Cloning and Temperature-Dependent Expression in *Escherichia coli* of a *Legionella pneumophila* Gene Coding for a Genus-Common 60-Kilodalton Antigen

PAUL S. HOFFMAN,* CHARLES A. BUTLER, AND FREDERICK D. QUINN†

Department of Microbiology and Immunology, University of Tennessee, Memphis, Memphis, Tennessee 38163

Received 19 November 1988/Accepted 15 February 1989

All *Legionella* species express a 60-kilodalton (kDa) protein which contains a genus-specific epitope recognized by murine monoclonal antibody GW2X4B8B2H6. A genomic cosmid library of *Legionella pneumophila* chromosomal DNA was constructed in pHCT9 and screened for 60-kDa antigen-expressing clones with the monoclonal antibody. A 3.2-kilobase *EcoRI* fragment from cosmid 14B11 expressing a 60-kDa protein was subcloned into pUC19 (pSH16), and deletion of a 1.2-kilobase *HindIII* fragment (pSH16A) generated a 33-kDa truncated polypeptide no longer reactive with the monoclonal antibody. Southern blot analysis of chromosomal DNA from selected *Legionella* species restricted with *EcoRI* and probed with the 1.2-kilobase fragment coding for the carboxyl region of the protein revealed DNA homology which was not observed with DNA from *Escherichia coli*. Maxicell analysis of pSH16 identified a second polypeptide of approximately 15 kDa expressed from a gene (*htpA*) upstream of the gene coding the 60-kDa protein (*htpB*). Both proteins were preferentially synthesized by *L. pneumophila* following heat shock (temperature shift from 25 to 42°C), and under steady-state growth conditions the relative level of 60-kDa protein was unaffected by temperature. In *E. coli*, expression of a 60-kDa protein from pSH16 also increased following heat shock (25 to 42°C), but under steady-state conditions expression was temperature dependent. Temperature-dependent expression from pSH16 was not observed in an *rpoH* (*htpR*) mutant strain of *E. coli*. The *Legionella* 60-kDa protein appears to be a heat shock protein which shares cross-reactive epitopes with the GroEL homolog of *E. coli*. In addition, a region of *htpB* encoding the 27-kDa carboxyl portion of the protein containing the monoclonal antibody-reactive epitope also contains DNA sequences unique to and conserved within the genus.

Legionella pneumophila and related species are a diverse group of environmental microorganisms which under appropriate conditions produce a severe pneumonitis in humans and experimental animals referred to as legionellosis. These invasive, facultative, intracellular parasites grow within the phagosomes of macrophages, where they abrogate phagolysosomal fusion (18, 19) and prevent acidification of the vacuole (20). Host cellular immune responses appear to be required for protective immunity (3, 17, 21, 29, 42), and this immunity involves activated macrophages which inhibit multiplication of *L. pneumophila* (1, 12, 21, 29). Macrophages can be activated in vivo by a variety of T-cell antigens, including BCG (12, 31, 42), and in vitro by gamma interferon treatment (1). However, specific *Legionella* antigens associated with cellular immunity have not been identified. Cellular immune responses are also considered to be important for such diseases as tuberculosis and leprosy (2, 28). In this regard, a 65-kilodalton (kDa) highly conserved heat shock protein (HSP) from *Mycobacterium tuberculosis* and *M. leprae* has been identified as an important T-cell antigen (28, 33, 34, 39). It is noteworthy that five of six major protein antigens of *M. tuberculosis* are stress induced (43). An operon coding a highly immunogenic 62-kDa HSP from *Coxiella burnetii* has been cloned, and the structural gene (*htpB*) contains sequence homology with the mycobacterial gene coding the 65-kDa protein and with *Escherichia coli* *groEL* (40). Young et al. (43) have suggested that the invading parasite may respond to the hostile environment of

its host by synthesizing stress-induced proteins. The eucaryotic homologs of procaryotic stress-induced proteins have been shown to provide essential cellular functions such as assisting in the posttranslational assembly of oligomeric proteins; participating in renaturation of partially denatured proteins; and, by binding to precursor polypeptides, assisting protein export from one organelle to another (5, 8, 9, 15). While the role of HSPs in disease has not been extensively investigated, it has been suggested that the parasite-derived homologous HSPs might potentiate autoimmune diseases (39).

As a first step in examining the role of stress-induced proteins in pathogenesis and immunity, we have cloned and characterized the *L. pneumophila* gene coding for the 60-kDa common antigen. The protein is preferentially synthesized upon heat shock (26) and is serologically cross-reactive with the GroEL protein of *E. coli* (25) and with the *M. tuberculosis* 65-kDa antigen (34). The protein is highly immunogenic and is the predominant *Legionella* protein reactive with human convalescent serum from confirmed cases of legionellosis (32). Expression of the 60-kDa protein in *E. coli* appears to require a functional heat shock sigma factor for increased expression at 37°C. Finally, the region of *htpB* coding the monoclonal antibody (MAB)-reactive epitope was highly conserved among the legionellae and exhibited no detectable homology with *E. coli* *groEL*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cosmid. *L. pneumophila* Philadelphia 1 (serogroup 1), *L. micdadei*, *L. dumoffii*, *L. oakridgensis*, *L. gormanii*, *L. jordanis*, *L. bozemanii* WIGA, *L. feeleeii*, and *Bordetella pertussis* were obtained from the

* Corresponding author.

† Present address: Department of Medical Microbiology, Stanford University School of Medicine, Stanford, CA 94305.

Centers for Disease Control, Atlanta, Ga., and maintained on ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid]-buffered charcoal-yeast extract agar medium (30). *L. pneumophila* SVir is a streptomycin-resistant virulent strain of *L. pneumophila* Philadelphia 1 which had been passaged intraperitoneally in guinea pigs, grown once on ACES-buffered charcoal-yeast extract agar medium, and frozen at -70°C . Plasmid vector pUC19 (41), cosmid pH79 (16), and various strains of *E. coli*, JM109 (41), LE392 (27), and SE5000 (35), were cultured on appropriate medium with selection. The phagemid vector bluescript (Stratagene, San Diego, Calif.) is derived from a pUC vector and contains the T7 and T3 promoters inserted into the *lacZ* gene and M13 sequences inserted into the pUC parent plasmid, which allows isolation of single-stranded DNA for sequencing. The *E. coli* host for bluescript vectors is XL1-blue (Stratagene) with the following genotype: *endA1 hsdR17* ($r_K^- m_K^+$) *supE44 thi-1 λ^- recA1 gyrA96 relA1* (*lac*) [*F'* *proAB lacIqZ M15 Tn10* (Tet^r)]. *E. coli* strains used for examining specific heat shock responses of cloned *Legionella* genes included K165 [*rpoH*(Am) *sup-4*(Ts)] (6), obtained from Graham C. Walker, and CG217 [*groES30*(Ts)] and CG218 [*groEL100*(Ts)], obtained from Costa Georgopoulos.

Protein purification and antibody production. Crude cell-free extract (French press, two passages), prepared from 10 g of cell paste of *L. pneumophila* SVir as described previously (22), was subjected to ultracentrifugation at $100,000 \times g$ for 90 min in 50 mM Tris hydrochloride buffer, pH 8.0. The soluble material was diluted to 150 ml in Tris buffer, applied to a DE-52 (Whatman, Inc., Clifton, N.J.) column (30-cm DE-52 in a 60-ml syringe), and washed with column buffer until no A_{280} -absorbing material was detected in the eluate. A shallow gradient of KCl (0 to 150 mM) in Tris buffer (300-ml total volume) was used to elute proteins, and fractions containing 60-kDa protein were identified by spotting 10- μ l samples onto nitrocellulose and using the MAb for identification by immunoblot. Pooled immunoreactive fractions were concentrated by ultrafiltration and applied to a Bio-Gel P1.5 column (90 by 2.4 cm; Bio-Rad Laboratories, Richmond, Calif.) equilibrated in Tris buffer. Fractions containing 60-kDa protein were again identified by immunoblot. Preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to purify the protein further. The 60-kDa protein was localized in a 7.5 to 15% gradient polyacrylamide gel by removing lanes and using a combination of immunoblotting with the MAb and Coomassie brilliant blue staining. The band containing the 60-kDa protein was excised from the remaining gel and extracted with bicarbonate buffer (10). Following dialysis against 3 liters of Tris buffer, the protein was concentrated by ultrafiltration and stored frozen at -70°C . The protein concentration was estimated by the biuret method, with bovine serum albumin as a standard (13).

The preparation of MAb GW2X4B8B2H6 has been described previously (14). The hybridoma cell line was grown in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.), and the MAb was purified by passage of tissue culture medium through a protein A-Sepharose column (Sigma Chemical Co., St. Louis, Mo.). Polyclonal antibody was prepared in New Zealand White rabbits by subcutaneous injection of 500 μ g of SDS-PAGE-purified, 60-kDa protein in Freund incomplete adjuvant. Boosters of 400 μ g of protein were given at 4-week intervals. MAb and polyclonal antibody titers were determined by enzyme-linked immunosorbent assay as described previously (14). Preimmune serum was found to have a low titer ($<10^3$),

while hyperimmune serum exhibited titers in the range of 10^7 . The immunoglobulin G fraction from rabbit serum was obtained by affinity column chromatography, using protein A-Sepharose.

SDS-PAGE and immunoblot procedures. SDS-PAGE was performed on a 7.5 to 15% gradient polyacrylamide gel by using the discontinuous buffer system of Laemmli (24). Samples were denatured by boiling for 5 min in sample buffer containing 10% 2-mercaptoethanol. Proteins were resolved by staining the polyacrylamide gel with Coomassie brilliant blue R250, and when autoradiograms were performed, the destained gel was dried and applied to Kodak XAR X-ray film.

Immunoblot procedures were performed as described by Towbin et al. (38). Proteins were electroeluted at 15 V overnight to nitrocellulose, and following blocking with a 3% gelatin solution in phosphate-buffered saline-Tween 20 (14), the MAb (1:200 to 1:1,000) or polyclonal antibody (1:1,000 to 1:10,000) in 100 ml of phosphate-buffered saline-Tween 20 containing 1% gelatin was added. Following overnight incubation and washing in phosphate-buffered saline-Tween 20, either biotinylated goat anti-mouse or goat anti-rabbit immunoglobulin (Organon Teknica Corp., Malvern, Pa.) was applied for 4 h. Following washings, avidin-horseradish peroxidase (1:1,000; Sigma) was added for 1 h, with subsequent development with hydrogen peroxide and 4-chloro-1-naphthol (Sigma) color substrate.

Cloning and preparation of a cosmid library. *L. pneumophila* SVir chromosomal DNA, purified as described previously (23), was partially restricted with *Sau3A1*, and high-molecular-weight fragments were ligated into the *Bam*HI site of cosmid vector pH79. The ligated DNA was packaged into lambda phage heads (Gigapak; Stratagene, San Diego, Calif.), and 100 μ l of packaged phage suspension was incubated with a suspension of *E. coli* LE392 for 30 min. The bacteria were plated on LB agar containing 200 μ g of ampicillin per ml, and approximately 2,200 Am^r colonies were picked and patched to wells of microdilution dishes containing LB broth supplemented with 200 μ g of ampicillin per ml and containing glycerol. The bank was screened by replica plating from the microdilution wells to nitrocellulose sheets with the aid of a 96-pin replicator. The nitrocellulose sheets were placed onto fresh LB agar plates (150 mm) containing 200 μ g of ampicillin per ml, and plates were incubated at 37°C for 16 h. The colonies were lysed by exposure to chloroform vapors, and immunoreactive colonies were identified by immunoblot, using the MAb.

Subcloning, genetic analysis, maxicell, and Southern blot procedures. Cosmid DNA was purified by a combination of alkaline lysis and cesium chloride gradient ultracentrifugation (27). Cosmid 14B11 was restricted with *Eco*RI, and DNA fragments were cloned into the *Eco*RI site of expression vectors pUC19 and bluescript. Transformants were screened by immunoblot for expression of the 60-kDa protein. Restriction endonuclease mapping was done by procedures outlined in reference 27. Restriction enzymes, buffers, and procedures followed those of the various manufacturers.

Maxicell analysis was performed as described in reference 35. Plasmids pSH16 and pUC19 were transformed into *E. coli* SE5000 and grown at 30°C because overexpression of the 60-kDa protein in this strain was found to be inhibitory for growth at 37°C . However, to ensure that factors required for transcription of this gene at 37°C (temperature used for radiolabeling) were synthesized, some of the cultures were shifted to 37°C for 30 min prior to exposure to UV light. All cultures were grown to a final optical density of 0.7 to 0.8

(A_{680} ; 1-cm light path in a Cary-14 spectrophotometer) and exposed to UV light (300 ergs/mm²). Following overnight incubation in the presence of D-cycloserine (50 µg/ml), the cells were washed once, suspended in M63 glucose medium to equivalent volume, and incubated at 37°C for 1 h. [³⁵S]methionine (1,134 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) was added to cell suspensions at 25 µCi/ml for a pulse time of 10 min. The cells were washed and suspended in SDS sample buffer, and 5- to 20-µl samples were applied to a SDS-polyacrylamide gradient gel; following electrophoresis, the gel was exposed to Kodak XAR X-ray film.

DNA hybridizations essentially followed the procedures of Southern (37). Chromosomal DNAs from various *Legionella* species, *B. pertussis*, and *E. coli* were restricted with *Eco*RI and subjected to electrophoresis in a 0.8% horizontal agarose gel. Two DNA fragments of equal size spanning the 1.2-kilobase (kb) *Hind*III-*Eco*RI region were gel purified from pSH16 and radiolabeled with a random priming kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Following prehybridization in 25% formamide-6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the DNA probe (10⁶ cpm) was added and incubated at 37°C overnight. The nitrocellulose blot was washed three times at 55°C in 2× SSC-0.1% SDS (20, 10, and 2 min) and once at 60°C in 2× SSC-0.1% SDS for 20 min. The blot was developed at -80°C, using Kodak XAR X-ray film and an intensifying screen. The percent mismatch in the DNA duplex was estimated by the method described by Davis et al. (7).

Heat shock and temperature-dependent expression of 60-kDa protein. HSPs were identified by autoradiography following temperature shift as described by Lema et al. (26). Briefly, *L. pneumophila* SVir and *E. coli* JM109(pSH16) were grown at 25°C to an optical density of 0.8 (A_{600}) in buffered yeast extract and LB broth, respectively, and each was divided into three 0.5-ml volumes (2-ml Microfuge tubes). Following addition of 20 µCi of [³⁵S]methionine (1,134 Ci/mmol) to each cell suspension, tubes were incubated at 25, 37, and 42°C for 10 min. The reactions were stopped by addition of an equal volume of ice-cold 10% trichloroacetic acid, and the collected pellets were washed once with acetone and suspended in SDS sample buffer. Proteins (20,000 cpm per lane) were separated by SDS-PAGE, and the radiolabeled proteins were visualized by autoradiography on Kodak XAR X-ray film. Relative differences in expression of HSPs at various temperatures were confirmed by laser densitometry (UltraScan XL; LKB, Bromma, Sweden). For *L. pneumophila*, data were normalized to either the 28-kDa major outer membrane protein or a 43-kDa protein whose synthesis was not affected by temperature. The effect of temperature on the steady-state levels of 60-kDa protein was determined for *L. pneumophila* SVir and *E. coli* JM109(pSH16) grown at 23, 30, and 37°C in buffered yeast extract and in LB broth plus ampicillin, respectively. The cells were harvested in mid- to late-log phase of growth, and the washed pellets were suspended in 50 mM Tris hydrochloride buffer (pH 7.2) to a common optical density (A_{680} of 5.0). Whole-cell suspensions were suspended in SDS sample buffer and subjected to SDS-PAGE, and stained proteins were scanned and compared with the aid of a densitometer. Proteins subjected to electrophoresis on a duplicate polyacrylamide gel were electroeluted to nitrocellulose and screened with the MAb (GW2X4B8B2H6).

In a related experiment, *E. coli* K165 strains containing pSH16, pSH16A, pUC19, bluescript, or pBSH35 were grown at the permissive temperature of 30°C, divided into

FIG. 1. Immunoblot analysis of the *Legionella* 60-kDa protein. MAb GW2X4B8B2H6 was used to identify 60-kDa antigen following transfer of proteins from an SDS-polyacrylamide gel to nitrocellulose, as described in the text. Lane A, *E. coli* JM109(pUC19) whole-cell lysate; lane B, *E. coli* JM109(pSH16) whole-cell lysate; lane C, *L. pneumophila* SVir whole-cell lysate; lane D, purified 60-kDa protein, 3 µg.

fractions of equal optical density, and subjected to heat shock at 37 and 42°C for 1 h. Following heat shock, the whole cells were treated as described previously and subjected to electrophoresis on a 7.5 to 15% gradient polyacrylamide gel. Following SDS-PAGE, the proteins were transferred to nitrocellulose, and the levels of 60-kDa protein were determined following immunoblot with the MAb.

RESULTS

Identification of cosmid clones expressing 60-kDa antigen. The cosmid bank constructed in pHC79 was screened by immunoblot for clones reactive with MAb GW2X4B8B2H6. Of approximately 2,200 colonies screened, 12 were found to be positive. Interestingly, two clones (14B11 and 11H5) which grew slowly at 37°C appeared to be grossly overproducing this protein. One of these cosmid clones (14B11) was chosen for further study. *Eco*RI-cleaved 14B11 DNA was initially cloned into pUC19 and later into bluescript. Again, small colonies were found to be expressing 60-kDa protein. Colonies expressing antigen were picked, and plasmid DNA (*Eco*RI restricted) was examined to determine the sizes of the cloned fragments. A 3.2-kb *Eco*RI fragment was found to express the 60-kDa protein as judged by immunoblot (Fig. 1). The apparent molecular weight of the pSH16-expressed protein ranged from 59,000 to 63,000 and appeared to be overexpressed at 37°C (lane B). The lower band of the doublet appeared to be identical to the 60-kDa protein expressed in whole cells of *L. pneumophila* (lane C) and to the purified 60-kDa protein (lane D). The slightly higher-molecular-weight band was not observed in subsequent experiments. The MAb did not cross-react with the GroEL protein of *E. coli* (pUC19) (lane A). However, polyclonal rabbit serum raised to purified 60-kDa protein of *L. pneumophila* exhibited cross-reactivity with a 62-kDa protein of *E. coli*, presumed to be GroEL (data not shown). The

FIG. 2. Restriction map of the *Legionella* DNA fragment expressing the 60-kDa antigen. pSH16 is pUC19 containing a 3.2-kb *EcoRI* fragment expressing 15- and 60-kDa antigens. pSH16A was derived from pSH16 following deletion of two 600-base-pair *HindIII* fragments. The deletion leads to expression of a 15-kDa protein and a truncated polypeptide of 33 kDa reactive with polyclonal anti-60-kDa serum but not with MAb GW2X4B8B2H6. The arrow shows the direction of transcription of *htpB*, though the location of the promoter has not been established. RAb, Rabbit anti-60-kDa serum. Symbols: +, antigen positive by immunoblot and enzyme-linked immunosorbent assay; -, negative by both tests.

cross-reactivity noted with polyclonal antibody is consistent with a previous report that anti-GroEL serum is reactive with the *L. pneumophila* 60-kDa protein (25).

Restriction mapping and maxicell analysis of polypeptides. Figure 2 depicts a restriction map of the 3.2-kb DNA fragment. A *HindIII* deletion of pSH16 (pSH16A) resulted in expression of a truncated 33-kDa polypeptide which was reactive with polyclonal antiserum, but not with the MAb. The deleted fragment (1.2 kb) was composed of two *HindIII* fragments of equal size and of the expected size to encode the deleted portion of the protein. Maxicell analysis of proteins expressed by pSH16 (Fig. 3) revealed that, in addition to the 60-kDa protein, pSH16 encoded a second protein exhibiting an apparent molecular weight of 15,000 to 17,000. Interestingly, as depicted in lane B, cells shifted to 37°C for 30 min prior to UV exposure synthesized in excess of 15-fold more protein over that seen in lane A. Fourfold less protein was loaded onto lane B as judged from the Coomassie-stained SDS-polyacrylamide gel, and densitometry scans of the autoradiogram indicated a 3.7-fold difference in intensity between the 60-kDa bands depicted in lanes A and B. While the temperature shift did not affect the expression of β -lactamase as noted in lanes C and D (pUC19 control), we could not rule out the possibility that some increased synthesis of β -lactamase occurred for maxicells (pSH16) shifted to 37°C. Maxicells containing pSH16A expressed the 15-kDa protein (not shown), suggesting that its structural gene must be upstream of the gene coding the 60-kDa protein. Since the expression of these two proteins appeared to be temperature dependent, we have used the *E. coli* nomenclature for HSPs and have designated the 15-kDa protein as the product of the *htpA* gene and the 60-kDa protein as the product of *htpB*. These two proteins may correspond to the 17- and 60-kDa proteins described previously by Lema et al. as HSPs of *L. pneumophila* (25, 26). While the direction of transcription for *htpB* appears to be toward *lacP* in pSH16, the location of *htpA* in the DNA fragment upstream of *htpB* and the direction of transcription have not been resolved.

Southern hybridization. Since the MAb (GW2X4B8B2H6) recognized a common epitope in a 60-kDa protein expressed by all *Legionella* species (14), we wanted to know whether the genes coding these proteins also exhibited sequence homology. It is known that considerable sequence homology and antigenic cross-reactivity exist for the GroEL class of HSPs (15, 34, 36). Further, since the MAb was found to cross-react with a similar protein of *B. pertussis* (14; unpublished observations), we desired to know whether a genetic basis for this cross-reactivity might exist between these

genera. Since deletion analysis (pSH16A) suggested that the MAb epitope was most likely within the carboxy-terminal 27 kDa of the 60-kDa protein, the *HindIII*-*EcoRI*-generated DNA fragments coding for this region were used as a probe. There are no plasmid sequences or *E. coli* DNA sequences in this probe. The probe did not hybridize with chromosomal sequences from either *B. pertussis* (Fig. 4, lane I) or *E. coli* (Fig. 4, lane J). However, weak hybridization was noted with *B. pertussis* chromosomal DNA following washings at low stringency (26% mismatch in duplex), but under these

FIG. 3. Maxicell analysis of recombinant proteins expressed from pSH16 and pUC19 in *E. coli* SE5000. Details of the maxicell experiment are described in the text. [³⁵S]methionine-labeled whole cells were suspended in sample buffer containing 2-mercaptoethanol and boiled for 10 min prior to loading onto the SDS-polyacrylamide gel. Lanes A and B contain 20 and 5 µl, respectively, of pSH16; lanes C and D contain 20 µl each of pUC19. The cells were grown at 30°C, and the samples loaded onto lanes B and D were from cells shifted to 37°C prior to UV irradiation. Bla, β -Lactamase.

FIG. 4. Southern analysis of chromosomal DNA from *Legionella* spp., *B. pertussis*, and *E. coli*, using the *Hind*III-*Eco*RI region as a probe. Chromosomal DNA was digested with *Eco*RI and transferred to nitrocellulose as described in the text. The DNA probe was derived from a 1.2-kb *Hind*III-*Eco*RI fragment (actually two 600-base-pair fragments) of *htpB* coding for the carboxy-terminal region of the 60-kDa protein. The following species were run (lanes): A, *L. pneumophila* Svir; B, *L. micdadei*; C, *L. dumoffii*; D, *L. oakridgensis*; E, *L. gormanii*; F, *L. jordanis*; G, *L. bozemanii* WIGA; H, *L. feeleii*; I, *B. pertussis*; and J, *E. coli* JM109(pUC19). The relative positions of the molecular weight standards were determined from the ethidium bromide-stained agarose gel prior to transfer of DNA to nitrocellulose. Stringency was calculated at approximately 18% mismatch in the DNA duplex.

conditions the probe also hybridized at multiple sites to chromosomal DNA from the various *Legionella* species (data not presented). At higher stringency (18% mismatch), the probe identified homologous sequences in *Eco*RI-restricted chromosomal DNA from all *Legionella* species examined. Both *L. pneumophila* and *L. feeleii* exhibited hybridizations at 3.2 kb, while hybridizations with DNA fragments from the other species differed in size. These results suggest that the DNA sequences coding the carboxy-terminal region of the 60-kDa protein must be unique to and conserved within the genus.

Temperature-dependent and heat shock expression of the 60-kDa protein in *E. coli* JM109 and in *L. pneumophila*. To confirm the 60-kDa protein as a HSP, *L. pneumophila* and *E. coli* JM109(pSH16), grown at 25°C, were subjected to heat shock at 37 and 42°C. Proteins of 15, 17, 60, and 70 kDa expressed by *L. pneumophila* were preferentially radiolabeled following heat shock (Fig. 5). In the absence of antibody to the 15-kDa protein, it was not possible to determine which protein of *L. pneumophila* corresponded to the *htpA* gene product expressed from pSH16. For *L. pneumophila*, a twofold increase in 60-kDa protein (determined by densitometry) was observed between cells incubated at 25 and 42°C, consistent with previous reports (25, 26). We noted increased expression of 15- and 60-kDa proteins in *E. coli*(pSH16) following heat shock. Interestingly, a twofold increase in expression of 60-kDa protein from pSH16 was noted between cells radiolabeled at 25 and 42°C. Several *E. coli*-derived HSPs were noted, including a 62.5-kDa protein believed to be GroEL.

Early in the course of this study, we noted that expression of 60-kDa protein from pSH16 in *E. coli* was dependent on temperature. Since a characteristic feature of the heat shock response is the return of HSPs to near basal levels following

FIG. 5. Heat shock expression of 60-kDa antigen in *L. pneumophila* and in *E. coli* JM109(pSH16). The bacteria were grown at 25°C to mid-log phase, and 500 μ l of suspension containing 20 μ Ci of [35 S]methionine was incubated for 10 min at 25°C (lanes A and D), 37°C (lanes B and E), or 42°C (lanes C and F). Each lane of an SDS-polyacrylamide gel was loaded with 20,000 cpm of the trichloroacetic acid precipitate suspended in SDS sample buffer. Densitometry scans of the autoradiogram were used to verify that increases in radiolabeled proteins were due to heat shock effects. Lanes A, B, and C depict *E. coli*(pSH16) proteins, and lanes D, E, and F depict *L. pneumophila* SVir proteins.

adaptation to the higher temperature (11), we decided to compare steady-state levels of 60-kDa protein in *E. coli* and *L. pneumophila* grown at different temperatures. Expression of the 60-kDa protein under steady-state growth conditions by *L. pneumophila* was relatively constant (Fig. 6A and B), though at 42°C there was a slight increase (data not presented). These bacteria do not grow above 42°C. These results suggest that the relative level of 60-kDa protein in unstressed *Legionella* cells remains constant over a wide range of temperatures. Even in cells grown at 23°C, the 60-kDa protein appeared to be the second or third most abundant protein in whole-cell extracts, as indicated by SDS-PAGE. In contrast, expression of the pSH16-coded 60-kDa protein in *E. coli* was temperature dependent (Fig. 6A and B). Little or no protein was observed in cells grown at 23°C, whereas at 37°C expression of this protein was increased approximately 20-fold. The gross overexpression of the 60-kDa protein in *E. coli* at elevated temperatures may be a consequence of both gene copy (pUC vector) and an inability by *E. coli* to modulate expression.

To distinguish between temperature-dependent or sigma factor-32-dependent expression of the plasmid-coded protein at elevated temperatures, pSH16, pSH16A, and pBSH35 (3.2-kb *Eco*RI fragment in opposite orientation from pSH16)

FIG. 6. Temperature-dependent expression of 60-kDa protein in *L. pneumophila* and *E. coli* JM109(pSH16). For steady-state experiments, bacteria were grown to mid-log phase at 23, 30, or 37°C and, following centrifugation, were suspended in Tris buffer to a common optical density. Equal portions were then mixed with SDS sample buffer, and 25 μ l each was loaded onto duplicate SDS-polyacrylamide gels. Following electrophoresis, one gel was stained with Coomassie brilliant blue (Δ) and the proteins in the other gel were transferred to nitrocellulose and screened for expression of the 60-kDa protein with the MAb (B). Lanes A, B, and C, *L. pneumophila* grown at 23, 30, and 37°C, respectively; lanes D, E, and F, *E. coli* JM109(pSH16) grown at 23, 30, and 37°C.

were transformed into an *rpoH* mutant strain of *E. coli*. This strain (K165) does not grow at elevated temperatures since it contains an amber mutation in *rpoH* (sigma-32) suppressed by a temperature-sensitive amber suppressor (6). If expression of 60-kDa protein is simply temperature dependent, expression in K165 should be elevated upon temperature shift. If expression at elevated temperatures requires increased levels of sigma-32, 60-kDa protein levels should remain unchanged. Expression of 60-kDa protein at 37 and 42°C following temperature shift from 30°C was essentially unchanged over basal levels (Fig. 7). These results suggest that transcription of *htpB* requires the function of the *rpoH* (*htpR*) sigma-32 factor. Alternatively, expression of *htpB* may require other factors whose expression may be dependent on the expression of the *rpoH* gene product in *E. coli*.

Since heat shock genes are highly conserved in all forms of life, we considered the possibility that the heat shock genes of *L. pneumophila* might complement conditional-lethal heat shock mutants of *E. coli*. pSH16 was transformed into *groES30* and *groEL100* strains of *E. coli* and examined for growth at 37 and 42°C. Ampicillin-resistant recombinants of both strains failed to grow at the nonpermissive temperatures. Immunoblots confirmed that 60-kDa protein was produced at 30°C by these strains. These results demonstrate that the *htpA* and *htpB* gene products of *L. pneumophila* do not complement these mutations.

DISCUSSION

We have cloned a gene coding the 60-kDa genus-common antigen of *L. pneumophila*. The gene was localized to a 3.2-kb *EcoRI* fragment subcloned into pUC19 (pSH16) and shown by maxicell analysis to code for two polypeptides of

FIG. 7. Expression of 60-kDa protein in *E. coli* K165 *rpoH*(Am) *sup-4*(Ts) following heat shock. Strain K165 contains a temperature-sensitive mutation affecting expression of the heat shock sigma-32 factor (6) and fails to grow at 37°C. Plasmids pSH16 (lanes A), pSH16A (lanes B), pUC19 (lanes C), bluescript (lanes D), and pBSH35 (bluescript containing the 3.2-kb *EcoRI* fragment in the opposite orientation from that in pSH16; lanes E) were transformed into K165 and grown on LB plus ampicillin at 30°C. Equal suspensions of these cells were incubated at 37 and 42°C for 1 h and subjected to SDS-PAGE and immunoblot with the MAb.

15 and 60 kDa, tentatively proposed as the products of *htpA* and *htpB*, respectively. In *L. pneumophila*, expression of *htpB* appears to be regulated by thermal stress in a transient manner, indicative of heat shock genes. Following heat shock from 25 to 42°C, the 60-kDa protein was one of several proteins preferentially and coordinately synthesized 10 min after temperature shift. As initially reported by Lema et al. (25, 26), the increase was approximately twofold over synthesis at 25°C. In contrast, the expression of 60-kDa protein in *L. pneumophila* under non-heat shock, steady-state growth conditions was unaffected by temperature except at the extreme permissive temperature of 42°C. The transient and steady-state responses suggest a second level of control typical of heat shock genes (11, 15). While the 60-kDa HSP of *Legionella* spp. was serologically cross-reactive with GroEL (25), we do not know whether the 15-kDa HSP is a homolog of GroES. Preliminary results from deletion analysis suggest *htpA* to be upstream of *htpB*, but whether these genes are arranged into an operon like *groE* (15) and a related heat shock operon of *C. burnetii* (40) has not been established. Sequence analysis of the 3.2-kb DNA fragment as well as analysis of mRNA transcripts should resolve questions regarding operon structure. Preliminary sequence data (manuscript in preparation) indicate that the open reading frame for *htpB* terminates approximately 260 bases from the *EcoRI* site and codes for the terminal amino acid repeat (-Gly-Gly-Met-Gly-Gly-Met-Gly-Gly-Met-Met) typical of the GroEL class of HSPs (15, 40).

The temperature-dependent expression of 60-kDa protein from pSH16 could be abolished in an *rpoH* (*htpR*) sigma-32 mutant of *E. coli* K165 (6). At nonpermissive temperatures (37°C or higher), reduced synthesis of sigma-32 has a pleiotropic effect on the transcription of heat shock genes (11). The results obtained in this study are consistent with the idea that transcription initiation for *htpB* requires a functional sigma-32 factor or perhaps a factor whose synthesis is dependent on sigma-32. It is not clear, however, why heat shock expression of 60-kDa protein is elevated only twofold in *L. pneumophila*. Generally, the HSPs of *E. coli* exhibit more substantial increases following heat shock (11, 15, 26). That a twofold increase in expression was also noted for pSH16 following heat shock suggests that differences in expression might be related to *htpB*, rather than to differences in the transcription machinery between these genera. Another characteristic of the HSPs of *E. coli* is their presence in low levels under non-heat shock conditions (11, 15). In contrast, the 60-kDa protein was one of the most abundant proteins detected in extracts of *L. pneumophila* grown at a variety of temperatures under steady-state growth conditions (23 to 37°C). Since expression of 60-kDa protein from pSH16 in *E. coli* was dependent on temperature, it is likely that differences in levels of expression of 60-kDa protein between these bacteria reflect differences in expression or activation of heat shock sigma factors. Certainly, the high level of 60-kDa protein in *L. pneumophila* under non-heat shock conditions indicates that this protein plays perhaps a more prominent role than do the GroE proteins in *E. coli*.

Synthesis of 60-kDa protein in *E. coli* at 37°C appeared to be uncontrolled, suggesting that the *E. coli* gene products which usually modulate the heat shock response may not recognize *htpB* or the 60-kDa product. The failure of *E. coli* to modulate the response may also be complicated by expression from a multicopy plasmid. Vodkin and Williams also noted that expression of *htp* genes of *C. burnetii* in *E. coli* was temperature dependent (40). The gross overexpres-

sion from multicopy plasmids might in itself provide the stress response which perpetuates the high-level expression. *E. coli* strains harboring pSH16 and pBSH35 grew poorly at 37°C, and revertants to normal growth no longer expressed 60-kDa antigen. We have observed that the overexpressed 60-kDa protein is susceptible to proteolysis by *E. coli* and that in resting cells most of the protein is degraded to 30-kDa fragments which retain the MAb-reactive epitope (unpublished observations). The fate of the 15-kDa protein, which is unreactive with either the MAb or polyclonal antibody raised to the 60-kDa protein, is unknown. Further studies on the regulation of expression of these genes will require their expression from either low-copy vectors or genes integrated into the *E. coli* chromosome to avoid problems due to gene copy.

Our interest in the *Legionella* 60-kDa protein resulted from a prior study in which we characterized a MAb that identified by enzyme-linked immunosorbent assay and immunoblot a protein of similar molecular weight expressed by all named species of *Legionella* (14). Interestingly, the MAb also identified a 58-kDa protein in all *Bordetella* species (14; unpublished results). That the MAb failed to react with so many different genera of bacteria led us to consider the possibility that *Legionella* and *Bordetella* spp. might share DNA sequence homology for this gene. At low stringency (approximately 26% mismatch in duplex), we noted no hybridization with *E. coli* sequences and weak hybridization with sequences from *B. pertussis*. The weak hybridization noted for *B. pertussis* does not rule out the possibility that some sequence homology exists in the region coding the MAb epitope. That the DNA probe did not hybridize to *E. coli* chromosomal sequences suggests that this region of the *htpB* gene may be less conserved among bacteria. We do not know whether there is significant DNA homology between *groEL* and *htpB* in the region coding the amino terminus or for *htpA* and *groES*. Further comparisons will require DNA sequence data for *htpB* and *htpA*.

The apparent lack of homology between the *HindIII*-*EcoRI* region of *htpB* (coding the carboxyl region of the 60-kDa protein) and *groEL* might also explain the inability of *htpA* and *htpB* from pSH16 to complement *groES30* and *groEL100* mutants of *E. coli*. The gene coding the mycobacterial 65-kDa protein was also unable to complement mutations in *E. coli* (34). While the GroEL class of proteins appear to be highly conserved through evolution, it is equally possible that these proteins have become so highly specialized as to be nonfunctional in a different genetic background. That the DNA probe hybridized to chromosomal sequences from the various species of *Legionella* tested suggests that the *htpB* gene may be conserved within this genus. The genomic DNA sequence homology among the various species of *Legionella* varies greatly, with many containing <7% homology with *L. pneumophila* (4). Therefore, the high degree of sequence homology predicted for the *htpB* genes suggests that the functional role(s) for the 60-kDa proteins may be both conserved and highly specialized within the genus.

In general, stress-induced proteins of procaryotic pathogens share cross-reactive epitopes and are highly immunogenic (15, 34, 36, 40). The mycobacterial 65-kDa protein, which is 54% homologous to GroEL (34), is recognized as a key humoral and cellular immune antigen (2) and a candidate for immunoprophylaxis (43). Since these proteins are expressed by normal flora as well as by pathogens, it is not surprising that the host immune system responds in a generalized nonspecific manner to these antigens. This nonspe-

cific immunity might provide the host with increased resistance to microbial infections. In this regard, nonspecific immunity to lethal challenge with *L. pneumophila* has been noted in guinea pigs vaccinated with BCG or infected with mycobacteria (12, 42). In contrast, production of nonspecific antibodies to GroEL-like antigens might diminish the specificity of serological tests, especially with whole-cell preparations, and as recently shown for the mycobacterial 65-kDa antigen, contribute to autoimmune diseases such as arthritis (39). Future studies must address whether the expression of stress-induced proteins is increased in human and animal hosts and whether these proteins exhibit biological activities related to pathogenesis. Since the *hspB* genes of the various *Legionella* species appear to possess substantial sequence homology, it will be important in future studies to characterize further epitopes common to the genus which may be involved in humoral and cell-mediated immunity and as targets for immunodiagnostic reagents.

ACKNOWLEDGMENTS

This project was supported by funds from the Molecular Resource Center, University of Tennessee, Memphis, and the MUREX Corp., Norcross, Ga., and by Public Health Service grant AI24279 from the National Institute of Allergy and Infectious Diseases to P.S.H. F.D.Q. was supported by Public Health Service training grant AI33702 from the National Institute of Allergy and Infectious Diseases.

We also acknowledge the helpful suggestions and strains from Roger Hendrix, Costa Georgopoulos, Graham Walker, Mike Lema, and Arnold Brown and the technical assistance of Laura Houston and Glen Soldan.

LITERATURE CITED

- Bhardwaj, N., T. W. Nash, and M. A. Horwitz. 1986. Interferon-gamma-activated human monocytes inhibit the intracellular multiplication of *Legionella pneumophila*. *J. Immunol.* **137**:2662-2669.
- Boom, W. H., R. N. Husson, R. A. Young, J. R. David, and W. F. Piessens. 1987. In vivo and in vitro characterization of murine T-cell clones reactive to *Mycobacterium tuberculosis*. *Infect. Immun.* **55**:2223-2229.
- Breiman, R. F., and M. A. Horwitz. 1987. Guinea pigs sublethally infected with aerosolized *Legionella pneumophila* develop humoral and cell-mediated immune responses and are protected against lethal aerosol challenge. *J. Exp. Med.* **164**:799-811.
- Brenner, D. J., A. G. Steigerwalt, G. W. Gorman, H. W. Wilkinson, W. F. Bibb, M. Hackel, R. L. Tyndall, J. Campbell, J. C. Feeley, W. L. Thacker, P. Skaliy, W. T. Martin, B. J. Brake, B. S. Fields, H. V. McEachern, and L. K. Corcoran. 1985. Ten new species of *Legionella*. *Int. J. Syst. Bacteriol.* **35**:50-59.
- Chirico, W. J., M. G. Waters, and G. Blobel. 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature (London)* **332**:805-810.
- Cooper, S., and T. Ruettinger. 1975. A temperature sensitive nonsense mutation affecting the synthesis of a major protein of *Escherichia coli* K12. *Mol. Gen. Genet.* **139**:167-176.
- Davis, R. W., D. Botstein, and J. R. Roth. 1980. A manual for genetic engineering: advanced bacterial genetics, p. 220-221. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (London)* **332**:800-805.
- Ellis, R. J. 1987. Proteins as molecular chaperones. *Nature (London)* **328**:378-379.
- Engvall, E., and P. Perlman. 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen coated tubes. *J. Immunol.* **109**:129-135.
- Erickson, J. W., V. Vaughn, W. A. Walter, F. C. Neidhardt, and C. A. Gross. 1987. Regulation of the promoters and transcripts of *rpoH*, the *Escherichia coli* heat shock regulatory gene. *Genes Dev.* **1**:419-432.
- Gibson, D. H., A. Baskerville, L. A. Ashworth, and R. B. Fitzgeorge. 1985. Non-specific protection against pulmonary *Legionella pneumophila* infection in guinea-pigs immunized and challenged with mycobacteria. *Br. J. Exp. Pathol.* **66**:333-344.
- Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**:751-766.
- Helsel, L. O., W. F. Bibb, C. A. Butler, P. S. Hoffman, and R. M. McKinney. 1988. Recognition of a genus-wide antigen of *Legionella* by a monoclonal antibody. *Curr. Microbiol.* **16**:201-208.
- Hemmingsen, S. M., C. Woolford, S. M. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgopoulos, R. W. Hendrix, and R. J. Ellis. 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature (London)* **333**:330-334.
- Hohn, B., and J. Collins. 1980. A small cosmid for efficient cloning of large DNA fragments. *Gene* **11**:291-298.
- Horwitz, M. A. 1983. Cell-mediated immunity in Legionnaires disease. *J. Clin. Invest.* **71**:1686-1697.
- 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.
- Horwitz, M. A. 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *J. Exp. Med.* **158**:2108-2126.
- Horwitz, M. A., and F. R. Maxfield. 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. *J. Cell Biol.* **99**:1936-1943.
- Horwitz, M. S., and S. C. Silverstein. 1981. Activated human monocytes inhibit intracellular multiplication of Legionnaires' disease bacteria. *J. Exp. Med.* **154**:1618-1635.
- Keen, M. G., and P. S. Hoffman. 1984. Metabolic pathways and nitrogen metabolism in *Legionella pneumophila*. *Curr. Microbiol.* **11**:81-88.
- Keen, M. G., E. D. Street, and P. S. Hoffman. 1985. Broad-host-range plasmid pRK340 delivers Tn5 into the *Legionella pneumophila* chromosome. *J. Bacteriol.* **162**:1332-1335.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- 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.
- 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.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Mustafa, A. S., H. K. Gill, A. Nerland, W. J. Britton, V. Mehra, B. R. Bloom, R. A. Young, and T. Godal. 1986. Human T-cell clones recognize a major *M. leprae* protein antigen expressed in *E. coli*. *Nature (London)* **319**:63-66.
- 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.
- Pascale, A. W., J. C. Feeley, R. J. Gibson, L. G. Cordes, R. L. Meyerowitz, C. M. Patton, G. W. Gorman, C. L. Carmack, J. W. Ezzell, and J. N. Dowling. 1980. Pittsburg pneumonia agent: direct isolation from human lung tissue. *J. Infect. Dis.* **141**:727-732.
- Rajagopalan, P., E. Dournon, J.-L. Vilde, and J.-J. Pocidalo. 1987. Direct activation of human monocyte-derived macrophages by a bacterial glycoprotein extract inhibits the intracellular multiplication of virulent *Legionella pneumophila* serogroup 1. *Infect. Immun.* **55**:2234-2239.

32. Sampson, J. S., B. B. Plikaytis, and H. W. Wilkinson. 1986. Immunologic response to patients with legionellosis against major protein-containing antigens of *Legionella pneumophila* serogroup 1 as shown by immunoblot analysis. *J. Clin. Microbiol.* **23**:92-99.
33. Shinnick, T. M. 1987. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. *J. Bacteriol.* **169**:1080-1088.
34. 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.
35. Silhavy, T. O., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. Sompolinsky, D., J. B. Hertz, N. Hoiby, K. Jensen, B. Mansa, and Z. Samra. 1980. An antigen common to a wide range of bacteria. 1. The isolation of a "common antigen" from *Pseudomonas aeruginosa*. *Acta Pathol. Microbiol. Scand. Sect. B* **88**:143-149.
37. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
38. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
39. Van Eden, W., J. E. R. Thole, R. van der Zee, A. Noordzij, J. D. A. van Embden, E. J. Hensen, and I. Cohen. 1988. Cloning of the mycobacterial epitope recognized by T-lymphocytes in adjuvant arthritis. *Nature (London)* **331**:171-173.
40. Vodkin, M. H., and J. C. Williams. 1988. A heat shock operon in *Coxiella burnetii* produces a major antigen homologous to a protein in both mycobacteria and *Escherichia coli*. *J. Bacteriol.* **170**:1227-1234.
41. Yanish-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103-119.
42. Yoshida, S., Y. Mizuguchi, Y. Nikaido, M. Mitsuyama, and K. Nomoto. 1987. Fate of *Legionella pneumophila* Philadelphia-1 strain in resident, elicited, activated, and immune peritoneal macrophages of guinea pigs. *Infect. Immun.* **55**:2477-2482.
43. Young, D., R. Lathigra, R. Hendrix, D. Sweetser, and R. A. Young. 1988. Stress proteins are immune targets in leprosy and tuberculosis. *Proc. Natl. Acad. Sci. USA* **85**:4267-4270.