

## Induced Expression of the *Legionella pneumophila* Gene Encoding a 20-Kilodalton Protein during Intracellular Infection

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The eukaryotic protein synthesis inhibitor cycloheximid has been used by many investigators to selectively radiolabel intracellular bacteria. Although cycloheximide has no direct effect on bacterial gene expression, there are concerns that long-term inhibition of the host cell protein synthesis may have secondary effects on bacterial gene expression. Therefore, prior to further identification and cloning of the macrophage-induced (MI) genes of *Legionella pneumophila*, the effects of cycloheximide on *L. pneumophila*-infected U937 cells were evaluated by transmission electron microscopy. Inhibition of protein synthesis of the host cell for 6 h had no major effect on the ultrastructure of the host cell, on the formation of rough endoplasmic reticulum-surrounded replicative phagosome, or on initiation of intracellular bacterial replication. In contrast, by 15 h of cycloheximide treatment, there was profound deterioration in the host cell as well as in the phagosome. To examine protein synthesis by *L. pneumophila* during the intracellular infection, U937 macrophage-like cells were infected with *L. pneumophila*, and intracellular bacteria were radiolabeled during a 2-h cycloheximide treatment or following 12 h of cycloheximide treatment. Comparison by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the protein profile of radiolabeled in vitro-grown *L. pneumophila* to that of intracellularly radiolabeled bacteria showed that 23 proteins were induced in response to the intracellular environment during 2 h of inhibition of host cell protein biosynthesis. Twelve MI proteins of *L. pneumophila* were artifactually induced due to prolonged inhibition of the host cell protein synthesis. The gene encoding a 20-kDa MI protein was cloned by a reverse genetics technique. Sequence analysis showed that the cloned gene encoded a protein that was 80% similar to the enzyme inorganic pyrophosphatase. Studies of promoter fusion to a promoterless *lacZ* gene showed that compared to in vitro-grown bacteria, expression of the pyrophosphatase gene (*ppa*) was induced fourfold throughout the intracellular infection. There was no detectable induction in transcription of the *ppa* promoter during exposure to stress stimuli in vitro. The *ppa* gene of *L. pneumophila* is the first example of a regulated *ppa* gene which is selectively induced during intracellular infection and which may reflect enhanced capabilities of macromolecular biosynthesis by intracellular *L. pneumophila*. The data indicate caution in the long-term use of inhibition of host cell protein synthesis to selectively examine gene expression by intracellular bacteria.

*Legionella pneumophila* is a ubiquitous organism in the natural aquatic environment where it is amplified by intracellular multiplication within protozoa (reviewed in reference 17). The ability of *L. pneumophila* to cause Legionnaires' disease is dependent on its capacity to survive and to multiply within host alveolar macrophages and possibly alveolar epithelial cells (14, 21, 35).

Part of the uptake of *L. pneumophila* by macrophages is mediated by the complement receptor (36). We have recently shown that uptake of *L. pneumophila* by the protozoan *Hartmannella vermiformis* is mediated by a  $\beta 2$  integrin-like galactose lectin, which undergoes a time-dependent tyrosine dephosphorylation upon bacterial attachment (46). Although the mechanisms of *L. pneumophila* uptake by macrophages and by protozoa are different (5, 25), the intracellular life cycles of the bacterium within mammalian macrophages and within protozoa are highly similar (2, 18a, 19). In both host cells, the bacterium replicates in a phagosome that is inhibited from maturation through the endosomal-lysosomal pathway and is surrounded by the rough endoplasmic reticulum (RER) (2, 10, 19, 20, 43).

Pathogenic bacteria such as *L. pneumophila* and *Salmonella typhimurium* respond and adapt to the various local environmental conditions that they encounter by coordinate regulation of gene expression (1, 3, 16, 42). Upon phagocytosis and during intracellular survival, facultative intracellular pathogens are exposed to a complex mixture of stimuli, and they respond to these stimuli by a profound alteration in gene expression (1, 3, 16). These phenotypic modulations allow intracellular bacteria to survive and adapt to environmental conditions that may be encountered intracellularly, including nutrient limitation, temperature, oxidative injury, osmolarity, pH, and other stimuli (6, 33, 41). The phenotypic alteration by intracellular pathogens is controlled by multiple regulons and is most probably a simultaneous response to a complex combination of unknown stimuli. Characterization of the bacterial macrophage-induced (MI) genes and examination of the kinetics of their expression during the intracellular infection will facilitate characterization of the phagosomal microenvironment that the organisms are exposed to within the host cell. We and others have shown that intracellular *L. pneumophila* manifests a stress response to the intracellular environment (3, 6, 16). We have recently shown that the global stress gene (*gspA*) of *L. pneumophila* is induced in response to in vitro stress stimuli and is also induced throughout the intracellular infection period (4, 6). Differential expression of *gspA* by a  $\sigma^{32}$ -regulated promoter throughout the intracellular infection is indicative of a contin-

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uous stress response manifested by intracellular bacteria (6). Besides the evidence of exposure of intracellular *L. pneumophila* to unidentified stress stimuli (3, 6, 16), the intracellular niche of *L. pneumophila* is not well identified.

To start understanding the nature of the microenvironment within the replicative vacuole and the biochemical signals that intracellular *L. pneumophila* is exposed to, the technique of reverse genetics was utilized to isolate and characterize one of the 23 *L. pneumophila* MI genes that are specifically induced during intracellular infection.

#### MATERIALS AND METHODS

**Bacterial strains and vectors.** The virulent AA100 strain of *L. pneumophila* has been described previously (3, 15). *Escherichia coli* XL1-Blue was used as the host for the genomic library. *E. coli* K-12 derivative strain  $\chi$ 2981 is an *asd* insertion mutant that renders it auxotrophic for diaminopimelic acid (7).

The plasmids pBluescript and pBC were purchased from Stratagene (La Jolla, Calif.). The plasmid pUC-4K was purchased from Pharmacia Biotechnologies (Piscataway, N.J.), and was the source of the kanamycin resistance gene in generating the insertional mutations. The single-copy plasmid pEU730 is a spectinomycin-resistant plasmid containing a promoterless *lacZ* gene, kindly provided by R. Perry (University of Kentucky, Lexington) (18). Bacteria harboring pEU730 or its derivative (pPPZ) were maintained by growth on agar plates supplemented with 0.5 mg of spectinomycin per ml. The high concentration of spectinomycin is required to select for extrachromosomal locus on the plasmid, since *L. pneumophila* contains a chromosomal spectinomycin resistance gene (12a). The plasmid pBOC20 (kindly provided by N. Cianciotto, Northwestern University, Chicago, Ill.) is a chloramphenicol-resistant plasmid that contains *oriT* and the *sacB* gene, which is lethal to *L. pneumophila* grown in the presence of sucrose (13). The conjugative plasmid pRK212.1 was used as a helper plasmid to mobilize the pBOC20 vector and its recombinant derivatives by triparental conjugation into *L. pneumophila* (8).

**DNA manipulations.** Chromosomal DNA preparations, transfection, in situ colony hybridization, end radiolabeling of oligonucleotides, restriction enzyme digestion, and DNA ligation were performed as described elsewhere (39) unless specified. Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were obtained from Bethesda Research Laboratories (BRL) (Gaithersburg, Md.).

Plasmid DNA preparations were done by using the QIAGEN (Chatsworth, Calif.) plasmid kit according to manufacturer's recommendations. Transformations were carried out by electroporation with a Bio-Rad (Hercules, Calif.) gene pulser as recommended by the manufacturer. Purification of DNA fragments from agarose gels for subcloning or labeling for Southern hybridization was done by using a QIAEX kit (QIAGEN) according to manufacturer's recommendations. Transfer of DNA from agarose gels onto membranes, fluorescein labeling of DNA probes, hybridizations, and detection were done as described previously (4). For DNA sequencing, the dideoxy chain termination method of Sanger et al. (40) was employed. Oligonucleotide primers were synthesized commercially (BRL).

**Tissue culture.** Macrophage-like U937 cells were maintained at 37°C and 5% CO<sub>2</sub> in RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated fetal calf serum (Sigma Chemical Co., St. Louis, Mo.). Prior to infection, the cells were differentiated with phorbol 12-myristate 13-acetate for 48 h, as described previously (3). Differentiated cells are nonreplicative adherent macrophage-like cells. Monolayers were washed three times with the tissue culture medium prior to infection. For infection of monolayers, *L. pneumophila* grown for 48 h at 37°C on BCYE agar plates were resuspended in RPMI 1640. The infection was carried out as described for each experiment.

**Transmission electron microscopy.** U937 cell monolayers were treated with cycloheximide for 30 min prior to the beginning of the infection by *L. pneumophila* at a multiplicity of infection (MOI) of 10, followed by extensive washing of extracellular bacteria with tissue culture medium. To examine phagocytosis, infection was carried out for 3 min at an MOI of 500. At several time intervals, infected U937 cell monolayers were fixed in 3.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h on ice followed by 1% OsO<sub>4</sub> in 0.1 M phosphate buffer for 1 h on ice. Dehydration was accomplished by serial exposure to ethanol (50 to 100%) for 10 min at room temperature. The cells were embedded in Eponate 12 resin (Ted Pella, Redding, Calif.) according to the manufacturer's recommendations. Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined with a Hitachi model H-7000/STEM electron microscope at 75 kV.

**Radiolabeling of bacteria and two-dimensional polyacrylamide gel electrophoresis (PAGE).** A modification of a previously reported protocol (3) was used to radiolabel intracellular *L. pneumophila*. Monolayers of differentiated U937 cells were infected for 1 h with *L. pneumophila* AA100 resuspended in RPMI 1640 (Gibco, Gaithersburg, Md.) at a bacterium-to-U937 ratio of 10:1. The monolayers were subsequently washed and treated with gentamicin (50 µg/ml) for 1 h to kill extracellular bacteria. Two sets of infected monolayers were used; in one set, cycloheximide (200 µg/ml) was added immediately to the monolayers

after the gentamicin treatment, while for the second infected monolayers, cycloheximide was added after 12 h of the infection, prior to radiolabeling of intracellular bacteria. [<sup>35</sup>S]methionine (150 µCi/ml) was added to both sets of infected monolayers for an additional 2 h to permit incorporation of the label. Uninoculated U937 cells monolayers and monolayers infected with heat-killed *L. pneumophila* were also radiolabeled under the same experimental conditions to confirm the complete inhibition of protein synthesis of the macrophage. Intracellular radiolabeled organisms were harvested as described previously (3). For comparison, bacteria were radiolabeled in vitro in defined medium during mid-log growth phase in a manner similar to that of intracellular bacteria, exactly as described previously (3).

Cell extracts of the radiolabeled bacterial pellet were prepared by the methods of O'Farrell, as described previously (3). Equal amounts of tricarboxylic acid-precipitated radiolabeled proteins were subjected to equilibrium isoelectric focusing prior to separation in the second dimension on sodium dodecyl sulfate (SDS)-11.5% PAGE slab gels. After electrophoresis, the gels were dried and autoradiographed by using Kodak BIO-MAX film.

**Protein isolation and partial N-terminal sequence analysis.** The protein spot on two-dimensional SDS-PAGE corresponding to the MI protein was isolated for N-terminal sequencing, as described previously (3). Briefly, after electrophoresis gels were soaked in transfer buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid-10% methanol (pH 11.0)] for 10 min and the proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore) for 30 min at 50 V (150 to 170 mA) in a Trans-Blot cell (Bio-Rad, Richmond, Calif.). The membranes were stained with Coomassie brilliant blue R-250 (Sigma), and the stained protein spots were subjected to direct, automated Edman degradation.

**Cloning.** A degenerate oligonucleotide corresponding to the amino acid sequence of the N terminus of the MI protein was synthesized (BRL). The sequence of the degenerate oligonucleotide was 5'-GGI(C/A)GIGA(T/C)GTICC IAA, where I is inosine. The oligonucleotide was radiolabeled and used as a probe for in situ colony hybridizations to screen the *L. pneumophila* partial genomic library (39). The partial genomic library was constructed by ligating gel-purified 2- to 2.5-kb *Eco*RI fragments into plasmid pBC and transformed into *E. coli*. The in situ colony hybridizations were performed exactly as described previously (4).

**Transposon mutagenesis.** To facilitate subsequent allelic exchanges of insertion mutations in the cloned 2.2-kb insert, it was subcloned into plasmid pBOC20 to generate pJA2a. Transposon mutagenesis of pJA2 was performed by using lambda 1105 containing mini-Tn10-kan, exactly as described previously (7). Selected insertions were introduced into *L. pneumophila* AA100 by triparental conjugation (8).

**Primer extension.** Total RNA was isolated from BCYE-grown *L. pneumophila* and from intracellular bacteria in U937 monolayers, exactly as described previously (7). The isolated RNA was used immediately or stored at -20°C. For 5' primer extensions, an oligonucleotide with the sequence 5'-CTTCATACTTTA CAGGTTCTCCGTGCATAG was used, and the reactions were carried out exactly as described previously (4).

**In vitro transcription-translation.** A coupled in vitro transcription-translation system was used to examine the polypeptides expressed by pJA2 (Promega Biotech, Madison, Wis.). [<sup>35</sup>S]methionine was used to radiolabel the polypeptides. The reaction products were resolved by SDS-PAGE and autoradiography.

**Fusion of the *ppa* promoter to a promoterless *lacZ* gene and  $\beta$ -galactosidase ( $\beta$ -Gal) assays.** The sequence encompassing the promoter region upstream of the initiation codon of *ppa* was amplified by PCR with the primers 5'-CGCTT AATTAAGTGGTAACTTCCTGCCC and 5'-GCGGGTACCTTACGCACC CTTAATAAT. The PCR reaction was carried out in the presence of 2.5 mM Mg<sup>2+</sup>-0.25 mM deoxynucleoside triphosphates-0.2 µg of each primer-50 ng of the template pJA2a-2.5 U of *Taq* polymerase (Stratagene) in a 100-µl reaction mixture. The PCR protocol included one step of denaturation at 94°C for 5 min, 5 cycles of annealing at 48°C for 1 min, and extension at 72°C for 30 s followed by 25 cycles of annealing at 58°C and extension at 72°C for 1 min each. The PCR product was purified from an agarose gel and directionally cloned into the *Pac*I-*Kpn*I sites of the single-copy plasmid pEU730 (18), which places the promoterless *lacZ* gene under the control of the *ppa* promoter. The in-frame construct was confirmed by DNA sequencing of the junction. The recombinant was designated pPPZ and electroporated into *L. pneumophila*.

$\beta$ -Gal expression was monitored during several stages of growth in vitro. Culture aliquots for each  $\beta$ -Gal determination were plated to determine the number of CFU per milliliter, and thus,  $\beta$ -Gal activity could be expressed as units per CFU (34). To assess  $\beta$ -Gal activity in response to stress stimuli, mid-log-phase *L. pneumophila* grown in BYE (optical density at 550 nm of 0.6 to 0.7) was exposed to stress stimuli, as described previously (3). Bacteria were pelleted immediately after exposure to the stress stimuli and resuspended in BYE for determination of  $\beta$ -Gal activity.

To determine  $\beta$ -Gal activity from intracellularly grown *L. pneumophila* at several stages of the intracellular infection, monolayers of differentiated U937 cells were infected with *L. pneumophila* harboring pPPZ or pEU730. The infection was accomplished with an MOI of 10 for 1 h followed by three washes with tissue culture medium to remove most of the extracellular bacteria. Gentamicin treatment (50 µg/ml) was done for 1 h to kill any remaining extracellular bacteria, followed by three washes with tissue culture medium. At several time

intervals, monolayers were washed three times with tissue culture medium and lysed hypotonically with distilled water. Aliquots of the lysate were diluted and plated to determine the number of intracellular CFU per milliliter.  $\beta$ -Gal activity was determined for each lysate and calculated in terms of Miller units per CFU (34). To determine whether the  $\beta$ -Gal assay was affected by debris of the lysed U937 cells, mid-log-phase BYE-grown *L. pneumophila* was pelleted and resuspended in a lysate of uninfected monolayers for 30 to 60 min prior to measurement of  $\beta$ -Gal activity.

**Nucleotide sequence accession number.** The nucleotide sequence of the predicted open reading frame encoding the 178-amino-acid protein identified in this study has been assigned GenBank accession no. AF031464.

## RESULTS

**Effects of long-term inhibition of host cell protein biosynthesis on phagosomal ultrastructure.** Cycloheximide is a protein synthesis inhibitor of eukaryotic cells that has been used by many investigators to examine protein synthesis by intracellular bacterial pathogens (1, 3). However, the long-term use of this inhibitor may have secondary effects on bacterial gene expression due to possible physiological changes in the host cell. To examine this possibility, the effect of inhibition of protein synthesis of the host cell was examined at several times during the intracellular infection of the U937 macrophage-like cells. In these experiments, cycloheximide was added to the monolayers 30 min prior to the infection. At several intervals (3 min and 1 h, 2, 4, 6, 8, and 15 h), the monolayers were fixed and processed for transmission electron microscopy. Control untreated, infected monolayers were also examined. Complete inhibition of host cell protein synthesis was confirmed by the failure of uninfected U937 cells to incorporate [ $^{35}$ S]methionine compared to that of untreated cells (data not shown). Compared to that in untreated infected cells, there was no detectable effect of cycloheximide on recruitment of the host cell organelles around the *L. pneumophila* phagosome or on formation of the RER-surrounded replicative vacuole during the first 6 h of treatment (Fig. 1) (19, 43). Inhibition of host cell protein synthesis for 8 h did not have any detectable effect on the ability of *L. pneumophila* to initiate bacterium replication as detected by transmission electron microscopy at 8 h postinfection (Fig. 1 and 2A) and by the number of intracellular bacteria, which had increased for at least two generations (data not shown). There were no major ultrastructural alterations in the host cell during the first 8 h of inhibition of protein synthesis, with the exception of increased frequency of fusion of cellular vesicles to the phagosome, and there may have been some minor changes in the host cell at 6 h as well (Fig. 1 and 2). These data showed that inhibition of protein synthesis of the host cell for 6 h during infection did not have a major effect on the ultrastructure of the replicative vacuole or the rest of the infected cell.

In contrast, after 15 h of inhibition of protein synthesis of the host cell, there was a clear evidence of deterioration of the ultrastructure of the replicative vacuole and the rest of the host cell as well (Fig. 1f and 2B). Although *L. pneumophila* appeared to be in clusters, there was no definitive visible phagosomal membrane and no visible RER around the vacuole. These observations indicated that long-term inhibition of host cell protein synthesis had dramatic effects on cell physiology and ultrastructure. These changes may have a secondary effect on bacterial gene expression. The data indicated that caution should be taken with interpretation of data obtained with the long-term use of this inhibitor to examine gene expression of other slow-growing intracellular bacterial pathogens, such as *Mycobacterium tuberculosis*, for which bacterial protein synthesis has been examined after 23 h of cycloheximide treatment of infected cells (31).

**Protein synthesis by intracellular *L. pneumophila*.** The protein profile of radiolabeled intracellular *L. pneumophila* has been previously reported (3). In that report, the U937 macrophage-like cells were treated with cycloheximide for more than 20 h during radiolabeling of intracellular bacteria. Since inhibition of protein synthesis of the host cell for 15 h had profound effects on the host cell biology, we reevaluated protein synthesis by intracellular *L. pneumophila* radiolabeled during cycloheximide treatment of infected cells. In the previous study cycloheximide was added to the monolayers at the beginning of the infection, and intracellular bacteria were radiolabeled at mid-log phase (3). In this study, mid-log-phase intracellular bacteria were radiolabeled for 2 h in U937 cells treated with cycloheximide at the beginning of the infection or just prior to the 2 h radiolabeling of intracellular bacteria. Examination of infected cells at the end of the cycloheximide treatment showed that in contrast to cellular deteriorations after prolonged inhibition of protein synthesis of the host cell, this short-term treatment did not have any detectable effect on the ultrastructural characteristics of the replicative phagosome or on multiplication of intracellular bacteria (data not shown).

Extracts of intracellular radiolabeled *L. pneumophila* were subjected to two-dimensional SDS-PAGE, and the protein profile was compared to that of in vitro-grown bacteria radiolabeled during the same growth phase. Equal amounts of radioactivity (in counts per minute) were loaded for intracellular and in vitro-grown bacteria. Figure 3 shows the protein profile of radiolabeled intracellular *L. pneumophila* in which cycloheximide was added just prior to radiolabeling of intracellular bacteria. Compared to the protein profile of in vitro-grown organisms (3), the spots marked by arrowheads (Fig. 3) indicate the 23 proteins that were induced in response to the intracellular environment (3). In contrast to the detection of 35 MI proteins in intracellular *L. pneumophila* following 12 h of cycloheximide treatment of infected cells (3), there was an induction of only 23 of the 35 MI proteins induced during prolonged inhibition of the host cell protein biosynthesis (3). There was no detectable difference in the repressed proteins. It has been previously shown that there is no detectable difference in the protein profiles of intracellular bacteria labeled at 10 to 12 h postinfection compared to that radiolabeled at 20 to 22 h postinfection (3). Thus, long-term inhibition of protein synthesis of the host cell had a secondary effect of artifactual induction of expression of at least 12 *L. pneumophila* proteins (marked by small and large bold arrows in Fig. 3).

**Cloning of one of the MI genes of *L. pneumophila*.** The microenvironment of the RER-surrounded phagosome containing *L. pneumophila* and the biochemical signals to which the bacteria are exposed in their specialized and unique niche are not known. To start understanding this microenvironment, one of the MI genes that was specifically induced during the intracellular infection and not induced by in vitro stress stimuli was cloned and characterized (3). The protein spot marked by an open arrow (Fig. 3 and 4), which was shown by phosphorimaging semiquantitation to be induced by approximately 3.4-fold, was subjected to N-terminal amino acid sequencing by Edman degradation. The sequence obtained was SLMEIPSGRDVPNEVNVIIE, with serine as the N-terminal amino acid. A degenerate oligonucleotide corresponding to the underlined sequence was synthesized. In Southern hybridizations, the probe hybridized to single 1.8-kb *Bam*HI, 11-kb *Cla*I, 2.2-kb *Eco*RI, 3.2-kb *Eco*RV, and 8-kb *Hind*III fragments of *L. pneumophila* chromosomal DNA (Fig. 5). A partial genomic library of the ~2.2-kb *Eco*RI fragments was constructed in plasmid pBC. Screening was performed by in situ colony hybridizations with the degenerate oligonucleotide as a probe. Two positive



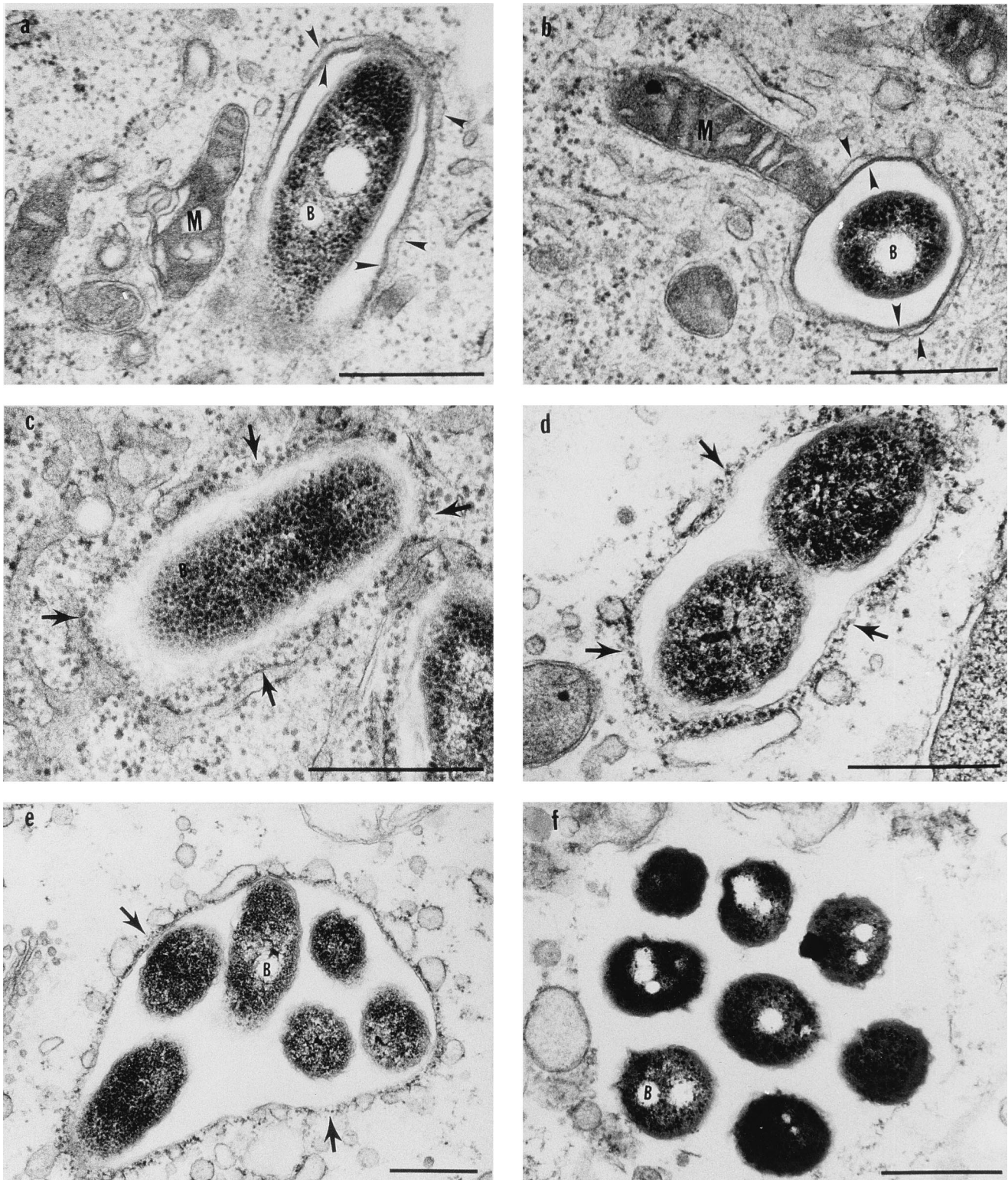


FIG. 1. Electron micrographs of infected U937 cells during inhibition of host cell protein synthesis at 1 h (a), 2 h (b), 4 h (c), 6 h (d), 8 h (e), and 15-h (f) postinfection. Arrowheads indicate the smooth multilayer phagosomal membrane, arrows indicate the RER surrounded phagosome, M indicates mitochondria, and B indicates bacteria. Note the vesicles making contact and possibly fusing with the phagosomal membrane at 8 h in panel e. Note the absence of a defined membrane around the phagosome at 15 h in panel f. Bars, 0.5  $\mu$ m.



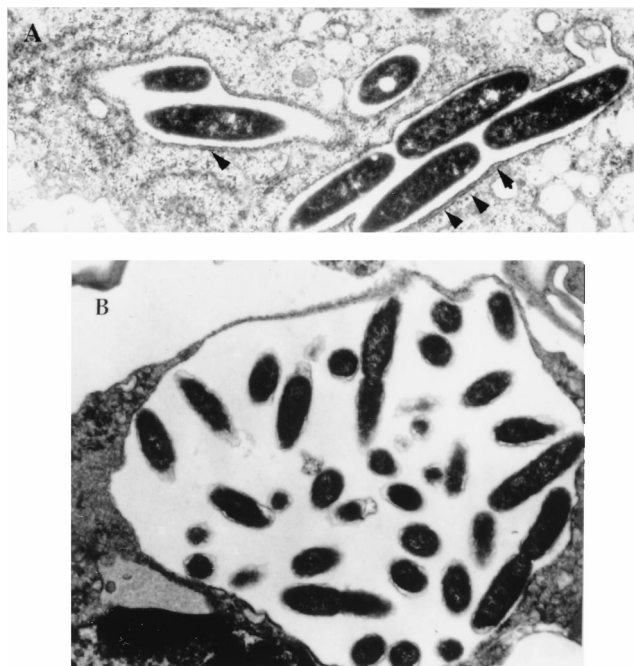


FIG. 2. Electron micrographs of untreated infected U937 cells at 8 h (A) and 15 h (B) postinfection. Arrowheads indicate the RER-surrounded phagosome.

clones were isolated, and one clone, designated pJA2, was chosen for further analysis (Fig. 5).

**Sequence analysis.** The degenerate oligonucleotide corresponding to the N-terminal sequence was used for an initial sequencing reaction, and subsequent oligonucleotides were synthesized. The DNA sequence revealed the presence of a predicted open reading frame consisting of 534 nucleotides encoding a 178-amino-acid protein (Gen Bank accession no. AF031464). There was a Shine-Dalgarno sequence 8 nucleotides upstream of the initiation codon, strongly suggesting that this initiation codon represented a translational initiation site.

To identify the promoter regulating transcription of the MI gene, we identified the transcriptional initiation site by 5' primer extension analysis. By comparing the synthesized cDNA to the DNA from a sequencing reaction primed with the same oligonucleotide, the 5' primer end of the cDNA was mapped to 30 nucleotides upstream of the initiation codon (Fig. 6). Analysis of the DNA sequence upstream from the transcriptional start site suggested the presence of potential -10 and -35 regions that appeared to be similar to  $\sigma^{70}$ -regulated promoters.

Translation of the predicted open reading frame revealed that it encoded a protein with a predicated molecular mass of 20 kDa and an estimated isoelectric point of 5.22. These results are in agreement with the size of the protein and its migration in the acidic portion of the two-dimensional SDS-PAGE (Fig. 3). Expression of the 20-kDa MI protein by the cloned fragment was confirmed by *in vitro* transcription-translation, which showed synthesis of the 20-kDa polypeptide encoded by pJA2 compared to the pBC vector (Fig. 7). Furthermore, the N-terminal sequence derived by Edman degradation completely matched the predicted N-terminal sequence of the open reading frame. It is possible that the protein is processed posttranslationally to remove the N-terminal methionine from the mature protein. These data confirmed that the predicted open reading frame encoded the 20-kDa MI protein.

Alignment with sequences in GenBank showed that the MI

protein was 62% identical and 80% similar to the inorganic pyrophosphatase (PPase) of *E. coli* (Fig. 8) (27). Similar identities to inorganic pyrophosphatase of other organisms were also observed (data not shown). Similarly, the N-terminal sequence of the mature PPase of *E. coli* is also posttranslationally processed by an aminopeptidase to remove the N-terminal methionine (27).

**Construction of null mutation in *ppa*.** A mini-Tn10::kan was used to construct an insertion mutation in the *ppa* gene in pJA2a. Three different insertions within the gene and many insertions in the sequences flanking the gene were obtained. The mutagenized plasmids were introduced into *L. pneumophila* by triparental conjugation (see Materials and Methods). Allelic exchange was achieved by selection on kanamycin-containing plates in the presence of sucrose. Allelic exchange was achieved for all four insertions (attempted) in the flanking regions of the *ppa* gene at a frequency of approximately  $10^{-4}$  to  $10^{-5}$ . In contrast, many trials for allelic exchange of the three different insertion mutations within the *ppa* gene were unsuccessful, with the exception of occasional integration of the whole plasmid into the chromosome, creating a merodiploid (data not shown). These allelic exchanges are performed routinely in our laboratory (6, 7), and this is the first example of our inability to achieve the proper allelic exchange. Although these data suggested but did not confirm that PPase was essential to *L. pneumophila*, the observations that PPase is essential for the viability of both *E. coli* and yeast and our inability to construct a mutation in *ppa* suggested that this gene is probably essential for the viability of *L. pneumophila* (12, 32). Considering the essential and ubiquitous role of this protein in macromolecular biosynthesis (see Discussion), it is not surprising that it would be essential for all living organisms.

**Induction in transcription of the *ppa* gene of *L. pneumophila* within macrophages.** The PPase gene (*ppa*) of *E. coli* is constitutively expressed and is not regulated *in vitro*. Interestingly, synthesis of the 20-kDa MI protein corresponding to PPase of *L. pneumophila* was induced in response to the intracellular environment but was not induced upon exposure to *in vitro* stress stimuli (3). These observations indicated that certain unique stimuli within the phagosomal microenvironment triggered expression of the 20-kDa protein. To examine the kinetics of transcription of *ppa* throughout the intracellular infection, we constructed a fusion of the *ppa* promoter to a promoterless *lacZ* gene. The promoter fusion construct (pPPPZ) or the vector (pEU730) was introduced into *L. pneumophila*.

Monolayers of differentiated U937 cells were infected with *L. pneumophila* harboring pPPPZ or pEU730, followed by gentamicin treatment to kill extracellular bacteria, and further incubated without gentamicin (see Materials and Methods). At several times, monolayers were lysed hypotonically and  $\beta$ -Gal activity was determined (34). To determine whether the  $\beta$ -Gal assay was affected by debris of the lysed U937 cells, BYE-grown *L. pneumophila* in growth phases similar to those of intracellular bacteria was pelleted and resuspended in a lysate of uninfected monolayers, prior to measurement of  $\beta$ -Gal activity. Compared to that of *in vitro*-grown bacteria, transcription of the *ppa* gene was induced during intracellular logarithmic replication by approximately fourfold (Fig. 9). There was an induction in *ppa* transcription upon entry to the logarithmic phase by approximately two- and eightfold by *in vitro*-grown and intracellular bacteria, respectively. These data indicated that expression of *ppa* was induced in response to the intracellular microenvironment. Since the density of the bacteria may affect the level of expression through the *lacZ* fusion, the  $\beta$ -Gal levels were evaluated for *in vitro* cultures of different densities ( $10^5$  to  $10^9$  CFU/ml), and the density of the culture had no

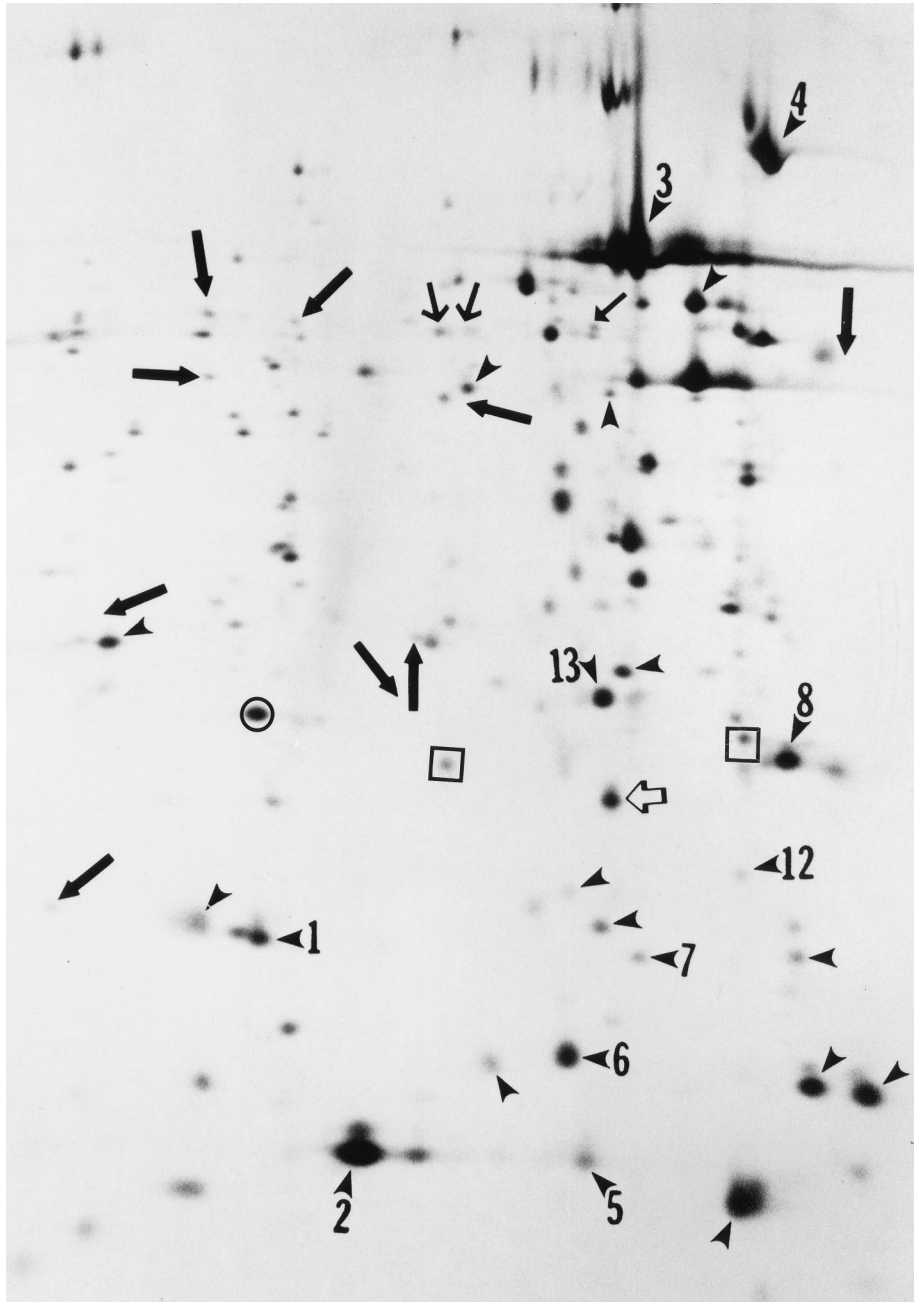


FIG. 3. Autoradiograph of two-dimensional SDS-PAGE of *L. pneumophila* proteins radiolabeled intracellularly in U937 cells during 2 h of cycloheximide treatment and compared to the protein profile of intracellular bacteria radiolabeled during the same growth phase after 12 h of inhibition of host cell protein synthesis and to *in vitro*-grown bacteria during the same growth phase (3). The alkaline-to-acid gradient is from left to right. Arrowheads indicate the proteins that were induced in response to the intracellular environment. Closed arrows indicate the proteins that were artificially induced as a result of prolonged inhibition of host cell protein synthesis (3). The open arrow indicates the 20-kDa PPase that is uniquely induced in response to the intracellular environment. The spots in squares represent the two major spots shown in Fig. 4 on both sides of the 20 kDa PPase. The spot in the circle represents the protein to which the levels of 20-kDa PPase was normalized.

detectable effect on the expression of  $\beta$ -Gal per CFU (data not shown).

In contrast to the intracellular induction of the *ppa* promoter, there was no induction in expression following exposure of *L. pneumophila* to heat shock, osmotic shock, acid shock, and oxidative stress (data not shown). These data further confirmed the stable expression of *ppa* upon exposure to stress stimuli and further showed that *ppa* was specifically

induced in response to the intracellular environment of the host cell.

#### DISCUSSION

Radiolabeling of intracellular bacterial pathogens during a period of inhibition of host cell protein synthesis has been used by many investigators to examine bacterial gene expression

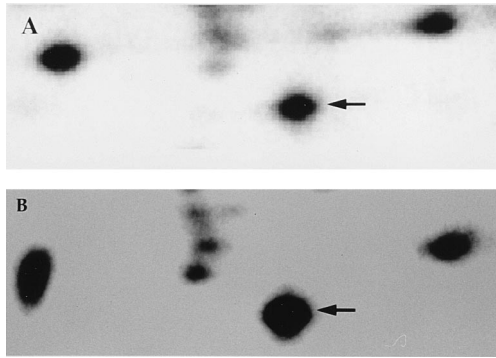


FIG. 4. Magnification of portions of two-dimensional gels to illustrate the protein level of the PPase protein expressed in vitro-grown bacteria (A) compared to that expressed by intracellular bacteria (B). The arrows indicate the 20-kDa PPase that is uniquely induced in response to the intracellular environment. Although three spots on the left of PPase seem to be slightly induced in this gel, they were not reproducible in multiple experiments.

during intracellular infection (1, 3). Cycloheximide has no direct effect on bacterial viability, growth rate, or pattern of protein synthesis in vitro (1, 3). However, prior to further cloning of *L. pneumophila* MI genes, the effects of this inhibitor on the host cell physiology were evaluated to ensure that some *L. pneumophila* MI proteins were not artifactually induced as a secondary effect of prolonged inhibition of protein synthesis of the host cell. Transmission electron microscopy was used to evaluate the effects of this inhibitor on the intracellular infection by *L. pneumophila* and on the ability of the bacterium to form the RER-surrounded replicative phagosome. Inhibition of protein synthesis of *L. pneumophila*-infected U937 cells for up to 6 h did not cause any detectable

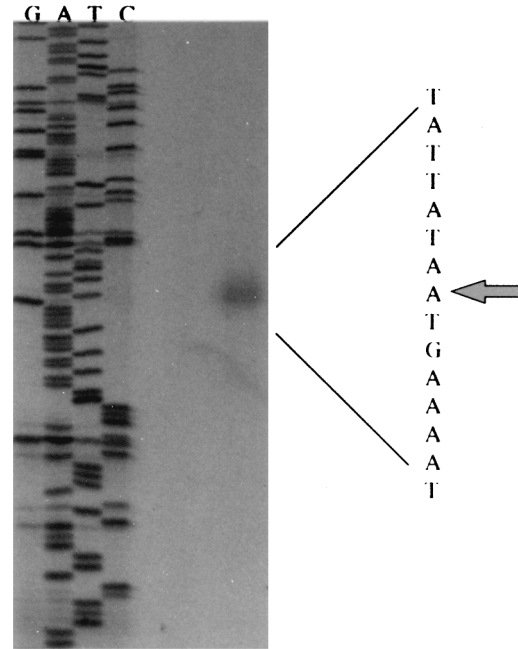


FIG. 6. Primer extension analysis of *ppa* transcript. A radiolabeled oligonucleotide was annealed to RNA isolated from BYE-grown bacteria, and reverse transcriptase was added to produce cDNA. The same oligonucleotide was used to prime the dideoxy sequencing reaction of pJA2 DNA. The letters above each lane indicate the dideoxy nucleotide used to terminate each reaction. The arrow indicates the 5' end of the mRNA specie corresponding to nucleotide 140.

structural changes in the host cell. By 15 h posttreatment, there was clear evidence of dramatic structural deteriorations in the host cell and in the vacuole containing intracellular bacteria. These detectable structural deteriorations in the host cell caused a secondary effect of an artifactual induction of 12

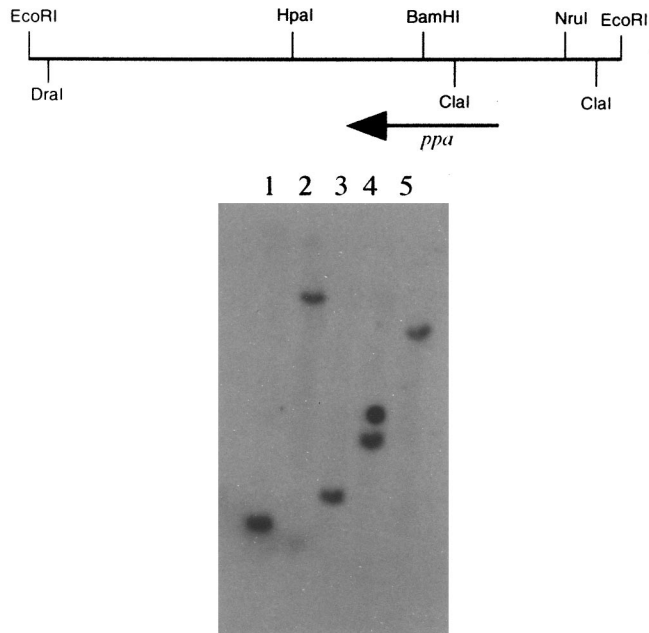


FIG. 5. (Top) Restriction map of pJA2 insert; (bottom) Southern hybridization of chromosomal DNA of *L. pneumophila* digested and probed with the degenerate oligonucleotide corresponding to the N-terminus sequence of the 20-kDa MI protein. Lanes: 1, 1.8-kb *Bam*HI; 2, 11-kb *Cla*I; 3, 2.2-kb *Eco*RI; 4, 3.2-kb *Eco*RV; 5, 8-kb *Hind*III. The second signal in lane 4 is an artifact. The sizes were based on a 1-kb ladder (BRL).

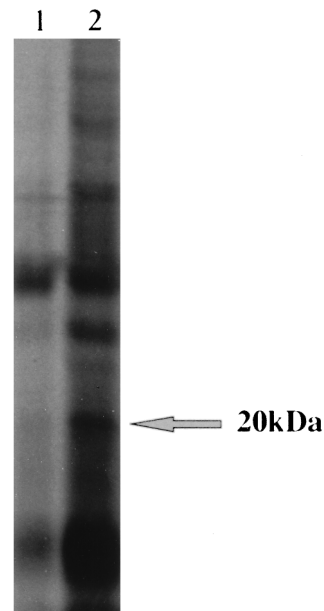


FIG. 7. In vitro transcription-translation of pJA2 (lane 2) and the vector pBC (lane 1). The arrow indicates the 20-kDa <sup>35</sup>S-labeled protein. Sizes were estimated based on Bio-Rad low-molecular-mass prestained protein standards.



```

                20                40                60
Lpn  MSLMEIIPSGRDVPNEVNIIEIIPPMHGEPVKYEVDKKTGALFVDRFMTTTAMFYPTNYGYIP
MSL:  :P:G:D:P  : : V:IEIP  : :P:KYE:DK::GALFVDRFM:TAMFYPTNYGYI
Ec    MSLLNVPAGKDLPEDIYVIEIPANADPIKYEIDKESGALFVDRFMSTAMFYPCNYGYIN

                80                100               120
Lpn  NTLSEDGDPVDVLVITPVPLISGAVISCRAVGMLKMTDESGVDAKILAVPTTKLSKMYQS
      :TLS DGDPVDVLV TP PL  G:VI CR VG:LKMTDE:G DAK::AVP :KLSK Y
Ec    HTLSLDGDPVDVLVPTPYPLQPGSVIRCRPVGVLKMTDEAGEDAKLVAVPHSKLSKEYDH

                140                160
Lpn  MQTYQDIPQHLLLSIEHFFKHYKDLEEGKWVKVEGVWGPDAAREEITSSINRYNHIKK
      : : D:P: L  I HFF:HYKDLE:GKWVKVEGW :AA: EI :S R
Ec    IKDVNDLPELLKAQIAHFFEHYKDLEKGKWVKVEGWENAEAAKAEIVASFERAKNK
    
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FIG. 8. Alignment of *L. pneumophila* (Lpn) PPase to the *E. coli* (Ec) homolog. Conservative substitutions are indicated by colons. The amino acid residues that have been shown to be crucial for structural and catalytic activity of the enzyme in *E. coli* (see Discussion) are indicated in bold.

bacterial proteins. Our data caution that extended use of this inhibitor to selectively radiolabel intracellular bacterial pathogens (3, 31) has profound effects on the host cell physiology, which may lead to changes in bacterial gene expression. It is recommended that to exclude any possible secondary effects on expression by intracellular bacteria, this inhibitor not to be used for more than 6 h.

Formation of the RER-surrounded replicative phagosome of *L. pneumophila* was not affected by inhibition of protein synthesis of the host cell. These data indicated that formation of the replicative vacuole is dictated at the biochemical level of signal transduction and that new host cell proteins are not required for this process. Bacterial replication within the RER-surrounded phagosome was also independent of the host cell protein synthesis. The data also excluded the possibility that degradation of newly synthesized proteins is required for bacterial replication.

Expansion of the phagosome was also independent of host cell protein synthesis. The data suggested that expansion of the membrane of the replicative phagosome was due to incorporation of host cell vesicles of presynthesized proteins or of bacterial components or both. The incorporation of bacterial components into the phagosomal membrane has been described previously. For example, protein antigens of the intracellular pathogens *Chlamydia trachomatis* and *Chlamydia psittaci* are incorporated into the phagosomal membrane (37, 44). Incorporation of bacterial proteins into the phagosomal

membrane by *L. pneumophila* has yet to be determined. Such incorporation may have significant effects on the biochemical interaction between *L. pneumophila* and the host cell.

The PPase of *L. pneumophila* was 80% similar to the *E. coli* PPase (EC 3.6.1.1). The “housekeeping” function of PPase may explain its highly conserved domains that are involved in catalytic and structural integrity. The PPase of *E. coli* has been extensively characterized at the biochemical, structural, and ultrastructural levels. Many residues of the PPase have been shown to be critical for activity and structural integrity of the enzyme. The *E. coli* PPase is composed of six identical subunits (47) arranged in an octahedral configuration. Residues that are involved in structural stability of the homohexamer (H136 and H140) (9, 45), in binding to the Mg<sup>2+</sup> cofactor (D65, D70, N24, D26, E98, Y141, and D102) (23, 28, 29, 38), and in catalytic activity of the enzyme (K29, R43, K142, D97, D102, Y55, and E20) (24, 28, 38) are all conserved in *L. pneumophila*. X-ray crystallography of the three-dimensional structure of the *E. coli* PPase clearly showed the presence of a group of functionally important residues that are conserved among all identified PPases (22). The consensus sequence is D-hyd-D-P-ali-D-ali (hyd is a small hydrophilic residue like S, G, or N; ali, an aliphatic hydrophobic residue like C, I, L, M, or V) (22). All of these residues are also conserved in the *L. pneumophila* PPase (D-G-D-P-V-D-V-L). These data clearly demonstrated that the 20-kDa MI protein of *L. pneumophila* is a PPase. These data further confirmed the highly conserved nature of this

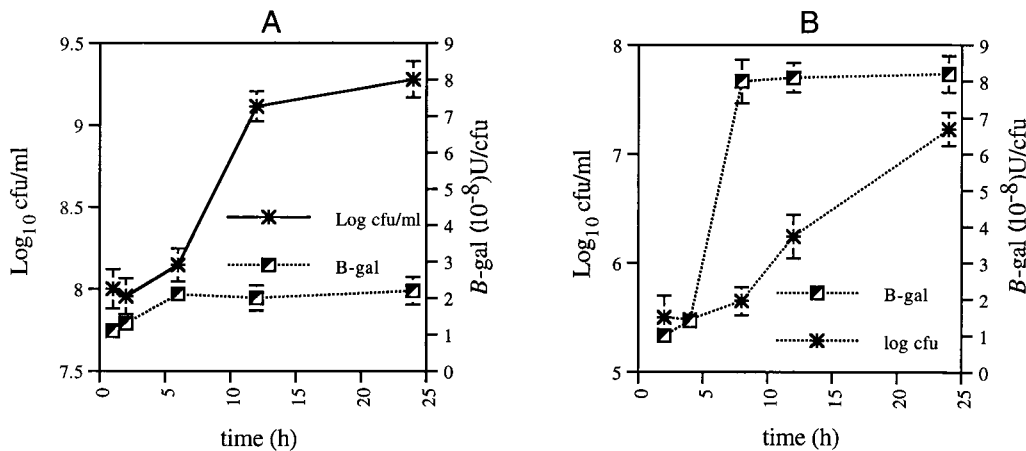


FIG. 9. Kinetics of β-Gal expression by in vitro-grown (A) and macrophage-grown (B) *L. pneumophila* harboring a *ppa* promoter (pPPZ) fused to the promoterless *lacZ* gene of pEU730. Data are expressed as the mean β-Gal activity for triplicate samples (34). *L. pneumophila* harboring the vector pEU730 did not show any detectable β-Gal activity (data not shown).



enzyme, which is underlined by its vital housekeeping functions in macromolecular biosynthesis.

The data suggested that the PPase may be essential for viability of *L. pneumophila*. PPase is essential for survival in *E. coli* as well as in yeast (12, 32). It catalyzes reversible transfer of the phosphoryl group from pyrophosphate to water, providing a thermodynamic pull for essential biosynthetic processes such as tRNA charging and DNA, RNA, protein, and polysaccharide biosynthesis (reviewed in reference 26). It may also have an important role in evolutionary events by affecting the accuracy by which DNA molecules are copied during chromosomal replication (30). Therefore, it may not be surprising that this enzyme is essential for viability (12, 32).

The predicted sequence of the *ppa* promoter had some similarity to  $\sigma^{70}$ -regulated promoters. Induction in transcription of *ppa* by intracellular *L. pneumophila* indicated the presence of other factors and/or other unidentified upstream sequences that are involved in transcriptional regulation of this gene. Mutational analysis of the upstream region may provide an explanation of the inducible nature of this gene and whether this induction is essential for survival of *L. pneumophila* within the intracellular environment.

Although the levels of PPase have been shown to be constitutive for *E. coli* (11), regulation in expression of none of the characterized PPase genes has been examined. *L. pneumophila ppa* is the first example of a regulated *ppa* gene during growth in a unique niche. Transcription of *L. pneumophila ppa* was specifically induced during intracellular multiplication but was not induced in response to in vitro stress stimuli. It is expected that actively growing organisms would require a higher rate of macromolecular biosynthesis including that of DNA, RNA, protein, and polysaccharides. Since pyrophosphate is a by-product of these reactions, higher levels of PPase activity would be required to provide the thermodynamic pull for these biosynthetic reactions. The short generation time of intracellular *L. pneumophila* compared to that of in vitro-grown bacteria in rich medium (21) indicates that intracellular *L. pneumophila* undergoes macromolecular biosynthesis at a higher rate than do in vitro-grown bacteria. These observations suggest that for replication of *L. pneumophila*, the microenvironment within macrophages is a more favorable niche than is rich medium. Whether enhanced macromolecular biosynthesis is the only factor required for induction of *ppa* transcription by intracellular bacteria or whether *ppa* induction is triggered by other signals to which intracellular *L. pneumophila* is exposed is still to be determined. Future studies to examine regulation of expression of *ppa* by other intracellular pathogens would shed light on whether induction of *ppa* in the intracellular environment is a general phenomenon among intracellular pathogens.

In summary, short-term radiolabeling of intracellular bacteria during inhibition of protein synthesis of the host cell does not have any detectable effect on the host cell biology, on the formation of the RER-surrounded replicative phagosome, or on replication of *L. pneumophila*. One of the MI proteins was identified as PPase, a protein highly conserved through evolution. The *ppa* gene of *L. pneumophila* is regulated intracellularly and is the first example of a regulated *ppa* gene. Regulation of *ppa* during intracellular infection may reflect enhanced capabilities of macromolecular biosynthesis by intracellular *L. pneumophila* compared to those of in vitro-grown bacteria.

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