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Characterization of Members of the *Legionellaceae* Family by Automated Ribotyping

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In order to implement a new and reliable method for characterizing different species of *Legionella*, a genetic fingerprinting study with an automated ribotyping system (RiboPrinter) was completed with members of this genus which were deposited at the American Type Culture Collection. The RiboPrinter examined the different patterns of *Eco*RI digestion fragments from the rRNA operons of 110 strains, representing 48 of the 49 described *Legionella* species as well as 70 serogroups of those species. Distinctive and consistent patterns were obtained for the type strains of the 48 species investigated. *Legionella pneumophila* subsp. *fraseri* and *L. pneumophila* subsp. *pascullei* each generated a specific pattern, whereas *L. pneumophila* subsp. *pneumophila* produced six different fingerprint patterns. No correlation seemed to exist between the ribotypes obtained and the 15 serotypes of *L. pneumophila*. For the other species, those with two known serogroups presented two distinctive patterns with the RiboPrinter with the exception of *L. hackeliae* and *L. quinlivanii*, which yielded only one pattern. We also encountered ribotypes for strains which were not identified to the species level. The ribotypes generated for these strains with the RiboPrinter did not match those generated for known type strains, suggesting the putative description of new serogroups or species. Although the automated system did not have sufficient discriminatory ability to serve as an epidemiological tool in a clinical setting, it appeared to be a powerful tool for general genomic analysis of the *Legionella* isolates (e.g., determination of new species) and assessment of the interrelationship among *Legionella* strains through the RiboPrinter database connection.

Since the first isolation of *Legionella pneumophila*, the causative agent of Legionnaires' disease, nearly 30 years ago (37), members of this genus have been isolated from a wide range of environments and geographical locations (17, 20). To date, members of the family *Legionellaceae* comprise 49 described species (Deutsche Sammlung von Mikroorganismen und Zellkulturen website [ftp://ftp.dsmz.de/pub/DSMZ/bactnom/bactname.pdf]), with 15 serogroups (Sgs) described for *L. pneumophila*; 2 Sgs each described for *L. bozemanii*, *L. longbeachae*, *L. feeleii*, *L. hackeliae*, *L. sainthelensi*, *L. spiritensis*, *L. erythra*, and *L. quinlivanii*; and a single Sg each described for the remaining species (3). Over the years, several species of *Legionella* that were initially isolated from environmental sources but that were not implicated as etiological agents have later been shown to be human pathogens (9, 22, 25, 35). Approximately 70 to 90% of *Legionella* infections are caused by *L. pneumophila* Sgs 1 and 6, and others species are responsible for between 5 and 30% of the cases of infection (18, 42). Nineteen species have been recognized to be pathogenic for humans (14), causing pneumonia (Legionnaires' disease) (11, 19), a mild febrile disease (Pontiac fever) (31, 55), and most recently, soft tissue abscesses (25). Pneumonia caused by *Legionella* is becoming a public health problem, since this organism has the potential to cause large outbreaks (1, 40) and to infect young immunocompetent adults (51) or newborns after water birth (21). Legionnaires' disease, if left untreated, leads to an average mortality rate of 15% (16).

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Various methods for typing of members of the family *Legionellaceae* have been developed in the past. These include antibiotic susceptibility testing (54), plasmid analysis (47), fatty acid profiling (29), multilocus enzyme electrophoresis (45), pulsed-field gel electrophoresis (39), and various DNA fingerprinting protocols by PCR (14, 34, 41, 53). Most of them have focused specifically on the subtyping of *L. pneumophila* Sgs 1 and 6 because these Sgs are responsible for the majority of legionellosis cases.

At present, the majority of the *Legionella* isolates are detected and typed by serological designation (3, 30, 36, 49). This has been satisfactory for the most commonly occurring species and serovars. However, antisera are not available commercially for many of the less well known species. Immunological cross-reactions among some species have also been reported to be troublesome (3, 6, 33, 49). With the increasing number of described *Legionella* species, methods based on serology will become more difficult and cumbersome to use in environmental and clinical studies.

Ribotyping, a molecular method based on the analysis of the restriction fragment length polymorphisms (RFLPs) of rRNA genes (23), was also used to characterize *Legionella* strains (2, 52). The feasibility of ribotyping for the differentiation of *Legionella* species was initially tested by Grimont et al. (24) with 28 members of the *Legionellaceae* family.

To further investigate this approach, we tested a larger number of *Legionella* species using an automated ribotyping system. A total of 110 strains representing 48 species and 3 subspecies, as well as a newly reported Sg for *L. londiniensis* (Sg 2; F. Lo Presti [Centre National de Référence des Légionelles, Lyon, France], personal communication to the American Type Culture Collection [ATCC]) were included in the study. *L.*

lytica (28) is a symbiont of amoeba and requires cocultivation with the host, so it was not feasible to include a culture of this isolate for testing. The results of that study are presented in this paper.

MATERIALS AND METHODS

Cultures, media, and growth conditions. A total of 110 *Legionella* strains comprising 48 species, including 3 subspecies and 70 Sgs deposited at ATCC, were used for this study (Table 1). Each culture was grown in an atmosphere of 5% CO_2 at 37°C for 24 to 48 h on ATCC 1099 Charcoal Yeast Extract buffered medium (the medium formulation is described at the website http://www.atcc.org/SearchCatalogs/MediaFormulations.cfm).

Sample preparation and processing. All *Legionella* strains were characterized by use of the RiboPrinter system (Qualicon Inc., Wilmington, Del.) as described previously (13) with respect to the procedures and conditions recommended by the manufacturer (12, 46). *Eco*RI was used as the restriction enzyme. At the end of the process, a densitometric scan depicting the distributions and molecular weights of the restriction fragments was obtained for each sample analyzed. This output was saved in the RiboPrinter's computer. Ribotype groups (ribogroups) were defined by the RiboPrinter's proprietary algorithm, which compared the pattern of each isolate to those of others in the database and assigned groups by the differences in band number, position, and signal intensity. A given ribogroup was defined as a group of ribotypes with similarity values >0.93. Strains used for repeatability testing were analyzed by using the RiboPrinter's proprietary algorithm, which was a modified version of the coefficient of simple correlation (38). Similarity values obtained are reported in Table 2.

Ribotype analysis. For each batch of eight samples, ribotypes were normalized to the positions of the molecular weight standards with Qualicon software. Computerized ribotypes were exported for analysis in .txt files and imported into BioNumerics software (version 2.5; Applied Maths, Sint-Martens-Latem, Belgium) by using the Qualicon macro. Clustering analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA) method based on the Dice (15) coefficient for band matching, with a position tolerance setting of 1.0% (default values are 1% of position tolerance and 0.5% of optimization). Bands for analysis with the Dice coefficient were assigned manually, according to densitometric curves and the accompanying hard-copy photograph.

RESULTS

All 110 *Legionella* strains in this study could be processed with the RiboPrinter, resulting in 100% typeability. Two dendrograms were derived by using the BioNumerics software (version 2.5). Figure 1 presents the results for all *L. pneumophila* strains included in this study, while Fig. 2 presents the results for all other strains. The second dendrogram also included eight *L. pneumophila* strains (ATCC 43108, ATCC 33734, ATCC 43736, ATCC 35096, ATCC 33152, ATCC 43130, ATCC 33736, and ATCC 33156), which corresponded to the eight ribogroups displayed in Fig. 1.

Pattern reproducibility was investigated by ribotyping of 30 isolates more than twice, which resulted in mean similarity values ranging from 0.95 to 1.00 (Table 2). The patterns obtained after *Eco*RI cleavage and probe hybridization contained two to six fragments (mainly three to four) in the range of 3 to 60 kb. Sixty-seven different patterns were generated for the 110 strains tested, and distinctive patterns were obtained for the type strains of 48 species.

Examination of 32 *L. pneumophila* strains allowed us to distinguish three separate clusters and eight different ribogroups within these clusters (Fig. 1). Two ribogroups, which clustered separately from each other and away from the major cluster of *L. pneumophila*, were formed by three strains each of *L. pneumophila* subsp. *fraseri* and *L. pneumophila* subsp. *pascullei*. We could distinguish six ribogroups within the major cluster of *L. pneumophila* strains. The first ribogroup was made up of ribotypes from three strains of *L. pneumophila* Sg 1 (ATCC 43106, ATCC 43108, ATCC BAA-74). The second ribogroup consisted of two strains of *L. pneumophila* subsp. *pneumophila* Sg 1 (ATCC 33733, ATCC 33734). The third ribogroup included identical ribotypes from nine strains (ATCC 35289, ATCC 43107, ATCC 43109, ATCC 43113, ATCC 43660, ATCC 43662, ATCC 43661, ATCC 43736, ATCC 700711), corresponding to three different Sgs (*L. pneumophila* Sg 1, *L. pneumophila* subsp. *pneumophila* Sg 9 and Sg 13). The fourth ribogroup comprised identical ribotypes from seven strains (ATCC 33155, ATCC 33153, ATCC 33823, ATCC 35096, ATCC 43112, ATCC 43283, ATCC 43703), corresponding to six Sgs (*L. pneumophila* Sg 1 and Sg 7, *L. pneumophila* subsp. *pneumophila* Sg 3, Sg 8, Sg 10, and Sg 14). Only four *L. pneumophila* subsp. *pneumophila* strains (ATCC 33152, ATCC 33154, ATCC 33215, ATCC 43290) made up of the fifth ribogroup, each with a different Sg (Sg 1, Sg 2, Sg 6, and Sg 12). The sixth ribogroup contained one strain of *L. pneumophila* subsp. *pneumophila* Sg 11 (ATCC 43130).

For 10 other species examined (Fig. 2) we were able to distinguish two ribogroups (*L. feeleii*, *L. dumoffii*) or ribotypes (*L. bozemanii*, *L. erythra*, *L. londiniensis*, *L. longbeachae*, *L. parisiensis*, *L. sainthelensi*, *L. spiritensis*, and *L. gormanii*). For the remaining 37 species, which included *Legionella* genomospecies 1 (ATCC 51913), a unique and distinctive ribogroup (*L. micdadei*, *L. gresilensis*, *L. oakridgensis*, *L. anisa*, *L. hackeliae*, *L. quinlivanii*, *L. jordanis*, and *L. birminghamensis*) or ribotype was observed for each species (Fig. 2). Finally, we also noted that three *Legionella* sp. strains (ATCC 700511, ATCC 700703, ATCC 700761) showed different fingerprint patterns which were not observed among other *Legionella* members.

DISCUSSION

The previous studies on the ribotyping of the members of the *Legionellaceae* family were done by traditional, time-consuming manual techniques and focused on a limited number of strains. In this study, we used an automated microbial genotyping system, the RiboPrinter (Qualicon), to investigate a large panel of *Legionella* species. We used the *Eco*RI restriction enzyme, which generated a number of fragments similar to the number observed by manual ribotyping of *Legionella* strains with various restriction enzymes (*Eco*RV, *Hin*dIII, and *Pst*I) (2, 24). There was evidence that in *Escherichia coli* automated riboprints correlated well with the fingerprinting patterns generated by traditional methods (13). Furthermore, the *Eco*RI ribotypes obtained manually by Schoonmaker et al. (44) for *L. pneumophila* strains (ATCC 33153, ATCC 33152, ATCC 33216) were identical to the corresponding ribotypes obtained in the present study. The reproducibility of our data is demonstrated in Table 2. These results indicate that the RiboPrinter is a powerful device with excellent reproducibility, in addition to a high throughput capacity, which allows the analysis of 32 isolates per day.

The main dendrogram that was derived from our study indicated that each of the 48 type strains produced a distinctive and consistent fingerprint pattern (Fig. 2). This suggested that the patterns for *Legionella* strains obtained with the RiboPrinter could be used to identify new isolates by comparison to the patterns generated for known species. However, they may

TABLE 1. *Legionella* strains included in the study[a]

Species	Subspecies	Sg	Other	Source	Original designation	ATCC no.
L. adelaidensis		1	TS	Cooling tower water, Adelaide, Australia	1762-AUS-E	49625
L. anisa		1	TS	Hot water, sink, Los Angeles, Calif.	WA-316-C3	35292
L. anisa				Sink faucet, Chicago, Il.	CH-47-C3	35291
L. anisa				Sink faucet, Chicago, Il.	CH-47-C1	35290
L. beliardensis		1	TS	Water from a calorifier in reanimation unit, Montbeliard, France	Montbeliard A1	700512
L. birminghamensis		1	TS	Lung biopsy, cardiac transplant recipient, Alabama	1407-AL-H	43702
L. birminghamensis				Water near Clermont-Ferrand, France	CF VII no. 3A	700709
L. bozemanii		1	TS	Lung tissue, pneumonia, Key West, Fla.	WIGA	33217
L. bozemanii		2	RS	Human lung aspirate, Toronto, Ontario, Canada	Toronto 3	35545
L. brunensis		1	TS	Cooling tower water, Brno, Czechoslovakia	444-1	43878
L. cherrii		1	TS	Thermally altered water, Michigan	ORW	35252
L. cincinnatiensis		1	TS	Lung tissue, pneumonia, Cincinnati, Ohio	72-OH-H	43753
L. drozanskii		1	TS	Tank of well water, Leeds, United Kingdom	LLAP-1	700990
L. dumoffii		1	TS	Water in cooling tower, New York, N.Y.	NY 23	33279
L. dumoffii				Human lung, Los Angeles, Calif.	Wadsworth 81-782A	35850
L. dumoffii				Thermal spa water	A3a3F	700714
L. erythra		1	TS	Water in cooling tower, Seattle, Wash.	SE-32A-C8	35303
L. erythra		2	RS	Strain isolated in Paris, France	LC217	BAA-536
L. fairfieldensis		1	TS	Cooling tower water, Fairfield, Victoria, Australia	1725-AUS-E	49588
L. fallonii		1	TS	Ship air-conditioning system, United Kingdom	LLAP-10	700992
L. feeleii		1	TS	Grinding machine coolant fluid, Windsor, Ontario, Canada	WO-44C-C3	35072
L. feeleii		2	RS	Human lung tissue, Wisconsin, Wis.	691-WI-H	35849
L. feeleii		1		Bronchoalveolar lavage, pneumonia, Savoy, France	Ly126.92b	700513
L. feeleii		1		Bronchoalveolar lavage, pneumonia and HIV, Lyon, France	Ly166.96	700514
L. geestiana		1	TS	Hot-water tap, Geest Office building, London, United Kingdom	1308	49504
L. genomospecies 1				Cooling-water tower, Adelaide, Australia	2055-AUS-E	51913
L. gormanii		1	TS	Soil from a creek bank, Atlanta, Ga.	LS-13	33297
L. gormanii				Bronchial brush, Pneumonia, Calif.	86A5796	43769
L. gratiana		1	TS	Thermal spa water, Savoy region, France	Lyon-8420412	49413
L. gresilensis		1	TS	Water from shower in a thermal spa. Gréoux, France	Gréoux 11 D13	700509
L. gresilensis				Water sample from a potash mine near Mulhouse, France	Mulhouse 12 A23	700759
L. hackeliae		1	TS	Human bronchial biopsy specimen, Ann. Arbor., Mich.	Lansing 2	35250
L. hackeliae		2	RS	Human lung aspirate, Pittsburgh, Pa.	798-PA-H	35999
L. israelensis		1	TS	Water, Israel	Bercovier 4	43119
L. jamestowniensis		1	TS	Wet soil, Janestown, N.Y.	JA-26-G16-E2	35298
L. jordanis		1	TS	Jordan River, Bloomington, Ind.	BL-540	33623
L. jordanis				Patient with pneumonia, France	Ly95.90	700762
L. lansingensis		1	TS	Bronchial aspirate pneumonia and leukemia, Lansing, Mich.	1677-MI-H	49751
L. londiniensis		1	TS	Office building cooling tower, London, United Kingdom	1477	49505
L. londiniensis		2	RS	Water, Mulhouse, France	Mulhouse B26	700510
L. longbeachae		1	TS	Human lung, pneumonia, Long Beach, Calif.	Long Beach 4	33462
L. longbeachae		2	RS	Human lung, Tucker, Ga.	Tucker 1	33484
L. maceachernii		1	TS	Water in home evaporator cooler, Phoeniz, Ariz.	PX-1-G2-E2	35300
L. micdadei		1	TS	Human blood, pneumonia, Fort Bragg, Calif.	TATLOCK	33218
L. micdadei				Lung tissue, pneumonia, Pittsburgh, Pa.	EK	33204
L. micdadei				Transtracheal aspirate, Pittsburgh, Pa.	VAMC-MCC	33344
L. micdadei				Showerhead, Pittsburgh, Pa.	VAM-7W	33345
L. micdadei				Ultrasonic nebulizer, Pittsburgh, Pa.	VAM-PGH-12	33346
L. moravica		1	TS	Cooling-tower water, Jihlava, Czechoslovakia	316-36	43877
L. nautarum		1	TS	Domestic hot-water tap, Greenwich, London, United Kingdom	1224	49506
L. oakridgensis		1	TS	Industrial cooling-tower water, Pennsylvania	Oak Ridge 10	33761
L. oakridgensis				Bronchoalveolar lavage, pneumonia, Nantes, France	Nantes-930101868	700515
L. oakridgensis				Bronchoalveolar lavage, pneumonia, Nantes, France	Nantes-930101937	700516
L. parisiensis		1	TS	Water in cooling tower, Paris, France	PF-209C-C2	35299
L. parisiensis				Tracheal aspirate, liver transplant, France	FLP2	700174
L. pneumophila	*fraseri*	4	TS	Human lung, pneumonia, Los Angeles, Calif.	Los Angeles-1	33156

Continued on following page

TABLE 1—*Continued*

Species	Subspecies	Sg	Other	Source	Original designation	ATCC no.
L. pneumophila	*fraseri*	5	RS	Cooling tower, Dallas, Tex.	Dallas 1E	33216
L. pneumophila	*fraseri*	15	RS	Human lung, fatal pneumonia, Royal Oak, Mich.	Lansing 3	35251
L. pneumophila	*pascullei*		TS	Water from showerhead, Pittsburgh, Pa.	U8W	33737
L. pneumophila	*pascullei*	13	RS	Water from showerhead, Pittsburgh, Pa.	U7W	33736
L. pneumophila	*pascullei*			Tap water, Pittsburgh, Pa.	MICU B	33735
L. pneumophila	*pneumophila*	1	TS	Human lung, pneumonia, Philadelphia, Pa.	Philadelphia-1	33152
L. pneumophila	*pneumophila*	1		Tap water, Pittsburgh, Pa.	684	33733
L. pneumophila	*pneumophila*	1		Tap water, Pittsburgh, Pa.	687	33734
L. pneumophila	*pneumophila*	2	RS	Human lung, Togus, Maine	Togus-1	33154
L. pneumophila	*pneumophila*	3	RS	Creek water, Bloomington, Ind.	Bloomington-2	33155
L. pneumophila	*pneumophila*	6	RS	Human lung biopsy specimen, Chicago, Ill.	Chicago 2	33215
L. pneumophila	*pneumophila*	8	RS	Human lung, Concord, Calif.	Concord 3	35096
L. pneumophila	*pneumophila*	9	RS	Tap water, Leden, Holland	IN-23-G1-C2	35289
L. pneumophila	*pneumophila*	11	RS	Human endotracheal tube, Pittsburgh, Pa.	797-PA-H	43130
L. pneumophila	*pneumophila*	10	RS	Respiratory tract secretions, Holland	Leiden 1	43283
L. pneumophila	*pneumophila*	12	RS	Human lung, pneumonia, Denver, Col.	570-CO-H	43290
L. pneumophila	*pneumophila*	14	RS	Bronchial aspirate, pneumonia, Minn.	1169-MN-H	43703
L. pneumophila	*pneumophila*	13	RS	Lung aspirate, pneumonia, Calif.	B2A3105	43736
L. pneumophila		1		Human lung, Knoxville, Tenn.	Knoxville-1	33153
L. pneumophila		7	RS	Showerhead, Illinois	Chicago 8	33823
L. pneumophila		1		CDC-Quebec	Allentown 1	43106
L. pneumophila		1		CDC-Quebec	Heysham 1	43107
L. pneumophila		1		CDC-Quebec	Benidorm 030 E	43108
L. pneumophila		1		CDC-Quebec	OLDA	43109
L. pneumophila		1		CDC-Quebec	France 5811	43112
L. pneumophila		1		CDC-Quebec	Camperdown 1	43113
L. pneumophila				Clinical sample, State Health Department California	RIO	43660
L. pneumophila				Hospital faucet, Pittsburgh Veterans Affairs Medical Center, Pittsburgh, Pa.	11EJ	43661
L. pneumophila				Hospital faucet, Calif.	FAUC 19	43662
L. pneumophila		1		Bronchoalveolar lavage fluid, pneumonia, France	CA1	700711
L. pneumophila		1		Transtracheal aspirate, 1978	F1724	BAA-74
L. quateirensis		1	TS	Shower in hotel bathroom, Quarteira, Portugal	1335	49507
L. quinlivanii		1	TS	Water in bus air conditioner, Australia	1442-AUS-E	43830
L. quinlivanii		2	RS	Cooling-tower pond, London, United Kingdom	LC870	BAA-538
L. rowbothamii		1	TS	Water and sludge from an industrial liquifier tower, United Kingdom	LLAP-6	700991
L. rubrilucens		1	TS	Tap water, Los Angeles, Calif.	WA-270A-C2	35304
L. sainthelensi		1	TS	Spring water, Mt. St. Helens, Wash.	MSH-4	35248
L. sainthelensi		2	RS	Human bronchial washings, pneumonia, Calif.	1489-CA-H	49322
L. santicrucis		1	TS	Tap water, St. Croix, U.S. Virgin Islands	SC-63-C7	35301
L. shakespearei		1	TS	Cooling-tower water, Stratford upon Avon, United Kingdom	214	49655
L. species				Water from a well, Montpellier, France	IB V no 3	700511
L. species				Water, Bourbonne-les-Bains, France	Nancy II no. 1	700703
L. species				Water, La Rochelle, France	La Rochelle A2.1	700705
L. species				Water, Bourbonne-les-Bains, France	Nancy II no. 3	700706
L. species				Environmental isolate, Venissieux, France	IBV no. 2	700761
L. species				Clinical isolate, France	ParisB1	700833
L. spiritensis		Sg1	TS	Spirit Lake, Mt. St. Helens, Wash.	MSH-9	35249
L. spiritensis		Sg2	RS	Cooling tower, United Kingdom	ML 76	BAA-537
L. steigerwalii			TS	Tap water, St. Croix, U.S. Virgin Islands	SC-18-C9	35302
L. taurinensis		Sg1	TS	Water from a hospital oxygen bubble humidifier, Turin, Italy	Turin no 1	700508
L. tucsonensis			TS	Pleural fluid, renal transplant, Tucson, Ariz.	1087-AZ-H	49180
L. wadsworthii		Sg1	TS	Human sputum, pneumonia, Los Angeles, Calif.	Wadsworth 81-716A	33877
L. waltersii		Sg1	TS	Drinking water distribution system, Adelaide, Australia	2074-AUS-E	51914
L. worsleiensis		Sg1	TS	Industrial cooling tower, Worsley, United Kingdom	1347	49508

[a] TS, type strain; RS, reference strain; CDC, Centers for Disease Control and Prevention HIV, human immunodeficiency virus.

not be suitable for phylogenetic purposes, as our dendrogram did not agree with the phylogenetic tree that was generated by 16S rRNA analysis (28). By examination of Fig. 1, two separate clusters for *L. pneumophila* subsp. *fraseri* and *L. pneumophila* subsp. *pascullei* were clearly observed, while the patterns for all *L. pneumophila* subsp. *pneumophila* strains clustered in a common group. This is in agreement with results based on DNA hybridization (10) as well as those of a previous study showing

TABLE 2. Strains used for repeatability testing

Strain	ATCC no.	No. of repeated tests	Similarity values[a]
L. dumoffii	33279	2	1.0, 0.99
L. longbeachae	43462	3	1.0, 0.99, 0.97
L. lansingensis	49751	3	1.0, 0.98, 0.97
L. birminghamensis	43702	4	1.0, 0.98, 0.98, 0.98
L. feeleii	35849	2	1.0, 0.95
L. anisa	35292	2	1.0, 0.95
L. pneumophila subsp. *fraseri*	33156	2	1.0, 0.96
L. pnemophila subsp. *pascullei*	33737	5	1.0, 0.99, 0.98, 0.96, 0.98
L. cincinnatiensis	43753	2	1.0, 0.98
L. fallonii	700992	2	1.0, 0.95
L. gratiana	49413	2	1.0, 0.98
L. parisiensis	700174	2	1.0, 0.96
L. dumoffii	700714	2	1.0, 0.95
L. pneumophila subsp. *pneumophila*	33152	3	1.0, 0.96, 0.95
L. pneumophila subsp. *pneumophila*	43283	2	1.0, 0.99
L. pneumophila subsp. *pneumophila*	43130	3	1.0, 0.99, 0.99
L. pneumophila	BAA-74	3	1.0, 0.99, 0.98
L. pneumophila subsp. *pneumophila*	35289	6	1.0, 0.95, 0.97, 0.98, 0.97, 0.97
L. cherrii	33252	2	1.0, 0.98
L. moravica	43877	4	1.0, 0.99, 0.97, 0.97
L. jordanis	33623	2	1.0, 0.97
L. quinlivanii	43830	3	1.0, 1.0
L. drosanskii	700990	3	1.0, 0.99
L. hackeliae	35250	2	1.0, 0.99
L. micdadei	33218	2	1.0, 0.98
L. maceachernii	35300	6	1.0, 0.97, 0.98, 0.98, 0.97, 0.98
L. bozemanii	33217	7	1.0, 0.98, 0.98, 0.98, 0.99, 0.99, 0.96
L. londinensis	49505	4	1.0, 0.98, 0.97, 0.96
L. nautarum	49506	2	1.0, 0.98
L. steigerwaltii	35302	2	1.0, 0.99

[a] Similarity values were generated by comparing all runs to the first one with the RiboPrinters proprietary algorithm (see Materials and Methods).

that *L. pneumophila* subsp. *fraseri* could be separated from other subspecies of *L. pneumophila* by four restriction enzymes (3). We also noticed that there did not seem to be any correlation between the 15 serotypes of *L. pneumophila* and their patterns obtained with the RiboPrinter. This is in accordance with previous reports indicating that the separation of *L. pneumophila* into different Sgs has no apparent relation to the underlying genetic structure of the microorganism (27, 45). Furthermore, strains of Sg 1 were present in five of the six ribogroups of *L. pneumophila*. These results are in agreement with previous findings which indicated that *L. pneumophila* Sg 1 is a fairly heterogeneous group (3). In addition, we noticed three strains (ATCC 700706, ATCC 700705, ATCC 700833) deposited without species names clearly clustered with three different *L. pneumophila* ribogroups (Fig. 2), which strongly suggests that these strains belong to this species. These results should be further confirmed by other molecular tests such as 16S rRNA gene sequencing or DNA-DNA hybridization studies.

A few *Legionella* species (*L. micdadei*, *L. gresilensis*, *L. oakridgensis*, *L. anisa*, *L. jordanis*, *L. birminghamensis*) from various origins seemed to display genetic homogeneity within the species, exhibiting one ribogroup per species, as seen previously with the subspecies of *L. pneumophila. L. hackeliae* (ATCC 35250, ATCC 33216) and *L. quinlivanii* (ATCC 43830, ATCC BAA-538), with two serotypes each, also displayed this feature (one ribogroup per species), which is in agreement with earlier studies based on manual ribotyping analysis (8, 24).

On the other hand, *L. bozemanii*, *L. erythra*, *L. londiniensis*, *L. longbeachae*, *L. parisiensis*, *L. sainthelensi*, *L. spiritensis*, *L. gormanii*, *L. dumoffii*, and *L. feeleii* displayed remarkable genetic diversity. Homologies of less than 16% were consistently observed between ribogroups (*L. feeleii*, *L. dumoffii*) or ribotypes (*L. bozemanii*, *L. erythra*, *L. londiniensis*, *L. longbeachae*, *L. parisiensis, L. sainthelensi*, *L. spiritensis*, *L. gormanii*) within a given species. Interestingly, for seven of these species (*L. bozemanii*, *L. erythra*, *L. londiniensis*, *L. longbeachae*, *L. sainthelensi*, *L. spiritensis*, and *L. feeleii*), two serogroups have previously been described or reported (5, 7, 26, 43, 48, 50). For these species, each Sg seemed to be associated with a given ribotype or ribogroup (*L. feeleii* Sg 1). This observation is in agreement with previous findings based on manual ribotyping (24) and randomly amplified polymorphic DNA analysis (34). *L. dumoffii*, *L. parisiensis*, and *L. gormanii* each has a single Sg; however, two different fingerprint patterns were observed for each species. Regarding *L. parisiensis* ATCC 700174 (35), *L. gormanii* ATCC 43769 (22), and *L. dumoffii* ATCC 700714 (M. Molmeret [Centre National de Référence des Légionelles, Lyon, France], personal communication to ATCC), our results suggest that these strains may correspond to new putative serotypes, having less than 16% of homology within their own ribogroup. This, however, needs to be confirmed by further serological studies.

For all the other species which were represented in the study by only one strain, each type strain produced a distinctive and consistent identifying pattern.

Automated ribotyping may represent an alternative tool for determination of putative new species before labor-intensive techniques are needed. For example, ATCC 700509 and ATCC 700761 were deposited at ATCC as *Legionella* spp. and clearly showed two unequivocal and distinctive patterns in our study. Recently, these two strains were described as two novel species with the names *L. gresilensis* sp. nov. (type strain, ATCC 700509) and *L. beliardensis* sp. nov. (type strain, ATCC 700761) (32). In the same way, the RiboPrinter displayed three distinctive patterns for three *Legionella* strains (ATCC 700511, ATCC 700703, ATCC 700761) in our dendrogram (Fig. 2), and thus, these strains may possibly represent new species of *Legionella* or new Sgs of known species. Interestingly, ATCC 700511 has been reported to produce a specific randomly amplified polymorphic DNA pattern (34), and our results could be considered new data to support this strain as a new species. Nevertheless, further investigation by 16S rRNA gene sequencing and DNA homology studies are necessary before a conclusion should be made.

In the same way, ATCC 51913, reported as *Legionella* genomospecies 1, displayed a distinctive pattern with the RiboPrinter. This strain was related to *L. quinlivanii* Sg 2 serologically and to *L. quinlivanii* Sg 1 and Sg 2 genetically (4). However, the pattern obtained with the RiboPrinter clearly differed from those associated with *L. quinlivanii* Sg 1 and Sg

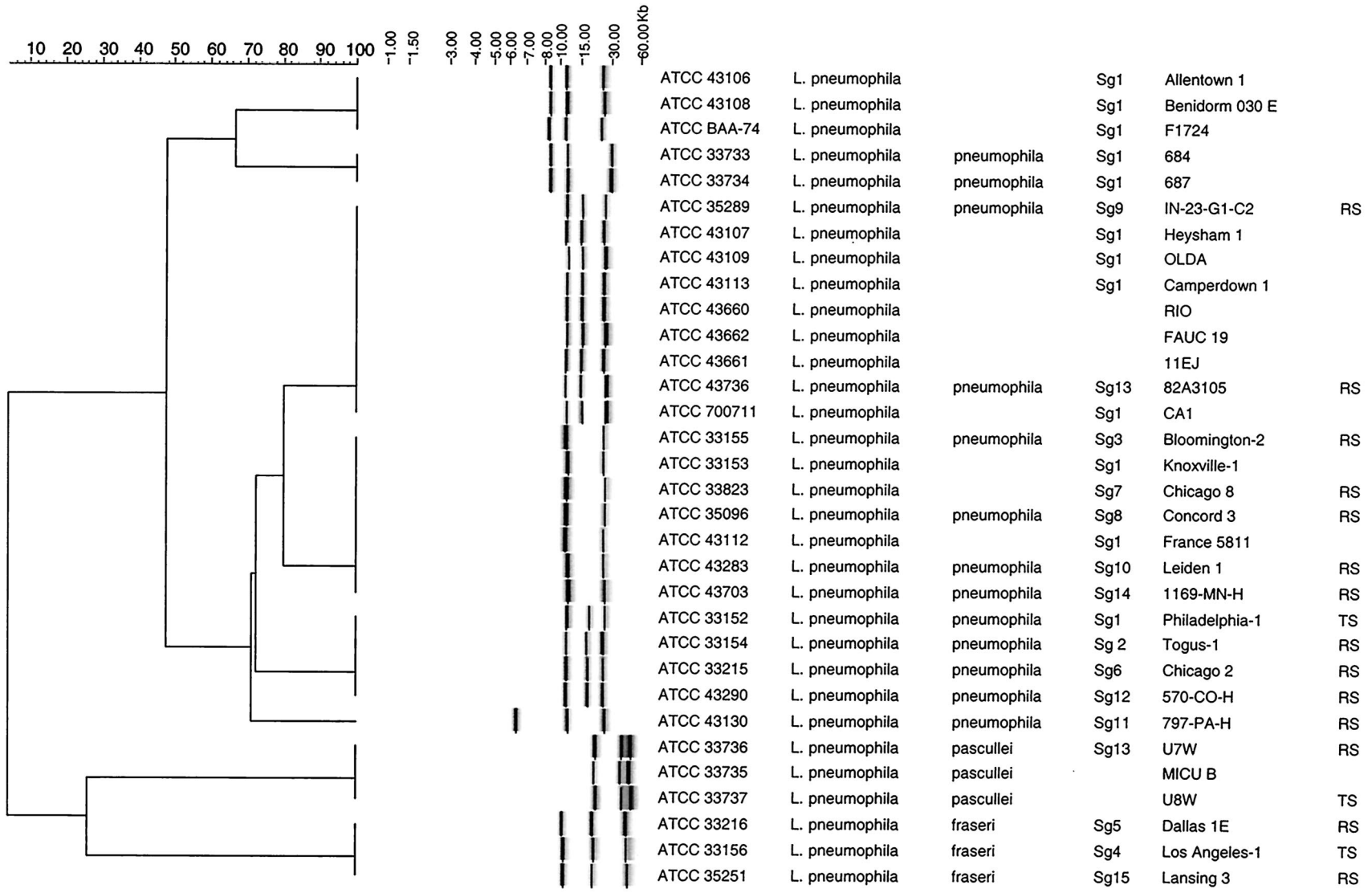

FIG. 1. Comparative analysis of the *Eco*RI ribotypes obtained with the RiboPrinter for the collection of *L. pneumophila* strains. Clustering was performed by the UPGMA method, and similarity analysis was based on the use of the Dice coefficient (see Materials and Methods). In the dendrogram scale, correlation levels were converted to percent homology levels. TS, type strain; RS, reference strain.

ATCC 43830 L. quinlivanii Sg1 1442-AUS-E TS
ATCC BAA-538 L. quinlivanii Sg2 LC870 RS
ATCC 700990 L. drozanskii Sg1 LLAP-1 TS
ATCC 35999 L. hackeliae Sg2 798-PA-H RS
ATCC 35250 L. hackeliae Sg1 Lansing 2 TS
ATCC 51913 L. genomospecies 1 Sg1 2055-AUS-E
ATCC 35304 L. rubrilucens Sg1 WA-270A-C2 TS
ATCC 49655 L. shakespearei Sg1 214 TS
ATCC 33623 L. jordanis Sg1 BL-540 TS
ATCC 700762 L. jordanis Ly95.90
ATCC 35302 L. steigerwaltii Sg1 SC-18-C9 TS
ATCC 33877 L. wadsworthii Sg1 Wadsworth 81-716A TS
ATCC 43878 L. brunensis Sg1 444-1 TS
ATCC 43753 L. cincinnatiensis Sg1 72-OH-H TS
ATCC 700992 L. fallonii Sg1 LLAP-10 TS
ATCC 33734 L. pneumophila pneumophila Sg1 687
ATCC 43108 L. pneumophila Sg1 Benidorm 030 E
ATCC 43736 L. pneumophila pneumophila Sg13 82A3105 RS
ATCC 700833 L. species ParisB1
ATCC 35096 L. pneumophila pneumophila Sg8 Concord 3 RS
ATCC 700705 L. species La Rochelle A2.1
ATCC 43130 L. pneumophila pneumophila Sg11 797-PA-H RS
ATCC 700706 L. species Nancy II no. 3
ATCC 33152 L. pneumophila pneumophila Sg1 Philadelphia-1 TS
ATCC 35849 L. feeleii Sg2 691-WI-H RS
ATCC 35249 L. spiritensis Sg1 MSH-9 TS
ATCC 49588 L. fairfieldensis Sg1 1725-AUS-E TS
ATCC 700511 L. species IB V no 3
ATCC 33484 L. longbeachae Sg2 Tucker 1 RS
ATCC 49751 L. lansingensis Sg1 1677-MI-H TS
ATCC 35303 L. erythra Sg1 SE-32A-C8 TS
ATCC 43877 L. moravica Sg1 316-36 TS
ATCC 700509 L. gresilensis Sg1 Gréoux 11 D13 TS
ATCC 700759 L. gresilensis Mulhouse 12 A23
ATCC 35248 L. sainthelensi Sg1 MSH-4 TS
ATCC 35300 L. maceachernii Sg1 PX-1-G2-E2 TS
ATCC 43769 L. gormanii 86A5796
ATCC 33204 L. micdadei EK
ATCC 33218 L. micdadei Sg1 TATLOCK TS
ATCC 33344 L. micdadei VAMC-MCC
ATCC 33346 L. micdadei VAM-PGH-12
ATCC 33345 L. micdadei VAM-7W
60.00 kb
40.00
30.00
20.00
15.00
10.00
8.00
7.00
6.00
5.00
4.00
3.50
3.00
2.50
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1.00
10 20 30 40 50 60 70 80 90 100

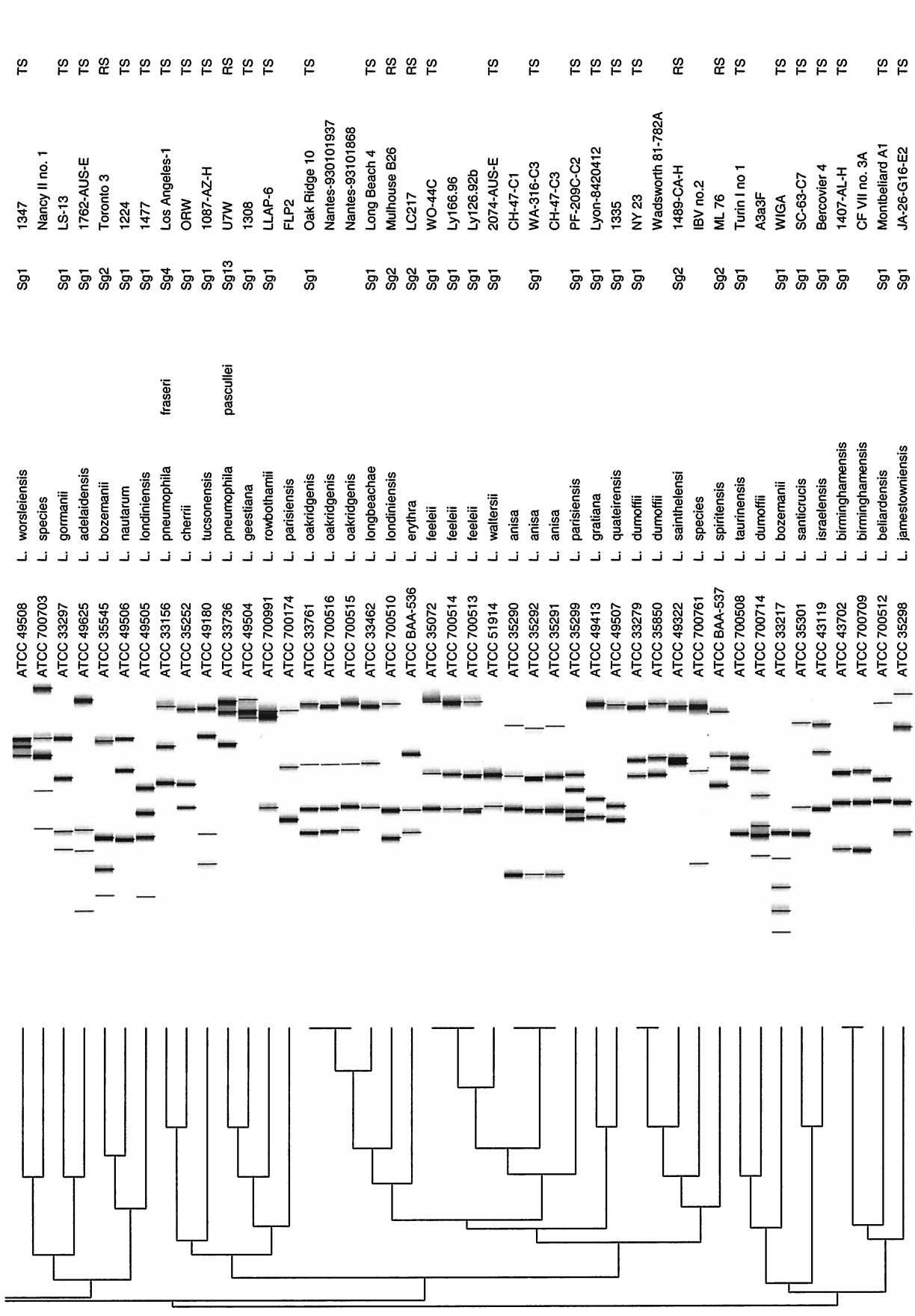

FIG. 2. Comparative analysis of the *Eco*RI ribotypes obtained with the RiboPrinter for the ATCC collection of *Legionella* strains. Clustering was performed by the UPGMA method, and similarity analysis was based on the use of the Dice coefficient (see Materials and Methods). In the dendrogram scale, correlation levels were converted to percent homology levels. TS, type strain; RS, reference strain.

2. Thus, this strain could possibly be considered a new Sg of *L. quinlivanii* or a novel species.

The automated ribotyping system with *Eco*RI restriction digestion has been shown to be a powerful tool for general genomic analysis of *Legionella* isolates (e.g., determination of new species or serotypes within a given species). However, this method lacked the discriminatory power required for routine analysis of nosocomial etiological agents, as epidemiologically unrelated strains within a given species may present identical ribotypes. This could be illustrated by examination of the major cluster of *L. pneumophila* strains in which six ribogroups have been identified. Four ribogroups (ribogroups 1, 3, 4, and 5) contained epidemiologically unrelated strains, as shown by their different serotypes, as well as strains with identical or unknown serotypes which were most unlikely related due to their very different geographical origins or sources (Table 1). For example, the nine isolates of ribogroup 3 came from Holland, the Centers for Disease Control and Prevention-Quebec, California, and Pennsylvania. Strains within ribogroups 4 and 5 were isolated from different parts of the United States as well as other countries.

When our study was initiated, *Eco*RI was the only enzyme available for use with the instrument. In the past few years additional restriction enzymes have been added, which may improve the discriminatory ability of the system. Enzymes such as *Cla*I, *Nci*I, *Pst*I, and *Hin*dIII have already been tested for use in the ribotyping of the *Legionellaceae* family manually (2, 24, 44), but a combination of enzymes was needed for good differentiation among the species (2).

Conclusion. Automated ribotyping can serve as a rapid and reproducible method for characterization of the members of the *Legionellaceae* family. Increased awareness of the diseases caused by *Legionella* has resulted in closer monitoring and investigation of potential sources of infection. This will no doubt increase the number of *Legionella* species being isolated and examined from environmental and clinical studies. Given its worldwide distribution and interconnection, the RiboPrinter system will enable immediate comparisons of ribotypes through connection of databases of ribotypes and assessment of interrelationships within the *Legionellaceae* family. Despite the limitations with the use of the RiboPrinter as a tool for epidemiological analysis of nosocomial members of the *Legionellaceae* family, this automated system holds promise as a very useful addition to the ever expanding molecular typing repertoire.

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