JOURNAL OF BACTERIOLOGY
Vol. 88, No. 4, p. 1065–1070 October, 1964

IDENTITY OF THE PINK-PIGMENTED METHANOL-OXIDIZING BACTERIA AS *VIBRIO EXTORQUENS*

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Received for publication 13 April 1964

ABSTRACT

STOCKS, PETER K. (Louisiana State University, Baton Rouge), AND C. S. MCCLESKEY. Identity of the pink-pigmented methanol-oxidizing bacteria as *Vibrio extorquens*. J. Bacteriol. **88**:1065–1070. 1964.—Pink-pigmented bacteria isolated from enrichment cultures of methane oxidizers were found to possess similar morphological, cultural, and physiological characteristics. All the strains utilized methanol, formate, oxalate, succinate, glycerol, and benzene as sole carbon sources; methanol, formate, and glycerol afforded best growth. Most strains utilized fructose and ribose; other carbohydrates tested were not available as carbon and energy sources. There was strain variation in the use of hexane, heptane, *n*-propanol, *n*-butanol, acetate, and propionate. Methane, ethane, *n*-propane, and *n*-butane were not utilized. Our isolates, and *Pseudomonas methanica* of Harrington and Kallio (not the methane-dependent *P. methanica* of Dworkin and Foster), *Pseudomonas* AM1 of Peele and Quayle, *Pseudomonas* PRL-W4 of Kaneda and Roxburgh, and *Protaminobacter ruber* den Dooren de Jong are nearly identical with *Vibrio extorquens* (Bassalik) Bhat and Barker, and should be considered the same species.

The crude bacterial cultures which oxidize methane almost invariably include pink-pigmented bacteria which oxidize methanol as their sole carbon and energy source. We isolated many of these organisms from methane enrichment cultures, and also found them frequently as contaminants gaining entrance to pure cultures of methane-oxidizing bacteria. Our isolates resemble the pink-pigmented methanol-oxidizing bacteria described in the literature as *Vibrio* (*Pseudomonas*, *Bacillus*) *extorquens* (Bassalik, 1913; Janota, 1950; Bhat and Barker, 1948); *Pseudomonas* AM1 (Peele and Quayle, 1961); *Pseudomonas* PRL-W4 (Kaneda and Roxburgh, 1959); *Protaminobacter ruber* (den Dooren de Jong, 1927; Breed, Murray, and Smith, 1957); and *P. methanica* (Harrington and Kallio, 1960). The *P. methanica* cultures of Dworkin and Foster (1956), Leadbetter and Foster (1958, 1960), and Johnson and Temple (1962) appear to differ from the other pink-pigmented methanol oxidizers, in that they were described as capable of oxidizing methane. Pink-pigmented bacteria in methane cultures were encountered by Brown (1958) and Holmes (1962), but apparently were not tested for their ability to utilize methanol.

It was desirable to bring all these similar organisms together for a careful evaluation of their relationships, as suggested by Quayle (1961).

MATERIALS AND METHODS

The cultures employed included our own isolates from crude cultures of methane oxidizers, and some strains of previously described species from the following sources: AS-P, methane-oxidizing enrichment culture from oil field soil; PBG-P, methane-oxidizing enrichment culture from coal mine waters in West Germany; Mc, a contaminant in a culture of *Methanomonas methanooxidans* Brown and Strawinski; Dor, a methane-oxidizing culture obtained from Doris Holmes (1962); JT, from the methane-oxidizing culture of Johnson and Temple (1962); Rum, methane-oxidizing enrichment culture from the rumen of a cow; Sew, methane-oxidizing enrichment from sewage; S, T, U, V, contaminants of methane-oxidizing cultures; Kal, *P. methanica* obtained from R. E. Kallio; VX, *V. extorquens* (Bassalik) Bhat and Barker from J. R. Quayle; P. rub., *Protaminobacter ruber* den Dooren de Jong from J. R. Quayle; and AM1, *Pseudomonas* AM1 from J. R. Quayle. Isolations were made from mineral salts-agar plates, streaked with methane-oxidizing cultures, and incubated in an atmosphere of methane, oxygen, and carbon dioxide in a 65:30:5 mixture (Stocks and McCleskey, 1964).

Determination of carbon and nitrogen sources suitable for growth were made in the mineral salts

medium of Brown (1958): KNO_3, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $K_2HPO_4 \cdot 3H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 0.05 g; and NaCl, 0.2 g per liter of distilled water. Mineral salts-agar was prepared by adding 15 g of agar (Difco) per liter. Tryptone Glucose Extract (TGE) agar (Difco) and other common bacteriological media were employed in tests for purity of cultures and in some of the biochemical tests. For purposes of comparison, the mineral salts media of Mevius (1953) and Jayasuriya (1955) were employed in some tests. Carbon sources were routinely added to give concentrations of 0.1 and 0.5%; in some cases, where inhibition was feared, concentrations of 0.01% were also used.

Inocula for testing carbon and nitrogen sources consisted of washed cells which had been grown in mineral salts-methanol (0.5%) medium on a rotary shaker at 28 C for 48 hr. Tests for the utilization of substrates were also incubated at 28 C, and observations were made at intervals for 21 days. Cultures showing no growth after 21 days were recorded as negative.

Antisera were prepared in rabbits. Washed methanol-grown cells were suspended in formolized physiological saline at a density corresponding to the McFarland no. 3 standard, and seven 1-ml injections were made intraperitoneally at intervals of 2 to 3 days. For the agglutination tests, the cell suspensions were adjusted to the density of the McFarland no. 2 standard. Some of the strains could not be tested because of spontaneous clumping. Tube agglutination tests were incubated at 50 C in a water bath for 24 hr; readings were made at 2 hr, 24 hr, and finally after an additional day at 5 C.

RESULTS AND DISCUSSION

Morphologically and culturally, all the strains were similar; all were gram-negative, motile, nonsporeforming rods. The cells successfully stained or observed with an electron microscope showed a single polar flagellum (Fig. 1). Capsules were not observed. All strains produced coral-pink to red colonies, with pigmentation deeper at the center and increasing with age. On mineral salts-methanol-agar, colonies were small (about 0.2 mm after 5 days), circular, entire, and butyrous (Fig. 2); on TGE agar, the colonies were larger, about 1 mm in 5 days. On nutrient agar, the colonies became tenacious so that the entire colony could be removed with the needle. An interesting fea-

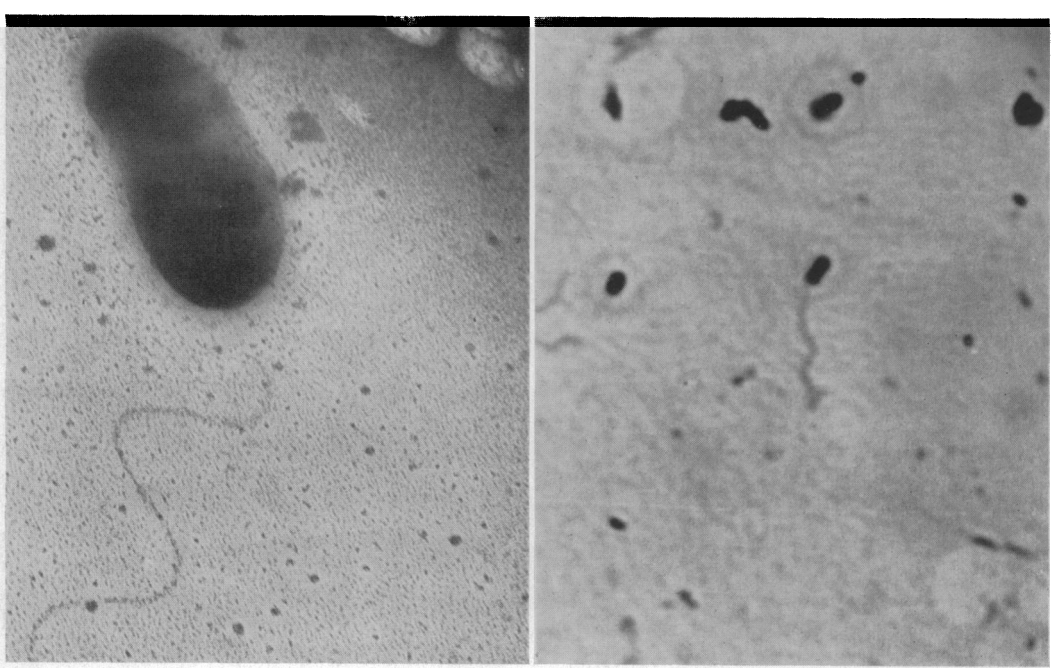

FIG. 1. *Flagellation of the pink-pigmented methanol-oxidizing bacteria. Left: electron micrograph of Pseudomonas methanica Kallio strain; right: strain Rumen, stained by the Bailey method.*

ture of these organisms was the presence in the cell of large fat bodies (Fig. 3), as described by Harrington and Kallio (1960). Cells of all the strains contained polyhydroxybutyrate, as determined by the method of Law and Slepecky (1961).

All strains showed abundant growth in mineral salts broth with methanol, glycerol, or oxalate as the source of carbon and energy; all except *Protaminobacter ruber* grew almost as well on formate. All the organisms grew moderately on benzene. No other carbon sources tested were satisfactory for all the strains (Table 1). None of the cultures grew on methane, ethane, *n*-propane, or *n*-butane; only the strain of Johnson and Temple (1962) grew on hexane and heptane. Growth was relatively abundant in ordinary peptone media without the addition of other carbon sources.

As nitrogen source, all the strains grew with peptone, ammonium salts, nitrate, alanine, asparagine, aspartic acid, uric acid, and leucine. Other organic nitrogen compounds afforded growth of some strains (Table 2).

Biochemically, the cultures were essentially identical; all reduced nitrate to nitrite, formed catalase, and were negative to all the other tests (gelatinase, caseinase, tyrosinase, amylase, H_2S, indole, acetoin, cellulase, deoxyribonuclease, urease, lipase, and oxidase). All were sensitive to chlortetracycline (6 μg/ml) and resistant to streptomycin (80 μg/ml). All the strains grew in 4% and some in 10% methanol. All grew in the presence of 1% NaCl and were inhibited by 2%.

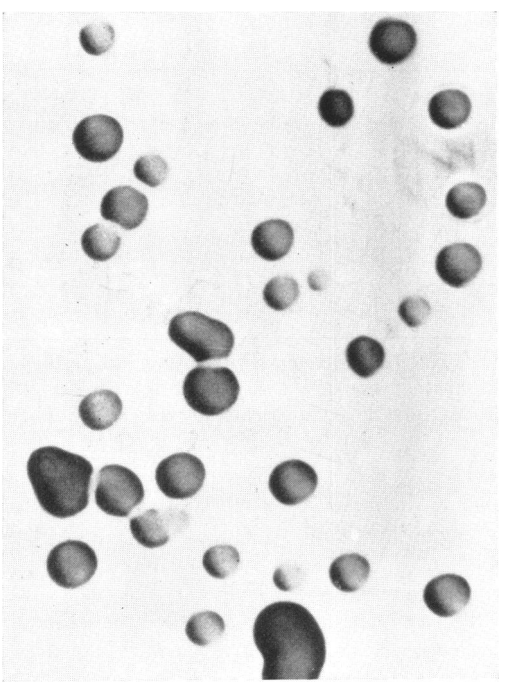

FIG. 2. *Colonies of pink-pigmented methanol oxidizer on mineral salts-methanol-agar. Incubated 5 days at 28 C.* × *9.*

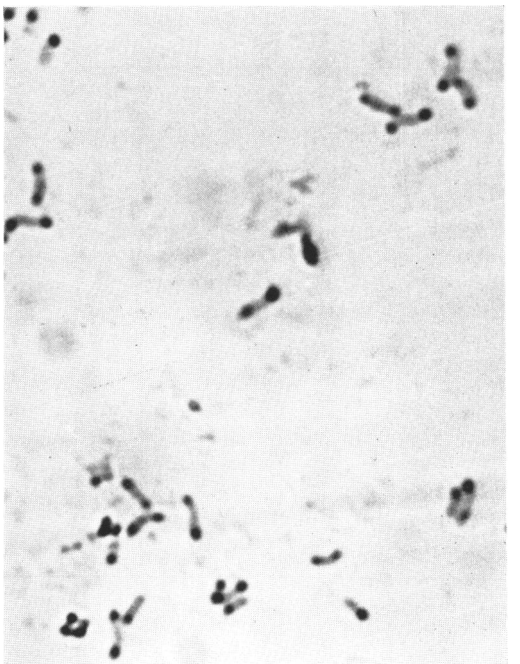

FIG. 3. *Fat bodies in the pink-pigmented organism, stained with Sudan black.*

Serological studies indicated that the pink-pigmented methanol oxidizers are not antigenically homogeneous (Table 3), but *V. extorquens* was agglutinated at low titer with antiserum prepared against strain AS-P, and *P. ruber* was agglutinated by antisera for strain PBG-P and strain S. The lack of homogeneity is indicated by the fact that no single antiserum agglutinated more than three heterologous strains, and one strain (AM1) was not agglutinated by any of the five antisera. It is significant, however, that strains from widely separated sources showed antigenic relationships.

There can be no doubt that the pink-pigmented methanol-oxidizing bacteria included in this study comprise a group of organisms similar in morpho-

TABLE 1. *Growth of pink-pigmented methanol-oxidizing bacteria on various carbon substrates with $(NH_4)_2SO_4$ as nitrogen source**

Substrate	AS-P	PBG-P	Mc	DOR	JT	Rum	Sew	S	T	U	V	KAL	VX	P. rub.	AM1
n-Hexane	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Heptane	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Propanol	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+
Butanol	+	−	−	−	+	+	+	+	+	+	−	−	+	+	
Acetate	+	+	−	±	+	+	+	±	+	+	+	+	+	−	+
Propionate	−	−	−	−	+	−	−	+	+	−	+	−	−	−	−
Citrate	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+
Fructose	+	−	−	−	++	++	++	++	++	++	+	++	+	+	+
Ribose	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+
Xylose	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Alanine	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−
Valine	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−
Leucine	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Aspartic acid	+	+	+	+	+	+	+	+	±	−	−	+	+	+	+
Asparagine	+	+	+	+	+	+	+		−	−	−	+	+	+	+

* All the strains grew with methanol, benzene, formate, oxalate, succinate, fumarate, and glycerol as sole carbon source. None of the strains grew with methane, ethane, *n*-propane, *n*-butane, octane, toluene, paraffin, butyrate, lactate, sorbitol, mannitol, inositol, glucose, galactose, mannose, sucrose, lactose, maltose, sorbose, glycine, methionine, cysteine, or cystine. Symbols: ++ indicates good growth; +, moderate growth; ±, doubtful growth; −, no growth.

TABLE 2. *Availability of various nitrogen sources for growth of methanol-oxidizing bacteria with methanol as carbon source**

Nitrogen source	AS-P	PBG-P	Mc	DOR	JT	Rum	Sew	S	T	U	V	KAL	VX	P. rub.	AM1
Glycine	+	+	+	+	+	±	±	++	+	++	+	+	+	+	+
Valine	±	±	±	±	±	+	+	+	+	+	+	+	+	±	+
Phenylalanine	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−
Urea	−	−	+	+	+	+	+	+	+	+	+	+	+	+	±
Methionine	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Cysteine	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Cystine	−	−	−	±		+	+	−	−	−	−	−	+	−	±

* All the strains utilized $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, KNO_3, peptone, hippurate, alanine, asparagine, aspartic, leucine, and uric acid as sole nitrogen sources. None of the strains grew with KNO_2 or CH_3NH_2 as nitrogen sources. Symbols: ++ indicates good growth; +, moderate growth; ±, doubtful growth; −, no growth.

logical, cultural, and physiological characteristics. Also, the ecology of the organisms is suggestive of their relationship. Their common occurrence in enrichment cultures of methane oxidizers is no doubt a consequence of their preference for methanol as a carbon and energy source, analogous to the yeast-*Acetobacter* relationship. In retrospect, we may confidently assume that the pink pellicle reported by Söhngen (1906) in the first description of methane-oxidizing bacteria was due to pink-pigmented methanol organisms. During the intervening years, other investigators of bacteria which oxidize methane have been confronted and sometimes confused by these pink-pigmented organisms.

To be distinguished from the methanol oxidizers employed in this study, which do not attack methane, but merely grow in the favorable environment created by the oxidation of methane, is the bacterium described by Dworkin and Foster (1956) and Leadbetter and Foster (1958, 1960) as pink-pigmented and methane-dependent. We

TABLE 3. *Serological reactions*

Antigen	AS-P	PBG-P	S	T	KAL
AS-P	640	—	—	—	—
PBG-P	—	5,120	—	—	—
Me	—	—	320	—	—
S	—	—	5,120	—	80
T	—	—	—	5,120	80
KAL	—	—	320	—	1,280
Vibrio extorquens	160	—	—	—	—
Protaminobacter ruber	—	20	1,280	—	—
AM1	—	—	—	—	—

have not encountered this organism among our isolates. Also, the *Pseudomonas methanica* of Dworkin and Foster (1956), which oxidizes methane, is not to be confused with the *P. methanica* of Harrington and Kallio (1960), which does not oxidize methane and is a typical pink-pigmented methanol oxidizer, as shown in this investigation.

The large number of pink-pigmented bacteria isolated from methane-oxidizing enrichment cultures by Brown (1958) and Holmes (1962) were no doubt methanol oxidizers, although they were not tested in this substrate. These organisms, because of their metabiotic relationship with the methane oxidizer, pose a problem in the isolation of the latter organisms in pure culture.

We feel that the pink-pigmented methanol oxidizers included in this study are sufficiently alike to be considered one species. The published description of *Pseudomonas* PRL-W4 of Kaneda and Roxburgh (1959) indicates that it also is a strain of the same species.

The first published description of an organism of this group was apparently that of *B. extorquens* by Bassalik (1913). This species was placed in the genus *Pseudomonas* Migula by Janota (1950), and in the genus *Vibrio* Muller by Breed et al. (1957), as suggested by Bhat and Barker (1948). According to *Bergey's Manual* (Breed et al., 1957), the borderline between the genera *Pseudomonas* and *Vibrio* is not sharp, since curved rods sometimes occur in species normally composed of straight rods. The pink-pigmented methanol oxidizers are not clearly vibrios, although some curved rods are observed. The movement of motile cells in a culture is vibriolike in some cases but not in all. Although these organisms are not typical vibrios, we suggest that they be considered as strains of *V. extorquens* (Bassalik) (Bhat and Barker, 1948) until a more appropriate generic affiliation for this species is found by further investigations.

ACKNOWLEDGMENTS

We express thanks to J. R. Quayle for providing us with cultures of *Pseudomonas* AM1, *Protoaminobacter ruber*, and *Vibrio extorquens*, and to R. E. Kallio for his strain of *Pseudomonas methanica*.

This work was supported in part by contract 1575(01) from the Office of Naval Research.

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