JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1991, p. 346–354
0095-1137/91/020346-09$02.00/0

Vol. 29, No. 2

Genus-Specific Epitope on the 60-Kilodalton *Legionella* Heat Shock Protein Recognized by a Monoclonal Antibody

IVO STEINMETZ,[1] CLAUDIA RHEINHEIMER,[1] IRIS HÜBNER,[2] AND D. BITTER-SUERMANN[1]*

Institute of Medical Microbiology, Hannover Medical School, 3000 Hannover 61,[1] and WFM Wasserforschung Mainz GmbH, 6500 Mainz,[2] Federal Republic of Germany

Received 28 August 1990/Accepted 21 November 1990

A monoclonal antibody (MAb) immunoglobulin G2a (2125) was produced against a 60-kDa *Legionella* heat shock protein (HSP), recognizing a unique epitope common to all species of the genus *Legionella*. The antibody reacted in the immunoblot with 59 *Legionella* species and serogroups that were tested and showed no cross-reactivity with other bacteria, including *Acinetobacter* spp., *Bordetella* spp., *Pseudomonas* spp., *Mycobacterium* spp., and *Escherichia coli*. Two other MAbs (2122 and 2130) reacted with the 60-kDa *Legionella* protein as well but showed different cross-reactivities with other gram-negative bacteria in the same molecular mass range. The genus-specific MAb 2125 as well as the cross-reacting MAbs 2122 and 2130 were shown to be reactive with the expressed protein of the cloned gene of the 60-kDa HSP of *Legionella micdadei* and *Legionella pneumophila*. These antibodies demonstrate that *Legionella*-specific and nonspecific epitopes are present on this protein. A sandwich enzyme-linked immunosorbent assay (ELISA) in which the genus-specific MAb is used both as a capture antibody and as a biotinylated second antibody has been established. With this test it is possible to detect *Legionella* whole cells, sonicated cells, and cell fractions containing the 60-kDa HSP. The main part of the 60-kDa HSP is found in the cytoplasmic fraction. The sandwich ELISA can be used to demonstrate the increased expression of the 60-kDa protein in *Legionella* cells following heat shock as well as marked differences in the detection of the 60-kDa HSP on whole cells of different *Legionella* strains. The high specificity and sensitivity of the sandwich ELISA for sonicated cells might be very useful to screen on a genus level for *Legionella* cells or the 60-kDa antigen in environmental isolates or body fluids of patients.

The genus *Legionella* currently contains 29 named species and multiple serogroups. Although *Legionella pneumophila* serogroup (SG) 1 is the most common causative agent of legionellosis (3, 26), many different serogroups and species which cause disease in humans have been described (2, 10, 17, 22, 30–34). The serodiagnosis of legionellosis is based mainly on the use of species-specific and serotype-specific monoclonal antibodies (MAbs) and antisera in indirect immunofluorescence. Because of the increasing number of species and serological variants, a routine screening with these tests is beyond the capabilities of most routine diagnostic laboratories. It is therefore desirable to start the serological diagnosis of legionellosis on a genus level for a time-saving and improved diagnostic procedure. Recently it has been shown by using antisera that a 60-kDa protein antigen of all *Legionella* species contains unspecific epitopes shared by many gram-negative bacteria as well as epitopes specific for the genus *Legionella* (25). This 60-kDa protein corresponds to the common antigen described for many different bacteria (16, 18). We reasoned that MAbs against the genus-specific epitopes of this *Legionella* protein would be of high diagnostic value. In addition, these antibodies might be useful to further characterize the 60-kDa heat shock protein (HSP) and to identify *Legionella*-specific oligopeptide domains which can be synthesized for diagnostic use.

Here we report the production and characterization of a MAb recognizing a genus-specific epitope on the 60-kDa HSP of all *Legionella* species. Two other MAbs reacting with different cross-reacting epitopes on the 60-kDa antigen confirm the presence of *Legionella*-specific and nonspecific epitopes on this protein. We established a sandwich enzyme-linked immunosorbent assay (ELISA), based on the genus-specific antibody, which exclusively detects all members of the genus *Legionella*.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and cultivation. Table 1 lists the strains used in this study. *Legionella* strains were grown on buffered charcoal-yeast extract (BCYE) agar plates (Biomérieux, Marcy l'Etoile, France) or in buffered yeast extract (BYE) broth containing 1% (wt/vol) yeast extract, 1% (wt/vol) *N*-(2-acetamido)-2-aminoethanesulfonic acid buffer, 0.1% (wt/vol) alpha-ketoglutarate, 0.04% (wt/vol) L-cysteine–HCl and 0.025% (wt/vol) ferric PP_i at 37°C (or as noted).

Production of monoclonal antibodies. Six-week-old female BALB/c Ann mice (Zentralinstitut für Versuchstierkunde, Hannover, Federal Republic of Germany) were weekly immunized intraperitoneally over a period of 6 weeks with 2.5×10^8 live *L. pneumophila* SG 1 (strain UM1) grown on BCYE agar plates. The primary injection was given with complete Freund adjuvant. The second injection was given with incomplete Freund adjuvant (Difco). The subsequent injections were given with physiological saline. At the end of the immunization regimen, the mice were splenectomized, and the spleen cells were fused with X63-Ag8.653 myeloma cells as described elsewhere (24). The culture supernatant fluids of growing clones were screened by ELISA with whole *L. pneumophila* SG 1 cells as antigens. The resulting hybridomas were cloned by limiting dilution. For production of ascitic fluid, hybridoma cells were injected intraperitoneally into pristane-pretreated BALB/c Ann mice. Animals were irradiated with 300 rad (3 Gy) preinjection.

ELISA for detecting MAbs against *L. pneumophila*. An ELISA was performed in U-shaped wells of flexible polyvi-

TABLE 1. Strains used

Legionella or other species	Source
L. pneumophila	
SG 1 (14 strains)	ATCC 33152, ATCC 33153, ATCC 43108, ATCC 43112, ATCC 43109, ATCC 43110, ATCC 43106, ATCC 43107, NCTC 11191, NCTC 11193, NCTC 11201, NCTC 11231, NCTC 11378, NCTC 11404
SG 2	ATCC 33154
SG 3	ATCC 33155
SG 4	ATCC 33156
SG 5	ATCC 33216
SG 6	ATCC 33215
SG 7	ATCC 33823
SG 8	ATCC 35096
SG 9	ATCC 35289
SG 10	ATCC 43283
SG 11	ATCC 43130
SG 12	ATCC 43290
SG 13	ATCC 43736
SG 14	ATCC 43703
L. pneumophila	ATCC 43660
L. steigerwaltii	ATCC 35302
L. anisa	ATCC 35291
L. cherrii	ATCC 35252
L. erythra	ATCC 35303
L. feeleii	ATCC 35072
L. hackeliae	ATCC 35250
L. jamestowniensis	ATCC 35298
L. maceachernii	ATCC 35300
L. parisiensis	ATCC 35299
L. israelensis	ATCC 43119
L. spiritensis	ATCC 35249
L. santicrusis	ATCC 35301
L. birminghamensis	ATCC 43702
L. bozemanii	ATCC 33217
L. dumoffii	ATCC 33279
L. gormanii	ATCC 33297
L. jordanis	ATCC 33623
L. longbeachae	ATCC 33462
L. micdadei	ATCC 33204
L. oakridgensis	ATCC 33761
L. sainthelensi (2 strains)	ATCC 49322, ATCC 35248
L. wadsworthii	ATCC 33877
L. bonensis	ATCC 43878
L. morawica	ATCC 43877
L. quinlivanii	ATCC 43830
L. tucsonensis	ATCC 49180
L. cincinnatiensis	ATCC 43753
L. rubrilucens	ATCC 35304
Bacteroides fragilis (3 strains)	ATCC 43936, ATCC 43937, ATCC 43935
Pseudomonas fluorescens (2 strains)	ATCC 49271, ATCC 49270
Pseudomonas aeruginosa (2 strains)	ATCC 49266, ATCC 49267
Acinetobacter lwoffii	ATCC 15309
Acinetobacter calcoaceticus	ATCC 19606
Alcaligenes faecalis	ATCC 15173
Mycobacterium smegmatis	ATCC 14468
Mycobacterium cookii	ATCC 49103
Mycobacterium gastri	ATCC 15754
L. pneumophila SG 1 (UM1)	Institute of Hygiene, University of Mainz, Mainz, Federal Republic of Germany
L. pneumophila SG 1 (RC1)	B. Wright, Dept. of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark

Continued

TABLE 1—*Continued*

Legionella or other species	Source
Staphylococcus aureus (3 strains)	IMM[a]
Staphylococcus epidermidis	IMM
Streptococcus agalactiae	IMM
Streptococcus faecalis (2 strains)	IMM
Streptococcus pyogenes	IMM
Streptococcus pneumoniae (2 strains)	IMM
Peptococcus magnus	IMM
Neisseria gonorrhoeae	IMM
Neisseria meningitidis	IMM
Salmonella typhimurium	IMM
Salmonella enteritidis	IMM
Shigella flexneri (2 strains)	IMM
Shigella sonnei	IMM
Escherichia coli (3 strains)	IMM
Citrobacter diversus (2 strains)	IMM
Enterobacter cloacae (2 strains)	IMM
Klebsiella pneumoniae	IMM
Klebsiella spp. (3 strains)	IMM
Serratia marcescens	IMM
Proteus mirabilis	IMM
Proteus vulgaris	IMM
Morganella morganii (2 strains)	IMM
Hafnia alvei	IMM
Yersinia enterocolitica	IMM
Yersinia pseudotuberculosis	IMM
Vibrio parahaemolyticus	IMM
Plesiomonas shigelloides	IMM
Aeromonas hydrophila	IMM
Pasteurella multocida	IMM
Haemophilus influenzae	IMM
Haemophilus parainfluenzae	IMM
Pseudomonas fluorescens	IMM
Pseudomonas cepacia (4 strains)	IMM
Pseudomonas aeruginosa (3 strains)	IMM
Pseudomonas maltophilia	IMM
Acinetobacter lwoffii	IMM
Flavobacterium sp.	IMM
Brucella abortus	IMM
Bordetella pertussis	IMM
Bordetella parapertussis	IMM
Bordetella bronchoseptica	IMM
Campylobacter jejuni (2 strains)	IMM
Bacteroides fragilis	IMM
Listeria monocytogenes	IMM
Listeria innocua	IMM
Fusobacterium nucleatum	IMM
Corynebacterium pseudodiphtheriticum	IMM
Erysipelotrix rhusiopathiae	IMM
Bacillus subtilis	IMM
Clostridium sp.	IMM
Nocardia sp.	IMM
Moraxella sp.	IMM
Providencia stuartii	IMM
Mycobacterium gordonae	IMM
Mycobacterium terrae	IMM
Mycobacterium bovis BCG	IMM
Mycobacterium tuberculosis H37	IMM
Escherichia coli JM 109(pBA 200)	J. Bangsborg, Dept. of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark
Escherichia coli JM 109(pLBL 3020)	J. Hacker, Inst. Genetik/Mikrobiologie University of Würzburg, Würzburg, Federal Republic of Germany

[a] IMM, Institute of Medical Microbiology, Hannover, Federal Republic of Germany.

nyl chloride microtiter plates (Falcon 3911; Becton Dickinson). Plates were coated with live bacteria (*L. pneumophila* SG 1 [strain UM1]) grown on BCYE agar plates as follows. Cells were washed with physiological saline and adjusted in buffer A (0.01 M potassium phosphate buffer made isotonic with saline, pH 7.5) to 2×10^8 cells per ml. Then 20 μl of the suspension per well was added and incubated for 2 h at room temperature. Plates were washed twice with 200 μl of buffer A and then incubated with buffer A-BSA (buffer A containing 1% [wt/vol] bovine serum albumin; Merck, Darmstadt, Federal Republic of Germany) for 30 min. Hybridoma culture supernatant fluids (20 μl) and culture medium as a negative control were then added, and plates were incubated for 2 h and then washed extensively with buffer A prior to the addition of 10 μl of peroxidase-labeled goat anti-mouse immunoglobulin (Jackson Immunoresearch Laboratories; diluted 1:2,000 in buffer A-BSA) for 30 min. The microtiter plates then were washed twice with buffer A and once with 0.01 M potassium phosphate buffer, pH 6.0. Substrate solution {20 μl of 2.7% 2,2′-azino[di(3-ethylbenzthiazolinesulfonic acid)] (Sigma) in potassium phosphate buffer, pH 6.0, containing 0.0025% H_2O_2} then was added to each well, and the enzyme reaction was measured by monitoring the increase in A_{414} with a Titertek Multiscan MC (Flow Laboratories, Meckenheim, Federal Republic of Germany).

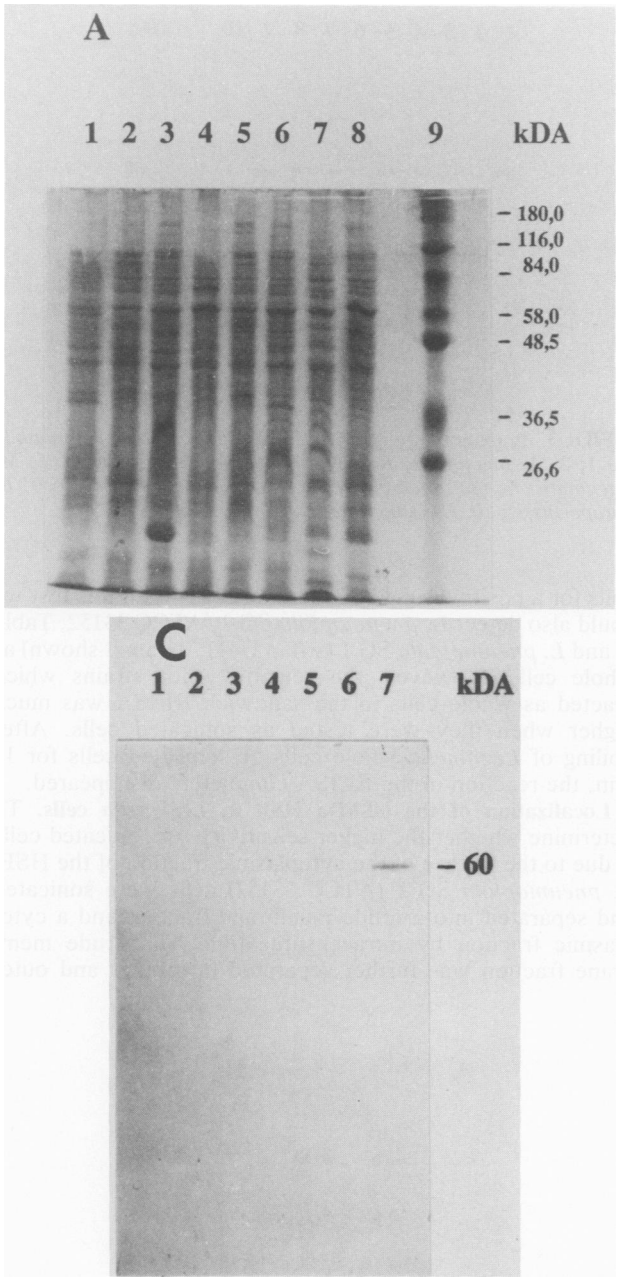
SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate (SDS) slab gel electrophoresis (with 12% SDS) was performed as described previously (24). Bacteria grown on agar plates were washed twice with physiological saline and adjusted to a concentration of 5×10^9 cells per ml. After centrifugation in an Eppendorf Biofuge at 10,000 × *g* for 5 min, the pellet from 1 ml of the bacterial suspension was suspended in 1 ml of sample buffer (0.0625 M Tris hydrochloride, pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 12.5% glycerol), sonicated four times for 15 s each time with the microtip of a Branson sonicator (Sonifier B-12) at a setting of 4 with 30-s intervals between sonications, and heated for 10 min at 100°C. A 20-μl sample (equivalent to 10^8 bacteria) was applied for each lane of the gel. Polyacrylamide gel electrophoresis (PAGE) was carried out until the bromophenol blue tracking dye had reached the bottom of the gel. Molecular weights were estimated by comparison with a prestained SDS marker kit (MW-SDS-Blue; Sigma). In every case in which a Western immunoblot was performed, a second slab gel was run in parallel, and proteins were revealed by Coomassie blue staining to ensure that equal amounts of proteins were added to each lane. The blotting procedure was done by previously described methods (24) with minor modifications by using 200 V for 3 h. The nitrocellulose sheet was saturated with 2% (wt/vol) instant dried milk in buffer A overnight. MAbs 2125, 2130, and 2122 were used as detecting antibodies (MAbs 2125 and 2122 were purified by affinity chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden) and diluted 1:2,000 in 2% instant dried milk–buffer A. MAb 2130 was used in ascitic fluid and diluted in the same manner. Peroxidase-conjugated goat anti-mouse immunoglobulin (Jackson Immunoresearch Laboratories; 1:2,000 in 2% dried milk–buffer A) was used for final development. The substrate was 4-chloro-1-naphthol (Sigma).

Cloning and expression of the *Legionella* 60-kDa antigen in *Escherichia coli*. Cloning of the genus-common 60-kDa protein of *Legionella micdadei* (1) and *L. pneumophila* SG 1 (12) was performed as described previously. *E. coli* clone JM109(pBA 200) expresses the 60-kDa antigen of *L. micdadei* (ATCC 33218). *E. coli* clone JM109(pLBL 3020) expresses the 60-kDa antigen of *L. pneumophila* SG 1 (ATCC 33152). The two recombinant clones were used in immunoblot analysis with the MAbs.

Heat shock and temperature-dependent expression of 60-kDa protein. *L. pneumophila* SG 1 (ATCC 33152) was grown at 30 or 42°C to mid-log phase in BYE broth before use. When the effect of temperature on the steady-state cultures was determined, cells were harvested, washed with physiological saline, adjusted to a common optical density, and tested as either intact cells or sonic extracts in the *Legionella* sandwich ELISA (see below). SDS-PAGE was performed to ensure that equal amounts of proteins which were not affected by temperature were found in the bacteria of different cultures. When heat shock experiments were performed, aliquots (500 μl) were removed from 30°C cultures, placed into sterile Eppendorf tubes, and then incubated at 30 or 42°C for different times as noted in the figure legends. Bacterial cells of the same optical density were then processed for SDS-PAGE and immunoblot or tested as either intact cells or sonic extracts in a *Legionella* sandwich ELISA (see below).

Localization of HSP. *L. pneumophila* SG 1 (ATCC 33152) was grown at 37°C to mid-log phase in BYE broth, and cells were collected from the suspension by centrifugation at 10,000 × *g* for 15 min at 4°C. Cells were suspended in Tris EDTA buffer (50 mM Tris hydrochloride, 1 mM EDTA, pH 7.2) and sonicated as described above. Sonicated cells were centrifuged in a Heraeus Biofuge 17 RS at 4°C for 10 min at 20,000 × *g*. The pellet was discarded and the low-speed supernatant (sonic extract) was then recentrifuged in a Beckman Ultracentrifuge at 100,000 × *g* for 90 min at 4°C. The resulting pellet resuspended in Tris buffer (total membranes, fraction A) and supernatant (crude cytoplasma, fraction B) were tested in the *Legionella* sandwich ELISA (see below). Outer membranes were prepared from the total membrane fraction by the Sarkosyl procedure (5). Briefly, the total membrane fraction was suspended in the Tris EDTA buffer containing 2% (wt/vol) sodium lauroyl sarcosinate (Sigma), incubated at 37°C for 1 h with shaking, and again subjected to ultracentrifugation at 100,000 × *g* for 90 min at 4°C. The Sarkosyl supernatant (cytoplasmic membrane, fraction C) was concentrated in a Centricon 10 Microconcentrator (Amicon), and the retained volume was diluted in Tris buffer. The pellet (Sarkosyl-insoluble material, fraction D) containing the outer membranes was resuspended in Tris buffer. Both fractions were also tested in the *Legionella* sandwich ELISA.

***Legionella* sandwich ELISA.** MAb 2125 was used both as a capture antibody and as a developing antibody. Briefly, U-shaped wells of unflexible polystyrol plates (Greiner, Nürtingen, Federal Republic of Germany) were coated with 10 μl of MAb 2125 (10 μg of protein per ml) in buffer A for 2 h at room temperature or overnight at 4°C. Plates were then washed with 200 μl of buffer A (see above), and 150 μl of buffer A-BSA was added for 30 min. Bacteria (whole cells, sonicated cells, or sonic extracts) diluted in buffer A-BSA were added (20 μl per well) for 2 h. The plates were washed, and 10 μl of biotinylated MAb 2125 diluted in buffer A-BSA was incubated for 1 h. Biotinylation of the MAb was performed as previously described (11, 35). Plates were then washed with buffer A, and 10 μl of streptavidin coupled to β-galactosidase (Boehringer GmbH, Mannheim, Federal Republic of Germany) diluted 1:2,000 in buffer A-BSA was added and incubated for 30 min. The microtiter plates were washed twice with buffer A and once with 0.01 M sodium phosphate buffer, pH 6.9. Substrate solution (20 μl, 0.1 mM



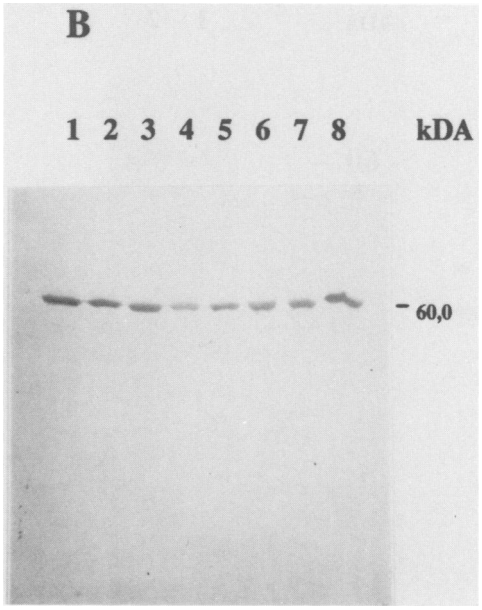


FIG. 1. (A) SDS-PAGE (12% SDS) of different *Legionella* species and SGs. Lanes: 1, *L. pneumophila* SG 11; 2, *L. pneumophila* SG 10; 3, *L. pneumophila* SG 12; 4, *L. birminghamensis*; 5, *L. pneumophila* SG 14; 6, *L. pneumophila* SG 13; 7, *L. cincinnatiensis*; 8, *L. pneumophila* SG 1; 9, molecular mass marker. (B) Immunoblot of 12% SDS-PAGE with MAb 2125 (lanes as in panel A). This immunoblot is representative for the reaction of MAb 2125 with all *Legionella* species and SGs listed in Materials and Methods. (C) Immunoblot with MAb 2125 of 12% SDS-PAGE with non-*Legionella* strains. Lanes: 1, *P. aeruginosa*; 2, *E. coli*; 3, *B. parapertussis*; 4, *A. lwoffii*; 5, *M. gordonae*; 6, *Bacteroides fragilis*; 7, *L. pneumophila* SG 1. This immunoblot is representative for all of the non-*Legionella* strains tested.

4-methylumbelliferyl-β-D-galactopyranoside [Boehringer] in 0.01 M sodium phosphate buffer, pH 6.9) was added to each well, and the fluorescent product was measured in a Titertek Fluoroskan 2 (excitation, 355 nm; emission, 460 nm) (Labsystems, Helsinki, Finland) as relative fluorescence units.

RESULTS

MAbs recognizing *Legionella*-specific and nonspecific epitopes on a 60-kDa protein. MAbs were obtained from fusions after immunization of BALB/c mice over a period of 6 weeks with live *L. pneumophila* SG 1 (UM 1) cells. During the hybridoma screening, 26 clones that produced antibodies reacting in an ELISA with *L. pneumophila* SG 1 whole cells were selected. Of these clones, 1 had specificity for a subgroup of *L. pneumophila* SG 1 and 22 were cross-reactive with other gram-negative bacteria, recognizing structures of different molecular weights. Further studies of these clones were not pursued.

One clone produced a MAb (immunoglobulin G2a, designated 2125) recognizing a genus-specific epitope on the 60-kDa protein present in all *Legionella* species and SGs tested (Fig. 1A and B). This antibody showed no cross-reactivity in the immunoblot (Fig. 1C) with *Acinetobacter lwoffii* ATCC 30159, *Acinetobacter calcoaceticus* ATCC 19606, *Alcaligenes faecalis* ATCC 15173, *Klebsiella pneumoniae*, *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchoseptica*, *Pseudomonas aeruginosa* ATCC 49266 and ATCC 49267, *Pseudomonas cepacia*, *Pseudomonas fluorescens* ATCC 49271 and ATCC 49270, *Mycobacterium gordonae*, *Mycobacterium tuberculosis* H37, *Mycobacterium gastri* ATCC 15754, *Mycobacterium cookii* ATCC 49103, *Mycobacterium smegmatis* ATCC 14468, *Neisseria meningitidis*, *Shigella flexneri*, *Shigella sonnei*, *E. coli*, *Haemophilus influenzae*, and *Bacteroides fragilis* ATCC 43936, ATCC 43937, and ATCC 43935. In addition, there was no cross-reactivity observed with all of the other non-*Legionella* strains (Table 1) tested as whole cells in the same ELISA used for hybridoma screening. To further exclude cross-reactivity with proteins of other bacteria that are not located on the surface, we prepared sonic extracts from all the other non-*Legionella* strains and tested them in a sandwich ELISA (see below) with MAb 2125. Only the extracts of *Legionella* species were positive.

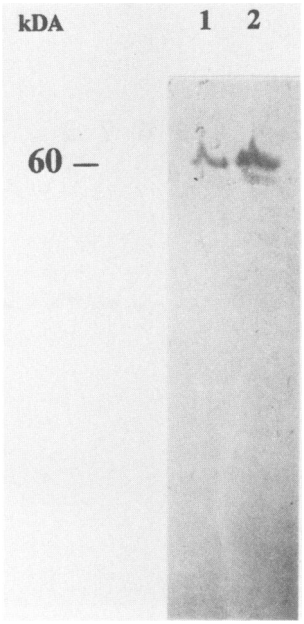


FIG. 2. Immunoblot of the 60-kDa antigen of *L. pneumophila* following heat shock. Bacteria were grown at 30°C to mid-log phase, incubated at 42°C (lane 2) or 30°C (lane 1) for 1 h, and subjected to SDS-PAGE and immunoblot as described in the text.

MAb 2125 was used in the immunoblot to identify the heat shock nature of the 60-kDa protein. *L. pneumophila* grown at 30°C was subjected to heat shock at 42°C for 1 h. Immunoblot after SDS-PAGE revealed an increased detection of the 60-kDa protein following heat shock (Fig. 2). For the heat shock response in the sandwich ELISA, see below and Fig. 6.

The other two cell lines produced MAbs (2122 [immunoglobulin G2a] and 2130 [immunoglobulin M]) that also reacted with the 60-kDa antigen in all *Legionella* species tested but, unlike MAb 2125, cross-reacted with different gram-negative bacteria. These cross-reactions were shown in the immunoblot where MAbs 2122 and 2130 reacted with proteins of about 60-kDa molecular mass. MAbs 2122 and 2130 cross-reacted with *E. coli*, *H. influenzae*, *P. aeruginosa*, *P. cepacia*, *P. fluorescens*, *A. calcoaceticus*, *Alcaligenes faecalis*, *N. meningitidis*, *K. pneumoniae*, and *B. pertussis*. Unlike MAb 2130, only MAb 2122 reacted with *Mycobacterium* species, among them *M. tuberculosis*, *M. smegmatis*, *M. cookii*, *M. gordonae*, and *M. gastri*. Figure 3 shows some representative reactions in the immunoblot with MAb 2122.

Reaction of MAbs with the recombinant 60-kDa antigen of *L. micdadei*. To definitely characterize the genus-specific MAb 2125 as well as the cross-reactive MAbs 2122 and 2130 as HSP specific, we tested them with the cloned 60-kDa common antigen of *L. micdadei* ATCC 33128 (Fig. 4) and *L. pneumophila* ATCC 33152 (data not shown) in *E. coli*. A strong reaction of the three MAbs with the cloned 60-kDa HSP could be demonstrated. In contrast to MAb 2125, which showed no reaction with the *E. coli* control strain, MAbs 2122 (data not shown) and 2130 reacted as expected with the *E. coli* analog of the *L. micdadei* common antigen.

***Legionella* sandwich ELISA based on the genus-specific MAb.** The sandwich ELISA was highly specific for the genus *Legionella* and the sensitivity for sonicated cells was about 3 $\times 10^3$/ml (Fig. 5). This corresponds to an absolute number of cells for a positive signal of about 60 cells. With this test we could also detect *L. pneumophila* SG 1 (ATCC 33152; Table 2) and *L. pneumophila* SG 1 (strain UM1; data not shown) as whole cells. However, the sensitivity for strains which reacted as whole cells in the sandwich ELISA was much higher when they were tested as sonicated cells. After boiling of *Legionella* whole cells or sonicated cells for 10 min, the reaction in the ELISA completely disappeared.

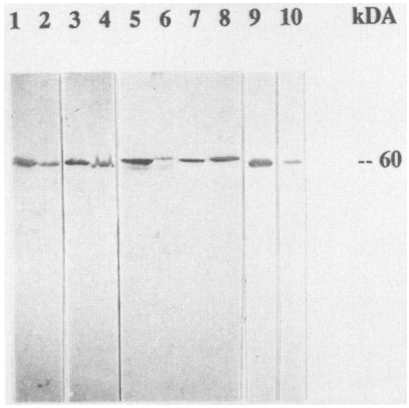


FIG. 3. Immunoblot with MAb 2122. Lanes: 1, *L. pneumophila* SG 1; 2, *A. faecalis*; 3, *K. pneumoniae*; 4, *P. aeruginosa*; 5, *M. smegmatis*; 6, *M. tuberculosis*; 7, *M. cookii*; 8, *M. gastri*; 9, *B. parapertussis*; 10, *H. influenzae*.

Localization of the 60-kDa HSP in *Legionella* cells. To determine whether the higher sensitivity for sonicated cells is due to the release of the cytoplasmic fraction of the HSP, *L. pneumophila* SG 1 (ATCC 33152) cells were sonicated and separated into a crude membrane fraction and a cytoplasmic fraction by ultracentrifugation. The crude membrane fraction was further separated into inner and outer

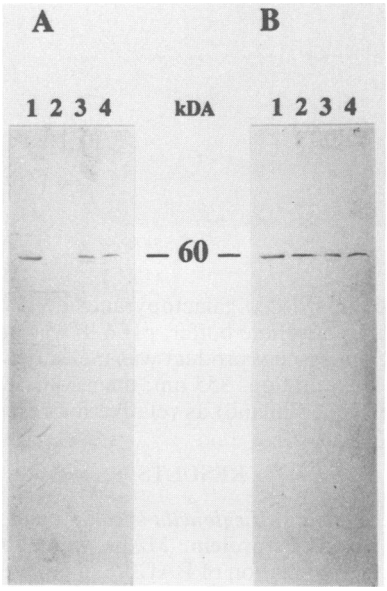


FIG. 4. Reactivity of MAbs 2125 (A) and 2130 (B) with recombinant *E. coli* clone JM 109(pBA200) expressing the 60-kDa *L. micdadei* antigen in immunoblot. Lanes: 1, JM 109(pBA200); 2, JM 109(pBGS18+) (*E. coli* control strain); 3, *L. micdadei*; 4, *L. pneumophila* SG 1.

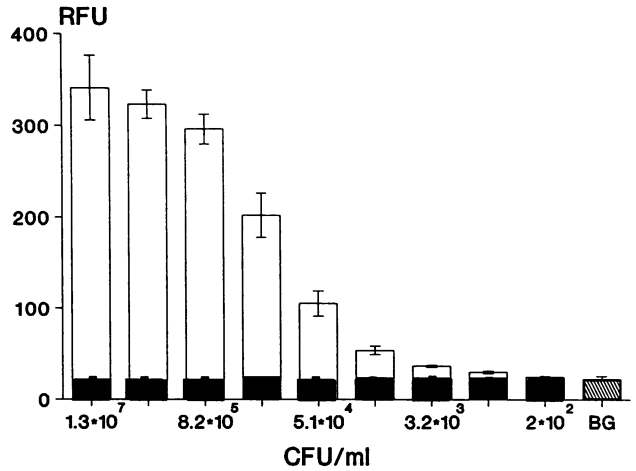


FIG. 5. Sandwich ELISA specific for all *Legionella* species and SGs tested. The reaction of *L. pneumophila* SG 1 (ATCC 33152) is shown. Cell numbers are given as CFU. Cells were grown at 37°C, sonicated, and tested in the sandwich ELISA as described in the text. □, *L. pneumophila* SG 1; ■, control *E. coli*; BG, background; RFU, relative fluorescence units. Mean values and standard deviations from four determinations are given. Signals exceeding the mean background plus three standard deviations were considered positive.

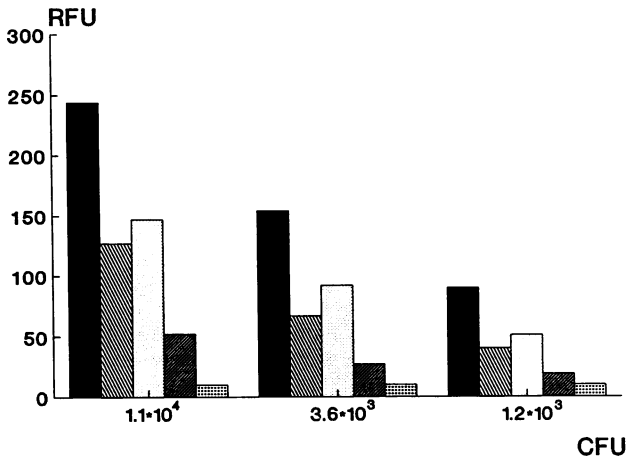


FIG. 6. Heat shock and temperature-dependent expression of the 60-kDa HSP of *L. pneumophila* SG 1 (ATCC 33152). Cell numbers are given as CFU. Bacteria were grown at 30°C steady state (▨) or 42°C steady state (▩) to mid-log phase. Bacteria grown at 30°C were subjected to heat shock for 10 min at 42°C with subsequent incubation for 50 min at 30°C (▧) or for 60 min at 42°C (■). Sonic extracts of the steady-state cultures and the cultures subjected to heat shock were prepared and tested in the sandwich ELISA as described in the text. Mean values from four determinations of three different cell numbers and background (■) are given. RFU, Relative fluorescence units.

membranes by the Sarkosyl procedure (5). All fractions were tested in the sandwich ELISA. The HSP could be identified in the cytoplasmic fraction as well as in the membrane fractions. An equivalent of only 60 cells resulted in a positive signal of the membrane-free fraction (fraction B), whereas 2 × 10^5 cells were needed for a positive signal of the total-membrane fraction (fraction A). It is therefore obvious that the predominant amount of the 60-kDa HSP can be found in the membrane-free fraction. The sandwich ELISA with the separated membrane fractions showed that the 60-kDa HSP could be found in the outer membrane fraction (fraction D) but gives a weaker signal than the cytoplasmic membrane fraction (fraction C).

Heat shock response and temperature-dependent expression of the 60-kDa protein demonstrated in the sandwich ELISA. With the described sandwich ELISA we tried to demonstrate the heat shock response of the 60-kDa protein in *L. pneumophila* SG 1 (ATCC 33152). Figure 6 shows the increased detection of the 60-kDa protein following a temperature shift from 30 to 42°C for 10 min with subsequent incubation for 50 min at 30°C (more than threefold, as estimated from the different fluorescence values in the linear portion of the sandwich ELISA curve) compared with detection after continuous growth at 30°C. A temperature shift from 30 to 42°C for 60 min produced a more pronounced increase (more than 20-fold). We also compared the temperature-dependent expression under steady-state culture conditions. The sandwich ELISA revealed an increased (about ninefold) expression of the HSP at 42°C steady-state growth conditions compared with expression in a 30°C culture. The increased (about threefold) expression of the 60-kDa protein after 60 min at 42°C compared with expression in the 42°C steady-state culture indicates that synthesis of the 60-kDa protein returns to a lower level than does the heat shock response but remains elevated compared with levels in the 30°C culture. We could also detect a temperature-dependent expression and a heat shock response in the sandwich ELISA with whole cells, demonstrating that the heat shock response also produces a higher expression on the surface of the cell. However, the differences seen between steady-growth cultures at 30 and 42°C and cultures after heat shock seemed to be less marked (data not shown).

TABLE 2. Reactivity pattern of different *Legionella* species and SGs when tested as whole cells or sonic extracts in the sandwich ELISA

Strain[a]	RFU[b] ± SD for:	
	Whole cells	Sonic extract
L. pneumophila SG 1 (ATCC 33152)	176.4 ± 2.6	423.7 ± 23.3
L. pneumophila SG 1 (RC1)	16.7 ± 1.3	435.4 ± 20.0
L. cherrii ATCC 35252	15.5 ± 1.4	420.9 ± 23.6
L. micdadei ATCC 33204	14.5 ± 0.9	434.9 ± 11.8
Background	15.3 ± 2.0	8.1 ± 0.6

[a] For each strain, 3 × 10^5 cells from mid-log phase were tested.
[b] RFU, Relative fluorescence units. Values are means from four determinations.

Comparison of 60-kDa protein expression between different species and SGs and within SG 1. In order to determine the total 60-kDa protein expression of the cell and its location on the cell surface, we used the sandwich ELISA to examine sonicated cells as well as intact cells of different species and SGs. The strains were grown under steady-state growth conditions at 37°C.

Table 2 shows that only *L. pneumophila* SG 1 (ATCC 33152) reacted strongly when tested as whole cells. *Legionella cherrii* ATCC 35252, *L. micdadei* ATCC 33204, and *L. pneumophila* SG 1 (RC1) did not seem to express the 60-kDa HSP on the surface. *Legionella anisa* ATCC 35291 and *L. pneumophila* SG 8 (ATCC 35096) were also tested and did not react as whole cells either (data not shown). No signifi-

cant differences were seen when sonic extracts were tested (Table 2). The *L. pneumophila* SG 1 strain (UM1) used for immunization and screening of the hybridoma supernatant showed a strong reaction, comparable to that of *L. pneumophila* SG 1 (ATCC 33152), when tested as whole cells (data not shown). We could not detect *Legionella* whole cells with immunofluorescence methods.

DISCUSSION

The serological relationship of the common antigen of different genera has been shown by crossed immunoelectrophoresis (6, 16), by immunoblot with antisera to the *E. coli* GroEL protein (20), with antisera to the common antigen of *P. aeruginosa* (13), and with MAbs to the 65-kDa HSP of mycobacteria (28). A MAb reacting with a protein in the same molecular mass range present in all *Legionella* species, all *Bordetella* species, and *A. lwoffii* has been recently described (14).

In this study, we report the production and characterization of a MAb recognizing a genus-specific epitope on the 60-kDa HSP of *Legionella* species. This antibody reacted with all *Legionella* species tested either in a sandwich ELISA or in an immunoblot and showed no cross-reactivity with other bacteria, including *P. fluorescens* and *Bacteroides fragilis* strains, which were shown to cross-react with a polyclonal immunofluorescence kit for the detection of *Legionella* species (8). The strong reaction of the genus-specific MAb with the cloned 60-kDa antigen of *L. micdadei* (1) and *L. pneumophila* (12) will be of great use when the *Legionella*-specific domain on this protein is mapped to possibly produce *Legionella*-specific peptides for diagnostic use. The cross-reaction of MAbs 2130 and 2122 with bacteria from different genera, including *Mycobacterium* (2122 only), demonstrated the conserved nature of the 60-kDa HSP. The unique *Legionella*-specific (MAb 2125) and the *Legionella*-nonspecific (MAbs 2122 and 2130) epitopes detected by these MAbs on the 60-kDa protein are in accordance with previous results obtained with polyclonal antisera (25). The distribution of the shared epitopes among different bacteria will provide clues to possible DNA homologies between different species and genera. Because of the different reactivity patterns of the cross-reacting MAbs reported in this study and the cross-reacting MAb previously reported (14), one can conclude that at least three different epitopes shared by the genus *Legionella* and the genus *Bordetella* exist.

In order to put the diagnosis of legionellae on a genus level, we established a sandwich ELISA based on the genus-specific MAb 2125. In this test we take advantage of the fact that the 60-kDa protein is an abundant protein of all *Legionella* species. The high specificity and sensitivity of the *Legionella* sandwich ELISA should improve and shorten the screening for different *Legionella* species and SGs.

It has been shown previously that the 60-kDa *Legionella* protein can be isolated under nondenaturing conditions as a homomultimer with a molecular mass of 650 kDa. It appears that the native 650-kDa protein consists of 10 to 11 subunits of 60 kDa (23). The isolation and characterization of the common antigen of *P. aeruginosa* also revealed a native protein of similar size consisting of 59- to 62-kDa subunits (29). Our findings that after boiling of the sample a positive reaction in the sandwich ELISA completely disappeared is in accordance with data on a heat-labile *L. micdadei* common antigen in crossed immunoelectrophoresis (1). These results correlate well with the assumption that the native 650-kDa protein complex or at least aggregates of the 60-kDa subunit are necessary for a positive signal in the sandwich ELISA (especially in a membrane-free fraction [see below]) because the recognized epitope which is not heat labile (as shown with the immunoblot) is found only once on the 60-kDa subunit.

The 60-kDa protein of the genus *Legionella* is produced in response to a sudden increase in temperature (21). In these studies the heat shock nature of *Legionella* proteins was identified by autoradiography. Cells were subjected to heat shock, and newly synthesized HSPs were preferentially labeled by incubation with radioactively labeled amino acids. We found that the sandwich ELISA with sonicated cells can be used for the demonstration of the heat shock response of the 60-kDa protein as an alternative to the autoradiography commonly used. The fact that an elevated expression of the 60-kDa protein following heat shock is also observed in the sandwich ELISA with *L. pneumophila* SG 1 (ATCC 33152) whole cells indicates that the surface exposure (see below) of this strain is also increased.

We detected an increased level of expression of the 60-kDa HSP of *L. pneumophila* SG 1 (ATCC 33152) under steady-state growth conditions at 42°C compared with the level at 30°C. When we compared the expression of the 60-kDa HSP under 42°C steady-state growth conditions with the heat shock response after a shift from 30 to 42°C for 1 h, we found a lower expression under steady-state 42°C conditions. This is in contrast to other studies: when the temperature-dependent expression of the 60-kDa HSP of *Legionella* species was determined by immunoblot, the expression was found to be essentially unchanged (15), or when determined by autoradiography, the expression in 42°C steady-state culture was found to be the same as after heat shock (21). We found that the sandwich ELISA can be used to quantify the HSP and to characterize the heat shock response pattern.

The exact localization of the common antigen in different genera has not been finally determined. In the case of *Mycobacterium leprae*, the common antigen was found to be associated only with the cell wall (9), whereas the common antigen of *Mycobacterium bovis* was present in the soluble cellular extract and only 2% was extracted from the pellet fraction after ultracentrifugation (7). In *L. pneumophila*, the 60-kDa protein was found to be equally distributed between the supernatant and pellet fraction after ultracentrifugation, as analyzed by autoradiography following SDS-PAGE (20). We also separated sonicated cells into membrane and cytoplasmic fractions by ultracentrifugation. The HSP could be identified by the sandwich ELISA in both fractions. Our results show that the 60-kDa protein is located predominantly in the cytoplasm. The detection of two *L. pneumophila* SG 1 strains (ATCC 33152 and UM1) as whole cells in the sandwich ELISA indicates that the epitope can also be located on the surface of the cell. We therefore separated inner and outer membranes of *L. pneumophila* SG 1 cells (ATCC 33152) and tested them in the sandwich ELISA. The 60-kDa HSP could be found in both membrane fractions. The cytoplasmic membrane gave a higher 60-kDa HSP signal than the outer membrane. We could not detect *Legionella* whole cells with immunofluorescence methods by using the *Legionella*-specific MAb 2125. It is possible that the conditions under which these experiments were performed altered the accessibility of the epitope or the epitope itself. Another possibility is that the amount of protein found on the surface is not sufficient for a positive signal in immunofluorescence, whereas in the sandwich ELISA many cells together result in a positive signal.

We investigated the expression of this protein in different

Legionella strains further. Marked differences were demonstrated by using the sandwich ELISA with whole cells, indicating differences in surface exposure between *Legionella* strains. No significant differences were seen when sonicated cells were tested. It is therefore very unlikely that the detection of whole cells in the sandwich ELISA and the differences seen are artifacts due to lysis of cells and the detection of cytoplasmic 60-kDa HSP. The differences in the amount of the 60-kDa HSP detectable on whole cells are interesting because the 60-kDa HSP of *Legionella* species and the corresponding common antigen of other bacteria are strong immunogens inducing humoral and cellular immune responses (13, 27, 28). Whether the difference in expression of the 60-kDa HSP is of any significance for the bacterium in the interaction with the immune system of the host, and thus of potential relevance to pathogenicity, has to be investigated in the future.

ACKNOWLEDGMENTS

We thank Jette Bangsborg, Copenhagen, Denmark, and Jörg Hacker, Würzburg, Federal Republic of Germany, for kindly providing the recombinant *E. coli* clones expressing the common antigen of *L. micdadei* and *L. pneumophila* SG 1, respectively.

REFERENCES

1. **Bangsborg, J. M., M. T. Collins, N. Høiby, and P. Hindersson.** 1989. Cloning and expression of the *Legionella micdadei* common antigen in *Escherichia coli*. APMIS **97**:14–22.
2. **Best, M. G., J. E. Stout, V. L. Yu, and R. R. Muder.** 1985. *Tatlockia micdadei* (Pittsburgh pneumonia agent) growth kinetics may explain its infrequent isolation from water and the low prevalence of Pittsburgh pneumonia. Appl. Environ. Microbiol. **49**:1521–1522.
3. **Brindle, R. J., T. N. Bryant, and P. W. Draper.** 1989. Taxonomic investigation of *Legionella pneumophila* using monoclonal antibodies. J. Clin. Microbiol. **27**:536–539.
4. **Burger, R., U. Deubel, U. Hadding, and D. Bitter-Suermann.** 1982. Identification of functionally relevant determinants on the complement component C3 with monoclonal antibodies. J. Immunol. **129**:2042–2050.
5. **Butler, C. A., E. D. Street, T. P. Hatch, and P. S. Hoffman.** 1985. Disulfide-bonded outer membrane proteins in the genus *Legionella*. Infect. Immun. **48**:14–18.
6. **Collins, M. T., F. Espersen, N. Høiby, S.-N. Cho, A. Friis-Møller, and J. S. Reif.** 1983. Cross-reactions between *Legionella pneumophila* (serogroup 1) and twenty-eight other bacterial species, including other members of the family *Legionellaceae*. Infect. Immun. **39**:1441–1456.
7. **De Bruyn, J., R. Bosmans, M. Turneer, M. Weckx, J. Nyabenda, J.-P. Van Vooren, P. Falmagne, H. G. Wiker, and M. Harboe.** 1987. Purification, partial characterization, and identification of a skin-reactive protein antigen of *Mycobacterium bovis* BCG. Infect. Immun. **55**:245–252.
8. **Edelstein, P. H., and M. A. C. Edelstein.** 1989. Evaluation of the Merifluor-*Legionella* immunofluorescent reagent for identifying and detecting 21 *Legionella* species. J. Clin. Microbiol. **27**:2455–2458.
9. **Gillis, T. P., R. A. Miller, D. B. Young, S. R. Khanolkar, and T. M. Buchanan.** 1985. Immunochemical characterization of a protein associated with *Mycobacterium leprae* cell wall. Infect. Immun. **49**:371–377.
10. **Griffith, M. E., D. S. Lindquist, R. F. Benson, W. L. Thacker, D. J. Brenner, and H. W. Wilkinson.** 1988. First isolation of *Legionella gormanii* from human disease. J. Clin. Microbiol. **26**:380–381.
11. **Guesdon, J.-L., T. Ternyck, and S. Avrameas.** 1979. The use of avidine-biotin interaction in immunoenzymatic techniques. J. Histochem. Cytochem. **27**:1131–1139.
12. **Hacker, J., B. Ludwig, R. Marre, U. Rdest, and E. Wintermayer.** 1990. Cloning of genes coding for *Legionella* specific antigens and hemolysins and expression in *Escherichia coli*. Zentralbl. Bakteriol. **273**:92.
13. **Hansen, K., J. M. Bangsborg, H. Fjordvang, N. S. Pedersen, and P. Hindersson.** 1988. Immunochemical characterization of and isolation of the gene for a *Borrelia burgdorferi* immunodominant 60-kilodalton antigen common to a wide range of bacteria. Infect. Immun. **56**:2047–2053.
14. **Helsel, L. O., W. F. Bibb, C. A. Butler, P. S. Hoffmann, and R. M. McKinney.** 1988. Recognition of a genus-wide antigen of *Legionella* by a monoclonal antibody. Curr. Microbiol. **16**:201–208.
15. **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.
16. **Høiby, N.** 1975. Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. Scand. J. Immunol. **2**:187–196.
17. **Joly, J. R., P. Dery, L. Gauvreau, L. Cote, and C. Trepanier.** 1986. Legionnaires' disease caused by *Legionella dumoffii* in distilled water. Can. Med. Assoc. J. **135**:1274–1277.
18. **Kaijser, B.** 1975. Immunological studies of an antigen common to many gram-negative bacteria with a special reference to *E. coli*. Int. Arch. Allergy Appl. Immunol. **48**:72–81.
19. **Kaufmann, S. H. E.** 1990. Heat shock proteins and the immune response. Immunol. Today **11**:129–136.
20. **Lema, M. W., A. Brown, C. A. Butler, and P. S. Hoffman.** 1988. Heat-shock response in *Legionella pneumophila*. Can. J. Microbiol. **34**:1148–1153.
21. **Lema, M. W., A. Brown, and G. C. C. Chen.** 1985. Altered rate of synthesis of specific peptides in the *Legionellae* in response to growth temperature. Curr. Microbiol. **12**:347–352.
22. **Lindquist, D. S., G. Nygaard, W. L. Thacker, R. F. Benson, D. J. Brenner, and H. W. Wilkinson.** 1988. Thirteenth serogroup of *Legionella pneumophila* isolated from patients with pneumonia. J. Clin. Microbiol. **26**:586–587.
23. **Pau, C.-P., B. B. Plikaytis, G. M. Carlone, and I. M. Warner.** 1988. Purification, partial characterization, and seroreactivity of a genuswide 60-kilodalton *Legionella* protein antigen. J. Clin. Microbiol. **26**:67–71.
24. **Peters, H., M. Jürs, B. Jann, K. Jann, K. N. Timmis, and D. Bitter-Suermann.** 1985. Monoclonal antibodies to enterobacterial common antigen and to *Escherichia coli* lipopolysaccharide outer core: demonstration of an antigenic determinant shared by enterobacterial common antigen and *E. coli* K5 capsular polysaccharide. Infect. Immun. **50**:459–466.
25. **Plikaytis, B. B., G. M. Carlone, C.-P. Pau, and H. W. Wilkinson.** 1987. Purified 60-kilodalton *Legionella* protein antigen with *Legionella*-specific and nonspecific epitopes. J. Clin. Microbiol. **25**:2080–2084.
26. **Reingold, A. L., B. M. Thomason, B. J. Brake, L. Thacker, H. W. Wilkinson, and J. N. Kuritsky.** 1984. *Legionella pneumophila* in the United States: the distribution of serogroups and species causing human illness. J. Infect. Dis. **149**:819.
27. **Sampson, J. S., B. B. Plikaytis, and H. W. Wilkinson.** 1986. Immunologic response of patients with legionellosis against major protein-containing antigens of *Legionella pneumophila* serogroup 1 as shown by immunoblot analysis. J. Clin. Microbiol. **23**:92–99.
28. **Shinnick, T. M., M. H. Vodkin, and J. C. Williams.** 1988. The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* GroEL protein. Infect. Immun. **56**:446–451.
29. **Sompolinsky, D., D. Hertz, N. Høiby, K. Jensen, B. Mansa, and Z. Samra.** 1980. An antigen common to a wide range of bacteria. I. The isolation of a 'common antigen' from *Pseudomonas aeruginosa*. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **88**:143–149.
30. **Tang, P. W., S. Toma, and L. G. MacMillan.** 1985. *Legionella oakridgensis*: laboratory diagnosis of a human infection. J. Clin. Microbiol. **21**:462–463.
31. **Thacker, W. L., H. W. Wilkinson, R. F. Benson, S. C. Edberg, and D. J. Brenner.** 1988. *Legionella jordanis* isolated from a

patient with fatal pneumonia. J. Clin. Microbiol. **26**:1400–1401.
32. **Thacker, W. L., H. W. Wilkinson, B. B. Plikaytis, A. G. Steigerwalt, W. R. Mayberry, C. W. Moss, and D. J. Brenner.** 1985. Second serogroup of *Legionella feeleii* strains isolated from humans. J. Clin. Microbiol. **22**:1–4.
33. **Wilkinson, H. W., W. L. Thacker, D. J. Brenner, and K. J. Ryan.** 1985. Fatal *Legionella maceachernii* pneumonia. J. Clin. Microbiol. **22**:1055.
34. **Wilkinson, H. W., W. L. Thacker, A. G. Steigerwalt, D. J. Brenner, N. M. Ampel, and E. J. Wing.** 1985. Second serogroup of *Legionella hackeliae* isolated from a patient with pneumonia. J. Clin. Microbiol. **22**:488–489.
35. **Yolken, R. H., F. J. Leister, L. S. Whitcomb, and M. Santosham.** 1983. Enzyme immunoassays for the detection of bacterial antigens utilizing biotin-labeled antibody and peroxidase biotin-avidin complex. Immunol. Methods **56**:319–327.