New Pseudomonad Utilizing Methanol for Growth

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A bacterium capable of rapid growth on methanol as sole carbon source was isolated and classified as a new pseudomonad. Its doubling time was about 100 min at 32 to 37 C, and it grew well at methanol concentrations up to 2%. The organism was sensitive to phosphate, but reasonable cell densities could be obtained by using pH control. Cell yields of about 31%, based on methanol consumed, were obtained. The amino acid pattern of the protein indicated that the bacterium holds promise as a source of single-cell protein.

During studies of methanol as substrate for microbial growth, a contaminant which rapidly utilized this alcohol as sole carbon and energy source appeared in the cultures. This bacterium was isolated because of its possible application to the production of single-cell protein. A preliminary investigation of its characteristics was carried out, the purpose being to identify the bacterium so as to facilitate its comparison with other bacteria known to grow on methanol or methane, to gain some understanding of its nutritional requirements, and to obtain an indication of its suitability as a source of single-cell protein.

MATERIALS AND METHODS

Microorganisms. The bacterium, obtained as a contaminant and isolated, was designated as pseudomonad C. Pseudomonas aeruginosa B-771, P. aeruginosa B-12, and P. fluorescens B-10 were obtained from the Northern Regional Research Laboratory, Peoria, Ill., and were stored as slants on nutrient agar at 4 C, except for strain C, which was stored on minimal salts medium (MSM) agar containing methanol.

Media. MSM was prepared by the method of Foster and Davis (4). The production of pigments was tested on King media (11). Ability to oxidize gluconate to 2-ketogluconate was tested by the method of Haynes (8). Other media were prepared by procedures described previously [Manual of Microbiological Methods, Society of American Bacteriologists (ed.), McGraw-Hill Book Co., New York, 1957]. Gelatin liquefaction was tested with MSM containing 15% gelatin and 2% methanol. For some experiments, MSM was modified to contain (NH4)2SO4 (1.0 g/liter) instead of NaNO3, and the concentrations of several salts were changed as follows: Na2HPO4, 2.1 g/liter; Na2HPO4, 0.9 g/liter; and MgSO4·7H2O, 0.1 g/liter. This modified medium was designated MSM-M.

Experiments were carried out in shaker flasks, either 1-liter baffled Erlenmeyer flasks containing 100 ml of medium or 250-ml unbaffled Erlenmeyer flasks, with side arm, with 50 ml of medium. Incubation at the appropriate temperature was carried out in a New Brunswick gyratory shaker, model G-25. Culture densities were measured at 650 nm with a Spectronic 20 photometer. Some studies were carried out in a 5-liter New Brunswick fermentor equipped with an Ingold combined pH electrode, Radiometer pH meter, and titrator.

Methanol was added to the medium after autoclaving.

Assays. Methanol was determined by direct injection of medium into a column (50/80 mesh, Porapak Q). A Varian Aerograph 1200 gas chromatograph with a hydrogen flame detector was used. For injections of 3 uliters, a direct relation was observed between peak height and methanol concentrations between 0 and 2%. The dry weight of bacteria was determined by centrifuging 50 ml of broth, suspending the cells in 10 ml of distilled water, centrifuging once more, resuspending in a small quantity of water for transfer to a test tube (100 by 16 mm), drying overnight at 85 to 90 C in a vacuum oven, and weighing.

The amino acid analysis was carried out on a Beckman Spinco model 120B amino acid analyzer after hydrolysis of the whole cells in 6 N HCl for 22 hr at 110 C, in vacuo.

Reducing sugars, as well as 2-ketogluconate, were determined by the method of Sumner and Somers (16).

Electron microscopy. Specimens for electron microscopy were prepared by placing a drop of the bacterial culture on a collodion-carbon-coated grid for 60 sec. The drop was then removed, and the preparation was negatively stained for 30 sec with a 1% aqueous solution of ammonium molybdate, pH 7.0. The stain was removed, and the specimen was viewed in a Philips EM 300 electron microscope operating at 60 kv.
RESULTS

Characteristics of the organism. The bacterium is a gram-negative, nonsporeforming rod, measuring approximately 0.5 to 0.7 by 1.5 to 2.0 μm. In liquid cultures, it is highly motile, with a single polar flagellum, and appears either singly or in pairs. On an inorganic salts agar medium (4), with methanol as the sole carbon source, white, raised, circular, smooth, opaque colonies of about 1-mm diameter are obtained after 3 days of incubation at 37°C.

At a concentration of 0.2%, growth, but no acid production, occurs on the following carbon sources: glucose, lactose, mannitol, maltose, inositol, sucrose, cellobiose, starch, and ethanol. Indole is not produced, nitrate is not reduced, and gelatin is not liquefied. The pH of litmus milk is unchanged, and pyocyanin is produced (11).

Growth was not observed when C₄ compounds other than methanol were tested. No growth was obtained with methane on either liquid or solid medium. Careful determinations of growth on Oxoid Ionagar plates of MSM-salts when carbon dioxide and hydrogen were supplied led to the conclusion that no more growth occurred than could be found in the controls not exposed to carbon dioxide, whereas no growth was observed in liquid medium. No growth was found in liquid medium when sodium formate (0.01 to 0.5%) was the sole carbon source. When present at concentrations above 1%, sodium formate prevented growth on 1% methanol. Formaldehyde (0.001%) did not support growth, and concentrations higher than 0.01% inhibited growth on other substrates.

Two strains of P. aeruginosa and a strain of P. fluorescens failed to grow on methanol as sole carbon source.

Electron microscope examination. Electron microscope observations were carried out to determine number and location of flagella. When the bacteria were grown on nitrate, the polysaccharide which was produced interfered with the staining. However, ammonium ion as the nitrogen source caused only small amounts of polysaccharide to be produced. When methanol was the carbon source, the flagella proved to be very fragile and were readily lost during preparation, although high motility was observed with the light microscope. Good photographs could be obtained by using very young cultures (Fig. 1). No bacteria were observed with more than a single flagellum.

When glucose was used as the carbon source, the flagella were less fragile and could be observed on rods that were in the middle- and late-log phase of growth. The general appearance of the organism was identical with that of organisms grown on methanol.

Growth. The growth characteristics of the organism growing on methanol as carbon source were quite different, depending on whether the source of nitrogen was ammonium or nitrate ions. When the source of nitrate was nitrate, the culture became extremely viscous during growth, a result of production of polysaccharide. Under these conditions, the bacteria did not appear to be motile and could not readily be separated from the fluid by centrifugation because of the high viscosity. When the culture was blended and extracted (2), the polysaccharide could be recovered in a partly purified form. After acid hydrolysis in sealed evacuated ampoules (1 M H₂SO₄, 100°C, for 4 hr), preliminary analyses with glucose oxidase indicated that the polymer contained approximately 33% glucose; however, the polymer has not yet been further characterized.

When ammonia was the nitrogen source, culture densities were limited because of the resultant drop in pH. The buffer strength could not readily be increased because of a sensitivity to phosphate (Table 1). When pH control could be used (for instance, when growth was carried out in fermentors), optical densities of 2.5 to 3.0 at 650 nm were obtained. In shaker flasks, a minimum degree of agitation was necessary for homogenous growth to be obtained. Below 140 rev/min, aggregates of bacteria were produced even during the log phase of growth. This prevented quantitation of growth by optical measurements. On the other hand, at shaking rates of 200 rev/min or higher, aggregates were not produced, and the culture remained homogenous. In fermentors, where agitation rates of at least 300 rev/min were used, aggregate formation was not observed.

A relationship was noted between temperature of growth, methanol concentrations, and the resulting growth rate. Table 2 presents data obtained from a series of shaker flask experiments. Whereas at 30 and 37°C concentrations of methanol in the range 0.5 to 2% had little, if any, effect on doubling time, at 40°C the higher concentrations had an inhibitory effect. It may be seen that the optimal temperature for growth is in the range of 32 to 37°C at methanol concentrations of 2%.

For the preparation of moderate quantities of cells and for estimation of cell yields and composition, a series of fermentations was carried out in 5-liter fermentors equipped with pH controls. A typical run is shown in Fig. 2.
TABLE 1. Effect of phosphate concentration on growth of pseudomonad C in modified minimal salts medium

| Phosphate conc (mm) | Optical density at 650 nm* | pH* |
|---------------------|---------------------------|-----|
| 4.1                 | 0.34                      | 3.8 |
| 10.3                | 0.68                      | 4.3 |
| 20.6                | 0.72                      | 4.7 |
| 24.7                | 1.37                      | 5.3 |
| 26.7                | 1.37                      | 5.5 |
| 28.7                | 0.59                      | 6.5 |
| 31.0                | 0.01                      | 7.0 |
| 41.1                |                           | 7.1 |
| 51.5                |                           | 7.1 |
| 61.8                |                           | 7.1 |

* After 48 hr of growth.

TABLE 2. Combined effects of temperature and methanol concentration on the doubling time of pseudomonad C grown in shaker flasks on modified minimal salts medium a

| Temp (°C) | Methanol conc | 0.5% | 1.0% | 2.0% |
|-----------|---------------|------|------|------|
| 28        | NT*           | 169  | 165  |      |
| 32        | 101 ± 7c      | 93 ± 10 | 111 ± 14 | |
| 37        | 112 ± 8       | 114 ± 7 | 121 ± 4  | |
| 40        | 165 ± 15      | 219 ± 47 | 398 ± 59 | |
| 43        | NGc           | NG   | NG   |      |

*a Repeated measurements were made and data were taken from the logarithmic phase of growth. Data are expressed in minutes. Mean and standard deviations are indicated.

b Not tested.
c No growth.
Data from a series of such runs are given in Table 3. Addition of methanol towards the end of log-phase growth did not increase cell concentrations.

The cells from several runs were pooled, lyophilized, and subjected to amino acid analysis. The results of replicate analysis are given in Table 4. Kjeldahl analysis resulted in a value of 9.95% nitrogen, which corresponds to approximately 62.2% protein in the cells. The figure estimated from the amino acid analysis was 67.2% protein, which is in reasonably good agreement with the Kjeldahl analysis.

**DISCUSSION**

Identity of *pseudomonad C*. Stocks and McCleskey (15) classify bacteria that grow on methane or methanol in two groups: one that grows on methanol but cannot grow on methane, and another that can grow on both of these substances. Harrington and Kallio (7), on the other hand, propose that, after transfers on methanol-containing media, bacteria that originally could grow on both methanol and methane can lose their ability to grow on methane. Thus, it appeared desirable to compare our bacterium, which appeared to be a species of *Pseudomonas*, to bacteria that have been reported to grow on either methanol or methane as sole carbon source. In Table 5, the salient properties of *pseudomonad C* are compared with reported properties of other bacteria that grow on methanol or methane. *Pseudomonad C* is not a member of *Methylomonas* or *Methylococcus*, based on its ability to grow on carbon sources other than C1 compounds. It appears to be most similar to *Pseudomonas* AM-1 (14) but differs from this organism in its lack of ability to utilize formate and its more rapid rate of growth on methanol. In addition, preliminary experiments indicate that *pseudomonad C* lacks D-glycerate-nicotinamide adenine dinucleotide oxidoreductase (hydroxy-pyruvate reductase, EC 1.1.1.29), a key enzyme in the serine assimilatory pathway of *Pseudomonas* AM-1 (9). However, as with other methanol-utilizing bacteria (6), glycine does affect the growth rate of *pseudomonad C* (B. Stieglitz, personal communication).

**Growth characteristics.** Polysaccharide production was much less when ammonium ion served as the nitrogen source than when nitrate was the nitrogen source. The reason for this difference was not clear, but may be attributable to a slower rate of growth on nitrate. This hypothesis was difficult to check because

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**Figure 2.** Pattern of typical fermentation carried out in a fermentor with 1% methanol as carbon source and ammonium ion as nitrogen source. The pH was maintained at 7 by addition of sodium hydroxide.

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**Table 3.** Effects of growth of *pseudomonad C* on modified minimal salts medium in fermentors with 2% methanol and pH controla

| pH  | Final conc of cells [g (dry wt)/liter] | Yield (%) based on methanol |
|-----|--------------------------------------|-----------------------------|
| 7.0 | 1.92                                 | 31.0                        |
| 7.0 | 2.47                                 | 31.2                        |
| 7.0 | 2.30                                 | 24.2                        |

a (NH₄)₂SO₄ (2 g/liter) was the sole nitrogen source.

**Table 4.** Amino acid composition of cells of *pseudomonad C*

| Amino acid | Analysis (g/100 g of amino acids)       |
|------------|----------------------------------------|
|            | Sample 1 | Sample 2 | Sample 3 | Mean     |
| Alanine    |          |          |          |          |
| Arginine   | 8.55     | 9.77     | 9.26     | 9.19     |
| Aspartic   | 7.87     | 5.82     | 5.13     | 6.27     |
| Acid       | 9.55     | 10.77    | 10.83    | 10.38    |
| Half cysteine | 1.97   |          | 0.76     | 1.10     |
| Glutamic acid | 12.20  | 13.70    | 13.35    | 13.08    |
| Glycine    | 5.87     | 6.95     | 6.09     | 6.30     |
| Histidine  | 3.09     | 2.55     | 2.13     | 2.52     |
| Isoleucine | 5.53     | 5.92     | 5.50     | 5.85     |
| Leucine    | 7.95     | 7.50     | 8.77     | 8.07     |
| Lysine     | 10.20    | 7.89     | 6.86     | 8.32     |
| Methionine | 2.64     | 3.11     | 1.90     | 2.55     |
| Phenylalanine | 3.79  | 4.19     | 4.14     | 4.04     |
| Proline    | 3.33     | 3.81     | 4.09     | 3.74     |
| Serine     | 2.85     | 2.99     | 4.63     | 3.49     |
| Threonine  | 6.75     | 5.01     | 5.91     | 5.89     |
| Tyrosine   | 2.04     | 2.97     | 3.78     | 2.93     |
| Valine     | 6.24     | 6.92     | 6.97     | 6.71     |

a Samples 1 and 2 were analyzed at the Hebrew University-Hadassah Medical School, Jerusalem. Sample 3 was analyzed at the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass.
Table 5. Comparison of various bacteria able to utilize methanol as sole carbon source

| Determination                | Pseudomonad C | Pseudomonas methanica (3) | P. methanica (7) | P. methanica (13) | Pseudomonas AM-1 (14) | Pseudomonas PRL-W4 (10) | Rod; with rounded ends | Rod; single polar flagellum * | Rod; single polar flagellum | Rod; single polar flagellum | Diplococcus; capsule |
|------------------------------|---------------|---------------------------|-----------------|-------------------|----------------------|------------------------|-------------------------|--------------------------|---------------------------|---------------------------|---------------------|
| Morphology                   | Rod; single polar flagellum 0.5 to 0.7 by 1.5 μm | Rod; single polar flagellum 0.6 by 1 to 1.5 μm | Rod; nonmotile 0.3 by 0.7 μm | Rod; single polar flagellum 0.6 by 1 μm | Rod; single polar flagellum 0.8 by 2 μm | Rod; with rounded ends 0.5 by 1.2 μm | Rod; single polar flagellum | 1 by 1 μm | 1 by 1 μm | 1 by 1 μm | 1 by 1 μm |
| Size                         | Pink; pyocyanin | Pink | Pink | Pink | Pink | Pink | Pink | Pink | Pink | Pink | Pink |
| Color                        | 2 | 28-40 | 30 | 25-30 | 30-37 | 30 | 30 | 28 | 30-50 | 30-50 | 30-50 |
| Doubling time (hr)           | 2 | 30 | 30-37 | 4 to 10 | 7.2 | * | 3.5 to 13 | 30-50 | 30-50 | 30-50 | 30-50 |
| Growth temp (C)              | 28-40 | 30 | 25-30 | 30-37 | 30 | 30 | 28 | 30-50 | 30-50 | 30-50 | 30-50 |
| Sole carbon source           | Methane | Methanol | Formaldehyde | Formate | Ethanol | Glucose | Fructose | Starch | Peptone |
|                              | – | ++ | – | – | ++ | +* | – | – | – |
|                              | ++ | ++ | ++ | + | ++ | ++ | – | – | – |
|                              | – | – | – | – | – | – | – | – | – |
|                              | – | – | – | – | – | – | – | – | – |
|                              | ++ | – | – | – | – | – | – | – | – |
|                              | – | – | – | – | – | – | – | – | – |
|                              | – | – | – | – | – | – | – | – | – |
|                              | – | – | – | – | – | – | – | – | – |
|                              | – | – | – | – | – | – | – | – | – |
|                              | – | – | – | – | – | – | – | – | – |

* Symbols: ++, strong growth; +, weak growth; *, variable; –, no growth; *, not reported.
of the problem of measuring growth rate in the presence of the large amount of polysaccharide produced.

The formation of aggregates seemed to depend largely on the amount of agitation. At higher shear rates, obtained at higher agitation rates, aggregates were not seen. It has been postulated previously (3) that aggregates resulted only when small amounts of polysaccharide were produced, whereas growth was homogeneous when large amounts were produced. This explanation does not appear to be valid for pseudomonad C in the experiments reported here.

The interrelationship between the effects of temperature and methanol concentration on growth rate can be explained by the fact that methanol is toxic to these bacteria and also serves as a substrate for them. At temperatures near the upper limit for growth, the toxic effects become more apparent, and an increase in methanol concentration led to a substantial reduction in the growth rate. Comparison with the results of Håggström (5) indicates that the mixed culture used in the latter study was very much more sensitive to methanol inhibition than was pseudomonad C.

The yield of cells obtained was approximately 31%, based on methanol supplied. This figure, although somewhat low in comparison to the figure of 41% reported by Håggström (5), must be considered as a minimum because no correction was made for methanol lost by evaporation. If necessary for economic reasons, this loss could be reduced by feeding the methanol during the fermentation so that its concentration in the medium is maintained at a low level, thus reducing the fraction that is lost by evaporation.

The protein content of the cells grown with ammonia was typical of bacteria. The amino acid pattern found is similar to that of the mixed culture of gram-negative rods (culture TM-20) grown on methanol (5), but is quite different from the pattern resulting from the growth of P. aeruginosa on hexadecane, glucose, or xylose (17). The latter cells contained relatively low amounts of aspartic and glutamic acids, and their lysine content varied markedly with pH of the growth medium. Although gram-negative bacteria are sometimes associated with toxicities arising from their polysaccharides, at least one large-scale feeding study (12) with mice, chickens, and pigs indicated that pseudomonads are an acceptable source of single-cell protein.

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