

## Distribution, Expression, and Long-Range Mapping of Legiolysin Gene (*lly*)-Specific DNA Sequences in Legionellae

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The legiolysin gene (*lly*) cloned from *Legionella pneumophila* Philadelphia 1 confers the phenotypes of hemolysis and browning of the culture medium. An internal *lly*-specific DNA probe was used in Southern hybridizations for the detection of *lly*-specific DNA in the genomes of legionellae and other gram-negative pathogenic bacteria. Under conditions of high stringency, the *lly* DNA probe specifically reacted with DNA fragments from *L. pneumophila* isolates; by reducing stringency, hybridization was also observed for all other *Legionella* strains tested. No hybridization occurred with DNAs isolated from bacteria of other genera. The *lly* gene was mapped by pulsed-field gel electrophoresis to the respective genomic *NotI* fragments of *Legionella* isolates. By using antilegiolysin monospecific polyclonal antibodies in Western blots (immunoblots), *Lly* proteins could be detected only in *L. pneumophila* isolates.

Legionellae are the causative agents of the pulmonary illness known as Legionnaires disease and the nonpneumonic Pontiac fever (28). To date, more than 30 different species of the genus *Legionella* have been described (28). *Legionella* infections are characterized by multiplication of the bacteria in lung macrophages (7). In vitro, intracellular replication of virulent legionellae can be observed by using human cell lines. Spontaneously arising avirulent variants are unable to cause disease in animal models and do not multiply in cell culture systems (8, 15, 17). Various exoproducts have been implicated in the pathogenic process (27). Recently, the major secretory protein (Msp), a metalloprotease exhibiting hemolytic and cytolytic activity was cloned, but it seems to have no influence on virulence (2, 19, 24).

We have recently described the cloning and characterization of another hemolytic protein (39 kDa) termed legiolysin (*Lly*) from a genomic library of *Legionella pneumophila* Philadelphia 1 (20). This protein additionally confers the phenotypic characteristic of browning of the culture medium, which is likely due to pigment production (29).

In order to evaluate the distribution and expression of *lly*-specific DNA sequences among legionellae, we analyzed various strains including bacteria of other genera. The strains were first tested for hemolysin production using human, dog, and sheep erythrocytes and for the ability to cause browning of the culture medium. The data are summarized in Table 1.

To test for the presence of *lly*-specific DNA sequences in the genomes of *Legionella* strains, *NotI*-cleaved genomic DNA separated by pulsed-field gel electrophoresis was hybridized with the 0.7-kb *SphI-NcoI* fragment of plasmid pEWL 114, which is an internal *lly* DNA probe, starting from 60 bp in front of the ATG start codon of the gene and spanning over two-thirds of the coding region to the 3' end (28a, 29). Under conditions of high stringency, only *L. pneumophila* isolates displayed hybridization and it can be

seen (Fig. 1B and Table 1) that the strains generally hybridized to the *lly* probe in *NotI* fragments of different size. However, the virulent and avirulent variants (Fig. 1B, lanes 2 and 3) of the *L. pneumophila* Philadelphia 1 strain and the environmental isolates U1S1 and U22S3 (Fig. 1B, lanes 4 and 6) showed hybridization with fragments of the same size, respectively. Under conditions of reduced stringency, hybridization could be observed with all other *Legionella* strains tested. Also, these strains exhibited hybridization with fragments of different size with only a few exceptions: the strains of *Legionella longbeachae* (Fig. 1C, lanes 8 and 9), *L. feeleii*, and *L. hackeliae* belonging to different serogroups exhibited signals with *NotI* fragments of identical size (cf. Fig. 1C and Table 1). In contrast, chromosomal DNA isolated from *Serratia marcescens*, *Serratia liquefaciens*, *Aeromonas sobria*, *Salmonella typhimurium*, *Bordetella pertussis*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Shigella sonnei*, and *Escherichia coli* did not hybridize even under reduced stringency (Table 1). The same results were obtained by using the 2.4-kb *EcoRI-SmaI* fragment of plasmid pEWL 113 (29), which contains the whole gene and flanking regions, for hybridization (data not shown). Furthermore, we used the antilegiolysin monospecific antibodies (29) to detect legiolysins in legionellae. Western blots (immunoblots) were performed by using whole-cell extracts of the strains described above. All *L. pneumophila* strains, except the environmental isolate U21S6 (Fig. 2, lane 13), reacted with the antilegiolysin antibodies (Fig. 2 and Table 1), showing proteins of approximately the same size as described for the legiolysin of the Philadelphia 1 strain. The virulent and avirulent variants of this strain did not differ in *Lly* expression (Fig. 2, lanes 2 and 3). Legiolysin protein was not detected in non-*L. pneumophila* species or in the bacteria from other genera (cf. Fig. 2 and Table 1).

Our findings offer evidence for the conservation of the *lly* gene in legionellae, with the highest degree of homology maintained in the species *L. pneumophila*. Therefore, this DNA probe can be useful in differentiating between *L. pneumophila* and other legionellae or between legionellae

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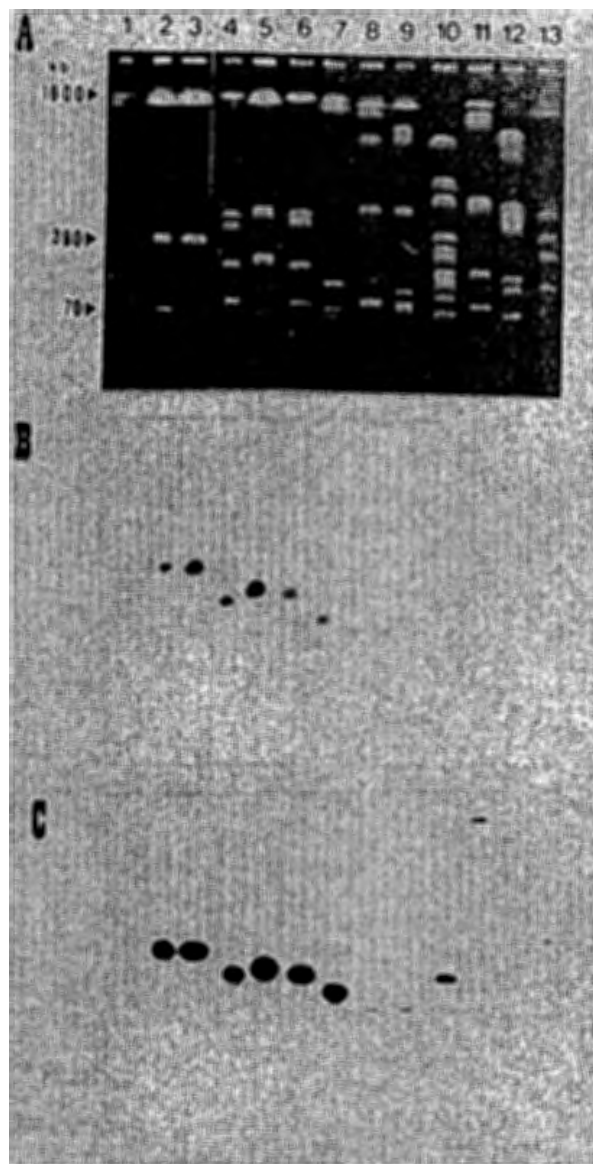


FIG. 1. Pulsed-field gel electrophoresis of *NotI*-cleaved genomic DNA (A) and Southern hybridization by using the *lly*-specific 0.7-kb *SphI*-*NcoI* DNA fragment of plasmid pEWL 114 <sup>32</sup>P labelled by the random priming technique (10) under conditions of high (B) and low (C) stringency. The probe was electroeluted from agarose gels (21). Genomic DNA for pulsed-field gel electrophoresis is isolated and cleaved as previously described (11). Electrophoresis was performed with the CHEF DrII system (Bio-Rad Laboratories, Richmond, Calif.) in 1% agarose gels at 14°C in 0.5× Tris-borate-EDTA buffer. Constant voltage of 200 V was applied with an increasing pulse time of 60 to 90 s over a period of 18 h, followed by a constant pulse time of 90 s for 6 h. *Saccharomyces cerevisiae* chromosomes (lane 1) (WAY 5-4A; Biometra, Göttingen, Germany) and lambda concatemers (not shown; Pharmacia, Freiburg, Germany) were used as high-molecular-weight DNA markers. Lanes: 2, *L. pneumophila* Philadelphia 1; 3, *L. pneumophila*, avirulent XXXV; 4, *L. pneumophila* U1S1; 5, *L. pneumophila* U21S6; 6, *L. pneumophila* U22S3; 7, *L. pneumophila* MSP19S1; 8, *L. longbeachae* S1; 9, *L. longbeachae* S2; 10, *L. dumoffii*; 11, *L. bozemanii*; 12, *L. micdadei*; 13, *L. gormanii*.

following hybridization under high-stringency conditions (8, 18). A DNA probe derived from the *htpAB* gene locus encoding the 60-kDa heat shock protein of *L. pneumophila* (13) was also shown to hybridize under conditions of high stringency only with DNAs isolated from *L. pneumophila* strains (1a). Since it has been shown previously that the *NotI* profiles of *legionellae* differ markedly from strain to strain (16), it could be expected that the *lly* genes of different isolates were also located on fragments of dissimilar size. However, in some strains, fragments of identical size could be detected. Such experiments can be useful for fine epidemiological studies and for establishing genome maps, as shown recently (3, 5, 22).

All *L. pneumophila* strains, except for the environmental isolate U21S6, produced Lly proteins that were detected in Western blots by using anti-Lly antibodies and that varied only slightly in size. Since the protein was not detected in non-*L. pneumophila* strains or in other gram-negative bacteria tested, legiolysin seems to be unique to *L. pneumophila*, as has been also reported for Msp (18). Therefore, hemolysis detected in a large number of *Legionella* species (4) may be due to factors other than *lly* or *msp*, as also suggested by Quinn et al. (18). Furthermore, the phenotypic characteristic of browning the culture medium, which occurs in many of the non-*L. pneumophila* strains, is not necessarily associated with legiolysin. These findings show that in *legionellae* similar phenotypes may be determined by different factors which are unrelated on the DNA or protein level.

The environmental *L. pneumophila* isolate (U21S6) which did not express the Lly protein had also lost the ability to cause browning of the culture medium, whereas an unaltered hemolysis phenotype was still detected which may result from the action of further hemolytic factors not yet identified. This strain will be helpful in the construction of the isogenic Lly<sup>+</sup> and Lly<sup>-</sup> strains. In this way, the contribution

and non-*Legionella* strains. A similar conservation of DNA structure has been observed for two other cloned *L. pneumophila* genes, the *msp* determinant and the *mip* gene cluster, the latter coding for a membrane-associated protein which plays a role in the uptake of *Legionella* species by macrophages (7, 8, 18). Using *msp*- and *mip*-specific sequences as probes, homologous sequences were also detected exclusively in the genomes of *L. pneumophila* strains

FIG. 2. Western blot analysis of whole-cell extracts using anti-Lly specific antibodies. Lanes: 2, *L. pneumophila* Philadelphia 1; 3, *L. pneumophila*, avirulent XXXV; 4, *L. pneumophila* 685S1; 5, *L. pneumophila* 664S6; 6, *L. longbeachae* S1; 7, *L. longbeachae* S2; 8, *L. micdadei*; 9, *L. dumoffii*; 10, *L. bozemanii*; 11, *L. gormanii*; 12, *L. pneumophila* U1S1; 13, *L. pneumophila* U21S6; 14, *L. pneumophila* U22S3; 15, *L. pneumophila* MSP19S1. As a control, *E. coli* DH5α harboring plasmid pEWL 4 was used (lane 1). SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (14), using 10% polyacrylamide gels. Equal amounts of extracts (determined by optical density at 600 nm) were applied to each lane.

TABLE 1. Characteristics of *Legionella* strains and other gram-negative bacteria

Strain <sup>a</sup> [serotype <sup>b</sup> ]	Reference	Hybridization <sup>c</sup> with <i>lly</i> with stringency		Size (kb) of <i>NotI</i> fragment hybridizing to <i>lly</i>	Hemolysis <sup>d</sup>	Browning <sup>e</sup>	Reaction with anti- <i>Lly</i> antibodies <sup>f</sup>
		High	Low				
<i>L. pneumophila</i>							
Philadelphia 1 ATCC 33152 [S1]		+ <sup>g</sup>	+	390	++	++	+
XXXV, avirulent, Philadelphia 1 [S1]	1b	+	+	390	++	++	+
U1S1 (environmental isolate)	1b	+	+	207	++	+	+
U21S6 (environmental isolate)	1b	+	+	272	++	-	-
U22S3 (environmental isolate)	1b	+	+	207	++	+	+
MSP19S1 (environmental isolate)	1b	+	+	144	++	+	+
68S1 (patient isolate)	29	+	+	227	++	+	+
667S4 (patient isolate)	This study	+	+	825	++	+	+
640S5 (patient isolate)	This study	+	+	211	++	+	+
664S6 (patient isolate)	This study	+	+	318	++	+	+
<i>L. longbeachae</i>							
ATCC 33462 [S1]		-	+	100	+	++	-
ATCC 33484 [S2]		-	+	100	-	-	-
<i>L. dumoffii</i> ATCC 33279							
		-	+	215	+	+	-
<i>L. bozemanii</i> ATCC 33217 [S1]							
		-	+	1,430	+	+	-
<i>L. micdadei</i> ATCC 33218							
		-	(+)	960	-	-	-
<i>L. gormanii</i> ATCC 33297							
		-	+	420	+	+	-
<i>L. feeleii</i>							
ATCC 35072 [S1]		-	+	260	-	(+)	-
ATCC 35849 [S2]		-	+	260	-	-	-
<i>L. hackeliae</i>							
ATCC 33250 [S1]		-	+	442	+	++	-
ATCC 35999 [S2]		-	+	442	+	++	-
<i>L. jordanis</i> ATCC 33623							
		-	+	172	+	+	-
<i>L. oakridgensis</i> ATCC 33761							
		-	+	150	+	++	-
<i>Serratia marcescens</i> W225							
	4a	-	-		(+)	+	-
<i>Serratia liquefaciens</i> DSM 30064							
	4a	-	-		-	-	-
<i>Aeromonas sobria</i> AB3							
	6	-	-		++	-	-
<i>Salmonella typhimurium</i> 05-1/4,12:i:1,2							
	12b	-	-		-	-	-
<i>Bordetella pertussis</i> 6564/85							
	12a	-	-		+ <sup>h</sup>	ND <sup>i</sup>	-
<i>Pseudomonas aeruginosa</i> SS 712							
	1	-	-		-	-	-
<i>Shigella sonnei</i> 5542/89							
	12a	-	-		-	-	-
<i>Shigella flexneri</i> 1265/89							
	12a	-	-		-	-	-
<i>E. coli</i> 536							
	3	-	-		++	-	-

<sup>a</sup> *Legionella* strains were cultivated on BCYE agar plates (Oxoid, Wesel, Germany) at 37°C in a 5% CO<sub>2</sub> atmosphere for 2 to 3 days (12). *E. coli* strains and all other gram-negative bacteria were grown overnight in Luria-Bertani (LB) medium at 37°C, except *Bordetella pertussis* which was cultivated on Bordet-Gengou plates (BG; Difco, Detroit, Mich.) containing 15% sheep blood for 5 days at 37°C.

<sup>b</sup> S designates the serogroup, determined as previously described (12).

<sup>c</sup> Southern hybridization was performed as previously described (23). For high-stringency conditions, 50% formamide, 42°C, and 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were used. The filters were washed three times with 0.1× SSC-0.1% sodium dodecyl sulfate (SDS) at 56°C. For low-stringency conditions, the formamide concentration of the hybridization solution was reached to 25%, and hybridization was carried out at 37°C with 6× SSC (21). The filters were washed three times in 2× SSC-0.1% SDS at 56°C. These two sets of conditions allow approximately 10 or 25% mismatches, respectively, as estimated by the method of Davis et al. (9).

<sup>d</sup> Hemolysis was determined by using human, canine, and sheep erythrocytes. Blood agar plates to test hemolysis were prepared as previously described (19). Hemolysin production of *Legionella* strains was detected by cultivation for 2 days at 37°C on BYE agar plates (12, 19) containing 5% erythrocytes from either humans, dogs, or sheep. Hemolysis of all other strains was assayed on LB blood agar plates containing either human, sheep, or dog erythrocytes, except *B. pertussis* which was tested only on BG plates containing sheep blood.

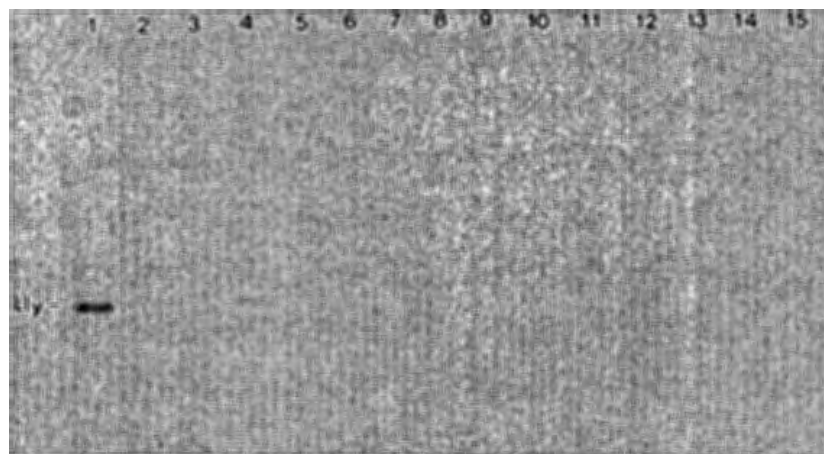
<sup>e</sup> Color production (browning of the culture medium) of *Legionella* strains was assayed after cultivation on BYE agar plates containing 4 mM tyrosine (26). This property was tested for non-*Legionella* strains (except for *B. pertussis*) after growth on LB agar plates containing 4 mM tyrosine.

<sup>f</sup> Determined in Western blots (25) using whole-cell extracts (cf. data in this column with Fig. 2). Antilegionin monospecific polyclonal antibodies were prepared in rabbits, using the LacZ-*Lly* fusion protein as described previously (29).

<sup>g</sup> Properties: -, negative; (+), weak; +, normal; ++, strong.

<sup>h</sup> Determined only with sheep blood.

<sup>i</sup> ND, not determined.



of this gene to survival processes in the environment as well as to pathogenicity of *Legionella* species can be evaluated.

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