INFECTION AND IMMUNITY, Nov. 1987, p. 2701–2705
0019-9567/87/112701-05$02.00/0

Vol. 55, No. 11

Influence of Growth Temperature on Virulence of *Legionella pneumophila*

PAUL H. EDELSTEIN,[1,2]†* KAREN B. BEER,[1] AND ELAINE D. DEBOYNTON[1]

Infectious Diseases Section, Research and Medical Services, Veterans Administration Medical Center, Los Angeles, California 90073,[1] and Department of Medicine, University of California School of Medicine, Los Angeles, California 90024[2]

Received 7 May 1987/Accepted 23 July 1987

The effect of growth temperature on the virulence of a strain of broth-grown serogroup 1 *Legionella pneumophila* (Wadsworth F889) was examined by growing the bacterium at different temperatures and then infecting guinea pigs (by intratracheal injection) and guinea pig alveolar macrophages. The 50% lethal dose for guinea pigs infected with 25°C-grown F889 was $\log_{10}$ 5.0 CFU and that for 41°C-grown F889 was $\log_{10}$ 5.7 CFU, or a fivefold difference. Guinea pig alveolar macrophages were infected in quadruplicate with $\log_{10}$ 3.8 CFU of F889 cells grown at either 25 or 41°C. Counts of F889 in the alveolar macrophages infected with 25°C-grown bacteria were 40% greater after 1 day of incubation ($P = 2 \times 10^{-4}$) than were counts in the alveolar macrophage suspensions inoculated with 41°C-grown bacteria. However, the counts were not significantly different after 3 days of incubation. Examination of cover slip cultures of guinea pig alveolar macrophages infected with 25°C-grown or 41°C-grown bacteria showed that the bacteria grown at the lower temperature were twice as likely to be macrophage-associated after 1 h of incubation than were the bacteria grown at the higher temperature. Growth at the lower temperature was also associated with a change in reactivity with monoclonal antibodies, but not with a change in plasmid content. Thus, environmental temperature may play an important role in modulating the virulence of *L. pneumophila*, possibly by affecting bacterial adherence to host cells.

Legionella pneumophila is ubiquitous in our aqueous environment, yet rarely causes disease, even when present in high numbers in potable water systems (5, 11, 15, 25). While host factors, organism concentration in water and air, and mechanical factors responsible for aerosol generation are all probably important pathogenic factors, organism virulence is also an extremely important factor. Monoclonal subtype has been shown to relate to organism virulence (2, 21; E. Dournon and P. Rajagopalan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, B279, p. 71), as has resistance to killing by serum and phagocytes (14, 17, 20). However, it is unclear whether there are naturally occurring intrinsically different populations of bacteria with varying degrees of virulence or whether some extrinsic factor(s) causes virulence shifts within the natural reservoir of the microorganism. This study was designed to determine whether the virulence of a single bacterial strain of *L. pneumophila* could be influenced by variation of growth temperature.

MATERIALS AND METHODS

Infecting bacterium and its growth conditions. *L. pneumophila* serogroup 1 strain Wadsworth F889 was used as the infecting bacterium. This is a clinical isolate derived from a single bacterial colony and is stable in virulence, as detailed elsewhere (7). It was stored in suspensions of infected guinea pig lung in skim milk at −70°C. A new vial was thawed for each experiment.

Two different broth media were used to grow strain F889. Buffered charcoal-yeast extract broth (BCYEB) was used to grow the bacterium from the skim milk vial, as this organism will not grow from the frozen state in broth devoid of charcoal (6). Once mid-log-phase growth was obtained, 0.1 ml of the BCYEB was subcultured to buffered yeast extract broth (BYEB), which is sterilized by filter sterilization rather than by autoclaving and is devoid of activated charcoal (6). The bacteria were again grown to the mid-log phase and then used for the various described studies; all experiments used bacteria grown in identical fashion. Broth volumes were always 10 ml, and the broth was contained in glass test tubes covered with a gas-permeable, water-impermeable cap.

Growth temperatures of 25, 35, or 41°C were used. All incubations were performed in shaking incubators, which were temperature calibrated with a National Bureau of Standards traceable thermometer. The time required to reach the mid-log phase for the first passage was about 90 h for 25°C growth, about 40 h for 35°C growth, and about 27 h for 41°C growth. The second passage required about 48 h at 25°C to achieve mid-log-phase growth and about 20 h for 35 and 41°C incubation. The bacterial cells were short bacilli or coccobacilli at this stage, with fewer than 1% filamentous forms.

Bacterial suspensions were checked for purity by plating on buffered charcoal-yeast extract medium supplemented with alpha-ketoglutarate (BCYE) and on chocolate and blood agar media (6). Quantitation was performed by duplicate plating of serial 10-fold dilutions on BCYE medium; this was incubated at 35°C in air and counted after 4 days of incubation. A Petroff-Hausser chamber was also used to count bacteria and to adjust inocula; viable counts were always used to determine the actual bacterial concentra-

* Corresponding author.
† Present address: Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, Philadelphia, PA 19104-4283.

tions. Phase-contrast microscopy was used with the counting chamber, and otherwise to assess bacterial morphology.

Animal studies. Male Hartley strain guinea pigs, 300 to 350 g, were used to determine the 50% lethal dose of bacterial strain F889 inoculated intratracheally, as previously described (7). Briefly, a 0.3-ml volume of bacteria suspended in normal saline was inoculated directly into the trachea with a syringe and needle after aseptic surgical exposure of the trachea under general and local anesthesia. The animal was then shaken while in the upright position to ensure distal distribution of the inoculum. The guinea pig was propped upright on gauze pillows for about 5 min and then returned to its cage, where it rested in the supine position until it awoke. Ten animals were studied at each of three dose levels or a total of 30 animals for each incubation temperature of bacterial inoculum. Animals were observed for 17 days or until their death, which usually occurred within 5 days postinfection. All dead animals were necropsied and lung cultures were taken to confirm the cause of death. Survivors at 17 days postinfection were killed by carbon dioxide gas inhalation without subsequent necropsy.

Plasmid analysis. The plasmid content of bacterial strain F889 grown at different temperatures was determined in triplicate by a modification of the Takahashi procedure, as detailed previously (8). Electrophoresis conditions, use of standards, and gel analysis methods were the same as in a prior study (8). Restriction endonuclease digestions were not performed.

Monoclonal subtyping. The Oxford and International panels of monoclonal antibodies to *L. pneumophila* serogroup 1 were used to type strain F889, which was grown at different temperatures before Formalin fixation (16, 27). The reactions were graded on a fluorescence intensity scale of 0 to 4, with 4 being maximally bright and 0 being no visible fluorescence.

Purification of alveolar macrophages. Alveolar macrophages were obtained from male Hartley strain guinea pigs weighing 300 to 350 g. The animals were killed with pentobarbital given intraperitoneally. The trachea was exposed by aseptic surgical technique, cannulated with a sterile 16-gauge, 3.2-cm-long catheter, and ligated proximal to the catheter. The catheter was secured with a distal ligature and connected to a sterile plastic three-way stopcock. Room temperature (20 to 30°C) sterile phosphate-buffered saline (PBS; 00.1 M, pH 7.4) was used to lavage the lung in 10-ml volumes, with interspersed chest massage, for a total lavage volume of 100 ml. The lavage fluid was collected in two 50-ml sterile plastic centrifuge tubes and chilled in ice. The fluid was then centrifuged at approximately 350 × *g* for 10 min at 4°C in a swinging-bucket rotor. The supernatant was discarded, and the pellets were gently suspended in 2 ml of cold (4°C) RPMI 1640 medium with L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). The tube contents were pooled and then recentrifuged under the same conditions. The supernatant was discarded, and the cells were resuspended in 2 ml of the same medium. A hemacytometer was used to quantitate the macrophages. About 9×10^6 macrophages were obtained from each animal. The cell suspension was diluted in the same diluent so that it contained about 10^6 cells per ml, and 250 μl was added to each well of 21-mm-diameter 24-well rigid tissue culture plates (Linbro; Flow Laboratories, Inc., McLean, Va.) to which an equal volume of the same diluent had previously been added (final concentration of 5×10^5 cells per ml in each well). The plates were gently agitated and then incubated for 1 h at 35°C in 5% CO_2 and 95% humidity. The wells were then aspirated dry and washed three times with 500-μl volumes of warm (35°C) RPMI 1640 medium with L-glutamine. The wells were then filled with 1,000 μl of warm (35°C) RPMI 1640 with 20% fresh fetal calf serum. Initial studies used macrophages incubated in 5% CO_2 at 35°C for up to 5 days before infection. Later studies used macrophages infected on the day of collection. For studies utilizing cover slips, ethanol-sterilized and flamed round glass cover slips (Goldseal) were placed in the tissue culture dish wells before the macrophages were added.

Infection of alveolar macrophage monolayers. The BYEB-grown *L. pneumophila* cells were suspended in RPMI 1640 with L-glutamine to achieve an approximate concentration of 10^5 cells per ml for growth studies and 10^8 cells per ml for phagocytosis studies. For some of the cover slip culture studies, the inoculum of 41°C-grown bacteria was deliberately increased. The suspension (100 μl) was added to tissue culture tray wells containing approximately 10^5 alveolar macrophages in 1 ml of RPMI 1640 with L-glutamine and 20% fresh fetal calf serum. Thus, the bacteria/macrophage ratios were 0.1:1 for growth studies and 100:1 for macrophage-bacterium association studies (see Tables 1 and 2). The bacteria-macrophage mixture was shaken at 37°C in 5% CO_2 in air for 1 h. Base-line bacterial counts were taken at this time by sampling 100 μl and plating 10-fold dilutions on BCYE agar in duplicate. The tissue culture plates were then incubated without shaking for three more days at 35°C in 5% CO_2 in air. Daily samples (100 μl) were taken for quantitative cultures. Since no extracellular growth occurs in this system, increases in supernatant bacterial concentration represent growth within the macrophages and subsequent macrophage lysis with release of free bacteria (13). All experiments were performed in quadruplicate.

Cover slip cultures. Cover slip cultures were incubated with bacteria for 1 h and then washed three times with RPMI 1640. The cover slips were then removed and fixed in 10% Formalin in distilled water for 5 min. They were then rinsed with PBS (0.01 M, pH 7.4), soaked in distilled water for 5 min, and air dried. The cover slips were stained for 30 min with *L. pneumophila* serogroup 1 antibody conjugated with fluorescein isothiocyanate (Centers for Disease Control, Atlanta, Ga.), diluted one part of antibody in three parts of PBS. The stain was washed off with PBS, after which the cover slips were soaked in PBS for 5 min, rinsed with distilled water briefly, air dried, and mounted on glass slides with pH 9 carbonate-buffered glycerol.

Cover slips were examined by incident light UV fluorescence microscopy with a Leitz Dialux EB20 microscope, 100-W mercury bulb, K2 filter cube, and ×660 magnification. Approximately 400 macrophages were viewed in each cover slip, and the numbers of macrophages with adherent bacteria and the numbers of cell-associated bacteria were counted. Intracellular as well as extracellular bacteria were stained by this method (13, 19). All studies were performed in at least triplicate.

Statistical testing. Descriptive statistics were calculated with standard definitions and assumption of Poisson distributions (18). Comparison of mean (parametric) values was done by paired and nonpaired two-tailed Student *t* tests. Calculation of 50% lethal doses and their confidence intervals was done by the method of moving averages (18). Power analysis was performed by the method of Cohen (4). Comparison of nominal nonparametric values was performed by chi-square testing.

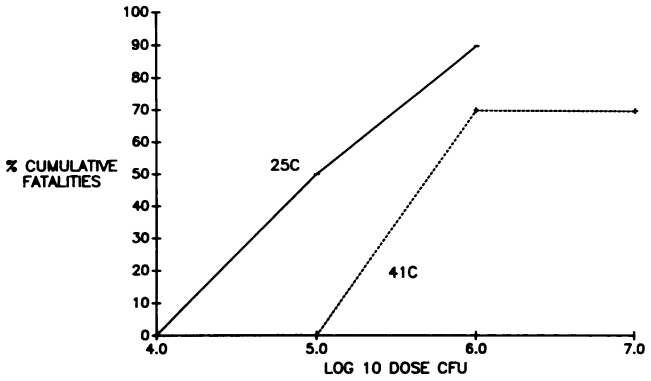

FIG. 1. Cumulative percent fatalities versus $\log_{10}$ CFU dose of *L. pneumophila* F889 grown at 25 and 41°C before intratracheal inoculation of guinea pigs. Ten animals were used at each dose level in each group.

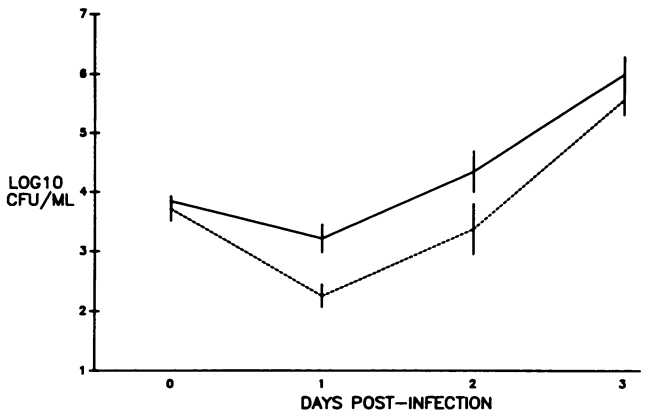

FIG. 2. Colony counts ($\log_{10}$ CFU/ml) of *L. pneumophila* F889 versus days postinoculation of explanted guinea pig alveolar macrophages. The *L. pneumophila* strain was grown at either 25 (solid line) or 41°C (dotted line) before infection of the macrophage monolayers, which were incubated at 37°C in 5% CO_2 before and after infection with the bacteria. Each data point represents the mean of five separate experiments, each performed in quadrupilicate. The vertical bars represent 95% confidence intervals. Significant differences between counts occurred at days 1 ($P < 0.001$) and 2 ($P = 0.002$) postinfection, but not on days 0 ($P > 0.20$) and 3 ($P = 0.09$).

RESULTS

Guinea pig virulence. *L. pneumophila* F889 grown at 25°C was significantly more virulent for guinea pigs than was the same strain grown at 41°C (Fig. 1). The 50% lethal dose value for 25°C-grown F889 was $\log_{10}$ 5.0 CFU (95% confidence interval, 4.6 to 5.4), and it was $\log_{10}$ 5.7 CFU (95% confidence interval, 5.1 to 6.3) for 41°C-grown F889. In an earlier pilot study with smaller numbers of animals, *L. pneumophila* grown at 25°C also had significantly higher virulence than *L. pneumophila* grown at 41°C (data not shown). In that study, bacteria grown at 41 or 35°C had comparable virulence. All dead animals had positive lung cultures for *L. pneumophila* and gross pathologic findings of dense bilateral pulmonary consolidation and hepatosplenomegaly. Comparisons at 25 and 41°C were used to accentuate any potential differences in subsequent studies.

Growth of *L. pneumophila* in alveolar macrophages. Preliminary experiments showed no significant differences in intracellular growth rates between strain F889 grown at different temperatures when the macrophages were cultured for 5 days before infection.

However, in all five experiments conducted with freshly explanted macrophages, the 25°C-grown bacteria multiplied better than the 41°C-grown bacteria (Table 1; Fig. 2). The initial inocula of 41°C-grown and 25°C-grown bacteria were not significantly different in all five experiments ($P > 0.20$ by the nonpaired t test, with $a_2 = 0.05$, $1 - B = 0.20$). Regardless of insignificant differences in initial inocula, viable counts of 25°C-grown bacteria were significantly higher than those of 41°C-grown bacteria at 1 and 2 days postinfection ($P = 2 \times 10^{-4}$ and 1.5×10^{-3}, respectively, by nonpaired t tests). Three days after infection, there was no significant difference in viable counts ($P = 0.09$ by nonpaired t test, with $1 - B = 0.60$ for $a_2 = 0.05$).

Cell-associated bacteria in first hour. All four experiments showed that more 25°C-grown F889 bacteria were macrophage associated than were 41°C-grown bacteria (Table 2). Considerable experiment-to-experiment variability was observed in absolute phagocytosis rates, although intraexperimental variability was small. Despite this, the relative phagocytosis ratios were reasonably constant, even over broad ranges in inoculum size. Overall, 7.4% of macrophages had one or more cell-associated bacteria when incubated with 25°C-grown bacteria, compared with 3.4% of macrophages with cell-associated bacteria when incubated with 41°C-grown bacteria (chi square = 77; $P < 1 \times 10^{-5}$).

Significantly more macrophages (10.7%) incubated with 25°C-grown bacteria had more than one cell-associated bacterium than those incubated with 41°C-grown bacteria (4.3%) ($P = 0.001$ by chi-square analysis). Even when the inoculum of 41°C-grown bacteria was two to five times greater than that of 25°C-grown bacteria, the number of macrophage-adherent bacteria was greater with 25°C-grown bacteria.

TABLE 1. Mean bacterial counts of *L. pneumophila* F889 grown at different temperatures and then inoculated to guinea pig alveolar macrophages

Expt	$\log_{10}$ CFU/ml at day:			
	0 (25°C/41°C)	1 (25°C/41°C)	2 (25°C/41°C)	3 (25°C/41°C)
1	3.9/3.6	3.9/2.6	5.0/4.8	6.2/6.0
2	4.0/3.6	3.3/1.6	4.5/3.2	6.5/5.8
3	3.9/3.2	3.4/2.1	4.6/2.7	6.3/<4.0
4	3.5/4.3	2.4/2.4	3.2/3.5	5.1/5.2
5	3.9/3.9	3.2/2.6	4.3/3.5	6.1/5.6

TABLE 2. Mean percentage of macrophages with cell-associated bacteria after 1 h of incubation with various inoculum sizes of *L. pneumophila* F889 grown at 25 and 41°C

Expt	Inoculation ratio[a]	% Macrophages with associated bacteria		
		25°C	41°C	Ratio[b]
1	1.0	16.7	7.2	2.3
2	1.0	3.0	1.6	1.9
3	0.2	1.3	0.8	1.5
4	0.5	8.7	3.8	2.3

[a] Ratio of inoculum of 25°C-grown F889 to that of 41°C-grown F889 for a particular experiment; in experiments 1 through 3 the inoculum of 25°C-grown bacteria was approximately 1×10^7 CFU/ml (final concentration), and 5×10^7 CFU/ml were used for experiment 4.

[b] Ratio of mean percentage of macrophages with cell-associated bacteria grown at 25°C to mean percentage of macrophages with cell-associated bacteria grown at 41°C.

TABLE 3. Reactions with monoclonal antibodies of *L. pneumophila* F889 grown at three different temperatures

Growth temp (°C)	Reaction strength with monoclonal antibody[a]:									
	W29	JR5	32A12	1315	9C3	4C3	MAB1	MAB2	MAB3	33G2
25	0	0	2	4	4	4	4	4	1	4
35	1	1	3	4	4	4	4	4	1	4
41	3	3	3	4	4	4	4	4	1	4

[a] 4 denotes maximum fluorescent intensity; 0 denotes no visible fluorescence. No reactions were detected with monoclonal antibodies W32, K1G6, and 144C2 regardless of growth temperature.

Plasmid typing. No differences in plasmid content were observed for strain F889 grown at different temperatures. Bacteria grown at 25, 35, and 41°C all contained a single 28-megadalton plasmid.

Monoclonal typing. Two monoclonal antibodies, W29 and JR5, reacted differently with bacterial strain F889 grown at different temperatures (Table 3). Eleven other monoclonal antibodies showed no differences in their reactions with the same bacteria. Strain F889 was classified as a Pontiac 3a subgroup when grown at 25°C and as a Pontiac 4b subgroup when grown at 41°C. The international standardized panel classified the strain as a Philadelphia subgroup, regardless of its growth temperature.

DISCUSSION

We demonstrated that the same strain of *L. pneumophila* varies in virulence when grown at different temperatures. Bacteria grown at 25°C were about fivefold more virulent for guinea pigs than those grown at 41°C. This difference was maintained qualitatively when the bacteria were grown in alveolar macrophages and may be explained by greater initial adherence or uptake of the 25°C-grown bacteria. We detected slight changes in cell surface markers, although whether these changes cause alterations in virulence is unknown.

Slopes of the cumulative fatality rate versus $\log_{10}$ dose curves for the 25°C- and 41°C-grown bacteria were approximately the same, with a difference only in intercept. This implies that the difference in virulence is explained by a relatively early phenomenon, such as initial growth or macrophage uptake. This is supported by our study of macrophage-adherent bacteria after 1 h of incubation, in which about 2.5 times more 25°C-grown bacteria were cell associated than were 41°C-grown bacteria.

Results of the studies of bacterial growth in macrophages are perhaps best understood by greater initial uptake of 25°C-grown bacteria, thus increasing the population of intracellular (and viable) bacteria. The decrease in bacterial count at 24 h postinfection may represent die off of extracellular bacteria, which will not grow extracellularly in this experimental system (10, 13, 19).

It is unlikely that the enhanced virulence of 25°C-grown bacteria is due to differences in growth lag phase or rough-to-smooth dissociation. When strain F889 is grown in broth at 25, 35, or 41°C and then plated and incubated at 35°C, no differences occur in colonial morphology, size, number, or time to first visible growth. In addition, there is no difference in the homogeneity of the bacterial suspensions when grown in broth at the various temperatures. Analysis of whole-cell digests by sodium lauryl sulfate-gel electrophoresis shows no changes in major band amounts or presence for bacteria grown in broth at each of the three temperatures (data not shown).

Monoclonal antibody reactivity correlated with the virulence change associated with different growth temperatures. This points to bacterial cell surface changes, as these monoclonal antibodies react with different bacterial lipopolysaccharide constituents (26). Nonreactivity of Oxford monoclonal antibodies W29 and JR5 with strain F889 grown at 25°C is similar to the reactivity pattern found with the majority of clinical strains in the Pontiac subgroup that are isolated in the United Kingdom (23, 27). It is possible that such nonreacting strains are more likely to cause disease in humans. Nonreactivity with monoclonal antibody type MAB2, which is equivalent to the Oxford Pontiac subgroup, was not noted in this study (23). Failure to react with MAB2 has been correlated with a more marked decrease in virulence than apparently occurs within the MAB2-positive subgroups that we studied (21; Dournon and Rajagopalan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987); this suggests that several different bacterial cell surface changes occur that modulate virulence. These surface changes could possibly correlate with hydrophobicity and overall bacterial adhesiveness, which would be important in the natural aqueous habitat of legionella, as well as in disease causation. Another possibility is that affinity of the bacterial cell surface for the third component of complement, which has been shown to be important in phagocytosis, is altered by growth temperature (N. R. Payne and M. A. Horwitz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D86, p. 86).

Growth temperatures of environmental *L. pneumophila* causing human disease are not precisely known, but are generally believed to be in the range of 35 to 45°C, the temperatures of some water heaters and cooling tower ponds. In fact, we originally thought that because of this *L. pneumophila* would be more virulent when grown at 41°C than at 25°C, and our finding to the contrary was surprising. Several possibilities exist to explain this discrepancy. One is that there are temperature gradients in legionella amplifiers and disseminators and that subpopulations of different virulence exist within any one system. Temperature gradients are well-recognized phenomena in hot water heaters, in hot water lines, and at distal plumbing fixtures (12, 15, 25). Another possibility is that our perception of elevated (>25°C) water temperature as a risk factor for Legionnaires disease is incorrect. It is true that hot water systems hotter than 60°C are unlikely to contain *L. pneumophila*, but it is also true that many environmental legionellae are found at temperatures ranging from 25 to 35°C (5, 11, 22) and that *L. pneumophila* multiplies and survives in 25°C tap water (24). However, the most likely possibility is that growth of *L. pneumophila* in BYEB at 25°C is more representative of the restricted growth requirements found in the environment, where doubling times are measured in days and not hours (9, 24). Work reported by others using a different, but similarly restrictive, culture system supports this last possibility (M. A. Faghri, R. D. Miller, and J. H. Wallace, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D80, p.85).

It has been suggested that plasmidless strains of *L. pneumophila* are more virulent than those containing plasmids (3). No such correlation was noted in this study, as growth at 25°C did not result in curing of the plasmid present in bacteria grown at 35 or 41°C.

One prior study found no correlation between growth temperature of *L. pneumophila* and guinea pig virulence, as measured by response to the intraperitoneal route of infection (1). However, in that study growth temperatures of 37 and 44°C were compared; virulence of bacteria grown at much lower temperatures, such as 25°C, was not studied.

Regardless of the correlation of our growth system and incubation temperatures with those found naturally, we demonstrated that growth temperatures modulate organism virulence. This may have important implications for future virulence studies, which have generally been performed only at a temperature optimal for growth, but perhaps not virulence.

ACKNOWLEDGMENTS

This study was supported by the Medical and Research Services of the Veterans Administration.

We thank Marcus Horwitz for technical and other advice and John Tobin for performing monoclonal typing.

LITERATURE CITED

1. **Berg, J. D., J. C. Hoff, P. V. Roberts, and A. Matin.** 1985. Growth of *Legionella pneumophila* in continuous culture. Appl. Environ. Microbiol. **49**:1534–1537.
2. **Bollin, G. E., J. F. Plouffe, M. F. Para, and R. B. Prior.** 1985. Difference in virulence of environmental isolates of *Legionella pneumophila*. J. Clin. Microbiol. **21**:674–677.
3. **Brown, A., R. M. Vickers, E. M. Elder, M. Lema, and G. M. Garrity.** 1982. Plasmid and surface antigen markers of endemic and epidemic *Legionella pneumophila* strains. J. Clin. Microbiol. **16**:230–235.
4. **Cohen, J.** 1980. Statistical power analysis for the behavioral sciences, revised ed. Academic Press, Inc., New York.
5. **Desplaces, N., K. Nahapetian, and E. Dournon.** 1984. Inventaire des *Legionella* dans l'environnement parisien: implications pratiques. Presse Med. **13**:1875–1879.
6. **Edelstein, P. H.** 1985. Legionnaires' disease laboratory manual, 3rd ed. Document no. 86-129871. National Technical Information Service, Springfield, Va.
7. **Edelstein, P. H., K. Calarco, and V. K. Yasui.** 1984. Antimicrobial therapy of experimentally induced Legionnaires' disease in guinea pigs. Am. Rev. Respir. Dis. **130**:849–856.
8. **Edelstein, P. H., C. Nakahama, J. O. Tobin, K. Calarco, K. B. Beer, J. R. Joly, and R. K. Selander.** 1986. Paleoepidemiologic investigation of Legionnaires' disease at Wadsworth Veterans Administration Hospital by using three typing methods for comparison of legionellae from clinical and environmental sources. J. Clin. Microbiol. **23**:1121–1126.
9. **Edelstein, P. H., R. E. Whittaker, R. L. Kreiling, and C. L. Howell.** 1982. Efficacy of ozone in eradication of *Legionella pneumophila* from hospital plumbing fixtures. Appl. Environ. Microbiol. **44**:1330–1334.
10. **Elliott, J. A., and W. C. Winn, Jr.** 1986. Treatment of alveolar macrophages with cytochalasin D inhibits uptake and subsequent growth of *Legionella pneumophila*. Infect. Immun. **51**: 31–36.
11. **Fliermans, C. B., W. B. Cherry, L. H. Orrison, S. J. Smith, D. L. Tison, and D. H. Pope.** 1981. Ecological distribution of *Legionella pneumophila*. Appl. Environ. Microbiol. **41**:9–16.
12. **Groothuis, D. G., H. R. Veenendaal, and H. L. Dijkstra.** 1985. Influence of temperature on the number of *Legionella pneumophila* in hot water systems. J. Appl. Bacteriol. **59**:529–536.
13. **Horwitz, M. A., and S. C. Silverstein.** 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J. Clin. Invest. **66**:441–450.
14. **Jacobs, R. F., R. M. Locksley, C. B. Wilson, J. E. Haas, and S. J. Klebanoff.** 1984. Interaction of primate alveolar macrophages and *Legionella pneumophila*. J. Clin. Invest. **73**:1515–1523.
15. **Joly, J.** 1985. *Legionella* and domestic water heaters in the Quebec City area. Can. Med. Assoc. J. **132**:160.
16. **Joly, J. R., R. M. McKinney, J. O. Tobin, W. F. Bibb, I. D. Watkins, and D. Ramsay.** 1986. Development of a standardized subgrouping scheme for *Legionella pneumophila* serogroup 1 using monoclonal antibodies. J. Clin. Microbiol. **23**:768–771.
17. **Kishimoto, R. A., J. D. White, F. G. Shirey, V. G. McGann, R. F. Berendt, E. W. Larson, and K. D. Hedlund.** 1981. In vitro response of guinea pig peritoneal macrophages to *Legionella pneumophila*. Infect. Immun. **31**:1209–1213.
18. **Meynell, G. G., and E. Meynell.** 1970. Theory and practice in experimental bacteriology. Cambridge University Press, Cambridge.
19. **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.
20. **Plouffe, J. F., M. F. Para, and K. A. Fuller.** 1985. Serum bactericidal activity against *Legionella pneumophila*. J. Clin. Microbiol. **22**:863–864.
21. **Plouffe, J. F., M. F. Para, W. E. Maher, B. Hackman, and L. Webster.** 1983. Subtypes of *Legionella pneumophila* serogroup 1 associated with different attack rates. Lancet **ii**:649–650.
22. **Plouffe, J. F., L. R. Webster, and B. Hackman.** 1983. Relationship between colonization of hospital buildings with *Legionella pneumophila* and hot water temperatures. Appl. Environ. Microbiol. **46**:769–770.
23. **Tobin, J. O., I. D. Watkins, S. Woodhead, and R. G. Mitchell.** 1986. Epidemiological studies using monoclonal antibodies to *Legionella pneumophila* serogroup 1. Isr. J. Med. Sci. **22**:711–714.
24. **Wadowsky, R. M., R. Wolford, A. M. McNamara, and R. B. Yee.** 1985. Effect of temperature, pH, and oxygen level on the multiplication of naturally occurring *Legionella pneumophila* in potable water. Appl. Environ. Microbiol. **49**:1197–1205.
25. **Wadowsky, R. M., R. B. Yee, L. Mezmar, E. J. Wing, and J. N. Dowling.** 1982. Hot water systems as sources of *Legionella pneumophila* in hospital and nonhospital plumbing fixtures. Appl. Environ. Microbiol. **43**:1104–1110.
26. **Watkins, I. D., and J. O. Tobin.** 1984. Studies with monoclonal antibodies to *Legionella* species, p. 259–262. *In* C. Thornsberry, A. Balows, J. C. Feeley, and W. Jakubowski (ed.), Legionella. Proceedings of the 2nd International Symposium. American Society for Microbiology, Washington, D.C.
27. **Watkins, I. D., J. O. Tobin, P. J. Dennis, W. Brown, R. Newnham, and J. B. Kurtz.** 1985. *Legionella pneumophila* serogroup 1 subgrouping by monoclonal antibodies—an epidemiological tool. J. Hyg. **95**:211–216.