INFECTION AND IMMUNITY, Sept. 1991, p. 3333–3336
0019-9567/91/093333-04$02.00/0

Vol. 59, No. 9

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

LARISA BENDER,[1] MANFRED OTT,[1] ANETTE DEBES,[1] URSULA RDEST,[1] JÜRGEN HEESEMANN,[2] AND JÖRG HACKER[1]*

Institut für Hygiene und Medizinische Mikrobiologie[2] and Institut für Genetik und Mikrobiologie,[1] University of Würzburg, Röntgenring 11, W-8700 Würzburg, Germany

Received 9 April 1991/Accepted 25 June 1991

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 *Not*I 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, *Not*I-cleaved genomic DNA separated by pulsed-field gel electrophoresis was hybridized with the 0.7-kb *Sph*I-*Nco*I 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 *Not*I 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 *Not*I 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 *Eco*RI-*Sma*I 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*

* Corresponding author.

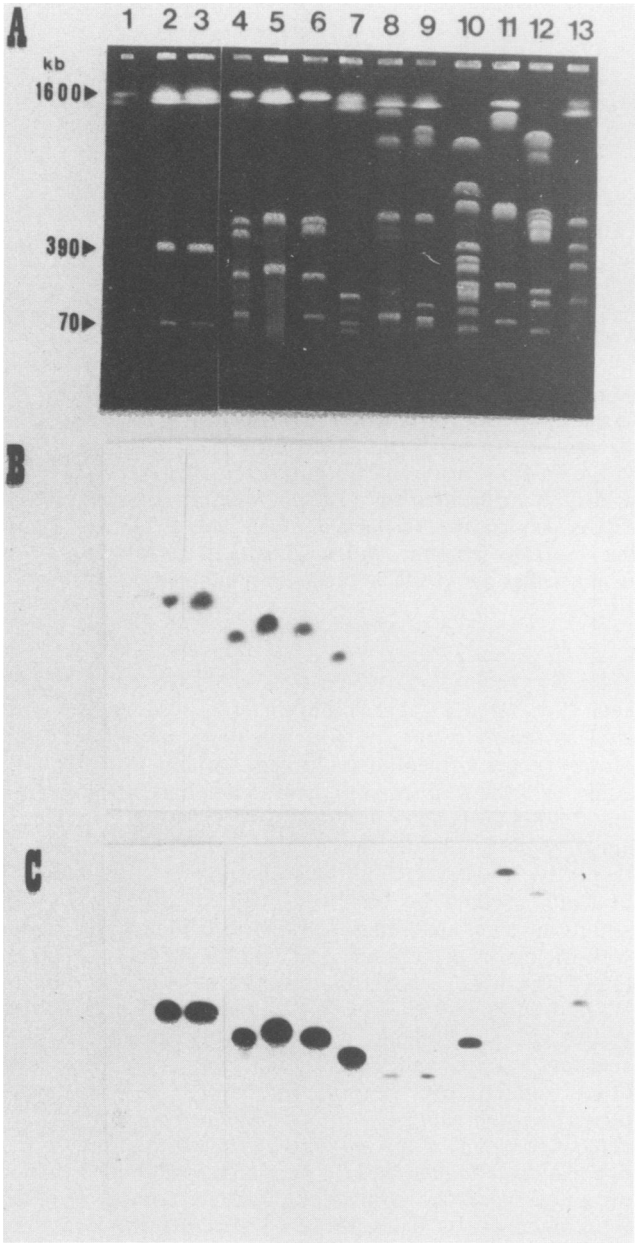


FIG. 1. Pulsed-field gel electrophoresis of *Not*I-cleaved genomic DNA (A) and Southern hybridization by using the *lly*-specific 0.7-kb *Sph*I-*Nco*I DNA fragment of plasmid pEWL 114 ^{32}P labelled by the random priming technique (10) under conditions of high (B) and low (C) stringency. The probe was electroeluted from agarose gels (21). Genomic DNA for pulsed-field gel electrophoresis was 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*.

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 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 *Not*I 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$^+$ and Lly$^-$ strains. In this way, the contribution

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[a] [serotype[b]]	Reference	Hybridization[c] with *lly* with stringency		Size (kb) of *Not*I fragment hybridizing to *lly*	Hemolysis[d]	Browning[e]	Reaction with anti-Lly antibodies[f]
		High	Low				
L. pneumophila							
Philadelphia 1 ATCC 33152 [S1]		+[g]	+	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	++	+	+
685S1 (patient isolate)	29	+	+	227	++	+	+
667S4 (patient isolate)	This study	+	+	825	++	+	+
640S5 (patient isolate)	This study	+	+	211	++	+	+
664S6 (patient isolate)	This study	+	+	318	++	+	+
L. longbeachae							
ATCC 33462 [S1]		−	+	100	+	++	−
ATCC 33484 [S2]		−	+	100	−	−	−
L. dumoffii ATCC 33279		−	+	215	+	+	−
L. bozemanii ATCC 33217 [S1]		−	+	1,430	+	+	−
L. micdadei ATCC 33218		−	(+)	960	−	−	−
L. gormanii ATCC 33297		−	+	420	+	+	−
L. feeleii							
ATCC 35072 [S1]		−	+	260	−	(+)	−
ATCC 35849 [S2]		−	+	260	−	−	−
L. hackeliae							
ATCC 33250 [S1]		−	+	442	+	++	−
ATCC 35999 [S2]		−	+	442	+	++	−
L. jordanis ATCC 33623		−	+	172	+	+	−
L. oakridgensis ATCC 33761		−	+	150	+	++	−
Serratia marcescens W225	4a	−	−		(+)	+	−
Serratia liquefaciens DSM 30064	4a	−	−		−	−	−
Aeromonas sobria AB3	6	−	−		++	−	−
Salmonella typhimurium 05-1/4,12:i:1,2	12b	−	−		−	−	−
Bordetella pertussis 6564/85	12a	−	−		+[h]	ND[i]	−
Pseudomonas aeruginosa SS 712	1	−	−		−	−	−
Shigella sonnei 5542/89	12a	−	−		−	−	−
Shigella flexneri 1265/89	12a	−	−		−	−	−
E. coli 536	3	−	−		++	−	−

[a] *Legionella* strains were cultivated on BCYE agar plates (Oxoid, Wesel, Germany) at 37°C in a 5% CO_2 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.

[b] S designates the serogroup, determined as previously described (12).

[c] 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).

[d] 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.

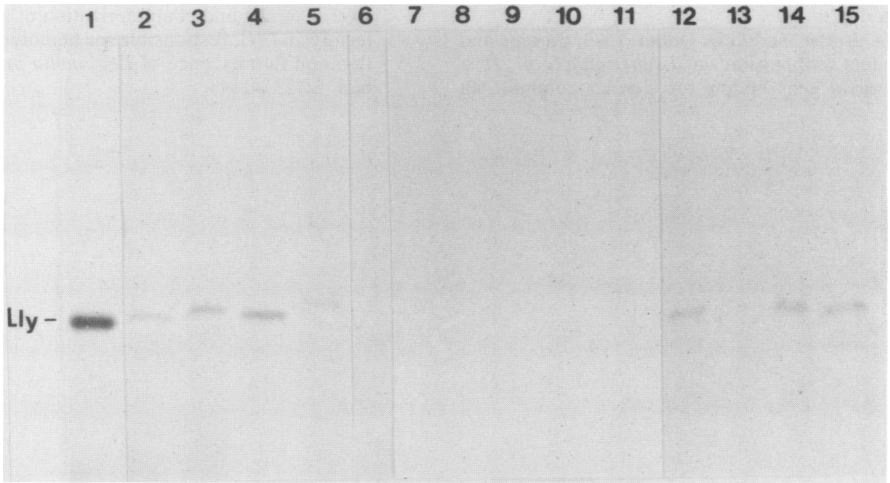
[e] Color production (browning of the culture medium) of *Legionella* strains was assayed after cultivation on BYE agar plates containing 4 mM tyrosine (26). This property was tested for non-*Legionella* strains (except for *B. pertussis*) after growth on LB agar plates containing 4 mM tyrosine.

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

[g] Properties: −, negative; (+), weak; +, normal; ++, strong.

[h] Determined only with sheep blood.

[i] ND, not determined.



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

We are grateful to M. Wuenscher for critical comments on the manuscript. H. Kurz is acknowledged for editorial assistance.

The work was supported by the Bundesministerium for Forschung und Technologie (BMFT 01K; 8829) and Fonds der Chemischen Industrie.

REFERENCES

1. **Behringer, H.** Unpublished data.
1a. **Bender, L.** Unpublished data.
1b. **Bender, L., M. Ott, R. Marre, and J. Hacker.** 1990. Genome analysis of *Legionella* spp. by orthogonal field alternation gel electrophoresis. FEMS Microbiol. Lett. **72**:253–258.
2. **Blander, S. J., L. Szeto, H. A. Shuman, and M. A. Horwitz.** 1990. An immunoprotective molecule, the major secretory protein of *Legionella pneumophila*, is not a virulence factor in a guinea pig model of Legionnaires' disease. J. Clin. Invest. **86**:817–824.
3. **Blum, G., M. Ott, A. Cross, and J. Hacker.** 1991. Virulence determinants of *Escherichia coli* O6 extraintestinal isolates analysed by Southern hybridizations and DNA long range mapping techniques. Microb. Pathog. **10**:127–136.
4. **Bornstein, N., M. Nowicki, and J. Fleurette.** 1988. Haemolytic activity in the genus *Legionella*. Ann. Inst. Pasteur/Microbiol. (Paris) **139**:325–329.
4a. **Braun, V.** Unpublished data.
5. **Butler, P. D., and E. R. Moxon.** 1990. A physical map of the genome of *Haemophilus influenzae* type b. J. Gen. Microbiol. **136**:2333–2342.
6. **Chakraborty, T., S. Kathariou, J. Hacker, H. Hof, B. Huhle, W. Wagner, M. Kuhn, and W. Goebel.** 1987. Molecular analysis of bacterial cytolysins. Rev. Infect. Dis. **9**(Suppl.):456–466.
7. **Cianciotto, N., B. I. Eisenstein, N. C. Engleberg, and H. Shuman.** 1989. Genetics and molecular pathogenesis of *Legionella pneumophila*, an intracellular parasite of macrophages. Mol. Biol. Med. **6**:409–424.
8. **Cianciotto, N. P., J. M. Bangsborg, B. I. Eisenstein, and N. C. Engleberg.** 1990. Identification of *mip*-like genes in the genus *Legionella*. Infect. Immun. **58**:2912–2918.
9. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. A manual for genetic engineering: advanced bacterial genetics, p. 220–221. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specificity. Anal. Biochem. **132**:6–13.
11. **Grothues, D., and B. Tümmler.** 1987. Genome analysis of *Pseudomonas aeruginosa* by field inversion gel electrophoresis. FEMS Microbiol. Lett. **48**:419–422.
12. **Harrison, T. G., and A. G. Taylor (ed.).** 1988. A laboratory manual for *Legionella*. Wiley Interscience, Chichester, Great Britain.
12a. **Heesemann, J.** Unpublished data.
12b. **Hof, H.** Unpublished data.
13. **Hoffman, P. S., C. A. Butler, and F. D. Quinn.** 1989. Cloning and temperature-dependent expression in *Escherichia coli* of a *Legionella pneumophila* gene coding for a genus-common 60-kilodalton antigen. Infect. Immun. **57**:1731–1739.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Marra, A., M. A. Horwitz, and H. A. Shuman.** 1990. The HL-60 model for the interaction of human macrophages with the Legionnaires' disease bacterium. J. Immunol. **144**:2738–2744.
16. **Ott, M., L. Bender, R. Marre, and J. Hacker.** 1991. Pulsed field electrophoresis of genomic restriction fragments for the detection of nosocomial *Legionella pneumophila* in hospital water supplies. J. Clin. Microbiol. **29**:813–815.
17. **Pearlman, E., A. H. Jiwa, N. C. Engleberg, and B. I. Eisenstein.** 1988. Growth of *Legionella pneumophila* in a human macrophage-like (U937) cell line. Microb. Pathog. **5**:87–95.
18. **Quinn, F. D., M. G. Keen, and L. S. Tompkins.** 1989. Genetic, immunological and cytotoxic comparisons of *Legionella* proteolytic activities. Infect. Immun. **57**:2719–2725.
19. **Quinn, F. D., and L. S. Tompkins.** 1989. Analysis of a cloned sequence of *Legionella pneumophila* encoding a 38 kd metalloprotease possessing haemolytic and cytotoxic activities. Mol. Microbiol. **3**:797–805.
20. **Rdest, U., E. Wintermeyer, B. Ludwig, and J. Hacker.** 1991. Legiolysin, a new hemolysin from *L. pneumophila*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **274**:471–474.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Smith, C. L., and G. Condemine.** 1990. New approaches for physical mapping of small genomes. J. Bacteriol. **172**:1167–1172.
23. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
24. **Szeto, L., and H. A. Shuman.** 1990. The *Legionella pneumophila* major secretory protein, a protease, is not required for intracellular growth or cell killing. Infect. Immun. **58**:2585–2592.
25. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
26. **Vickers, R. M., and V. L. Yu.** 1984. Clinical laboratory differentiation of *Legionellaceae* family members with pigment production and fluorescence on media supplemented with aromatic substrates. J. Clin. Microbiol. **19**:583–587.
27. **Williams, A., A. Baskerville, A. B. Dowsett, and J. E. Conlan.** 1987. Immunocytochemical demonstration of the association between *Legionella pneumophila*, its tissue-destructive protease, and pulmonary lesions in experimental Legionnaires' disease. J. Pathol. **153**:157–164.
28. **Winn, W. C.** 1988. Legionnaires disease: historical perspective. Clin. Microbiol. Rev. **1**:60–81.
28a. **Wintermeyer, E., et al.** Unpublished data.
29. **Wintermeyer, E., U. Rdest, B. Ludwig, A. Debes, and J. Hacker.** 1991. Cloning and characterization of a DNA sequence, termed legiolysin (*lly*), responsible for hemolytic activity, color production and fluorescence of *Legionella pneumophila*. Mol. Microbiol. **5**:1135–1143.