Microbial Growth on C₁ Compounds

1. ISOLATION AND CHARACTERIZATION OF *PSEUDOMONAS* AM 1

By D. PEEL AND J. R. QUAYLE

Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

(Received 28 April 1961)

It has long been known that some non-photo-synthetic micro-organisms can grow on organic C₁ compounds as their sole source of energy and carbon. The means whereby such organisms grow has been the subject of speculation. It has been suggested (Bhat & Barker, 1948; van Niel, 1954) that they might couple the energy of oxidation of the organic substrate to the reduction of carbon dioxide and the synthesis therefrom of all cell constituents. This would then be a variant of chemo-synthetic autotrophy in which the organic compound would merely play a role similar to the inorganic electron donor. The occurrence of this autotrophic type of metabolism has been established by Quayle & Keech (1959*a, b*) in *Pseudomonas oxalaticus* grown on formate as sole carbon and energy source. In this organism, the cellular carbon is incorporated as carbon dioxide by the ribulose diphosphate cycle of carbon dioxide fixation (Bassham *et al.* 1954). More data are needed before the extent of occurrence of this cycle in general microbial growth on organic C₁ compounds can be assessed. It might be expected, especially with a C₁ substrate more reduced than formate, that the reduction level of the organic substrate might be conserved by at least some assimilation of carbon as reduced C₁ units, rather than synthesis of all cell material from carbon dioxide. Evidence has been obtained which indicates that such a heterotrophic type of metabolism operates during growth of *Pseudomonas methanica* on methane (Leadbetter & Foster, 1958) and *Pseudomonas* PRL-W4 on methanol (Kaneda & Roxburgh, 1959*a, b, c*). Few details of the process are known. For use in investigation of this problem, an organism has been isolated which grows rapidly on methylamine, methanol, formate, or a wide range of other compounds, in an otherwise mineral medium. Its isolation and characteristics are described in this paper.

A preliminary account of part of this work has been presented (Quayle & Peel, 1960).

METHODS

Maintenance and growth of the organism. All growth media contained the same concentrations of inorganic salts as those used by Jayasuriya (1955) for the growth of *Pseudomonas* OD1. For liquid culture the only addition

was 0.5% (v/v) methanol (A.R.). Stock cultures were grown at 30°, on slopes of 0.1 M-methylamine hydrochloride with 1% (w/v) of agar-agar (Davis, New Zealand), stored at 2° and subcultured every 4 weeks. For growth on a large scale an inoculum was prepared by incubating 10 ml. of liquid medium for 24–48 hr. at 30° after sterile transfer of bacteria from the slope. The inoculum was introduced into a 1 l. conical culture flask (Fearnbach flask), containing 200 ml. of liquid medium. The cells were then grown at 30° on a reciprocal shaker for 24–36 hr. and harvested by centrifuging whilst in the logarithmic growth phase at a cell density of 0.1–0.2 mg. dry wt./ml. Growth was determined by measurements with a nephelometer (Evans Electro-selenium Ltd., Essex) previously calibrated in terms of dry wt. of cells/ml. of bacterial suspension.

Manometric methods. Consumption of O₂ was measured in conventional Warburg manometers at 30° with air as the gas phase. The cells were washed with, and resuspended in, aq. 0.9% KCl to a final concn. of about 4 mg. dry wt./ml.; 1 ml. of this cell suspension was placed in each main compartment of Warburg flasks, to each of which was added 100 μmoles of phosphate buffer (pH 7.2), prepared by adjusting 0.1 M-KH₂PO₄ to this pH by addition of 0.1 M-Na₂HPO₄. The volume of the contents of the main compartment was adjusted with water to give the flask contents a total volume of 2.2 ml. The centre well contained 0.2 ml. of 2 N-KOH. Sufficient substrate was added from the side arm to give a total theoretical O₂ uptake of 300–400 μl.

Electron microscopy. Dilute suspensions of the washed organism were fixed with 1% (w/v) OsO₄, placed on a Formvar-coated grid by the blot technique, and shadowed with gold-palladium (60:40). The photographs were taken in the Akashi Tronoscope, TRS 50.

Chemicals. A sample of β-hydroxy[¹⁴C]butyric acid was a gift from Dr H. L. Kornberg.

Chromatographic analysis of pigments. The pigments were extracted from 1 g. of wet cells in two 10 ml. portions of chloroform-methanol (1:1, v/v). This solution was evaporated to dryness under N₂ at 60° and dissolved in 2 ml. of chloroform, ready for loading on a chromatography column. The column (250 mm. × 18 mm. internal diameter) was packed with 20 g. of silicic acid (Mallinckrodt Analytical Reagent, 100 mesh) slurried in chloroform. The silicic acid had been dried overnight at 120°. The column was developed with approx. 100 ml. portions of chloroform-methanol mixtures containing respectively 0, 1, 5 and 50% (v/v) of methanol. Fractions of 8–10 ml. were collected. During development the pigments separated into ten distinct bands all of which were red. The fractions containing these bands were evaporated to dryness under N₂ at 60°. The residues were dissolved in 4 ml. of absolute ethanol and their spectra measured over the range 250–650 mμ with a Cary recording spectrophotometer.

Identification of poly- β -hydroxybutyrate. Digestion of methanol-grown cells of organism AM1 with alkaline hypochlorite at 37° (Williamson & Wilkinson, 1958) left a white, amorphous material which, after successive washing with water, acetone-ether (1:1, v/v) and ether, amounted to 6% of the dry weight of the organism. It was identified as poly- β -hydroxybutyrate on the basis of its melting point of 158–160°, and its hydrolysis to β -hydroxybutyric acid. This hydrolysis was performed in 2.5N-NaOH at 100° for 3 hr. After neutralization with HCl, samples of the resulting solution were chromatographed in the solvent systems described by Kornberg (1958): phenol-90% (w/v; A.R.) formic acid-water (500 g.: 13 ml.: 167 ml.) and by Benson *et al.* (1950): butanol-propionic acid-water (47:22:31, v/v). The acidic product, located by spraying with ethanolic bromocresol green (0.1%, w/v), co-chromatographed with authentic radioactive β -hydroxybutyric acid.

RESULTS

Isolation. During an investigation of bacterial growth on C_1 compounds as sole source of carbon and energy, heavy, pink growth, presumably due to some airborne contaminant, was observed in a medium containing 0.1M-methylamine hydrochloride and inorganic salts. This organism was isolated by repeated subculture in liquid medium and selection of single colonies from agar plates containing the same carbon source. The organism was designated AM1.

Description. The organism is a Gram-negative rod measuring $0.8 \mu \times 2.0 \mu$ and is motile with a single polar flagellum (Fig. 1). Colonies on agar are pink, raised, moist, circular and have smooth edges. They are pin-point within 2 days and reach a maximum diameter of 1 mm. after 1 week. The organism is strictly aerobic. Unshaken cultures produce a thin pellicle and shaken cultures have a tendency to clump which is particularly marked in growth on methylamine hydrochloride.

Growth characteristics

pH Optimum. This was determined in the medium of Jayasuriya (1955) except that the phosphate concentration was increased from 25 to 50 mM, and methanol was present as carbon source. The organism grows over the range pH 6.0–8.5 with a broad optimum at pH 7.0. No growth was observed at pH 5.0.

Temperature. The organism grows slowly at 25°, well at 30° and not at all at 37°.

Substrate concentration. Growth is reduced at methanol concentrations greater than 1% (v/v) and totally inhibited at 5% (v/v). Below these concentrations a mean generation time of 4–10 hr. is normally observed.

Growth on other substrates. Many compounds were tested as sole sources of carbon and energy at a concentration of 0.1% (w/v). The results of these

experiments are shown in Table 1. Rapid growth to a cell density of about 0.2 mg. dry wt./ml. was observed on methanol, methylamine hydrochloride, sodium formate, ethanol, lactate, succinate, malate and fumarate. Slower growth was observed on glucose, fructose, aspartate, glutamate, glycollate and citrate. Negligible growth occurs on oxalate, glutarate, tartrate, alanine and mineral medium with no added carbon source. No trace of growth was found in the presence of formaldehyde (0.1 and 0.01%, w/v), glyoxylate, acetate, glycine, serine, propionate, glutarate and ribose. No growth was observed when the organism was incubated in liquid or on solid media without a carbon source other than that contained in an atmosphere of either methane (National Coal Board, Point of Ayr Unit, Flintshire, Wales)-air (1:1, v/v) or carbon dioxide-hydrogen-air (1:4:15, by vol.).

Growth factors. A requirement for added growth factors was not found since the organism grew normally after ten serial subcultures in liquid medium at a cumulative dilution of about 10^{-30} . The mean generation time of the organism was unchanged in the presence of 0.01 or 0.1% of yeast extract.

Pigment. The organism produced red intracellular

Fig. 1. Electron micrograph of *Pseudomonas* AM1.

Table 1. *Comparison of the properties of organism AM1 and other similar organisms*

		<i>Pseudomonas methanica</i> (<i>Methanomonas methanica</i> ; Bergey)		<i>Pseudomonas</i> PRL-W 4	<i>Pseudomonas aminovorans</i> (7 strains)	<i>Protaminobacter alboflavus</i> (4 strains)	<i>Protaminobacter ruber</i> (2 strains)	<i>Bacillus extorquens</i> (<i>Vibrio extorquens</i>)	Organism AM 1
References	...	Dworkin & Foster (1956), Leadbetter & Foster (1958), Harrington & Kallio (1960)	Kaneda & Roxburgh (1959a)	den Dooren de Jong (1926, 1927)	Basselik (1913), Bhat & Barker (1948)	Approx. 1.5 × 3.0	Single polar	Single polar 4-10	
Dimensions (μ)	...	0.4 × 1.5	0.3 × 0.7	0.5 × 1.2					
Flagella									
Mean generation time (hr.)									
Growth-factor require- ments									
Pigmentation	...	Red, brown, yellow, colourless	Red	Red	Colourless or yellow	Red	Red	Red	
Utilization of carbon sources									
Methane		+	+	+	+	+	+	+	+
Methanol		+	+	+	+	+	+	+	+
Methylamine		.	.	.	.	.	.	.	.
Dimethylamine		.	.	.	.	.	.	.	.
Trimethylamine		-	-	-	-	-	-	-	-
Formaldehyde		.	.	.	.	.	.	.	.
Formamide		.	.	.	.	.	.	.	.
Formate		-	-	-	-	-	-	-	-
Ethanol		+	+	+	+	+	+	+	+
Ethylamine		.	.	.	.	.	.	.	.
Diethylamine		.	.	.	.	.	.	.	.
Lactate		-	-	-	-	-	-	-	-
Succinate		-	-	-	-	-	-	-	-
Malate		-	-	-	-	-	-	-	-
Fumarate		-	-	-	-	-	-	-	-
Tartrate		-	-	-	-	-	-	-	-
Acetate		.	.	.	.	.	.	.	.
Glyoxylate		.	.	.	.	.	.	.	.
Glycollate		.	.	.	.	.	.	.	.
Oxalate		.	.	.	.	.	.	.	.
Propionate		.	.	.	.	.	.	.	.
Glutarate		.	.	.	.	.	.	.	.
Citrate		-	-	-	-	-	-	-	-
Glycine		.	.	.	.	.	.	.	.
Serine		.	.	.	.	.	.	.	.
Alanine		.	.	.	.	.	.	.	.
Aspartate		.	.	.	.	.	.	.	.
Glutamate		.	.	.	.	.	.	.	.
Glucose		-	-	-	-	-	-	-	-
Fructose		-	-	-	-	-	-	-	-
H ₂ -CO ₂ -air		-	-	-	-	-	-	-	-

* Weaver, Samuels & Sherego (1938).

+ P. J. Large, unpublished observations with *Protaminobacter ruber* (N.C.I.B. No. 2879) and *P. alboflavus* (N.C.I.B. No. 8167).

pigments in the presence of all the carbon sources tested. The spectra of all of the fractions obtained by chromatography had absorption maxima at 526, 497, 472, 388, 315 and 270 $m\mu$ in ethanol, but the relative extinction coefficients at these maxima varied from one fraction to another. The spectrum of the main fraction is shown in Fig. 2. The spectra

Fig. 2. Absorption spectrum of main pigment fraction from *Pseudomonas* AM1. The solvent was absolute ethanol.

and solubility of the pigments resemble those of highly unsaturated carotenoid pigments (Karrer & Jucker, 1950).

Presence of poly-β-hydroxybutyrate. Methanol-grown cells of organism AM1 contained 6% of their dry weight in the form of a white, amorphous material which was identified as poly-β-hydroxybutyrate on the basis of its melting point and alkaline hydrolysis to β-hydroxybutyric acid.

Oxidative properties. The oxidation of various substrates by washed, whole cells of the organism grown on methanol, formate, or succinate is recorded in Table 2. When grown on any of these substrates, the organism is able to oxidize both formate and formaldehyde. With all substrates tested, the total oxygen uptake was 50–70% of the theoretical value for complete combustion. An increase of about 10% in the total oxygen uptake with methanol, formaldehyde and formate was observed in the presence of 1 mM-2,4-dinitrophenol. The oxidation of formate was partially inhibited by 1 mM-sodium hypophosphite, a compound which is an inhibitor of formic dehydrogenase in some organisms (Takamiya, 1953). Tris hydrochloride was not oxidized by the cells, nor did its presence alter the rate of oxidation of methanol.

DISCUSSION

The properties of organism AM1 lead us to classify it as a species of *Pseudomonas* (Bergey, 1957), and we propose to refer to it as *Pseudomonas* AM1. A culture so named has been deposited with the National Collection of Industrial Bacteria, Torry, Aberdeen.

Table 2. *Oxidative properties of washed suspensions of organism AM1 grown on methanol, formate and succinate*

Manometer cups contained: main compartment, 1.0 ml. of cell suspension (4 mg. dry wt.); 100 μ moles of $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 7.2; side arm, substrate; centre well, 400 μ moles of KOH. Total volume of contents was 2.2 ml. Incubation was at 30° for a maximum of 3–4 hr. (For further details see Methods.)

Substrate for oxidation	Growth substrate					
	Methanol		Formate		Succinate	
	Q_{O_2} in presence of substrate	Endogenous Q_{O_2}	Q_{O_2} in presence of substrate	Endogenous Q_{O_2}	Q_{O_2} in presence of substrate	Endogenous Q_{O_2}
Methanol	-187	-13	-146	-8	-32	-29
Formaldehyde	-199		-121		-65	
Formate	-99		-53		-39	
Methylamine	-163		-57*		-73	
Succinate	-43		-52		-81	
Malate	-48		-55	-19		
Fumarate	-45		-37			
Citrate	-13		-21			
Glycine	-13		-41			
Serine	-13		-32			

* After a lag of 3 hr.

The organisms listed in Table 1 are similar to *Pseudomonas* AM1 in various respects. They are all Gram-negative rods which can grow aerobically on C_1 compounds as sole sources of carbon and energy. Most of them are motile with a single polar flagellum and some of them contain carotenoid-like pigments. With the exception of *Pseudomonas methanica* (*Methanomonas methanica*; Bergey, 1957), they will also grow aerobically on a wide range of organic acids, simple amines or glucose as sole sources of carbon and energy. *P. methanica*, like *Pseudomonas* AM1, has been found to contain poly- β -hydroxybutyrate (Kallio & Harrington, 1960). In view of the important similarities between these organisms, their classification into different genera may not in fact be justified. Indeed, Dworkin & Foster (1956) and Slepecky & Doetsch (1954) have questioned the establishment of the genera *Methanomonas* and *Protaminobacter*, and suggest that organisms so named could equally well be classified as *Pseudomonas*.

The relative ease with which *Pseudomonas* AM1 grows on several C_1 compounds in a defined medium makes it a valuable organism for study of microbial growth on such compounds, and the following paper describes initial studies on the metabolism of methanol and formate by cells grown on these substrates.

SUMMARY

1. An organism has been isolated which grows aerobically on methylamine, methanol, formate or formamide as sole sources of carbon and energy. Neither methane nor formaldehyde serves as growth substrate, nor can the organism grow autotrophically at the expense of oxidation of hydrogen. Certain mono- and di-carboxylic acids, amino acids, amines and glucose will also support growth in an otherwise mineral medium.

2. The organism is a pink-coloured, Gram-negative, motile rod ($0.8 \mu \times 2.0 \mu$) with a single polar flagellum. It is identified as a species of *Pseudomonas* and is referred to as *Pseudomonas* AM1.

3. With methanol as growth substrate, the optimum growth conditions of *Pseudomonas* AM1 are obtained at pH 7, at a temperature of 30°, and at a methanol concentration of 0.5–1% (v/v). No requirement for any growth factor was found.

4. The oxidative abilities of the methanol-, formate- and succinate-grown organism have been examined manometrically.

5. The solubility and spectral characteristics of the pink pigments suggest that they are unsaturated carotenoids.

6. Methanol-grown *Pseudomonas* AM1 contains poly- β -hydroxybutyrate to the extent of about 6% of its dry weight.

7. The properties of *Pseudomonas* AM1 are compared with those of other similar organisms.

We wish to thank Professor Sir Hans Krebs, F.R.S., for his interest and encouragement, Dr D. E. Hughes for performing the electron microscopy, Professor S. R. Elsdon for the loan of a copy of den Dooren de Jong's thesis, and Miss A. West for technical assistance. We also wish to acknowledge many helpful discussions with Dr June Lascelles and Dr J. G. Morris. This work was supported in part by the Rockefeller Foundation and the United States Public Health Service, and also by the Office of Scientific Research of the Air Research and Development Command of the United States Air Force, through its European Office, under Contract no. AF 61 (052)–180 and was done while D. P. held a Medical Research Council Scholarship for training in research methods.

REFERENCES

- Bassalik, K. (1913). *Jb. wiss. Bot.* **53**, 255.
 Bassham, J. A., Benson, A. A., Kay, L. D., Harris, A. Z., Wilson, A. T. & Calvin, M. (1954). *J. Amer. chem. Soc.* **76**, 1760.
 Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C. Haas, V. A. & Stepka, W. (1950). *J. Amer. chem. Soc.*, **72**, 1710.
Bergey's Manual of Determinative Bacteriology (1957). 7th ed. Ed. by Breed, R. S., Murray, E. G. D. & Smith, N. R. London: Baillière Tindall and Cox.
 Bhat, J. V. & Barker, H. A. (1948). *J. Bact.* **55**, 359.
 den Dooren de Jong, L. E. (1926). Thesis: Delft.
 den Dooren de Jong, L. E. (1927). *Zbl. Bakt. (Abt. 2)*, **71**, 193.
 Dworkin, M. & Foster, J. W. (1956). *J. Bact.* **72**, 646.
 Harrington, A. A. & Kallio, R. E. (1960). *Canad. J. Microbiol.* **6**, 1.
 Jayasuriya, G. C. N. (1955). *J. gen. Microbiol.* **12**, 419.
 Kallio, R. E. & Harrington, A. A. (1960). *J. Bact.* **80**, 321.
 Kaneda, T. & Roxburgh, J. M. (1959a). *Canad. J. Microbiol.* **5**, 87.
 Kaneda, T. & Roxburgh, J. M. (1959b). *Canad. J. Microbiol.* **5**, 187.
 Kaneda, T. & Roxburgh, J. M. (1959c). *Biochim. biophys. Acta*, **33**, 106.
 Karrer, P. & Jucker, E. (1950). *Carotenoids*. London: Elsevier Publishing Co. Inc.
 Kornberg, H. L. (1958). *Biochem. J.* **68**, 535.
 Leadbetter, E. & Foster, J. W. (1958). *Arch. Mikrobiol.* **30**, 91.
 Quayle, J. R. & Keech, D. B. (1959a). *Biochem. J.* **72**, 623.
 Quayle, J. R. & Keech, D. B. (1959b). *Biochem. J.* **72**, 631.
 Quayle, J. R. & Peel, D. (1960). *Biochem. J.* **76**, 3P.
 Slepecky, R. A. & Doetsch, R. N. (1954). *Bact. Proc.* **p. 44**.
 Takamiya, A. (1953). *J. Biochem., Tokyo*, **40**, 415.
 van Niel, C. B. (1954). *Annu. Rev. Microbiol.* **8**, 105.
 Weaver, R. H., Samuels, T. C. & Sherago, M. (1938). *J. Bact.* **35**, 59.
 Williamson, D. H. & Wilkinson, J. F. (1958). *J. gen. Microbiol.* **19**, 198.