Methylophaga marina gen. nov., sp. nov. and *Methylophaga thalassica* sp. nov., Marine Methylophages

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After enrichment in a medium containing seawater and methanol, 42 methylophagous 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 methanol utilizers 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 methylophages 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 *Methylomonas* (1, 14, 26, 30, 34, 46, 50), "*Methylophilus*" (33, 51), or *Methylobacillus* (59). Recently, the name *Methylomonas* was revived for a category of strict methane utilizers (28, 56). For the category of bacteria defined above, only the genus *Methylobacillus* is mentioned on the Approved Lists of Bacterial Names (48), with one species, *Methylobacillus glycogenes*. These terrestrial methylophages are characterized by deoxyribonucleic acid (DNA) guanine-plus-cytosine contents which range from 50 to 53 mol%. A DNA relatedness study (10) of terrestrial methylophages identified two groups, corresponding to *Methylobacillus glycogenes* and "*Methylophilus methylophilus*."

The taxonomic position of gram-negative, marine methylophages that are unable to utilize methane is even more confused. The strains described in 1978 by Yamamoto et al. (58) 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 "*Alteromonas thalassomethanolica*," whereas the strains that were claimed to be unable to utilize fructose were designated "*Methylomonas thalassica*" (57).

Recently, Strand and Listrom (49) described a similar marine obligate methylophage (strain FMD) which utilizes methanol through the RuMP pathway. Strand and Listrom did not name their strain, but suggested that it might belong to the genus "*Methylophilus*." The guanine-plus-cytosine content of the DNA of marine methylophages is about 45 mol% (49, 57).

The purposes of the present work were to (i) characterize phenotypically 42 new marine methylophagous isolates, (ii) delineate species by DNA-DNA hybridization, and (iii) compare our strains with available marine or terrestrial methylophagous 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 (T = type strain); "*Alteromonas thalassomethanolica*" ATCC 33145; and "*Methylomonas thalassica*" ATCC 33146^T. The following strains were received from the National Collection of Industrial and Marine Bacteria: "*Methylomonas thalassica*" NCMB 2163^T (= ATCC 33146^T); "*Methylomonas thalassica*" NCMB 2162; *Alteromonas macleodii* NCMB 1963^T; and "*Methylophilus methylophilus*" NCIB 10515. Methylophagous strains AM1 and M27, *Pseudomonas* sp. strain C, and "*Methylomonas 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- μ m 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 $\times$ stock mixture (solution A) adjusted to pH 7.4 and autoclaved at 120°C. Methanol was added to a concentration

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TABLE 1. Utilization of fructose and methylated amines by marine methylotrophic bacteria

Strain ^a	Utilization of:				Enzymes of monomethylamine oxidation	
	Fructose	Monomethylamine	Dimethylamine	Trimethylamine	Methylamine dehydrogenase	N-Methylglutamate dehydrogenase
<i>Methylophaga thalassica</i>						
" <i>Alteromonas thalassomethanolica</i> " ATCC 33145	+	+	+	+	+	—
" <i>Methylomonas thalassica</i> " ATCC 33146 ^T	+ ^b	+	—	—	—	+ (W) ^c
" <i>Methylomonas thalassica</i> " NCMB 2162	—	+	+	+	—	+
New strain 310	+	+	+	+	—	+
New strain 150	+	+	+	+	—	+
New strain 2b	+	+	+	—	—	+
New strain 1d	+	+	+	+	—	+
<i>Methylophaga marina</i>						
222 ^T	+	+	—	—	+	—
92b	+	+	—	—	—	+
81	+	+	—	—	+	—
290	+	+	—	—	—	+
232	+	+	—	—	+	—
312	+	+	—	—	+	—
173	+	+	+	—	+	—

^a ATCC, American Type Culture Collection; NCMB, National Collection of Marine Bacteria.

^b See text.

^c (W), Weak reaction.

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 solution of Bacto-Agar (Difco) in water (32 g/liter) was added to an equal volume of double-strength ASW medium.

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 that were centrifuged and suspended in ASW. The volume of inoculum was calculated to obtain a starting absorbance at 600 nm of 0.05 (ca. 10⁸ cells per ml). Growth with methylated amines was tested without NH₄Cl in the medium. The methylated amines used were hydrochloride salts obtained from Sigma. Formic acid was used as a sodium salt provided by Sigma. All of the cultures were incubated at 30°C with shaking. Carbon source utilization tests were done with API-CH, API-AO, and API-AA strips (API System, La Balme-les-Grottes, France) (20) with the inocula described above. Growth on methane was tested in liquid cultures and on solid cultures incubated under an atmosphere containing 20% methane and 80% air, and autotrophic growth was tested under an atmosphere containing 85% H₂, 10% CO₂, and 5% oxygen.

Enrichments and isolations. For enrichment seawater medium was inoculated with pieces of red or green algae or with marine 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

(pore size, 0.45 µm), which were then washed with sterile seawater before transfer in enrichment medium. When growth was visible (after 2 to 6 days at 30°C under slow agitation), the cultures were streaked onto the same 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 4°C 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

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 Crosa 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 methylamine dehydrogenase and methylglutamate dehydrogenase assays methylamine was used as the growth substrate. Freshly harvested cells were suspended in cold 50 mM potassium phosphate buffer (pH 7.2), passed twice through a French pressure cell at 16,000 lb/in², and centrifuged for 2 h at $200,000 \times g$. The supernatant was used for all assays unless stated otherwise. Protein concentrations were determined by using Coomassie blue G-250 (47) and bovine serum albumin as the standard. Methanol dehydrogenase (EC.1.99.8) was assayed by the method of Anthony and Zatman (4) (modified by adding 10 mM KCN to the cuvette). Hexulose phosphate synthase was assayed by the method of Ferenci et al. (16), and hydroxypyruvate reductase (EC.1.1.1.49) was assayed as described by Large and Quayle (32). For glucose-6-phosphate dehydrogenase (EC 1.1.1.49), activity was followed at 340 nm in an assay mixture containing 25 μ mol of tris(hydroxymethyl)aminomethane hydrochloride (pH 7.8), 5 μ mol of MgCl₂, 10 μ mol of D-glucose 6-phosphate, and 2 μ mol of oxidized nicotinamide adenine dinucleotide in a total volume of 1 ml. The presence of nicotinamide adenine dinucleotide (NAD)-independent methylamine 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. (24), using N-methyl-L-glutamate as the substrate. The addition of 2.5 mM KCN was often necessary to reveal the activity of this enzyme. Methylglutamate dehydrogenase was found in part in the supernatant and in part in the pellet in varying proportions depending on the extract. All of the enzymatic assays were performed at 28°C. The specific activities were expressed as nanomoles of substrate consumed or of product formed per minute per milligram of protein.

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_f).

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_f 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 NH₄Cl in 100 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 9). After 1 h the

FIG. 1. Percentage of lysis of marine methylotrophic bacteria following osmotic shock. The protein contents were assayed in the supernatants and are expressed as percentages of the total cellular protein content. The cross-hatched bar indicates the results obtained with terrestrial strains used as controls (see text).

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 reisolation and larger, lightly pink-pigmented colonies (diameter, 0.5 to 2 mm) of various morphological types, which became more uniform after reisolation. 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 guanine-plus-cytosine content was 38 to 46 mol% (calculated for 33 strains).

Osmotic sensitivity and salt requirement. When transferred into distilled water after NaCl treatment, the cells of all of our marine isolates broke open and liberated their protein contents, as shown in Fig. 1. Terrestrial heterotrophic or methylotrophic bacteria of various types (RuMP or serine pathway) did not solubilize their protein contents after such an osmotic shock. The NaCl treatment was a necessary step before osmotic shock to obtain good lysis. Direct transfer of the cell pellet in distilled water and repeated washing in water did not produce appreciable lysis. The divalent cations Mg²⁺ and Ca²⁺ at concentrations as low as 50 mM added to the 0.5 M NaCl washing solution prevented lysis.

For all of the strains tested, optimal growth was obtained with minimum concentrations of 20 mM Na⁺ and 5 mM Mg²⁺ in otherwise normal ASW-methanol medium. This moderate halophily is comparable to the salt requirement of many marine bacteria (39).

Structural characteristics. The main morphological feature of thin sections (Fig. 2a and b) was the thickness (20 to 30 nm) of the granular layer between the cytoplasmic and outer membranes. Such thickness is unusual for a gram-negative organism (11). This characteristic was observed in four different strains, whatever the nature of the growth substrate (methanol or fructose). A similar thickness of cell envelopes was observed by Rokem et al. (41) in terrestrial methylo-

trophs which use the RuMP pathway. We have not observed the protrusions of the outer membrane reported by these authors in *Pseudomonas* sp. strain C.

Silver proteinate staining revealed a thin layer of polysaccharide along the outer face of the outside membrane (also lightly stained with phosphotungstic acid) and large clusters of glycogen-like material localized in the cytoplasm (Fig. 2c and d). The exact nature of this reserve material has not been determined. Peptidoglycan, as revealed by phosphotungstic acid, appeared as a weak dark brown deposit in the periplasmic layer (Fig. 2e and f). A cell wall preparation of strain 92b extracted and analyzed by O. Kandler, Botanische Institut der Universität München, Munich, Federal Republic of Germany, confirmed the very low content of peptidoglycan observed by electron microscopy. The peptidoglycan contained *meso*-diaminopimelic acid and muramic acid together with alanine and glutamic acid. These findings fit with the assumption that strain 92b contains the same murein as the other gram-negative organisms (O. Kandler, personal communication).

Nutritional and biochemical characteristics. None of the 42 isolates grew on glucose, acetate, ethanol, pyruvate, lactate, succinate, or citrate in ASW medium or in ASW medium 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, strains 92b and 222^T. This survey showed that fructose is the only non-C₁ 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. Methylamine 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). Dimethylamine 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 dimethylamine. 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. (58) 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 .

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

Source of unlabeled DNA	Source of labeled DNA					
	"A. thalassomethanolicus" ATCC 33145		"Methylomonas thalassica" ATCC 33146 ^T ; % relative binding	Strain 222 ^T ; % relative binding	Strain 92b	
	% Relative binding	ΔT_m (°C)			% Relative binding	ΔT_m (°C)
<i>Methylophaga thalassica</i>						
" <i>Alteromonas thalassomethanolicus</i> " ATCC 33145	100	0	61	31	26	
" <i>Methylomonas thalassica</i> " ATCC 33146 ^T	66	6	100	32	29	
" <i>Methylomonas thalassica</i> " NCMB 2162	71	5.5	71	25	25	
New strain 310	74		74	32	25	
New strain 150	70	5.9		31	25	
New strain 2b	71			34	31	
New strain 1d	75			37	31	
<i>Methylophaga marina</i>						
222 ^T	36	>16	31	100	94	0
92b	21		24	114	100	0
81	25			81	84	1.29
290	23			82	84	
232	30			86	83	2.04
312	32	10.7		103	103	
173	20		33	86	85	2.17
<i>Alteromonas macleodii</i> NCMB 1963 ^T	8		1		2	
<i>Methylobacillus glycogenes</i> ATCC 29475 ^T	2		1		3	
" <i>Methylophilus methylotrophus</i> " NCIB 10515	8		6		5	

Two DNA hybridization groups were distinguished (Table 2). The first group contained the suggested type strain of "*A. thalassomethanolicus*" and two strains of "*Methylomonas 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 ΔT_m 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 "*Methylophilus methylotrophus*" NCIB 10515. These two strains are representative of the two DNA hybridization groups described by Byrum (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 (R_f , 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 cor-

related well with the hybridization groups; their R_f 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 seashores 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 *Hyphomicrobium* (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

FIG. 3. Electrophoretic migration patterns of methanol dehydrogenases and glucose-6-phosphate dehydrogenases of marine methylotrophic bacteria. The lanes contained extracts of the strains. Lane a, Strain ATCC 33146^T; lane b, strain 310; lane c, strain 150; lane d, strain ATCC 33145; lane e, strain 2b; lane f, strain 1d; lane g, strain 290; lane h, strain 232; lane i, strain 312; lane j, strain 173; lane k, strain 222^T; lane l, strain 92b. Extracts of strains NCMB 2162 and 81 (data not shown) migrated like extracts of strains ATCC 33145 (lane d) and 222 (lane k), respectively. (A) Revelation achieved without glucose 6-phosphate and without NAD. The thick bands of nothing dehydrogenases were identified as methanol dehydrogenases (see text); their R_f values were 0.22 (lanes a through c), 0.27 (lanes d through f), 0.30 (lanes g through k), and 0.19 (lane l). No additional band appeared when methanol was added as a substrate to the revealing mixture. (B) Results with glucose 6-phosphate and NAD as the substrates. Numerous indistinct bands appeared in the lower half of the gel, and sharp bands with two distinct R_f values (0.35 [lanes a through f] and 0.33 [lanes g through l]) were present in front of the methanol dehydrogenases. The results were identical when nicotinamide adenine dinucleotide phosphate was used instead of NAD. Omission of NAD or nicotinamide adenine dinucleotide phosphate resulted in the pattern obtained in (A).

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 "*Methylomonas*," 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 "*Methylomonas 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 "*Methylomonas 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 *Methylomonas* 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 *Methylomonas* 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 *Methylomonas* 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 *Methylomonas* (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 *Methylophaga* gen. nov. *Methylophaga* gen. nov. (Me.thyl.o.pha'ga. French n. *methyl*, methyl radical; Gr. n. *metha* fermented beverage; Gr. n. *hyle* wood; Gr. v. *phagein* to eat; *Methylophaga* 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 methanol 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 *Methylophaga marina*.

Description of *Methylophaga marina* sp. nov. *Methylophaga marina* sp. nov. (ma.ri'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 (*R_f* values) are 0.33 for glucose-6-phosphate dehydrogenase and 0.30 or 0.19 for methanol dehydrogenase. The guanine-plus-cytosine content of the DNA is 43 mol%. The type strain is strain 222 (= ATCC 35842 = NCMB 2244).

Description of *Methylophaga thalassica* sp. nov. *Methylophaga thalassica* sp. nov. (tha.las'si.ca. Gr. adj. *thalassica* 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.

Most strains grow on fructose and dimethylamine; some grow on trimethylamine. Most dissimilate methylamine through an *N*-methylglutamate dehydrogenase. The *R_f* for glucose-6-phosphate dehydrogenase is 0.35, and the *R_f* for methanol dehydrogenase is 0.22 or 0.27. The guanine-plus-cytosine content of the DNA is 44 mol%. The level of hybridization with DNA of type species *Methylophaga marina* is around 30 to 35% (S1 nuclease method).

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

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