# CHARACTERIZATION OF PINK-PIGMENTED FACULTATIVE METHYLOTROPHIC BACTERIA ISOLATED FROM OIL RESERVOIRS

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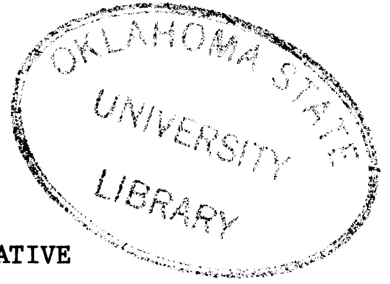

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FROM OIL RESERVOIRS

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# CHAPTER I

## INTRODUCTION

Seven strains of aerobic bacteria were isolated from produced water or crude oil samples from several oil reservoirs in California, Illinois, Texas, and Oklahoma which had undergone polymer floods. These strains were isolated along with sulfate-reducing bacteria and appeared, at the time of isolation, to have a mutualistic relationship with them. This was shown by an increased resistance to certain biocides in mixed culture (Grula, McManus, Sewell, and Williams, 1984). In addition, plates cross-streaked with sulfate-reducing bacteria and the aerobic strains showed markedly enhanced sulfate reduction in the proximity of the aerobic growth, and enhanced growth of aerobic strains close to the sulfate reducers (Grula, Williams, and Grula, unpublished).

The same reservoirs from which the aerobic strains were isolated have been examined and shown to contain sulfate-reducing bacteria in significant numbers. These organisms were observed in especially large numbers in samples of water from the Wilmington Field in Long Beach, California, taken at a depth of 1000 m.

Although these organisms in pure culture are aerobic, they have been shown to grow anaerobically in a mixed culture with sulfate-reducing bacteria. Because of their apparent enhancement of growth of sulfate-reducing bacteria, and because they apparently co-exist with sulfate-reducing bacteria in oil reservoirs, it was of interest to learn more about their properties, and to identify them if possible.

In many ways these organisms resemble the genus *Pseudomonas*. Major characteristics of *Pseudomonas* are as follows: Gram negative rods, aerobic (strictly respiratory), motile by polar flagella, and oxidase and catalase positive. Many strains are psychrotrophic, with growth occurring at 4° - 10°C. Maximum temperature for most strains is about 43°C. The mole percent G+C of the DNA ranges between 58 and 70 (Doudoroff and Pallenroni, 1974).

Many species can carry out dissimilatory nitrate reduction by using nitrate anaerobically as an alternate electron acceptor to oxygen. *Pseudomonas* sp. can utilize a large number of, and wide variety of organic substances as sole sources of carbon and energy. These substrates include many low molecular weight organic acids such as lactic, pyruvic, malonic, acetic, propionic, and glyoxylic acids as well as tricarboxylic acid cycle intermediates. They can also use a large number of alcohols like ethanol, 1-propanol, 1-butanol, and 2-methyl,1-butanol. Many sugars and sugar alcohols can also serve as carbon and energy sources. These include glucose, sucrose, galactose, fructose, lactose, and mannitol. Many species can use other substrates such as amino acids, aromatic hydrocarbons, phenols, acids, and other derivatives of aromatic hydrocarbons, lipids, fatty acids, and a number of straight chain hydrocarbons, both saturated and unsaturated. Many strains possess dehalogenating enzymes, and thus can utilize halogenated compounds that are resistant to degradation by, or toxic to other microorganisms. When a strain capable of degrading persistent pesticides, or compounds such as polychlorinated biphenyls is finally isolated, it usually turns out to be a *Pseudomonas*.

Another interesting feature of pseudomonads is their resistance to tetracycline, a broad spectrum antibiotic. Pseudomonads, however, are sensitive to carbenicillin (Davis, Dulbecco, Eisen, Ginsberg, and Wood, 1973).

A group of microorganisms characterized by their ability to use, as sole carbon source for growth, compounds that are more reduced than carbon dioxide and contain no carbon-carbon bonds are called methylotrophs (Colby, Dalton, and Whittenbury, 1979). Methylotrophic bacteria, both obligate and facultative, are a diverse group taxonomically. A number of strains, particularly of facultative methylotrophs, have been placed in the genus *Pseudomonas*.

Some, but not all, methylotrophs can utilize methane as sole carbon-energy source. It was believed for a long time that only obligate methylotrophs could utilize methane, whereas facultative methylotrophs could not. Obligate methylotrophs are classified as type I and type II organisms, a classification by membrane morphology based on the pathway used for incorporation of reduced $C_1$ compounds (Patt, Cole, Bland, and Hanson, 1974). Methylotrophic bacteria use one of two pathways of assimilation of formaldehyde for growth on one carbon compounds, the serine-iso-citrate lyase pathway or the ribulose-monophosphate cycle (Gottschalk, 1979). The end result of the serine-isocitrate lyase pathway is the formation of acetyl-CoA from formaldehyde and $CO_2$. Key enzymes of this cycle are malyl-CoA synthetase, malyl-CoA-lyase, serine-glyoxylate transaminase and hydroxypyruvate reductase. In the ribulose-monophosphate cycle, three molecules of formaldehyde are used to generate one molecule of dihydroxyacetone phosphate. Key enzymes in this pathway are transketolase and transaldolase (Gottschalk, 1979). Most facultative

methylotrophic bacteria utilize the serine pathway for formaldehyde incorporation (Patt et al., 1974).

Methane utilization depends on the presence of methane monooxygenase, an enzyme found on intracytoplasmic membranes. These membranes are not present in non-methane oxidizers. The intracytoplasmic membranes of type I methane oxidizers contain bundles of vesicular discs. These organisms use the ribulose monophosphate pathway for carbon assimilation. Type II methane oxidizers contain paired membranes around the cell periphery. These organisms use the serine pathway for carbon assimilation (Colby el at., 1979).

The type of organisms, pink pigmented facultative methylotrophic bacteria (PPFM), which is the object of this study, have been studied for 80 years. In 1906, Söhngen (Dworken and Foster, 1956) isolated a pink-pigmented bacterium from aquatic plant material and named it *Bacillus methanicus*. Dworkin and Foster (1956) isolated a very similar bacterium and named it *Pseudomonas methanica*. This organism was a Gram negative rod, oxidase and catalase positive, and motile by a single polar flagellum. Because of these properties, Dworkin and Foster felt it belonged in the genus *Pseudomonas*. This organism was an obligate methylotroph, only capable of growing on methanol and methane. They did not feel that methylotrophy alone warranted the creation of a new genus, therefore deciding on a *Pseudomonas* identification rather than including it in a genus of existing methylotrophs.

In 1974, Hanson and co-workers described an organism that could oxidize methane in addition to more complex substrates such as citric acid cycle intermediates and carbohydrates (Patt et al., 1979). Patt, Cole and Hanson (1976) suggested the formation of a new genus,

Methylobacterium. These bacteria produce colonies that are pink, circular, and convex with entire margins. When grown on methane, they contain intracytoplasmic membranes similar to those described as type II. These membranes were absent when the organisms were grown on substrates other than methane. They use the serine pathway for $C_1$ metabolism (Patt et al., 1976).

Methylobacterium is similar to Pseudomonas in a number of respects. They are Gram negative aerobic rods, with polar flagellation, and are oxidase and catalase positive. These bacteria are capable of utilizing the one-carbon compounds methane, methanol, formaldehyde, formic acid, and methylformate. In the latter case, two carbon atoms are present in the compound, but there are no carbon-carbon covalent bonds. Five sugars, glucose, galactose, sucrose, lactose, and ribose are used. In addition, they grow well on the citric acid cycle intermediates malate, succinate, and fumarate. Acetate can also serve as a sole source of carbon and energy. The deoxyribonucleic acid base composition is 66 mole % guanine plus cytosine.

The question of how the genus Methylobacterium differs from Pseudomonas has been raised. This is indicative of the confusion that still persists in this area. Authors frequently mention that certain Pseudomonas species are capable of growth on one-carbon compounds. Evidently the authors and editors of Bergey's Manual of Systematic Bacteriology (Palleroni, 1984) want to clarify the situation by completely excluding $C_1$ utilizers from the genus. In our opinion, the rationale for this is not clear, since they have included some facultative chemolithotrophs (able to use $H_2$ or CO as source of energy) in Pseudomonas. In any event, facultative methylotrophs are not included

in Pseudomonas. A careful study of the paper by Patt et al. (1976) showed no feature that would unequivocally exclude Methylobacterium spp., except for facultative methylotrophy, from the genus Pseudomonas.

The generic name Methylobacterium was proposed, indicating methane utilization by a small rod. The type species is Methylobacterium organophilum which denotes the preference of this organism for organic carbon and energy sources more complex than methane. Since it was believed for such a long time that no facultative methylotroph could utilize methane, the discovery of these microorganisms necessitated a new genus. This physiological trait was thus considered to be important enough to warrant a new genus. The authors (Patt et al., 1976) felt, however, that the strain belonged in the family Methylomonadaceae rather than a new family.

It has been proposed that the genus description of Methylobacterium should be broadened to include pink pigmented facultative methylotrophic bacteria which do not oxidize methane since methane oxidation is most likely plasmid borne and therefore readily lost (Green and Bousfield, 1979).

As a result of numerical taxonomic analysis performed on 150 strains of pink pigmented methylotrophic bacteria, it appears that the genus Methylobacterium would be the most suitable classification for these organisms (Green and Bousfield, 1982). Within the PPFM complex there were at least two clusters, referred to as 3A and 3B. These clusters were differentiated by a number of features, including the ability of 3B, and the inability of 3A, to utilize certain monosaccharides. Green and Bousfield (1982) described in detail the two major clusters of pink pigmented facultative methylotrophic bacteria mentioned above. They were

described as showing branching L-shaped, pleomorphic cells, and Gram variability.

Since our strains have some features in common with the PPFM's and since they have all come from similar environments, it was thought to be of interest to characterize them further. Therefore we undertook this study. As mentioned earlier, the PPFM bacteria discussed in this manuscript were isolated along with sulfate-reducing bacteria. The growth of a strictly aerobic bacterium and a strictly anaerobic bacterium in mixed culture is not without precedent. Balba and Evans (1980) grew a strain of *Pseudomonas aeruginosa* in a mixed culture with *Desulfovibrio vulgaris* and together, they carried out the dissimilation of benzoate through anaerobic sulfate respiration. They concluded that the *Desulfovibrio* initially produces small quantities of organic acids capable of acting as electron acceptors for the pseudomonad to attack the benzoate.

# CHAPTER II

# MATERIALS AND METHODS

## Isolation and Maintenance of Cultures

The seven strains were first isolated on plates of Medium E Agar (Postgate, 1979; Personal communication with G. Sewell, 1985). They were seen on plates prepared from enrichment cultures for sulfate-reducing bacteria which were allowed to incubate aerobically.

Duplicate stock cultures were maintained on slants of Postgate's Medium F Agar and in liquid Postgate's Medium C. They were grown at 37°C for 24 hours then kept at 6°C. Stock cultures were transferred approximately once every month. Duplicate working cultures were also maintained on Medium F and Medium C. They were grown at 37°C for 24 hours then maintained at room temperature. Working cultures were transferred at least once a week.

## Media

Postgate's Medium E contains (per liter of tap water): $KH_2PO_4$, 0.5 g; $NH_4Cl$. 1.0 g; $Na_2SO_4$, 1.0 g; $CaCl_2 \cdot 6H_2O$, 1.0 g; $MgCl \cdot 7H_2O$, 2.0 g; Sodium lactate, 6.0 ml; yeast extract, 1.0 g; ascorbic acid, 0.1 g; thioglycollic acid, 0.1 g; $FeSo_4 \cdot 7H_2O$, 0.5 g; Agar, 15.0 g. The pH was adjusted to 7.6. Medium E was made for counting populations of sulfate-reducing bacteria as black colonies in deep agar, and for isolation of pure cultures (Postgate, 1979). The composition of Postgate's Medium C

(per one liter of distilled water): $KH_2PO_4$, 0.5 g; $NH_4Cl$, 1.0 g; $NaSO_4$, 4.5 g; $CaCl \cdot 6H_2O$, 0.06 g; $MgSo_4 \cdot 7H_2O$, 0.06 g; sodium lactate, 10.0 ml; yeast extract, 1.0 g; $FeSO_4 \cdot 7H_2O$, 4.0 mg; sodium citrate, 0.3 g. The pH was adjusted to 7.5 $\pm$ 0.2. Medium C was designed for mass culture of sulfate-reducing bacteria for research; the citrate prevents precipitation of the iron salt (Postgate, 1979). The composition of Postgate's Medium F Agar (per one liter of distilled water): Iron sulfite Agar (Oxoid) 23.0 g; sodium lactate, 6.0 ml; $MgSO_4 \cdot 7H_2O$, 2.0 g. The pH was adjusted to 7.1. Medium F is widely used in food microbiology to diagnose Desulfotomaculum nigrificans, supplemented to make it suitable for most other sulfate-reducing bacteria as well (Postgate, 1979).

Substrates were tested using a chemically defined medium consisting of (per liter of distilled water): $K_2HPO_4$, 1.74 g; $KH_2PO_4$, 1.36 g; $NH_4Cl$, 0.8 g; $MgSO_4$, 30 mg; $H_3BO_3$, 5.0 μg ; $CaCO_3$, 100 μg ; $CuSO_4 \cdot 5H_2O$, 10.0 μg; $Fe(NH_4)_2(SO_4)_2$, 0.5 mg; KI, 20.0 μg; $MoO_3$, 10.0 μg; $ZnSO_4 \cdot 7H_2O$, 50.0 μg . The pH was adjusted to 7.2 (Grula, 1960).

## Morphological Characteristics

Gram reactions and morphology of the seven strains were determined from cultures grown at 37°C for 24 hours in Postgate's Medium C. Colonial morphologies were determined from streak plates on Postgate's Medium F grown at 37°C for 24 hours.

Motility was determined by observing a wet mount of each culture under oil immersion. Flagella number and position were determined from transmission electron micrographs.

## Biochemical and Physiological Characteristics

To determine if the organisms were aerobic or facultatively anaerobic, tubes of Postgate's Medium C were inoculated and incubated aerobically at 37°C and observed for one week. An identical set of tubes were inoculated and incubated in an anaerobic jar (BBL) at 37°C for one week.

Temperature growth range was determined by incubating cultures on Medium F at 5°C, 15°C, 37°C, 45°C, and 50°C. The plates were observed for two weeks or until growth occurred.

Effect of initial pH in the range 5.0 to 8.5 on total growth was determined by the pH of Postgate's Medium C, using 1.0 M Hcl and 1.0 N NaOH to adjust to values of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. The tubes were inoculated with one drop from a washed suspension of cells. They were incubated aerobically in a 37°C shaking water bath and observed for seven days. Presence of catalase was determined by transferring some cells from a colony on Medium F to a clean glass slide, then adding a drop of 3.0% hydrogen peroxide. The oxidase tests were performed using oxidase discs (Difco). A disc was placed onto a 24 hour culture on a plate of Medium F, a drop of water was placed on the disc which was observed for 45 minutes or until blackening around the disc occurred.

Since these bacteria resembled *Pseudomonas* in several ways, the possible ability to produce a fluorescent pigment was checked by streaking the cultures on *Pseudomonas* Agar F (Difco). This medium enhances the production of pigments. After incubation, the plates were observed in a U. V. box under short wave ultra-violet light.

The indole test was done by inoculating tubes of tryptone (Difco) for 24 hours, after which Kovacs' reagent was added. Cultures were observed for the presence of a bright red ring around the top indicating indole formation.

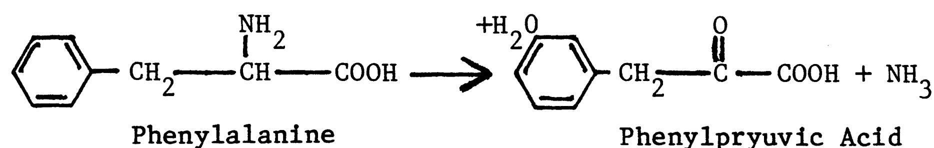
Ability to ferment glucose and lactose were determined using carbohydrate fermentation tubes with a 1.0% concentration of the sugar, bromthymol blue pH indicator, and an inverted durham tube.

Reduction of nitrate to nitrite was tested for in a chemically defined medium (Grula, 1960) with sodium lactate (0.6% v/v) as the carbon and energy source, and containing 0.1% (w/v) sodium nitrate. Duplicate cultures were incubated in an anaerobic jar (BBL), and aerobically in a shaking water bath, both at 37°C for one week. This test would detect both assimilatory and dissimilatory nitrate reduction. Uninoculated controls and controls containing no nitrate were also prepared and incubated. Growth was estimated visually and nitrite was tested for using sulfanilic acid and $\alpha$-naphthylamine, which should form a red azo dye if nitrite is present. If no red color was formed, it may have meant either that the nitrate was not reduced or that it all had been reduced to free $N_2$ and escaped from the medium. A small amount of zinc dust added to the cultures would chemically reduce any nitrate present to nitrite, and thus would produce the red color.

Since these organisms were isolated along with sulfate-reducing bacteria, the ability to carry out dissimilatory sulfate reduction was also tested. This was done in the same manner as dissimilatory nitrate reduction but with sodium sulfate added in place of sodium nitrate. Concentrations of 4.5 g/l, 2.5 g/l, and 0.45 g/l were added.

Inoculation, incubation and controls were identical to those used in the nitrate reduction test. All tubes were examined for visible growth.

The bacteria were also tested for the ability to oxidatively deaminate phenylalanine. This was done on phenylalanine agar slants (phenylalanine, 1.0 g; yeast extract, 3.0 g; sodium chloride, 5.0 g; disodium phosphate, 1.0 g; agar, 12.0 g; and distilled water, 1000 ml; pH 7.3). Slants were streaked and incubated at 37°C for 24 hours. Following incubation, 10% ferric chloride was added to the slants. A green color indicates a positive result.




Hydrogen sulfide production from cysteine, as opposed to dissimilatory sulfate reduction, can be determined by using Postgate's Medium F agar slants. A blackening of the slant indicates hydrogen sulfide production.

Ability to hydrolyze urea was determined by streaking the organisms on plates of urea agar (peptone, 1.0 g; dextrose, 1.0 g; sodium chloride, 5.0 g; monopotassium phosphate, 2.0 g; urea, 20 g; phenol red, 0.012 g; and distilled water, 1000 ml). The plates were incubated for 24 hours at 37°C. Urea hydrolysis produces ammonia, which raises the pH to 8.0 or higher, resulting in alkaline color (rose-red) of phenol red.

Ability to hydrolyze esculin was tested using plates containing a chemically defined medium with sodium lactate (0.6%) and a 0.1% (w/v) of esculin. The plates were inoculated with a single streak and incubated at 37°C for 48 hours. A zone of clearing around the growth indicates hydrolysis.

Gelatin agar plates (peptone, 5.0 g; beef extract, 3.0 g; gelatin, 20 g; agar 15 g; and distilled water, 1000 ml) were used to detect ability to hydrolyze gelatin. The plates were inoculated with a single streak and incubated at 37° C for 24 hours. Saturated ammonium sulfate which produces a precipitate with an intact protein is used as the indicator. A clear zone around bacterial colonies indicates hydrolysis of gelatin.

Starch hydrolytic ability was tested for on plates of starch agar (peptone, 5.0 g; beef extract, 3.0 g; soluble starch, 2.0 g; agar, 15 g; and distilled water, 1000 ml). The plates were inoculated with a single streak and incubated at 37°C for 24 hours. Lugol's iodine was used as the indicator; the dark blue of the starch-iodine complex shows the presence of unhydrolyzed starch.

Lipid hydrolysis was detected with tributyrin agar plates (peptone, 5.0 g; beef extract, 3.0 g; tributyrin, 10 ml; agar, 15 g; and distilled water, 1000 ml). The plates were inoculated with a single streak and incubated at 37°C for 24 hours. Unhydrolyzed tributyrin forms a cloudy emulsion in water. The plates were examined for a clearing around the streak.

When first isolated, the strains, while not requiring added NaCl, were stimulated to some extent by relatively low levels of NaCl (3% w/v), indicating a possible marine origin. To determine the degree of salt tolerance of the seven strains used in this study a rough quantitative test was done of their ability to grow in the presence of added NaCl. The chemically defined medium, with lactate, containing four concentrations of NaCl, 4%, 5%, 6%, and 7%, was used. Plates were inoculated with a single streak and incubated for 14 days.

The agar diffusion test was employed to determine antibiotic sensitivity. The plates were inoculated to produce a "lawn" of growth and discs containing one of 13 antibiotics were aseptically placed on the plates. Antibiotics used were: Gentamycin, Erythromycin, Vancomycin, Streptomycin, Penicillin, Ampicillin, Methicillin, Carbenicillin, Polymixin B, Chloramphenicol, Bacitracin, Cephalothin, and Tetracycline. The plates were incubated at 37°C for 24 hours, and presence of zones of inhibition was noted.

## Nutritional Characteristics

The bacteria were tested for the ability to utilize various compounds as sole nitrogen or carbon sources. Ammonium chloride, sodium nitrate and atmospheric nitrogen were examined as potential sources of nitrogen. The chemically defined medium was used as the test medium. A 0.1% w/v concentration of sodium nitrate was added as the only source of nitrogen to one set of tubes. To a second set of tubes, no source of nitrogen was added. This tested for the ability to fix atmospheric nitrogen. A positive control containing 0.8 g/liter of ammonium chloride was done, and uninoculated controls of each set were run. The tubes were incubated aerobically in a 37°C shaking water bath for two weeks.

Seventy-nine organic substrates were examined as potential sole sources of carbon and energy. They were tested in the chemically defined medium containing the test compound.

All tubes, for both nitrogen and carbon source tests, were inoculated with one drop of a 24 hour culture grown in Postgate's Medium C. The inoculum cultures were grown in 5.0 ml of the medium, after which they were centrifuged in sterile saline 3 times. The final suspension

was made in 5.0 ml sterile saline and had an optical density of approximately 0.5-0.6. One drop of this suspension was used as an inoculum. Volumes of test cultures were 5.0 ml in 14 mm test tubes. (Positive controls containing 0.6% sodium lactate, negative controls containing no carbon source, and uninoculated controls of each sample were also run. A variety of different types of compounds were examined including: carbohydrates, amino acids, alcohols, organic acids, citric acid cycle intermediates, aromatics, hydrocarbons, and one carbon compounds.)

Eight alcohols were tested, each one at a 0.1% v/v concentration. A 1.0% solution of each alcohol was prepared and filter sterilized using a 0.45μ filter. One-half milliliter of the 1.0% alcohol was added to 4.5 ml of the autoclaved minimal media. The alcohols examined were: 1-butanol, 2-butanol, tertiary butyl alcohol, isobutyl alcohol, ethyl alcohol, 1-propanol, 2-propanol, and isoamyl alcohol.

The following organic acids, in the form of their sodium salts, were tested: acetic, butyric, glyoxylic, malonic, propionic, and pyruvic acids. Each was added to the minimal medium in a final concentration of 0.1% (w/v). Sodium lactate again was used as a control. All were added before autoclaving.

Eight intermediates of the citric acid cycle were tested. The sodium salts of citric, cis-aconitic, isocitric, α-keto-glutaric, succinic, fumaric, malic, and oxalacetic acids were prepared individually as a 1.0% solution and filter sterilized with a 0.45μ filter. 0.5 ml of each was added to 4.5 ml of sterile chemically defined minimal media to bring the final volume to 5.0 ml and the final concentration of each intermediate to 0.1% v/v.

Twenty-one amino acids were tested to see if they could individually serve as a sole source of carbon and energy. A 1.0% solution of each amino acid was prepared and filter sterilized with a 0.45μ filter. A volume of 0.5 ml of the sterile solution was added to 4.5 ml of autoclaved medium. The final concentration of each amino acid was 0.1% and the final volume was 5.0 ml. Amino acids tested were glycine, the L-forms of alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, lysine, histidine, arginine, aspartic acid, glutamic acid, asparagine, cysteine, methionine, serine, threonine, tyrosine, glutamine, and ornithine.

The bacteria were tested for ability to utilize 21 sugars or sugar alcohols. A 5.0% concentration of each sugar was prepared and filter sterilized with a 0.45μ filter. A volume of 0.5 ml of each sugar solution was added to 4.5 ml of sterile minimal media, to give a final concentration of 0.5% (w/v). The 21 carbohydrates that were examined were glucose, maltose, lactose, sucrose, galactose, fructose, cellobiose, melezitose, melibiose, raffinose, mannose, rhamnose, arabinose, ribose, xylose, inositol, adonitol, dulcitol, sorbitol, mannitol, and trehalose.

Aromatic compounds examined as potential sources of carbon and energy were sodium benzoate, sodium p-hydroxybenzoate, phenol, as a representative of phenolic compounds, and toluene, as representative of aromatic hydrocarbons. Sodium benzoate and p-hydroxybenzoate were tested at concentrations of 0.1% and 0.05% (w/v). Each compound was filter sterilized and added after autoclaving. A 1.0% solution of phenol was prepared and filter sterilized. The sterile phenol solution was added to sterile minimal media at a final concentration of 0.1%. Toluene was

considered to be self sterilizing; and 0.5 ml was added to 5.0 ml of sterile minimal medium.

Two aliphatic hydrocarbons, heptadecane and kerosene (refined to 99% pure aliphatic hydrocarbons--all aromatics removed) were examined as sources of carbon and energy. To test heptadecane, 0.1 ml was added to 25 ml of sterile minimal media in a 125 ml flask. The flasks were inoculated and incubated on a shaker at 37°C. Kerosene is composed primarily of hexadecane, a 16 carbon saturated hydrocarbon. Kerosene was tested using the same procedure as heptadecane.

Several one-carbon compounds, methanol, formaldehyde, sodium formate, monomethylamine, trimethyl amine (trimethyl amine has three carbons but no carbon-carbon bonds), and methane were tested as potential sources of carbon and energy. Solutions were prepared at a 5.0% concentration, filter sterilized and added at concentrations, 0.05% and 0.1%. Cultures were incubated in a 37°C shaking water bath.

It was originally believed that methane utilizers were obligate methylotrophs, but in 1974 Hanson and his coworkers discovered that certain strains of microorganisms were facultative methlyotrophs, and yet could utilize methane as a sole carbon-energy source (Patt et al., 1974).

These strains resembled ours in certain ways; therefore it became important to test for ability to utilize methane. In order to test for methane utilization, methane had to be present in the gas phase, in concentrations ranging from 20 to 25% v/v. To achieve the required atmosphere, an anaerobic jar (BBL) was used. Three holes were drilled in the lid of the jar and valves inserted into each hole. After the jar was closed, all three valves were opened. One valve was used to flush with methane at one liter per minute. The second valve was used to flush with

air at four liters per minute. The third valve was used as an exhaust valve. The system was flushed with the methane/air mixture for 20 minutes in a table top hood. After 20 minutes, all three valves were closed leaving a final concentration of approximately 20% methane inside the container (Personal communication with G. Sewell, 1985).

The strains were tested on a solid chemically defined minimal medium with no carbon source present. The plates were inoculated with a single streak from a 24 hour culture that was washed three times. Three sets of controls were run: plates containing no carbon source not inoculated, uninoculated plates containing lactate, and plates containing lactate and inoculated. In addition to these controls, a positive control was also run. Methylobacterium organophilum (ATCC #27886), a known methane oxidizer was inoculated on a plate containing a chemically defined minimal medium with no carbon source present. This control was run to ensure proper growth conditions for methane oxidation was present inside the chamber.

$CO_2$, a necessity for growth on methane, was provided by filling a 5 ml beaker with dry ice and inverting it in the chamber prior to flushing.

After the plates and beaker of dry ice were placed into the jar, it was sealed, flushed, and incubated at 37°C for 14 days.

One polyol, glycerol and polyethylene glycol, were examined. A 1.0% solution of each compound was prepared, filter sterilized, and added to sterile minimal medium in a final concentration of 0.1%.

The ability to utilize all compounds was determined by visually observing cultures and recording the results as follows: ϕ = no growth;

+ = poor growth; ++ = fair growth; +++ = good growth; and ++++ = excellent growth.

## Isolation of DNA

DNA was isolated from each strain by the Marmur method of DNA isolation (Marmur, 1961; Johnson, 1981).

A culture of each strain was grown in Postgate's Medium C in two one liter Erlenmeyer flasks (500 ml per flask) for 18 hours in a shaking (230 rpm) water bath at 37°C.

The cultures were centrifuged in four 250 ml centrifuge bottles in a RC2-B centrifuge at 10,000 rpm for 20 minutes using a GSA-B rotor at 0-4°C. Supernatants were discarded and the pellets were suspended in 20 ml of saline-EDTA buffer (0.15 M NaCl, 0.01 M sodium-EDTA, pH 8.0). The suspensions were combined into one ground glass-stoppered bottle. Fifteen milliliters of saline-EDTA buffer was added to this, bringing the final volume to 95 ml.

The cells were disrupted using sodium dodecyl sulfate (SDS). Five milliliters of a 20% solution of SDS was added to the 95 ml of cell suspension bringing the concentration of SDS to 1.0% and the volume to 100 ml. The suspension was incubated in a 60°C water bath until it became clear and viscous (3-5 minutes). It was removed from the water bath and 25 ml of a 5.0 M sodium perchlorate solution was added. The final concentration of sodium perchlorate was 1.0 M and the volume of the solution was 125 ml. Chloroform-isoamyl alcohol (24:1 v/v) was added at 0.5 volumes (62.5 ml). The solution was shaken on a wrist action shaker (Burrell) for 30 minutes at a speed just sufficient to provide an emulsion (a setting of 5 on the shaker). The emulsion was centrifuged at

12,000 rpm in a RC2-B centrifuge in a SS-34 rotor for 10 minutes at 0-4°C. The upper aqueous phase from each tube was carefully pipetted off using an inverted 10 ml pipette and a pipette bulb. It is important to avoid collecting any of the protein precipitate at the interphase. The aqueous phases were collected in a 250 ml beaker. This process was repeated and the aqueous phases were again collected in a 250 ml beaker. The chloroform-isoamyl alcohol extractions were done to remove the protein from the solution. The aqueous phase was then slowly overlaid with 2.0 volumes of cold 95% ethyl alcohol.

The precipitated DNA was collected with a glass stirring rod by gently stirring the two phases while spinning the rod. The DNA adhered or "spooled" onto the rod. The excess ethanol was removed by pressing the rod against the side of the beaker, then holding the rod vertically while inverted to allow drainage.

The spooled DNA was dissolved in 1.0X saline-sodium citrate (SSC) (0.15 M sodium chloride, 0.015 M trisodium citrate, pH 7.0). The SSC concentration was adjusted from 0.1X to 1.0X by adding 0.8 ml of 20x SSC for a final volume of 20.8 ml. Ribonucleic acid was removed by adding 1.04 ml of ribonuclease (bovine pancreatic RNase from Sigma, 1 mg/ml in 0.15 M NaCl, pH 5.0, heated at 80°C for 10 minutes) to a concentration of 50 g/ml. The mixture was incubated at 37°C for 30 minutes.

After the RNase treatment, 10.92 ml of chloroform-isoamyl alcohol solution (0.5 volumes) was added. The mixture was shaken on a wrist action shaker for 15 minutes. The emulsion was removed and centrifuged at 12,000 rpm for 10 minutes. The upper aqueous phases were removed, as before, and collected in a 250 ml beaker. The chloroform-isoamyl alcohol

extraction was repeated until very little protein was seen at the interphase.

The aqueous phases were then collected into a 250 ml beaker. This was slowly overlaid with 2.0 volumes of cold 95% ethyl alcohol. The precipitated DNA was then spooled onto a glass rod. It was then dissolved into 20 ml of 0.1X SSC. This was repeated two or three times to remove any possible remaining ribonucleotides. The SSC concentration was adjusted to 1.0X by adding 0.8 ml of 20X SSC prior to each precipitation. Finally the DNA was dissolved in 0.1X SSC and stored at 6°C.

The concentration of each sample of DNA was determined spectrophotometrically. A 2.0 ml sample of each DNA preparation was removed and its optical density measured on a Perkin-Elmer spectrophotometer at 260 nm with a deuterium lamp and 1 cm pathlength cell. Saline-sodium citrate (0.1%) was used as the reference sample. The following formula was used to calculate the concentration of DNA:

$$mg/ml = \frac{\text{Absorbance at 260 nm}}{20} .$$

## Determination of Guanine Plus Cytosine Content of DNA

The percentage of guanine plus cytosine of the DNA was determined by a CsCl density gradient ultracentrifugation (Schildkraut, Marmur, and Doty, 1962; Mandel, Schildkraut, and Marmur, 1968). A Beckman Model E analytical ultracentrifuge with monochromator, photoelectric scanner, and electronic speed control were used with an An-D rotor and a single, double sector cell with 12 mm thick, aluminum-filled epon centerpiece. The cells were assembled and centrifuge operated as described in the

instrument manual, except that the current was limited to 11 amperes during acceleration of the rotor.

The CsCl gradient was prepared by dissolving 6.5 g of optical grade CsCl (Sigma) in 3.5 ml of 0.02 M tris buffer, pH 8.5. Reference cell solution and sample cell solution were prepared as follows: reference cell solution; 0.84 ml CsCl, and 0.23 ml H O for a total of 1.07 ml; sample cell solution; 0.84 ml CsCl, 0.17 ml H O, 0.02 ml reference 0.1% DNA, and 0.04 ml unknown DNA for a total of 1.07 ml. A volume of 0.4 ml of the solutions were injected into the cell. Viewed from the cell top the left sector of the cell contained the reference solution and the right sector the sample solution.

Centrifugation at 44,000 rpm was carried out at room temperature for approximately 28 hours. Absorbance recordings were obtained using 260 nm light. Pertinent operating conditions for this instrument were 60 volts, 3 amp for the drive motor and a camera lens position of 7.5 in.

## Isolation of Protein

Whole cell protein samples were prepared from each bacterium for polyacrylamide tube gel electrophoresis.

A 500 ml culture was grown in Postgate's Medium C for 18 hours in a 37°C shaking water bath. This was centrifuged at 12,000 rpm in a RC2-B centrifuge in a GSA rotor for 20 minutes. The supernatant was discarded and the pellet was resuspended in 50 ml of phosphate buffer (0.01 M, pH 7.0). This was centrifuged at 10,000 rpm in a SS-34 rotor for 10 minutes. The supernatant was discarded and the pellet was resuspended in 50 ml of 3.0 mM tris buffer. This was again centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the

pellet was resuspended in 25 ml of 3.0 mM tris buffer. The suspension was poured into a 50 ml glass beaker and sonicated (Branson Sonifier Model 350) in 20 second bursts until the suspension was clear. The suspension was cooled on ice between sonications. The sonicate was centrifuged at 10,000 rpm for 10 minutes to spin down cell fragments (wall, etc.) and the supernatant collected.

## Protein Determination

A Bio-Rad protein determination was performed on each sample before electrophoresis. A standard curve was done using bovine serum albumin (BSA) (Table I) in concentrations ranging from 0.0 to 1.4 mg/ml and protein concentration for each bacterial preparation using Bio-Rad reagent (Bio-Rad). The Bio-Rad reagent was diluted 1:4 with water prior to use. The BSA standard curve was performed using the volumes shown in Table II (Figure 1).

The determination of protein concentration of each individual strain was performed using the volumes shown in Table III.

TABLE I

BSA STANDARD CURVE

| μg/ml of BSA | $A_{595}$ (nm) |
|---|---|
| 0.0 | 0.000 |
| 28.0 | 0.369 |
| 56.0 | 0.690 |
| 84.0 | 0.998 |
| 112.0 | 1.238 |
| 140.0 | 1.432 |

TABLE II

VOLUMES (ml) OF COMPONENTS OF STANDARD CURVE

| Tube No. | Bio-Rad Reagent | $H_2O$ | BSA[1] |
|---|---|---|---|
| 1 | 5.0 | 0.10 | 0.00 |
| 2 | 5.0 | 0.08 | 0.02 |
| 3 | 5.0 | 0.06 | 0.04 |
| 4 | 5.0 | 0.04 | 0.06 |
| 5 | 5.0 | 0.02 | 0.08 |
| 6 | 5.0 | 0.00 | 0.10 |

[1]BSA was made at a concentration of 1.4 mg/ml.

TABLE III

VOLUMES (ml) OF COMPONENTS OF PROTEIN DETERMINATION

| Tube No. | $H_2O$ | Bio-Rad Reagent | Protein Sample |
|---|---|---|---|
| 1 | 0.08 | 5.00 | 0.02 |
| 2 | 0.06 | 5.00 | 0.04 |
| 3 | 0.05 | 5.00 | 0.05 |

## Polyacrylamide Disc Gel Electrophoresis

Electropherograms of whole cell protein extracts were determined by polyacrylamide tube gel electrophoresis (Kersters and DeLey, 1975; Clark and Switzer, 1977; and Wood, 1981).

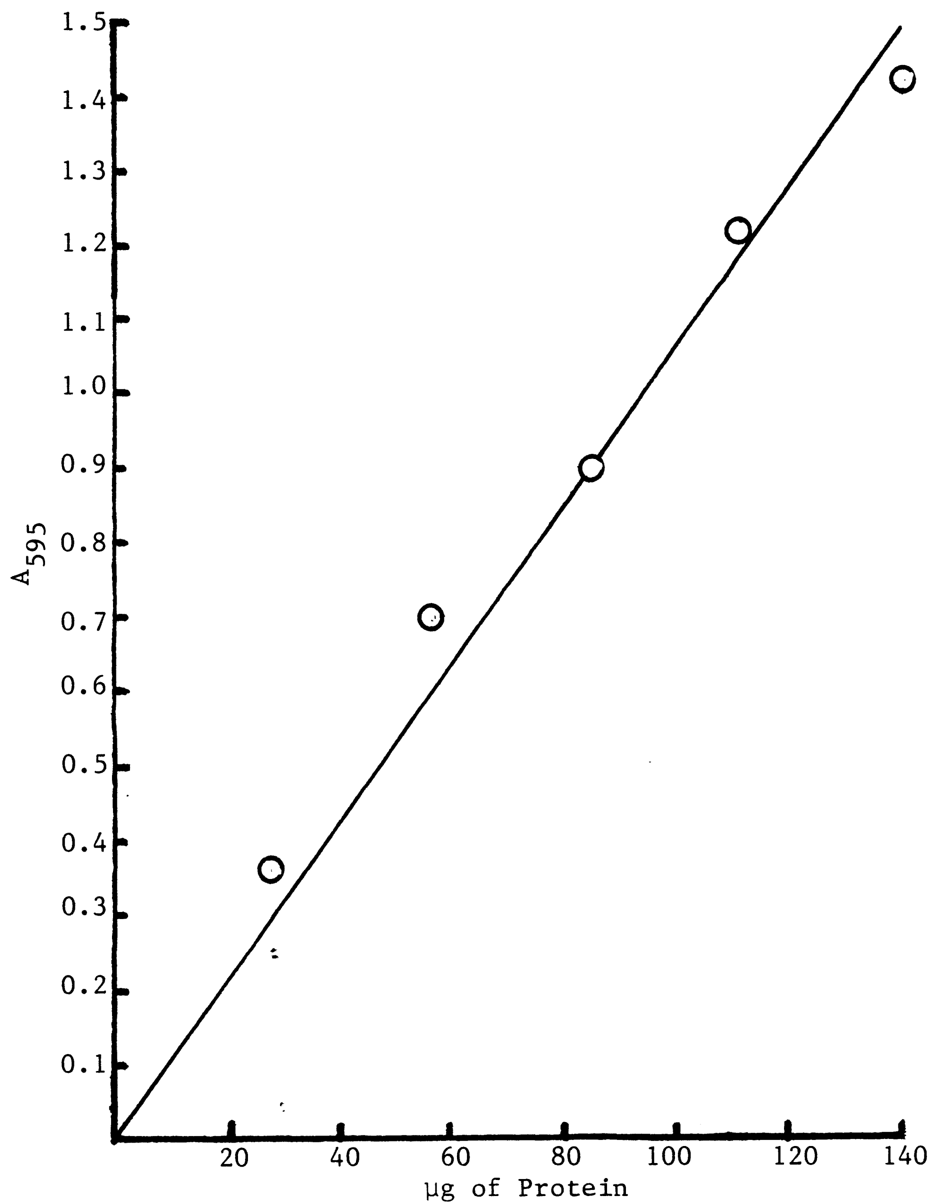



Figure 1. BSA Standard Curve

Gels were prepared as follows. Two parts of a solution containing 28% (w/v) acrylamide and 0.735% (w/v) N,N'-methylene bisacrylamide were mixed with: (1) one part of a solution containing 0.23% (v/v) N, N,N', N'-tetramethyl ethylenediamine and 6.1% (w/v) tris (adjusted to pH 8.8 with 2N HCl); (2) one part of distilled water; and (3) four parts of 0.14% (w/v) ammonium persulfate. Glass tubes 77 mm long with an internal diameter of 6 mm and one end (bottom end) wrapped with parafilm were placed in a vertical position prior to filling. The tubes were filled with 1.2 ml of gel prewarmed to 24°C. The gels were overlaid with 0.1 ml of distilled water. Polymerization was carried out at 24°C for 120 minutes. Following polymerization, the water was removed with a Pasteur pipette and the final drops were wiped out with a Kimwipe. The parafilm was removed and the tubes placed in the electrophoresis system. A 0.1 ml volume of undiluted protein solution was mixed with 0.1 ml of 0.002% bromphenol blue in 5% sucrose solution. From this mixture, 10 µl was layered onto the gels. A standard containing 1.4 mg/ml BSA and 0.002% bromophenol blue in 5% sucrose was also run. Tris buffer (a stock solution containing 77.5 g tris-HCl, 0.64 M; 1000 ml distilled water, and pH 8.7) was used as the electrode buffer. The reservoirs were filled with the tris buffer diluted 1/10. Electrophoresis was performed with the anode in the lower chamber at a constant current of 1.25 mA/gel for 15 minutes, followed by 3.8 mA/gel until the tracking dye (bromophenol blue) had moved to within 1.0 cm of the bottom of the tube, which took approximately 2 hours. The gels were removed with a needle supplied with a gentle stream of water. The gels were cut at the front edge of the tracking dye and placed in screw cap test tubes and stained at room temperature overnight with amido black staining solution. The staining

solution contained: 0.6 g amido black 10B, 7.0 ml glacial acetic acid, 93 ml distilled water. Gels were destained in 7.0% (v/v) acetic acid until the gels were clear and the bands left stained (Davis, 1964).

## Biocide Sensitivity

Three strains of the pink-pigmented facultative methylotrophs were tested for their ability to grow in the presence of two different commercial biocides commonly used by the oil industry for controlling microbial growth in various operations, strain 79 Pink isolated from Oklahoma, strain 2EP isolated from Texas, and strain Pink isolated from California were tested. The two biocides examined were 2,2-dibromo-3-nitrilopropionamide (DBNPA)(DOW) and glutaraldehyde. Postgate's Medium C was used as the test medium. A 1.0% solution of DBNPA was prepared and added to the media. Four concentrations were tested: 100 ppm, 500 ppm, 1000 ppm, and 2000 ppm. Positive controls containing no biocide and uninoculated controls were run. The tubes were incubated in a 37°C shaking water bath and observed for seven days.

The same three strains were used to test glutaraldehyde. This experiment was carried out in the same manner at the DBNPA experiment.

# CHAPTER III

# RESULTS

## Morphological Characteristics

All seven strains of the pink-pigmented facultative methylothrophic (PPFM) bacteria are Gram negative rod shaped bacteria. Each strain produces copious amounts of an extracellular polysaccharide material. All are motile by means of a single polar flagellum (Figure 2). As the name implies, the organisms produce a pink non-diffusible pigment. Colonies are round, convex, and mucoid with entire margins. Size of the cells range from 1.9 μ to 2.3 μ x 0.7 μ to 1.1 μ.

The metabolism of these strains is strictly respiratory. This is shown by the fact that under normal conditions, the organisms will only grow aerobically. No growth occurred in any of the Postgate's media or in the chemically defined minimal medium under anaerobic conditions.

The organisms grow at temperatures from 15°C to 45°C while they appear to grow best at 37°C. No growth occurred at 5°C or 50°C (Table IV). The strains were tested for growth temperature range in Postgate's Medium C and observed for 2 weeks or until growth occurred.

The organisms are capable of growth over a wide range of pH's (Table V). This was tested by adjusting the initial pH of Postgate's Medium C and observing the cultures for 2 weeks or until growth occurred. All seven strains were capable of growth from pH 5.5 to 8.5. All but

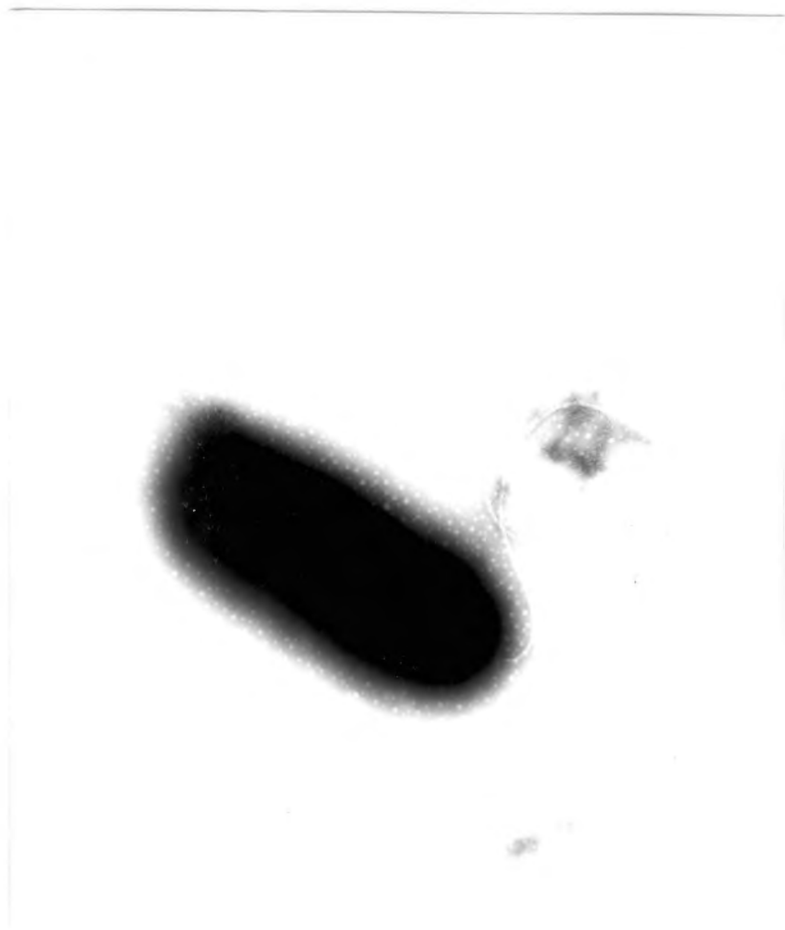


Figure 2. Transmission Electron Micrograph of Strain Pink (magnified 19,900x)

two, 79 Pink and Met, grew at pH 5.0. Good growth of all strains occurred over the range 6.0 to 7.5.

TABLE IV

GROWTH AS A FUNCTION OF TEMPERATURE

| | 5°C | 15°C | 37°C | 45°C | 50°C |
|---|---|---|---|---|---|
| 79 Dark Pink | ϕ | ++ | ++++ | ++ | ϕ |
| 79 Pink Black | ϕ | ++ | ++++ | ++ | ϕ |
| 79 Pink | ϕ | ++ | ++++ | ++ | ϕ |
| Pink | ϕ | ++ | ++++ | ++ | ϕ |
| Fuzz | ϕ | ++ | ++++ | ++ | ϕ |
| Met | ϕ | ++ | ++++ | ++ | ϕ |
| 2EP | ϕ | ++ | ++++ | ++ | ϕ |

TABLE V

GROWTH AS A FUNCTION OF INITIAL pH

| | 5.0 | 5.5 | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 | 8.5 |
|---|---|---|---|---|---|---|---|---|
| 79 Dark Pink | + | + | +++ | +++ | +++ | +++ | ++ | ++ |
| 79 Pink Black | + | ++ | +++ | +++ | +++ | +++ | ++ | ++ |
| 79 Pink | ϕ | ++ | +++ | +++ | +++ | +++ | ++ | ++ |
| Pink | + | ++ | ++++ | ++++ | +++ | +++ | +++ | +++ |
| Fuzz | + | +++ | +++ | ++++ | +++ | +++ | ++ | ++ |
| Met | ϕ | ++ | +++ | ++++ | +++ | +++ | ++++ | ++ |
| 2EP | + | ++ | ++++ | ++++ | ++++ | +++ | ++++ | ++ |
| Negative Control | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |

## Biochemical Characteristics

All seven organisms are catalase and oxidase positive. None of the seven bacteria produce a fluorescent pigment.

As mentioned before, the metabolism of all seven bacteria is strictly respiratory. They are unable to grow anaerobically under ordinary conditions. Growth is achieved anaerobically, however, under two specialized conditions. The first is in a mixed culture with sulfate-reducing bacteria. Slight growth of the PPFM strains was seen when they were inoculated with a single streak perpendicular to a single streak of *Desulfovibrio* on plates of Postgate's Medium E agar (personal communication with G. Sewell, 1985). The plates were incubated in an anaerobic jar at 37°C for 2 weeks. Growth of the PPFM strains was seen only in the area immediately next to the sulfate-reducing bacteria.

The second method of anaerobic growth involves dissimilatory nitrate reduction. All seven strains are capable of growing anaerobically in the presence of nitrate, using nitrate as an alternate electron acceptor to oxygen. The nitrate is reduced beyond the level of nitrite, presumably to nitrogen gas (Table VI). Although growth was much greater aerobically with or without nitrate, growth occurred anaerobically with nitrate while none occurred without nitrate.

None of the seven bacteria was able to use sulfate as an alternate electron acceptor to oxygen and carry out dissimilatory sulfate reduction. Sodium sulfate was tested at 4.5 g/l (the amount used in Postgate's Medium C), 2.25 g/l, and 0.45 g/l (Table VII). Tubes were observed for one week.

TABLE VI

ANAEROBIC GROWTH AS INFLUENCED BY PRESENCE OF NITRATE

| | A540* | | | |
|---|---|---|---|---|
| | Aerobic | | Anaerobic | |
| | With $NO_3$ | Without $NO_3$ | With $NO_3$ | Without $NO_3$ |
| 79 Dark Pink | 1.48 | 1.70 | 0.50 | 0.01 |
| 79 Pink Black | 1.94 | 1.94 | 0.35 | 0.01 |
| 79 Pink | 1.99 | 1.80 | 0.39 | 0.03 |
| Pink | 1.92 | 2.0+ | 0.36 | 0.01 |
| Fuzz | 1.99 | 2.0+ | 0.23 | 0.00 |
| Met | 1.88 | 1.85 | 0.57 | 0.01 |
| 2EP | 1.53 | 1.82 | 0.36 | 0.02 |

*Absorbance readings taken at 5 days.

TABLE VII

DISSIMILATORY SULFATE REDUCTION

| | Aerobically | | | Anaerobically | | |
|---|---|---|---|---|---|---|
| | 4.5 g/l | 2.25 g/l | 0.45 g/l | 4.5 g/l | 2.25 g/l | 0.45 g/l |
| 79 Dark Pink | ++++ | ++++ | ++++ | ϕ | ϕ | ϕ |
| 79 Pink Black | ++++ | ++++ | ++++ | ϕ | ϕ | ϕ |
| 79 Pink | ++++ | ++++ | ++++ | ϕ | ϕ | ϕ |
| Pink | ++++ | ++++ | ++++ | ϕ | ϕ | ϕ |
| Fuzz | ++++ | ++++ | ++++ | ϕ | ϕ | ϕ |
| Met | ++++ | ++++ | ++++ | ϕ | ϕ | ϕ |
| 2EP | ++++ | ++++ | ++++ | ϕ | ϕ | ϕ |

None of the seven strains of PPFM organisms was capable of fermenting glucose or lactose. All seven bacteria were negative for indole production and phenylalanine deaminase production. When the organisms are grown on slants of Postgate's Medium F, a black precipitate was formed indicative of $H_2S$ production.

All but one organism were capable of hydrolyzing tributyrin; only strain Pink was negative. In contrast, only strain Pink was capable of hydrolyzing urea whereas the other six strains were negative. None of the seven strains could hydrolyze starch or esculin. Four of the seven strains were capable of hydrolyzing gelatin, strains 79 Dark Pink, 79 Pink Black, Pink, and 2EP. Strains 79 Pink, Fuzz, and Met were incapable of hydrolyzing gelatin. A summary of results on biochemical tests is given in Table VIII. A summary of all morphological, and cultural characteristics is given in Table IX. If numerical taxonomy were applied, they would undoubtedly constitute one cluster (a very closely related group of organisms).

All seven strains were capable of growth in the presence of 6% NaCl, and all but one, strain Met, grew with 7% added NaCl. Agar plates containing the chemically defined minimal medium were used as the test medium and observed for 2 weeks (Table X).

All seven strains were inhibited by 5 of the 13 antibiotics that were examined. These were: gentamycin, streptomycin, chloramphenicol, ampicillin, and carbenicillin. Gentamycin and streptomycin inhibit protein synthesis by binding to the 30s ribosomal fraction, thereby causing the production of non-sense proteins. Chloramphenicol inhibits protein synthesis by binding to the bacterial ribosome and interfering with the formation of peptide bonds between amino acids. Ampicillin and

carbenicillin, inhibit cell wall synthesis by preventing the crosslinking of peptidoglycan.

TABLE VIII

BIOCHEMICAL TESTS

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP |
|---|---|---|---|---|---|---|---|
| Catalase | + | + | + | + | + | + | + |
| Oxidase | + | + | + | + | + | + | + |
| Glucose Fermentation | - | - | - | - | - | - | - |
| Lactose Fermentation | - | - | - | - | - | - | - |
| Indole | - | - | - | - | - | - | - |
| Phenylalanine Deaminase | - | - | - | - | - | - | - |
| $H_2S$ | + | + | + | + | + | + | + |
| Lipid Hydrolysis | + | + | + | - | + | + | + |
| Urea Hydrolysis | - | - | - | + | - | - | - |
| Starch Hydrolysis | - | - | - | - | - | - | - |
| Esculin Hydrolysis | - | - | - | - | - | - | - |
| Gelatin Hydrolysis | + | + | - | + | - | - | + |

All seven strains were resistant to 5 of the antibiotics tested. Vancomycin inhibits cell wall synthesis by blocking subunit transfer from carrier lipid to peptidoglycan. Methicillin inhibits cell wall synthesis by preventing crosslinking of peptidoglycan. Bacitracin inhibits cells wall synthesis by blocking dephosphorylation of undecaprenol-PP. Tetracycline inhibits protein synthesis by blocking the binding of the amino acid-tRNA complex to ribosomes. And Polymyxin B causes nonselective increase in membrane permeability. Three antibiotics showed

TABLE IX

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP |
|---|---|---|---|---|---|---|---|
| Gram Reaction | - | - | - | - | - | - | - |
| Cell Morphology | rod | rod | rod | rod | rod | rod | rod |
| Cell Size (μ) | 1.9x0.8 | 2.2x0.8 | 2.2x.75 | 2.1x0.7 | 2.3x1.1 | 2.1x0.9 | 2.1x0.69 |
| Motility | + | + | + | + | + | + | + |
| Flagella Number | one | one | one | one | one | one | one |
| Flagella Position | polar | polar | polar | polar | polar | polar | polar |

varied results within the strains. Erythromycin inhibits proteins synthesis by binding to one of the ribosomal fractions and blocks formation of peptide bonds between amino acids. Penicillin G and cephalothin inhibit cell wall synthesis by preventing crosslinking of peptidoglycan (Table XI). Postgate's Medium F was used as the test medium. The plates were incubated at 37°C for 24 hours.

Sensitivity to the β-lactam antibiotics indicates that the molecule with an intact β-lactam ring structure is getting inside the cell, and that crosslinking of N-acetyl-glucosamine-N-acetylmuramic acid chains is being inhibited. The high degree of sensitivity of all strains to ampicillin and carbenacillin, combined with what appears to be a very high degree of resistance to methicillin can only be explained in terms of the cells being impermeable to methicillin.

With any antibiotic, ability to reach, and interact with, susceptible structures or enzymes is a key factor. An antibiotic which acts by disrupting or somehow damaging the cell membrane could possibly inhibit growth, or kill cells, by getting into the periplasmic space, if susceptible sites were located on the outer surface of the cell membrane. These (membrane associated) antibiotics are extremely potent for many bacteria completely inhibiting growth at levels of the order of 0.1-0.01 μg/l. Our strains are relatively resistant to polymyxin B; only small inhibition zones were formed.

TABLE X

EFFECTS OF SODIUM CHLORIDE ON GROWTH

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP |
|---|---|---|---|---|---|---|---|
| 4% NaCl | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| 5% NaCl | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| 6% NaCl | ++ | ++ | ++ | +++ | +++ | +++ | ++++ |
| 7% NaCl | ++ | + | + | ++ | ++ | ϕ | ++ |
| Control | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |

Note: Time of incubation, 14 days.

Nitrogen Sources

All strains will grow on a complex medium, such as TSA or casein hydrolysate medium. It is known that they can utilize ammonium salts as a sole nitrogen source in the chemically defined medium. It seemed to be of interest to test for abilities to utilize nitrate as a sole nitrogen source in this medium and also dinitrogen ($N_2$). No growth of any strain occurred after 14 days incubation with either $NaNO_3$ or $N_2$ as sole nitrogen sources. These observations indicate that: (a) none of the strains carry out assimilatory nitrate reduction, and (b) none can fix atmospheric nitrogen.

As will be seen later, none of 21 amino acids could serve as sole sources of carbon for any of the strains, even L-alanine. Therefore it is likely that none could serve as a nitrogen source, because ability to deaminate (yielding, in the case of alanine, pyruvic acid, which can serve as a carbon source as well as ammonia) is a prerequisite for an

TABLE XI

ANTIBIOTIC RESISTANCE

| | Diameter of Zones of Inhibition (nm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP |
| Gentamycin (10 μg) | 26(S) | 22(S) | 24(S) | 28(S) | 24(S) | 24(S) | 20(S) |
| Erythromycin (15 μg) | 12(R) | 14(I) | 16(I) | 26(S) | 26(S) | 22(S) | 22(S) |
| Vancomycin (30 μg) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) |
| Streptomycin (10 μg) | 22(S) | 24(S) | 24(S) | 28(S) | 26(S) | 24(S) | 22(S) |
| Penicillin G (10 u) | 18(I) | 14(I) | 12(I) | 30(S) | 12(I) | 16(I) | 0(R) |
| Ampicillin (10 μg) | 28(S) | 28(S) | 28(S) | 40(S) | 22(S) | 26(S) | 14(S) |
| Methicillin (5 μg) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) |
| Carbenicillin (50 μg) | 36(S) | 36(S) | 34(S) | 42(S) | 26(S) | 26(S) | 18(S) |
| Polymyxin B (300 u) | 8(R) | 8(R) | 8(R) | 8(R) | 8(R) | 8(R) | 8(R) |
| Chloramphenicol (30 μg) | 28(S) | 24(S) | 26(S) | 36(S) | 30(S) | 30(S) | 24(S) |
| Bacitracin (10 u) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) |
| Cephalothin (30 μg) | 12(R) | 14(R) | 14(R) | 24(S) | 0(R) | 28(S) | 0(R) |
| Tetracycline (30 μg) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) | 0(R) |

Note: R - resistant; I - Intermediate; and S - Susceptible.

amino acid to serve as a nitrogen source. Growth on complex media (TSA, casein hydrolysate medium) is in another category, because in these cases the need for many synthetic reactions has been eliminated.

## Carbon Source Utilization

The ability to utilize specific chemical compounds as sole carbon source is a useful characteristic in the classification of microorganisms. Since our strains, in many respects, resemble the genus *Pseudomonas*, it appeared to be important to study their abilities to use various compounds as carbon sources.

Seventy-nine organic compounds were examined as possible sole sources of carbon and energy. The chemical classes to which the compounds belong were: sugars and sugar alcohols, $\alpha$-amino acids, low molecular weight amino and dicarboxylic acids, Krebs cycle intermediates, aliphatic hydrocarbons, aromatics, and alcohols.

None of the eight alcohols could serve as a sole source of carbon and energy (Table XII).

Three organic acids were able to serve as sole sources of carbon and energy (Table XIII). All seven strains could use the sodium salts of acetic acid, lactic acid, and pyruvic acid. Lactic acid appears to be the carbon source of choice and is also the carbon source in Postgate's Media with which the PPFM's are maintained. In some cases the original isolation was done on Postgate's Medium B. Therefore sodium lactate was used as the positive control for all carbon utilization tests.

TABLE XII

ABILITY OF STRAINS TO UTILIZE ALCOHOLS

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP |
|---|---|---|---|---|---|---|---|
| Positive Control | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Negative Control | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| 1-Butanol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| 2-Butanol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Tertiary Butyl Alcohol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Isobutyl Alcohol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Ethanol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| 1-Propanol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| 2-Propanol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Isoamyl Alcohol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |

Note: Time of incubation, 7 days.

TABLE XIII

ABILITY OF STRAINS TO UTILIZE ACIDS*

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP |
|---|---|---|---|---|---|---|---|
| Acetate | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Butyrate | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Glyoxylate | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Lactate | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Malonate | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Propionate | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Pyruvate | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Negative Control | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |

*Sodium salts.

Note: Incubation time, 7 days.

Heavy growth developed first in sodium lactate followed by growth in sodium pyruvate after 24 hours. No growth was apparent on sodium acetate until after 48 hours, then heavy growth developed. Even though no growth occurred on glyoxylate, ability to grow on acetate indicates the presence of the key enzymes of the glyoxylate cycle, malate synthase, and isocitrate lyase.

In testing the di- and tricarboxylic acids of the Krebs cycle, it was found that malate and succinate supported fairly good growth after 24 hours (Table XIV).

In carrying out tests of this kind, in which the ability of a compound to support growth is determined, it should be kept in mind that it is essential for the compound to get inside the cell in order for it to support growth. It is quite probable that (especially in the case of the TCA cycle intermediates) the bacteria lack the mechanisms (active transport, specific permease, etc.) for transporting the compounds that do not support growth. They probably do not lack the enzymes necessary for metabolizing the compounds; for example, oxalacetic acid and acetyl CoA could be condensed via citrate synthase, and then the citrate further metabolized. To our knowledge, all aerobic bacteria that have been studied possess the enzymes of the TCA cycle, and these bacteria, as indicated previously, are highly aerobic.

TABLE XIV

ABILITY OF STRAINS TO UTILIZE TCA CYCLE INTERMEDIATES*

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP |
|---|---|---|---|---|---|---|---|
| Positive | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Negative | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Citrate | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Cis-Aconitate | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Isocitrate | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Keto-Glutarate | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Succinate | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Fumarate | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Malate | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Oxalacetate | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |

*Sodium salts.

Note: Incubation time, 7 days.

None of the seven PPFM's could use any amino acid as a sole source of carbon and energy (Table XV).

None of the sugars or sugar alcohols tested could serve as a sole source of carbon and energy for any of the seven PPFM's (Table XVI).

None of the seven strains were capable of using any aromatic compound tested as a sole source of carbon and energy (Table XVII). The fact that none of these compounds could be used makes it highly unlikely that any more complex aromatic compounds, either hydrocarbons, phenols, or carboxylic acids, could be used.

Neither one of the aliphatic hydrocarbons examined could serve as a sole source of carbon and energy for any of the seven strains (Table XVIII).

TABLE XV

ABILITY OF STRAINS TO UTILIZE AMINO ACIDS*

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP |
|---|---|---|---|---|---|---|---|
| Positive | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Negative | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Glycine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Alanine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Leucine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Valine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Isoleucine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Proline | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Phenylalanine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Tryptophane | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Lysine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Histidine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Arginine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Aspartate** | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Glutamate | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Asparagine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Cysteine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Methionine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Serine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Threonine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Tyrosine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Glutamine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Ornithine | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |

*All present as L-forms (except glycine).

**Sodium salts.

Note: Incubation time, 14 days.

The reason that we chose heptadecane and kerosene (aside from the fact that they are readily available) was that intermediate molecular weight alkanes, in about the $C_{10}$ - $C_{19}$ range, are most readily utilized by the largest number of microorganisms. Lower molecular weight alkanes (roughly the $C_4$ - $C_9$ range) tend to be quite toxic, partly because they are more water soluble than those of higher molecular weight. This does not apply to methane and ethane, which are usually utilized from the gas phase. Alkanes with higher molecular weights ($C_{20}$ and greater) are very difficult for microbes to utilize because of their extreme water insolubility, as well as the fact that they are solids at temperatures compatible with life.

All seven strains were shown to be able to utilize methanol and formate but not formaldehyde, methylamine, or trimethylamine. Growth was not as heavy on these compounds as on most of the other carbon sources utilized. Results are in Table XIX.

Since the pathway of utilization of methanol involves oxidation to formaldehyde by methanol dehydrogenase, it seemed reasonable that formaldehyde should also be utilized by these bacteria. Since formaldehyde is toxic, it is possible that the initial concentration tried was too high. Decreasing the formaldehyde concentration from 0.1% to 0.05% did not allow growth.

TABLE XVI

ABILITY OF STRAINS TO UTILIZE SUGARS AND SUGAR ALCOHOLS

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP |
|---|---|---|---|---|---|---|---|
| Positive | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Negative | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Glucose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Maltose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Lactose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Sucrose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Galactose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Fructose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Cellobiose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Melezitose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Melibiose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Raffinose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Mannose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Rhamnose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Arabinose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Ribose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Xylose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Trehalose | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Inositol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Adonitol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Dulcitol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Sorbitol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Mannitol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Glycerol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |
| Polyethyleneglycol | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ | ϕ |

Note: Incubation time 3 days.

TABLE XVII

ABILITY OF STRAINS TO UTILIZE REPRESENTATIVE AROMATIC COMPOUNDS

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP |
|---|---|---|---|---|---|---|---|
| Positive Control | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Negative Control | φ | φ | φ | φ | φ | φ | φ |
| Benzoate* | φ | φ | φ | φ | φ | φ | φ |
| p-Hydroxybenzoate* | φ | φ | φ | φ | φ | φ | φ |
| Phenol | φ | φ | φ | φ | φ | φ | φ |
| Toluene | φ | φ | φ | φ | φ | φ | φ |

*Sodium salt.

Note: Incubation time, 7 days.

TABLE XVIII

ABILITY OF STRAINS TO UTILIZE REPRESENTATIVE ALIPHATIC HYDROCARBONS

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP |
|---|---|---|---|---|---|---|---|
| Positive Control | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Negative Control | φ | φ | φ | φ | φ | φ | φ |
| Heptadecane | φ | φ | φ | φ | φ | φ | φ |
| Kerosene | φ | φ | φ | φ | φ | φ | φ |

Note: Incubation time, 7 days.

TABLE XIX

UTILIZATION OF ONE CARBON COMPOUNDS*

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP | *M. organophilum* |
|---|---|---|---|---|---|---|---|---|
| Positive Control | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++** |
| Negative Control | φ | φ | φ | φ | φ | φ | φ | NT*** |
| Methylamine | φ | φ | φ | φ | φ | φ | φ | NT |
| Trimethylamine | φ | φ | φ | φ | φ | φ | φ | NT |
| Methanol | ++ | ++ | ++ | ++ | ++ | ++ | +++ | NT |
| Formaldehyde | φ | φ | φ | φ | φ | φ | φ | NT |
| Formate | + | + | + | + | + | + | + | NT |
| Methane | - | - | - | - | - | - | - | ++++ |

*Sodium salt.

**Tested on glucose.

***Not tested.

Note: Incubation time, 7 days.

Ability to grow on one-carbon compounds more reduced than $CO_2$ (trimethylamine is included in this category because it does not contain any carbon-carbon covalent bonds) by definition, means that a microorganism is a methylotroph. This definition excludes phototrophic bacteria (cyanobacteria and the various types of anaerobic photosynthetic bacteria) which can utilize $CO_2$ as a sole carbon source. Haber, Allen, Zhao, and Hanson (1983), in a review article, pointed out that, except for a few species of yeasts and molds that grow on methane or methanol, all methylotrophs are prokaryotes. Known methylotrophic bacteria are all Gram negative, obligate aerobes, and exist in a variety of shapes (rods,

vibrios, and cocci) (Haber et al., 1983). Methylotrophic bacteria which can use methane for carbon and energy are known as methanotrophs.

Since the strains used in this study possessed all these characteristics of known methylotrophs, we designated them henceforth as pink-pigmented facultative methylotrophs, or "PPFMs".

The term "methylotroph" does not designate a taxonomic category. Methylotrophs belong to a number of genera.

Methylotrophs may be either obligate (growing only on one carbon compound) or facultative. Obviously, the strains described here are facultative. It was thought for a long time that all strains utilizing methane were obligate autotrophs, and conversely, that no facultative methylotroph could utilize methane, but this distinction has been rendered invalid in recent years by the report by Hanson and his co-workers (Patt, Cole, and Bland) of the discovery of a number of methane utilizing strains that could also utilize a fairly large number of compounds such as sugars, various alcohols, and organic acids.

Therefore, it became important for us to determine whether or not our strains could utilize methane. The procedures used to make this determination were given in Part II of this thesis. Several trials confirmed that none of the strains could utilize methane as a sole carbon-energy source. However, abundant growth by *M. organophilum* was visible after 48 hours.

This lack of ability to utilize methane would exclude our strains from the genus *Methylobacterium*, as proposed by Patt, Cole, and Hanson (1976).

## Isolation of DNA

The absorbance of DNA isolated from each strain was determined spectrophotometrically (Table XX) and the concentration (Table XIX) was determined using the following equation: mg/ml = $\frac{A_{260}}{20}$.

TABLE XX

CONCENTRATION OF DNA PREPARATIONS FROM PPFM'S

| | $A_{260}$ | mg/ml DNA |
|---|---|---|
| 79 Dark Pink | 0.186 | 0.0093 |
| 79 Pink Black | 0.208 | 0.0104 |
| 79 Pink | 0.000 | 0.000 |
| Pink | 1.000+* | 0.050** |
| Fuzz | 1.000+* | 0.050** |
| Met | 0.235 | 0.01175 |
| 2EP | 0.019 | 0.00095 |
| Reference Buffer | 0.000 | 0.000 |

*$A_{260}$ was greater than 1.0.

**Calculated as $A_{260}$ = 1.000.

## Determination of Mole Percent Guanine Plus Cytosine in DNA

The method of density gradient centrifugation was employed. This method is based on the fact that the buoyant density of DNA in CsCl is directly proportional to its guanine-cytosine (G+C) content (Schildkraut

et al., 1962). Before determining the percent of G+C, the density of each sample had to be calculated. This was done using the equation:

$$\rho = \rho_0 + 4.2\ {}^2(r^2 - r_0^2) \times 10^{-10} \text{ g/ml}$$

where $\rho$ = density of unknown DNA,

$\rho_0$ = density of known DNA,

$\omega$ = speed of rotation in radians/sec,

$r$ = peak distance of unknown DNA from center of rotation, and

$r_0$ = peak distance of known DNA from center of rotation.

The reference DNA used was from E. coli B (Sigma) which has a density of 1.710 g/ml. The $\omega^2$ is $2.123 \times 10^7$ at 44,000 rpm. The equation now reads: $\rho = 1.710 + 0.0089(r^2 - r_0^2)$. After determining the density of the unknown DNA, the guanine plus cytosine content of the unknown DNA can then be calculated from the equation: $\% \text{ G+C} = \frac{\rho - 1.660 \text{ g/ml}}{0.098} \times 100$.

For the An-D rotor, the distance from the center of rotation to the top of the cell sector, $r_t$ = 5.810 cm. Themagnification factor of the recordings, MF, was calculated as the ratio of the apparent sector length from each recording to the true length, 1.40 cm. Using these, the true peak distance r, from the center of rotation is

$$r = \frac{\Delta R}{MF} + r_t$$

where $\Delta R$ is the distance on the recording from the top of the sector to the peak.

Because of a rotor explosion during another application of the Model E, which seriously damaged the instrument, we were able to complete the % G+C content determinations for only three strains, 79 Dark Pink, 79 Pink Black, and Pink (Table XXI). The largest uncertainty in these

TABLE XXI

MOLE PERCENT GUANINE PLUS CYTOSINE

| | 79 Dark Pink | | 79 Pink Black | | Pink | |
|---|---|---|---|---|---|---|
| Scan Speed* | 1x | 4.75x | 1x | 4.75x | 1x | 4.75x |
| Chart Length (cm)** | 22 | 4.6 | 22 | 6.0 | 22 | 4.6 |
| Sample Peak (cm)** | 10.3 | 2.2 | 14.18 | 3.02 | 13.3 | 2.6 |
| Reference Peak (cm)** | 13.8 | 2.8 | 16.7 | 3.57 | 15.5 | 3.25 |
| Sample Peak Actual Distance (cm) | 6.47 | 6.48 | 6.71 | 6.51 | 6.66 | 6.60 |
| Reference Peak Actual Distance (cm) | 6.69 | 6.66 | 6.87 | 6.64 | 6.80 | 6.80 |
| Density ($\rho$) | 1.684 | 1.689 | 1.691 | 1.695 | 1.693 | 1.686 |
| G+C mole % | 24.5 | 29.6 | 31.6 | 35.7 | 33.7 | 26.5 |

*Scan speed is the speed that the u.v. scanner scans the cell. This was done at two separate speeds: 1x and 4.75x.

**These distances are averages of at least three scans.

values is in the positions of the reference peaks as discussed on page 58.

The mole percent G+C content of DNA from a bacterial cell is recognized as an essential component of the description of any bacterium. It is not a precise determinant of the primary structure (base sequence) of the DNA. Two organisms may have very similar % G+C values, and yet be different in DNA primary structure and in phenotypic characteristics. On the other hand, two organisms with widely different G+C contents are different taxonomically. For example, Bradley (1980) gives examples of organisms similar in many respects, and formerly believed to be related, and classified together that have % G+C values differing by 5 to 30 percent.

## Bio-Rad Assays of Isolated Protein

A standard protein curve was prepared using bovine serum albumin (BSA) (Table I). The absorbance of the BSA and isolated protein sample from each strain was determined spectrophotometrically in triplicate. In addition to the seven strains of PPFM's, protein was isolated from *Pseudomonas aeruginosa* (provided by Oklahoma State University, Department of Botany and Microbiology Culture Collection) and its absorbance determined spectrophotometrically.

A volume of 0.02 ml of each isolated protein sample was measured spectrophotometrically. The concentrations of each sample (Table XXII) were interpolated from the BSA standard curve.

TABLE XXII

CONCENTRATIONS OF ISOLATED PROTEIN SAMPLES

| | $A_{595}$ (nm) | Protein (mg/ml) |
|---|---|---|
| 79 Dark Pink | 0.817 | 3.7 |
| 79 Pink Black | 0.910 | 4.2 |
| 79 Pink | 0.810 | 3.7 |
| Pink | 1.038 | 4.7 |
| Fuzz | 1.040 | 4.7 |
| Met | 1.045 | 4.8 |
| 2EP | 0.660 | 3.0 |
| *P. aeruginosa* | 1.045 | 4.8 |

## Electrophoresis

Electropherograms for each strain of PPFM and the strain of *Pseudomonas aeruginosa* were obtained from the polyacrylamide tube gel electrophoresis. At least four gels of each strain were run. The migration distances shown in Table XXIII are the averages from those gels.

Electropherograms can play an important role in determining the degree of similarity between different strains of bacteria. As expected, these electropherograms gave us additional evidence that these seven strains are very closely related.

## Biocide Sensitivity

Since biocides are often used in enhanced oil recovery, we thought it interesting to determine the reaction of selected PPFM's to the presence of two biocides currently employed in the oil field. All three

strains examined were much more sensitive to glutaraldehyde than DBNPA (Table XXIV).

TABLE XXIII

AVERAGE BAND POSITIONS (RELATIVE MOBILITY)

| | 79 Dark Pink | 79 Pink Black | 79 Pink | Pink | Fuzz | Met | 2EP | *P. aeruginosa* |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.017 | 0.017 | 0.017 | 0.018 | 0.017 | 0.018 | 0.032 | 0.047 |
| 2 | 0.085 | 0.034 | 0.051 | 0.054 | 0.083 | 0.045 | 0.048 | 0.063 |
| 3 | 0.119 | 0.052 | 0.068 | 0.107 | 0.150 | 0.055 | 0.081 | 0.094 |
| 4 | 0.186 | 0.103 | 0.085 | 0.143 | 0.317 | 0.073 | 0.097 | 0.109 |
| 5 | 0.237 | 0.121 | 0.102 | 0.214 | 0.367 | 0.127 | 0.113 | 0.156 |
| 6 | 0.288 | 0.138 | 0.119 | 0.250 | 0.400 | 0.145 | 0.161 | 0.203 |
| 7 | 0.339 | 0.190 | 0.136 | 0.286 | 0.433 | 0.182 | 0.210 | 0.250 |
| 8 | 0.356 | 0.241 | 0.203 | 0.321 | 0.483 | 0.218 | 0.258 | 0.281 |
| 9 | 0.373 | 0.310 | 0.237 | 0.411 | | 0.255 | 0.290 | 0.313 |
| 10 | 0.407 | 0.397 | 0.271 | 0.429 | | 0.309 | 0.323 | 0.406 |
| 11 | 0.458 | 0.431 | 0.424 | | | 0.364 | 0.419 | 0.469 |
| 12 | 0.508 | | | | | 0.455 | 0.484 | |
| 13 | 0.525 | | | | | 0.527 | | |
| 14 | | | | | | | | |

TABLE XXIV

BIOCIDE SENSITIVITY

| | 79 Pink | Pink | 2EP |
|---|---|---|---|
| No Biocide | ++++ | ++++ | ++++ |
| DBNPA* | | | |
| 100 ppm | ++ | ++ | ++ |
| 500 ppm | + | + | + |
| 1000 ppm | ϕ | ϕ | ϕ |
| 2000 ppm | ϕ | ϕ | ϕ |
| Glutaraldehyde** | | | |
| 100 ppm | ϕ | ϕ | ϕ |
| 500 ppm | ϕ | ϕ | ϕ |
| 1000 ppm | ϕ | ϕ | ϕ |
| 2000 ppm | ϕ | ϕ | ϕ |

*Incubation time 7 days.

**Incubation time 14 days.

## CHAPTER IV

## DISCUSSION

The taxonomic status of the pink-pigmented facultative methylotrophs is somewhat of a puzzle, even after the isolation and numerical taxonomic study of hundreds of strains. P. N. Green, of Torry Research Station, Aberdeen, Scotland, is one of the leading researchers in this field. Some of the major factors which have contributed to this taxonomic confusion have been given by Green, Hood, and Dow (1984), as follows: (1) difficulty in isolating the organisms in pure culture. This is especially true for methane oxidizers; (2) difficulty in preserving cultures; (3) availability of cultures; and (4) use of many names in the literature which have been proposed prematurely.

On the basis of a numerical taxonomic analysis of 150 strains of PPFM's and 28 non-pigmented facultative methylotrophs (Green and Bousfield, 1982; Green et al., 1984), recommended that the genus _Methylobacterium_, first proposed by Patt et al. (1976) would be the most suitable for these organisms. Within the PPFM complex there are at least two clusters, which will be referred to as 3A and 3B, differentiated in part by the ability of 3B, and the inability of 3A organisms to utilize certain monosaccharides, notably fructose. The type species, _Methylobacterium_ _organophilum_ (the strain originally isolated, described, and named by Hanson and his co-workers in 1976), was included in cluster 3A. This PPFM was the first Gram negative organism to be isolated which

could utilize methane as well as more complex carbon compounds as sole carbon sources. Previous to this isolation, it was thought that all methane utilizers were obligate methylotrophs. Today, however, the ability to utilize methane as a sole carbon source is not assigned so much weight as a taxonomic characteristic since, in the facultative strains, it is a trait which is easily lost and may be plasmid-borne (Green and Bousfield, 1979).

Our strains were very similar to one another in all the characters we have examined. The only differences among our seven strains were: (1) lipid hydrolysis, strain Pink was negative, the other six were positive; (2) gelatin hydrolysis, strains 79 Dark Pink, 79 Pink Black, Pink, and 2EP were positive while strains 79 Pink, Fuzz, and Met were negative; (3) urea hydrolysis, strain Pink was positive while the other six were negative; (4) growth in the presence of 7% NaCl, strain Met was negative while the other six were positive; (5) erythromycin sensitivity, strain 79 Dark Pink was resistant, strains 79 Pink Black and 79 Pink were intermediate, and the other four were susceptible; (6) penicillin sensitivity, strain Pink was susceptible, strain 2EP was resistant, and the other five were intermediate; and (7) cephalothin sensitivity, strains Pink and Met were susceptible, while the other five were resistant. Therefore, all seven of our strains would belong in the same group or cluster. Our strains all originated from oil fields and were isolated either from crude oil or produced water.

However, they differ in a number of ways from all other groups of PPFM's so far described in the literature. Some of the differences are: (1) inability to use any monosaccharide or other carbohydrate or sugar alcohol, including fructose and glycerol, as a carbon-energy source. One

hundred percent of the 150 PPFM strains examined by Green et al. (1984) utilized glycerol, and 112 out of 150 utilized fructose; (2) all 150 strains of PPFM's studied by Green and Bousfield (1982) hydrolyzed urea, whereas only one of seven strains in this study was urease positive; (3) did not utilize malonate, whereas 100 percent of Green's strains did utilize malonate. This is also true of fumarate and 2-oxoglutarate. Ethanol and propylene glycol were also utilized by Green's strains.

There were also a number of differences in antibiotic resistance between Green's strains and ours, most notably, carbenicillin and tetracycline. All of Green's strains were resistant to carbenicillin whereas all seven of our strains were sensitive to it. On the other hand, all of Green's strains were sensitive to tetracycline whereas all seven of our strains were resistant to it.

The most glaring difference between our strains and all other PPFM's in the literature is the mole percent guanine plus cytosine content of their DNA. Of the selected PPFM's examined by Green et al. (1984), their G+C mole % ranged from 67.1% to 68.8%. *Methylobacterium organophilum* has a G+C mole % of 66%. The G+C mole % from the species of the genus *Pseudomonas* ranges from 58 to 70%. Three of our strains were examined and found to have a G+C content of 25% to 35%.

The biggest uncertainty in the % G+C values results from the uncertainty in the positions of the reference peaks. The reference DNA solutions have suffered extensive degradation judging from the very low and variable peak amplitudes, broad peak widths, and multiple peak nature of the bands recorded in the different experiments by the u.v. scanner of the ultracentrifuge (see the Figures and Appendix). Even in the best of reference samples (*E. coli* B DNA alone on November 19, 1984) the broad

peak width is indicative of low molecular weight or heterogeneous DNA (Dr. Melcher, personal communication, 1984). Since this stock solution was used after 11 months storage as a solution, it is likely that the poorer peaks in experiments in 1985 are due to further degradation of the DNA.

It is still likely that the reference bands are primarily nucleic acids, since proteins have very much lower densities (ca. 1.35). It is possible that these nucleic acids are those of contaminating micro-organisms, whose densities are unknown. This would make calculations of % G+C meaningless. Assuming however, that the reference band is degraded *E. coli* DNA, we can calculate with reasonable confidence a maximum value for the G+C content of the bacteria under test by taking the lowest density position of the reference band. THese are the value summarized in Table XXI on page 51.

The % G+C values should be redetermined with undegraded DNA of known density. Narrow peaks characteristic of such DNA should be obtained. Under such conditions, the precision of the measurements should be about 1% in % G+C values (Mandel et al., 1968). It may be useful to know the relation between errors in peak positions, $\delta r$ or $\delta \Delta R$, and errors in % G+C, $\delta y$. For equal uncertainties in r and $r_0$ (see equations on page 50),

$$\delta y = 169\, \delta\Delta\, R[cm]MF$$

For a MF of 3.5, $\delta y = 48\delta\Delta R[cm]$. Hence errors of 0.02 cm in $\Delta R$ and $\Delta R_0$ would give 1% error in % G+C for this MF.

In summary, I regret to say that the rate of error for these G+C values is not less than 10%. In any event, even with this high rate of

error, the mole % G+C content of the DNA from these three PPFM's is still significantly less than that of *E. coli* B.

Green et al. (1984) formally proposed the redefinition of the genus *Methylobacterium* and the transfer of all PPFM's to it. They gave two reasons for this. First, they feel the sooner the PPFM's are recognized as a well-characterized body of closely related organisms with one name, instead of their many confusing labels, the less likely it will be for complications in nomenclature associated with the group. They also feel that the instability of facultative methane utilization makes it unreliable as a primary generic criterion.

Just where these bacteria would fit best into the currently recognized scheme of bacterial classification (1) is not clear. They differ from every described genus with similar characteristics in at least two, and in many instances, more important ways.

One characteristic which has become recognized as reflecting true phylogenetic relatedness is DNA homology. While our data on mole % G+C are only a beginning in the analysis of DNA relationships, they are an important beginning and have some interesting implications. First, even allowing for an error of $\pm$10%, the G+C content of these strains is much lower than any value reported for any of the phenotypically similar bacteria. For example, values of mole % G+C for obligate methanotrophs range from 50 to 63.5, obligate methanol utilizers range from 49.8 to 67.3, and PPFM's range from 60.5 to 70.5.

Obviously the G+C content cannot be used as necessarily indicating that two strains or clusters are closely related, but it is generally agreed that widely differing G+C contents indicate that the strains or clusters are not closely related. Our strains, which show a 90%, or

more, similarity over the total number of characters examined form a cluster which has a very different G+C content from groups of organisms similar in other respects.

This statement is based on the validity of the G+C data; we have considered this aspect, and feel that the values obtained are correct within a range of $\pm 10\%$. We followed recognized procedures for isolating and purifying the DNA; these involved repeating the step for protein removal three times, as well as treatment with RNase. It is impossible to rule out some degradation of the DNA, but in order to invalidate the results of a density gradient centrifugation, such a degradation would have to be quite extensive.

Certainly the yield of DNA, in at least four cases, was very low, but in the other three it was acceptable; in any case, the validity of the G+C content determination does not depend on the amount of DNA tested.

Assuming, then, that the relatively low G+C contents of three strains are valid, what conclusions can be drawn? First, these strains are not closely related to any previously described group of microorganisms. Before proposing a new genus, however, it is essential to have more data, or more strains, from various sources. It would be very desirable to have DNA hybridization studies done, among these strains, and between various kinds of methylotrophs isolated by other laboratories.

It would be desirable to work toward a consensus among microbiologists on the relative weights that should be given various phenotypic characters in establishing genera, families, etc., of bacteria.

At present, it would be premature to assign these seven strains to any existing genus (there are too many significant differences) or to propose a new genus, although it appears as if that may be necessary. Much more information, about more isolates, is needed.

It is interesting that seven such very similar strains should have come from sources widely separated geographically, and yet were very similar environmentally, including the presence of sulfate-reducing bacteria. The connection, if any, *in situ* between these two very different types of bacteria remains a mystery.

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# APPENDIX

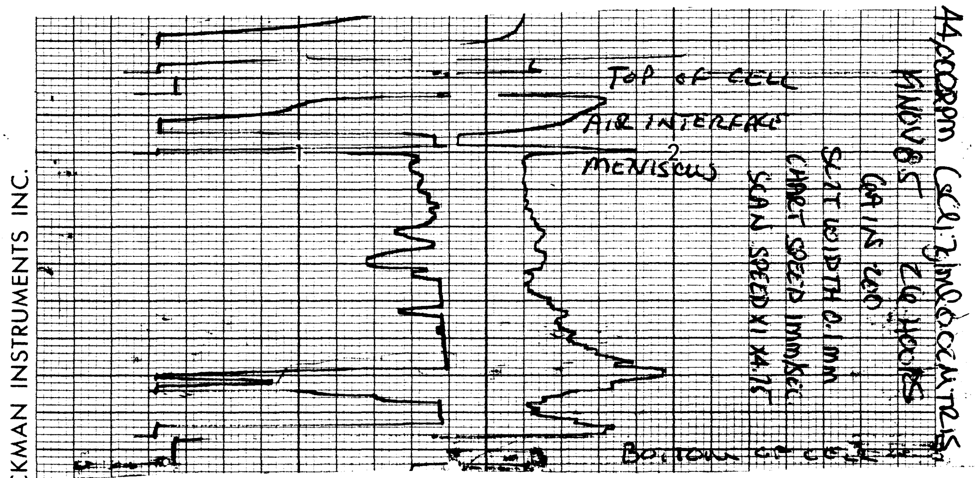

Figure 3. U-V Scan of DNA from E. coli B

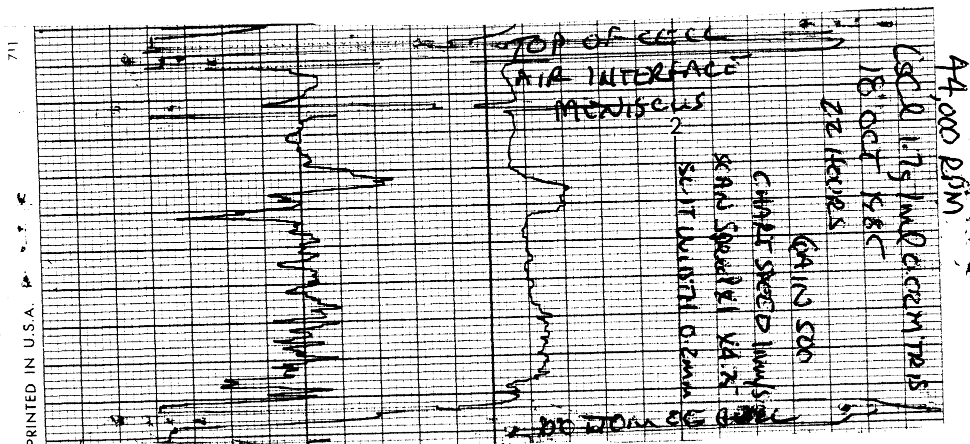

Figure 4. U-V Scan of DNA from Strain Pink

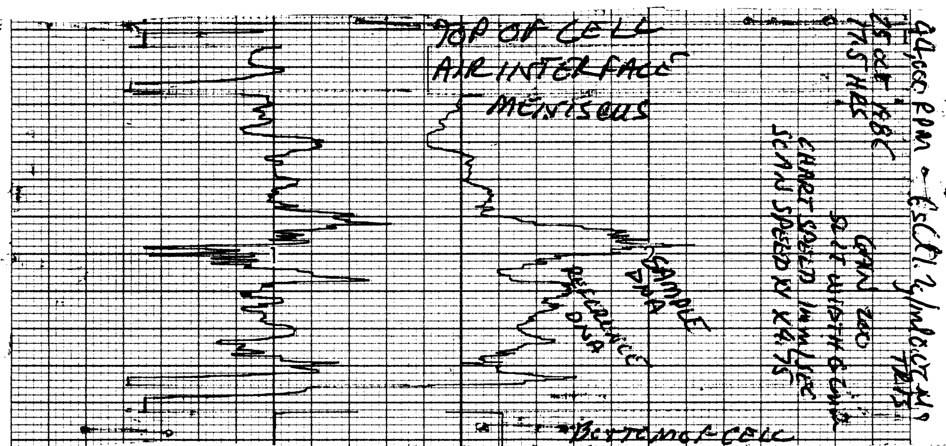


Figure 5. U-V Scan of DNA from E. coli B and DNA from Strain Pink

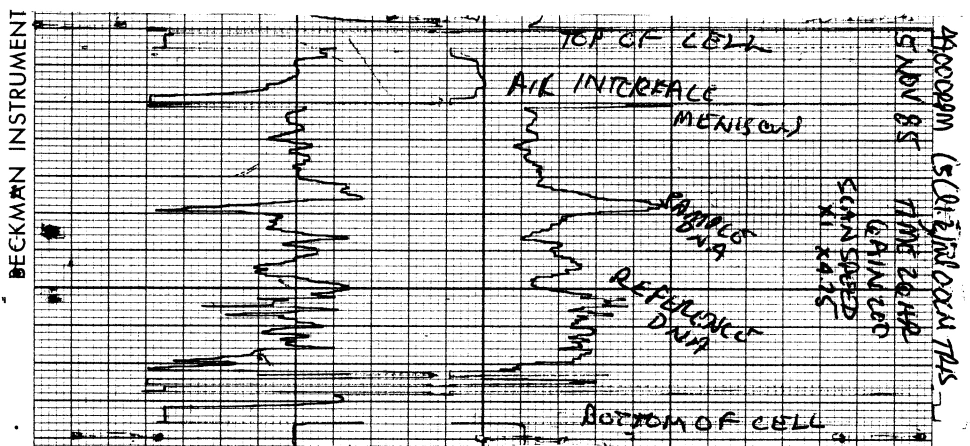


Figure 6. U-V Scan of DNA from E. coli B and DNA from Strain 79 Dark Pink

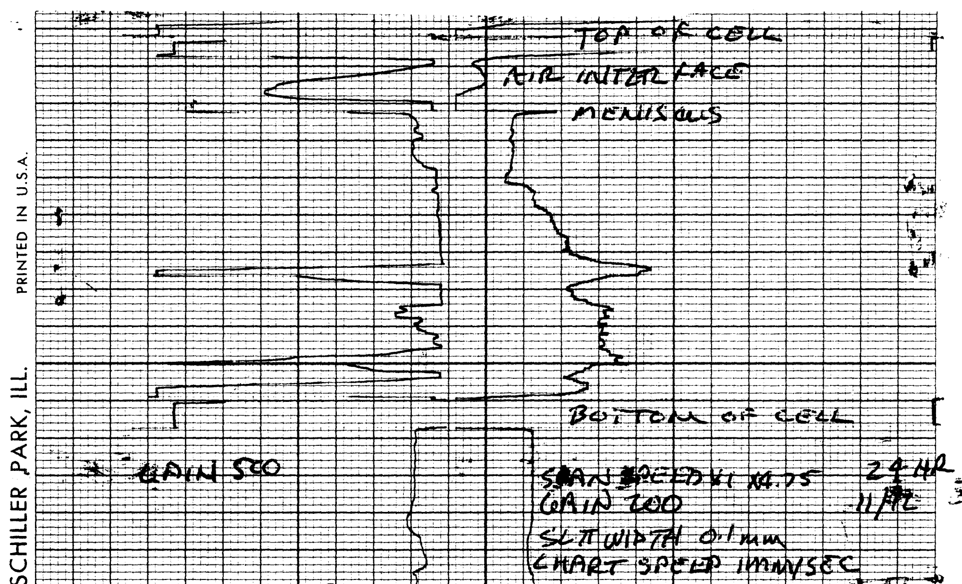


Figure 7. U-V Scan of DNA from E. coli B and DNA from Strain 79 $\overline{\text{Pink}}$ $\overline{\text{Bl}}$ack

## VITA

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