JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1982, p. 290–297
0095-1137/82/020290-08$02.00/0

Vol. 15, No. 2

Legionella jordanis: a New Species of *Legionella* Isolated from Water and Sewage

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Received 27 August 1981/ Accepted 10 November 1981

Legionella jordanis sp. nov., as found in two cultures, is described. One isolate was from river water in Indiana and the other isolate was from sewage in DeKalb County, Ga. The former is the type strain of the species, and is designated BL-540 (ATCC 33623). *L. jordanis* had a partial relationship to *L. bozemanii* by direct fluorescent-antibody tests but was unrelated to *L. pneumophila, L. dumoffii, L. micdadei, L. gormanii*, or *L. longbeachae*. *Legionella* phenotypic characteristics, including large amounts of branched-chain cellular fatty acids, were shown by the isolates. Studies of DNA relatedness showed that the two cultures of *L. jordanis* were only slightly related to the six previously described species of *Legionella* but were more than 90% related to each other. Indirect fluorescent-antibody tests with human sera suggested that unrecognized human infections with *L. jordanis* may be occurring.

The recognized species of *Legionella* are *L. pneumophila* (4), *L. bozemanii* (3), *L. micdadei* (16, 27), *L. dumoffii* (3, 17), *L. gormanii* (20), and *L. longbeachae* (18). In this report, we describe a new species for which the name *Legionella jordanis* sp. nov. is proposed. The type strain of *L. jordanis* is BL-540 (ATCC 33623). Although *L. jordanis* has not been isolated from human infections, seroprevalence data indicate that it may be involved in human disease.

MATERIALS AND METHODS

Isolation of cultures. The type culture of *L. jordanis*, BL-540, was isolated from a water sample taken from the Jordan River near the Indiana Memorial Union at the University of Indiana in Bloomington, where an outbreak of Legionnaires disease occurred in early 1978 (28). A portion of the water sample was injected into a guinea pig. A homogenate of the infected animal's spleen was then injected into embryonated hen eggs, and the culture designated BL-540 was isolated from the yolk sac of the embryo (19) on charcoal-yeast extract (CYE) agar (9).

A second culture of this species, which was designated strain ABB-9, was isolated in June, 1980, from sewage collected from the dispensing arm of a trickling filter bed at a disposal plant in DeKalb County, Ga. About 1 liter of the sewage was pressure filtered, and the bacteria were collected, centrifuged, and concentrated to 5 ml as described elsewhere (26). A guinea pig was injected intraperitoneally with 0.3 ml of this sample. After its temperature rose, the guinea pig was sacrificed on the seventh day after the injection, and the peritoneal fluid and spleen were cultured on CYE agar. Fluorescent-antibody stains of the tissues were negative, but colonies resembling *Legionella* spp. appeared on the plate 7 days after inoculation with peritoneal fluid.

DFA tests. In an attempt to identify the two isolates, we performed direct fluorescent-antibody (DFA) tests as described previously (26). Labeled antibodies for the following species and serogroups were used: *L. pneumophila* serogroups 1 through 6, *L. bozemanii, L. dumoffii, L. micdadei, L. gormanii*, and *L. longbeachae* serogroups 1 and 2.

Preparation of antisera and fluorescent-antibody conjugates for DFA tests. Heavy cell suspensions (40 IU of turbidity or 4×10^9 cells per ml) were prepared from the growth on CYE agar and killed with 1% Formalin in 0.85% NaCl. These antigens were used to immunize rabbits by a published protocol, except that Freund adjuvant was not used (7). Immunoglobulin G was separated from the serum on a column of *Staphylococcus* protein A Sepharose (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) as described by Goding (12). The antibody was labeled with fluorescein isothiocyanate, and the protein was adjusted to 5 mg/ml by methods previously described (15).

Antibody absorption studies. Antigens for each absorbing culture were prepared by harvesting the cells from 10 plates of CYE agar that had been inoculated and incubated for 48 h at 35°C. Cells were harvested in a total of 20 ml of 1% Formalin in 0.85% NaCl. The cells were collected by centrifugation, washed once by being blended (with a Vortex mixer) with the saline-Formalin solution, and packed by recentrifugation. This cell pack was adequate to absorb about 2.0 ml of conjugate containing about 10 mg of immunoglobulin G.

Homologous and heterologous titrations of all conjugates were performed by DFA tests with formalinized (1%) cell suspensions of relevant cultures in

0.85% NaCl. The cultures consisted of BL-540, ABB-9, *L. bozemanii* (WIGA), serogroups 1 through 6 of *L. pneumophila*, a culture (OR-2) of *L. pneumophila* antigenically related to serogroup 4, *L. dumoffii* (TEX-KL), *L. micdadei* (TATLOCK), *L. gormanii* (LS-13), four isolates of *L. longbeachae* belonging to serogroup 1 (LB-4, LA-24, Concord 1, and Atlanta 5), and one culture of *L. longbeachae* of serogroup 2 (Tucker 1) (2). In addition, the conjugate for ABB-9 was tested against three non-*Legionella* cultures serologically related to *L. pneumophila* of serogroup 1 (two cultures of *Pseudomonas fluorescens*, CDC-93 and Evans blue, and a culture of the *Flavobacterium-Xanthomonas* group designated CDC-65).

IFA assay for antibodies against BL-540 and ABB-9 in human sera. The indirect immunofluorescence assay was done as described previously (35) with a polyimmunoglobulin antihuman conjugate and heat-killed antigens of *L. pneumophila* serogroups 1 through 6, *L. bozemanii, L. dumoffii, L. gormanii, L. micdadei, L. longbeachae* serogroups 1 and 2, strains BL-540 and ABB-9, and eight additional *Legionella*-like organisms that are currently under investigation. Titers were expressed as the reciprocal of the highest serum dilution giving 1+ fluorescence intensity. Interpretive criteria for positive titers were as described previously for *L. pneumophila* (34).

Morphological, staining, and physiological tests. The two isolated cultures, BL-540 and ABB-9, were stained by the Gram method (Hucker modification), by a fat stain (Sudan black B) for the presence of storage material, and by the Wirtz-Conklin method for demonstrating spores. Acid-fast stains were performed by the Ziehl-Neelsen procedure. The cultures were streaked on Trypticase soy agar containing 5% of sheep blood (BBL Microbiology Systems, Cockeysville, Md.) and on CYE agar (9). The incubation temperature for all tests was 35 to 36°C unless otherwise stated.

Plate cultures were incubated in candle extinction jars. The methods of Weaver and Feeley (33) were used to test 24- to 48-h cultures on CYE slants for oxidase and catalase. Urea hydrolysis was determined by heavy inoculation of Christensen urea agar slants with 48-h cultures grown on CYE agar. The cultures were tested for the production of a brown, water-soluble pigment after growth on yeast extract agar medium which had been absorbed with charcoal by the method of Ristroph et al. (29) and supplemented with 2.0 mmol of L-tyrosine per liter (1). The chromogenic cephalosporin test for the detection of β-lactamase production was performed as described by Thornsberry et al. (32). Cultures on CYE were examined for autofluorescence at a 366-nm wave length by use of a Woods lamp (3, 8). The isolates were grown on CYE agar slants containing 0.2% KNO_3 to test for nitrate reduction (33). The ability to hydrolyze gelatin was tested in a CYE medium in which the agar was replaced with 3% gelatin (25). The ability to hydrolyze sodium hippurate was tested by the method of Hébert (13). A heavy loopful of culture from a CYE slant was suspended in 1.0 ml of disodium-*p*-phenyl phosphate to test for alkaline phosphatase as described by Orrison et al. (25). The ability to hydrolyze diacetylfluorescein, 4-methylumbelliferyl propionate, and 4-methylumbelliferyl nonanonate was tested in similar fashion (25). Appropriate positive and negative controls were used in all tests.

GLC studies of cellular fatty acids. Cells for fatty acid analysis were obtained after overnight growth on CYE agar slants. Growth from one slant was removed with about 1 ml of sterile distilled water and transferred to a test tube (20 by 150 mm) containing 5 ml of 5% NaOH in 50% aqueous methanol. The tubes were sealed with Teflon-lined caps, and the cellular lipids were saponified for 30 min at 100°C. Fatty acids were then extracted and methylated as described previously (21). The fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) on both packed and capillary columns by using a flame ionization detector (21). The peaks were tentatively identified by retention time data, and quantitation was done with a computing integrator (Hewlett-Packard, Avondale, Pa.). All of the peaks were positively identified from the results obtained from hydrogenation, acetylation, and GLC-mass spectrometry (23).

DNA relatedness studies. Each of the strains of the various species (3–5, 16, 18, 20) used in DNA relatedness studies was grown on 12 to 20 standard plastic petri dishes containing CYE agar. The plates were incubated at 36 ± 1°C either in candle jars or in an atmosphere containing 2.5% CO_2 until growth was confluent (4 to 5 days). Cells from each of the plates were harvested, pooled, and suspended in 10 ml of buffer containing 0.1 M NaCl, 0.05 M Tris, 0.05 M EDTA, and 50 μg of pronase per ml. The extraction and purification of DNA, the in vitro labeling method, and details of the hydroxyapatite procedure used to determine DNA relatedness have been described elsewhere (5).

RESULTS

DFA tests of isolates with conjugates for recognized serogroups and species of *Legionella*. When BL-540 and ABB-9 cells were tested with the diagnostic dilutions of polyvalent and monovalent conjugates for the six species, including all six serogroups of *L. pneumophila* and both serogroups of *L. longbeachae* listed in Materials and Methods, only very slight cellular fluorescence was observed, with one exception. The conjugate for *L. bozemanii* (WIGA) gave significant cellular staining (1+ to 3+), with visibly stained flagella.

Antibody absorption studies with DFA conjugates. The results of homologous and heterologous DFA staining tests with absorbed and unabsorbed conjugates for BL-540, ABB-9, and *L. bozemanii* (WIGA) are shown in Table 1. The ABB-9 conjugate had a titer of 1,024 for both BL-540 and ABB-9 cells. Against the BL-540 conjugate, the homologous antigen titer was 1,024, and against ABB-9 it was 512. *L. bozemanii* WIGA and GA-PH were the only other cultures among those tested that were stained appreciably by either conjugate. The GA-PH strain (3) and the MI-15 strain (8) are presumed to be identical because they were isolated from the same patient and have the same characteristics.

When conjugates for BL-540 and ABB-9 were

TABLE 1. Relationship of *L. jordanis* BL-540 and ABB-9 to each other and to *L. bozemanii* (WIGA), as determined by DFA staining tests

Antigen[b]	Titer for indicated conjugate[a]							
	WIGA		BL-540			ABB-9		
	Unabsorbed	Absorbed with BL-540	Unabsorbed	Absorbed with WIGA	Absorbed with ABB-9	Unabsorbed	Absorbed with WIGA	Absorbed with BL-540
WIGA	256	512	32	<4		256	<2	
BL-540	16	<4	1,024	64	<4	1,024	64	<2
ABB-9	32	<4	512	64	<4	1,024	256	<2

[a] Numbers represent highest dilution factors at which staining intensity was at least 2+ to 3+ on a scale of 0 to 4+.

[b] Cell suspensions (10^7/ml) were in 0.85% NaCl containing 1% Formalin.

cross-absorbed and tested against their homologous and heterologous antigens, no cellular fluorescence was seen at the 1:4 dilutions (Table 1). These results show that the two isolates were serologically identical, as determined by DFA tests.

The relationship of BL-540 and ABB-9 to *L. bozemanii* was explored by absorbing WIGA conjugate with BL-540 and BL-540 and ABB-9 conjugates with WIGA antigen. These results indicate a partial relationship between WIGA and cultures of the new species (Table 1).

IFA with human sera. Of 444 serum pairs from cases of suspected Legionnaires disease that were tested against multiple *Legionella* antigens in a seroprevalence survey, 5 showed evidence of specific antibody titers against the ABB-9 and BL-540 antigens (i.e., titers against *L. jordanis* that were fourfold or greater than those against any other antigen tested [H. W. Wilkinson, A. L. Reingold, B. J. Fikes, D. L. McGiboney, T. A. Thompson, C. V. Broome, and G. W. Gorman, manuscript in preparation]). In all cases, titers against the two cultures of *L. jordanis* agreed within the acceptable twofold dilution factor, although there was a tendency for any discrepant titers to be one tube dilution factor higher against the ABB-9 antigen. A greater than fourfold rise in titer (seroconversion) occurred for two cases within 25 days after onset of illness: one from 64 to 2,048 and the other from 64 to 8,192. Two additional cases had both acute-phase (within 2 days) and convalescent-phase (21 ± 1 day) titers that were 1,024, presumptive evidence of infection at an unknown time. The remaining case showed a greater than fourfold decrease in IFA titer, from the first serum titer of 1,024 to the second one of 128, over a 2-month period. The date of onset was unknown for the latter case. The five sera came from four states: Florida, Indiana, Missouri, and Oklahoma.

Morphological, staining, and physiological characteristics of the species. Cultures of BL-540 and ABB-9 are thin, gram-negative rods consistent in size with members of *Legionella*, i.e., 0.3 to 0.9 μm wide by 2 to 20 μm long (6). Motile by polar flagella, they are non-spore-forming, nonencapsulated, and non-acid-fast organisms. When stained with Sudan black B, very few cells contained fat deposits.

The cultures failed to grow on either blood agar or Trypticase soy agar but gave growth typical of legionellae on CYE agar slants and plates. Colonies appeared about the third day of incubation and continued to increase in size for several days. They were grey, with a smooth but textured surface (ground glass appearance), and raised, with an entire edge. In Table 2, the important phenotypic characteristics of BL-540 and ABB-9 are compared with those of the six other species of *Legionella*. In addition, an esterase produced by cultures of *L. jordanis* readily hydrolyzed 4-methylumbelliferyl propionate and 4-methylumbelliferyl nonanoate to produce the intensely blue fluorescent 4-methylumbelliferone (25, 30). Diacetylfluorescein was hydrolyzed, resulting in the production of a brownish-green color different from that seen in tests with other *Legionella* spp. (25). Alkaline phosphatase activity was not demonstrated.

Characterization of the cellular fatty acids by GLC-mass spectrometry. Previous studies have established that all known species of *Legionella* characteristically contain relatively large amounts of branched-chain cellular fatty acids (14, 17, 22, 24). The data in Table 3 show that the fatty acids of strains BL-540 and ABB-9 were qualitatively similar to those of other legionellae. The single most abundant acid in each strain of the new species was a saturated, branched-chain, 15-carbon acid (a-15:0)—the methyl branch was at the *anteiso* (antipenultimate) carbon atom—and the second most abundant was an a-17:0 acid. The next most abundant acids were a saturated, branched-chain, 16-carbon acid (i-16:0)—the methyl branch was at the *iso* (penultimate) carbon atom—a mono-unsaturated, 16-carbon, straight-chain acid (16:1), and a normal saturated, straight-chain, 16-carbon acid

TABLE 2. Phenotypic characteristics of *Legionella* species[a]

Characteristic	*L. jordanis*	*L. pneumophila*	*L. bozemanii*	*L. micdadei*	*L. dumoffii*	*L. gormanii*	*L. longbeachae*
Growth on agar							
CYE	+	+	+	+	+	+	+
Blood (BAB or TSA)[b]	−	−	−	−	−	−	−
Blue fluorescence (CYE agar)[c]	−	−	+	−	+	+	−
Browning of FG or YE agar[d]	+	+	+	−	+	+	+
Stain							
Gram	−	−	−	−	−	−	−
Acid-fast	−	−	−	−			
Leifson or DFA[e]	+	+	+	+	+	+	+
Motility	+	+	+	+			
Biochemical reaction							
Oxidase	+	+ or −	−	+	−	−	+
Catalase	+	+	+	+	+	+	+
Urease	−	−	−	−	−	−	−
Gelatin liquefaction	+	+	+	+	+	+	+
$NO_3 \rightarrow NO_2$	−	−	−	−	−	−	−
β-lactamase	+	+	±	−	+	+	+ or −
Hippurate hydrolysis	−	+	−	−	−	−	−

[a] Data for *L. longbeachae* and certain data for species other than *L. jordanis* were taken from prior publications (1, 3, 7, 8, 13, 14, 18, 20, 31).
[b] BAB, Blood agar base; TSA, Trypticase soy agar.
[c] Long wave length (366 nm); UV light.
[d] FG, Feeley-Gorman (10); YE, yeast extract.
[e] DF Antibody or Leifson stains of flagella. Flagella are polar.

(16:0). Thus, the overall composition of the fatty acids of the new species is quantitatively similar to the composition of the acids of *L. bozemanii, L. dumoffii, L. gormanii*, and *L. micdadei* but is distinct from the composition of the acids of *L. pneumophila*, which contains i-16:0 as the major acid (Table 3).

DNA relatedness. *Legionella*-like organisms received by our laboratory are first tested for DNA relatedness to the type strains of existing species. Unlabeled DNA from BL-540, therefore, was tested against labeled DNAs from the six recognized *Legionella* species. Relatedness of BL-540 to the other legionellae was between 4 and 20% when reactions were done at a 60°C incubation temperature and from 0 to 10% when reactions were done at the more stringent 75°C incubation temperature (Table 4). These data indicated that BL-540 was a new species of *Legionella*. Because the phenotypic characteristics of ABB-9 were extremely similar to those of BL-540, DNA from ABB-9 was labeled and tested against BL-540 DNA. The two strains were almost identical on the basis of relatedness at both 60 and 75°C, as well as on the basis of the lack of appreciable divergence within related sequences (Table 4). ABB-9 was less than 10% related to DNAs from other *Legionella* species (Table 4). Thus, BL-540 and ABB-9 belonged to the same new species.

DISCUSSION

The two environmental isolates, BL-540 and ABB-9, of *L. jordanis* were identical in their most important attributes. In reciprocal cross-absorption tests with direct fluorescent-antibody conjugates, each antigen completely exhausted the antibody of the heterologous conjugate. Each had a minor serological relationship to *L. bozemanii* (WIGA) but was unrelated to any of the other described species or serogroups of *Legionella* or to any of several *Legionella*-like organisms or non-*Legionella* species except for MI-15 and GA-PH, cultures similar if not identical to WIGA.

Biochemically, the characteristics of the two isolates conformed to those described for other *Legionella* spp., except that the hydrolysis of diacetylfluorescein was atypical. The cultures failed to hydrolyze sodium hippurate, but among the six *Legionella* species, only *L. pneumophila* has been shown to have this ability (13). Also, we were unable to demonstrate alkaline phosphatase activity by cultures of *L. jordanis*. Among the other six described species of *Legionella*, only *L. micdadei* lacks this ability.

TABLE 3. Cellular fatty acid composition of *L. jordanis* BL-540 and ABB-9, *L. pneumophila*, *L. bozemanii*, *L. micdadei*, *L. dumoffii*, and *L. gormanii*

Species	% of total fatty acid composition													
	i-14:0[a]	14:0	a-15:0	15:1	15:0	i-16:1	i-16:0	16:1	16:0	a-17:1	a-17:0	17 CYC[b]	17:0	18:0	19:0	20:0
L. jordanis BL-540	T[c]	T	42	2	T	2	17	7	4	T	18	2	2	T	T	2
L. jordanis ABB-9	2	T	45	T	T	T	18	6	3	T	22	T	T	T	T	T
L. bozemanii (4)[d]	4	T	31	T	T	T	17	11	12	T	24	2	T	T	T	T
L. micdadei (6)	T	T	40	T	T	T	11	10	10	3	24	T	T	T	T	T
L. dumoffii (2)	2	T	26	T	T	T	14	16	9	T	22	5	2	T	T	T
L. gormanii (1)	5	T	24	2	T	T	20	15	10	T	12	3	3	2	T	T
L. pneumophila (280)	8	T	14	T	T	2	32	13	10	T	11	3	T	2	T	2

[a] Numbers to left of the colon are the number of carbon atoms; numbers to the right are the number of double bonds; i- indicates a methyl branch at the *iso* carbon atom; a- indicates a methyl branch at the *anteiso* carbon atom.

[b] CYC, A cyclopropane acid.

[c] T, <2%.

[d] Numbers in parentheses are the number of strains tested.

GLC analysis of the cellular fatty acids of BL-540 and ABB-9 showed a profile more closely resembling that of *L. bozemanii, L. dumoffii, L. gormanii*, and *L. micdadei* than that of *L. pneumophila*. The most abundant acid was a saturated, branch-chain, 15-carbon acid (a-15:0).

DNA relatedness studies showed that BL-540 and ABB-9 were only slightly related to the six described species of *Legionella* and that they were more than 90% related to each other. These two environmental isolates, therefore, constituted a new species of *Legionella*.

Indirect fluorescent antibody tests with human sera strongly suggest that unrecognized human infections with *L. jordanis* may be occurring. Even though it has not yet been isolated from human infections, suitable reagents should be available for its detection. Meanwhile, the degree of relationship of *L. jordanis* to *L. bozemanii* (WIGA) is sufficient to permit detection of this species when specimens are screened with a WIGA conjugate in DFA tests but not with the WIGA antigen in IFA tests.

Some data were available on the results of IFA tests on rabbit antisera (P. S. Hayes, unpublished data). The strains and species used to produce these sera were the WIGA and MI-15 strains of *L. bozemanii, L. dumoffii, L. gormanii*, and BL-540. When these sera were tested against the homologous and heterologous antigens, comparable titers were obtained with WIGA and MI-15. Titers of *L. bozemanii* antisera (WIGA and MI-15) for BL-540 antigen suggested a partial serological relationship. Reciprocally, tests with the BL-540 antiserum indicated a partial relationship of BL-540 to both WIGA and MI-15.

Legionella jordanis sp. nov. is proposed as the name for the species represented by strains BL-540 and ABB-9; jore · da' nis, L. gen. n. *Jordanis* of the Jordan river in Bloomington, Ind., where the organism was first isolated. The type strain of *L. jordanis* is BL-540 (ATCC 33623).

There is a general requirement that strains of any given species show 70% or more DNA relatedness. There is no such agreement with regard to the amount of relatedness that could be used to define a genus or a family. We are of the opinion that all designations above the species level are basically artificial and must be made arbitrarily. There is no problem when the boundaries of a genus seem clear by both genetic and phenotypic criteria. If the two sets of data are not consistent, phenotype should take precedence. Species identification occurs at the laboratory bench, and a genus should consist of a group of phenotypically similar strains that can be differentiated diagnostically from one another.

TABLE 4. DNA relatedness of BL-540 and ABB-9 to *Legionella* species and to each other

Source of unlabeled DNA	RBR of the following source of labeled DNA at indicated temp (°C)[a]														
	ABB-9			*L. pneumophila* Philadelphia 1		*L. bozemanii* WIGA		*L. micdadei* TATLOCK		*L. dumoffii* NY-23		*L. gormanii* LS-13		*L. longbeachae* Long Beach 4	
	60	% D[b]	75	60	75	60	75	60	75	60	75	60	75	60	75
BL-540	93	0.7	99	19		8	4	6	2	4	0	7	1	17	
ABB-9	100 (55)	0.0	100 (49)												
L. pneumophila Philadelphia 1	10		2	100 (65)											
L. bozemanii WIGA	6		0			100 (56)	100 (53)								
L. micdadei TATLOCK	9		1					100 (45)	100 (42)						
L. dumoffii NY-23	9		3							100 (62)	100 (67)				
L. gormanii LS-13	8		2									100 (59)	100 (51)		
L. longbeachae Long Beach 4	5		0											100 (71)	

[a] RBR, Relative binding ratio. RBR = [(% heterologous DNA bound to hydroxyapatite)/(% homologous DNA bound to hydroxyapatite)] × 100. Numbers in parentheses are the actual percentages of DNA bound to hydroxyapatite in homologous reactions. All reactions were done at least twice. Control reactions containing only labeled DNA gave 1 to 2% binding to hydroxyapatite with ABB-9 DNA and 3 to 9% binding with other labeled DNAs. These control values were subtracted before the data were normalized.

[b] % D, Percent divergence. % D was calculated by assuming that each 1°C decrease in the thermal stability of a heterologous DNA duplex, compared with that of a homologous DNA duplex, is caused by 1% unpaired bases within the heterologous duplex (4).

DNA relatedness among species of the family *Legionellaceae* is low—usually 25% or less. Their phenotypic characteristics are very similar. Furthermore, all species have directly or indirectly (*L. gormanii* and *L. jordanis*) been implicated as causes of human pneumonia. They constitute a practical phenotypic genus. The phenotypic concept must take precedence because few laboratories can, at present, separate the species of *Legionella* (3). With experience, it may be possible to create genera that reflect genetic and phenotypic similarity among legionellae, but at present, a proposal to create additional genera in the family *Legionellaceae* (11) is premature, impractical, and unwarranted.

ACKNOWLEDGMENTS

We thank Charlotte M. Patton for assistance in the primary isolation of the BL-540 strain and Peggy S. Hayes for furnishing the IFA data on the rabbit antisera. We thank David J. Dodd for expert technical assistance and Barbara Gary for processing the manuscript. We are indebted to Thomas Ozro MacAdoo (Department of Foreign Languages and Literatures, Virginia Polytechnic Institute and State University, Blacksburg, Va.) for his advice on how to name *L. jordanis* in accordance with the rules of Latin grammar and the requirements of the *Bacteriological Code (1975 Revision)*.

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