Methylophaga marina gen. nov., sp. nov. and Methylophaga thalassica sp. nov., Marine Methylotrophs  

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After enrichment in a medium containing seawater and methanol, 42 methylotrophic strains were isolated. All of these strains were gram-negative, strictly aerobic, motile, rod-shaped organisms that required vitamin B₁₂. None grew on methane or on complex nutrient media supplemented or not supplemented with NaCl. All but 2 strains grew on methanol, methylamine, and fructose. 17 strains grew on dimethylamine, and 10 strains grew on trimethylamine. Fructose was the only multicarbon compound tested that was used as a growth substrate. All 11 strains tested used the ribulose monophosphate pathway of carbon assimilation. Depending on the strain, methylamine was oxidized either through a methylamine dehydrogenase or through a methylglutamate dehydrogenase. The mean guanine-plus-cytosine content of 33 strains was 43 mol%. Based on deoxyribonucleic acid-deoxyribonucleic acid hybridization, two related groups were identified among 11 strains examined. We propose a new genus, Methylophaga, with two species, Methylophaga marina (type species) and Methylophaga thalassica. There was no significant deoxyribonucleic acid hybridization between Methylophaga and the terrestrial obligate methane utilisers tested. The type strains of M. marina and M. thalassica are strains ATCC 35842 (= NCMB 2244) and ATCC 33146 (= NCMB 2163), respectively.

A number of terrestrial strains of gram-negative, obligate or restricted facultative methylotrophs which use the ribulose monophosphate pathway (RuMP) for carbon source utilization and are unable to utilize methane have been described previously (for a review, see reference 2). Some of these strains did not receive generic designations. Others have been referred to as Methylophilus (1, 14, 26, 30, 34, 46, 50), "Methylophilus" (33, 51), or Methylobacillus (59). Recently, the name Methylophilus was revived for a category of terrestrial methylotrophs (28, 56). For the category of methylotrophs that are unable to utilize methane is even more confused. The strains described in 1978 by Yamamoto et al. (57) were later assigned to two different genera on the basis of the ability of the strains to utilize fructose as a growth substrate. The fructose-utilizing strains were designated "Methylophilus methylotrophus." The purposes of the present work were to (i) characterize phenotypically 42 new marine methylotrophic isolates, (ii) delineate species by DNA-DNA hybridization, and (iii) compare our strains with available marine or terrestrial methylotrophic strains representative of previously published studies. The outcome of this study was the description and designation of a new genus, Methylophaga, with two new species, Methylophaga marina and Methylophaga thalassica.

MATERIALS AND METHODS

Bacterial strains. The newly isolated strains used for taxonomic characterization are listed in Table 1. The following strains were received from the American Type Culture Collection: Methylobacillus glycogenes ATCC 29475 (T = type strain); "Alteromonas thalassamethanolica" ATCC 33145; and "Methylophilus thalassica" ATCC 33146. The following strains were received from the National Collection of Industrial and Marine Bacteria: "Methylophilus thalassica" NCMB 2163 (= ATCC 33146); "Methylophilus thalassica" NCMB 2162; Alteromonas macleodii NCMB 1963; and "Methylophilus methylotrophus" NCIB 10515. Methylotrophic strains AM1 and M27, Pseudomonas sp. strain C, and "Methylophilus methanolica" were received from I. Goldberg, Hebrew University, Hadassah Medical School, Jerusalem, Israel. Pseudomonas fluorescens CIP 7325 and Escherichia coli K-12 CIP 54117 were obtained from the Collection de l’Institut Pasteur, Paris, France.

Media. For enrichments and isolations, natural seawater was used as a mineral base. The seawater was kept in the dark for 2 weeks, filtered through 0.45-μm membranes (Millipore Corp., Bedford, Mass.), and autoclaved for 30 min at 120°C. The final seawater medium contained (per liter) 0.14 g of KH₂PO₄, 2 g of NH₄Cl, 60 mg of ferric ammonium citrate, and 2 g of Bis-Tris (Sigma Chemical Co., St. Louis, Mo.). These ingredients were added separately as a 50× stock mixture (solution A) adjusted to pH 7.4 and autoclaved at 120°C. Methanol was added to a concentration
of 0.3% (vol/vol). For solid media, 16 g of Bacto-Agar (Difco Laboratories, Detroit, Mich.) per liter was added. Subcultures were made in artificial seawater (ASW) medium composed of ASW supplemented with solution A and vitamin B12 (1 μg/liter). ASW contained (per liter) 24 g of NaCl, 3 g of MgCl2·6H2O, 2 g of MgSO4·7H2O, 0.5 g of KCl, 1 g of CaCl2·2H2O, and 0.5 g of Bis-Tris. The trace element solution of Pfennig (36) was added to a concentration of 1% (vol/vol). The final pH was 7.4. For solid media, a sterile suspensions of muds taken from zones exposed at low tide on the coasts of France and Iraq. Suspensions of muds (5 g in 40 ml of seawater) were centrifuged for 10 min at 200 × g, and 6 ml of each supernatant was filtered on Millipore membranes.

**Growth substrates and growth conditions.** All of the growth substrates were used as 20 or 10% (vol/vol) solutions in water, sterilized by filtration, and added aseptically to ASW medium at a final concentration of 0.3% for methanol and fructose and 0.2% for other substrates, unless otherwise stated. Inocula were overnight cultures in ASW-methanol medium. After several successive reisolations of single colonies, the presence of heterotrophic colonies was checked by spreading samples of a well-grown liquid culture in methanol medium onto plates of Difco nutrient agar containing 1% NaCl and plates of Difco marine agar 2216.

**Electron microscopy.** Bacterial cells grown in ASW-methanol or ASW-fructose medium were centrifuged and suspended in 100 mM diethylbarbituric acid–sodium acetate buffer (pH 6.8) containing 2.5% glutaraldehyde (45). After 1 h, the samples were concentrated in 2.5% agar (55), post-fixed overnight as described by Ryter and Kellenberger (45), dehydrated with acetone, and embedded in Epon. Thin sections were stained with lead citrate. Polysaccharides were revealed by two different methods, phosphotungstic acid staining and silver proteinate treatment. Phosphotungstic acid staining was achieved by using the method of Rambourg (37), as modified by Roland et al. (42), and was used after the procedure of Frehel and Ryter (19), which was slightly modified by extending to 20 min the phosphotungstic acid treatment, followed by thorough rinsing (twice for 10 s and twice for 10 min). The detailed procedure of the silver proteinate treatment of Thiery (52) has been described previously (18). Phosphotungstic acid staining preferentially reveals peptidoglycan, and silver proteinate staining shows the polymers containing α-glycol bonds, such as glycogen and teichoic acid (18, 44, 52).

**Estimation of lysis.** A culture in ASW-methanol medium corresponding to 80 to 100 mg (dry weight) of bacterial cells was centrifuged at 4ºC during the early stationary phase. The pellet was suspended in 25 ml of cold 0.5 M NaCl, and the total protein content (T) of this suspension was estimated by the Biuret method as modified for whole cells (23). This cell suspension was centrifuged at 12,000 × g for 30 min, and the pellet was suspended in 8 ml of cold distilled water and was

<table>
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<th>Strain*</th>
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<th>Enzymes of monomethylamine oxidation</th>
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<tr>
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<td>New strain 1d</td>
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* ATCC, American Type Culture Collection; NCMB, National Collection of Marine Bacteria.
* See text.
* (W), Weak reaction.

**TABLE 1. Utilization of fructose and methylated amines by marine methylotrophic bacteria**
kept on ice for 1 h. The lysed suspension was centrifuged at 12,000 \times g for 30 min, and the protein contents in the residual pellet (P) and the supernatant (S) were estimated after concentration with trichloroacetic acid (final concentration, 12.5%). The percentage of lysis was calculated as follows: S/T \times 100. We verified that T = S + P and that the supernatant after NaCl treatment did not contain an appreciable amount of protein. The same procedure was applied to the following gram-negative methylotrophic strains: *Pseudomonas* sp. strain AM1 (35), *Pseudomonas* sp. strain M27 (3), *Pseudomonas* sp. strain C (40), and *Methylomonas methanolica* (1) cultivated in M3 medium (40) with methanol as the growth substrate; *P. fluorescens* CIP 7325 and *E. coli* K-12 cultivated in mineral medium containing 0.2% glucose.

**DNA studies.** Bacterial DNA was prepared by the method of Brenner et al. (9). The method used to determine guanine-plus-cytosine contents has been described previously (27). The procedures used for in vitro labeling of DNA with tritium-labeled nucleotides and for hybridization experiments (S1 nuclease-trichloroacetic acid method) were those of Grimont et al. (22). The temperature at which 50% of the reassociated DNA became hydrolyzable by S1 nuclease (T_m) was determined by the method of Cossa et al. (12). The value called ΔT_m was the difference between the T_m of homologous reaction and the T_m of the heterologous reaction. Such a value is an estimate of divergence between two DNAs (7).

**Enzyme assays.** For all assays except the methylamine dehydrogenase and methylglutamate dehydrogenase assays bacteria were grown in ASW-methanol medium; for the enzymatic assays were performed at 28°C. The specific activity of this enzyme. Methylglutamate dehydrogenase (EC 1.4.99.3) was tested by the method of Eady and Large (15). Methylglutamate dehydrogenase (EC 1.5.99.5) was assayed by the method of Hersh et al. (16), and hydroxypyruvate reductase (EC 1.1.1.49) was assayed as described by Ferenci et al. (17). Controls without dehydrogenase substrates (NAD and glucose 6-phosphate, 6-phosphogluconate, or formate) were used to reveal the bands of "nothing dehydrogenases." Such bands (see Fig. 3) were identified as methanol dehydrogenases by the following procedure: after electrophoresis, a region of the gel which was supposed to contain the enzyme was cut out, and the piece of gel (ca. 1 cm \times 3 mm) was eluted at room temperature in a cuvette containing 2 ml of 15 mM NaCl in 100 mM tris (hydroxymethyl)aminomethane hydrochloride (pH 9). After 1 h the

Electrophoresis.** Electrophoresis on 7.5% polyacrylamide gels was performed by the method of Laemmli (31), except that sodium dodecyl sulfate and mercaptoethanol were omitted and the crude extract samples were not heated. Bromophenol blue was used as the front marker. Electrophoresis was carried out at 4°C at a constant current of 25 mA. After electrophoresis, dehydrogenases were revealed by treatment for 30 min at room temperature with the following mixtures (total volume, 50 ml): for glucose 6-phosphate dehydrogenase, a solution containing 50 mM tris (hydroxymethyl)aminomethane hydrochloride (pH 7.8), 5 mM MgCl₂, 10 mM glucose 6-phosphate (disodium salt; Sigma), 1 mM NAD, 2 mg of phenazine methosulfate, and 20 mg of Nitro Blue Tetrazolium; for 6-phosphogluconate dehydrogenase, a solution containing 50 mM glycylglycine (pH 7.5), 5 mM MgCl₂, 10 mM 6-phosphogluconate (trisodium salt; Sigma), 1 mM NAD, 2 mg of phenazine methosulfate, and 20 mg of Nitro Blue Tetrazolium; and for formate dehydrogenase, a solution containing 50 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 8.4), 1 mM NAD, 10 mM sodium formate, 2 mg of phenazine methosulfate, and 20 mg of Nitro Blue Tetrazolium. The distance of migration of each enzyme was measured relative to the migration of the front marker (√R).

Controls without dehydrogenase substrates (NAD and glucose 6-phosphate, 6-phosphogluconate, or formate) were used to reveal the bands of "nothing dehydrogenases." Such bands (see Fig. 3) were identified as methanol dehydrogenases by the following procedure: after electrophoresis, a region of the gel which was supposed to contain the enzyme according to its √R value was cut out, and the piece of gel (ca. 6 by 3 mm) was eluted at room temperature in a cuvette containing 2 ml of 15 mM NaCl in 100 mM tris (hydroxymethyl)aminomethane hydrochloride (pH 9). After 1 h the
piece of gel was removed, and the methanol dehydrogenase assay was performed directly in the cuvette as described above.

RESULTS

Six samples of algae and 33 samples of marine mud were examined. All of the enrichment cultures gave visible growth in ASW-methanol medium after 3 to 6 days. On primary isolations, after 5 to 6 days of incubation, colonies of two different sizes were present; there were numerous punctiform colonies which were not viable after resolation and larger, lightly pink-pigmented colonies (diameter, 0.5 to 2 mm) of various morphological types, which became more uniform after resolation. Eventually, 42 strains exhibiting good growth on seawater-methanol medium were kept for further examination.

General characteristics. All of the organisms isolated were gram-negative small rods (ca. 1 by 0.2 μm). Some of the isolates appeared coccoid in older (3- to 5-day) cultures. Under phase-contrast microscopy no cytoplasmic granules were observed. In ASW-methanol medium, all of the strains except two were motile by a single polar flagellum. The colonies were translucent, pale pink, and round with glossy surfaces and entire edges; they were 1 to 2 mm in diameter after 4 days at 30°C on solid ASW-methanol medium. No growth occurred on nutrient agar (Difco) supplemented with 10 g of NaCl per liter and on marine agar 2216 (Difco). The temperature range for growth extended from 10 to 40°C, with optimal growth between 30 and 37°C. All strains required vitamin B₁₂ for growth, were strict aerobes, produced catalase, oxidase, and urease, and did not reduce nitrate. The divalent cations Mg²⁺ and Ca²⁺ at concentrations as low as 50 mM added to ASW-methanol medium after 3 to 6 days. On primary isolation, only 13 of 42 strains exhibited growth on marine agar 2216 (Difco) supplemented with 0.1% tryptone and 0.1% yeast extract. A survey of 147 compounds, including amino acids, organic acids, sugars, and polyalcohols distributed in API-CH, API-AO, and API- AA strips, was conducted with two strains, 92b and 222°. This survey showed that fructose is the only non-C₄ compound that is utilized by these strains. In fact, all of the strains (except two) utilized fructose as a sole carbon and energy source in ASW medium. However, depending on the strain, growth conditions, such as aeration and temperature, may be critical; some strains which failed to utilize fructose at 30°C in shaken culture were able to do so at room temperature without agitation.

Autotrophic growth was not observed. Methane (20%) and formate (0.1 and 0.01%) did not support growth. Methyamine was a suitable carbon and nitrogen source for all of the strains. This compound was dissimilated either directly through an NAD-independent methylamine dehydrogenase or through the cycle which involves a methylglutamate dehydrogenase (24). Dimethyamine was utilized by 17 strains, and trimethylamine was utilized by 10 of the 42 new isolates. All of the strains which utilized trimethylamine also utilized dimethyamine. Details of these results for 11 strains are shown in Table 1.

Methanol dehydrogenase, hexulose phosphate synthase, and glucose-6-phosphate dehydrogenase were detected in crude extracts of the strains listed in Table 1. Hydroxypyruvate reductase activity was not found. Hexulose phosphate synthase, a key enzyme of the RuMP pathway, was present at a high specific activity; for example, 3,400 U of hexulose phosphate synthase was detected in crude extracts of strain 92b, along with 728 U of methanol dehydrogenase and 261 U of glucose-6-phosphate dehydrogenase.

DNA-DNA hybridizations. For DNA-DNA hybridization experiments 14 strains were selected, including 3 strains described by Yamamoto et al. (38) and 11 of our isolates.

FIG. 2. Thin sections of strain 92b. (a and b) Lead citrate staining. The periplasmic space appears as a thick layer with a granular structure (detail in b) located between the outer membrane (OM) and the cytoplasmic membrane (CM). (a) Bar = 0.1 μm. (b) Bar = 0.04 μm. (c and d) Silver proteinate staining. A thin, dark, granulated layer of polysaccharide overlays the whole cell surface. Clusters of glycogen-like material are present in the cytoplasm. (c) Bar = 0.1 μm. (d) Bar = 0.2 μm. (e and f) Phosphotungstic acid staining. The peptidoglycan was revealed by this treatment as a faintly stained layer (arrowhead) in the periplasmic space. (e) Bar = 0.1 μm. (f) Bar = 0.2 μm.
Two DNA hybridization groups were distinguished (Table 2). The first group contained the suggested type strain of "A. thalassomethanolica" and two strains of "Methylophilus thalassica," including the suggested type strain (strain ATCC 33146) (fructose positive) and strain 2162 (fructose negative). Four newly isolated strains also belonged to this group. Levels of DNA homology of 66 to 75% among the members of this group suggested some genetic diversity at the subspecific level. The ΔTm values (less than 6°C), confirmed that they belong to a single genospecies (7). A second hybridization group, composed of seven newly isolated strains, was more homogeneous based on their high levels of DNA hybridization.

The levels of DNA relatedness between the two groups (from 20 to 36%) are compatible with their assignment to the same genus (8).

DNAs from representative marine strains belonging to the two hybridization groups mentioned above did not hybridize consistently with DNAs from two terrestrial strains, the type strain of Methylobacillus glycogenes and "Methylphilus methylotrophus" NCIB 10515. These two strains are representative of the two DNA hybridization groups described by Byrom (10).

**Electrophoretic migration of enzymes.** The data in Table 1 show that none of the phenotypic characters reported supports without exception the separation into two species obtained by DNA-DNA hybridization. The migration patterns of the dehydrogenases for glucose-6-phosphate, 6-phosphogluconate, methanol, and formate were studied. Without substrate many bands of nothing dehydrogenases were revealed (Fig. 3A). The most intense bands (Rf, 0.19 to 0.30) were identified as methanol dehydrogenases; in each hybridization group, two patterns of migration of this enzyme were recognized. Only the migration distances of the NAD-dependent glucose-6-phosphate dehydrogenases correlated well with the hybridization groups; their Rf values were 0.33 and 0.35 for the first and second hybridization groups, respectively (Fig. 3B). As for the NAD-dependent 6-phosphogluconate and formate dehydrogenases, the variations observed in the migration distances within each hybridization group did not allow any clear grouping.

**DISCUSSION**

The bacteria described by Yamamoto et al. (57), strain FMD of Strand and Listrom (49), and our strains were isolated from marine environments as diverse as the shores of Japan, Norway, South California, Iraq, and France (English Channel and Atlantic Ocean). All are gram-negative rod-shaped obligate methylotrophs (except some which utilize fructose) that use the RuMP pathway and are unable to grow on methane. All of the strains which we examined belong to a single taxonomic group, as shown in Table 2. Methylotrophic bacteria with the same characteristics are well known in terrestrial environments. We did not find in marine samples any of the gram-negative pink-pigmented facultative methylotrophs (21) which use the serine pathway and are so frequently found in terrestrial environments. Until now the only known marine methylotrophs which use the serine pathway belong to the genus Syphomicibium (5, 25).

The seacoast location where our samples were taken raises the question of the marine nature of these bacteria. An interesting property of marine bacteria is cell lysis in distilled water after NaCl treatment (13, 38). Rayman and MacLeod have shown that the sparse peptidoglycan of marine bacteria is strengthened by divalent cation bridges (38). The displacement of divalent cations by monovalent cations weakens the cell wall, making it sensitive to osmotic shock. This behavior toward osmotic shock is found only in marine bacteria, not

**TABLE 2. DNA reassociation among marine methylotrophic bacteria and reference strains**

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<th>Source of unlabeled DNA</th>
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<td>71</td>
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<tr>
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in terrestrial bacteria. The low peptidoglycan content, which was demonstrated by electron microscopy and by chemical estimation, is also consistent with the marine nature of our isolates (38).

The question raised by Strand and Listrom (49) about the taxonomic similarity of marine and terrestrial strains of gram-negative rods which use the RuMP pathway received a clear answer in our study (Table 2); no significant hybridization was detected between representative strains of the two groups.

In order to identify their isolates, Yamamoto et al. (57) considered the utilization of fructose a key character; they assigned the fructose-positive strains to a heterotrophic genus, *Alteromonas*, and the fructose-negative strains to "*Methylocalmonas*," which is considered a genus of strictly methylotrophic bacteria. This opinion has been criticized by Anthony (2), who remarked that the important point was that all of these bacteria are unable to grow on ordinary nutrient medium and so should be considered similar to obligate methylotrophs. Moreover, the ability to grow on fructose does not always give clear-cut results. Cultures of the type strain of "*Methylocalmonas thalassica*" received from the American Type Culture Collection (strain ATCC 33146) and from the National Collection of Industrial and Marine Bacteria (strain NCMB 2163) grew perfectly on fructose at 30°C in shaken cultures. This was confirmed by P. N. Green, National Collection of Industrial and Marine Bacteria (personal communication). Another strain of "*Methylocalmonas thalassica*" (strain NCMB 2162) failed repeatedly to grow on fructose; however, this strain belongs to the DNA-DNA hybridization group containing the suggested type strain of "*A. thalassomethanolica*".

The genus *Alteromonas* was created by Baumann et al. (6) as a group of gram-negative, heterotrophic, rod-shaped bacteria of marine origin. The lack of homology between *A. macleodii* (the type species of the genus *Alteromonas*) and "*A. thalassomethanolica*" was expected since Van Landschoot and De Ley (54) demonstrated that the latter species did not show any relatedness with any of the four DNA-ribosomal ribonucleic acid hybridization groups that they recognized among *Alteromonas* species.

The name *Methylocalmonas* was initially proposed by Foster and Davis (17) to replace "*Methanomonas*" in order to avoid confusion between methane-producing and methane-oxidizing microorganisms. Recently, Whittenbury and Krieg (56) assigned to the genus *Methylocalmonas* the strict methanotrophs that use the RuMP pathway and possess a type I intracellular membrane. Romanovskaya et al. (43) used the same definition for a formal description of genera and species of methane-utilizing bacteria. As a consequence, the name *Methylocalmonas* should not be used for those methylotrophic bacteria which do not have intracytoplasmic membranes and do not grow in methane. This view has been confirmed by the recent validation of the revived name *Methylocalmonas* (28).

Formal proposals for the creation of a new genus with two species (based on the data in Table 2) are given below. Nevertheless, the real taxonomic problem with the obligate methylotrophs, whatever their marine or terrestrial origins, is determination of relevant phenotypic characters that are
easy to handle and correlate with genomic similarities (such as those detected by DNA-DNA hybridization). For this purpose, the relative electrophoretic migration distances of isofunctional enzymes have been used with success in other groups of bacteria (29). In terrestrial methylotrophic bacteria, results have been reported by Urakami and Komagata (53), but these results were not correlated with DNA hybridization studies. Figure 3 shows that for several enzymes, variable patterns of migration were found within each hybridization group. Such variations could be used for division at the subspecific level.

Description of Methyllophaga gen. nov. Methyllophaga gen. nov. (M.et.hyl.o.pha'ga. French n. methyl, methyl radical; Gr. n. metha fermented beverage; Gr. n. lyle wood; Gr. v. phagein to eat; Methyllophaga methyl eating) cells are gram-negative rods that are motile by means of single polar flagella. Very thick (20- to 30-nm) periplasmic space. Cells can be broken by osmotic shock after washing with 0.5 M NaCl. Strictly aerobic, moderately halophilic, and auxotrophic for vitamin B12. Strains do not grow on peptone-yeast extract medium containing (or not containing) NaCl. Except fructose, the only growth substrates that are used are C1 compounds, such as methyl alcohol and methylamine, which are dissimilated by the RuMP pathway. Do not grow on methane.

The range of guanine-plus-cytosine contents of the DNAs is 38 to 46 mol%.

Isolated from marine environments.

The type species is Methyllophaga marina.

Description of Methyllophaga marina sp. nov. Methyllophaga marina sp. nov. (ma.r'i'na. L. fem. n. mare sea; L. fem. adj. marina of the sea) cells are short straight rods 0.2 by 1 μm. Colonies on ASW-methanol agar are pale pink. Catalase and oxidase positive; reduction of nitrate negative. Optimum temperature, 30 to 37°C. Grows at 10 and 40°C. Na+ and Mg2+ are required for growth. Grows on fructose and methylamine. Some strains grow on dimethylamine; none grows on trimethylamine. Methylamine is dissimilated by an N-methylamine dehydrogenase or through a methylglutamate dehydrogenase. On polyacrylamide gels, the electrophoretic migration distances relative to bromophenol blue values) are 0.33 for glucose-6-phosphate dehydrogenase and 0.35, and the guanine-plus-cytosine content of the DNA is 44 mol%. The level of hybridization with DNA of type species Methyllophaga marina is around 30 to 35% (S1 nuclease method).

The type strain is strain ATCC 33146 (= NCMB 2163).

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Literature Cited

20. Gavini, F., D. Izard, H. Leclerc, M. Desmonceaux, and J. P.


