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Australian Isolates of *Legionella longbeachae* Are Not a Clonal Population

J. C. MONTANARO-PUNZENGRUBER,[1,2]* L. HICKS,[2] W. MEYER,[1,2] AND G. L. GILBERT[1,2]

Department of Medicine, University of Sydney, New South Wales 2006,[1] and Center for Infectious Diseases and Microbiology, Institute of Clinical Pathology and Medical Research, Westmead Hospital, Westmead, New South Wales 2145,[2] Australia

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***Legionella longbeachae* is almost as frequent a cause of legionellosis in Australia as *Legionella pneumophila*, but epidemiological investigation of possible environmental sources and clinical cases has been limited by the lack of a discriminatory subtyping method. The purpose of this study was to examine the genetic variability among Australian isolates of *L. longbeachae* serogroup 1. Pulsed-field gel electrophoresis (PFGE) of *Sfi*I fragments revealed three distinct pulsotypes among 57 clinical and 11 environmental isolates and the ATCC control strains of *L. longbeachae* serogroups 1 and 2. Each pulsotype differed by four bands, corresponding to <65% similarity. A clonal subgroup within each pulsotype was characterized by >88% similarity. The largest major cluster was pulsotype A, which included 43 clinical isolates and 9 environmental isolates and was divided into five subgroups. Pulsotypes B and C comprised smaller numbers of clinical and environmental isolates, which could each be further divided into three subgroups. The ATCC type strain of *L. longbeachae* serogroup 1 was classified as pulsotype B, subtype B3, while the ATCC type strain of *L. longbeachae* serogroup 2 was identified as a different pulsotype, LL2. *Sfi*I macrorestriction analysis followed by PFGE showed that the Australian *L. longbeachae* strains are not a single clonal population as previously reported.**

Legionellae are environmental organisms that can cause disease in humans (2). Clinical manifestations of legionella infection range from no symptoms to potentially fatal pneumonia and multisystem disease. There are 42 species in the genus *Legionella* (5), more than half of which have been implicated in human disease (2).

Transmission of the bacteria from the environment to humans occurs via inhalation or aspiration of *Legionella*-containing aerosols (6, 8). A suspected cluster or outbreak of cases of legionellosis requires careful epidemiological investigation to identify possible sources of infection. Such investigations also require a sensitive and discriminatory subtyping technique to identify similarities and differences between possibly related strains (3).

Legionella longbeachae is an uncommon pathogen in most parts of the world (17) but causes up to half the cases of legionellosis in many regions in Australia (1, 11). The reason for this is not clear. It has commonly been isolated from soil and decomposing materials, such as bark or sawdust used in potting mixes (33), and has been detected occasionally in water (29). *L. longbeachae* has caused at least two outbreaks of legionellosis in Australia, one in Western Australia (7) and the other in South Australia (19). Studies of Australian clinical strains of *L. longbeachae* by multienzyme electrophoresis (19), ribotyping (19), and random amplified polymorphic DNA (RAPD) typing (9) have suggested that *L. longbeachae* serogroup 1 strains are largely clonal. This similarity between strains has thwarted attempts to develop a discriminatory subtyping method, which would be useful to link environmental isolates to cases of clinical disease.

Legionella pneumophila serogroup 1 causes up to 95% of the cases of legionellosis worldwide and most outbreaks and sporadic cases in Australia (1, 11). For this reason, it has been the focus of most subtyping techniques, including typing with different panels of monoclonal antibodies (20), plasmid analysis (10, 26), multienzyme or alloenzyme electrophoresis (18), restriction fragment length polymorphism (15, 16), ribotyping (18), arbitrary primed PCR (13, 14), RAPD typing (30), and macrorestriction enzyme digestion followed by pulsed-field gel electrophoresis (PFGE) (28, 31). At present, PFGE, following restriction digestion with the enzymes *Sfi*I or *Not*I, is the most discriminatory method. This technique has also been used to subtype epidemiologically linked strains of *L. pneumophila* serogroup 6 (24), *Legionella bozemanii* (22), and *Legionella micdadei* (21) but was not used previously to type isolates of *L. longbeachae*.

In this study we used macrorestriction enzyme digestion followed by PFGE to investigate the genetic variability of clinical and environmental isolates of *L. longbeachae* serogroup 1 from five states in Australia over a period of 10 years.

MATERIALS AND METHODS

Bacterial isolates. The 68 isolates of *L. longbeachae* serogroup 1 investigated in this study included 24 clinical isolates from New South Wales, 16 clinical and 5 environmental isolates from Queensland, 1 clinical isolate from Tasmania, 4 clinical isolates from South Australia, and 12 clinical and 6 environmental isolates from Western Australia (Table 1). One environmental isolate which had been identified as *L. longbeachae* serogroup 2 from Western Australia was also tested. Controls were selected from the American Type Culture Collection (ATCC): *L. pneumophila* serogroup 1 (Philadelphia 1; ATCC 33152), *L. longbeachae* serogroup 1 (Long Beach 4; ATCC 33264), and *L. longbeachae* serogroup 2 (Tucker 1; ATCC 33484).

Bacterial cultures were grown for 48 h on buffered charcoal yeast extract agar with alpha ketoglutarate (BCYEα agar; Oxoid, Ltd., Basingstoke, Hampshire, England) and incubated in a humidified atmosphere with 5% CO_2 at 35°C (31).

Identification of isolates. A rapid latex test (Serobact; Disposable Products, Adelaide, South Australia) was used for presumptive identification of the isolates as *L. longbeachae* serogroup 1. These results were confirmed by direct immunofluorescence with a panel of pooled monovalent *Legionella* antibodies (MarDx Diagnostics, Scotch Plains, N.J.) and a monoclonal antibody to *L. pneumophila* groups 1 to 14 (Genetic Systems, Seattle, Wash.) according to the manufacturers' recommendations. Direct immunofluorescence with eight species- or serogroup-

* Corresponding author. Mailing address: Center for Infectious Diseases and Microbiology, Level 3, Institute of Clinical Pathology and Medical Research, Westmead Hospital, Westmead, New South Wales 2145, Australia. Phone: 61-2-9845-6895. Fax: 61-2-9893-8659. E-mail: jacquiem@blackburn.med.usyd.edu.au.

TABLE 1. Clinical and environmental isolates of *L. pneumophila* and *L. longbeachae* used in this study[a]

Species and ICPMR reference no.	Country or state of origin	Source	Yr of isolation	Pulsotype	Estimated genome size (kb)
Legionella pneumophila subsp. *pneumophila* Brenner					
ATCC 33152* (type strain, Philadelphia 1)	USA	Human lung	1979	Not assigned	3,629
Legionella longbeachae McKinney					
ATCC 33462* (type strain, Long Beach 4)	USA	Human lung	1981	B3	3,436
ATCC 33484* (type strain, Tucker 1, Georgia)	USA	Human lung	1981	LL2	3,514
147, 150, 287*, 288, 273, 460	NSW	Clinical	1992–1998	A1	3,569
149, 158, 280, 283, 284	NSW	Clinical	1990–1992	A2	
144, 272*, 275	NSW	Clinical	1992	A3	3,514
143	NSW	Clinical	1993	B1	
459*	NSW	Sputum	1998	B2	4,041
159, 279, 281*, 285, 286	NSW	Clinical	1992–1993	C2	3,495
142*, 274, 282	NSW	Clinical	1992–1994	C3	3,674
151, 157, 160, 163, 164, 302, 303*, 354	QLD	Clinical	1988–1992	A1	3,429
155	QLD	Environmental	1992	A2	
156, 165	QLD	Clinical	1992	A2	
153*	QLD	Environmental	1992	A3	3,723
162, 304, 305, 306, 356	QLD	Clinical	1989–1995	A4	
141, 154	QLD	Environmental	1992	A4	
161*	QLD	Clinical	1992	A5	3,673
152*	QLD	Environmental	1992	B1	3,297
260	TAS	Clinical	1993	A1	
389	SA	Clinical	1988	A3	
387, 401*	SA	Clinical	1983	B1	3,297
390*	SA	Clinical	1992	C3	3,655
361, 362, 363, 364, 365, 370, 371, 374, 375	WA	Clinical	1992–1993	A1	
367, 368, 376	WA	Potting mix	1992–1994	A1	
377*	WA	Potting mix	1994	A2	3,531
378	WA	Sputum	1994	A3	
366*	WA	BAL	1992	A4	3,436
359*	WA	Potting mix	1992	A4	3,436
373*	WA	Potting mix	1993	C1	3,468
379*	WA	Sputum	1994	C1	3,591
369* (atypical strain)	WA	Potting mix	1993	Not assigned	2,421

[a] BAL, bronchoalveolar lavage; NSW, New South Wales; SA, South Australia; QLD, Queensland; TAS, Tasmania; WA, Western Australia; ICPMR, Institute of Clinical Pathology and Medical Research, Westmead Hospital, Westmead, NSW. All *L. longbeachae* isolates were serogroup 1 except ATCC 33484 and 369 (atypical strain), which were serogroup 2. The asterisks denote isolates for which an estimated genome size is given in the right-hand column.

specific monovalent antibodies, including *L. longbeachae* serogroups 1 and 2 (MarDx Diagnostics), was also performed according the manufacturer's protocol. Isolate identification was confirmed as *L. longbeachae* with a positive reaction to reagents *L.* species b to j and *L.* omni species b to p and a negative reaction to both *L. pneumophila* reagents 1 to 6 and *L. pneumophila* 1 to 14, in addition to a positive reaction to *L. longbeachae*-specific monovalent antibody.

Preparation of PFGE plugs. PFGE plugs were prepared according to a modified version of the methods of Smith and Cantor (32) and Gautom (12). Briefly, bacterial cells were harvested into approximately 3 ml of Pett IV buffer (1.0 M NaCl, 10 mM Tris-HCl [pH 7.6]) and the bacterial suspensions were adjusted to exactly 20% transmittance (equivalent to 3×10^{10} organisms/ml) with a calibrated bacterial nephelometer (Vitek colorimeter; Hach Company, Loveland, Colo.). After centrifugation, a 400-μl aliquot of the bacterial suspension was concentrated to half its volume and mixed with an equal volume of molten 2.4% low-melting-point agarose (Bio-Rad, Hercules, Calif.) in Pett IV buffer and dispensed into a disposable plug mold (Bio-Rad). The final concentration of the bacterial DNA in the plug was 10 μg (1 μg of DNA/plug slice). The usually recommended preliminary RNase and lysozyme digestion step at 37°C (4, 23, 31) was omitted, as it was found that this step did not affect digestion with the enzyme *Sfi*I. The bacterial plugs were incubated in 2-ml Eppendorf tubes containing 1.5 ml of ESP solution (0.5 M EDTA [pH 8.0], 1.0% *N*-lauryl sarcosine, 2 mg of proteinase K/ml) and incubated overnight at 55°C.

PFGE plug digestion and electrophoresis. Prior to digestion, the plugs were incubated in a solution of 2 ml of 10 mM Tris–0.1 M EDTA and 1.0 mM phenylmethylsulfonyl fluoride (pH 7.5) (Sigma-Aldrich, St. Louis, Mo.) for 1 h at room temperature, washed in 1× TE (10 mM Tris, 0.1 mM EDTA [pH 7.5]), and then stored in 1× TE at 4°C until required. For restriction enzyme digestion, plug slices were digested overnight at 50°C for *Sfi*I or 37°C for *Not*I in a 50-μl reaction mixture which contained 2.5 U of *Sfi*I or *Not*I restriction enzyme/ml of buffer (New England Biolabs, Beverly, Mass.). *L. longbeachae* serogroup 1 (ATCC 33462) and *L. pneumophila* serogroup 1 (ATCC 33152) were used as internal controls and digested in parallel with the test organisms. These were included as controls in every gel along with at least three lanes of *Saccharomyces cerevisiae* chromosomes (catalog no. 345; Promega, Madison, Wis.) as fragment size standards. The fragments were electrophoretically separated by PFGE with a contour-clamped homogeneous electric field system (Bio-Rad Chef Mapper) in 1% PFGE grade agarose (Bio-Rad) and 0.5× TBE running buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA [pH 8.0]). The initial pulse time of 3.51 s was increased linearly to a final switch time of 93.56 s over 24 h at 6 V/cm at 14°C. The gels were then stained with 0.5 g of ethidium bromide/ml for 10 min, destained in water, and photographed under UV transillumination.

Evaluation of reproducibility of the PFGE results. Electrophoretic bands for the PFGE restriction fragments were sized and compared with the software program GelCompar version 4.1 (Applied Maths, Kortrijk, Belgium). Computer comparison was based on the algorithm of the unweighted pair group method for arithmetic averages and the Dice coefficient (25) with 3.2% band tolerance. Band tolerance statistics were calculated on the basis of differences in band positions of a list of identical internal control patterns with the GelCompar program. The lowest band tolerance required to have identical isolates typed as identical by the GelCompar program was 3.2%, and this value was applied to the entire band-matching comparison. No other computer-enhanced optimization or smoothing was used.

RESULTS

Analysis of PFGE typing. Preliminary results showed that digestion with *Not*I produced too few (three to four) restriction fragments to allow discrimination between strains of *L. longbeachae* (results not shown). Further analyses were confined to PFGE with *Sfi*I. The number of *Sfi*I fragments varied from four to seven, which ranged in size from approximately 400 to 1,500 kb (Fig. 1, 2, and 3). Interpolation of the known chromosome sizes of *S. cerevisiae* gave a standard reference curve for the

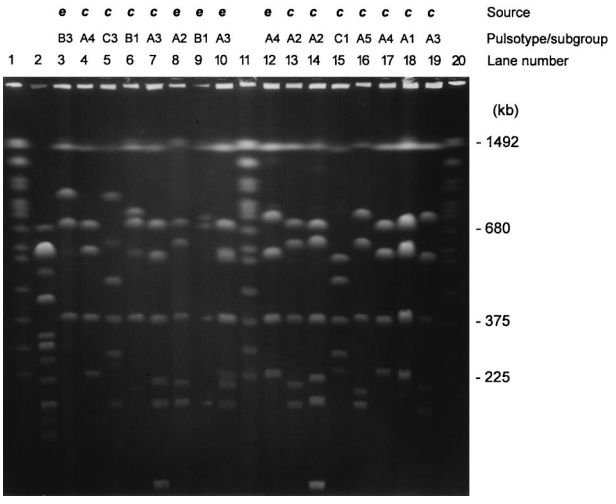

FIG. 1. PFGE of *Sfi*I-cleaved DNAs from *L. longbeachae* serogroup 1 isolates from Australia. Lanes: 2, *L. pneumophila* serogroup 1 (ATCC 33152); 3, *L. longbeachae* serogroup 1 (ATCC 33462); 4, 141; 5, 142; 6, 143; 7, 144; 8, 149; 9, 152; 10, 153; 12, 154; 13, 155; 14, 158; 15, 159; 16, 161; 17, 162; 18, 163; 19, 272. Lanes 1, 11, and 20 contained *S. cerevisiae* chromosomes as a molecular size standard. e, environmental isolate; c, clinical isolate. The letters A to C and the numbers 1 to 5 indicate pulsotypes and subgroups, respectively.

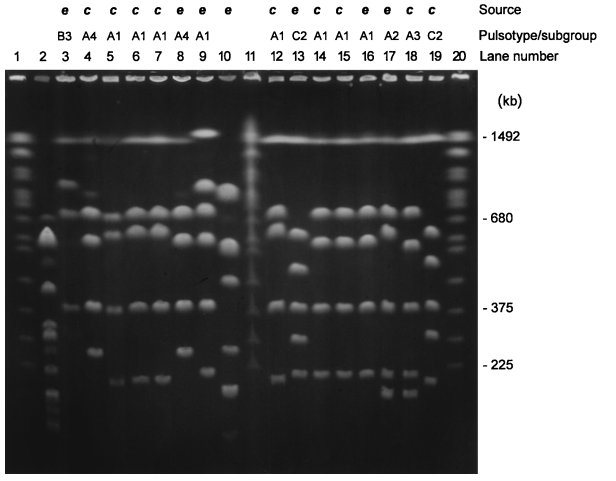

FIG. 3. PFGE of *Sfi*I-cleaved DNAs from *L. longbeachae* serogroup 1 isolates from Western Australia. Lanes: 2, *L. pneumophila* serogroup 1 (ATCC 33152); 3, *L. longbeachae* serogroup 1 (ATCC 33462); 4, 359; 5, 361; 6, 364; 7, 365; 8, 366; 9, 368; 10, 369; 12, 371; 13, 373; 14, 374; 15, 375; 16, 376; 17, 377; 18, 378. Lane 19, isolate 369, was typed by the MIDI system as an atypical *L. longbeachae* strain. Lanes 1, 11, and 20 contained *S. cerevisiae* chromosomes as a molecular size standard. e, environmental isolate; c, clinical isolate. The letters A to C and the numbers 1 to 4 indicate pulsotypes and subgroups, respectively.

comparison of sample fragment sizes. The genome sizes of the *L. longbeachae* isolates were calculated by adding the sizes of individual fragments for each strain. They ranged from 3,300 to 4,300 kb (Table 1). A fragment of 1,493 kb was common to all *L. longbeachae* serogroup 1 isolates and both serogroups 1 and 2 ATCC strains but was absent from the one environmental isolate that had been identified as *L. longbeachae* serogroup 2 and from the *L. pneumophila* control strain. The next most common fragments of the *L. longbeachae* isolates were 389 (94% of isolates) and 701 kb (79% of isolates).

The two *L. longbeachae* ATCC serogroup 1 and 2 control strains showed patterns that were distinguishable from each other, with the former being similar to the pulsotypes obtained from the Australian *L. longbeachae* serogroup 1 strains. The type strains of *L. longbeachae* serogroups 1 and 2 (ATCC 33462 and 33484) showed a similarity of 64%, using the Dice coefficient. *L. longbeachae* and *L. pneumophila* serogroup 1 (Philadelphia 1) were <40% similar, and the Australian *L. longbeachae* serogroup 1 strains showed a similarity of 52%. When the Australian *L. longbeachae* serogroup 1 isolates were considered together, there were three distinct patterns, resulting in three dendrogram clades that could be separated by a four-band difference and <65% similarity with the Dice coefficient (Fig. 4). The percentage of similarity between different pulsotypes varied from 52 to 65%. Type A was the commonest pattern, with 52 of 68 isolates, and was divided into five subgroups, A1 to A5, which differed in one or two bands. The number of fragments shared between subgroups within a pulsotype varied from five to seven fragments. Within each of these subgroups, the fragment patterns were >88% similar by the Dice coefficient and could be clearly distinguished from each other on a PFGE gel.

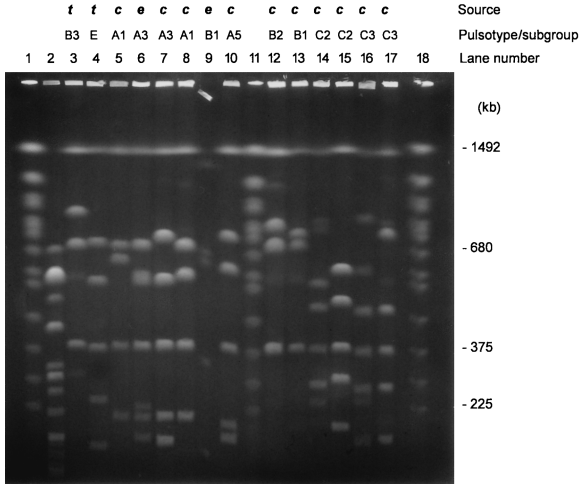

FIG. 2. PFGE of *Sfi*I-cleaved DNAs from *L. longbeachae* serogroup 1 isolates from Australia. Lanes: 2, *L. pneumophila* serogroup 1 (ATCC 33152); 3, *L. longbeachae* serogroup 1 (ATCC 33462); 4, *L. longbeachae* serogroup 2 (ATCC 33484); 5, 287; 6, 153; 7, 272; 8, 303; 9, 152; 10, 161; 12, 459; 13, 401; 14, 159; 15, 379; 16, 142; 17, 390. Lanes 1, 11, and 18 contained *S. cerevisiae* chromosomes as a molecular size standard. t, type strains; e, environmental isolate; c, clinical isolate. The letters A to C and the numbers 1 to 5 indicate pulsotypes and subgroups, respectively. The DNA block in lane 9 moved from its original position on the comb prior to the gel being run.

Most pulsotype A subgroups were found over periods of several years, and some were widely distributed geographically (Fig. 2 and Table 1). Type A1 was represented by 27 isolates from four states (Table 1). It was isolated repeatedly from two patients (isolates 361 to 365 and 370 to 371), and there were single isolates from individual patients presented over a period of at least 10 years, which suggests that the PFGE patterns are genetically stable. Types A2, A3, and A4 were represented by nine, six, and nine isolates, respectively, and there was one clinical isolate in pulsotype A5. The designation LL2 was given to the type strain of *L. longbeachae* serogroup 2, which showed 65% similarity to the pulsotype A clade of the Australian clinical *L. longbeachae* serogroup 1 isolates.

Six *L. longbeachae* isolates were designated pulsotype B. Four of the six isolates, which were geographically widespread, were designated pulsotype B1. There was only a single representative of pulsotype B2. Pulsotype B was most similar (74%

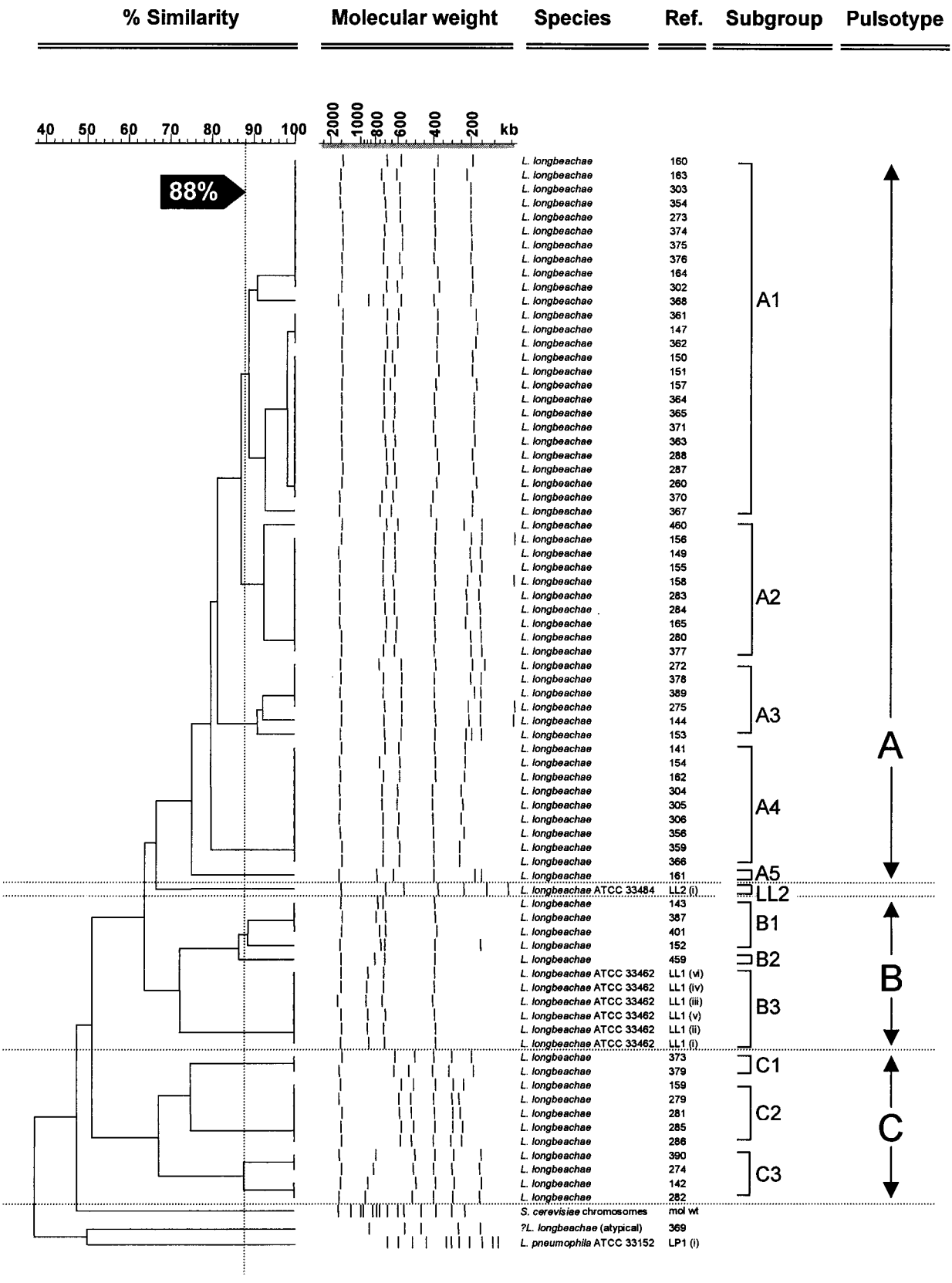

FIG. 4. Cluster dendrogram of Australian *L. longbeachae* isolates generated from *Sfi*I restriction fragments separated by PFGE and analyzed with the program GelCompar ver 4.1. Similarity of >65% and more than a four-band difference in the band pattern divide the different types and clades. Subtype divisions have >88% similarity and band pattern differences of two or three bands. The type strain for *L. longbeachae* serogroup 1 was designated pulsotype B3, and the type strain for *L. longbeachae* serogroup 2 was designated pulsotype LL2. Isolates LL1 (i to vi) are representative internal controls of ATCC 33462, which were run on separate PFGE gels.

similarity) to the *L. longbeachae* ATCC serogroup 1 control strain, which was designated pulsotype B3.

Eleven *L. longbeachae* isolates designated pulsotype C were divided into three subgroups: pulsotype C1 was found only in Western Australia (two isolates), pulsotype C2 was found only in New South Wales (five isolates), and pulsotype C3 was found in South Australia (one isolate) and in New South Wales (three isolates).

The Western Australian isolates included 12 clinical isolates from six patients and 6 environmental isolates (Fig. 3), which belonged to pulsotypes A and C. Five isolates from patient 1 (361 to 365), two from patient 3 (370 and 371), two isolates from patient 4 (374 and 375), and two potting mix isolates (368 and 376) showed pattern A1. Pulsotype A4 was found in one patient isolate (366; patient 2) and from a potting mix sample (359). Pulsotype C1 (379) was isolated from one patient (patient 6) and was also represented among potting mix isolates. Thus, all four subtypes isolated from Western Australian patients were also found among the environmental isolates over periods of 1 to 3 years.

One Western Australian environmental isolate (369) did not fit any of the three pulsotypes (Fig. 3 and 4), and its genome size was smaller (2,421 kb). None of the major fragments was present in any other isolate investigated. The 1,493-kb band, common to all other *L. longbeachae* isolates, was absent. Initial investigation of this isolate by routine methods with direct and indirect immunofluorescence indicated that it was *L. longbeachae* but was only weakly reactive with serogroup 2 antiserum. PFGE also showed this strain to be very different (38% similarity by the Dice coefficient) (Fig. 4) from other *L. longbeachae* isolates but more similar to *L. pneumophila*. Further tests to confirm its identification were undertaken. It failed to react with both *L. pneumophila* monovalent serum pools and reacted weakly with monovalent immunofluorescence pooled sera. When this isolate was tested with the MIDI bacterial identification system (Sherlock MIS; MIDI Inc., Newark, Del.) with bacterial fatty acid analysis and compared to a commercial database, it was identified as *L. longbeachae*, but based on its similarity index (0.437) it was an atypical strain.

DISCUSSION

The purpose of our study was to examine the genetic variability of Australian *L. longbeachae* serogroup 1 isolates and develop a practicable method for subtyping. Using macrorestriction digestion with *Sfi*I followed by PFGE, we demonstrated three distinct patterns (dendrogram clades) that could be separated by four-band differences and <65% similarity with the Dice coefficient (Fig. 1) among 68 clinical and environmental isolates. The three major *L. longbeachae* serogroup 1 pulsotypes were subdivided into 11 subgroups, most of which were widely distributed geographically throughout Australia and over significant periods.

These results are in contrast to those of previous studies, in which alloenzyme electrophoresis, ribotyping, and RAPD analyses failed to distinguish among Australian strains of *L. longbeachae* serogroup 1 and showed only minor differences among strains of *L. longbeachae* serogroup 2 (9, 19). The results of previous studies have been interpreted as indicating widespread distribution of a single clone of *L. longbeachae* in Australia (9, 19).

However, by adding the sizes of fragments obtained after digestion (Table 1), we estimated that the genome sizes of different strains varied from 3,300 to 4,300 kb. This is similar to the degree of variation in genome size among strains of *L. pneumophila* serogroup 1, which has been reported to range from 2,600 to 3,900 kb (31). A previous study had also reported the genome size of *L. pneumophila* Philadelphia 1 as 3,900 kb (4). This suggests that *L. longbeachae* is more variable than was previously believed.

This apparent variability is unlikely to be due to incomplete lysis of bacteria or digestion of DNA, since consistent results were obtained on repeat testing up to five times. The largest (1,493-kb) fragment was present in all *L. longbeachae* serogroup 1 strains and both *L. longbeachae* ATCC control strains, and the number of sizes of fragments did not vary when lower concentrations of DNA were used (data not shown). Moreover, the *L. pneumophila* PFGE internal control strain, which was processed in the same way as *L. longbeachae* isolates, gave the same number and size of fragments as previously described (31).

The appearance of the same pulsotypes and subgroups in different parts of Australia over a period of 10 years may be explained by a low mutation rate of *L. longbeachae* strains (19). Alternatively, it could be due to the widespread distribution of a common vehicle, such as potting mix, with persistence of *L. longbeachae* in the environment or in unused potting mix. It has been shown that *L. longbeachae* is able to survive in potting mix for up to 7 months (33).

Ideally, for PFGE, a restriction enzyme should be chosen that will generate at least 10 fragments per isolate (34). The enzyme *Not*I generated too few fragments to produce a useful profile, and *Sfi*I generated only four to seven fragments from *L. longbeachae* DNA. However, in defining their criteria for the use of PFGE for bacterial subtyping, Tenover et al. (34) conceded that modification of the criteria may be necessary for defining pulsotypes among large numbers of isolates over extended periods. We suggest that modification of the criteria is justified to extend the use of *Sfi*I for PFGE to *L. longbeachae* as well as *L. pneumophila* (31), *L. micdadei* (21), and *L. bozemanii* (22), for which its use has been described. PFGE typing of *L. bozemanii* with *Sfi*I produced a similar number of fragments (four to eight), and pulsotypes were defined by criteria similar to those used in our study, namely, four or more band differences and <65% band similarity by the Dice coefficient (22).

The routine use of PFGE as a typing method is often limited by the fact that it is time-consuming and labor intensive (12). The standard procedure (23) was reduced to 3 days by using a turbidity standard rather than an optical density reading to estimate bacterial numbers for agarose plugs, and the omission of the lysozyme digestion step eliminated an overnight incubation. Although interpretation of subtyping data is most useful when multiple techniques are used (31), no other sufficiently discriminatory method for subtyping *L. longbeachae* has been described. However, it is likely that the use of other restriction enzymes, which cleave at different sites within the genome, could provide complementary patterns for subtyping.

*Sfi*I digestion followed by PFGE showed that the Australian *L. longbeachae* strains are not a single clonal population. The results of this study contribute to an understanding of the distribution of *L. longbeachae* serogroup 1 strains in Australia. A computer database with a number of mainly unrelated environmental and clinical isolates from five Australian states was established and can now be used in, and supplemented by, investigations of future cases and outbreaks. The PFGE method developed is discriminatory, could be applied to other *Legionella* species, and is sufficiently rapid to allow a timely investigation of a potential outbreak of legionellosis.

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