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Genome analysis of *Legionella* ssp. by orthogonal field alternation gel electrophoresis (OFAGE)

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1. SUMMARY

Various *Legionella* isolates from different sources and origins were analysed by orthogonal field alternation gel electrophoresis of *NotI* cleaved genomic DNA. The genome of *L. pneumophila* Philadelphia I, the original isolate of the epidemics in 1976, exhibits only five *NotI* fragments. Two virulent derivatives, derived from *L. pneumophila* Philadelphia I, which were obtained by prolonged passage on artificial culture media, did not differ from their isogenic virulent strain according to the *NotI* fragment pattern. By summing the lengths of the *NotI* fragments, the genome size of *L. pneumophila* Philadelphia I was calculated as approximately 3.9 Mb. Environmental *L. pneumophila* strains exhibited different *NotI* patterns, as did *Legionella* strains not belonging to the species *pneumophila*. The usefulness of DNA long range mapping of *Legionella* ssp. with *NotI* for epidemiology and evaluation of their evolutionary relationships is discussed.

2. INTRODUCTION

Legionella pneumophila, the causative agent of Legionnaires' disease, is capable of producing severe pneumonia. *L. pneumophila* multiplies intracellularly in lung macrophages, leading to tissue damage [1]. The natural environment of *L. pneumophila* is the water habitat, since *L. pneumophila* bacteria can be easily isolated from water tanks and plumbing fixtures [2,3]. Distinct serogroups within the species *L. pneumophila* have been identified and many serologically distinguishable separate species of *Legionella* have been described in recent years [1,3]. Some of the non-pneumophila strains were isolated from human disease, whereas others originated from the environment [1].

Cultivation of *Legionella* bacteria must be performed on special media containing charcoal [4]. Prolonged passage of *Legionella* strains in the laboratory leads to loss of virulence [5]. Avirulent derivatives obtained in this way exhibit a reduced infectivity of the guinea pig peritoneum; other animal tests for evaluation of virulence have also been used [1].

At present, limited information exists on the genomic structure of *Legionella* ssp. A first approach to genome analysis with the help of orthogonal field attenuation gel electrophoresis

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(OFAGE) of *NotI* cleaved genomic DNA is presented here. Different *Legionella* isolates are compared, and the genome size of *L. pneumophila* Philadelphia I has been determined.

3. MATERIALS AND METHODS

3.1. Bacterial strains

Bacterial strains used for this study are listed in Table 1. The avirulent derivative *L. pneumophila* SMR was obtained by culturing *L. pneumophila* Philadelphia I on BCYE agar, containing streptomycin (200 µg/ml). One of the spontaneous arising streptomycin resistant colonies was further subcultured on BCYE. Strain *L. pneumophila* XXXV was cultured for 35 passages over supplemented Mueller-Hinton agar. The loss of virulence of both strains was tested by intraperitoneal infection of guinea pigs. While the original virulent strain could be passaged 2–3 times, no bacteria

could be recovered after infection with the avirulent variants (data not shown). The environmental *L. pneumophila* isolates were isolated from warm water tanks [6]. All other strains were obtained from the American Type Culture Collection (ATCC). All strains, except the avirulent *L. pneumophila* variants, have not been passaged more than five times on BCYE agar, after receipt. Strains were stored at -70°C in 50% glycerol.

3.2. Media and reagents

All *Legionella* strains were cultured on buffered charcoal yeast extract (BCYE) agar at 37°C with 5% CO_2 atmosphere for 2–3 days, as previously described [4]. Restriction enzyme *NotI* was purchased from Beckmann, F.R.G. Streptomycin was obtained from Merck, F.R.G. Reagents for *Legionella* growth media were a gift of Oxoid, F.R.G. All other chemicals were obtained from Sigma, F.R.G.

Table 1
Legionella strains

Strain	Source	Reference
<i>L. pneumophila</i> Philadelphia I	Human lung	ATCC 33152
<i>L. pneumophila</i> SMR	Avirulent derivative of strain Philadelphia I	This study
<i>L. pneumophila</i> XXXV	Avirulent derivative of strain Philadelphia I	This study
<i>L. pneumophila</i> U ₁ S ₁ *	Environment, warm water tank	[6]
<i>L. pneumophila</i> U ₂ S ₁	Environment, warm water tank	[6]
<i>L. pneumophila</i> U ₁ S ₆	Environment, warm water tank	[6]
<i>L. pneumophila</i> MSP ₁₉ S ₁	Environment, warm water tank	[6]
<i>L. gormanii</i>	Environment, soil from a creek bank	ATCC 33297
<i>L. micdadei</i>	Human blood	ATCC 33218
<i>L. dumoffii</i>	Environment, cooling tower	ATCC 33279
<i>L. bozemanii</i> serogroup 1	Human lung	ATCC 33217
<i>L. longbeachea</i> serogroup 1	Human lung	ATCC 33462
<i>L. longbeachea</i> serogroup 2	Human lung	ATCC 33484

* S designates the serogroup.

3.3. OFAGE

Bacterial cells were harvested and resuspended in SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.4) and OD_{600} was adjusted to 0.8. DNA isolation was performed essentially as described by Grothues and Tümmler [7]. For cleavage with restriction enzyme, the agarose blocks containing genomic DNA were equilibrated at 50°C for 2 h in restriction enzyme buffer. Cleavage was performed in fresh buffer, according to the manufacturers instructions. OFAGE analysis was performed with Consort equipment (F.R.G.) in 0.25 × TBE as running buffer at 8°C. The electric field alternated every 60 s for at least 72 h at a constant voltage of 250 V. Lambda concatamers (Pharmacia, F.R.G.) and yeast chromosomes (*Saccharomyces cerevisiae* WAY 5-4A, Biometra, F.R.G.) were used as DNA size markers. Gels were stained with ethidium bromide and photographed under UV light.

4. RESULTS

4.1. Genome size of *L. pneumophila* Philadelphia I and comparison of virulent and avirulent variants

Genomic DNAs of *L. pneumophila* Philadelphia I and the avirulent variants were isolated and cleaved with *Not*I. OFAGE analysis revealed four *Not*I fragments. The genomes of avirulent variants, derived from the Philadelphia I strain, exhibited an identical *Not*I pattern (see Fig. 1A, lanes 1–3). Two very large fragments of 1570 kilobases (kb) and 1430 kb, as well as two smaller fragments of 390 kb and 70 kb were visible. Further analysis of the Philadelphia I strain and the avirulent derivatives revealed that the 390 kb fragment represents a double band, visible after a longer period of electrophoresis (see Fig. 1B, lane 1, for avirulent strains, data not shown). The prolonged time of electrophoresis leads to a co-migration of the two larger fragments side by side,

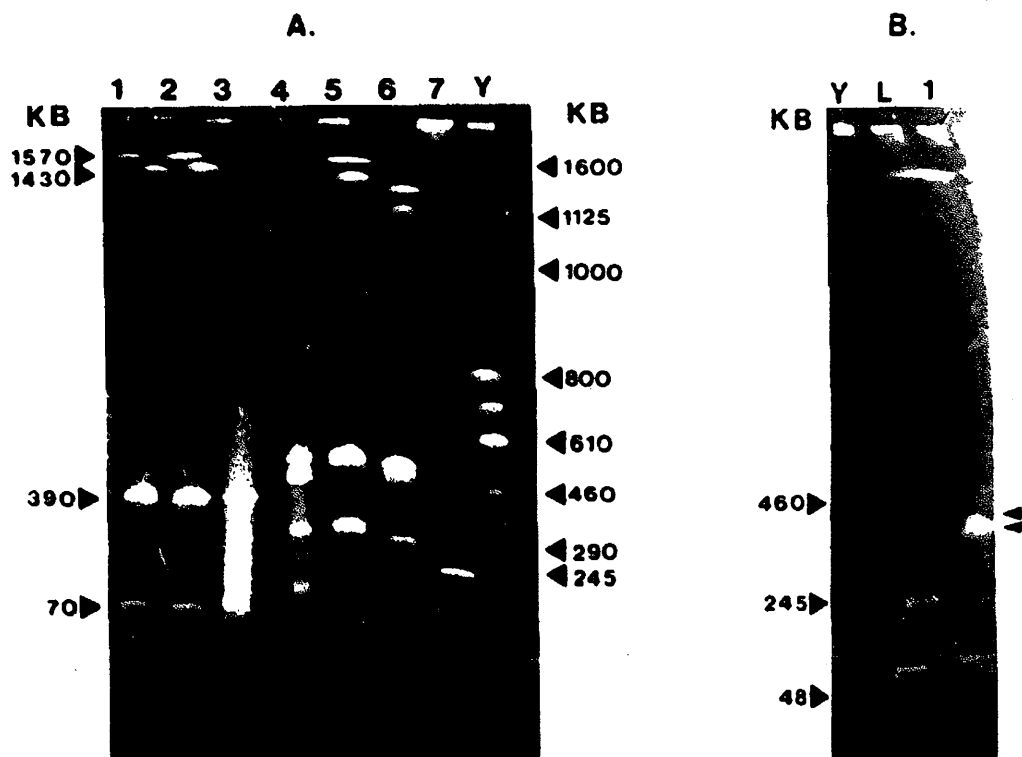


Fig. 1. Orthogonal field alternation gel electrophoresis of *Not*I cleaved genomic DNAs. (A) 1, *L. pneumophila*, Philadelphia I; 2, *L. pneumophila* SMR, avirulent; 3, *L. pneumophila* XXXV, avirulent; 4, *L. pneumophila* U₁S₁; 5, *L. pneumophila* U₂₁S₆; *L. pneumophila* U₂₂S₃; 7, *L. pneumophila* MSP₁₉S₁; Y, yeast chromosomes. DNA sizes are indicated. Electrophoresis was performed for 72 h. (B) 1, *L. pneumophila*, Philadelphia I; Y, yeast chromosomes; L, lambda concatamers. DNA sizes are indicated. The double band is marked by two arrows. Electrophoresis was performed for 120 h.

as the electric field is most discontinuous in the upper part of the gel, resulting in a loss of quality of the separation of longer fragment. Nevertheless, the existence of the two fragments, approximately 390 and 380 kb, could be shown in this way. By summing the length of the five *NotI* fragments, a genome size of approximately 3.9 Megabases (Mb) plus or minus 100 kb (± 100 kb) can be calculated for the Philadelphia I strain, and for the avirulent derivatives.

4.2. Analysis of the genomes of *L. pneumophila* isolates from the environment

L. pneumophila strains isolated from warm water tanks, belonging to serogroups 1 and 6 (cf. Table 1) were analysed according to their *NotI* pattern. From Fig. 1, it is obvious that these strains exhibit a markedly different *NotI* patterns compared to the Philadelphia I strain, and compared to each other (see Fig. 1A, lanes 4–7), although in some cases fragment of similar length exist (see Fig. 1A, lanes 4 and 6). We did not calculate the genome size of these strains, as we cannot exclude that some of the smaller fragments represent double or even triple bands. However, we can see from Fig. 1 that at least some of the environmental isolates seem to have a smaller genome than the *L. pneumophila* Philadelphia I isolate (lanes 4, 6).

4.3. Analysis of the genomes of *L. non-pneumophila* isolates

In order to analyse the genomes of *Legionella non-pneumophila* strains, genomic DNAs of *L. longbeachae*, serogroup 1 and 2, *L. dumoffii*, *L. bozemanii*, serogroup 1, *L. micdadei*, and *L. gormanii* were analysed according their *NotI* restriction fragment patterns. From Fig. 2, it can be seen that these strains also display only a few *NotI* fragments, but differ in size compared to each other, and compared to the fragments of the *L. pneumophila* isolates. *L. longbeachae* isolates of serogroup 1 and 2 share some fragments (Fig. 2, lanes 2 and 3), but also exhibit differences according the *NotI* pattern. In general a highly heterogeneous *NotI* fragment pattern can be observed for the strains not belonging to the species *pneumophila*. As in the case of the environmental *L.*

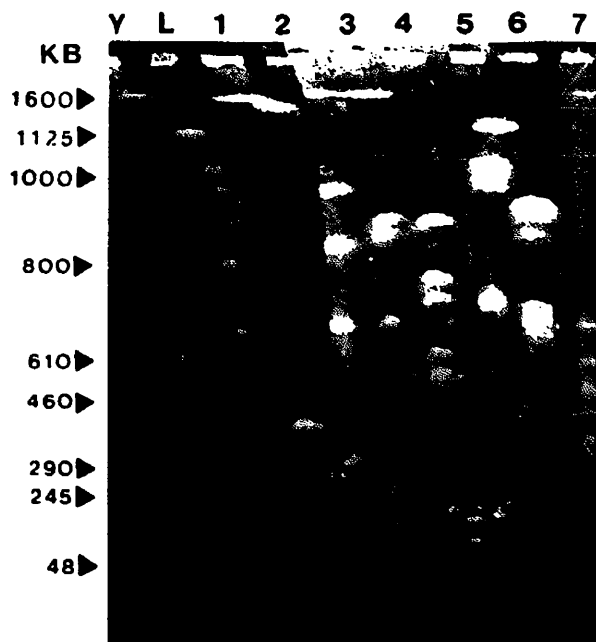


Fig. 2. Orthogonal field alternation gel electrophoresis of *NotI* cleaved genomic DNAs, strains are as follows: 1, *L. pneumophila*, Philadelphia I; 2, *L. longbeachae*, serogroup 1; 3, *L. longbeachae*, serogroup 2; 4, *L. dumoffii*; 5, *L. bozemanii*, serogroup 1; 6, *L. micdadei*; 7, *L. gormanii*; Y, yeast chromosomes; L, lambda concatamers. DNA sizes are indicated. Electrophoresis was performed for 72 h.

pneumophila isolates, we have not estimated a genome size for these strains.

5. DISCUSSION

In this report, the *NotI* restriction fragment patterns of the genomes of various *Legionella* isolates were analysed by OFAGE. For *L. pneumophila* Philadelphia I, the calculated size of the genome is approximately 3.9 Mb (± 0.1). This value is similar to the size of the *E. coli* chromosome. Avirulent variants derived from this strain exhibited an identical *NotI* pattern of their genomic DNA. In contrast, the *Legionella* strains not belonging to the species *pneumophila*, as well as *L. pneumophila* isolates from the environment, showed markedly different restriction enzyme patterns, compared to *L. pneumophila* Philadelphia I and compared to each other. These data are in agreement with the low percentage of DNA homology among the various species of *Legionellae*

which has been reported [8]. The genome sizes of *Legionella* strains other than the *L. pneumophila* Philadelphia I isolate have not been determined, since for each strain the individual parameters for DNA separation must be determined to obtain clear-cut results. This includes the variation of pulse times and the duration of electrophoresis. Nevertheless, it seems that the genomes of some *Legionella* isolates are smaller than the genome of *L. pneumophila* Philadelphia I.

With regard to the avirulent variants of *L. pneumophila* Philadelphia I which were obtained by prolonged passage on laboratory media, it is evident that the loss of virulence is not associated with marked differences in the genome structure. Although one should be careful by interpreting only the *NotI* pattern, large deletion events or rearrangements should have been detectable, as has been demonstrated for *E. coli* strains with reduced virulence which spontaneously arise in vitro and in vivo [9].

Interestingly, the *L. pneumophila* strains isolated from the environment exhibited a totally different restriction fragment pattern compared to *L. pneumophila* Philadelphia I originating from human disease. From these data it can be concluded that *L. pneumophila* strains represent a heterogeneous family of isolates, at least according to their genome structure. Since it has been shown that environmental *L. pneumophila* are the source of infectious disease, further comparative analysis of the genomes of patient isolates and strains derived from the environment will be helpful for epidemiological studies and for evaluating genetic relationships. Further studies should include the use of other rare cutting enzymes, as they have to be extended to the remaining non-*pneumophila* *Legionella* strains, which have not been analysed here.

Besides biochemical and serological methods for the classification of *Legionella* strains [10], genome analysis has become important in recent years. Southern hybridization studies with special DNA probes were performed, and plasmid profiles have been analysed [11–14]. In this report we introduced a first approach to genome analysis of *Legionella* by DNA long range mapping via OFAGE using the rare cutting enzyme *NotI*. It has been shown for many other bacterial species

that this technique is useful for physical mapping of virulence determinants, and for calculation of genome sizes [7,15–18].

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REFERENCES

- [1] Winn, W.C. (1988) Clin. Microbiol. Rev. 1, 60–81.
- [2] Best, M., Yu, V.L., Stout, J., Goetz, A., Muder, R.R. and Taylor, F. (1983) Lancet ii, 307–310.
- [3] Cianciotto, N., Eisenstein, B.I., Engleberg, N.C. and Shuman H. (1989) Mol. Biol. Med. 6, 409–424.
- [4] Edelstein, P.H. (1982) J. Clin. Microbiol. 16, 697–699.
- [5] Catrenich, C.E. and Johnson, W. (1988) Infect. Immun. 56, 3121–3125.
- [6] Ludwig, B., Bender, L., Chirinos, E., Ehret, W., Marre, R., Schmitt, A. and Hacker, J. (1990) Microb. Pathogen., submitted.
- [7] Grothues, D. and Tümmler, B. (1987) FEMS Microbiol. Lett. 48, 419–422.
- [8] Brenner, D.J. (1986) Isr. J. Med. Sci. 22, 655–661.
- [9] Hacker, J., Bender, L., Ott, M., Wingender, J., Lund, B., Marre, R. and Goebel, W. (1990) Microb. Pathogen. 8, in press.
- [10] Harrison, T.G. Taylor, A.G. (1988) A Laboratory Manual for *Legionella*, Wiley-Interscience, U.K.
- [11] Edelstein, P.H. (1986) J. Clin. Microbiol. 23, 481–484.
- [12] Pasculle, A.W., Veto, G.E., Krystofiak, S., MeeKelvey, K. and Vrsalovic, K. (1989) J. Clin. Microbiol. 27, 2350–2358.
- [13] Saunders, N.A., Harrison, T.G., Haththotuwa, A., Kachwalla, N. and Taylor, A.G. (1990) J. Med. Microbiol. 31, 45–55.
- [14] Maher, W.E., Plouffe, J.F. and Para, M.F. (1983) J. Clin. Microbiol. 18, 1422–1423.
- [15] Allardet-Servent, A., Bourg, G., Ramuz, M., Pages, M., Bellis, M. and Roizes, G. (1988) J. Bacteriol. 170, 4603–4607.
- [16] Smith, C.L. and Condemine, G. (1990) J. Bacteriol. 172, 1167–1172.
- [17] Colman, S.D., Hu, P.-C., Litaker, W. and Bott, K.F. (1990) Mol. Microbiol. 4, 683–687.
- [18] Ely, B., Ely, T.W., Gerardot, C.J. and Dingwall, A. (1990) J. Bacteriol. 172, 1262–1266.