	211 ± 59
F_{420} dependent		
Methanol	75 ± 42	439 ± 68
Ethanol	150 ± 76	290 ± 75
1-propanol	155 ± 63	242 ± 54
Glycerol	76 ± 47	194 ± 54

Cells grown aerobically or anaerobically in medium C at 37ºC for 48 h, harvested and resuspended in 0·1 M phosphate buffer pH 7·4. Oxygen uptake by the cell suspension measured in the presence of methanol using flavin cofactors as indicated.
Values are averages ± SEM of three independent experiments.

could be well suited to enrich, preserve and propagate many methanogenic and other obligate anaerobic species that are otherwise difficult to maintain under normal laboratory conditions. Involvement of microbial consortia or communities composed of two or more taxa to effect efficient catabolism of different compounds were shown by many studies including those from our laboratory (Krishnan and Lalitha 1990; Lalitha *et al* 1994a; Wolfaardt *et al* 1994). Such populations also enable degradation of xenobiotics, particularly of many of the environmental pollutants (Hrywna *et al* 1999; Hubert *et al* 1999).

Though stable coexistence of methanogens and non-methanogens in methanogenic mixed cultures, on various growth substrates have been extensively studied (Bryant *et al* 1977; Stams 1994), there are no reports on such cultures with methanol as the growth substrate. The present study clearly reveals the methylotrophic, methanogenic and metabolic partnership between the isolated facultative *Pseudomonas MC* with the methanogen(s) under anaerobic conditions.

The isolated *Pseudomonas MC* proved versatile in metabolizing a variety of substrates including many alcohols. Though the cells of *Pseudomonas MC* anaerobically utilized sugars efficiently, it metabolized aliphatic acids and aminoacids as well. The present study conforms to the earlier observations (Palleroni 1984) on the growth of *Pseudomonads* in simple mineral medium devoid of trace elements or organic growth factors and with either NH_4^+ ion or NO^{3-} as sole nitrogen source. Significant enhancement in growth ensued by addition of yeast extract and tryptone suggestive of the probable requirement of some growth factor(s).

Evidence for a constitutive oxidative activity of the membrane system emerged from the high specific oxygen uptake of *Pseudomonas MC* apart from the high nitrate reductase activity seen in anaerobically grown cells. Cells grown anaerobically on glycerol displayed effective oxidation of methanol in the presence of added FAD and F_{420}. The pronounced enhancement in the rate of methanol oxidation in the presence of added flavin, might be due to (i) synthesis of the enzyme methanol dehydrogenase without the flavin cofactor or, (ii) the lability of flavin as a result of loose binding. Such lability was revealed in the NADPH dehydrogenase of *Entamoeba histolytica*, an eukaryote and the activity was reported to be low in the absence of added FMN/FAD (Lo and Reeves 1980). Evidence for several coenzyme F_{420}-dependent enzymes systems have emerged in recent times including those of several alcohol dehydrogenases (Reid and Fewson 1994). The operation of methanol dehydrogenation mediated by factor F_{420} emerges as a novel feature in *Pseudomonas MC*. More aspects of this mode of methanol oxidation are currently under investigation.

Anaerobic growth on glycerol was notable in the presence of nitrate and it is significant that the activity of nitrate reductase was highest with methanol as growth substrate, pointing to the effective electron-transfer at the membrane level coupled to energy recovery. With glucose as the growth substrate, cells take up the sugar by specific transport mechanisms for energy yield primarily by glycolysis. In contrast, during anaerobic growth of *Pseudomonas*, the action of the membrane-bound nitrate reductase in oxidizing ubiquinol by nitrate is coupled to the translocation of protons across the cytoplasmic membrane resulting in energy capture. Nitrate reductases of *Escherichia coli* has been characterized and the regulatory mechanisms have been studied in detail (Cole 1996).

The isolate *Pseudomonas MC* possesses characteristics conforming to the generally accepted metabolic versatility of the species. But in addition, this study reveals in particular, an efficient alcohol utilizing capacity of the isolate. A marked propensity for anaerobic methanol utilization by *Pseudomonas MC* warrants special mention. Further, studies are in progress with an aim to understand the nature of metabolic partnership between *Pseudomonas MC* and methanogen(s) in the methylotrophic methanogenic enrichment. The results of these studies suggest that it was the *Pseudomonas MC* that had the capability to utilize methanol, with effective contribution of F_{420} from the methanogen(s), while inter-species hydrogen and formyl group transfer mechanisms could also operate to effect the methylotrophic methanogenic partnership between these organisms.

The present study is an example of syntrophic metabolic interactions in an artificially developed ecosystem that was operative toward achieving an uphill task of methanogenic

anaerobic growth on a simple one-carbon substrate, methanol. Further, investigations on the specific nature of metabolic interactions in this association (to be communicated), are suggestive of efficient formyl group transfer mechanisms being operative within this syntrophic association.

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