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# The biology of PM-1, a methyl tert-butyl ether-degrading bacterium

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# THE BIOLOGY OF PM-1, A METHYL TERT-BUTYL ETHER -DEGRADING BACTERIUM

A Thesis

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The Faculty of the Department of Biological Sciences

San Jose State University

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## ABSTRACT

THE BIOLOGY OF PM-1, A METHYL TERT-BUTYL ETHER-DEGRADING BACTERIUM

by Mrinalini Aswath

This thesis describes a detailed ultrastructural and biochemical observation of PM-1, a bacterial isolate from the environment that degrades methyl *tert*-butyl ether (MTBE). MTBE is an oxygenate in gasoline that has been found to contaminate groundwater and soil. PM-1 degrades MTBE with high efficiency and forms aggregates in the presence of MTBE. Electron microscopy revealed the presence of an exopolysaccharide layer that is involved in the formation of aggregates. Also reported are morphological differences between PM-1 and SP-Y, which also degrades MTBE albeit slowly, and *Sphingomonas*, once thought to be the genus to which PM-1 belonged. Detailed molecular analyses and biochemical tests of PM-1 identified the isolate as an *Aquabacterium* species. An enrichment was set up to investigate the presence of the MTBE degraders in nature.

## ACKNOWLDGEMENTS

I dedicate this thesis to my grand-uncle Mr. S. R. Ramaswamy who first introduced me to the concept of "environment", who instilled in me, the habit of reading, and in his quiet ways, inspired me to explore ways to make this a better planet. His tireless efforts to save trees in southern India is continuing to bring much awareness among common people otherwise engaged in destructive felling activities. This work would not have been possible without the unending support from Dr. Grilione, Dr. Bruck, and Dr. Scow. I wish to thank Dr. Grilione for her enthusiasm and dedication that made this research a pleasurable experience.

Special thanks to my husband, Murali Venkat, who has supported me in every way possible and to Swapna Mayya for her encouragement and friendship all along graduate school.

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# LITERATURE REVIEW

Bioremediation is a term used in reference to a variety of technologies that exploit the metabolic actions of biological organisms, usually microorganisms, to alter (degrade and/or detoxify) chemical wastes. Soil, sediment, sludge, ground and wastewater, and even contaminated gas streams are detoxified using these methods (21). The decomposition is achieved by the existing microbial metabolic potential or by the introduction of genes encoding those functions. Bioremediation is used for post-contamination treatment and as a preventative measure. Because of potential cost savings and public concern over incineration and placement into landfills, bioremediation is playing an increasing role in the remediation marketplace (28). Bacteria and fungi mediate the majority of degradative pathways of organic compounds in nature. Rapid growth and metabolism, genetic plasticity, and the ability to survive in various environments have made bacteria most useful in bioremediation (3). Pollutants that have been biologically decontaminated include polychlorobiphenyls, pesticides and insecticides, petroleum, and dioxins.

## Methyl *tert* -butyl ether (MTBE).

MTBE is a synthetic chemical (Fig. 1) used as a blending component in gasolines. It is an oxygenate manufactured by the chemical reaction of methanol and isobutylene. The chemical was first introduced in the late 1970s to areas identified with high levels of air pollution, California being one of them. MTBE improves air quality by reducing

emissions of ozone-forming compounds, air toxics, and carbon monoxide. It dilutes or displaces gasoline components such as aromatics and sulfur (48). Table 1 summarizes select features of MTBE that have direct and indirect consequences on the environment. Only recently have researchers begun to identify the health hazards and the ecological impact of MTBE in soil and groundwater.

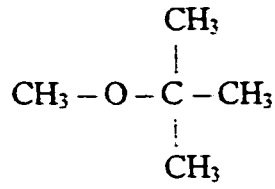


**FIG. 1.** Elemental composition of MTBE.

**TABLE 1.** Selected features of MTBE (48)

| | |
|---|---|
| Molecular Weight | 88.14 g/mole |
| Boiling and Freezing points | 55.2 °C/ 108.6 °C |
| % in gas | 15% |
| Water solubility in pure phase | ~43,000 ppm |
| Water solubility in gas phase | ~5100 ppm |
| Density | 0.741 g/ml at 20 °C |
| Vapor Pressure | 313 Torr at 30 °C |
| Henry's law constant | 0.018 |
| Log $K_{oc}$ | 1.05 |
| Taste threshold in water | ~40 – 140 ppb |
| Odor threshold in water | 15-40 ppb |
| CA state limit regulation in water | 35 ppm |

**Health and ecological impacts of MTBE**

Acute inhalation effects in humans are caused by breathing small amounts of MTBE. The symptoms include nausea, headache, and irritation of the eyes, sinus and throat. Evidence suggests carcinogenic effects among mice upon repeated exposure to high levels of MTBE (31).

There are many ecological problems associated with MTBE. The oxygenate quickly evaporates from fuel exhaust, open-air containers, and surface water. It is highly persistent in groundwater following release to the subsurface. Possible point sources of contamination of groundwater include leakage from underground storage systems (tanks and pipes), overfill and spills at gasoline stations, pipelines, landfill sites and dumps, spillage at industrial and refueling facilities, accidental spills during transport, and aboveground storage tanks. At room temperature, it is a volatile, flammable, and colorless liquid that is highly soluble in water. MTBE is poorly removed through volatilization by air-stripping and adsorptive removal by activated carbon (12). Current methods of remediating contaminated sites include ozone/oxygen sparging and natural attenuation (48). Cheaper and more effective methods are desired and microbial remediation is considered a potentially promising technology.

**Bioremediation of MTBE, an historical perspective**

Many laboratories have been actively involved in addressing the issue of biodegradability of MTBE since the early 1990s when the problem of groundwater contamination was first identified. In 1993, Sulfita and Mormille (44) reported MTBE

degradation only under methanogenic conditions with a very low yield of biomass of cells. MTBE was converted to *tert*-butyl alcohol, but this intermediate was not degraded further. In 1994, researchers reported the discovery of an aerobic bacterial consortium, BC-1, from sludge that mineralized MTBE to $CO_2$. The organisms within the consortium were not characterized and pure cultures were not obtained (42). Another mixed, aerobic culture which grew on MTBE was discovered by Cowan and Park (12) through enrichments, but the rates of degradation were not significant. Mo, *et al.* (34) reported the ability of pure cultures of *Rhodococcus*, *Methylobacterium*, and *Arthrobacter* to degrade MTBE. They observed only partial degradation and very slow growth on MTBE. More recently, propane-oxidizing bacteria have been successfully used to cometaboloize MTBE (43). The ability of propanotrophs to degrade MTBE in the presence of gasoline is currently being investigated. Anaerobic degradation of MTBE by a consortium was reported in soil containing a low concentration of organic matter and with a pH around 5.5 (53). MTBE was not degraded in organic-rich soil.

In addition to bacterial cultures, fungi and plants have been explored as MTBE-degraders. An isolate of *Graphium* was reported by Hardison, *et al.* to degrade MTBE (20). Phytoremediation of MTBE using poplars and eucalyptus trees is also being investigated (35).

Thus far, the bacteria identified as MTBE-degrading have one or all of the following drawbacks:

1. They exist in mixed cultures. The members of the consortia have not been characterized or the organisms cease to degrade MTBE if isolated from the consortium.
2. The cultures yield very low biomass and the rates of degradation are too slow to be useful for bioremediation.
3. Many of them operate only under anaerobic conditions that are impractical for industrial applications.

**Recent research on MTBE degradation**

In 1997, a research team at University of California, Davis (UCD), isolated two strains of MTBE-degrading bacteria from a mixed microbial consortium obtained from a compost-filled biofilter at the Joint Water Pollution Control Plant in Los Angeles County, CA. The two isolates were named PM-1 and SP-Y. Isolate PM-1 completely mineralized MTBE considerably faster than any other previously described microorganism. SP-Y degraded MTBE much more slowly. Preliminary rDNA analyses of a small portion of the 16S ribosome indicated a high degree of homology with *Sphingomonas* sp. Results from 16S rRNA sequencing and the Ribosomal Database Project similarity searches led to the conclusion that SP-Y resembled *Sphingomonas* sp. (86-90%), while PM-1 was closest to *Rubrivivax gelatinosus* (80-88%). Other close matches to PM-1 were *Leptothrix discophora* (86%) and *Ideonella dechlorotans* (82%).

The National Center for Biotechnology Information BLAST searches matched PM-1 to *Aquabacterium commune* (99%). Fingerprinting approaches indicated that PM-1 was the dominant member of the consortium and, based on the above data, it was concluded that PM-1 belongs to the genus *Aquabacterium* (8).

PM-1 aggregates into clumps when exposed to MTBE (8). For successful bioremediation of contaminated sites, accurate enumeration of the organism is required and aggregation results in inaccurate quantification by plate counts. Thus it became imperative that an investigation be conducted to determine causes of aggregation among PM-1. Many factors, including the presence of a capsule, flagella, and response to environmental stress have been suggested as the cause(s) of clumping of other microorganisms (1).

**Bacterial structures associated with aggregation**

Exopolysaccharide. Bacterial exopolysaccharides (EPS) can be present in two forms, capsules (capsular polysaccharide, CPS) and a slime layer. Many gram-positive and negative bacteria have covalently attached capsules as superficial layers that are diverse in composition, thickness, and function (52). CPS generally has a high molecular weight and a net negative charge, although CPS of a few genera has cationic side chains (25). Entire microcolonies may sometimes be ensheathed in capsular material (52).

Bacterial slimes are composed of homo- and heteropolymers but, unlike the rigid capsules, slimes are often found as a loose network of gel and fibrils extending from the cell surface (5). Some bacteria overproduce capsules and, for various reasons, the

polymers become detached from the cell to form extracellular slime. These slime polysaccharides have a low molecular weight (6). The term glycocalyx is a general term for bacterial polysaccharides and is defined as the polysaccharide-containing material external to the cell (30). If the glycocalyx is rigid, it is called a capsule, whereas, if the polysaccharide is easily deformed, it is called slime.

The EPS layer plays important roles in bacterial functions. Capsules provide protection to invading pathogenic bacteria and help cells adhere to surfaces. Many drugs have been designed to prevent the adhesion of pathogens to their target organs (4). The EPS protects cells from drying and prevents fluctuation in water potential in the environment of the cell. The hygroscopic nature of the polysaccharides also increases nutrient availability within the bacterial colony. Desiccation has been found to enhance EPS production in *Pseudomonas* sp. (39). The EPS mediates sorption of cells to soil particles and thus hinders movement within soil. This can ultimately affect the rate of degradation of pollutants (18). Many bacteriophages bind to EPS during the first step in infection (27). Exopolysaccharides are involved in bacterial attachment to solid phases of iron, magnesium silicate, copper, nickel, and cobalt-containing substrates. This property is effectively employed in bioleaching and to detoxify sulfur-containing mine water and other industrial effluents (17, 19). *Dextran, Xanthan, Scleroglucan, Pullulan, Curdlan, Zanflo* are some examples of microbial polysaccharides which are commercially important rheological agents (50). Capsular material has been found to act as glue to hold cells together to form aggregates that on repeated cell divisions lead to biofilms. Biofilm formation leads to reduced effectiveness in industrial systems and

expensive cleaning operations. On the other hand, biofilms are useful in wastewater treatment (10).

The chemical structures of many of the polysaccharides have been determined by mass spectrometry, infrared resonance, nuclear magnetic resonance, and chemical methods (2). The amount of EPS produced is greatly influenced by the composition of the medium used to culture the organisms (9). Concentrations of yeast extract, glucose, potassium phosphate, and trace elements, pH of the medium, and the temperature of incubation have significant influence on the nature and quantity of EPS (7).

Pili/fimbriae. Pili and fimbriae are non-flagellar bacterial appendages that range from 30 to 250 A° in diameter and are beyond the resolving power of the light microscope. They differ morphologically from flagella in that they are less rigid, thinner, and straighter, and are more numerous than the flagella. There is confusion in the literature in differentiating pili from fimbriae. Both are thought to participate in attachment to natural substrates and to other members of the community. They appear to enhance membrane-associated functions, such as respiration and nutrient uptake. Specific types of pili (sex pili) participate in DNA transfer during conjugation and act as specific receptor sites for some bacteriophages. Fimbriae are classified as thick or thin based on the diameter of the organelle. Both pili and fimbriae occur mainly among gram-negative bacteria (37). These structures have been identified in many biofilms that are involved in bioremediation (40).

**Bacterial cell structural changes during growth**

Many structural changes occur in bacteria during the lag, logarithmic (log), stationary, and death phases of growth. Changes in shape, accumulation of storage materials [poly β-hydroxybutyrate (PHB) and polyphosphates], and decreased levels of EPS are thought to occur with the gradual deterioration of cell contents leading to death. The duration of each phase, the synthesis and quantity of bacterial EPS, and the average size of a bacterium vary with the genus and growth conditions (38). In some bacteria growing in a broth, EPS production does not start until the end of the log phase and is then released into the environment during the stationary phase (7, 26, 44, 47). In others, the maximum rate of EPS synthesis occurs during the log phase (32). Clusters of many genes tightly control the dislodging process (52).

PHB. Many microorganisms accumulate PHB as their principal intracellular reserve of carbon and energy. The amount of PHB accumulation depends on the composition of the medium and the physiology of the cells. In *Bacillus megaterium*, large amounts of PHB accumulate when the medium contains a high ratio of carbon to nitrogen and when the cells are in exponential phase. Levels of PHB decrease when the cells move to stationary phase (29, 37).

Polyphosphate. Polyphosphate serves as a source of reserve phosphate and pyrophosphate. There is evidence indicating that polyphosphate replaces ATP in some

reactions, maintains electrochemical potential across membranes, and acts as chelator of divalent cations. Polyphosphates accumulate during the log phase among bacteria (38).

## OBJECTIVES OF THIS RESEARCH

The objectives of this research are (1) to study the gross and ultrastructural differences between PM-1 and SP-Y, the two MTBE-degrading isolates, and *Sphingomonas*, a tentatively matched genus of PM-1, (2) to attempt to demonstrate the presence of an EPS layer surrounding the aggregates, (3) to study the morphological differences at the electron microscope and light microscope levels along the growth curve of PM-1 grown on MTBE and ethanol as sole carbon sources, (4) to characterize the biochemical properties of PM-1 and compare them to those of *Aquabacterium* sp., a genus of apparent identity based on rRNA sequencing with PM-1, and (5) to conduct an enrichment to investigate the ubiquity of MTBE-degraders in a soil environment.

## MATERIALS AND METHODS

### Bacterial cultures

The original isolates of PM-1 and SP-Y obtained from Kate Scow, Department of Land, Air, and Water Resources, University of California, Davis, were received growing in mineral salts medium with MTBE as sole carbon source, in tryptic soy broth diluted 1:10, and on tryptic soy agar plates. *Sphingomonas paucimobilis* strain 29837 was purchased from the American Type Culture Collection as a lyophilized culture and was reconstituted according to instructions.

### Growth conditions

All media used for culturing and characterizing the organisms were obtained from Difco and prepared according to the Difco Manual (15) unless otherwise noted. The pH of all media was checked and adjusted to 7.0. Liquid cultures were incubated on a waterbath shaker at 30°C for 24-96 h at 100 rpm. Agar plates were incubated in a 30°C incubator for 24-96 hours. Frozen stocks were maintained at –70°C in sterile nutrient broth containing 30% glycerol.

Culture and growth media. PM-1 was routinely grown on nutrient agar (NA) and nutrient broth (NB), SP-Y on trypticase soy agar (TSA) and trypticase soy broth (TSB), and *Sphingomonas* sp. on yeast-malt (YM) agar diluted 1:4. A modified Davis minimal medium broth (MM) containing 0.1 g $MgSO_4$, 1 g $(NH4)_2SO_4$, 7 g $K_2HPO_4$ and 2 g $KHPO_4$ per liter of distilled water was used when growing cultures with MTBE

(20 ppm) as the sole carbon source. Special glassware fitted with Mini-Nert valves from Alltech Biosciences (Deerfield, IL) was used when growing PM-1 on MTBE in liquid culture.

To identify an alternative carbon source for PM-1, washed 24 h cultures were inoculated into 100 ml sterile MM. One of the following was added as a sole carbon source: pyruvate, acetate, citrate, L-asparagine, DL-alanine, or glycine at 0.01% (w/v) or butanol, ethanol, or methanol at 0.1% (w/v). The cultures were monitored regularly for turbidity and aggregation. The relative turbidity of cultures was measured with a Spectrometer 20 at 525 nm.

Characterization media for PM-1. Media for detecting starch, casein, DNA, and gelatin hydrolysis and for nitrate reduction were prepared according to the Difco Manual (15). Modified esculin agar contained 0.01% (w/v) esculin, 0.05% (w/v) iron (III) citrate, beef extract, peptone, and agar. Urea hydrolysis was determined using commercially available Remel urea agar (Lenexa, KS). Cells for catalase and oxidase tests were grown on NA. API 20NE biochemical tests were conducted according to the manufacturer's instructions (bioMerieux, Paris).

Enrichment media. The chemical composition of minimal salts agar (MSA) plates was similar to that of MM except for the addition of 15g/L agar. MTBE was supplied by placing 100% MTBE-saturated filter papers in the lids of inverted glass Petri dishes containing MSA. Subsequent volatilization made the MTBE available to the organism.

**Light and phase contrast microscopy**

The size, shape, motility, and Gram reaction of PM-1, SP-Y, and *Sphingomonas* cells were observed by wet mounts, negative stains, and Gram stains using a Leitz phase contrast microscope. PHB granules were detected with Sudan Black (24). Aggregates of PM-1 growing in MTBE were lifted from the liquid medium with a sterile 10 ml serological pipette, placed on a glass slide, and covered with a coverslip. A few microliters of 10% (v/v) skim milk were allowed to flow under the coverslip; the mount was then observed for the presence of an exopolysaccharide layer around the clump (16). Anthony's capsule stain (33) was used on the dispersed cells of PM-1.

**Transmission electron microscopy**

Negative staining. The organisms were suspended in Millipore-filtered, distilled water, and single drops of the cell suspension were placed on carbon and Formvar-coated copper grids. The cells were allowed to settle onto to the grid surface for 1 min before blotting with filter paper. Single drops of 1% (w/v) aqueous uranyl acetate were added to the grid for 10-25 s. These specimens were observed with a Zeiss EM 109 transmission electron microscope (TEM) at 80 kV and filmed with Kodak Technical Pan 120 film.

Thin sectioning. Electron microscopy chemicals were purchased from Electron Microscopy Sciences (Fort Washington, PA) or Sigma Chemical Co. (St. Louis, MO). Bacteria grown on the various carbon sources were centrifuged at 2500 xg for 5 min.

The pellets were resuspended in 0.1 M sodium cacodylate buffer, pH 7.0. Centrifugation and resuspension were repeated twice more. Primary fixation was carried out for 2 h without agitation at room temp in 5% glutaraldehyde in the same buffer. The specimens were washed 5 times with buffer and then immobilized in sterile, cooled 4% Bacto Difco agar. The agar was cut into approximately 1 $mm^3$ blocks and postfixed at room temp for 2 h with osmium tetroxide in cacodylate buffer without agitation. These samples were washed 5 times by gently swirling in the buffer, dehydrated for 5 min each in a graded series of acetone (10, 25, 40, 50, 70, 85, 95, and 3x in 100%), infiltrated and embedded in Epon 812 resin, and cured at 60°C for 48 h. The blocks were thin sectioned on a Sorvall Porter-Blum ultramicrotome with glass knives. Thin sections were poststained with 2% (w/v) aqueous uranyl acetate and Reynold's lead citrate and examined under the TEM (20, 49). Thin sections from a minimum of 6 blocks per culture were observed.

Clumps of PM-1 in MTBE were extracted from the medium and glutaraldehyde was added prior to the washes with sodium cacodylate. The clumps were then prepared for electron microscopy as described above.

**Growth curve experiment and cell sampling**

Growth curves were determined for PM-1 growing in ethanol and in MTBE. Data were collected as described below, after the cells were transferred 4 successive times into MM containing either ethanol or MTBE. This was done to assure the cells were acclimated to the carbon source. From these data, the time points at which the cells entered log and stationary phases were determined.

Ethanol growth curve determination and cell sampling. The optical density (OD) of a 3 ml sample of the acclimated 24 h ethanol culture was measured with a Spectrometer 20 at 525 nm. A separate 3 ml sample was used as the seed inoculum. This cell suspension was centrifuged at 1600 xg for 5 min, washed in sterile distilled water, recentrifuged, and inoculated into 200 ml MM containing 200 µL absolute ethanol. The contents of the flask were mixed by gentle swirling, and a 3 ml sample was immediately taken and its OD was measured (time=0). The culture was incubated in a 30°C water bath shaker. OD was measured on 3 ml samples every 15 min for a total of 300 min. The growth curve generated is shown in Fig. 2. In a subsequent procedure, samples of cells were taken at t = 120, 128, 135, 165 and 210 min and immediately fixed for TEM.

MTBE growth curve determination and cell sampling. Growth of PM-1 on MTBE was monitored by measuring the disappearance of MTBE with a Shimadzu C14A gas chromatograph (GC) fitted with a photoionization detector. PM-1 was grown to maximum density in a bottle fitted with Mini-Nert valves containing 100 ml MM by feeding 60 ppm MTBE every 24 h for 3 successive days on a shaker at 100 rpm. The suspension was then centrifuged, washed and resuspended in sterile water. Two ml of the suspension was added to 25 ml MM containing 20 ppm MTBE. Fifty µL of this suspension were injected into the GC and sampling was conducted every 60 min until the concentration of MTBE fell below detectable limits (Table 2 and Fig. 3). The values were compared to a standard curve generated by injecting known concentrations of

MTBE (Fig. 4). Based on the GC data, 20 ml cell samples were collected and immediately fixed for TEM.

**Enrichment of MTBE-degraders from the environment**

Soil samples and isolates. Soil was collected from two sources. One was from a gas station under reconstruction (labeled Spartan soil) and the other from an unfertilized, untreated backyard soil (labeled Backyard soil). The samples were collected in 100 ml sterile collection containers with 3 holes bored at the base for drainage of excess liquid. Immediately after collecting the samples, total colony forming units per gram (cfu/g) were determined for each by means of standard plate count technique. The samples were then treated as shown in Table 3. The containers were loosely capped and incubated at room temperature for 7 days.

**TABLE 3.** Protocol for enriching for MTBE-degraders from soil

| Soil Sample | Treatment |
|---|---|
| Spartan soil | 25 ml sterile water |
| Spartan soil | 25 ml sterile NB |
| Backyard untreated soil | 25 ml sterile water |
| Backyard untreated soil | 25 ml sterile NB |
| Backyard treated soil | 25 ml 500 ppm MTBE in sterile water |
| Backyard treated soil | 25 ml 500 ppm MTBE in sterile NB |

To facilitate isolation, serial dilutions were performed on each treated sample and plated onto NA and MSA in glass Petri dishes. The soil samples were enriched with the appropriate treatment every 3 days (Table 3). The filter papers used to supply MTBE were saturated every 3 days and plates were inspected for growth every 24 h.

Fourteen days after the enrichments were set up, a second sampling was attempted, and the dilution and plating procedure described above was followed. Eleven well-isolated colonies were chosen from the MSA plates on the basis of their plate morphology. The cultures were streaked for isolation twice onto NA plates, and challenged to grow on MSA plates with MTBE supplied by the filter papers. NA and MSA plates without MTBE were used as controls. Stock and working slants were prepared on NA slants.

# RESULTS

## Growth curves and sampling

Ethanol. The growth curve for ethanol-grown cells is shown in Fig. 2. The seed inoculum had an OD of 0.12 (Fig. 2). There was a lag period of 120 min before the cells entered exponential phase. The short logarithmic phase was followed by an extended stationary phase. From this curve, it was determined that sampling at t=120, 128, 135, 165, and 210 min would represent the cells in early log, mid log, late log, early to mid stationary, and late stationary phases, respectively.

MTBE. The GC data based on the disappearance of MTBE (Table 2) were used to determine sampling intervals for MTBE-grown cells. From the graph shown in Fig. 3, sampling at t = 5, 8, 10, 12, 14, and 17 h would represent early log, mid log, late log, early to mid stationary, mid to late stationary, and late stationary stages of growth, respectively. A standard curve (Fig. 4) was generated using known MTBE concentrations (100, 200, and 300 ppm) prior to measuring the rate of disappearance. The standard curve was generated to find the concentration of MTBE (in ppm) of each sample and to assure the proper functioning of the lamp in the GC.

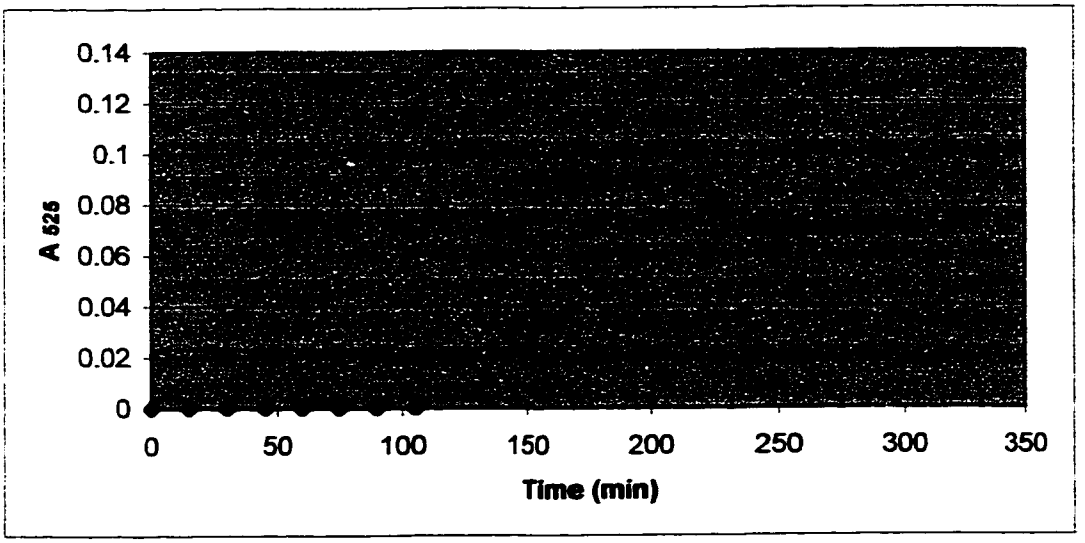



**FIG. 2.** Growth curve of PM-1 in ethanol as the sole carbon source.

**TABLE 2.** GC data of PM-1 grown on MTBE as the sole carbon source indicating the disappearance of MTBE

| Time (h) | GC area | Conc. (ppm) |
|---|---|---|
| 0 | 27031 | 207.61 |
| 1 | 14244 | 109.40 |
| 2 | 11463 | 88.04 |
| 3 | 10346 | 79.46 |
| 4 | 9446 | 72.55 |
| 5 | 8156 | 62.64 |
| 9 | 3836 | 29.46 |
| 20 | 120 | 0.92 |

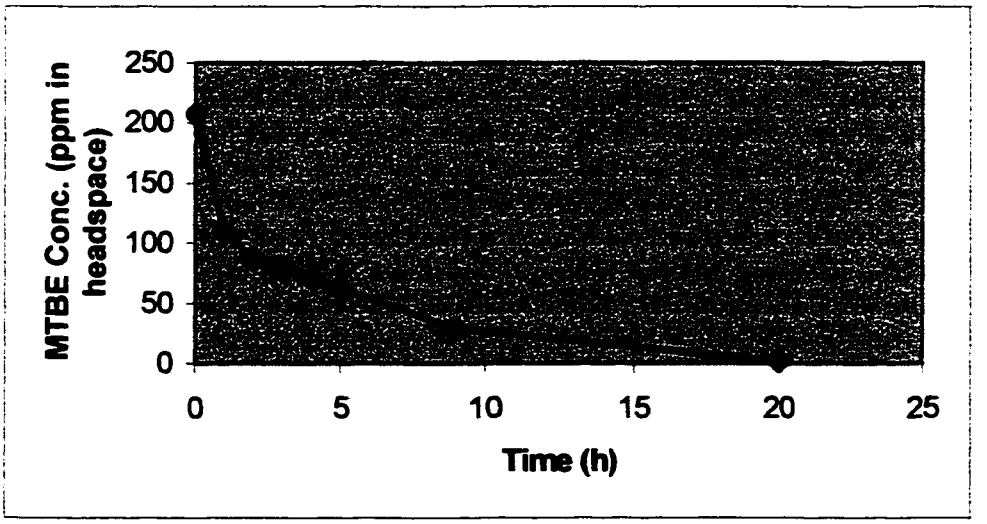



**FIG. 3.** Growth curve of PM-1 in MTBE as the sole carbon source; decrease in MTBE headspace concentration with time due to degradation by PM-1.

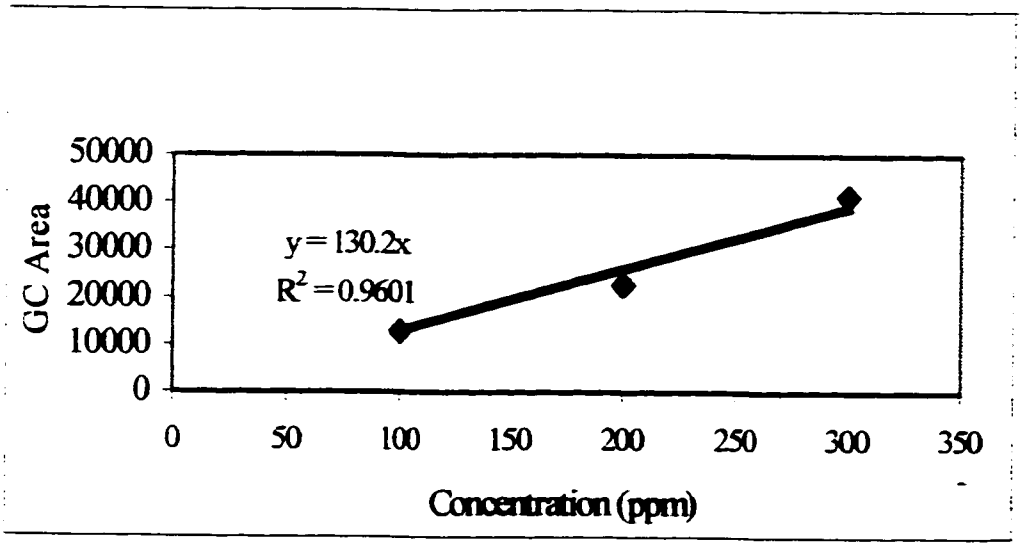



**FIG. 4.** Standard curve for the growth estimation of PM-1 on MTBE as the sole carbon source.

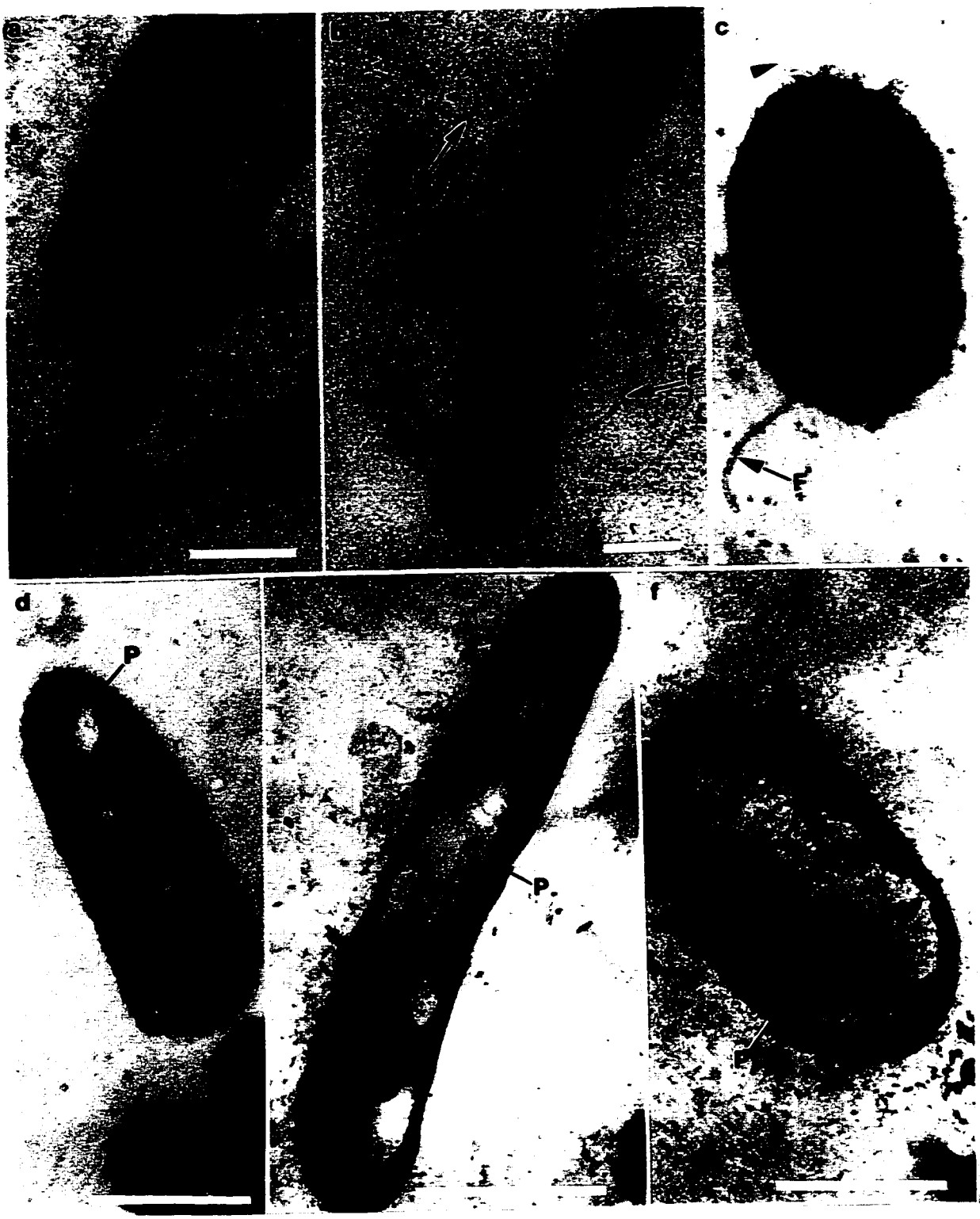
**Morphology of PM-1, SP-Y, and *Sphingomonas* sp.**

On NA, PM-1 formed cream-colored, flat colonies 2-3 mm in diameter and with smooth margins. SP-Y and *Sphingomonas* sp. grew at a faster rate on TSA than on NA and formed yellow, raised colonies 5-6 mm in diameter with smooth margins. The most rapid growth of PM-1 occurred at 25-30°C. Of the various substrates tested, PM-1 grew on the following compounds as sole carbon sources (in decreasing order of turbidity): ethanol, pyruvate, L-asparagine, acetate, butanol, DL-alanine, and methanol. Aggregation was not observed in these carbon sources. Growth was not present on citrate and DL-glycine.

Negatively stained cells of PM-1 (Fig. 5a) grown on NA were rod shaped with a single polar flagellum and multiple long, narrow fimbriae. The PM-1 cells averaged 1.5 μm in length. SP-Y cells were rods with multiple flagella (Fig. 5b) but lacked fimbriae. The flagella were not polar and varied in number per cell. The SP-Y cells (2.3 μm) were longer than the PM-1 cells. *Sphingomonas* sp. cells were rods (2.7 μm) bearing a single flagellum and multiple, short, thick fimbriae (Fig 5c). Thin sections of PM-1 in Fig. 5c revealed the presence of both PHB and polyphosphate, while SP-Y and the *Sphingomonas* sp. contained only PHB (Fig. 5d and 5e, respectively).

EPS was seen between cells and surrounding the clump in thin sections (Fig. 6) and under the light microscope.

**FIG. 5.** (a)-(c) Negative stains of (a) PM-1. x34,000, (b) SP-Y. x24,000, and (c) *Sphingomonas* sp. x21,000. The distribution of flagella (F) and fimbriae (arrowheads) varied among the three isolates. (d)-(f) Thin sections of (d) PM-1. x53,000, (e) SP-Y. x63,000, and (f) *Sphingomonas* sp. x54,000. All three organisms accumulated PHB granules (P) when grown on nutrient agar. All bars, 0.5 μm.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P
f

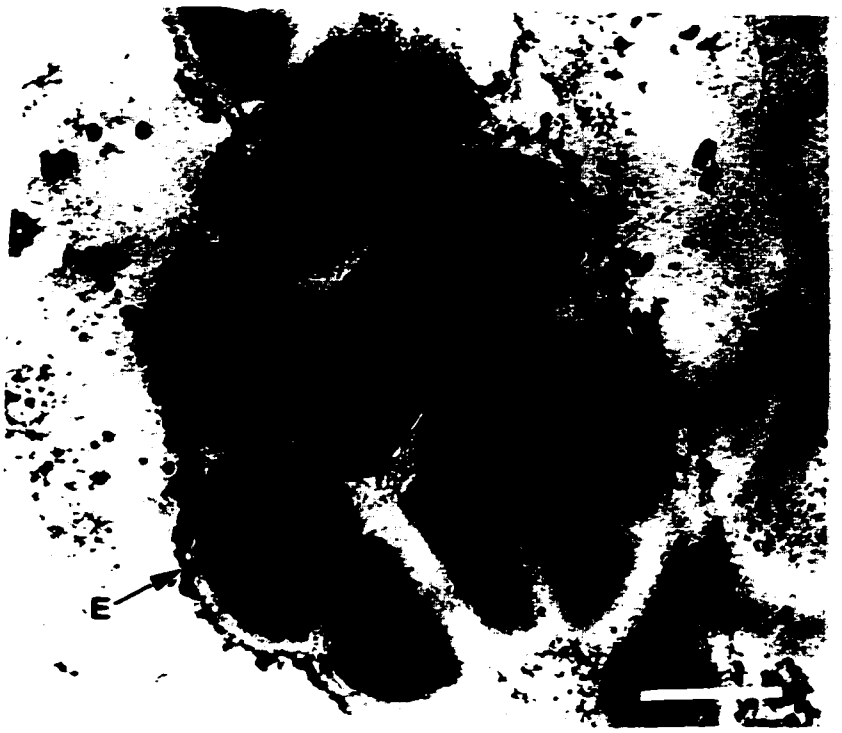


**FIG. 6.** Thin section of an aggregate of PM-1 grown in MTBE. The EPS layer (E) envelops the clump and is present between cells. x37,000. Bar, 0.5 μm.

**Biochemical properties of PM-1**

A comparison of the biochemical properties of PM-1 and *Aquabacterium* sp. (Table 4) suggest that the two organisms are identical.

**Thin sections of the growth curve samples**

Electron micrographs in Fig. 7 represent the ethanol-grown cells in various phases of growth. The cells in all phases were dispersed and did not appear to produce EPS. Accumulations of polyphosphate granules were seen during early logarithmic phase and were stored through stationary phase. PHB accumulation began during mid to late log phase and continued to late stationary phase. The cells were the longest at mid log phase (1.6 μm) and shortest (0.9 μm) during stationary phase.

MTBE-grown cells produced EPS from the early log phase to the stationary phase (Fig. 8). The amount of EPS gradually increased with age and became dispersed in the medium during the late stationary phase. In contrast to the ethanol-grown cells, PHB and polyphosphate materials were not evident during any of the phases. During the log and early stationary phases, the cells measured 0.5-0.7 μm and grew as much as 1.05 μm during mid stationary phase.

**Environmental enrichment of MTBE degraders**

Table 5 indicates the source of the 11 isolates obtained from the enrichment for MTBE-degraders. All soil sources yielded cells capable of growing on MTBE. Eight isolates were obtained from the backyard soil and 3 were from Spartan soil. A summary

of the biochemical properties of these isolates is shown in Table 6. Eight isolates were gram-positive rods, 2 isolates were gram-negative rods and 1 isolate was gram positive coccus.

**TABLE 4.** A comparison of biochemical characteristics of *Aquabacterium* sp. and PM-1

| Test | *Aquabacterium* [a] | PM-1 |
|---|---|---|
| | | |
| Motility | + [b] | + |
| Gram Reaction | - | - |
| Oxidase Test | + | + |
| Catalase Test | - | - |
| **Hydrolysis Tests** | | |
| DNA | - | - |
| Urea | +/- | + |
| Esculin | - | - |
| Starch | - | - |
| Casein | +/- | - |
| Gelatin | - | - |
| **API 20NE results** | | |
| ONPG | - | - |
| Lysinie decarboxylase | - | - |
| Ornithine decarboxylase | - | - |
| Citrate | - | - |
| Indole test | - | - - |
| Voges-Prausker test | - | - |
| Glucose | - | - |
| Rhamnose | - | - |
| Sucrose | - | - |
| Melibiose | - | - |
| Arabinose | - | - |
| Xylose | - | - |
| **Other tests** | | |
| Nitrate reduction | + | + |
| Nitrite reduction | - | - |
| Matrix around cells | + | + |
| Cream colonies | + | + |
| Pyruvate, butanol utility | + | + |

[a] The biochemical properties of *Aquabacterium* were summarized from Kalmbach, *et al.*, 1999 (23).

[b] + indicates the presence or a positive reaction, - indicates a negative reaction, and +/- indicates variability of reaction among strains.

**FIG. 7.** (a)-(e) Electron micrographs of thin sections of PM-1 grown in ethanol and at various stages of growth. (a) Early log phase. x34,000. (b) Mid log phase. x53,000. (c) Mid log phase. x37,000. (d) Early/mid stationary phase. x54,000. (e) Late stationary phase. x55,000. Polyphosphate granules (G) accumulated during early log phase and occurred throughout growth. PHB granules (P) appeared after late log phase. All bars, 0.5μm.

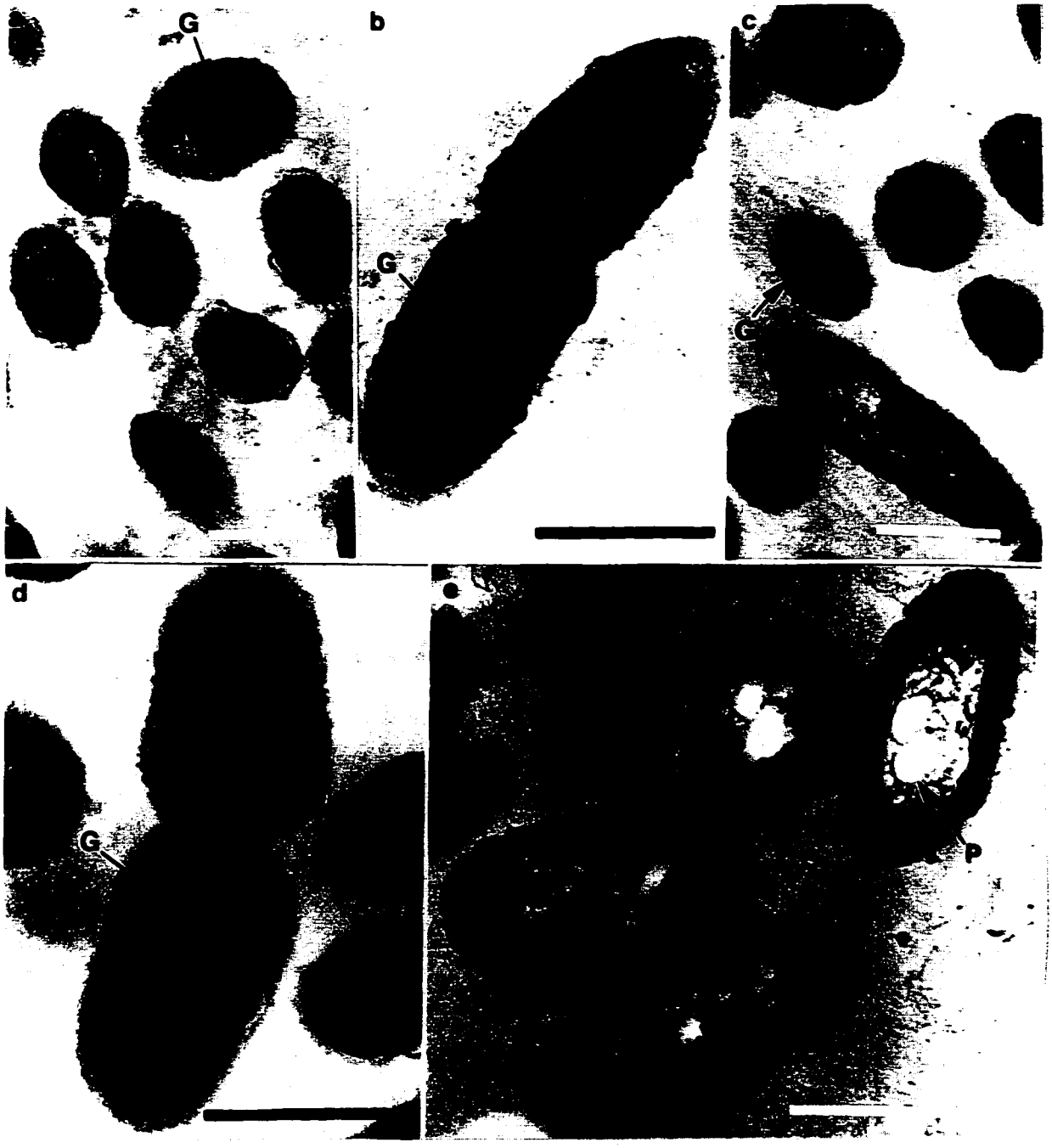
a
G
b
G
c
G
d
G
e
P

**FIG. 8.** (a)-(f) Electron micrographs of thin sections of PM-1 grown in MTBE at various stages of growth. (a) Early log phase. x58,000. (b) Mid log phase. x51,000. (c) Late log phase. x37,000. (d) Early stationary phase. x60,000. (e) Mid stationary phase. x37,000. (f) Late stationary phase. x50,000. EPS (arrowheads) is seen throughout growth surrounding the cells. All bars, 0.5μm.

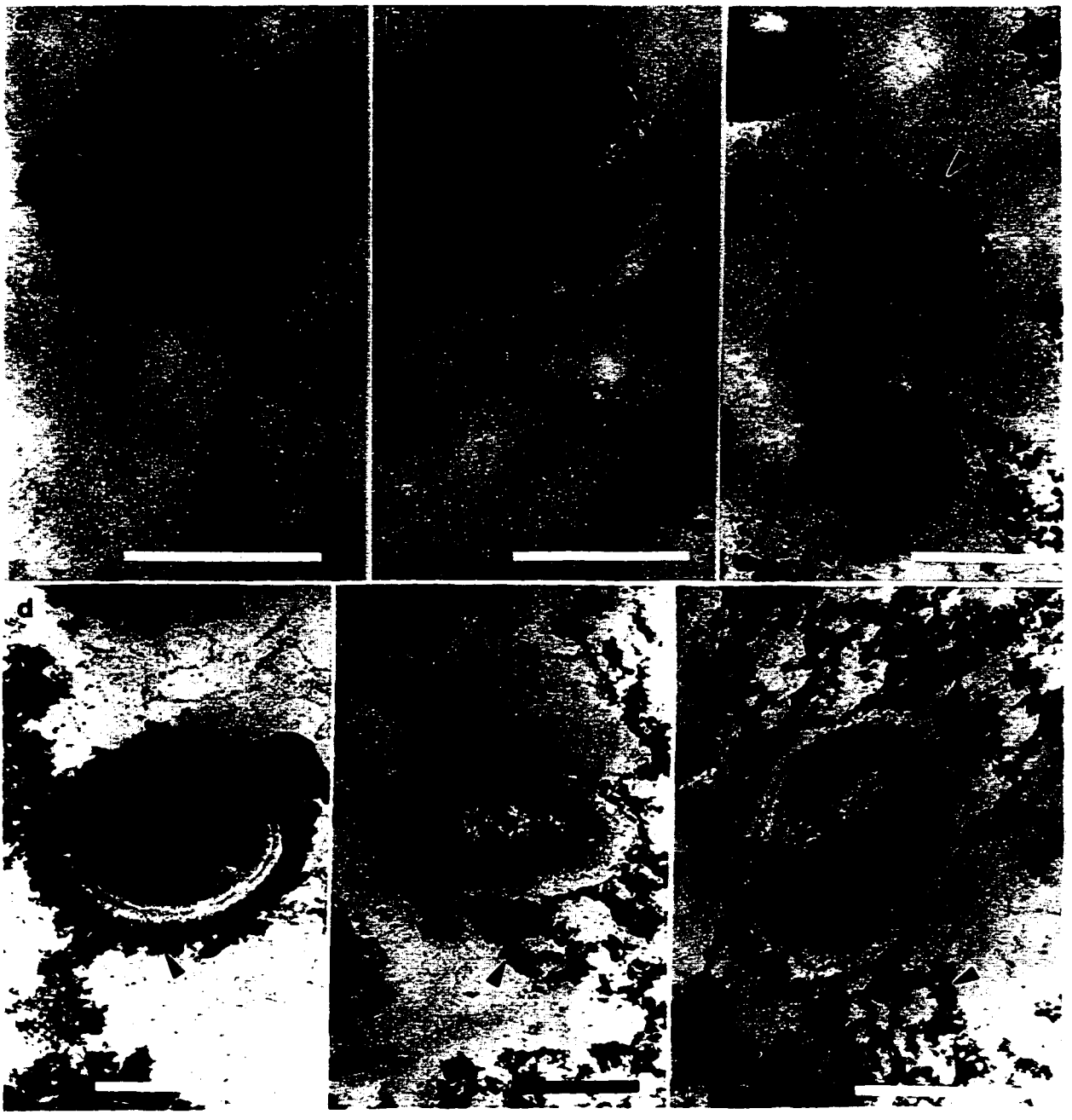
a

**TABLE 5.** A summary of the source and the plate counts of the environmental enrichment isolates

| Isolate Number | Source | Cfu/g |
|---|---|---|
| 1 | Backyard soil treated with NB[a] and MTBE[b] | 35 X10$^4$ |
| 2 | Backyard soil treated with NB and MTBE | 35 X10$^4$ |
| 3 | Spartan soil treated with NB | 48 X10$^5$ |
| 4 | Spartan soil treated with NB | 48 X10$^5$ |
| 5 | Backyard soil treated with water and MTBE | 20 X10$^4$ |
| 6 | Backyard soil treated with water and MTBE | 20 X10$^4$ |
| 9 | Backyard soil treated with NB | 25 X10$^3$ |
| 10 | Backyard soil treated with NB | 25 X10$^3$ |
| 11a | Backyard soil treated with NB | 25 X10$^3$ |
| 11b | Spartan soil treated with water | 54 X10$^4$ |
| 13 | Backyard soil treated with water | 25 X10$^3$ |

[a] Nutrient broth.
[b] 500 ppm MTBE.
[c] The total number of colonies on the plate from which the isolates were obtained after 14 days incubation.

**TABLE 6.** A summary of biochemical tests conducted on the environmental enrichment isolates

| Isolate | Gram Stain | Shape | RDS | Citrate | MRVP | Urea hydrolysis | Mannitol fermentation | Maltose fermentation | Glycerol fermentation | Starch hydrolysis | Gelatin hydrolysis | SIM | Nitrate reduction | Oxidase |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | + | Rods | Glc only[a] | - | MR-, VP-[c] | - | +/ no gas | -/ no gas | -/ no gas | + | + | $H_2S$-[d], indole+, non-motile | Complete | - |
| 2 | + | Rods | Glc + Lac | - | MR-, VP- | - | +/ no gas | +/ no gas | -/ no gas | - | + | $H_2S$-, indole-, motile | Complete | - |
| 3 | + | Rods | Glc + Lac[b] | - | MR-, VP- | - | +/ no gas | +/ no gas | +/ no gas | + | + | $H_2S$-, indole-, motile | Complete | - |
| 4 | + | Cocci | Neither | - | MR-, VP- | - | -/ no gas | -/ no gas | -/ no gas | - | - | $H_2S$-, indole-, motile | To $NO_2^-$ | - |
| 5 | + | Rods | Glc only | - | MR+, VP- | - | -/ no gas | +/ no gas | -/ no gas | + | - | $H_2S$-, indole-, motile | To $NO_2^-$ | - |
| 6 | + | Rods | Neither | - | MR-, VP- | + | -/ no gas | +/ no gas | -/ no gas | - | - | $H_2S$-, indole-, motile | To $NO_2^-$ | - |
| 9 | - | Rods | Neither | - | MR-, VP- | - | -/ no gas | -/ no gas | -/ no gas | - | - | $H_2S$-, indole-, nonmotile | - | - |
| 10 | + | Rods | Glc only | - | MR+, VP- | - | -/ no gas | +/ no gas | -/ no gas | - | - | $H_2S$-, indole-, motile | To $NO_2^-$ | - |
| 11a | + | Rods | Glc only | - | MR-, VP- | - | -/ no gas | -/ no gas | -/ no gas | + | - | $H_2S$-, indole-, motile | Complete | - |
| 11b | - | Rods | Glc + Lac | - | MR+, VP- | - | -/ no gas | +/ no gas | +/ no gas | + | - | $H_2S$-, indole-, nonmotile | To $NO_2^-$ | - |
| 13 | + | Rods | Glc + Lac | - | MR+, VP+ | - | +/ no gas | +/ no gas | -/ no gas | - | - | $H_2S$-, indole-, motile | To $NO_2^-$ | - |

[a] glucose is fermented.
[b] glucose and lactose are fermented.
[c] MR= methyl red test, VP= Voges-Prausker test.
[d] $H_2S$ is not produced.

## DISCUSSION

Aggregation is a common phenomenon among bacteria. PM-1 formed aggregates in the presence of MTBE but did not in an alternative carbon source. The clumps of PM-1 in MTBE showed an outer EPS layer surrounding the cells and thick layers between individual cells (Fig. 6). Exopolysaccharide production varies with the source of carbon, the carbon-to-nitrogen ratio, and the stage of the growth curve (45). Maximum EPS production was seen during stationary phase in my material (Fig. 8). Degeest and Vuyst reported similar results among streptococci (14). The onset of stationary phase, the secretion of EPS lyases (45), unbalanced growth conditions, and the genetics of the organism may all cause the release of capsular EPS (40). Fimbriae are also involved in aggregation of bacterial cells (1). Flocculation in MTBE-grown PM-1 most likely was caused by the presence of the EPS and not by fimbriae, as ethanol-grown cells bearing fimbriae but lacking EPS did not aggregate.

PM-1 cells grown in ethanol did not secrete EPS (Fig. 7). Certain other bacteria grown in an ethanol medium were reported to produce copious amounts of EPS and were found to aggregate (40). The differences between PM-1 grown on ethanol and on MTBE may be explained by the elucidation of the pathways and genes involved in metabolism of those compounds.

The EPS layer in PM-1 may protect the individual cells from fluctuations in the environment, prevent the cells from desiccation, and increase viability of the cells in soil (38). As a result, the presence of the EPS layer may benefit the remediation of MTBE by maintaining the viability of the cells.

Because it was impractical to measure growth of PM-1 aggregates in MTBE, two different methods were used to generate growth curves for the two substrates (ethanol and MTBE). When cultured in ethanol, the cells grew to a measurable turbidity and growth could be monitored by spectrophotometry. In MTBE, the cells did not grow to turbidity and aggregates formed during the mid to late logarithmic phases. Consequently, OD measurement was not a feasible method to measure growth in MTBE. A more accurate, though indirect, means for measuring growth among cellular aggregates is by following the disappearance of MTBE by gas chromatography (11). No MTBE degradation was detected during the lag phase and a steep decrease in the concentration of MTBE was observed during the exponential phase (Fig. 8).

From Fig. 2 and 3, it is evident that MTBE degradation was slower than ethanol degradation. Many factors are responsible (36, 41, 51). The ether bond cleavage is the rate-limiting step for most microorganisms. The C1 and C2 carbon bonds of MTBE are difficult to break, and MTBE oxidation yields very little energy in the form of ATP.

Preliminary studies on PM-1 and SP-Y, the two MTBE-degrading isolates, suggested that they belonged to the genus *Sphingomonas* (8). Electron microscopy of PM-1, SP-Y, and *S. paucimobilis* strain 29837 revealed differences in flagellation and the distribution of fimbriae (Fig. 5a-c). Work at UCD revealed differences in rRNA sequencing. This led to the identification of PM-1, the more efficient degrader of MTBE, as *Aquabacterium* (8). Biochemical characteristics of PM-1 corresponded to many of those recently reported (23) for *Aquabacterium* species (Table 4). One difference was

that PM-1 utilized ethanol and methanol, while *Aquabacterium* species did not (23). This difference is not significant, as there is variability in metabolic preference among strains.

The success of the enrichment experiment showed that MTBE degraders are present in nature and can be enriched and isolated. A large number of isolates were obtained from non-MTBE enriched soil samples due to the elevated organic content of those samples from frequent application of pesticides and manure. Of the 11 isolates, there were only two gram-negative isolates, but the biochemical properties of the isolates did not match those of PM-1. The ability to degrade MTBE can be confirmed by growing the isolates in liquid culture.

**Future investigation**

The structure and composition of the EPS produced by PM-1 is worthy of investigation by resonance studies. This EPS could have industrial applications and can be added to the growing number of natural products. The lack of EPS production among ethanol-grown PM-1 needs further investigation. A mutant of PM-1 that lacks EPS yet degrades MTBE at an equivalent or higher rate might be easier to quantify and might be more useful for bioremediation. Factors that contribute to the release of EPS during stationary phase also could be examined.

Other special methods are needed for labeling aggregates of cells with ferritin. The capsular layer was labeled with polycationized ferritin, according to the method of Jacques and Foiry (22). This method did not yield suitable material for thin sectioning as the blocks contained water. This may have been because of poor infiltration owing from the presence of the EPS layer.

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