Taxonomy of *Microcycclus* and Other Nonmotile, Ring-Forming Bacteria

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The genetic relatedness of some nonmotile, ring-forming bacteria was determined by deoxyribonucleic acid (DNA) base composition and base sequence homology experiments. It is proposed that four gas vacuolate strains with DNA base compositions of 67.1 to 68.8 mol% guanine plus cytosine (G + C) be placed in *Microcycclus aquaticus*. Nonmotile vibrioid bacteria of 51% G + C (*Microcycclus flavus* Raj and spiro-soma strains 1 and 2) were found to be more closely related to one another than to *M. aquaticus*, and it is suggested that the taxonomic position of *M. flavus* be reconsidered in relation to the genus *Spirosoma*. None of the nonmotile ring-forming strains were found to be related to two *Pseudomonadaceae*. A motile variant of a gas vacuolated strain of *M. aquaticus* was isolated, indicating that immotility is a questionable taxonomic criterion for inclusion in the genus *Microcycclus*.

Weibel (19) was apparently the first to isolate and describe nonmotile vibrios which were subsequently placed in the genus *Spirosoma* by Migula in 1894 (12). These isolates were eventually lost and, because of disuse, the genus was eliminated from *Bergey's Manual of Determinative Bacteriology* after the sixth edition. In 1928 Ørskov reported the isolation of nonmotile vibrioid bacteria that formed closed rings prior to division. He introduced the name *Microcycclus aquaticus* for these bacteria (14). Other nonmotile vibrios have been described in the past 25 years. Gromov proposed the name *Microcycclus major* for a large nonmotile vibrio that forms closed rings (6). Raj reported two ring-forming bacteria, a yellow-pigmented isolate from freshwater that he named *Microcycclus flavus* and a pink-pigmented isolate from seawater (16). Claus and co-workers also obtained nonmotile vibrioid bacteria in pure culture, but they concluded that their strains more closely resembled the original description of *Spirosoma* than *Microcycclus* (2, 3).

Deoxyribonucleic acid (DNA) base composition analysis of isolates from this group (3) suggested that the strains fell into three groups: *M. aquaticus* Ørskov (67.3 mol% guanine plus cytosine [G + C]), *M. major* Gromov (39.5 mol%), and Raj's *M. flavus* and the spiro-somas of Claus (51 to 51.5 mol%). On the basis of the morphological characteristics and DNA base composition data, Claus et al. (3) concluded that (i) there were at least three genera

represented and (ii) ring formation was not sufficiently restrictive as a sole criterion for the delineation of one genus. Furthermore, Claus et al., suggested that Raj's *M. flavus* and Claus' strains be placed in the genus *Spirosoma* although no formal taxonomic proposals to this effect were made. Nonetheless, Raj named his isolate *M. flavus*, contending that the results of extensive physiological studies were more meaningful than the DNA base composition data (16).

Recently two independent laboratories isolated gas vacuolate strains of nonmotile vibrioid bacteria. Nikitin (13) reported isolates from Russian soils and placed them in the newly created genus *Renobacter vacuolatum*. Van Ert and Staley also reported isolates of gas vacuolate, nonmotile vibrios obtained from aquatic habitats which they considered strains of *M. aquaticus* (18).

In the present communication, the taxonomy of these organisms was examined by DNA homology procedures to determine (i) the relationship among *M. aquaticus* Ørskov, *M. flavus* Raj, and the spiro-somas of Claus, (ii) the relationship between the gas vacuolate organisms and *M. aquaticus*, and (iii) the relationship of motile organisms to these nonmotile vibrios.

MATERIALS AND METHODS

Bacterial strains and growth conditions. H. D. Raj provided *M. flavus*, *M. aquaticus* Ørskov, and a nonmotile vibrio obtained from a marine habitat. Spirosoma strains 1 and 2 were obtained from D. Claus. *Comamonas terrigena* RH247 was provided by R. Hugh. Gas vacuolate strains M and B were described previously (18) and strains H and W were

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isolated from aquatic habitats (Hall Lake, Seattle, Wash., and Weyerhaeuser aeration lagoon, Everett, Wash.).

Cells for DNA extraction were grown in TGEY medium (0.3% beef extract, 0.5% tryptone, 0.1% glucose, and 0.1% yeast extract). The cells were harvested during early stationary phase of growth after incubation at 25 to 30°C in an aerated culture vessel. Strain M6 was grown in CAGV medium (8). Raj's marine vibrio was cultured in 0.5% peptone, 0.1% glucose, 2% Hutner mineral salts, and 1% vitamin solution (8) in artificial seawater.

DNA preparation. DNA was prepared by the procedure of Marmur (11). Cell lysis was enhanced by the use of lysozyme (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 100 µg/ml for 30 min at 37°C or by treating the cultures with 50 µg of penicillin G per ml (Glaxo, Toronto, Ontario) for 15 min before harvesting. The ratio of absorbance at 260 nm to that at 280 nm was at least 1.8 for all DNA preparations. Radiolabeled DNA was obtained by growing the cells for at least four generations in the presence of 10 to 100 µCi of [8-³H]adenine per ml (Amersham/Searle, Arlington Heights, Ill., 21 Ci/mmol). Both labeled and nonlabeled DNA preparations were passed through a French pressure cell at 15,000 lb/in² to give DNA fragments with a molecular weight of approximately 600,000. The samples were then exhaustively dialyzed against three changes of distilled water followed by three changes of 0.1 M NaCl.

DNA reassociation. The method for determining the base sequence homologies between the various DNAs was based on the S₁ endonuclease procedure as described by Crosa et al. (4). One-tenth microgram of heat-denatured DNA (105°C, 10 min, 0.1 M NaCl) from *M. aquaticus* Ørskov (22,100 cpm/µg), *M. aquaticus* strain M (9,900 cpm/µg), or *M. flavus* Raj (15,100 cpm/µg) was incubated at 73°C for 16 h with 150 µg of heat-denatured DNA from each of the strains to be examined. The total volume of the reaction mixture was 1.1 ml. Since there is a difference in the base composition of the test DNAs, reassociation with *M. aquaticus* Ørskov DNA was carried out in 0.17 M NaCl, reassociation with strain M was done in 0.21 M NaCl, and 0.45 M NaCl was used in the reactions with *M. flavus* DNA to obtain the optimal rate of reassociation (*T_m* - 25°C) and similar conditions for base-pairing at the single incubation temperature employed (7, 20).

After incubation, 0.3 ml of the reaction mixture was added to 0.7 ml of a solution containing S₁ endonuclease for digestion of unpaired single-stranded DNA which may remain. The final composition of this enzyme solution was 0.1 mM ZnSO₄, 0.17 M NaCl, 30 mM NaAc (pH 4.5), 20 µg of heat-denatured salmon sperm DNA, and enough S₁ endonuclease to solubilize 95% of the single-stranded DNA present in the mixture. The enzyme assays were incubated at 50°C for 20 min, chilled in an ice bath, and precipitated by the addition of 0.25 ml of cold 20% trichloroacetic acid. The precipitate was collected on nitrocellulose membrane filters (type B-6, Schleicher and Schuell, Keene, N.H.), washed with cold 5% trichloroacetic acid, and dried. Radio-

activity measurements were made in toluene scintillation fluid with a Beckman LS-300 liquid scintillation counter. Enzyme assays were performed in triplicate for each of the reaction mixtures. Under these reaction conditions, there was 0 to 5% hydrolysis of double-stranded DNA. S₁ endonuclease was prepared from crude α-amylase powder (Sigma Chemical Co., St. Louis, Mo.) as described by Sutton (17).

DNA base compositions. The base compositions of the DNAs of strains H, W, marine strain, and *Pseudomonas aeruginosa* were determined from their *T_m* values by the thermal denaturation procedure (10) on a Gilford 2400-S spectrophotometer.

RESULTS

Polynucleotide sequence relationships. The genetic relatedness among ring-forming non-motile bacteria and two members of the *Pseudomonadaceae* was determined from the extent to which the denatured DNA of these organisms reannealed with denatured radiolabeled DNA (Table 1). The DNAs of the four gas vacuolate strains (M, B, H, and W) and *M. aquaticus* Ørskov have a similar base composition, suggesting the strains might be related. The relationships among these strains was examined by heteroduplex formation with *M. aquaticus* Ørskov or *M. aquaticus* M-labeled DNA. The DNAs of the four gas vacuolate strains were 28 to 45% homologous with that of the Ørskov strain, whereas the gas vacuolate strains B and W were 43 and 40% related to strain M. The other test DNAs exhibited less than 5% heteroduplex formation with *M. aquaticus* Ørskov, except for spirosona strain 1, which was 14% related.

The only DNAs to show significant base sequence complementarity to *M. flavus* DNA were spirosona strains 1 and 2. These were 38 and 24% related, respectively. These results are similar to those using *M. aquaticus* DNA in that those organisms of similar DNA base composition exhibited significant heteroduplex formation (*M. flavus* and the two spirosona strains have G+C contents of 51.0 to 51.5 mol%). The *M. aquaticus* group, Raj's marine strain, and the two *Pseudomonadaceae* exhibited 2% or less heteroduplex formation with *M. flavus*.

Motile variant of strain M. Since immotility is one of the primary characteristics of the genus *Microcyclops*, it was of considerable interest when a motile mutant of *M. aquaticus* M was observed during passage of the culture. The high degree of base sequence homology between the DNA of the variant, designated M6, and strain M (Table 1) suggests that M6 arose by a spontaneous mutation in the M strain.

When grown below 20°C, M6 possessed gas

TABLE 1. Genetic relatedness of *Microcyclus* strains to other selected bacteria

Source of unlabeled DNA	Genetic relatedness ^a			
	<i>M. aquaticus</i> Ørskov ^b	<i>M. aquaticus</i> M ^b	<i>M. flavus</i> ^b	Mol% G+C
<i>Microcyclus aquaticus</i> Ørskov (ATCC 25396)	100		1	67.3 ^c
<i>M. aquaticus</i> M (ATCC 27068)	45	100	2	67.9 ^d
<i>M. aquaticus</i> M6	46	100		
<i>M. aquaticus</i> B (ATCC 27069)	28	43	2	68.4 ^d
<i>Microcyclus</i> sp. H	43		0	68.8
<i>Microcyclus</i> sp. W	34	40	0	67.1
Spirosoma 1 (DSM 74)	14		38	51.5 ^c
Spirosoma 2 (DSM 75)	5	6	24	51.0 ^c
<i>M. flavus</i> Raj (ATCC 23276)	0	0	100	51.0 ^c
Marine strain (ATCC 25205)	4		0	33.7
<i>Pseudomonas aeruginosa</i>	1			67.3
<i>Comamonas terrigena</i> (ATCC 8461)	2	0	0	64.0 ^e

^a Values are the relative binding expressed as a percentage and have been corrected by the background counts obtained in reactions with coliphage T₄ DNA (9.7% of input).

^b Source of ³H-labeled DNA.

^c From Claus et al. (3).

^d From Van Ert and Staley (18).

^e From Leifson and Mandel (9).

FIG. 1. Electron micrograph of *M. aquaticus* M1, fixed with Lugol iodine, showing the cell morphology. Note the ring-shaped cell and gas vesicles within some cells. Bar represents 1 μ m.

vacuoles and resembled strain M (Fig. 1) except for the single polar flagellum (Fig. 2). Above 20°C many of the cells were motile, but did not contain gas vacuoles. Attempts to find other variations in the different strains of *M. aquaticus* have so far proven unsuccessful.

DISCUSSION

The DNA base compositions and polynucleotide sequence relationships among the nonmotile vibrios we examined indicated that there are two groups of related organisms. *M. aquati-*

FIG. 2. Electron micrograph of *M. aquaticus* M6 grown at 15°C and stained with 1% phosphotungstic acid. The cell is polarly flagellated and contains gas vesicles (indicated by arrows). Because the stain obscures the vesicles, the left half of the photograph was underexposed to render the vesicles more apparent, and the right half was exposed to illustrate the flagellum.

cus Ørskov and the four gas vacuolate strains are related to one another. *M. flavus* Raj and the spirosona strains of Claus form another group of related organisms. This agrees with the suggestions of Claus et al. (3), who subdivided the members of this group on the basis of DNA base composition.

To assess genetic relatedness by DNA heteroduplex formation, the stringency of the reaction conditions had to be considered. Our heteroduplex reactions were performed at 25°C below the T_m of the radiolabeled DNA, the optimal conditions of heteroduplex formation (7, 20). Conditions corresponding to $(T_m - 25)$ were obtained by incubating all reactions at 73°C and adjusting the concentration of NaCl according to the formula $\text{mol\% G+C} = 2.44 (T_m - 81.5 - 16.6 \log M)$, where M equals the concentration of NaCl in moles per liter (10).

Although there was heteroduplex formation

between *M. aquaticus* Ørskov DNA and DNAs of the gas vacuolate strains (M, B, H, and W), the extent of the reactions (28 to 45%) was low in strains of the same species. DNA homology studies on the *Enterobacteriaceae* indicate that strains of one species may exhibit 75 to 100% similarity (1). Somewhat lower values, 50 to 100%, were found among strains of some *Pseudomonas* species (15). However, for two reasons we believe that the gas vacuolate strains should be considered strains of *M. aquaticus*, despite the low DNA homologies within this group as compared to homologies within species of *Pseudomonas* or the *Enterobacteriaceae*. First, there were phenotypic similarities between *M. aquaticus* Ørskov and some of the gas vacuolate strains (18). Secondly, the gas vacuolate strains could not be differentiated from *M. aquaticus* Ørskov by means of polynucleotide sequence relationships, since the DNAs of the

gas vacuolate strains diverged as much among themselves as with respect to *M. aquaticus* Ørskov. This was shown by the reactions between labeled strain M DNA and strains B and W. Thus, we believe it prudent to classify all gas vacuolate strains as *M. aquaticus*, at least until a larger number of isolates can be characterized. Unfortunately, we were unable to obtain a culture of *Renobacter vacuolatum* to compare it to the morphological similar gas vacuolate *M. aquaticus* strains.

Although the DNA base composition data of Claus indicated that *M. flavus* and the spiro-soma might be related and *M. aquaticus* is unrelated to these strains, he made no formal proposals concerning the taxonomy of the spiro-soma strains. Our results confirm that spiro-soma strains 1 and 2 are more closely related to *M. flavus* than to *M. aquaticus*. Furthermore, despite biochemical and morphological evidence that *M. flavus* and *M. aquaticus* are 42% related by numerical taxonomy (16), our heteroduplex reactions show that *M. aquaticus* is genetically unrelated to *M. flavus*, as would be expected from the difference in DNA base compositions (5). These data indicate that the taxonomic position of *M. flavus* should be reconsidered with relation to the genus *Spirosoma*. However, it is impossible to make direct comparisons, since the original strains of *Spirosoma* described by Migula have been lost. Two methods might be used to resolve the problem. The strains in question could be cultured under the conditions delineated by Migula in his description of *Spirosoma* (12) to determine if the strains resemble his description. Alternatively, *M. flavus* could be compared with Claus' strains that seem to resemble *Spirosoma*. The results of DNA heteroduplex reactions between these strains were not completely satisfactory, as the extent of reaction was somewhat lower than expected for strains of one species. However, physiological comparisons might be of value in resolving the question, but such studies have not as yet been performed.

Since we have not studied *M. major* Gromov, we are not making any proposals concerning its taxonomic status. It cannot be genetically related to *M. aquaticus*, however, because of the large difference in the DNA base compositions of the two organisms. Likewise, the marine strain of Raj with a DNA base composition of 33.7 mol% is genetically unrelated to *M. aquaticus* or *M. flavus*.

The confusion in the classification of the *Microcyclus* can be largely attributed to the use of immotility and ring formation as the sole criteria for inclusion in this genus. Ring formation

is a variable characteristic that is dependent upon nutritional conditions in *M. aquaticus* and *M. flavus*, so that these organisms can appear to be curved rods. In addition, we have described a motile variant of *M. aquaticus*. Thus, motility is also a variable characteristic within this group. Since *M. aquaticus* can appear to be an aerobic, motile, curved rod, perhaps it is related to organisms that are classified as *Pseudomonadaceae*. To test this hypothesis, we examined its genetic relatedness to *P. aeruginosa*, the type species of the genus, and *C. terrigena*, a motile curved rod that is presently classified as conforming to the description of *Pseudomonas*. These two organisms are similar in DNA base composition to *M. aquaticus*. However, they are genetically unrelated to *M. aquaticus*. Thus we cannot show any relation of *M. aquaticus* to the *Pseudomonadaceae* at this time. Nonetheless, the possibility exists that some other strain presently classified in the *Pseudomonadaceae* is related to *M. aquaticus*.

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REPRINT REQUESTS

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