INFECTION AND IMMUNITY, May 1996, p. 1842–1845 Vol. 64, No. 5
0019-9567/96/$04.00+0

Spectrum of *Legionella* Species Whose Intracellular Multiplication in Murine Macrophages Is Genetically Controlled by *Lgn1*

HIROSHI MIYAMOTO,[1] KOJI MARUTA,[1] MIDORI OGAWA,[1] MARIE-CLAIRE BECKERS,[2] PHILIPPE GROS,[2] AND SHIN-ICHI YOSHIDA[1]*

Department of Microbiology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807, Japan,[1] and Department of Biochemistry, McGill University, Montreal, Canada H3G 1A4[2]

Received 21 August 1995/Returned for modification 3 October 1995/Accepted 13 February 1996

We examined the intracellular growth of 20 strains within six species of *Legionella* in thioglycolate-elicited peritoneal macrophages from A/J and C57BL/6 mice and a congenic strain derived from them (A.B *Lgn1*). With the exception of *Legionella pneumophila* Togus-1 and Bloomington-2, the intracellular growth of the 15 *L. pneumophila* strains tested was controlled by *Lgn1*. However, the intracellular growth of five *Legionella* species other than *L. pneumophila* was not under *Lgn1*'s control.

Legionella pneumophila, the causative agent of Legionnaires' disease, is a facultative intracellular bacterium which can grow in human monocytes (12), alveolar macrophages (17), and the macrophages of guinea pigs, hamsters, and rats (13, 26). In mice, the bacterium cannot proliferate in peritoneal macrophages from many mouse strains, such as C57BL/6, C3H/HeN, and BALB/c (23, 26), but it can proliferate in thioglycolate-elicited peritoneal macrophages from A/J mice (23). This mouse strain difference offers a useful tool for studying the mechanism which enables some *L. pneumophila* strains to grow intracellularly. In a previous study (25), we analyzed the genetic control of the innate resistance of macrophages from C57BL/6 (nonpermissive) and A/J (permissive) mice to *L. pneumophila* Philadelphia-1. The study clearly showed that the nonpermissive trait is dominant and that a single gene, designated *Lgn1*, controls the trait. Further studies on *Lgn1* revealed that the gene was located on the distal portion of mouse chromosome 13 (1, 3). *Lgn1* is distinct from other genes known to influence the capacity of the host macrophage to control multiplication of intracellular parasites, such as *Ity*/*Bcg*/*Lsh* on mouse chromosome 1 (2, 7, 18), *Lps* on mouse chromosome 4 (20, 24), and others (8, 9). Therefore, the identification and study of the *Lgn1* gene may reveal a novel mechanism by which nonpermissive macrophages can suppress intracellular multiplication of this bacterium (1, 3).

To analyze the nature of the *Lgn1* gene's function, it is necessary not only to clone the *Lgn1* gene but also to characterize the spectrum of the bacterial species and strains on which the gene product functions. Understanding the parasite characteristics required for *Lgn1* control may be useful for understanding its mechanism of action. It remains unknown whether the bacteriostatic activity of the $Lgn1^r$ allele of the gene is effective against different *Legionella* species and strains. In addition, as far as we know, there is no report of intracellular growth of *Legionella* species other than *L. pneumophila* in murine macrophages.

Male and female A/J and C57BL/6 mice were obtained from Shizuoka Experimental Animals (Hamamatsu, Japan). Progeny of these strains were bred in the animal center at the University of Occupational and Environmental Health, Kitakyushu, Japan. Construction of the congenic mouse strain (A.B *Lgn1*) was performed as follows. In brief, the resistance-susceptibility phenotypes of macrophages from the progeny of a (A/J × C57BL/6)F_1 × A/J backcross were determined by methods described previously (25). The progeny that exhibited less than a 1.0 $\log_{10}$ increase in *L. pneumophila* Philadelphia-1 CFU per well after 3 days were selected as possessing the resistance allele ($Lgn1^r$) of C57BL/6 mouse origin. The progeny bearing $Lgn1^r$ were then backcrossed with the A/J mouse. The procedures of backcrossing the progeny and of typing their macrophages for resistance or susceptibility to *L. pneumophila* Philadelphia-1 were performed for 18 generations. The backcross progeny with the $Lgn1^{r/r}$ genotype were used as the congenic-strain mice (A.B *Lgn1*) in this study. These mice were used when they were 8 to 10 weeks old; both sexes were used.

The bacterial strains used in this study are listed in Table 1. The bacterial strains were passaged once intraperitoneally in Hartley guinea pigs as described previously (24) or in cultured peritoneal macrophages of guinea pigs (26) before they were used in this study. Fresh isolates from the spleen on day 3 postinoculation or from the macrophages on day 3 postinfection were grown on buffered charcoal-yeast extract agar plates (6). The bacteria were stored at −80°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) supplemented with 20% (vol/vol) glycerol until use. By using rabbit polyclonal antisera against *L. pneumophila* serogroups 1 to 6 (Denka Seiken Co., Ltd., Tokyo), the serogroups of *L. pneumophila* were confirmed by slide agglutination tests following the instructions of the manufacturer.

The intracellular growth of legionellae in thioglycolate-elicited peritoneal macrophages from A/J, C57BL/6, and congenic mice was examined after in vitro phagocytosis. Peritoneal exudate cells were collected by lavage of the peritoneal cavities of mice 4 days after intraperitoneal injection of 2.5 ml of 4% thioglycolate medium (Difco). The preparation of peritoneal-macrophage monolayers from the peritoneal exudate cells, the in vitro phagocytosis, and the microscopic observation of Gimenez-stained macrophage monolayers were carried out exactly as described previously (25). The postphagocytic determination of *Legionella* CFU was performed as described previously (16).

We examined the intracellular growth of 15 strains of *L. pneumophila* (Table 1) in thioglycolate-elicited peritoneal macrophages of A/J, C57BL/6, and congenic mice. In *L. pneumophila* Philadelphia-1 (serogroup 1) (Fig. 1A), after a 48-h in-

* Corresponding author. Mailing address: Department of Microbiology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807, Japan. Phone: 81-93-691-7242. Fax: 81-93-602-4799. Electronic mail address: yoshida@med.uoeh-u.ac.jp.

TABLE 1. Bacterial strains used in this study

Strain	Serogroup	Source
L. pneumophila		
Philadelphia-1	1	ATCC 33152
Nagasaki 80-045	1	Clinical isolate
GIFU 9799	1	Clinical isolate
GIFU 9888	1	Clinical isolate
GIFU 10067	1	Clinical isolate
GIFU 10068	1	Clinical isolate
GIFU 12438	1	Clinical isolate
Togus-1	2	ATCC 33154
Bloomington-2	3	ATCC 33155
GIFU 10073	3	Clinical isolate
GIFU 11491	4	Clinical isolate
GIFU 10841	4	Clinical isolate
GIFU 11395	5	Clinical isolate
GIFU 12293	5	Clinical isolate
KO 127	6	Environmental isolate
L. micdadei TATLOCK		ATCC 33218
L. dumoffii Tex-KL		ATCC 33279
L. bozemanii WIGA	1	ATCC 33217
L. oakridgensis OR-10		ATCC 33761
L. feeleii WO-44C	1	ATCC 35072

cubation, there was about a 300-fold increase in macrophages of A/J mice bearing the susceptibility allele (*Lgn1*^{s}). In macrophages of C57BL/6 and congenic (A.B *Lgn1*) mice bearing the resistance allele (*Lgn1*^{r}), the bacterial growth was suppressed, and there was less than a 10-fold increase in the number of bacteria in each of these cultures. These mouse strain differences were confirmed by light microscopy after Gimenez staining of macrophage monolayers cultured on chamber slides (data not shown). With the exception of the Togus-1 (serogroup 2) (Fig. 1B) and Bloomington-2 (serogroup 3) (Fig. 1C) strains, all of the strains of *L. pneumophila* tested here (Table 1) could grow intracellularly in A/J macrophages but could not grow in C57BL/6 or congenic-mouse macrophages (data not shown but same as for Philadelphia-1 strain in Fig. 1A). The mouse strain differences observed with the Philadelphia-1 strain were not observed with *L. pneumophila* Togus-1 and Bloomington-2 (Fig. 1B and C), indicating that the bacteriostatic activity of *Lgn1*^{r} is not specific to the species as a whole.

To determine whether bacteriostatic activity of *Lgn1* is specific to certain serogroups of *L. pneumophila*, we examined the intracellular growth of the GIFU 10073 strain, which belongs to the same serogroup as Bloomington-2 (serogroup 3) (Fig. 1D). The bacterial growth in C57BL/6 and congenic-strain macrophages was suppressed less than 10-fold, which is different from the growth of the Bloomington-2 strain, suggesting that the bacteriostatic activity of *Lgn1*^{r} is not specific to serogroups of *L. pneumophila*.

Legionella micdadei, *Legionella bozemanii*, *Legionella dumoffii*, and *Legionella feeleii* are most frequently implicated in cases of pneumonia or Pontiac fever caused by *Legionella* species other than *L. pneumophila* (5). Using these strains, we investigated intracellular bacterial growth in thioglycolate-elicited peritoneal macrophages of A/J, C57BL/6, and congenic mice. For *L. micdadei* TATLOCK (Fig. 2A), there was about a 20-fold increase in the number of CFU in A/J macrophages after a 24-h incubation. The viable counts leveled off thereafter. In C57BL/6 and congenic-mouse macrophages, the time courses of intracellular bacterial growth were slightly slower than that in A/J macrophages, but the levels of bacterial growth

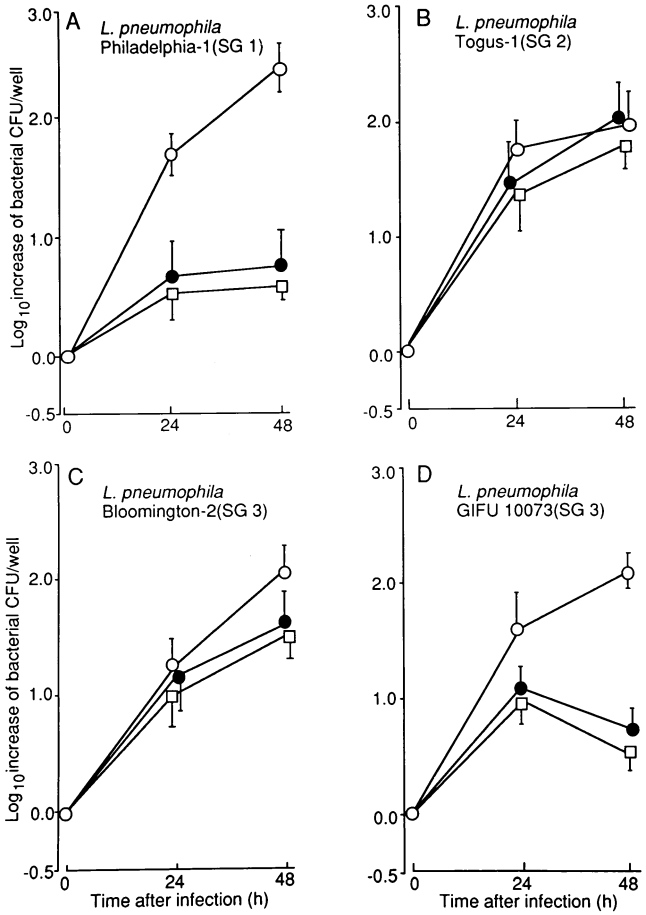

FIG. 1. Time courses of intracellular growth of *L. pneumophila* strains in peritoneal macrophages of A/J (○), C57BL/6 (●), and congenic (A.B *Lgn1*) (□) mice. The data are the means ± the standard deviations of representative results for four individual experiments, each using five mice. SG, serogroup.

after a 48-h incubation were the same as that in A/J macrophages. For *L. bozemanii* WIGA (serogroup 1) (Fig. 2B), there was about a 300-fold increase in the number of CFU in macrophages of A/J mice after a 48-h incubation. In C57BL/6 and congenic-mouse macrophages, the time courses and the levels of intracellular bacterial growth were almost the same as those in A/J macrophages. *L. dumoffii* Tex-KL (Fig. 2C), *L. feeleii* WO-44C (serogroup 1) (Fig. 2D), and *Legionella oakridgensis* OR-10 (data not shown) could also grow as well in macrophages of C57BL/6 and congenic mice as they did in A/J mouse macrophages. The mouse strain differences demonstrated for *L. pneumophila* were not observed with the other five *Legionella* species. Microscopic observation of Gimenez-stained macrophage monolayers 48 h after infection also clearly confirmed the lack of mouse strain differences (data not shown).

It has not yet been examined whether the multiplication of many *Legionella* species and serogroups in mouse macrophages is genetically controlled by *Lgn1*. Hence, it has generally been accepted that C57BL/6 mouse macrophages do not permit intracellular growth of *L. pneumophila* (22, 23, 26). However, we found that *L. pneumophila* Togus-1 (serogroup 2) (Fig. 1B) and Bloomington-2 (serogroup 3) (Fig. 1C) could grow intracellularly in thioglycolate-elicited peritoneal macrophages of C57BL/6 and congenic mice bearing *Lgn1*^{r}. In addition, C57BL/6 and congenic-mouse macrophages as well as A/J

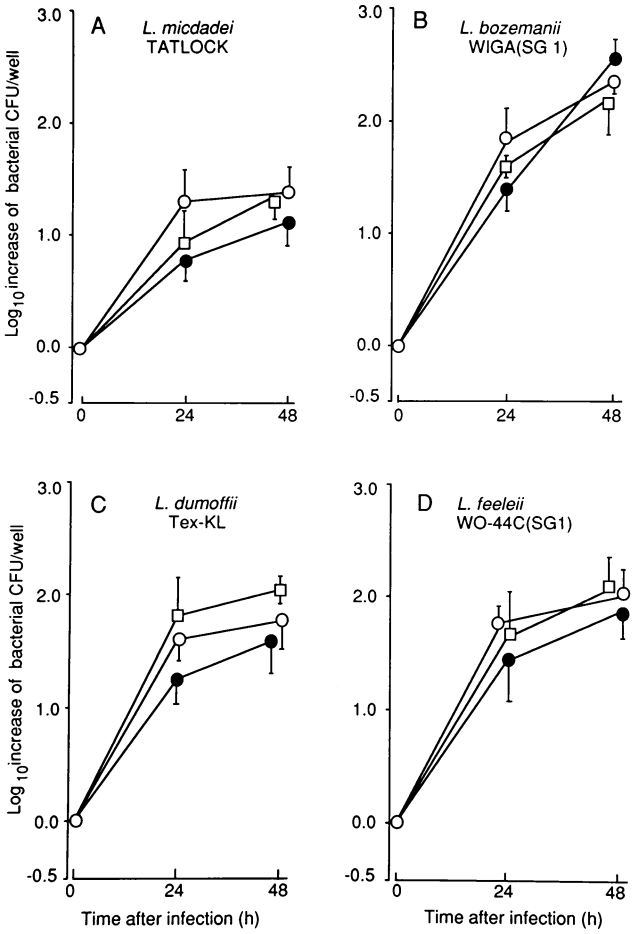

FIG. 2. Time courses of intracellular growth of *L. micdadei*, *L. bozemanii*, *L. dumoffii*, and *L. feeleii* in peritoneal macrophages of A/J (○), C57BL/6 (●), and congenic (A.B *Lgn1*) (□) mice. The data are the means ± the standard deviations of representative results for three individual experiments, each using three mice. SG, serogroup.

macrophages permitted bacterial growth of all five other *Legionella* species tested here (Fig. 2). In this study we used thioglycolate-elicited peritoneal macrophages of mice. It was reported that thioglycolate-elicited peritoneal macrophages show impairment of intracellular killing of a number of microorganisms due to loading with thioglycolate (4, 15). Further studies, using resident peritoneal macrophages of C57BL/6 mice, are required for assessing the permissiveness of C57BL/6 mouse macrophages for the seven *Legionella* strains used in this study.

The congenic strain used here was established after 18 generations of backcrossing with A/J mice, and it holds approximately a 15-centimorgan gene fragment of C57BL/6 origin on chromosome 13 (unpublished data). Macrophages of the congenic strain (A.B *Lgn1*) showed the same pattern as macrophages of C57BL/6 (Fig. 1 and 2). This supports the theory that the strain difference between A/J and C57BL/6 mice observed here is controlled by *Lgn1* and that the seven *Legionella* strains which grew in the macrophages of the congenic strain are not controlled by *Lgn1*.

Macrophages of the congenic mouse strain did not permit bacterial growth, although bacteria could survive in these macrophages (Fig. 1A and D). These results suggest that the *Lgn1* gene product has a bacteriostatic activity rather than a bactericidal activity and that A/J mouse macrophages may be defective in their ability to inhibit bacterial growth. Since the genetic defect can be overcome by gamma interferon activation of A/J macrophages (14), the *Lgn1* gene product may affect a very early step of the macrophage-*Legionella* interaction, such as macrophage signal transduction in response to the infecting bacterium.

Since there are some reports suggesting that diverse intracellular growth mechanisms are used by *Legionella* strains and species (10, 11, 19, 21), it is reasonable to speculate that expression of the bacteriostatic activity controlled by the *Lgn1*^{r} allele may reflect the development of adaptive mechanisms which enable *Legionella* strains and species to grow intracellularly. To study possible sites of action of *Lgn1* gene products, it is necessary to elucidate the modes of survival and multiplication within phagocytes specific to the different strains analyzed in this study.

The positional cloning of the *Lgn1* gene is currently under way. The spectrum of bacterial strains identified in this study that are or are not influenced by the presence of the *Lgn1*^{r} allele not only provides a valuable tool with which to analyze the gene's effect but also may point at the bacterial gene products on which the *Lgn1*^{r} allele acts. Gene transfer experiments using different *Legionella* species may identify such genes and shed light on *Lgn1*'s mechanism of action.

We are grateful to Hiroyuki Yamamoto (Gifu University), Fumiaki Kura (National Institute of Health, Japan) and Keiji Okuda (Mihagino College of Medical Technology) for kindly providing *Legionella* strains. We also acknowledge the technical assistance of Kumiko Mizue, Chizu Morita, and Yukiko Suzuki.

This study was supported by Grant-in-Aid for Scientific Research 06670312 from the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

1. **Beckers, M.-C., S. Yoshida, K. Morgan, E. Skamene, and P. Gros.** 1995. Natural resistance to infection with *Legionella pneumophila*: chromosomal localization of the *Lgn1* susceptibility gene. Mamm. Genome **6:**540–545.
2. **Bradley, D. J., B. A. Taylor, J. Blackwell, E. P. Evans, and J. Freeman.** 1979. Regulation of *Leishmania* populations within the host. III. Mapping of the locus controlling susceptibility to visceral leishmaniasis in mice. Clin. Exp. Immunol. **37:**7–14.
3. **Dietrich, W. F., D. M. Damron, R. R. Isberg, E. S. Lander, and M. S. Swanson.** 1995. *Lgn1*, a gene that determines susceptibility to *Legionella pneumophila*, maps to mouse chromosome 13. Genomics **26:**443–450.
4. **Eichner, R. D., and T. C. Smeaton.** 1983. Agar accumulates in rat peritoneal macrophages elicited with thioglycollate broth. Scand. J. Immunol. **18:**259–263.
5. **Fang, G. D., V. L. Yu, and R. M. Vickers.** 1989. Disease due to the Legionellaceae (other than *Legionella pneumophila*). Historical, microbiological, clinical, and epidemiological review. Medicine **68:**116–132.
6. **Feeley, J. C., R. J. Gibson, G. W. Gorman, N. C. Langford, J. K. Rasheed, D. C. Mackel, and W. B. Baine.** 1979. Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. J. Clin. Microbiol. **10:**437–441.
7. **Forget, A., and E. Skamene.** 1985. Genetic regulation of early host defense mechanisms controlling mycobacterial infection, p. 265–277. *In* E. Skamene (ed.), Genetic control of host resistance to infection and malignancy. Alan R. Liss, Inc., New York.
8. **Gervais, F., M. Stevenson, and E. Skamene.** 1984. Genetic control of resistance to *Listeria monocytogenes*: regulation of leukocyte inflammatory responses by the *Hc* locus. J. Immunol. **132:**2078–2083.
9. **Groves, M. G., D. L. Rosenstreich, B. A. Taylor, and J. V. Osterman.** 1980. Host defenses in experimental *Scrub typhus*: mapping of the gene that controls natural resistance in mice. J. Immunol. **125:**1395–1402.
10. **Horwitz, M. A.** 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. J. Exp. Med. **158:**1319–1331.
11. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:**2108–2126.
12. **Horwitz, M. A., and S. C. Silverstein.** 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J. Clin. Invest. **66:**441–450.
13. **Kishimoto, R. A., J. D. White, F. G. Shirey, V. G. McGann, R. F. Berendt,**

E. W. Larson, and K. W. Hedlund. 1981. In vitro response of guinea pig peritoneal macrophages to *Legionella pneumophila*. Infect. Immun. **31:**1209–1213.
14. **Klein, T. W., Y. Yamamoto, K. Brown, and H. Friedman.** 1991. Interferon-gamma induced resistance to *Legionella pneumophila* in susceptible A/J mouse macrophages. J. Leukocyte Biol. **49:**98–103.
15. **Leijh, P. C. J., T. L. van Zwet, M. N. ter Kuile, and R. van Furth.** 1984. Effect of thioglycolate on phagocytic and microbicidal activities of peritoneal macrophages. Infect. Immun. **46:**448–452.
16. **Miyamoto, H., M. Ogawa, K. Maruta, Y. Nikaido, C. Yamamoto, H. Taniguchi, and S. Yoshida.** 1995. Temperature effects on *Legionella pneumophila* killing by and multiplication in phagocytes of guinea pigs. Microbiol. Immunol. **39:**647–654.
17. **Nash, T. W., D. M. Libby, and M. A. Horwitz.** 1984. Interaction between the Legionnaires' disease bacterium (*Legionella pneumophila*) and human alveolar macrophages. Influence of antibody, lymphokines, and hydrocortisone. J. Clin. Invest. **74:**771–782.
18. **Nauciel, C., E. Ronco, M. Deschenes, and M. Guenounou.** 1985. Immune response during infection with a temperature-sensitive mutant of *Salmonella typhimurium* in susceptible and resistant mice, p. 257–264. *In* E. Skamene (ed.), Genetic control of host resistance to infection and malignancy. Alan R. Liss, Inc., New York.
19. **Rechnitzer, C., and J. Blom.** 1989. Engulfment of the Philadelphia strain of *Legionella pneumophila* within pseudopod coils in human phagocytes. Comparison with other *Legionella* strains and species. Acta Pathol. Microbiol. Immunol. Scand. **97:**105–114.
20. **Watson, J., K. Kelly, M. Largen, and B. A. Taylor.** 1978. The genetic mapping of a defective LPS response gene in C3H/HeJ mice. J. Immunol. **120:**422–424.
21. **Weinbaum, D. L., R. R. Benner, J. N. Dowling, A. Alpern, A. W. Pasculle, and G. R. Donowitz.** 1984. Interaction of *Legionella micdadei* with human monocytes. Infect. Immun. **46:**68–73.
22. **Yamamoto, Y., T. W. Klein, C. A. Newton, R. Widen, and H. Friedman.** 1987. Differential growth of *Legionella pneumophila* in guinea pig versus mouse macrophage cultures. Infect. Immun. **55:**1369–1374.
23. **Yamamoto, Y., T. W. Klein, C. A. Newton, R. Widen, and H. Friedman.** 1988. Growth of *Legionella pneumophila* in thioglycolate-elicited peritoneal macrophages from A/J mice. Infect. Immun. **56:**370–375.
24. **Yoshida, S., Y. Goto, H. Miyamoto, H. Fujio, and Y. Mizuguchi.** 1991. Association of *Lps* gene with natural resistance of mouse macrophages against *Legionella pneumophila*. FEMS Microbiol. Immunol. **4:**51–56.
25. **Yoshida, S., Y. Goto, Y. Mizuguchi, K. Nomoto, and E. Skamene.** 1991. Genetic control of natural resistance in mouse macrophages regulating intracellular *Legionella pneumophila* multiplication in vitro. Infect. Immun. **59:**428–432.
26. **Yoshida, S., and Y. Mizuguchi.** 1986. Multiplication of *Legionella pneumophila* Philadelphia-1 in cultured peritoneal macrophages and its correlation to susceptibility of animals. Can. J. Microbiol. **32:**438–442.

Editor: B. I. Eisenstein