Phenotypic and Genetic Diversity of Chlorine-Resistant *Methylobacterium* Strains Isolated from Various Environments

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Strains of pink-pigmented facultative methylotrophs which were isolated previously from various environments and assigned tentatively to the genus *Methylobacterium* were characterized in comparison with authentic strains of previously known species of this genus. Most of the isolates derived from chlorinated water supplies exhibited resistance to chlorine, whereas 29 to 40% of the isolates from air, natural aquatic environments, and clinical materials were chlorine resistant. None of the tested authentic strains of *Methylobacterium* species obtained from culture collections exhibited chlorine resistance. Numerical analysis of phenotypic profiles showed that the test organisms could be divided into 19 clusters at a similarity level of 80%, at which all established *Methylobacterium* species tested were separated from each other except *M. organophilum* and *M. rhodesianum*. The chlorine-resistant isolates were randomly distributed among all clusters. The 16S ribosomal DNA (rDNA) sequence-based phylogenetic analyses showed that representatives of the isolates together with known *Methylobacterium* species formed a line of descent distinct from that of members of related genera in the alpha-2 subclass of the *Proteobacteria* and were divided into three subclusters within the *Methylobacterium* group. These results demonstrate that there is phenotypic and genetic diversity among chlorine-resistant *Methylobacterium* strains within the genus.

The genus *Methylobacterium* (40) is a group of strictly aerobic, facultatively methylotrophic, gram-negative, rod-shaped bacteria that are able to grow on one-carbon compounds more reduced than carbon dioxide as sole carbon and energy sources (for a review, see reference 19). Mass cultures of these facultative methylotrophs are pink to red because of the presence of carotenoids. They also produce bacteriochlorophyll *a* under aerobic growth conditions (39, 45, 54), and some of them have proven to contain a photochemical reaction center similar to the reaction center of purple phototrophic bacteria (38, 50). Because of their production of photopigments with photochemical activity under aerobic conditions, *Methylobacterium* strains are categorized as aerobic phototrophic bacteria (47, 48). The genus *Methylobacterium* now consists of nine species, with *M. organophilum* as the type species (20, 21, 40, 53, 54). They are phenotypically and chemotaxonomically quite similar, and phenotypic differences among the species are found in only limited properties, such as carbon source utilization. In recent years, 16S rRNA sequence information has been used for phylogenetic placement and identification of different physiological groups of methylotrophic proteobacteria, including *Methylobacterium* species (4, 6, 27, 51, 52). The molecular data have shown that the genus *Methylobacterium* represents a line of descent in the alpha-2 subclass of the class *Proteobacteria*.

Members of the genus *Methylobacterium* are distributed in a wide variety of natural habitats, including soil, dust, air, fresh water, and aquatic sediments. These bacteria also occur in human-made environments, including potable water supplies (10–13, 18, 33), bathrooms (14), and washstands (14), where they sometimes produce pink ropy masses of growth. It is important to note that most of the *Methylobacterium* strains isolated from these environments are highly resistant to chlorine (11–13, 33). The capacity of the methylotrophic bacteria for chlorine resistance may explain why these organisms frequently occur in human environments. Moreover, some pink-pigmented bacteria that are now assigned to the genus *Methylobacterium* were isolated as opportunistic pathogens from clinical sources (15, 16, 22, 43), including the blood of a patient with AIDS (31), and from hospital environments (10). In Japan, therefore, the frequent occurrence and colonization of these bacteria in potable water systems have in recent years received much attention as a potential public health hazard. Nevertheless, while many species of aerobic chemoheterotrophic bacteria have been isolated from drinking water systems and other chlorinated environments throughout the world (8, 36, 37, 41, 42), little attention has been paid to the incidence, taxonomic identity, and chlorine resistance capacity of *Methylobacterium* strains possibly predominating in those environments.

We have hitherto isolated large numbers of chlorine-resistant *Methylobacterium* strains from various environments, including potable water supplies, in Japan. However, the classification of our isolates was tentative, and their taxonomic identity at the species level has remained unclear. Detailed systematic studies on these isolates are indispensable for elucidating the ecological and public health significance of the facultative methylotrophs in chlorinated environments. The present study was undertaken to characterize our isolates more

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thoroughly in this respect and to determine whether the capacity for chlorine resistance is widespread among known members of the genus *Methylobacterium* or is characteristic of particular species. Our approaches to this investigation were twofold: one involved physiological and biochemical characterization of the isolates, followed by numerical analysis of phenotypic profiles, and the other involved genetic analysis by PCR sequencing of 16S ribosomal DNA (rDNA). A previous phylogenetic study has shown that some *Methylobacterium* species, including those closely related to *M. extorquens*, are responsible for the formation of pink biofilms in a potable water treatment system (27).

MATERIALS AND METHODS

Bacterial strains and cultivation. A total of 77 strains isolated previously from a variety of environments (10–14, 33) were studied (see Fig. 1). These test strains consisted of 30 strains from potable water tanks (strains F01 to F30), 28 strains from the air (F31 to F58), 10 strains from wells and ponds (F59 to F68), 7 strains from clinical materials (F69 to F75), and 2 strains from a potable water treatment system (GK101 and GK118). All of these isolates were strictly aerobic, facultatively methylotrophic, pink-pigmented, chemoorganotrophic bacteria that had gram-negative or gram-variable, non-spore-forming, motile, rod-shaped cells. They were also positive for catalase and oxidase and produced bacteriochlorophyll *a* under aerobic growth conditions. These phenotypic data led to tentative classification of the isolates as members of the genus *Protomonas* (now reclassified as *Methylobacterium* [3]) or *Methylobacterium* (10–14, 27, 33). As reference organisms, the following seven strains were also used: *M. extorquens* JCM 2802^T (the superscript T indicates the type strain), *M. mesophilicum* JCM 2829^T, *M. organophilum* JCM 2833^T, *M. radiotolerans* JCM 2831^T, *M. rhodesianum* JCM 2810, *M. rhodinum* JCM 2811^T, and *M. zatmanii* JCM 2819. All strains with JCM numbers were obtained from the Japan Collection of Microorganisms, Riken, Wako, Japan, and were originally isolated from nonchlorinated environments, including soil, lake, leaf surface, rhizosphere, and fermentors (20, 21, 40, 54). All test organisms were maintained on agar slants at 10°C and subcultured every 3 months. Complex medium PBY (28) or standard agar (SA) medium (Nissui, Tokyo, Japan) was used for cultivation of the isolates. PBY medium was solidified by adding 1.5 and 1.8% agar when it was used for agar slants and plates, respectively.

Chlorine resistance activity. Colonies of the test organisms grown on SA medium at 30°C for 5 days were harvested, washed three times with autoclaved distilled water, and resuspended in this water. These cell suspensions were used immediately for tests for chlorine resistance. For these tests, 200-ml portions of a chlorine solution (0.1 mg as free residuals per liter) were prepared in 300-ml Erlenmeyer flasks by diluting and autoclaving a stock 10% aqueous solution of sodium hypochlorite in 10 mM phosphate buffer (pH 7.0). To this solution, the washed cells were resuspended to give a cell density of ca. 10^5 CFU/ml and incubated at 25°C for 5 min with gentle stirring. Then, 5-ml portions of the cell suspensions were taken and mixed with 0.05 ml of 0.3 M sodium thiosulfate to be neutralized. Viable cells in the suspensions were counted on SA medium which was incubated at 30°C for 7 days. Upon chlorine treatment, strains giving viable cell counts of more than 10% of the initial counts were considered chlorine-resistant strains. In these tests, most of the chlorine-resistant strains had viable-cell counts equivalent to the initial counts, suggesting that the thiosulfate used as a neutralizer had no effect on the viability of these organisms.

Phenotypic characterization and numerical analysis. Cells grown aerobically in PBY medium or on SA medium at 30°C for 2 to 3 days were subjected to a set of 88 phenotypic tests. This set of tests consisted of carbon source utilization tests for 49 organic compounds, biochemical reaction tests for 19 enzymes, and susceptibility tests for 20 antibiotics. In all tests, incubation was at 30°C. Tests for carbon source utilization and enzyme activities were performed with the API 50CH and API ZYM systems (BioMerieux, Montalieu-Vercieu, France), respectively; the final reading was made after 3 weeks of incubation for the former and 4 h for the latter. Antibiotic susceptibility was determined with the TRIDISC system (Eiken Chemical Co., Tokyo, Japan) after 1 week of incubation. For numerical analysis, tests for which all test strains were positive or negative were excluded, and the results for the remainder applied to each strain were coded as 0 (negative), 1 (weakly positive), 2 (moderately positive), or 3 (strongly positive). The codes were entered into a data matrix, which was then used to calculate a simple matching coefficient. A cluster analysis for the similarity coefficient was performed by the unweighted pair group mathematical averaging (UPGMA) method (49). Calculation of the similarity values and clustering by the UPGMA method were performed on an NEC personal computer with the Lotus 1-2-3 Multivariate Analysis program (Audemain Co., Tokyo, Japan).

Analyses of fatty acids and quinones. Cells grown in PBY medium were harvested by centrifugation from cultures at the early stationary phase of growth, washed, and lyophilized. Fatty acids were extracted as their methyl esters from lyophilized cells and analyzed by gas-liquid chromatography as described previously (29). Quinones were extracted with an organic solvent mixture from freeze-

dried cells, purified by thin-layer chromatography, and analyzed by reverse-phase high-performance liquid chromatography (26).

Amplification and sequencing of 16S rDNA. Colonies grown on SA medium were harvested, washed with sterilized 1% saline, resuspended in sterilized pure water, and then stored at −20°C. For PCR experiments, crude cell lysates were prepared by treating cells with proteinase K as described previously (30), but the procedure was modified by introducing sonic treatment prior to protease digestion. Stock cell suspensions were thawed, sonicated for 20 s with 2-s intermittent bursts (20 kHz; output power, 50 W), digested with proteinase K in the presence of detergents, and then heated at 95°C for 5 min, followed by centrifugation to remove unbroken cells and large debris. The resultant cell lysates as sources of template DNA were used directly for PCR. The 16S rDNA fragments that corresponded to positions 8 to 1510 of *Escherichia coli* 16S rRNA (5) were amplified by PCR, purified by agarose gel electrophoresis with resin binding, and sequenced directly by the linear PCR sequencing (cycle sequencing) method modified for automated fluorescence detection with a Pharmacia DNA sequencer. The PCR and sequencing procedures used have been described in previous papers (25, 30).

Phylogenetic analysis. Sequences were compiled from overlapping sequence data, and binary sequence similarities were calculated on an Apple Macintosh personal computer with the GENETYX-MAC program (Software Development Co., Tokyo, Japan). Multiple alignments of sequence, calculation of nucleotide substitution rates (K_{nuc}) (34), and construction of neighbor-joining phylogenetic trees (44) were performed with the CLUSTAL V program (24). Alignment gaps and unidentified base positions were not taken into consideration for the calculations. The topology of the phylogenetic tree was evaluated by bootstrap analysis (9) with 1,000 bootstrapped trials.

Nucleotide sequence accession numbers. The sequence determined in this study has been deposited in the DDBJ, GSDB, EMBL, and NCBI nucleotide sequence databases under accession numbers D32224 to D32237. The accession numbers for the sequences used in phylogenetic analysis are as follows: *Acidiphilium cryptum*, D30773; *Afipia felis*, M65248; *Agrobacterium tumefaciens*, M11223; *Azorhizobium calinodans*, X67221; *Bartonella quintana*, M11927; *Caulobacter crescentus*, M83799; *Erythrobacter longus*, M59062; *Hirschia baltica*, M52909; *Magnetospirillum magnetotacticum*, M58171; *Methylosinus sporium*, M95665; *Methylobacterium* sp. strain GK101, D25305; *Methylobacterium* sp. strain GK118, D25306; *Rhodobacter capsulatus*, D16428; *Rhodobium marinum*, D30790; *Rhodopila globiformis*, M59066; *Rhodoplanes roseus*, D25313; *Rhodopseudomonas palustris*, D25312; *Rhodospirillum rubrum*, D30778; *Rhodospirillum salexigens*, M59070; *Rhodovulum sulfidophilum*, D16423; *Roseobacter denitrificans*, M59063; and *Sphingomonas paucimobilis*, D13725.

RESULTS

Phenotypic characterization and numerical analysis. The 77 isolates and 7 authentic strains of *Methylobacterium* species were subjected to a set of 88 phenotypic tests. As a result of these tests, 40 characteristics could be taken into consideration in numerical analysis. A simplified dendrogram constructed on the basis of the results of our phenotypic tests is shown in Fig. 1. At a similarity level of 80%, the test organisms were divided into 19 clusters (designated phena 1 to 19), each of which contained more than two strains. Although phenon 7 included only one strain, we designated it a cluster because the strain was the type strain of *M. rhodinum*. The established species of the genus *Methylobacterium* could be separated from one another at an 80% level of similarity. For example, in addition to *M. rhodinum* in phenon 7, we found *M. zatmanii* in phenon 1, *M. extorquens* in phenon 2, *M. mesophilicum* in phenon 13, and *M. radiotolerans* in phenon 19. The exceptional cases were *M. organophilum* and *M. rhodesianum*, both of which fell into a single cluster (phenon 3) at an 80% level of similarity. Thus, each of the 19 phena which we defined may correspond to a species or a similar taxonomic level within the genus *Methylobacterium*.

The frequencies of positive characteristics in the 19 phena are shown in Table 1. As already described as key characteristics for the identification of *Methylobacterium* species (19), carbon source utilization tests for L-arabinose, D-xylose, D-fucose, fructose, glucose, and some other substrates were found to be useful for the classification of the test strains belonging to different phena. These strains showed resistance to a number of antibiotics tested, but most of them were susceptible to josamycin, tetracycline, and gentamicin. All of
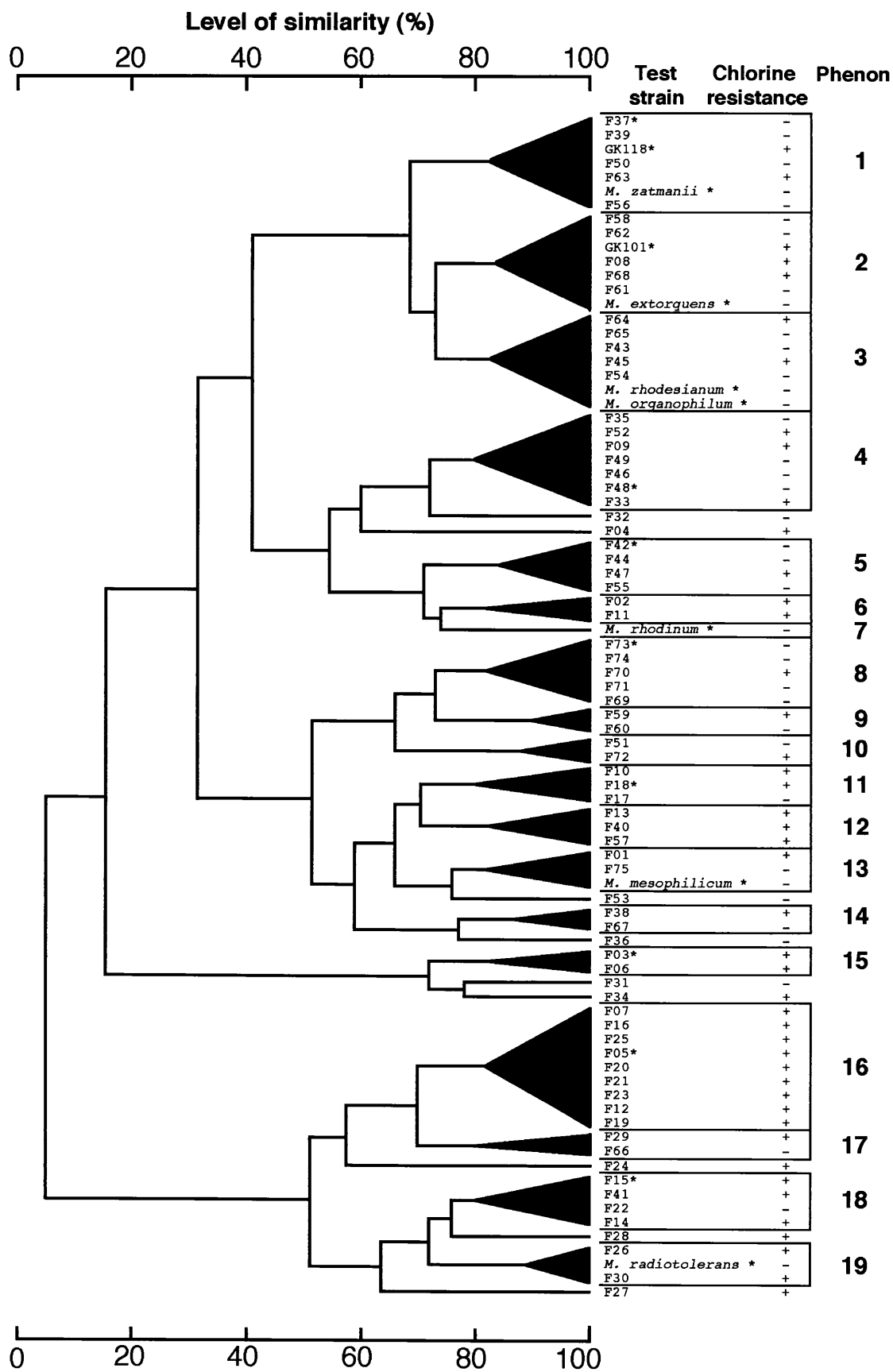
FIG. 1. Simplified dendrogram showing phenotypic clusters of *Methylobacterium* strains obtained by UPGMA linkage of similarity values. Test strains which were used for 16S rDNA analysis are marked with an asterisk.

TABLE 1. Characteristics of *Methylobacterium* strains belonging to different phena

Test	% of isolates positive in phenon:																		
	1 (7)[a]	2 (7)	3 (7)	4 (7)	5 (4)	6 (2)	7 (1)	8 (5)	9 (2)	10 (2)	11 (3)	12 (3)	13 (3)	14 (2)	15 (2)	16 (9)	17 (2)	18 (4)	19 (3)
Carbon sources																			
Glycerol	100	100	100	29	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
D-Arabinose	0	0	0	14	0	100	0	0	0	0	0	67	33	0	50	100	50	100	100
L-Arabinose	0	0	0	0	0	0	0	100	100	100	100	100	100	100	50	100	100	100	100
D-Ribose	0	0	0	0	0	0	0	0	0	0	33	0	67	50	0	22	0	25	33
D-Xylose	0	0	0	0	0	50	0	0	0	50	100	100	100	100	50	100	100	100	100
L-Xylose	0	0	0	0	0	0	0	0	0	0	0	0	67	0	0	56	0	0	67
D-Galactose	0	0	0	0	0	0	0	100	100	100	33	100	100	50	0	100	100	100	100
D-Glucose	0	0	86	29	0	100	100	80	50	0	100	100	0	0	100	89	100	100	100
D-Fructose	71	57	100	43	100	50	100	0	0	0	0	33	0	100	0	100	50	25	0
D-Turanose	0	0	0	0	0	0	0	0	50	0	0	0	0	0	0	0	0	0	0
D-Lyxose	0	0	0	0	0	0	0	0	0	0	100	33	100	100	0	100	0	100	100
D-Fucose	0	0	0	0	0	0	0	100	100	100	100	100	100	100	0	100	100	100	100
L-Fucose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	33
Gluconate	0	0	0	14	75	100	0	0	0	0	100	100	100	100	100	100	100	100	100
2-Ketogluconate	0	0	0	0	0	0	0	0	0	0	0	0	67	0	0	0	0	0	67
5-Ketogluconate	0	0	0	0	0	0	0	0	0	0	0	0	33	0	0	0	0	0	0
Enzyme activity																			
α-Glucosidase	0	0	0	0	0	0	0	80	0	0	33	0	0	0	0	22	0	25	33
Alkaline phosphatase	14	14	14	14	0	50	100	0	0	0	0	0	0	50	50	0	0	25	33
Acid phosphatase	29	0	14	14	50	100	0	100	100	50	33	67	67	0	0	56	50	100	33
Phosphoamidase	14	14	43	14	0	0	100	100	100	50	33	0	67	0	0	22	100	50	0
Esterase (C4)	71	100	86	71	100	100	100	100	100	100	100	100	67	50	100	100	100	100	100
Esterase (C8)	100	100	100	100	100	100	100	100	100	100	33	67	100	50	0	44	50	100	100
Leucinearylamidase	57	71	71	29	75	0	0	20	0	0	0	33	0	100	0	0	50	25	0
Valinearylamidase	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0
Trypsin	14	0	29	0	25	0	0	100	0	0	0	0	0	50	0	11	50	50	0
Antibiotics																			
Penicillin	0	14	0	14	25	0	0	0	0	50	0	0	0	0	100	56	100	50	0
Carbenicillin	0	29	57	57	100	100	100	80	0	100	33	67	67	0	100	100	100	100	100
Amoxicillin	29	57	86	100	100	100	100	100	0	100	67	100	100	50	100	100	50	100	100
Erythromycin	43	71	43	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Josamycin	71	86	86	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Chloramphenicol	0	14	0	0	75	50	0	0	0	50	0	33	33	0	100	89	100	100	100
Thiamphenicol	0	14	0	43	100	50	0	60	0	100	0	33	67	0	100	100	100	100	100
Tetracycline	86	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Gentamicin	57	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Cephalothin	0	0	29	43	75	0	0	60	0	100	0	0	33	0	100	100	100	100	67
Cephalexin	0	43	29	29	50	100	0	100	0	100	67	0	100	100	100	100	100	100	100
Cefazolin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50	0	50	0	0
Cephapirin	0	14	0	43	25	0	0	20	0	50	0	0	0	0	100	78	100	100	0

[a] Numbers in parentheses indicate the number of strains isolated and tested.

the test organisms were susceptible to minocyclin and kanamycin (data not shown).

Gas-liquid chromatographic analyses of whole-cell fatty acids showed that all of the test strains contained saturated and monounsaturated straight-chain fatty acids, with $C_{18:1}$ predominating (>80% of the total content). They also contained 3-hydroxy fatty acids, with 3-OH $C_{14:0}$ predominating. Quinone profiling by high-performance liquid chromatography revealed that all test strains contained ubiquinone-10 as the sole quinone component. These findings confirmed the results of previous studies on the chemotaxonomic characteristics of the methylotrophic bacteria (54).

Relationships of phenotypes, chlorine resistance, and sources. Figure 1 shows the results of tests for chlorine resistance in addition to the results of numerical analysis of phenotypic profiles. Also, the relationships between the chlorine resistance capacity and the sources of the test organisms are summarized in Table 2. The two isolates obtained from a raw-water treatment system and almost all of the isolates from the potable-water tanks showed chlorine resistance, whereas

29 to 40% of those from the air, wells, ponds, and clinical materials had this property. None of the authentic strains of known *Methylobacterium* species obtained from the culture collections were resistant to chlorine. The isolates from tank

TABLE 2. Capacity for chlorine resistance of isolates from different sources and authentic *Methylobacterium* strains

Test organism and source	No. of strains tested	No. resistant[a]	% Resistant
Isolates			
Potable-water tanks	30	28	93
Air	28	9	32
Wells and ponds	10	4	40
Clinical materials	7	2	29
Raw-water treatment system	2	2	100
Authentic strains from culture collections	7	0	0

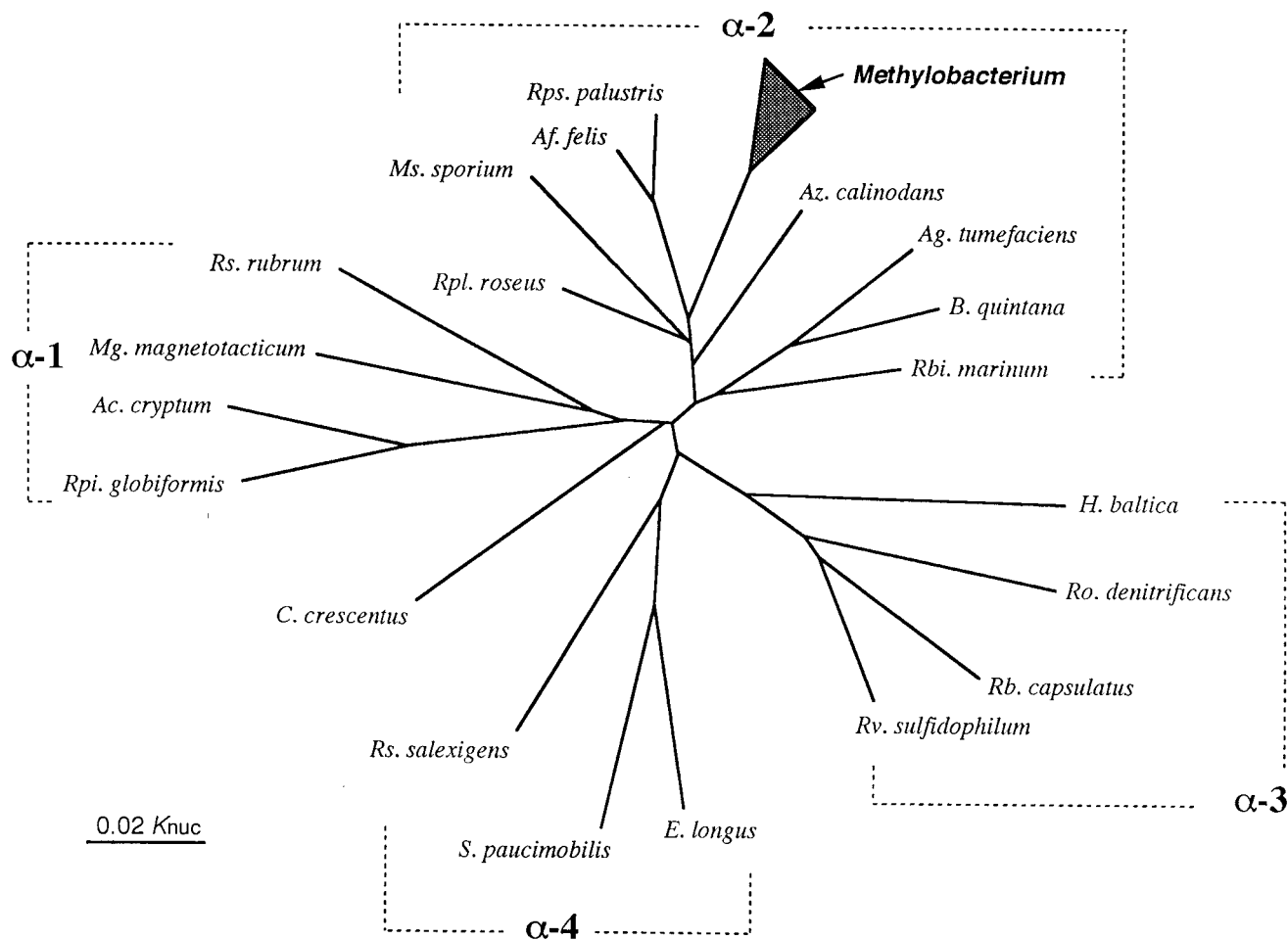[a] Strains resistant to treatment with chlorine (0.1 mg/liter) for 5 min.

FIG. 2. Distance matrix tree showing phylogenetic positions of *Methylobacterium* strains among members of the alpha subclass of the *Proteobacteria*. The cluster including the *Methylobacterium* strains tested is shown as a shaded triangle. The species given on the tree are as follows: *Ac. cryptum, Acidiphilium cryptum*; *Af. felis, Afipia felis*; *Ag. tumefaciens, Agrobacterium tumefaciens*; *Az. calinodans, Azorhizobium calinodans*; *B. quintana, Bartonella quintana*; *C. crescentus, Caulobacter crescentus*; *E. longus, Erythrobacter longus*; *H. baltica, Hirschia baltica*; *Mg. magnetotacticum, Magnetospirillum magnetotacticum*; *Ms. sporium, Methylosinus sporium*; *Rb. capsulatus, Rhodobacter capsulatus*; *Rbi. marinum, Rhodobium marinum*; *Rpl. roseus, Rhodoplanes roseus*; *Rps. palustris, Rhodopseudomonas palustris*; *Rs. rubrum, Rhodospirillum rubrum*; *Rs. salexigens, Rhodospirillum salexigens*; *Rv. sulfidophilum, Rhodovulum sulfidophilum*; *Ro. denitrificans, Roseobacter denitrificans*; *S. paucimobilis, Sphingomonas paucimobilis*; *Rpi. globiformis, Rhodopila globiformis*.

water, most of which were chlorine resistant, were relatively abundant in phenon 16 but also occurred in 10 other phena. The isolates from the other sources were also randomly distributed in different phena. As a result, all recognized phena included at least one chlorine-resistant strain, except phenon 7, which consisted of only one strain, *M. rhodinum* JCM 2811ᵀ. These results suggest that the capacity for chlorine resistance of the isolates is related to their source but not to their taxonomic properties.

16S rDNA sequencing. For 16S rDNA sequence studies, representative isolates with or without the chlorine resistance capacity were selected from 8 of the 19 clusters, i.e., phena 1, 4, 5, 8, 11, 15, 16, and 18. All the phena from which the test organisms were selected are different from the clusters including the established *Methylobacterium* species except phenon 1 (see Fig. 1). Of the nine species of the genus *Methylobacterium* currently established, five species have been studied for 16S rRNA or rDNA sequences (6). However, we found that all of the published sequence data for the methylotrophs include a number of undetermined positions and possibly erroneous gaps of more than 20 positions in each sequence. Exclusion of these undetermined positions may have serious effects on the

elucidation of interspecies relationships, because the sequences of *Methylobacterium* strains have high levels of similarity to one another, as described below. Therefore, we attempted to determine the 16S rDNA sequences not only for our isolates but also for most *Methylobacterium* species, including those species whose sequences are already available, and we used our new versions of the sequences in phylogenetic analyses.

The 16S rRNA genes from crude lysates of the eight isolates and seven established *Methylobacterium* species were amplified successfully by PCR except for one strain (strain F03), which yielded a very small PCR product, not sufficient for full sequencing. The amplified double-stranded 16S rDNAs were sequenced directly by the cycle sequencing method followed by automated fluorescence detection. The sequences which we determined included 1,400 to 1,407 residues of a continuous nucleotide stretch, covering 95% of the entire 16S rRNA gene. The 16S rDNAs of all test strains exhibited large nucleotide deletions at about positions 80, 210, and 470, features placing them in the alpha subclass of the *Proteobacteria*.

Phylogenetic analysis. Our sequence data were aligned with a data set consisting of 20 sequences from representative spe-

cies within the alpha subclass of the *Proteobacteria*, and the evolutionary distance (K_{nuc}) values were calculated for the 1,226 positions that could be aligned. On the basis of the K_{nuc} values obtained, a phylogenetic tree was reconstructed by the neighbor-joining method (Fig. 2). All *Methylobacterium* test strains formed a tight cluster (as indicated by the shaded triangle) in the alpha-2 subclass of the *Proteobacteria*.

Since the tested *Methylobacterium* strains showed high levels of binary sequence similarity (93.8 to 99.6%) (see Table 3) and represented a line of descent distinct from that of other members of the alpha-2 subclass, it was necessary to take as many sequence positions as possible into consideration to elucidate interspecies relationships more accurately. Thus, we made another data set consisting of sequences for only the *Methylobacterium* strains and *Rhodospirillum rubrum* as a representative of outgroups and calculated K_{nuc} values for the alignable 1,394 positions of this set. The evolutionary distance (K_{nuc}) values thus obtained and the levels of binary sequence similarity are shown in Table 3. A neighbor-joining phylogenetic tree which was reconstructed on the basis of these distance matrix data is shown in Fig. 3. The test organisms could be divided into three major groups that were designated subclusters I, II, and III. The known *Methylobacterium* species fell into two of the subclusters, one of which included *M. extorquens*, *M. organophilum*, *M. rhodesianum*, and *M. zatmanii* (subcluster I), and one of which encompassed *M. mesophilicum* and *M. radiotolerans* (subcluster II). Highly chlorine-resistant strains GK101 and GK118, both of which were studied previously for 16S rDNA sequences (27), belonged to subcluster I together with *M. extorquens* and related species. Five of our tested isolates were placed in subcluster II, and the remaining two isolates (strains F37 and F48) formed another lineage (subcluster III) distinct from the above two clusters. However, the relationships between subcluster III and the other two clusters appeared to be unstable, as suggested by a low level of bootstrap confidence.

DISCUSSION

Many species of aerobic chemoheterotrophic bacteria have been isolated from potable water and chlorinated environments. LeChevallier et al. (36, 37) isolated members of the genera *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, and *Pseudomonas* as the major constituents of aerobic heterotrophs from chlorinated and raw-water supplies and distribution system biofilms. Ridgway and Olson (42) isolated *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Moraxella*, and *Pseudomonas* strains from potable-water distribution systems. Reasoner et al. (41) detected high populations of pigmented bacteria, including members of the genera *Chromobacterium*, *Flavobacterium*, and *Serratia*, and of gram-positive organisms from potable water. Other investigators isolated similar species of aerobic chemoheterotrophs from drinking water (2, 8, 23) and also found a large number of antibiotic-resistant strains among the bacteria detected (2, 8). These observations suggest that a wide variety of bacterial species have the potential to resist chlorine or to survive under chlorinated conditions. Some studies have provided evidence for the persistence in chlorinated water of aerobic heterotrophic bacteria, such as mycobacteria (7, 17), *Legionella pneumophila* (35), and *Pseudomonas aeruginosa* (46).

On the other hand, until recently, there have been only a few reports in the literature concerning the isolation and chlorine resistance of *Methylobacterium* strains from chlorinated environments. One of the reasons for this is that the standard method used previously for heterotrophic plate counts of bacteria involved incubation at 37°C for 48 h on standard agar

TABLE 3. Sequence similarities and evolutionary distances for 16S rDNAs of isolates and authentic *Methylobacterium* strains

Strain no.	Species or strain no.	Sequence similarity (%) or evolutionary distance (K_{nuc}) with strain[a]:																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*M. extorquens*		99.5	99.1	98.9	98.8	97.7	96.3	95.1	95.2	94.9	95.0	95.0	94.9	94.2	95.1	95.2	86.3
2	*M. rhodesianum*	0.0057		99.1	98.7	98.5	97.3	96.1	95.6	94.9	94.7	94.7	94.7	94.6	93.9	94.8	95.5	86.1
3	GK101	0.0086	0.0086		98.9	99.0	97.7	96.9	95.1	95.2	94.8	94.9	94.9	94.7	94.3	95.6	95.2	86.7
4	GK118	0.0093	0.0122	0.0093		98.6	97.2	96.7	95.0	94.8	94.7	94.7	94.8	94.6	94.2	95.5	95.2	86.4
5	*M. zatmanii*	0.0122	0.0151	0.0100	0.0129		97.5	96.2	94.6	94.7	94.5	94.7	94.5	94.4	93.8	95.0	95.1	86.7
6	*M. rhodinum*	0.0230	0.0258	0.0230	0.0265	0.0244		96.4	95.6	95.8	95.7	95.8	95.3	95.7	93.8	94.9	95.2	85.9
7	*M. organophilum*	0.0352	0.0380	0.0301	0.0337	0.0373	0.0373		96.6	96.3	96.1	96.2	95.9	96.0	95.2	96.7	95.9	86.7
8	*M. radiotolerans*	0.0502	0.0531	0.0509	0.0531	0.0552	0.0445	0.0373		99.6	99.4	99.3	98.1	97.7	96.6	96.9	95.6	87.1
9	F73	0.0481	0.0509	0.0488	0.0509	0.0531	0.0423	0.0366	0.0050		99.4	99.3	97.9	97.9	96.7	96.7	95.3	86.7
10	F05	0.0488	0.0516	0.0495	0.0516	0.0531	0.0416	0.0373	0.0057	0.0050		99.6	97.9	97.4	96.4	96.6	95.2	86.8
11	F15	0.0481	0.0509	0.0488	0.0509	0.0524	0.0409	0.0366	0.0079	0.0057	0.0036		97.8	97.5	96.4	96.4	95.3	86.9
12	*M. mesophilicum*	0.0495	0.0524	0.0502	0.0509	0.0545	0.0473	0.0387	0.0187	0.0194	0.0201	0.0222		99.4	95.7	96.4	95.2	85.9
13	F18	0.0509	0.0538	0.0516	0.0524	0.0560	0.0430	0.0402	0.0244	0.0222	0.0244	0.0237	0.0057		95.6	95.9	95.2	85.9
14	F42	0.0595	0.0610	0.0574	0.0595	0.0624	0.0624	0.0466	0.0373	0.0323	0.0344	0.0337	0.0416	0.0430		95.1	94.8	86.5
15	F37	0.0509	0.0538	0.0459	0.0481	0.0531	0.0509	0.0337	0.0344	0.0344	0.0359	0.0352	0.0380	0.0395	0.0502		96.4	87.2
16	F48	0.0473	0.0502	0.0438	0.0459	0.0488	0.0473	0.0409	0.0473	0.0459	0.0452	0.0445	0.0452	0.0466	0.0516	0.0337		86.8
17	*R. rubrum*	0.1370	0.1392	0.1334	0.1370	0.1370	0.1413	0.1306	0.1334	0.1327	0.1320	0.1313	0.1399	0.1413	0.1356	0.1313	0.1313	

[a] The values on the upper right are percent sequence similarity, and the values on the lower left are evolutionary distances (K_{nuc} values).
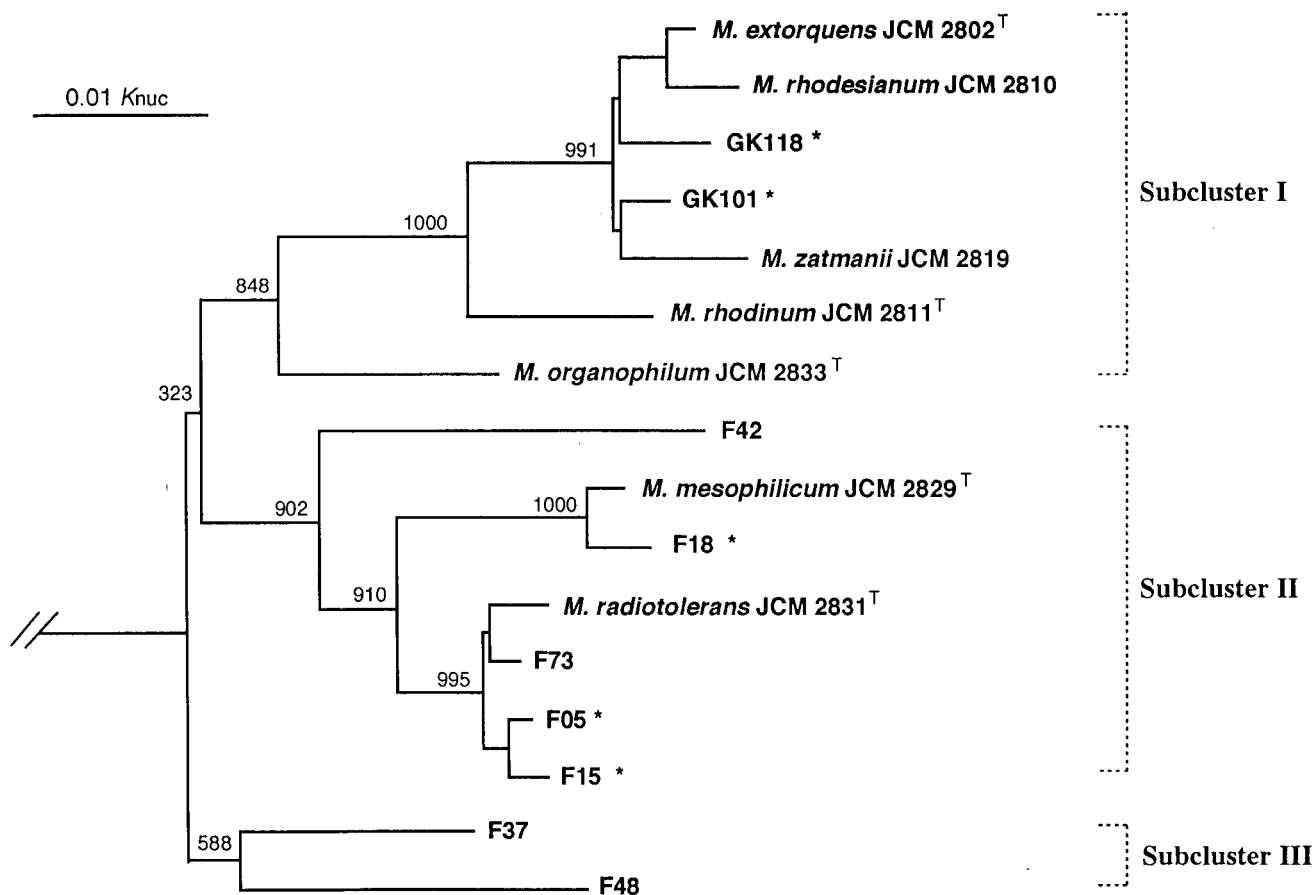
FIG. 3. Distance matrix tree showing phylogenetic relationships among the *Methylobacterium* strains within the genus. *Rhodospirillum rubrum* was used as a member of outgroups. Chlorine-resistant strains are marked with an asterisk. Bootstrap confidence values are shown at branching points of interest.

medium (1), although these culture conditions are unfavorable for the detection of *Methylobacterium* strains. Since the methylotrophic bacteria are relatively slow growers (19, 22), it is necessary to prolong incubation time to recover these bacteria from the environment. Thus, it can be assumed that methylotrophs as major inhabitants of drinking water and chlorinated environments have been overlooked for a long time. In fact, improved isolation techniques with prolonged incubation yielded high numbers of *Methylobacterium* strains from water supplies and other chlorinated environments (11, 14, 33).

One of our approaches to the present study, which was designed to determine the identity of chlorine-resistant *Methylobacterium* strains, was phenotypic characterization with numerical analysis. By this approach, the test organisms, including seven known species of the genus *Methylobacterium*, were found to fall into 19 clusters at an 80% level of similarity, at which the established species could be differentiated from each other with a few exceptions. The chlorine-resistant strains which we isolated were distributed among most of these clusters. These results suggest that chlorine-resistant *Methylobacterium* strains are phenotypically diverse and may be classified into a number of species, including those other than the seven authentic species within the genus.

The other approach, involving 16S rRNA gene sequence comparisons, has shown that the *Methylobacterium* test strains, including seven known species, form a tight cluster at interspecies similarity levels of more than 93% within the alpha-2 subclass of the *Proteobacteria* and that they fall into three

major lines of descent, termed here subclusters I, II, and III, within the *Methylobacterium* cluster. Among the established species of *Methylobacterium*, *M. extorquens*, *M. organophilum*, *M. rhodesianum*, *M. rhodinum*, and *M. zatmanii* are positioned in subcluster I, whereas *M. mesophilicum* and *M. radiotolerans* are included in subcluster II. The topology of the 16S rDNA-based phylogenetic tree is similar to the topology of the dendrogram obtained by UPGMA linkage of DNA-DNA hybridization values for the methylotrophs reported previously (32). Our test isolates, including chlorine-resistant strains, were distributed randomly among the three phylogenetic subclusters. These results demonstrate that chlorine-resistant *Methylobacterium* strains are phylogenetically diverse as well as phenotypically heterogeneous.

Why members of the genus *Methylobacterium* occur frequently in and colonize potable water and chlorinated environments has been a subject of major concern. The data presented here and elsewhere (11–13, 33) demonstrate that the capacity for chlorine resistance is one of the most important factors. In view of the results of the present study, this property of *Methylobacterium* strains is independent of their taxonomic and phylogenetic positions but closely related to their sources. Our data show that strains derived from chlorinated water supplies are most remarkable for chlorine resistance. This fact suggests that *Methylobacterium* strains acquire the capacity for chlorine resistance by adapting to chlorinated environments, and therefore they can compete with coexistent chemoheterotrophs, survive, and in some cases exhibit massive growth in

these environments. The mechanism for acquiring chlorine resistance is unknown, and our attempts to demonstrate that this trait is plasmid borne have given negative results so far.

Another important factor may be that *Methylobacterium* strains are able to produce bacteriochlorophyll *a* under aerobic conditions, suggesting their capacity for yielding energy by photophosphorylation. Potable water and chlorinated water systems are oligotrophic environments, in which carbon energy sources for the growth of chemotrophic bacteria are very scant. However, the fact that *Methylobacterium* strains have a cyclic photosynthetic electron transport system, like the phototrophic purple bacteria (50), indicates that their ability to acquire ATP helps them to survive without the need for carbon energy sources.

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