Deoxyribonucleic Acid Base Compositions, and Homology of "Microcycylus," Spirosoma, and Similar Organisms

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The nonmotile, vibrioid, curly or ring-forming bacteria were examined to assess their systematic relationships through determination of the guanine-plus-cytosine contents of their deoxyribonucleic acids and the degrees of homology among the deoxyribonucleic acids of the various genera and species. Our results verify that *Runella*, *Flectobacillus*, *Spirosoma*, and *Ancylobacter* ("Microcycylus") are separate genera. However, our results also indicate that (i) "*Ancylobacter eburneus*" is not a separate species but is in fact a strain of *Ancylobacter aquaticus*, (ii) *Flectobacillus marinus* should not be used to create the new genus "*Cyclobacterium*" and the latter name should be discarded, and (iii) "*Renobacter*" is very similar to *Ancylobacter*, but these two genera exhibit very low levels of deoxyribonucleic acid-deoxyribonucleic acid homology and should probably remain separate at least until additional information is available.

In *Bergey's Manual of Determinative Bacteriology*, 8th ed. (27), all nonmotile, curly or ring-forming vibrios are included in the genus "*Microcycylus*" Ørskov 1928. This genus is a heterogeneous collection of the following three species: "*Microcycylus aquaticus*" Ørskov (20) (the type species), "*Microcycylus major*" Gromov (8), and "*Microcycylus flavus*" Raj (22). Each of these species was described on the basis of a single isolate, and the major unifying characteristics of these organisms were their curved shape and lack of motility. Several investigators (4-6, 11, 15) found that this grouping is unsatisfactory, and after additional isolates were obtained and examined, it was proposed that the three species actually represent separate genera; "*Microcycylus flavus*" was placed in the revived genus *Spirosoma* as *Spirosoma linguale* (15), "*Microcycylus major*" was transferred to the genus *Flectobacillus* as *Flectobacillus major* (15), and "*Microcycylus aquaticus*" remained unchanged.

In later developments within this morphological group, (i) a marine form, "*Microcycylus marinus*" Raj, was described (23) and then moved to the genus *Flectobacillus* (4) as *Flectobacillus marinus*, (ii) two new isolates formed the basis for the establishment of the new genus *Runella* (14), (iii) the new organism "*Renobacter*" was found (19), (iv) a new species of "*Microcycylus*," "*Microcycylus eburneus*" (10), was named, and (v) the family *Spirosomaceae* (12) was created to include the genera *Spirosoma*, *Runella*, and *Flectobacillus* and to separate these genera from the genus "*Microcycylus*."

Early attempts to determine the relationships among these organisms by using deoxyribonucleic acid (DNA)-DNA hybridization demonstrated that "*Microcycylus aquaticus*" was not related to *Spirosoma* sp. or to *F. marinus* (11). Later, on the basis of an unpublished numerical taxonomic study, it was suggested that *F. marinus* is not closely related to *F. major* and should be treated as a separate genus, "*Cyclobacterium*" (H. D. Raj, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, 131, p. 100). It has been suggested (24) that the affiliations among these curly organisms are uncertain and that molecular data are needed to clarify these relationships (25).

Recently, the generic name "*Microcycylus*" Ørskov 1928 has been found to be illegitimate because of precedence of a

fungal genus having the same name. The name *Ancylobacter* has been proposed as a substitute (26) and is used below.

The purpose of this investigation was to determine whether the present classification scheme, which is based primarily on morphology and physiology, is justified by similarities in the DNA compositions of the species and by the levels of DNA homology.

MATERIALS AND METHODS

Bacterial strains. Cultures were obtained from the Deutsche Sammlung von Mikroorganismen (DSM) through the kindness of D. Claus, Gottingen, Federal Republic of Germany and from private collections. The cultures of type strains obtained from the DSM included the Ørskov strain of *Ancylobacter aquaticus* ("*Microcycylus aquaticus*" strain DSM 101), the Gromov strain of *F. major* ("*Microcycylus major*" DSM 109), a *S. linguale* strain (*Spirosoma* strain DSM 74), "*Renobacter vacuolatum*" DSM 1277, and "*Ancylobacter eburneus*" ("*Microcycylus eburneus*") DSM 1106. *A. aquaticus* ("*Microcycylus aquaticus*") strain B was obtained from J. Staley, *F. major* strain S-1 was obtained from R. Metcalf, *F. major* strain 014 was obtained from H. Gude, and *F. marinus* was obtained from H. Raj. *F. major* strains LAH and 3, *S. linguale* strains SLE, EB, and 5, and *Runella slithyformis* strains 4 and 6 were isolated in our laboratory. All cultures were maintained at room temperature on MS agar slants (14) and were transferred biweekly. For *F. marinus*, 3.0% NaCl was added to all growth media.

Extraction and purification of DNA. Cells were harvested after 48 h of growth at 25°C in MS broth and were suspended in 10 ml of a 0.5 M NaCl-0.1 M ethylenediamine-tetraacetic acid solution at pH 8.0. DNA was isolated primarily by the method of Marmur (17), but with the following modification. Lysozyme (0.04%) was added, and after 30 min of incubation at 37°C a 1/800 titer of *Pseudomonas aeruginosa* strain LSU 1 hemolysin (supplied by A. D. Larson) was added to a final concentration of 1.0% (vol/vol), and the preparation was incubated for an additional 1 h. Sodium dodecyl sulfate was then added to a final concentration of 1.0% (vol/vol), and the suspension was heated at 60 to 65°C until lysis occurred. The solution was cooled, and sodium perchlorate was added to a final concentration of 1.0 M. The lysate was extracted with chloroform-isoamyl alcohol (24:1), and the DNA was precipitated in 95% ethyl alcohol.

Additional purification was done by the method of Beh-

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rens et al. (3). The purified DNA was dialyzed twice against distilled deionized water and lyophilized until it was needed.

Determination of DNA base composition. Purified DNA was dissolved in 0.1× standard saline citrate (17) to an optical density at 260 nm of 0.4 to 0.6. A melting profile was obtained by thermal denaturation as described by Mandel and Marmur (16), using a Gilford model 2400 spectrophotometer equipped with a Gilford model 2527 thermoprogrammer. The control DNAs, which were obtained from Sigma Chemical Co., St. Louis, Mo., were *Clostridium perfringens* type XII, *Escherichia coli* type VIII, and *Micrococcus lysodeikticus* DNAs.

DNA-DNA hybridization in free solution. The procedure used for DNA-DNA hybridization was that of Barksdale et al. (2, 12). DNA was dissolved in 0.1× standard saline citrate to a concentration of 150 µg/ml, fragmented by 10 passages through a 26-gauge hypodermic needle, and suspended in an equal volume of 50% formamide in 12× standard saline citrate. The DNAs were melted separately and in pairs by increasing the temperature from 60 to 95°C at a rate of change of 1°C/min. After the absorbance leveled off, the samples were rapidly cooled to the renaturation temperature (melting temperature - 25°C), and the change in absorbance was measured at 270 nm. Levels of homology were calculated from the renaturation rates by using the equation of DeLey et al. (7). All assays were conducted at least twice (usually three times).

RESULTS

Table 1 shows the melting temperatures and the guanine-plus-cytosine (G+C) contents of the DNAs of all of the strains examined, as determined by us or by other workers or both. Three clusters were identified. One cluster contained the two species of *Flectobacillus* (G+C content, 37 to 41 mol%), another contained the genera *Runella* and *Spirosoma* (G+C content, 47 to 51 mol%), and the third contained "Renobacter" and the two species of *Ancylobacter* (G+C content, 65 to 67 mol%). These values confirm and expand the results of previous studies (6, 11, 15).

The results of the DNA-DNA hybridization experiments are shown in Table 2. Three clusters of interstrain and

interspecies homology were detected. Cluster I consisted of the two species of pink-pigmented vibrios (*Flectobacillus*). The four strains of *F. major* showed levels of homology of 82 to 97%, whereas the level of homology between the type strains of *F. major* and *F. marinus* was 71%. Cluster II consisted of the yellow-pigmented, curly *Spirosoma* strains, which had interstrain levels of homology ranging from 86 to 94%. Cluster III consisted of the two nonpigmented, ring-forming *Ancylobacter* species, which had levels of homology of 83 to 93%. "Renobacter" did not cluster with any of these groups. To the left of the 100% homology diagonal line are the levels of homology obtained between strains representing various pairs of genera; these levels ranged from 14 to 55%.

Using these data, we examined the genetic relationship of each organism to every other organism. Figures 1 through 3 group the strains both on the basis of the G+C contents of their DNAs and on the basis of their levels of DNA-DNA homology. Figure 1 shows the relationships of the *F. major* strains to the type strain of each of the other pigmented genera and to *Ancylobacter*. The strains of *F. major* formed a cluster, and very near this cluster was *F. marinus*, which had a level of homology of 71% and the same G+C content in its DNA. A similar analysis for *S. linguale* (Fig. 2) showed that the three strains used for hybridization formed a single cluster that was separated from the other organisms. The two strains of *A. aquaticus* and the single strain of "A. eburneus" also formed a single cluster, which was separated from the other organisms (Fig. 3). "Renobacter" did not cluster with any of the other organisms when both criteria were used.

DISCUSSION

The results described above verify the basic aspects of the classification of the curly or vibrioid, nonmotile bacteria as it has been developed on morphological and physiological considerations over the last decade. It is clear that *Ancylobacter*, *Spirosoma*, *Runella*, and *Flectobacillus* are separate genera and that *Ancylobacter* has little in common with the other organisms. Moreover, because the type strains of *F. major* and *F. marinus* displayed a DNA-DNA homology

TABLE 1. Melting temperatures and G+C contents of the DNAs of the strains examined

Strain	Melting temp of DNA (°C)	G+C content of DNA (mol%)	Previously reported G+C content of DNA (mol%)	Reference(s)
<i>F. major</i>				
DSM 109 ^T (= Gromov ^T)	68.30	37	40	6, 15
LAH	69.70	40		
S-1	69.00	39		
014	69.75	40		
3			40	15
<i>F. marinus</i> Raj ^T	70.30	41	34, 38	11, 23
<i>Runella slithyformis</i>				
4 ^T	73.55	48	50	15
6			49	15
<i>S. linguale</i>				
DSM 74 ^{Ta}			53	15
DSM 75	74.55	50	51, 52	5, 6, 15
SLE	74.70	50		
EB	75.00	50		
<i>A. aquaticus</i>				
DSM 101 ^T (= Ørskov ^T)	82.55	65	67	5, 6, 15, 23
B			68	11
"A. eburneus" DSM 1106	82.10	65		
"Renobacter vacuolatum" DSM 1277	82.50	65		

^a Neotype strain.

TABLE 2. Levels of homology of the DNAs of the strains examined

Strain	% Homology with DNA from:													
	<i>F. major</i> DSM 109 ^T	<i>F. major</i> LAH	<i>F. major</i> S-1	<i>F. major</i> 014	<i>F. mar-</i> <i>inus</i> Raj ^T	<i>Runella</i> <i>slithy-</i> <i>formis</i> 4 ^T	<i>S. lin-</i> <i>guale</i> DSM 74 ^T	<i>S. lin-</i> <i>guale</i> DSM 75	<i>S. lin-</i> <i>guale</i> SLE	<i>S. lin-</i> <i>guale</i> EB	<i>A. aquat-</i> <i>icus</i> DSM 101 ^T	<i>A. aquat-</i> <i>icus</i> B	" <i>A. ebur-</i> <i>neus</i> " DSM 1106	" <i>Renobacter</i> <i>vacuolatum</i> " DSM 1277
<i>F. major</i> DSM 109 ^T	100	93	86	97	71									
LAH		100	92	90	50	I								
S-1			100	82	40									
014				100	63									
<i>F. marinus</i> Raj ^T					100									
<i>Runella</i> <i>slithyfor-</i> <i>mis</i> 4 ^T	50				45	100								
<i>S. linguale</i> DSM 74 ^{Ta}	50					42	100	91	86					
DSM 75								100	93	94	II			
SLE									100					
EB										100				
<i>A. aquaticus</i> DSM 101 ^T	54						50				100	91	83	
B												100	93	III
" <i>A. ebur-</i> <i>neus</i> " DSM 1106							14						100	
" <i>Renobacter</i> <i>vacuola-</i> <i>tum</i> " DSM 1277							55				42	35	32	100

^a Neotype strain.

level of 71% and were very similar in morphology and physiology, as determined previously (4), we feel that it is best to retain *F. marinus* as a member of the genus *Flectobacillus* and that the creation of the new genus "*Cyclobacterium*" for this organism is unwarranted (Raj, Abstr. Annu.

Meet. Am. Soc. Microbiol. 1979). The results of this study, when coupled with the physiological data obtained previously (5, 11, 13-15), verify that the three pigmented genera *Spirosoma*, *Flectobacillus*, and *Runella* should be separated from the nonpigmented, physiologically different genus *An-*

FIG. 1. Relationship of *Flectobacillus* strains to each other and to the type strains of species of *Runella*, *Spirosoma*, and *Ancylobacter*. 1, *F. major* Gromov^T (T = type strain); 2, *F. major* 014; 3, *F. major* LAH; 4, *F. major* S-1; 5, *F. marinus* Raj^T; 6, *Runella* *slithyformis* 4^T; 7, *S. linguale* DSM 74^T; 8, *A. aquaticus* Ørskov^T.

FIG. 2. Relationship of *Spirosoma* strains to each other and to the type strains of species of *Flectobacillus*, *Runella*, "*Renobacter*," and *Ancylobacter*. 1, *S. linguale* DSM 74^T; 2, *S. linguale* DSM 75; 3, *S. linguale* SLE; 4, *F. major* Gromov^T; 5, *Runella* *slithyformis* 4^T; 6, "*Renobacter vacuolatum*" DSM 1277; 7, *A. aquaticus* Ørskov^T; 8, "*A. eburneus*" DSM 1106.

FIG. 3. Relationship of *A. aquaticus* strains to each other and to the type strains of species of "*Renobacter*," *Spirosoma*, and *Flectobacillus*. 1, *A. aquaticus* Ørskov^T; 2, *A. aquaticus* B; 3, "*A. eburneus*" DSM 1106; 4, "*Renobacter vacuolatum*" DSM 1277; 5, *S. linguale* DSM 74^T; 6, *F. major* Gromov^T.

cylobacter. To this end, we previously created the family *Spirosomaceae* to include only *Spirosoma*, *Flectobacillus*, and *Runella*.

"*A. eburneus*" is nearly identical to *A. aquaticus* in its morphology and physiology (R. Borrall, M.S. thesis, Louisiana State University, Baton Rouge, 1981). The molecular data presented here clearly show that "*A. eburneus*" should be considered a strain of *A. aquaticus* and not a separate species.

We were surprised that "*Renobacter*" did not exhibit a higher degree of DNA-DNA homology with *Ancylobacter*. "*Renobacter*" is morphologically and physiologically similar to *A. aquaticus* (Borrall, M.S. thesis), and the two genera have the same G+C content in their DNAs. Moreover, "*Renobacter vacuolatum*" and *A. aquaticus* strain B both have gas vacuoles. Because the level of hybridization of the DNAs of "*Renobacter*" and *A. aquaticus* strain B was less than the level of hybridization obtained between "*Renobacter*" and *Spirosoma*, we feel that it is best for the present to maintain "*Renobacter*" as a separate genus, while recognizing its probable close affinity to *Ancylobacter*.

It is clear that ring formation or a curly shape can no longer be considered a unifying characteristic in taxonomy. Other recently described but unrelated ring-forming or curly bacteria include the photosynthetic organisms *Rhodocyclus* (21), *Clostridium cocleatum*, and *Clostridium spiroforme* (9), as well as strains of *Bacillus subtilis* (18, 28) and *Bacillus stearothermophilus* (1).

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