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A Methane-Dependent Coccus, with Notes on Classification and Nomenclature of Obligate, Methane-Utilizing Bacteria

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ABSTRACT

FOSTER, J. W. (The University of Texas, Austin), AND RICHARD H. DAVIS. A methane-dependent coccus, with notes on classification and nomenclature of obligate, methane-utilizing bacteria. J. Bacteriol. **91**:1924–1931. 1966.—A new coccus-shaped bacterium capable of aerobic growth at the expense of methane or methanol in a mineral salts medium is described. The organism did not grow at the expense of any of the conventional substrates or homologous hydrocarbons tested. It is gram-negative, nonmotile, and thermotolerant. It grows well at 50 C, optimally at 37 C, but does not grow at 55 C. The cells are encapsulated and have a characteristic diplococcoid arrangement. Washed, "resting-cell" suspensions oxidized certain primary alcohols and short-chain alkanes, an example of "nongrowth oxidation." Of the methane-C utilized, 86% was "fixed" in organic form; the remainder was oxidized to CO_2. The guanine-cytosine content of the extracted deoxyribonucleic acid was 62.5%. Obligate methane-utilizing bacteria are considered as "one-carbon" organisms rather than hydrocarbon utilizers. The assimilation pathway in the obligate methane-methanol bacteria is different from that in the facultative methanol utilizers. Nomenclatural problems arising from the use of the prefix "*Methano-*" to denote both bacteria that oxidize methane and bacteria that produce methane are discussed. The obligate, one-carbon, methane-methanol bacteria are considered as "methyl" utilizers, and the prefix "*Methylo-*" is suggested as a solution to the problem of generic cognomens. "*Methylococcus capsulatus*" gen. n., sp. n. is the name proposed for the new methane coccus.

One of the best documented cases of organisms obligatorily dependent on a particular compound is the bacterium *Pseudomonas methanica* (9), which consumes methane for growth. Of numerous other organic substances tested, none excepting methanol—an intermediate in the biological oxidation of methane—can support growth of that organism (for description of a similar organism, *see* 50).

Another case of obligate methane-methanol dependence is now reported. An additional feature of the new bacterium is its ability to develop at 50 C.

MATERIALS AND METHODS

Bacteriological procedure. The following mineral salts solution was used in the media: $NaNO_3$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; KCl, 0.04 g; $CaCl_2$, 0.015 g; Na_2HPO_4, 0.21 g; NaH_2PO_4, 0.09 g; $FeSO_4 \cdot 7H_2O$, 1.0 mg; $CuSO_4 \cdot 5H_2O$, 5 μg; H_3BO_4, 10 μg; $MnSO_4 \cdot 5H_2O$, 10 μg; $ZnSO_4 \cdot 7H_2O$, 70 μg; MoO_3, 10 μg; deionized water, 1 liter. For a solid medium, 2% water-washed agar (Difco) was added. Gas atmospheres of the appropriate compositions were provided in closed desiccators, or in flasks closed with rubber stoppers fitted with glass tubing through which gassing could be done. The desiccator was partially evacuated, and the air was replaced with the desired gas to a final pressure of 1 atm. Petri plates and cotton-plugged flasks or tubes were incubated in the desiccators at appropriate temperatures. Kinetic-growth studies were conducted in 250-ml flasks with test tube side arms fitting a Klett-Summerson photoelectric colorimeter. Physiological studies were ordinarily done with cultures grown in a rotary-shaker incubator at the desired temperature. Direct cell counts made with the use of a Petroff-Hauser chamber were used to establish a relation between turbidity and cell numbers. The hydrocarbons were of 99 mole % purity and were purchased from

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Phillips Petroleum Co., Bartlesville, Okla. Other specialized procedures are described in connection with the particular experiments.

RESULTS

Isolation. Enrichment cultures were incubated with continuous shaking at 50 and 55 C. Inocula used at each temperature were: (i) sludge from the Austin municipal sewage plant; (ii) oil-soaked soil from Port Arthur, Tex.; (iii) flower-bed soil from the University of Texas campus; (iv) local pond mud; and (v) water from Gila Hot Springs, N. Mex. A 1-ml or 1-g amount of these materials was added to 25 ml of mineral salts medium in flasks; 50% air-50% methane (v/v) was added. Inoculated controls were incubated in air without methane. After 2 weeks, the only flask exhibiting conspicuous bacterial growth was that inoculated with sewage and incubated with methane at 50 C. Microscopically, a coccus was prominent, along with various rod-shaped bacteria. One drop was transferred to fresh mineral salts-methane medium and incubated at 50 C. Growth equivalent to approximately 200 Klett units developed in 3 days. An inoculated control containing no methane developed no turbidity. Streaks were made from the secondary enrichment onto mineral salts agar. In 10 to 14 days, colonies had developed in the methane-containing atmosphere, but not in air without methane.

Description of methane utilizer. Almost all of the colonies appeared to be similar. They were approximately 1 mm in diameter, colorless to ivory, smooth, rounded, and even-bordered. Microscopic examination revealed them to be composed of nonmotile, gram-negative cocci, approximately 1.0 μ in diameter. Rodlike forms were not observed. Most of the cells had a distinctively diplococcoid arrangement. As judged by the India ink stain, they were encapsulated (Fig. 1). In spite of this, the supernatant liquid from a centrifuged culture reacted negatively for polysaccharide in the anthrone test (38). However, when the culture was first boiled, and then centrifuged, carbohydrate was readily detected. Evidently, the capsular polysaccharide is practically insoluble in water under growth conditions at 50 C but is dissolved at 100 C. It is possible that the hot water also extracted intracellular polysaccharide. In comparable tests with *P. methanica*, capsules could not be demonstrated by the India ink procedure, even though the methane culture supernatant liquid reacted positively for soluble carbohydrate. It appears that the coccus synthesizes polysaccharide which, because of its insolubility, is retained around the cell, whereas the pseudomonad synthesizes a soluble polysaccharide which, in consequence, is noncapsular.

Physiological studies. After 3 days of incubation in liquid, mineral salts medium, the coccus attained populations of more than 10^9 cells per milliliter. Colony formation on mineral salts agar was much slower; it took 10 to 14 days to attain a diameter of approximately 1 mm. Growth was slightly stimulated by small amounts of complex extracts or Casamino Acids. Ornithine, glycine, or lysine also stimulated growth somewhat, whereas serine, threonine, and histidine were toxic at relatively low concentrations. The amino acids did not noticeably influence the colonial or microscopic morphology. As sole sources of nitrogen, ammonium ion and Casamino Acids supported growth, but nitrate was far superior on an equivalent N basis.

The coccus was cultivated at different temperatures under uniform shaking conditions, and the generation times were computed from Petroff-Hauser counts made during the logarithmic phase of growth. The organism grew well at 30, 37 (*see* Fig. 2 for growth curve), and 50 C, but there were marked differences in growth rates. At 30 C the generation time was 13 hr; at 37 C, 3.5 to 5.0 hr; at 50 C, 6.5 hr. Thus, this organism is thermotolerant, not thermophilic. This is also

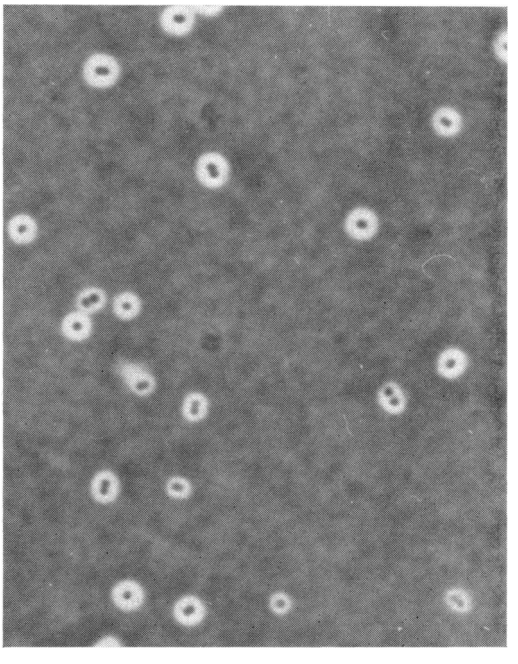

FIG. 1. *India ink wet mounts showing capsules and the diplococcoid tendency of the methane-utilizing bacterium.* × *900.*

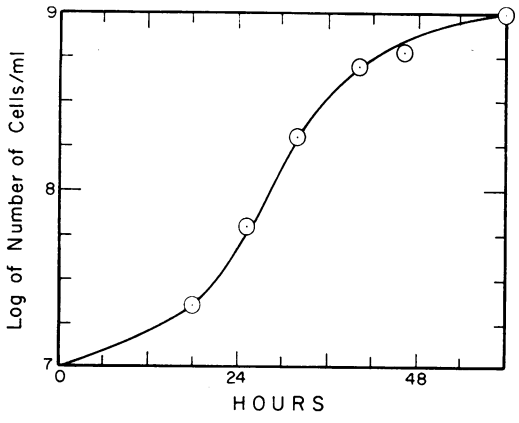

FIG. 2. *Growth of methane coccus at 37 C. A 250-ml side-arm flask containing 50 ml of mineral salts medium was fastened in a 10-liter desiccator which was placed on a shaking machine. At the indicated times, the desiccator was opened, a sample was removed for cell counts, the desiccator was regassed, and the incubation with shaking was resumed.*

indicated by its inability to adapt to growth at 55 C.

Only about 3% of the dry weight of cells from the stationary phase was lipid-extractable with ether (15). Poly-β-hydroxybutyrate could not be detected by the method of Law and Slepecky (29).

The results of manometric experiments with washed cells of the coccus which had been grown at 50 C and suspended in phosphate buffer (67 mM, *p*H 7.0) resembled the results with *P. methanica* (9). At 37 C, methane and methanol were oxidized rapidly and at similar rates. As with the pseudomonad, the O_2 uptake ceased almost abruptly at a point corresponding to 1 mole of O_2 consumed per mole of methanol supplied. Dworkin and Foster (9) showed that *P. methanica* produced formic acid according to the equation: $CH_3OH + O_2 \rightarrow HCOOH + H_2O$. The observed molar relation between O_2 consumed and methanol supplied suggests that the coccus, under these conditions, likewise carries out a stoichiometric oxidation of primary alcohols to the corresponding fatty acids.

The oxidation rates for methane were determined manometrically at 50, 55, and 60 C, by use of cells that had been grown at 37 and 50 C. The suspensions were adjusted to the same Klett reading. Regardless of the temperature at which the cells had grown, there was no oxidation of methane at 55 or 60 C. There was, however, vigorous oxidation at 50 C. Cells that had been grown at 37 C were decidedly more active in oxidizing methane at 50 C than the cells that had been grown at 50 C. For example, the pressure change (representing combined CH_4 and O_2 uptake) after 23 min was −265 mm for the 37 C cells and −131 mm for the 50 C cells (endogenous uptake deducted).

Ethane and propane were oxidized, but at rates considerably lower than those for methane. Glucose, sodium citrate, sodium α-ketoglutarate, sodium succinate, and D-ribose were oxidized at a negligible rate, if at all.

From extensive growth and oxidation experiments, which included a considerable number of conventional substrates, it was apparent that some compounds can be oxidized, but that only methane or methanol support growth at a rate that is significant. Of the compounds which were oxidized but which failed to substitute for methane in growth, short-chain alkanes and primary alcohols were the most noteworthy. This situation is comparable to that previously described as "nongrowth oxidation" (12, 30, 32, 33, 39).

Carbon balance. Analysis of the gas phase was done in an apparatus devised by Updegraff and Huckabay (53), with the use of *n*-hexadecane to absorb the methane. Organic carbon was determined by a modification of the van Slyke-Folch wet combustion procedure (45), and the carbon content of the samples was calculated according to Neish (38). The bulk of the methane consumed in a growth culture of the methane coccus was fixed as organic carbon (Table 1). Approximately 16% of the methane used was converted to CO_2. The rest was distributed between cellular carbon (centrifugable) and soluble carbon in a ratio of approximately 3:1. The conversion of methane

TABLE 1. *Carbon balance in a growth culture of the methane coccus**

Determination	Amt
Methane consumed	26.3 mmoles
O_2 consumed	15.2 mmoles
CO_2 produced	4.6 mmoles
Organic C synthesized	
Cellular†	18.3 matoms
Soluble C‡	6.8 matoms

* A 4-liter suction flask containing 500 ml of mineral salts solution was gassed with air containing 30% methane. After inoculation, the culture was incubated at 37 C on a mechanical shaker for 3 days. Samples were removed before and after incubation for gas and organic C analyses. Appropriate corrections were made for the gas pressure at the end of the experiment, and H_2SO_4 was added to release CO_2 in the solution.

† Centrifugable.

‡ This was mainly polysaccharide.

carbon to cellular carbon was high, namely, 69.7% (*see* 9).

Deoxyribonucleic acid (DNA) base composition. The shared physiological properties of the methane coccus and *P. methanica* raised the possibility that they might be genetically related, in spite of their morphological differences (*see* 34, 52).

Manley Mandel (Univ. Texas M. D. Anderson Hospital and Tumor Institute, Houston) analyzed the methane utilizers for the guanine-cytosine (GC) content of the DNA by means of the CsCl buoyant density method, with bacteriophage SP8 DNA as an internal reference (46). The values found were 62.5 and 52.1% GC for the methane coccus and *P. methanica*, respectively. The difference denotes genetic dissimilarity.

DISCUSSION

In view of the great diversity of organisms known to attack hydrocarbons (12, 13), the paucity of information on thermophilic forms may be more a consequence of neglect than of nonexistence. The new coccus is not a thermophile in the strict sense (14), but its ability to grow well at 50 C distinguishes it from hitherto described methane utilizers.

The curious methane dependence in two bacteria as diverse as *P. methanica* (cf. *Methanomonas methanooxidans*, 50) and the coccus described herein may yet be found in other morphological groups.

Classification of the coccus. Since sugars are not fermented, the family *Lactobacillaceae* is excluded. According to *Bergey's Manual* (3), three other families include organisms with spherical cells, namely, *Micrococcaceae*, *Neisseriaceae*, and *Brucellaceae* (genus *Moraxella*). The family *Micrococcaceae*, although basically comprised of gram-positive spheres, does contain organisms (genus *Micrococcus*) which decolorize in the staining procedures so readily that they appear gram-negative. However, the paired-cell character is not found in this genus. The pairing of the cells (with flattened adjacent sides), their size, and their gram-negativity would fit the genera *Neisseria* and *Moraxella*. Some genetic homology between these two has been suggested from reciprocal transformation studies (5). Murray and Truant (37) distinguish the two groups by the mode of division of the cells; *Neisseria* exhibits two planes of division, and *Moraxella*, one plane. Also, all known species of *Neisseria* are parasitic, but not all species of *Moraxella* (1); we presume the methane-oxidizing coccus is saprophytic. On these grounds, as well as the oxidative characteristics and ability to grow in nutritionally simple media, an affinity with the moraxellas may be indicated. We have not studied the methane coccus for the gliding motility found in some moraxellas (41).

Considering the rather arbitrary distinctions and the unclear lines of demarcation and perhaps overlapping in the classification currently employed for organisms of this general morphological group, and pending the obtaining of more definite characteristics of the coccus, it would be premature to commit the organism to one or another of the above genera. Furthermore, as seen below, it is unnecessary. In any case, the GC content of the methane coccus deviates widely from those of other cocci, including species of *Moraxella*, *Diplococcus*, *Micrococcus*, and *Neisseria* (6, 36, 51), with the exception of *Sarcina lutea* (64%). This criterion alone may be indicative of phylogenetic unrelatedness and, therefore, taxonomic nonidentity.

Other cocci capable of using hydrocarbons are known (17, 48), and gram-negative spherical cells have been found to attack long- and short-chain alkanes (10, 11); however, there is no indication that any of these organisms attack methane.

Nomenclatural problems with the prefix "Methano-." Organisms morphologically identical with common bacteria but possessing a unique mode of metabolism, i.e., the ability to *produce* methane, were assigned to new genera by Kluyver and van Niel (25) and Barker (2). These were created by prefixing "*Methano-*" to the appropriate morphological genus (forming *Methanobacterium*, *Methanobacillus*, *Methanococcus*, *Methanosarcina*); ". . . . the old form genera and new physiological groups could be combined, so that satisfactorily circumscribed genera would ensue" (54). The obligate, methane-oxidizing coccus can be treated according to this principle, the generic name thereby constructed from a suitable physiological prefix to, for example, "-coccus." We will return to this later.

In 1909, Orla-Jensen (40) first used the prefix "*Methano-*" to denote a genus of methane-oxidizing bacteria. Years later, Kluyver and van Niel's (25) choice of the same prefix to signify methane-producing bacteria has led to a certain amount of nomenclatural ambiguity, especially if one subscribes to the principle of a descriptive cognomen based on merging physiological and morphological features. Various methane producers and users have been described, each qualifying for the "*Methano-*" prefix. Carried to its logical conclusion, identical generic names would be constructed for morphologically similar organisms, one oxidizing methane aerobi-

cally, the other producing it anaerobically. The prefix "*Methano-*" thus stands incongruously for two opposing types of metabolisms. Although priority belongs to methane users, application of the prefix has become entrenched on a much wider scale in respect to methane producers. So far, the connotation of methane utilization is limited to the pseudomonads, i.e., *Pseudomonas* (*Methanomonas*).

Nomenclature of obligate, methane-utilizing pseudomonads. Dworkin and Foster (9; *see also* 26) made an elaborate case for relinquishing the cognomen *Methanomonas* in favor of *Pseudomonas*. It becomes increasingly clear, however, that obligateness itself is of taxonomic value, especially since a unique biochemical basis for it (discussed later) is now recognized. This, together with recent analytical data regarding GC contents, clearly sets the obligate methane utilizers apart from the classical pseudomonads (7, 35); accordingly, they warrant a corresponding taxon. If physiological and morphological features are merged nomenclaturally, the logical cognomen would be "*Methanopseudomonas*." This name has the advantage of denoting the clear-cut homology between Söhngen's (47) specialized organism and other monopolarly flagellated pseudomonads, and of conforming to a desirable nomenclatural pattern. Nevertheless, despite the sacrifice of those advantages it entails and (as seen in the following) despite the nomenclatural ambiguity its adoption engenders, reversion to Orla-Jensen's (40) truncation, "*Methanomonas*," is mandatory according to principles 8 and 9 and rule 23 in the *International Code of Nomenclature of Bacteria and Viruses* (20).

Semantics and rules notwithstanding, stemming from the common prefix "*Methano-*," the homology implied for the methane-oxidizing bacteria and the methanogenic bacteria is taxonomically awkward, if not actually confusing. As stated previously in another connection (10), two qualities any classification system should supply are consistency and an accurate means of referring to a taxonomic group. A revision of our concept of the nature of *P. methanica* may provide a way of circumnavigating the artificiality and inconsistency imposed by the rules in this situation.

Nature of "one-carbon" bacteria. Taxonomists will be aided in the resolution of this problem by an appreciation of the distinctiveness of the obligate methane utilizers. These bacteria cannot use other hydrocarbons for growth and, in consequence, are basically different from those which can (13). Their one-carbon predilection is further emphasized by the evidence that the overwhelming majority of hydrocarbon-utilizing bacteria are incapable of growth on methane (13). Seen in this light, the obligates are not primarily hydrocarbon-utilizing organisms but, rather, "one-carbon" organisms.

Methanol-utilizing bacteria. In natural habitats, the obligate methane-methanol bacteria have to compete with nonobligate (facultative) methanol users for the available one-carbon alcohol. However, bacteria recovered from methanol enrichment cultures do not necessarily grow at the expense of methane (16). Also, some 20 strains of pink, facultative methanol users obtained from Hayward (18) failed to use methane (J. J. Perry, *unpublished data*; *see also* 22). Similar results have been reported for 15 strains of facultative methanol-utilizing bacteria reclassified as *Vibrio extorquens* (49) and for several strains of the budding bacterium *Hyphomicrobium* (19). Thus, bacteria isolated by selective culture techniques as methanol utilizers invariably have been incapable of growth at the expense of methane. Accordingly, bacteria which use methane may be expected to be distinct from methanol isolates, as indeed they are in terms of substrate versatility.

Assimilation pathways in facultative versus obligate "one-carbon" bacteria. Total biosynthesis at the expense of methane or methanol (or other one-carbon compounds) presupposes assimilation pathways different from those of practically all other heterotrophic organisms. In such a facultative *Pseudomonas* organism, serine is the first stable product of methanol assimilation (23), and enzymes for its biosynthesis via the hydroxymethylation of glycine were demonstrated in a similar strain (28). The methanol was believed to be oxidized (4, 9) and assimilated as formaldehyde or formate, or both. A similar conclusion was reached in independent studies with another facultative methanol utilizer, *H. vulgare* (8, 27).

All methylamine utilizers thus far examined, including red, yellow, and nonpigmented forms, also will grow at the expense of methanol (E. R. Leadbetter, *personal communication*), but these are facultative, "one-carbon" bacteria (42). Quayle (42) considered the methylamine-utilizing *H. vulgare* to be "1-C specific," but Hirsch and Conti (19) showed that numerous methylamine hyphomicrobia were facultative and that they possessed the hydroxymethylation pathway.

The obligate one-carbon organisms thus far studied have an assimilation pattern distinctively different from that of the facultatives. *P. methanica* uses methane and methanol by a pathway in which the hexose allulose phosphate is a key intermediate, formed apparently via a

novel pentose phosphate cycle (21, 24). Enzymes of the allulose phosphate pathway have recently been demonstrated in the methane coccus (Kemp and Quayle, *personal communication*). This mechanism was absent in a facultative methanol *Pseudomonas*.

The allulose phosphate pathway may be a general mechanism for methane assimilation. In facultative bacteria capable of growth at the expense of either methane or other multicarbon substrates for carbon and energy, utilization of methane may also prove to occur via a constitutive or (more probable) an inducible allulose phosphate pathway. In any event, obligateness distinguishes *P. methanica* and the methane coccus from other (facultative) methane utilizers.

A unified view can now be considered for the assimilation of one-carbon substrates (including methylamine, 31), with the exception of CO_2 and a special case where formate is assimilated via CO_2 (43, 44).

Classification. The main problem is merely one of distinguishing the obligate from the facultative one-carbon organisms by substrate testing and determining the assimilation route. Such organisms may be assorted as described in Table 2.

It would be useful to have a physiological prefix denoting the capacity to utilize methane or methanol, yet automatically delimiting the obligates from the facultative methanol bacteria, which do not utilize methane (i.e., group II, Table 2).

A solution is offered by attributing the distinguishing substrate feature of the obligates to an ability to utilize methyl groups rather than the hydrocarbon methane per se. The taxonomic quandary discussed vanishes if the "methyl" character of these organisms in respect to substrates is acknowledged as important enough to supersede the "methane" character. Methane and methanol are, respectively, the hydride and the hydroxylated forms of the methyl group. For new organisms like the coccus described here, the prefix "*Methylo-*" is the obvious solution. Pending more critical disclosures of its morphological homology, the tentative genus name *Methylococcus* (gen. n.) is satisfactory. To denote the characteristic feature revealed in India ink mounts, the species epithet *capsulatus* (sp. n.) is proposed. A formal description of the new species follows. Cells are nonmotile, gram-negative cocci, approximately 1 μ in diameter, with a diplococcoid arrangement common amidst some single cells and irregular small clusters. The cells are markedly encapsulated when viewed in India ink mounts. The organism is an obligate aerobe capable of growth in a mineral salts medium containing methane or methanol as the only carbon source. Colonies on mineral salts-methane agar are slow in developing (ca. 7 to 10 days), eventually attaining a diameter of approximately 1 mm. Growth is faster in liquid cultures incubated with continuous agitation. The colonies are colorless to ivory, smooth, rounded, and even-bordered. Growth occurs best near neutrality and at temperatures ranging from 20 to 50 C, with optimal growth at 37 C. The organism cannot utilize other hydrocarbons or other conventional substrates as sole sources of carbon for growth, and is obligately dependent on reduced 1-carbon substrates, which are assimilated via formaldehyde and the allulose phosphate pathway. Preformed cells can oxidize certain short-chain alkanes. The organism was first isolated from sewage sludge.

Similarly, the use of "*Methylomonas*" in place of *Pseudomonas* (*methanica*) and *Methanomonas* would have the merit of precision, homology, and elimination of ambiguity stemming from the names of the methanogenic bacteria.

TABLE 2. *Assortment of "one-carbon" bacteria*

Property	Obligates	Facultatives	
		Group I[a]	Group II[b]
Growth on methane	+	+	−
Growth on methanol	+	+	+
Growth on multicarbon compounds	−	+	+
One-carbon assimilation pathway	Allulose-phosphate[c]	Serine[d]	Serine[d]

[a] Comprised of some versatile hydrocarbon-utilizing organisms, including some not well characterized and *Mycobacterium methanicum* and *M. flavum* var. *methanicum* (13). The serine pathway indicated for this group is a postulation.

[b] Group II includes strains of *Pseudomonas* (23, 28), several bacteria reclassified as *Vibrio extorquens* (49), and *Hyphomicrobium vulgare* (8, 27).

[c] From formaldehyde via modified pentose phosphate cycle (24).

[d] From hydroxymethylation of glycine (23, 27).

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