APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1991, p. 3226–3231 Vol. 57, No. 11
0099-2240/91/113226-06$02.00/0

Analysis of *Legionella pneumophila* Serogroup 6 Strains Isolated from a Hospital Warm Water Supply over a Three-Year Period by Using Genomic Long-Range Mapping Techniques and Monoclonal Antibodies

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Received 10 June 1991/Accepted 6 September 1991

Over a period of 3 years, *Legionella pneumophila* serogroup 6 strains were isolated from warm water outlets and dental units in the Dental Faculty and from the Surgery and Internal Medicine Clinics at the University of Dresden, Dresden, Germany. In the bacteriological unit of the above-mentioned facility, *L. pneumophila* serogroups 3 and 12 were grown from warm water specimens. The medical facilities are located in separate buildings connected with a ring pipe warm water system. All *L. pneumophila* serogroup 6 strains isolated from the warm water supply reacted with a serogroup-specific monoclonal antibody, but not with two other monoclonal antibodies which are subgroup specific, reacting with other serogroup 6 strains. The *Not*I genomic profiles obtained by pulsed-field gel electrophoresis of 25 serogroup 6 strains isolated from the Dental Faculty over a 3-year period, 1 isolate from the Internal Medicine Clinic, and 4 strains from the Surgery Clinic were identical. Furthermore, all these strains hybridized with a 300-kb *Not*I fragment when a legiolysin (*lly*)-specific DNA probe was used. The *Not*I pattern, however, differed from those of six serogroup 6 strains of other origins, one serogroup 12 strain from the bacteriological unit, and another six unrelated strains of serogroups other than serogroup 6. *L. pneumophila* serogroup 6 strains which can be divided into only two subgroups by the use of monoclonal antibodies are differentiated in at least six *Not*I cleavage types obtained by pulsed-field electrophoresis.

Legionella pneumophila is the causative agent of Legionnaires' disease, a severe pneumonia, and the nonpneumonic syndrome Pontiac fever (28). It is known that legionellae are ubiquitous in water environments (5, 7, 10, 13, 18, 19, 25). They have been isolated from warm water taps and faucets, humidifiers, warm water tanks, cooling towers, dental units, and other warm water reservoirs. The ecology and survival of legionellae in the environment are closely related to protozoa, such as *Acanthamoeba* spp., *Naegleria* spp., *Hartmanella* spp., and *Tetrahymena pyriformis* (6, 7), in which they can multiply, survive, and resist harmful environmental influences. Other factors influencing colonization of legionellae in warm water systems are heterotrophic bacteria, temperature, pH, oxygen level, and plumbing fixture materials (22, 24, 25).

Up to now, 14 serogroups of *L. pneumophila* have been defined by using absorbed polyclonal rabbit sera (27). *L. pneumophila* serogroup 1 is still the most common clinical and environmental isolate, followed by *L. pneumophila* serogroup 6 strains (17). By using monoclonal antibody typing and other techniques, these serogroups can be divided into subgroups or subtypes (5, 10, 15, 16, 19, 21, 23). In this way epidemiological linkage between water systems and patients has been established for *L. pneumophila* serogroup 1. It has been documented that in plumbing fixtures various *Legionella* species, serogroups, and subgroups can exist (2, 5, 16, 18, 19, 21, 23, 25). Factors which influence appearance and disappearance of defined subtypes are not understood. The present study was undertaken to determine whether there will be detectable changes in *L. pneumophila* serogroup 6 strains isolated during a 3-year surveillance from a water system at the School of Medicine in Dresden, Germany.

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MATERIALS AND METHODS

Water samples. After at least 16 h of stagnation, 0.3- to 0.5-liter water samples were collected from dental units and warm water taps in the Faculty of Dentistry, School of Medicine in Dresden over a 3-year period. In the Internal Medicine Clinic, Surgery Clinic, and the bacteriological unit, warm water specimens from taps and faucets were sampled once in the same manner. The four facilities mentioned above are separate buildings, connected with a ring pipe warm water system. This water system is fed by two separate hot water tanks, which were employed one after the other. The temperature in these tanks was adjusted to between 56 and 58°C. During this study, no measures for eliminating legionellae such as superheating to 70°C or chlorination were made. The water temperature in the warm water system ranged from 30 to 50°C at the time of water collection.

***Legionella* strains and culture conditions.** Water samples were concentrated by centrifugation at 5,500 × *g* for 30 min. Legionellae were isolated on ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid]-buffered charcoal-yeast extract (BCYE) agar medium (4, 8) supplemented with 0.1% α-ketoglutarate (Sigma Corp., Munich, Germany), 0.3% glycine (Serva, Heidelberg, Germany), 1 mg of vancomycin (Lilly, Giessen, Germany) per liter, 80,000 U of polymyxin B (Pfizer, Karlsruhe, Germany) per liter, and 50,000 U of amphotericin B (Squipp-van Heyden, Munich, Germany) per liter with or without acid pretreatment (8). Agar plates

were incubated at 36°C in humidified air with 2 to 3% CO_2 for at least 7 days. With a dissecting microscope, up to 10 arbitrary chosen colonies were picked and subcultured on BCYE agar without antibiotics and on LB blood agar. Colonies which grew on BCYE agar but not on LB blood agar were biotyped and serotyped. For genome analysis, a representative group of isolates was selected. Additional *L. pneumophila* strains were kindly supplied by R. Marre (Lübeck, Germany), W. Ehret, (Munich, Germany), and E. Kmety (Bratislava, Czechoslovakia). All strains used are listed in Table 1.

Biochemical and serological typing. All strains isolated in Dresden were tested for hippurate hydrolysis, gelatinase, katalase, oxidase, and browning on tyrosine agar by the method of Edelstein (3). By using absorbed (serogroup-specific) rabbit sera against all 14 serogroups of *L. pneumophila* and fluorescein isothiocyanate-conjugated anti-rabbit antibodies, all strains were tested by the indirect immunofluorescence test. All serogroup 6 strains were further subtyped with monoclonal antibodies (12).

PFGE. Bacteria were harvested from antibiotic-free BCYE agar in SE buffer (75 mM NaCl, 25 mM EDTA [pH = 7.4]), washed twice in the same buffer, and adjusted to an optical density at 600 nm of 1.5. Equal volumes of *Legionella* cell suspensions and molten 2% genetic technology-grade agarose (Biometra, Göttingen, Germany) in SE buffer were mixed, making the final concentration in the blocks equal to 1%. Suspensions were poured into forms and were allowed to harden at 6°C for 15 min. Solidified agarose blocks were immersed in a digestion solution containing 2 g of proteinase K (Merck, Darmstadt, Germany) per liter in NDS buffer (1% sodium lauroylsarcosine, 0.5 M EDTA [pH = 9.5]) and incubated at 50°C overnight. After the agarose blocks were solidified on ice, they were washed four times in TE buffer (10 mM Tris, 0.5 mM EDTA [pH = 7.4]) and stored in the same buffer at 4°C. For cleavage, the agarose blocks were first equilibrated at 50°C for 2 h in restriction enzyme buffer. After the addition of *Not*I (GIBCO, Berlin, Germany) and incubation for 15 h at 37°C, the blocks were loaded on 1% agarose (Bethesda Research Laboratories, Berlin, Germany) in 0.5× Tris–borate–EDTA buffer (pH = 8.3). Pulsed-field gel electrophoresis (PFGE) was performed by using CHEF-DR II (Bio-Rad, Munich, Germany) equipment at 14°C, with a constant voltage of 200 V. Runs were carried out with increasing pulse times (60 to 90 s) for 21 h and an additional 2 h at a constant pulse time of 80 s for the gel in Fig. 1a. The other two gels (Fig. 1b and c) were run for 22 h at 60 to 90 s and for 3 h at 80-s pulse time. Yeast chromosomes (*Saccharomyces cerevisiae* WAY 5-4A; Biometra) and lambda concatemers and *Hin*dIII-cleaved lambda DNA (Pharmacia, Freiburg, Germany) were used as DNA size markers. Gels were stained with ethidium bromide and photographed under UV light.

Southern hybridization and generation of the DNA probe. Southern hybridization was performed as described previously (20). A *lly*-specific fragment generated from plasmid pEWL 114 (1, 29) as a 0.7-kb *Sph*I-*Nco*I fragment was used as the DNA probe for hybridization under high-stringency conditions (20). DNA techniques, such as plasmid DNA isolation, restriction enzyme cleavage, agarose gel electrophoresis, and fragment isolation from agarose gels and radioactive labelling of the fragment, were performed by the standard protocols previously described by Sambrook et al. (20).

RESULTS

Monitoring of the water system. The *L. pneumophila* serogroup 6 was the only serogroup which was consistently isolated from 48 water samples collected from dental units and warm water taps in the Faculty of Dentistry, in the Internal Medicine Clinic, and in the Department of Surgery. In the bacteriological unit, serogroups 3, 12, and 6 of *L. pneumophila* were found at a ratio of 10:10:1, respectively. All strains isolated were positive for hippurate hydrolysis, katalase, gelatinase, and browning on tyrosine agar. Tests for oxidase showed variable results. *Legionella* germ count ranged from between 5×10^2 to 8×10^4 CFU/liter in the water samples from warm water taps. Water obtained from dental units contained fewer legionellae, 2×10^2 to 4×10^3 CFU/liter.

Monoclonal antibody subtyping. All *L. pneumophila* serogroup 6 strains included in this study reacted with the serogroup-specific monoclonal antibody MAb 9/4. Two subgroup-specific antibodies (MAb 4/5 and MAb 4/6) gave coinciding positive indirect immunofluorescence test results only with the type strain Chicago-2 and with one strain (S 664) isolated in Lübeck, Germany. All other strains included in this study and listed in Table 1 were negative with both monoclonal antibodies (Table 1).

Genome analysis. The *Not*I cleavage pattern of the genomic DNA obtained by PFGE is shown in Fig. 1. The DNA of all strains tested in this study was cleaved into four to nine fragments by *Not*I. The size of fragments varied from approximately 2,000 to 10 kb. The most discriminating bands were found between 100 and 500 kb. The two gels shown in Fig. 1b and 1c were therefore run 2 h longer than the gel in Fig. 1a.

It is clearly demonstrated in Fig. 1 that all *L. pneumophila* serogroup 6 strains isolated during this survey from the dental unit and the warm water supply in the Dental Faculty, the Internal Medicine Clinic, and the Department of Surgery exhibited an identical *Not*I restriction pattern. One *L. pneumophila* serogroup 12 strain isolated from the same warm water system, in the bacteriological unit, which cross-reacted with nonabsorbed *L. pneumophila* serogroup 6 serum, displayed a completely different cleavage pattern (Fig. 1a). Concerning *L. pneumophila* serogroup 6 strains isolated from seven different locations, six distinguishable cleavage patterns were found. Strain S 664 (isolated in Lübeck, Germany) could not be differentiated from the ATCC type strain Chicago-2. Strain U 21 isolated in Munich, Germany and the strains from the water system in Dresden as well as strain B 496 from Bratislava, Czechoslovakia, and three strains from another water system in Dresden (St 165/1, St 165/3, and St 162/3) seem to be related but not identical.

We also attempted to hybridize the *Not*I patterns to a DNA probe, specific for the legiolysin of *L. pneumophila* (1), which has been cloned recently (29). All the *L. pneumophila* serogroup 6 strains exhibiting an identical *Not*I cleavage pattern (see above; also see Fig. 1) hybridized with a 300-kb *Not*I fragment, whereas in the strains which were unrelated according to the *Not*I profile, the *lly* gene was located on fragments of different sizes (Fig. 2).

DISCUSSION

During the survey of the water system in the School of Medicine in Dresden, *L. pneumophila* of serogroup 6 was permanently isolated. All 30 arbitrary chosen *L. pneumophila* serogroup 6 strains from the Dental Faculty, from the

TABLE 1. *L. pneumophila* strains of different serogroups analyzed by PFGE and monoclonal antibodies

Designation in Fig. 1	Strain	Serogroup	Isolate or origin		Reactivity[a] against	
			Location or source	Date (mo yr)	Mab 9/4	MAb 4/5 and Mb 4/6
1a	St 16	6	Faculty of Dentistry	Nov. 1987	+	−
1b	St 25	6	Faculty of Dentistry	Nov. 1987	+	−
1c	St 37	6	Faculty of Dentistry	May 1988	+	−
1d	St 47	6	Faculty of Dentistry	Oct. 1988	+	−
1e	St 48	6	Faculty of Dentistry	Oct. 1988	+	−
1f	St 97/7	6	Faculty of Dentistry	May 1989	+	−
1g	St 100/3	6	Faculty of Dentistry	May 1989	+	−
1h	St 102/1	6	Faculty of Dentistry	May 1989	+	−
1i	St 102/2	6	Faculty of Dentistry	May 1989	+	−
1j	St 120/1	6	Faculty of Dentistry	Sept. 1989	+	−
1k	St 120/2	6	Faculty of Dentistry	Sept. 1989	+	−
1l	St 120/3	6	Faculty of Dentistry	Sept. 1989	+	−
1m	St 120/4	6	Faculty of Dentistry	Sept. 1989	+	−
1n	St 123/1	6	Faculty of Dentistry	Sept. 1989	+	−
1o	St 123/2	6	Faculty of Dentistry	Sept. 1989	+	−
1p	St 124	6	Faculty of Dentistry	Sept. 1989	+	−
1q	St 214	6	Faculty of Dentistry	Feb. 1990	+	−
1r	St 215/1	6	Faculty of Dentistry	Feb. 1990	+	−
1s	St 215/2	6	Faculty of Dentistry	Feb. 1990	+	−
1t	St 216	6	Faculty of Dentistry	Feb. 1990	+	−
1u	St 217	6	Faculty of Dentistry	Feb. 1990	+	−
1v	St 218/1	6	Faculty of Dentistry	Feb. 1990	+	−
1w	St 218/2	6	Faculty of Dentistry	Feb. 1990	+	−
1x	St 220	6	Faculty of Dentistry	Feb. 1990	+	−
1y	St 221	6	Faculty of Dentistry	Feb. 1990	+	−
2a	W 75/1	6	Surgery clinic	Oct. 1988	+	−
2b	W 75/2	6	Surgery clinic	Oct. 1988	+	−
2c	W 76/1	6	Surgery clinic	Oct. 1988	+	−
2d	W 76/4	6	Surgery clinic	Oct. 1988	+	−
3	W 214	6	Internal Medicine clinic	Nov. 1989	+	−
4	W 29/3	12	Bakteriological Unit	Oct. 1988	NT	NT
5a	St 162/3	6	Dental unit in another part of Dresden	Feb. 1990	+	−
5b	St 165/1	6	Dental unit in another part of Dresden	Feb. 1990	+	−
5c	St 165/3	6	Dental unit in another part of Dresden	Feb. 1990	+	−
6	B 420	6	E. Kmety, Bratislava, Czechoslovakia		+	−
7	B 496	6	E. Kmety, Bratislava, Czechoslovakia		+	−
8	Chicago-2	6	CDC[b] (ATCC 33 215)		+	+
9	U 21	6	W. Ehret, Munich, Germany		+	−
10	S 664	6	R. Marre, Lübeck, Germany		+	+
11	S 685	10	R. Marre, Lübeck, Germany		NT	NT
12	S 640	5	R. Marre, Lübeck, Germany		NT	NT
13	S 667	4	R. Marre, Lübeck, Germany		NT	NT
14	Los Angeles-1	4	CDC (ATCC 33 156)		NT	NT
15	Bloomington-2	3	CDC (ATCC 33 155)		NT	NT

[a] +, positive by indirect immunofluorescence test; −, negative by indirect immunofluorescence test; NT, not tested.
[b] CDC, Centers for Disease Control, Atlanta, Ga.

Internal Medicine Clinic, and from the Surgery Clinic were indistinguishable when two monoclonal antibodies, the genomic *Not*I profile, as well as the hybridization pattern obtained with the *L. pneumophila* legiolysin (*lly*)-specific DNA probe were used. Therefore, it seems that there were no observable changes in the *Legionella* population during the 3 years under study.

Most studies concerning *Legionella* populations or subtyping of *L. pneumophila* were done with *L. pneumophila* serogroup 1 strains. By using monoclonal antibodies, this serogroup can be divided into 12 different antigenic subgroups (11, 16, 21). In a few reports, there were changes in the *L. pneumophila* serogroup 1 population in the water system during eradication procedures (5, 16) defined by monoclonal antibody typing and by plasmid analysis or alloenzyme typing, whereas Stout et al. (21) and Ribeiro et al. (18) found no changes in the *Legionella* population over 3 or more years. It is known that several factors such as

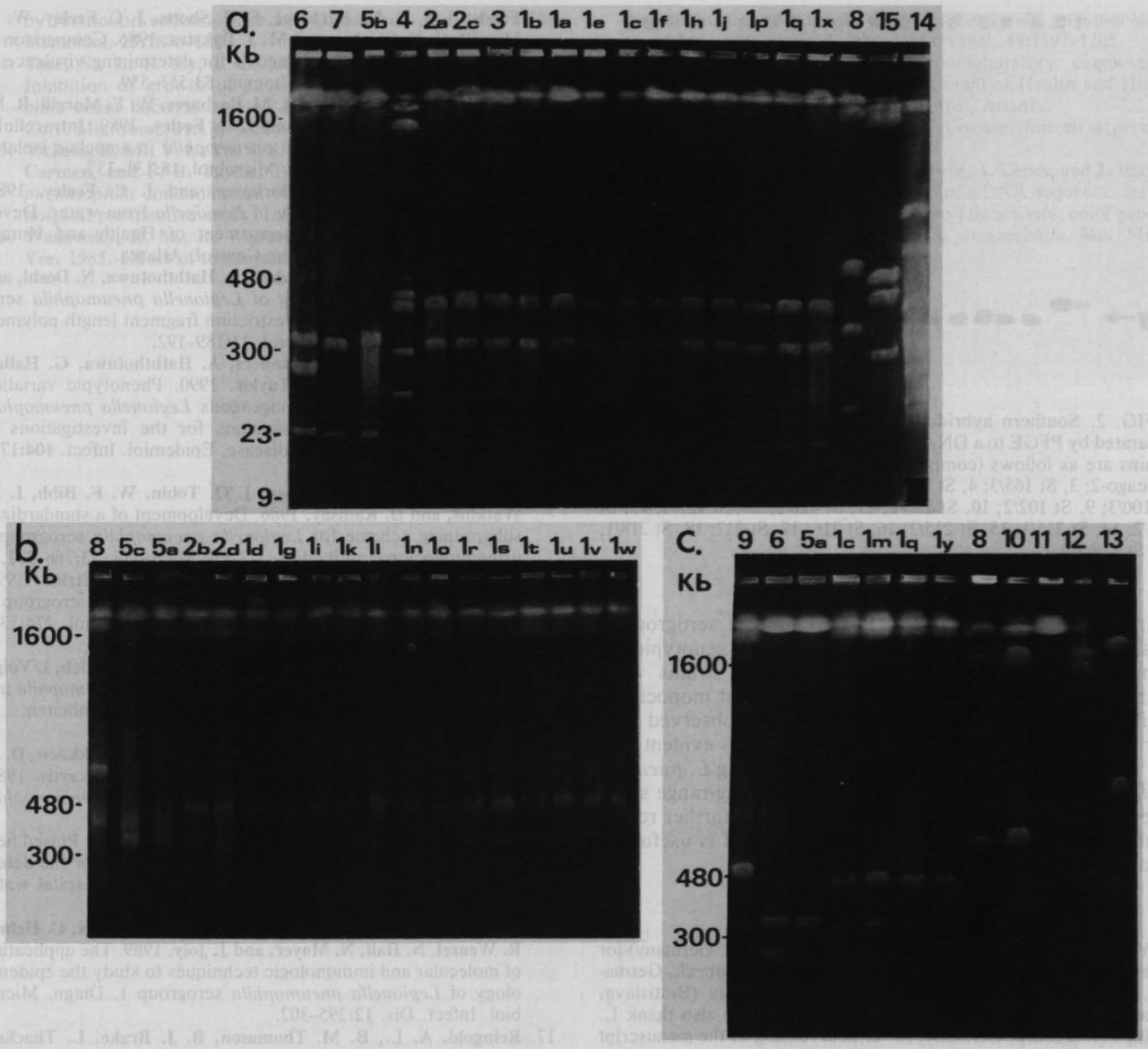

FIG. 1. PFGE of *Not*I-cleaved DNAs of *L. pneumophila* strains listed in Table 1. (a) Electrophoresis for 21 h at increasing pulse times (60 to 90 s) and for 2 h with 80-s pulse time. (b and c) Electrophoresis for 22 h at 60- to 90-s pulse time and for an additional 3 h with 80-s pulse time. DNA sizes are indicated to the left of the gels.

protozoa, plumbing material, construction pecularities, slime debris, symbiotic or antagonistic bacteria, temperature, pH, and oxygen level influence the ecology of *Legionella* spp. (4, 22, 24–26). However, it is not yet clear whether different subtypes or clones display different behavior in warm water systems, which may explain variations or constancy of *Legionella* populations in defined ecological systems. To answer this question, it is necessary to analyze *Legionella* strains genotypically and phenotypically.

In contrast to *L. pneumophila* serogroup 1, which can be divided into at least 12 subgroups by using monoclonal antibodies, serogroup 6 showed less antigenic diversity (12, 14). Up to now, only two different subgroups were found by McKinney et al. (14) and by us (12), using monoclonal antibodies. For epidemiological or ecological studies, it is necessary to subtype *Legionella* strains. Because of the limited discriminating power of monoclonal antibodies, subtyping *L. pneumophila* serogroup 6 strains should be complemented by at least one other subtyping method. In comparison to techniques such as elaborating genomic restriction fragment length polymorphism by hybridization to DNA probes (e.g., rDNA) (9) or alloenzyme typing (14), PFGE requires less time and laboratory work.

It should be emphasized that the number of *L. pneumophila* serogroup 6 strains tested in this study is not sufficient to establish completely the discriminating properties of this method, but the fact that *L. pneumophila* serogroup 6 strains isolated from seven different locations showed six different *Not*I cleavage patterns indicates that this technique is able to subtype strains of this serogroup in the desired manner. Taking into account the small amount of antigenic diversity of *L. pneumophila* serogroup 6 strains as defined by monoclonal antibody subtyping, we found complete agreement between phenotypic properties (monoclonal antibody reac-

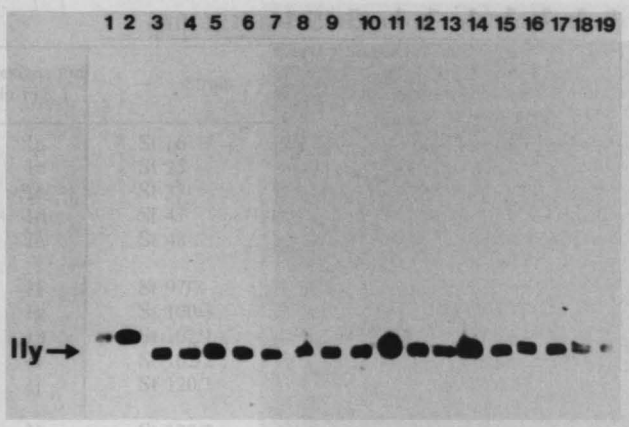

FIG. 2. Southern hybridization of *Not*I-cleaved genomic DNA separated by PFGE to a DNA probe specific for *lly*. *L. pneumophila* strains are as follows (compare with Table 1). Lanes: 1, S 664; 2, Chicago-2; 3, St 165/3; 4, St 162/3; 5, W 75/2; 6, W 76/4; 7, St 47; 8, St 100/3; 9, St 102/2; 10, St 120/2; 11, St 120/3; 12, St 123/1; 13, St 123/2; 14, St 215/1; 15, St 215/2; 16, St 216; 17, St 217; 18, St 218/1; 19, St 218/2.

tivity) and genome analysis (PFGE) for the serogroup 6 strains from Dresden. On the other hand, genotypically homogeneous *L. pneumophila* serogroup 1 strains were found to be different in their reactivity against monoclonal antibodies (10). Such a phenomenon was not observed with our strains. From the data presented here, it is evident that the *Not*I profile is a powerful tool for subtyping *L. pneumophila* serogroup 6 strains. Hybridization of long-range separated genomic DNA to specific DNA probes further represents a refinement for strain identification and is useful for establishing genome maps.

ACKNOWLEDGMENTS

We thank Jutta Möller and Sigrid Gäbler (Dresden, Germany) for technical assistance. We are grateful to R. Marre (Lübeck, Germany), W. Ehret (Munich, Germany), and E. Kmety (Bratislava, Czechoslovakia) for sending *Legionella* strains. We also thank L. Phillips (Würzburg, Germany) for critical reading of the manuscript and H. Kurz (Würzburg, Germany) for editorial assistance.

This study was supported by a DAAD fellowship (314/3 pe) to P.C.L. This work was also supported by the Bundesministerium für Forschung und Technologie (BMFT 01K; 8829) and Fonds der Chemischen Industrie.

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