Fluoribacter dumoffii (Brenner et al.) comb. nov. and *Fluoribacter gormanii* (Morris et al.) comb. nov.

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Strains of "atypical *Legionella*-like organisms" (ALLO) were studied by deoxyribonucleic acid homology. Strains WIGA (ALLO₁) and MI-15 (ALLO₂) were previously shown by Garrity et al. (G. M. Garrity, A. Brown, and R. M. Vickers, Int. J. Syst. Bacteriol. 30:609-614, 1980) to be related, and the name *Fluoribacter bozemanii* was proposed for them. We now show that strains NY-23 (ALLO₄) and TEX-KL are also closely related genetically. Studies with strain LS-13 demonstrate a lower, but significant, degree of homology of LS-13 with strains WIGA, MI-15, and NY-23, justifying inclusion of all of these strains in a single genus, distinct from *Legionella*. It is proposed, therefore, that because of phenotypic similarities and genetic relatedness, strains NY-23, TEX-KL, and LS-13 be classified as members of the genus *Fluoribacter*, which also includes strains WIGA and MI-15. Because NY-23 and LS-13 are the type strains of *Legionella dumoffii* and *L. gormanii*, respectively, we propose the transfer of these species to the genus *Fluoribacter* as *F. dumoffii* (Brenner et al.) comb. nov. and *F. gormanii* (Morris et al.) comb. nov.

Since the discovery of *Legionella pneumophila*, the agent of Legionnaires disease, two other groups of fastidious bacteria have been isolated (1, 7, 9, 13, 19, 21, 22) which share some phenotypic characteristics with *L. pneumophila* (6, 7, 11, 13). However, deoxyribonucleic acid (DNA) hybridization studies demonstrated no significant homology between these strains and *L. pneumophila* (5, 8-10, 13-15, 18), thereby excluding relatedness at the genus level. One group contains the strains known as the Pittsburgh pneumonia agent (PPA) (11, 19) and the TATLOCK strain (9, 11, 21), for which three scientific names, *Legionella micdadei*, *L. pittsburghensis* (18), and *Tatlockia micdadei* (8), have been proposed. Members of the second group are serologically more diverse (7, 13) and have been referred to as atypical *Legionella*-like organisms (ALLO) (7). Two of these bacteria (WIGA and MI-15) appear to share common antigenic determinants by the direct immunofluorescence antibody technique (7) and have been shown to be genetically related at the species level (5, 8). We proposed the name *Fluoribacter bozemanii* for these two isolates (8), whereas the name *Legionella bozemanii* [sic] was proposed by Brenner and co-workers (5). In our previous paper, we presented DNA homology data for WIGA (ALLO₁), MI-15 (ALLO₂), and NY-23 (ALLO₄) (8). In this paper, we now present data for two other members of this group, LS-13 (ALLO₃) (7) and TEX-KL (13),

data which clarify the relationship between these organisms.

MATERIALS AND METHODS

Bacteria. The bacterial strains used in this study are listed in Table 1. Methods of stock maintenance, media preparation, inoculation, and incubation have been previously described (8).

Preparation of DNA. For preparing DNA, a modification of the method of Brenner et al. (3) was used, as previously described (8). Briefly, late-log-phase bacteria were harvested and digested with proteinase K (80 µg/ml) and 0.2% (wt/vol) sodium dodecyl sulfate overnight at 37°C. Lysis was completed by increasing the sodium dodecyl sulfate concentration to 1% (wt/vol) and raising the temperature to 65°C. The lysed culture was then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1). The DNA in the aqueous phase was precipitated by the addition of 80 ml of 95% ethanol, resuspended in 5 ml of 0.1× SSC (saline citrate; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), and digested with deoxyribonuclease-free ribonuclease (50 µg/ml) for 30 min at 37°C and then proteinase K (40 µg/ml) for 2 h. Samples were reextracted once with phenol-chloroform-isoamyl alcohol and twice with chloroform-isoamyl alcohol (24:1). After re-precipitation with 80 ml of 95% ethanol, the DNA precipitate was washed sequentially with 70 and 90% ethanol and redissolved in 10 mM tris(hydroxymethyl)aminomethane (pH 7.2)-1 mM ethylenediaminetetraacetate at 25°C.

Thermal denaturation studies. The guanine-plus-cytosine (G+C) content of the *Fluoribacter* DNA

TABLE 1. *Strains used in this study*

Laboratory designation	Name	Strain	Received from
LDB 1	<i>Legionella pneumophila</i>	Philadelphia 1 (ATCC 33152)	CDC ^a
LDB 2	<i>L. pneumophila</i>	Bellingham	CDC
LDB 4	<i>L. pneumophila</i>	Knoxville 1 (ATCC 33153)	CDC
LDB 5	<i>L. pneumophila</i>	Pontiac	CDC
LDB 6	<i>L. pneumophila</i>	VAMC-L8W	VA ^b
TAT	<i>Tatlockia micdadei</i>	TATLOCK (ATCC 32218)	CDC
PPA 1	<i>T. micdadei</i>	PPA/EK (ATCC 33204)	AWP ^c
PPA 2	<i>T. micdadei</i>	PPA/LR	AWP
PPA 3	<i>T. micdadei</i>	VAMC-PGH 12 (ATCC 33364)	CDC, VA
PPA 4	<i>T. micdadei</i>	PPA/CRES	AWP
WIGA	<i>Fluoribacter bozemanae</i> (<i>L. bozemanii</i>)	WIGA (ATCC 33217)	AWP, LBC ^d
MI-15	<i>F. bozemanae</i>	MI-15	AWP, LBC
LS-13	Unclassified	LS-13 (ATCC 33342)	AWP, LBC
NY-23	Unclassified (<i>L. dumoffii</i>)	NY-23 (ATCC 33279)	AWP, LBC
TEX-KL	Unclassified	TEX-KL (ATCC 33343)	AWP, LBC
EC-1	<i>Escherichia coli</i>	VAMC-EC1	VA

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was determined spectrophotometrically as previously described (8).

Preparation of radioactively labeled DNA. Bacterial DNA was labeled with [α -³²P]deoxycytidine 5'-triphosphate (specific activity, 500 to 750 Ci/mmol) by the nick translation method of Rigby et al. (20). Nick translation reagents were obtained from Bethesda Research Laboratories, Rockville, Md. Labeled DNA was separated from unincorporated substrate by chromatography on a column of Sephadex G-50 fine (1 by 15 cm) equilibrated in 10 mM tris(hydroxymethyl)aminomethane at pH 8.0 (25°C). Probe DNA prepared in this manner had an average specific activity exceeding 8×10^7 cpm/ μ g.

DNA reassociation. Heat-denatured bacterial DNA (8 μ g) prepared from each test strain was added to radioactive probe DNA (0.29 ng) in 100 μ l of 0.28 M phosphate buffer (an equimolar amount of monobasic and dibasic sodium phosphate). Optimal conditions of reassociation were obtained by incubation of this mixture for 96 h at 64°C, at which time more than 85% of the labeled, input DNA had reannealed to homologous unlabeled DNA. To test for the extent of nucleotide mismatching between hybridized segments, DNA reannealing was also performed at 75°C. At 96 h, single- and double-stranded DNAs were separated by a modification of the batch hydroxyapatite method of Brenner et al. (3, 4, 8). Controls for self-annealing of the labeled probe DNA showed generally less than 5% of the reannealing observed in reactions containing an excess (27,500-fold) of homologous unlabeled DNA. These background values were subtracted from all test samples.

The degree of relatedness between organisms is expressed as the amount of DNA bound to hydroxy-

apatite in heterologous reactions as compared with that observed in homologous reactions (100%). Values for each hybridization reaction represent the average of at least four experimental measurements at each temperature.

RESULTS

The results of hybridization studies using DNA from the ALLO strains are presented in Table 2. Values represent the extent of hybrid formation in reciprocal reactions at 64 and 75°C.

The greatest base sequence homologies were observed between TEX-KL and NY-23 and between WIGA and MI-15 at 64°C. Furthermore, the extent of hybridization was essentially unchanged when reannealing was done at the more restrictive temperature (75°C).

Less homology was seen in the other reactions. With three of the combinations tested (LS-13 and MI-15, LS-13 and WIGA, and LS-13 and NY-23), homology ranged from 22 to 34%. In the hybridization reactions with DNA from NY-23 and either WIGA or MI-15, 14 to 22% of the labeled DNA was found to reanneal. The least homology in this group was observed between TEX-KL and either MI-15 or LS-13. In contrast to the reactions of TEX-KL and NY-23 and of WIGA and MI-15 reactions, which demonstrate a high degree of homology at both reaction temperatures, the other combinations exhibited low thermal stability as evidenced by signifi-

TABLE 2. DNA homology between the ALLO strains

Source of unlabeled DNA	Relative % of reannealing obtained with the following source of labeled DNA:									
	WIGA		MI-15		LS-13		NY-23		TEX-KL	
	64°C ^a	75°C ^a	64°C	75°C	64°C	75°C	64°C	75°C	64°C	75°C
WIGA	100 ^b	100	77	81	33	10	14	2	16	8
MI-15	74	76	100	100	34	7	15	2	5	7
LS-13	28	3	28	3	100	100	22	3	9	7
NY-23	16	2	22	1	25	6	100	100	91	94
TEX-KL	7	5	8	4	4	6	94	88	100	100

^a Temperature of reannealing reaction.

^b Homologous reactions have been normalized to 100%. Values are averaged from three to four individual experiments, with duplicate reactions in each experiment.

cantly less reannealing at 75°C compared with that at 65°C.

Studies with DNA from LS-13 and several *L. pneumophila* isolates demonstrated approximately 12% homology; in reactions with LS-13 and isolates of *T. micdadei*, an average of 6% homology was seen. The DNA of other ALLO strains showed no significant homology above background reannealing when reacted with DNA from *L. pneumophila* or *T. micdadei*, nor was significant homology seen between *L. pneumophila* and *T. micdadei* (8).

DISCUSSION

ALLOs are quite similar to *L. pneumophila* and *T. micdadei* with respect to their morphology and fastidious growth requirements. These organisms may be distinguished by several phenotypic differences (8). The colonies of all ALLO strains identified so far demonstrate an intense blue-white fluorescence when exposed to long-wave ultraviolet light (7, 8, 13, 22). In addition, ALLO strains can be distinguished from *L. pneumophila* and *T. micdadei* by differences in branched-chain fatty acid composition (7, 9, 13, 16, 17), by immunological methods (9), and by the absence of oxidase activity (9). Like *L. pneumophila*, the five *Fluoribacter* strains hydrolyzed starch, produced a brown pigment on charcoal-free buffered yeast extract agar containing 2.5 mM tyrosine, and possessed gelatinase activity; *T. micdadei* did not exhibit any of these activities (8).

In general, members of a species have been found to exhibit at least 70% DNA homology under optimal conditions for reannealing (2, 12); in addition, there usually is little difference between the degree of DNA hybrid formation at the optimum temperature and that at the more restrictive reannealing temperature. Genus-level relationships are characterized by much lower levels of DNA homology in the range of 20 to 60% (12). In addition, one frequently observes a large decrease in the thermal stability of the

hybrid DNA molecules formed when more distantly related organisms are studied by this technique; this is due to the presence of unpaired nucleotides in the hybrid (2).

We recently reported the results of DNA homology studies with several strains of *L. pneumophila* and the previously unclassified organisms PPA/TATLOCK and three ALLO strains (WIGA, MI-15, and NY-23). The PPA/TATLOCK strains were grouped as a single species, *T. micdadei*, and for WIGA and MI-15, the name *F. bozemanii* was proposed.

The results of this study show a high level of DNA homology between NY-23 and TEX-KL, suggesting a species-level relationship. Although LS-13 is more distantly related to the other ALLO strains, it occupies a position between WIGA/MI-15 and NY-23/TEX-KL in the genus *Fluoribacter*, with a sufficient degree of homology to justify considering this group as a single genus. Unlike the other *Fluoribacter* strains, LS-13 also demonstrates a low, but consistent, degree of homology with both *L. pneumophila* and *T. micdadei*.

In spite of the extremely close relationship between TEX-KL and NY-23, the degree of homology between the DNA of TEX-KL and that of either MI-15 or LS-13 (5 and 8% and 9 and 4%, respectively, for reciprocal reactions) is much less than that observed between NY-23 and these isolates (14 and 22% and 15 and 25%, respectively, for reciprocal reactions). This suggests that MI-15 and LS-13 have small areas of homology within the region common to TEX-KL and NY-23 and essentially complete homology to that segment unique to NY-23 (approximately 10% of the genome). Although this hypothesis is consistent with the data, it is also possible that some strains possess multiple copies of some genetic segments. Further studies to characterize common sequences are in progress and may lead to the construction of crude genetic maps.

Since this manuscript was submitted for pub-

lication, two papers have appeared in which were proposed the names *Legionella bozemanii* [sic] for WIGA (type strain) and MI-15 (5), *L. dumoffii* for NY-23 (type strain) and TEX-KL (5), and *L. gormanii* for LS-13 (type strain) (15). Significant phenotypic differences (8), as well as differences in DNA G+C content (8) and DNA hybridization data, argue strongly that these organisms do not belong in the same genus as *L. pneumophila*. Therefore, we propose that these organisms be transferred to the genus *Fluoribacter*, a taxon we previously proposed to include *F. bozemaniae*, whose type strain is WIGA (ATCC 33217). Therefore, *L. dumoffii* becomes *F. dumoffii* (Brenner et al.) comb. nov., and *L. gormanii* becomes *F. gormanii* (Morris et al.) comb. nov. The type strain of *F. dumoffii* is NY-23 (ATCC 33279), and that of *F. gormanii* is LS-13 (ATCC 33342). The argument that these organisms belong to the genus *Fluoribacter* is supported by phenotypic similarities and the DNA relatedness between the type strains.

All *Fluoribacter* species are gram-negative rods, 0.5 to 0.7 by 4.0 to 5.0 μm , with short filaments (up to $\sim 25 \mu\text{m}$) and have similar growth characteristics. Strain LS-13 forms slightly longer filaments (up to 50 μm) than do the other strains examined. All demonstrate colony fluorescence under ultraviolet illumination. The G+C content of the DNAs of these organisms is $40.7 \pm 0.7 \text{ mol}\%$ (8); the values for individual *Fluoribacter* strains do not differ significantly from this average. *F. gormanii* and *F. dumoffii* slightly darken charcoal-free yeast extract agar, unlike *F. bozemaniae*, which produces pigment only when the medium is supplemented with 2.5 mM tyrosine.

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REPRINT REQUESTS

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