Tatlockia and *Fluoribacter*: Two New Genera of Organisms Resembling *Legionella pneumophila*

G. M. GARRITY,^{1,2} A. BROWN,^{1,2} AND R. M. VICKERS¹

Medical, Laboratory, and General Medical Research Services, Veterans Administration Medical Center, Pittsburgh, Pennsylvania 15240,¹ and the School of Medicine and Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15261²

DNA homology studies were performed with strains of *Legionella pneumophila* Brenner et al., 1979, including the type strain, ATCC 33152, and a group of seven previously unclassified bacteria which resemble *L. pneumophila* in growth characteristics and colonial morphology and by their content of large amounts of branched-chain fatty acids. The organisms studied include three isolates of the Pittsburgh pneumonia agent (PPA), TATLOCK, WIGA (ALLO₁), MI-15 (ALLO₂), and NY-23 (ALLO₄). The purified DNAs were labeled with ³²P in vitro by a nick translation method, which made it possible to achieve very high specific activities. Results of our hybridization experiments suggest that these previously unclassified organisms are not related to *L. pneumophila* at the genus level, and thus two new genera within the family *Legionellaceae* are proposed for them: *Tatlockia* and *Fluoribacter*. The type species of these genera are *T. micdadei* (Hebert et al., 1980) comb. nov. and *F. bozemanii* sp. nov., respectively. The type strain of *T. micdadei* is strain TATLOCK (= ATCC 33218), and the type strain of *F. bozemanii* is strain WIGA (= ATCC 33217).

Legionnaires' disease, first recognized during an outbreak in Philadelphia in 1976 (9), has been shown to be due to infection with a bacterium (15) for which the name *Legionella pneumophila* has been proposed (5). This organism is gram negative and requires cysteine, iron salts, and a medium buffered at pH 6.9 for optimal growth (8).

Since the isolation of *L. pneumophila*, intensive study of previously unclassified organisms (1, 10, 16, 24) and a search for new isolates (7, 12, 14, 21, 25) from both human and environmental sources has led to the recognition of a number of organisms resembling *L. pneumophila* with respect to nutritional requirements and colonial morphology (7, 12, 14). Of these organisms, *L. pneumophila*, WIGA (ALLO₁), Pittsburgh pneumonia agent (PPA), HEBA, and TATLOCK possess common flagellar antigens (10, 12), a relationship which may exist among all flagellated members of this group of organisms and which may be of taxonomic importance. Cellular fatty acid analysis disclosed the presence of branched-chain fatty acids in unusually large amounts for gram-negative organisms (19), and characteristic fatty-acid patterns were described for *L. pneumophila* and for several others of these bacteria (7, 10, 14, 18, 19).

In this article, we present extensive deoxyribonucleic acid (DNA)-DNA homology data in an attempt to determine the relatedness among members of this group. Since the original sub-

mission of this manuscript for publication, two articles have appeared proposing scientific names for the organisms referred to as TATLOCK and PPA (11, 20); one of the articles also refers to the name *L. bozemanii* for the WIGA bacterium, but a formal proposal is not made (11). Both sets of authors have placed these organisms in the genus *Legionella*. Our data, as well as the available data presented in these other reports, do not support the classification of these organisms into a single genus, but argue strongly for the establishment of at least two new genera for these organisms.

MATERIALS AND METHODS

Bacterial strains. The strains of bacteria used in this study are listed in Table 1.

Media. Working stocks were maintained on buffered charcoal yeast extract (BCYE) agar (20). Media were buffered to pH 6.9 (20°C) with 1% ACES [N-(2 acetamido)-2-aminoethanesulfonic acid, (Research Organics, Inc., Cleveland, Ohio)]. Cultures for DNA extraction were prepared by making a suspension of agar-grown bacteria in buffered yeast extract broth (BYEB) (YEB) (prepared as described by Ristoph et al. [22] except that the charcoal adsorption step was omitted) with the turbidity adjusted to that of a number 5 McFarland standard. A 2-ml amount of this suspension was then inoculated into 500 ml of BYEB. Cultures were incubated for 24 h at 37°C with continuous shaking. Cells of *Escherichia coli* were similarly prepared.

Biochemical tests. Catalase and oxidase tests were performed by previously described techniques (26).

TABLE 1. *Strains used in this study*

Laboratory designation	Name	Strain	Received from ^a	Source of isolation	Reference
LDB ^b 1	<i>L. pneumophila</i>	Philadelphia 1 (= ATCC 33152)	1	Human	5
LDB 2	<i>L. pneumophila</i>	Bellingham	1	Human	5
LDB 3	<i>L. pneumophila</i>	PER	2	Human	
PPA 1	Unclassified	PPA/EK (= ATCC 33204)	3	Human	
PPA 2	Unclassified	PPA/LR	3	Human	
PPA 3	Unclassified	PPA/PGH-12	4	Environmental	
TAT	<i>L. micdadei</i>	TATLOCK (= ATCC 33218)	1	Guinea pig	10, 24
WIGA	Unclassified	WIGA (= ALLO ₁ = ATCC 33217)	1	Human	1, 7, 10
NY-23	Unclassified	NY-23 (= ALLO ₄ = ATCC 33279)	1	Environmental	7
MI-15	Unclassified	MI-15 (ALLO ₂)	1	Human	7
EC-1	<i>E. coli</i>	VAMC-EC1	2		

^a 1, Center for Disease Control, Atlanta, Ga.; 2, VA Medical Center, Pittsburgh, Pa.; 3, A. W. Pasculle, Presbyterian-University Hospital, Pittsburgh, Pa.; 4, Center for Disease Control, Atlanta, Ga., and VA Medical Center, Pittsburgh, Pa.

^b LDB, Legionnaires' disease bacterium.

Carbohydrate fermentation was performed in tubes of unbuffered BYEB containing 1.0% glucose, sucrose, maltose or lactose; 0.001% bromocresol purple was added as a pH indicator. API 20E (Analytabs, Plainview, N.Y.) was inoculated with a suspension of the organism in Mueller-Hinton broth (26) with a turbidity corresponding to a number 8 McFarland standard. These were incubated in air with 2.5% CO₂ at 35°C and were examined at 3 and 5 days. The presence of brown pigment was determined after incubation for 5 days on charcoal-free buffered yeast extract agar with and without added tyrosine (2.5 mM). To test for starch hydrolysis, the organisms were inoculated onto charcoal-free buffered yeast extract agar containing 0.15% soluble starch. After 5 days of incubation, the plates were flooded with Gram iodine solution. Starch hydrolysis resulted in clearing extending 3 to 4 mm beyond the area of growth.

Bromocresol purple spot test. One gram of bromocresol purple (Difco) was dissolved in 100 ml of 1 N KOH. This solution was diluted with distilled water to a final concentration of 0.04%. One drop of this solution was placed onto a piece of Whatman no. 1 filter paper, and a generous portion of growth from a BCYE agar culture was rubbed into the bromocresol purple-saturated area in a circular motion. The color was recorded after 30 s of contact with the dye. A yellow to dull-green reaction was considered negative; a positive reaction was defined as the development of an aquamarine color.

Preparation of DNA. DNA was prepared by a modification of the method of Brenner et al. (3). Late log-phase bacteria were harvested by centrifugation and were resuspended in 40 ml of phosphate-buffered saline (Ca²⁺- and Mg²⁺-free) containing 0.1 M ethylenediaminetetraacetic acid (EDTA). The bacteria were digested with proteinase K (80 µg/ml) and 0.2% (wt/vol) sodium dodecyl sulfate overnight at 37°C. At that time, the sodium dodecyl sulfate concentration was increased to 1% (wt/vol), and the temperature was raised to 65°C; as a result, the suspension cleared. The lysed culture was then extracted with an equal volume

of phenol-chloroform-isoamyl alcohol (24:24:1) for 5 min with continuous shaking, and the phases were separated by centrifugation (10,000 × *g* for 10 min). The aqueous phase was carefully removed, and the DNA was precipitated by the addition of 2 volumes (approximately 80 ml) of 95% ethanol. The DNA was allowed to dissolve overnight in 5 ml of 0.1× SSC (saline citrate, 1× is 0.15 M NaCl-0.015 M trisodium citrate, pH 7.0), after which it was digested sequentially with deoxyribonuclease-free ribonuclease (50 µg/ml) for 30 min at 37°C and with proteinase K (40 µg/ml) for 2 h. The DNA samples were re-extracted once with phenol-chloroform-isoamyl alcohol and twice with chloroform-isoamyl alcohol (24:1), after which the DNA was re-precipitated with ethanol. The precipitate was washed with 70 and 90% ethanol and was redissolved in 10 mM tris(hydroxymethyl)-aminomethane (Tris, pH 7.2 at 25°C)-1 mM EDTA.

Thermal denaturation studies. Samples of bacterial DNA, dissolved in 1× SSC, were adjusted to an absorbance of approximately 0.8 at 260 nm. A solution of deoxyadenosine triphosphate was similarly prepared and served as a reference which allowed for the automatic correction of absorbance changes due to thermal expansion. Samples (300 µl) were quickly brought to 78°C in tightly stoppered quartz cuvettes in a Gilford model 2000 recording spectrophotometer equipped with a model 2527 thermoprogrammer. The change in absorbance of each sample was followed as the temperature was increased at a rate of 0.5°C/min. The melting temperature (*T_m*) of each sample was calculated by using the equation of Marmur and Doty (15), with a corrected slope of 0.42°C/1% rise in guanine + cytosine (G+C) (based on our instrument calibration).

Preparation of radioactively labeled DNA. Bacterial DNA was labeled in vitro with [α -³²P]deoxycytosine triphosphate (specific activity, 500 to 750 Ci/mmol) by the nick translation method of Rigby et al. (22). Samples (20 µl) of each DNA solution, diluted to 10 to 12 µg/ml in Tris-hydrochloride, pH 7.2 (25°C), were added to a reaction mixture (80 µl) containing 18

μ M deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate, and 1.8 nM [α - 32 P]deoxycytosine triphosphate (specific activity, 500 to 750 Ci/mmol) in translation buffer (50 mM of Tris-hydrochloride, pH 7.8, 25°C; 5 mM of β -mercaptoethanol; and 50 μ g of nuclease-free bovine serum albumin per ml). After incubation with activated deoxyribonuclease I (EC 3.1.4.5) (1 ng/100- μ l reaction) for 10 min at 15°C, *E. coli* DNA polymerase I (EC 2.7.7.7) (2 U) was added, and incubation was continued for 75 min at 15°C. Reactions were terminated by the addition of 10 μ l of 0.3 M disodium EDTA (pH 8.0). Labeled DNA was separated from unincorporated substrate by chromatography on a column of Sephadex G-50 fine (1 by 15 cm) equilibrated with 10 mM Tris, pH 8.0 (25°C). Probe DNA prepared in this manner had an average specific activity exceeding 8×10^7 cpm/ μ g (nick translation reagents were obtained from Bethesda Research Laboratories, Rockville, Md.).

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 PB (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 (T_m , -25°C in 0.28 M PB for DNA containing 40% G+C), at which time more than 85% of labeled input DNA had reannealed to homologous unlabeled DNA. To test for the presence of unpaired bases in hybridized segments, DNA reannealing was also performed at 75°C for selected combinations (2, 5). At 96 h, single- and double-stranded DNAs were separated by a modification of the batch hydroxyapatite method of Brenner et al. (3, 4). Samples diluted to 0.12 M PB were added to 1.0 ml of freshly prepared hydroxyapatite equilibrated in the same buffer at 65°C. After vortex mixing, the samples were incubated at 65°C for 15 min, at which time they were centrifuged at $1,000 \times g$ for 60 s in centrifuge carriers heated to 75°C. The supernatant, containing single-stranded DNA, was decanted, and the hydroxyapatite pellets were resuspended in 3 volumes (3.0 ml) of 0.12

M PB and held at 65°C for 10 min with occasional mixing. This washing procedure was repeated six times. Double-stranded DNA was then eluted from hydroxyapatite by the addition of 3.0 volumes (3.0 ml) of 0.4 M PB followed by incubation in a boiling-water bath for 10 min. After centrifugation, the supernatant was collected in scintillation vials and counted in an aqueous, xylene-Triton X-100-based liquid scintillation cocktail. Controls for self-reannealing of the labeled probe DNA were generally less than 5% of that observed in reactions containing an excess (27,500 $\times$) of homologous unlabeled DNA, and these values were subtracted from all test samples.

The degree of relatedness between organisms is expressed as the amount of DNA bound to hydroxyapatite in heterologous reactions as compared with that observed in homologous reactions normalized to 100%. Values represent averages of at least two experiments, each of which was done with duplicate reactions.

RESULTS AND DISCUSSION

The results of the DNA homology studies are presented in Table 2. With these data, the organisms tested can be separated into three groups. The isolates of *L. pneumophila* form one group, as previously reported (5), and show a high degree of sequence homology ($\geq 80\%$) between the individual strains. However, these three *Legionella* isolates show little or no homology to the other bacteria tested. These results are in agreement with previously published studies in which strains TATLOCK and WIGA were shown to be unrelated to *L. pneumophila* by DNA homology (10) and by serological techniques (6, 7).

The PPA strains and TATLOCK comprise a second group of bacteria which show a high degree ($>70\%$) of intergroup sequence homology at both 64°C and the more stringent tempera-

TABLE 2. Results of DNA relatedness studies

Source of unlabeled DNA	Relative % reannealing obtained with (source of labeled DNA):											
	PPA		TAT		LDB 1		WIGA		MI-15		NY-23	
	64°C ^a	75°C ^a	64°C	75°C	64°C	75°C	64°C	75°C	64°C	75°C	64°C	75°C
PPA 1	100	100	72	77	0	2	2	0	0	0	0	0
PPA 2	82	85	84	90	4	NT ^b	0	NT	0	NT	0	NT
PPA 3	90	83	79	85	1	NT	0	NT	0	NT	0	NT
TAT	71	73	100	100	8	4	5	0	3	0	0	0
LDB 1 ^c	0	1	0	0	100	100	3	0	3	0	1	0
LDB 2	0	NT	0	NT	80	NT	0	NT	0	NT	0	NT
LDB 3	0	NT	0	NT	86	NT	0	NT	0	NT	0	NT
WIGA	4	0	10	4	13	7	100	100	77	81	14	0
MI-15	5	0	2	1	14	8	74	79	100	100	15	0
NY-23	5	0	7	3	14	8	16	1	22	0	100	100
<i>E. coli</i>	0	NT	2	NT	6	NT	3	NT	8	NT	0	NT

^a Reannealing temperature.

^b NT, Not tested.

^c LDB, Legionnaires' disease bacterium.

ture, 75°C. This group appears to be unrelated to either *L. pneumophila* or to the other organisms tested. Previous studies have shown that PPA, TATLOCK, and another isolate (HEBA) are related antigenically and possess similar cellular fatty acid compositions, which are different from those of *L. pneumophila* and WIGA (10, 12).

WIGA, MI-15 and NY-23 have been grouped together as "atypical *Legionella*-like organisms" (ALLO) based upon their growth and morphological similarities to *L. pneumophila* (7). A differentiating feature is the intense, nondiffusible, blue-white fluorescence emitted by ALLO colonies grown on charcoal yeast extract agar (7). Direct fluorescent-antibody studies by Cordes et al. (7) suggest an immunological relationship between WIGA and MI-15; however, NY-23 did not cross-react. Our study shows that of these organisms, WIGA and MI-15 show a high degree (>70%) of sequence homology at both temperatures, whereas NY-23 shows a low degree of homology to WIGA or MI-15 only at 64°C, thereby defining at least two subgroups.

Although the presently unclassified strains TATLOCK, PPA, WIGA, MI-15, and NY-23 share many phenotypic characteristics with *L. pneumophila*, our hybridization studies point out significant differences in DNA homology between these organisms. It has been suggested that members of a species usually show 70% or greater sequence homology, and members of the same genus show 25 to 60% homology (2, 13). On the basis of these criteria, our data suggest that those organisms currently identified as TATLOCK and PPA are members of the same species and should be placed in a genus different from *Legionella*. Similarly, the DNA homology between WIGA and MI-15 suggests species level relatedness of these organisms.

Thermal denaturation studies support the proposed separation of these bacteria into three genera. *L. pneumophila* strains had a T_m of $85.6 \pm 0.4^\circ\text{C}$, which corresponded to a mol% G+C of $38.8 \pm 0.9\%$ ($n = 8$). The DNAs of TATLOCK and the several PPA strains had T_m 's of $87.9 \pm 0.3^\circ\text{C}$, corresponding to $44.3 \pm 0.7\%$ mol% G+C ($n = 9$); and the ALLO strains tested had T_m 's of $86.4 \pm 0.3^\circ\text{C}$, corresponding to $40.4 \pm 0.7\%$ mol% G+C ($n = 6$). These differences are statistically significant by Student's *t* test at the $P < 0.01$ level.

Since submission of this manuscript, the names *Legionella micdadei* (11) and *L. pittsburgensis* (20) have been proposed for strains PPA and TATLOCK. We propose, based upon DNA homology, that *L. micdadei* Hebert et al., 1980 be transferred to a new genus, *Tatlockia*

(*Tat.lock'i.a*, M.L. gen. noun *Tatlockia*, of Tatlock; named for Hugh Tatlock, the individual who originally isolated this organism), as *Tatlockia micdadei* (Hebert et al., 1980) comb. nov. The type species of *Tatlockia* is *T. micdadei*, of which the type strain is strain TATLOCK (= American Type Culture Collection [ATCC] 33218). We believe that strains WIGA and MI-15 belong to a new genus and species, for which we propose the names *Fluoribacter* (*Flu.or.i.bac'ter*, L.V. *fluor*, to flow, because of the fluorescence of this organism; M.L. masc. n. *bacter*, the masculine form of the Gr. neut. n. *bactrum*, a rod or staff; M.L. masc. n. *Fluoribacter*, fluorescent rod) and *F. bozemaniae* (*boze.man'ae*, M.L. gen. noun *bozemaniae*, of Bozeman, named for F. Marilyn Bozeman, who was of importance in the recognition of this group of organisms), respectively. *F. Bozemaniae* is the type species of the genus, and strain WIGA (= ATCC 33217) is the type strain of the species. A name is not proposed for the NY-23 isolate, because further studies are required to elucidate its relationship to other members of this group.

Members of the family *Legionellaceae* produce colonies which are white to slightly grayish on BCYE agar. Occasionally, a faint pinkish or greenish sheen may be noted in confluent areas. After incubation for 5 days, *L. pneumophila* colonies become flatter with opaque centers and are slightly larger (2 to 4 mm) than colonies of *T. micdadei* or *F. bozemaniae* (2 to 3 mm). In addition, *Legionella* colonies are sticky and tenaceous compared with the buttery consistency of colonies of *Tatlockia* or *Fluoribacter*. Two colony types have been described for *Tatlockia*: a slowly growing, white colony which develops protuberences. These protuberences on subculture give rise to the second, more rapidly growing, yellow-green colony type (20). Colonies of *Fluoribacter* fluoresce brightly when illuminated with longwave UV light. When examined with a dissecting microscope, colonies of all three species have a clear, glistening to gelatinous surface with an opaque, ground-glass interior. On BCYE agar containing 0.001% bromocresol purple and 0.001% bromothymol blue, *Legionella* colonies are white to pale green, *Fluoribacter* colonies are a brighter green, and *Tatlockia* colonies are blue-gray (Vickers, Brown, and Garrity, submitted for publication). Although growth of these organisms is best on BCYE agar, adapted growth, although poor, may be occasionally obtained on a variety of nutritionally complex, charcoal-free media. However, growth has not been observed on unsupplemented sheep blood agar.

All members of this family are slender, pale,

TABLE 3. Phenotypic differentiation of species

Character	<i>L. pneumophila</i>	<i>T. micdadei</i>	<i>F. bozemanæ</i>
Color on dye-containing agar ^a	White-green	Blue-gray	Green
Brown pigment production:			
No added tyrosine	±	—	—
With added tyrosine	+	—	+
Catalase ^b	+	++	++
Oxidase ^c	+	±	—
Starch hydrolysis	+	—	+
Gelatinase	+	—	+
Colony fluorescence	—	—	+
Bromocresol purple spot test	—	+	—

^a BCYE agar with 0.001% bromothymol blue and 0.001% bromocresol purple.

^b ++, Immediate effervescence; +, effervescence after 10 s.

^c +, Dark blue in <60 s; ±, dark blue in >60 s.

gram-negative rods (4 to 5 µm by 0.5–0.7 µm) with blunt to slightly rounded ends. *Tatlockia* and *Fluoribacter* occasionally have tapered ends and may be slightly curved, with short filaments, to about 25 µm. *Legionella* cells are more pleomorphic and tend to form long, vine-like filaments in culture. From tissues, *Tatlockia* may be partly acid-fast (20). Although motility of some strains has been reported (10) and polar flagella have been demonstrated (10, 12, 20), these organisms are usually nonmotile after agar passage. Motility of some strains may be restored by passage on semisolid media.

Although growth occurs, no gas or acid production can be demonstrated in charcoal-free, unbuffered yeast extract broth containing glucose, maltose, lactose, or sucrose. All of these organisms are nitrate negative. *L. pneumophila* produces a poorly diffusible, brown pigment on charcoal-free yeast extract agar, whereas *F. bozemanæ* produces visible pigmentation only if the medium is supplemented with 2.5 mM tyrosine. On this medium, all *L. pneumophila* strains produce pigment. *T. micdadei* produces no pigment on either medium. *L. pneumophila* and *F. bozemanæ* are gelatinase positive and hydrolyze starch; *T. micdadei* is negative for these activities. No other biochemical reactivity could be detected by the API 20E. Table 3 shows those characteristics which may be used to differentiate *T. micdadei*, *F. bozemanæ*, and *L. pneumophila*.

Until further data are available, it is appropriate to include these organisms in the family *Legionellaceae* because of their phenotypic similarities.

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REPRINT REQUESTS

Address reprint requests to: Arnold Brown, M.D., Chief, Infectious Disease Section, VA Medical Center, University Drive C, Pittsburgh, PA 15240.

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