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Legionella bozemanii sp. nov. and *Legionella dumoffii* sp. nov.: Classification of Two Additional Species of *Legionella* Associated with Human Pneumonia

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Abstract. Deoxyribonucleic acid (DNA) relatedness was used to distinguish strains of *Legionella*-like organisms (LLO) from *Legionella pneumophila*. Two of these LLO strains, WIGA and MI 15, showed sufficient DNA relatedness to one another to be classified in the same species. The name *Legionella bozemanii* species nova is proposed for this new species. The type strain of *L. bozemanii* is WIGA (=ATCC 33217). Two other LLO strains, NY 23 and Tex-KL, were shown to represent a new species. The name *Legionella dumoffii* species nova is proposed for this species. The type strain of *L. dumoffii* is NY 23 (=ATCC 33279). These two species join *L. pneumophila* and *L. micdadei* in the genus *Legionella*.

In 1947, Jackson, Crocker, and Smadel injected blood from a patient with a mild febrile illness into guinea pigs and recovered "a rickettsia-like agent" from guinea pigs as well as from embryonated hen's eggs [9]. This organism was called OLDA. Another organism, termed WIGA, was isolated by Bozeman, Humphries, and Campbell in 1959 from lung tissue taken from a patient who died of bronchopneumonia [3]. A suspension of lung tissue from this patient was inoculated into guinea pigs, one of which developed fever and was sacrificed. A brain tissue suspension from this animal inoculated into embryonated hen's eggs killed the embryos within 5 days [3].

Shortly after *Legionella pneumophila* had been characterized, its deoxyribonucleic acid (DNA) was tested for relatedness to DNAs from OLDA and WIGA [11]. DNA relatedness between OLDA and *L. pneumophila* strain Philadelphia 1 (the type strain) was 90%—well within the range of relatedness seen among strains of *Legionella pneumophila* [1,2,11]. Similar experiments showed that *L. pneumophila* was only marginally related to WIGA [7,11]. Case histories for the patient from whom WIGA was isolated and for a second patient who was infected with a similar organism were recently published [4]. The second strain, isolated and studied independently by two groups, was designated MI 15 [4] and GA-PH [17].

Two additional strains, NY 23, from water from a cooling tower, and Tex-KL, from a postmortem lung specimen, were isolated by methods used for *L. pneumophila* [4,10]. In this report, we show that WIGA and MI 15 (GA-PH) are genetically separable from *L. pneumophila*, and that they belong to a new species: *Legionella bozemanii* sp. nov., the type strain of which is WIGA (=ATCC 33217). Similarly, NY 23 and Tex-KL belong to a new species: *Legionella dumoffii* sp. nov., the type strain of which is NY 23 (=ATCC 33279).

Materials and Methods

The strains used in this study are listed in Table 1. Their origin is given in references [1,2,3,4,7,9,10,11,12].

None of the strains grew on Trypticase soy blood agar, nutrient agar, or other commonly used laboratory media. They were cultivated on Mueller-Hinton agar supplemented with hemoglobin and IsoVitaleX (MH-IH), Feeley-Gorman (F-G) agar, or charcoal yeast extract (CYE) agar [5,6]. Biochemical tests and staining reactions were done as described by Weaver and Feeley [19], and serogrouping was done by direct immunofluorescence [12]. Antibiotic susceptibility patterns and β-lactamase production were assayed as described by Thornsberry and Kirven [18]. Cellu-

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Table 1. DNA relatedness of *Legionella*-like organisms (LLO) and *Legionella pneumophilia*.

	Source of labeled DNA								
	L. pneumophila strain Philadelphia 1			LLO strain WIGA			LLO strain MI 15		
Source of unlabeled DNA	RBR[a], 60°C	% D[b]	RBR, 75°C	RBR, 60°C	% D	RBR, 75°C	RBR, 60°C	% D	RBR, 75°C
L. pneumophila strain Philadelphia 1	100	0.0	100	4		0	5		0
L. pneumophila strain Flint 2	100		82						
L. pneumophila strain Burlington 1	100	0.0	94						
L. pneumophila strain Bloomington 1	75	1.5		2		2			
L. pneumophila strain Togus 1	82	1.0		5		9			
L. pneumophila strain Bellingham 1	92	2.5		8					
L. pneumophila strain Pontiac 1	91	2.0	90	13		6			
L. pneumophila strain Cambridge 2			94			5			
LLO strain WIGA (old preparation)	15			100	0.0	100	69	1.0	52
LLO strain WIGA (new preparation)	8			77	0.0	77			
LLO strain WIGA (gray colony type)	11			77	0.0	92	66	1.0	64
LLO strain MI 15	25			56	0.5	64	100	0.0	100
LLO strain NY 23	13		6	15		1	32		1
LLO strain LS 13	20			22		0	12		0
Proteus mirabilis	6			2			2		0
None	6[c]		6	3		12	30		28

[a] RBR = relative binding ratio = (% heterologous DNA bound to hydroxyapatite)/(% homologous DNA bound to hydroxyapatite) × 100.
[b] % D = % divergence = the decrease in thermal stability (in °C) of heterologous DNA duplexes compared to that of the homologous DNA duplexes. It can be expressed as % because each degree decrease in thermal stability is caused by approximately 1% unpaired bases in double-stranded DNA.
[c] None = no unlabeled DNA added. This reaction controls nonspecific reassociation and any double-stranded DNA in which both strands are labeled. These values are not normalized and have been subtracted from all other experimental values before normalization.

lar fatty acid composition was determined by gas-liquid chromatography [13,14]. Genetic relatedness was assayed by DNA-DNA hybridization [1,2].

Results and Discussion

A growing number of patient and environmental isolates that grow on media used to cultivate *Legionella pneumophila* differ from *L. pneumophila* in one or more important characteristics. WIGA and MI 15 are examples of two such human strains. It has been difficult to communicate information about these strains, since there was no term that could be used to group them or to distinguish between them. Hazel Wilkinson originated the term "atypical *Legionella*-like organisms" (ALLO) to refer to such strains. ALLO was used by Cordes et al. to refer to a group of four fastidious, water-associated strains, two of which were pathogenic for humans [4]. One of these strains was WIGA.

The word "atypical" has been used ambiguously or incorrectly in referring to several groups of organisms and diseases (atypical mycobacteria, primary atypical pneumonia, etc.). Furthermore, most of the "atypical" *Legionella*-like organisms will eventually be characterized as perfectly typical strains belonging to species other than *L. pneumophila*. It was therefore decided to use the term LLO (*Legionella*-like organisms) as an umbrella for all such organisms. It will be pronounced ell'o.

L. pneumophila is defined by its ability to grow on one or more of these media—MH-IH, F-G, and CYE—but not on common laboratory media; a

Gram-negative cell wall; the biochemical characteristics previously listed; and a characteristic fatty acid composition. LLO are defined as a group of Gram-negative bacteria that do not meet the definition of *L. pneumophila*, but will grow on at least one agar medium designed for the growth of *L. pneumophila* and fail to grow on other commonly used laboratory media. These are working definitions that may change as our knowledge and experience increase.

Using these definitions, a serologically unreactive strain that fulfills the definition of *L. pneumophila* is not an LLO. Similarly, a pseudomonad that cross-reacts with *L. pneumophila* antiserum is not an LLO.

Relatedness among LLO strains and between LLO strains and strains of *L. pneumophila* was determined by DNA hybridization. Sheared, denatured, in vitro ^{3}H-labeled DNAs from *L. pneumophila* strain Philadelphia 1 and from LLO strains WIGA and MI 15 were reacted with similarly treated, unlabeled DNAs from a series of *L. pneumophila* and LLO strains under conditions that allow DNA reassociation. The results of these experiments are shown in Table 1.

Six *L. pneumophila* strains were 75% or more related to *L. pneumophila* strain Philadelphia 1 in both 60°C and 75°C reactions (at 75°C only very closely related DNA sequences can reassociate). The % divergence (%D = unpaired nucleotide bases within related DNA sequences) is quite low: 0–2.5%. These values are similar to those previously obtained among strains of *L. pneumophila* [1,2,11] and are indicative of the close relatedness seen among strains of a single species (70% or higher).

L. pneumophila was 8–25% related to three preparations of WIGA DNA and DNAs from three other LLO strains. DNA from *Proteus mirabilis*, included as a negative control, was 6% related to *L. pneumophila*. These data indicate that none of the 4 LLO strains are the same species as *L. pneumophila*.

Labeled DNA from LLO strain WIGA was used to determine whether the 4 LLO strains were the same species or represented more than one species. Three unlabeled WIGA DNA preparations were used: a portion of the same DNA preparation that had been labeled (old preparation; homologous reaction), a new preparation, and a preparation from a second colony type that was gray on CYE agar. Both the new preparation and the gray colony DNA preparation were highly related to labeled WIGA DNA at both the optimal (60°C) and the stringent (75°C) criteria for DNA reassociation. As expected, there was no evidence of unpaired bases (%D = 0.0) within the related DNA sequences. WIGA was 56% related to MI 15 in 60°C reactions and 64% related to it in 75°C reactions. The %D was 0.5.

Similar results were obtained by using labeled MI 15 DNA. MI 15 was 66% and 69% related to two preparations of WIGA DNA at 60°C. %D was 1.0, and relatedness in 75°C reactions was 64% and 52%. Both WIGA and MI 15 were minimally related to *L. pneumophila* strains. They showed somewhat higher relatedness (12–32%) to LLO strains NY 23 and LS 13, but in 75°C reactions relatedness was essentially zero.

We have encountered a good deal of variability in reactions that used in vitro labeled DNA as compared to in vivo labeled DNA. For example:

1. Control reactions without unlabeled DNA are higher—as much as 30% with MI 15 DNA.
2. Reciprocal reactions are not similar: Labeled DNA from *L. pneumophila* strain Philadelphia 1 was 15% related to WIGA and 25% related to MI 15, whereas labeled WIGA and MI 15 DNAs were only 4% and 5%, respectively, related to *L. pneumophila*.
3. Unlabeled WIGA DNA prepared identically at different times did not show 100% relatedness to labeled WIGA DNA

Because of these technical problems, we hesitate to interpret these data quantitatively, especially for 60°C reactions. Nonetheless, we are satisfied that they are reliable qualitatively. There is no doubt that all *L. pneumophila* strains are highly related and show a low level of relatedness to all 4 LLO strains. Similarly, WIGA and MI 15 are preferentially related and mainly fit the definition of species relatedness (70% or more relatedness in 60°C reactions; %D of 5 or less; 60% or more relatedness in 75°C reactions). If we take into account the fact that two WIGA DNA preparations were only 77% related to the homologous WIGA preparation, the argument that WIGA and MI 15 are the same species is even more convincing. Finally, there is no doubt that NY 23 and LS 13 represent species separate from both *L. pneumophila* and WIGA.

Previously reported data showed that DNA from LLO strain Tex-KL was about 10% related to DNA from *L. pneumophila* and *L. bozemanii* [10]. We have now labeled DNA from LLO strains NY 23 and Tex-KL. These strains are more than 90% related in 60°C reactions and still show 90% relatedness in the 75°C reactions. They are 0–25% related to the type strains of *L. pneumophila*, *L. bozemanii*, and *L. micdadei* [8], and to LS 13. Therefore, NY 23 and

Table 2. Phenotypic characteristics of *Legionella bozemanii* and *Legionella pneumophila*.

Characteristic	*L. bozemanii* strain WIGA (type strain)[a]	*L. pneumophila* strain Philadelphia 1 (type strain)	*L. pneumophila*	
Growth			% +	Number of strains tested
Agar[b]:				
CYE	+	+	100	100
F-G	+[c]	+	100	100
MH-IH	+[c]	+		
Blood agar base	−	−	0	100
Broth[d]:				
Pine	+	+	100	7
MH	+	+		
F-G		+	100	5
Fluorescence[e]:				
CYE agar	Blue-white	Dull yellow (negative)	0	100
F-G agar	Blue-white	Yellow (positive)	90	30
Browning of F-G agar	+	+	95	100
Stains:				
Gram[f]	−	−	0	85
Giménez[g]	Red rods	Red rods		
Ziehl-Neelsen[h]	−	−		
Leifson[i]	+	+	80	41
Motility	+	+	45	33
Biochemical reactions:				
Oxidase	−	+[j]	97[j]	79
Catalase	+	+	100	81
Urease	−	−	0	15
Gelatin liquefaction	+	+	100	83
β-Lactamase[k]	±[l]	+	100	45
$NO_3 \rightarrow NO_2$	−	−	0	100
Starch utilization	+	+	100	77
Acid production from carbohydrates[m]	−	−	0	15

[a] The biochemical data for WIGA is taken from reference [7]. MI 15 exhibits exactly the same phenotypic reactions as WIGA.
[b] CYE = charcoal yeast extract. F-G = Feeley-Gorman. MH-IH = Mueller-Hinton supplemented with L-cysteine and IsoVitaleX. Some growth may occur on heavily inoculated areas, but no growth results on transfers to a second plate of blood agar base.
[c] Isolates are adapted to grow on these media after multiple transfers on CYE.
[d] Pine = Pine's chemically defined medium [15]. MH = Mueller-Hinton supplemented with 0.05% L-cysteine.
[e] Long wave length UV excitation using a Wood's light.
[f] Faint pink rods from CYE.
[g] Smears of infected yolk sac.
[h] Rods are stained blue from CYE.
[i] Straight or curly flagellae [16].
[j] Weakly positive reaction.
[k] Rapid chromogenic cephalosporin technique [18].
[l] Positive in one day; negative after one day.
[m] Negative in tests for acid production from D-glucose, D-xylose, mannitol, lactose, sucrose, and maltose.

Tex-KL belong to the same species, one that is distinct from all other described *Legionella* species and LLO strains.

We propose the name *Legionella bozemanii* (pronounced bows-mahn'-ee) sp. nov. for the species represented by strains WIGA and MI 15. This species is named in honor of F. Marilyn Bozeman who pioneered in the study of *Legionella* and LLO. The type strain of *L. bozemanii* is WIGA (=ATCC 33217), isolated by Bozeman et al. in 1959 from a man with bronchopneumonia [3]. The phenotypic characteristics of *L. bozemanii* strain WIGA were previously published [7] and are shown in Table 2. Also in Table 2 are the phenotypic characteristics of *L. pneumophila* strains and of Philadelphia 1, the type strain of *L. pneumophila* [1].

L. bozemanii is a Gram-negative, oxidase-negative, catalase-positive rod that liquefies gelatin, degrades starch, and fluoresces when exposed to long-wave (365 nm) ultra-violet light. It is negative in re-

actions for urease, reduction of nitrates to nitrites, and production of acid from D-glucose and other carbohydrates. It is motile by means of 1 or 2 polar flagella, which may be straight or curly. The major cellular fatty acid in *L. bozemanii* is a-15:0 [13].

Patients with *L. bozemanii* infection can respond immunologically with a specific rise in indirect immunofluorescence assay (IFA) titer during convalescence [4]. The antibody response can also be against antigenic determinants that are common to *L. bozemanii* and to multiple serogroups of *L. pneumophila* [20]. Like *L. pneumophila,* antigenic heterogeneity among strains of *L. bozemanii* is suggested by the fact that specific seroconversions against WIGA alone (in addition to those often found against WIGA and MI 15) have been demonstrated in IFA tests of human sera from patients with suspected legionellosis (H. W. Wilkinson, unpublished data).

As shown in Table 2, a few reactions are helpful in separating *L. bozemanii* from *L. pneumophila.* Fresh isolates of *L. pneumophila* grow on F-G and MH-IH agar, but *L. bozemanii* strains must be adapted by several passages on CYE before they grow on F-G or MH-IH. When viewed under a Wood's light, colonies on *L. bozemanii* fluoresce blue-white, but colonies of *L. pneumophila* appear dull yellow [13]. *L. bozemanii* is oxidase negative. The type strain of *L. pneumophila* is weakly oxidase positive, but some strains of *L. pneumophila* give a negative oxidase test.

A suspected *L. bozemanii* strain is best confirmed by direct immunofluorescence (DFA) and determination of cellular fatty acid composition. *L. bozemanii* cellular antigens are specific and do not cross-react with cellular antigens from the four described serogroups of *L. pneumophila* by DFA [12,17], or by the slide agglutination test [21]. *L. pneumophila* and *L. bozemanii* contain the same fatty acids; however, the major acid in *L. bozemanii* is a-15:0 and the major acid in *L. pneumophila* is i-16:0 [4,13,14].

One can argue that *L. bozemanii* should be in a separate genus from *L. pneumophila.* The two species showed 4–25% relatedness. These values may well be falsely high, since these two species showed 2–6% reaction with *Proteus mirabilis,* to which we assume they are not at all related. *L. bozemanii* was placed in the genus *Legionella* because the isolation procedures for *L. pneumophila* and *L. bozemanii* are identical and their phenotypic reactions are similar. We believe that genetic relatedness should be the basis for identification to species level. Ideally, a genus should contain a group of genetically and phenotypically related species. When both criteria cannot be met, phenotypic relatedness should take precedence to ensure that the genus designation is of practical use at the bench.

NY 23 was isolated from a sample of water from a cooling tower in New York, New York [4], and Tex-KL was isolated from a postmortem lung specimen obtained in Houston, Texas [10]. The cultural and phenotypic characteristics of these strains have been described [4,10]. We propose the name *Legionella dumoffii* (pronounced due-mahff'-ee) sp. nov. for the species represented by strains NY 23 and Tex-KL. This species is named in honor of the late Morris Dumoff who was, in all probability, the first to culture *L. pneumophila* on bacteriological media. The type strain of *L. dumoffi* is NY 23 (=ATCC 33279). The arguments for retaining *L. dumoffi* in the genus *Legionella* are similar to those already presented for *L. bozemanii.*

The WIGA strain of *L. bozemanii* was the second recognized species in *Legionella* [11]. A third species of *Legionella, L. micdadei* [8] has been described (the name *Legionella pittsburgensis* has also been used for this species; A. W. Pasculle et al., *Journal of Infectious Diseases,* in press). Thus, there are now four named species of *Legionella, L. pneumophila* [1], *L. micdadei* [8], *L. bozemanii,* and *L. dumoffi.* LLO strain LS 13, as yet unnamed, is genetically distinct from all of the named species.

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