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The *Legionella pneumophila* Major Secretory Protein, a Protease, Is Not Required for Intracellular Growth or Cell Killing

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The *Legionella pneumophila* major secretory protein (Msp) is a Zn^{2+} metalloprotease whose function in pathogenesis is unknown. The structural gene for the Msp protease, *mspA*, was isolated from an *L. pneumophila* genomic library. In *Escherichia coli* which contain plasmids with the *mspA* gene, Msp protein and activity are found in the periplasmic space and the cytoplasm. Transposon mutagenesis with Tn*9* of an *mspA*-containing plasmid in *E. coli* yielded mutants which no longer expressed protease activity and others with increased protease activity. These results suggested that *mspA* expression might be regulated. Msp was shown to be produced at a much higher level in *L. pneumophila* grown in rich compared to semidefined media. A Tn*9* insertion which abolishes Msp expression was introduced into the *L. pneumophila* genome. This *mspA*::Tn*9* *L. pneumophila* strain showed no detectable production of Msp by immunoblot analysis, and it had less than 0.1% of the protease activity found in the wild-type strain. This mutant was fully capable of growing within and killing human macrophages derived from the HL-60 cell line.

Legionella pneumophila, the causative agent of Legionnaires disease, is a gram-negative bacterium which grows within and kills human macrophages (18). The major secretory protein (Msp) of *L. pneumophila* is the most abundant protein found in culture supernatants of *L. pneumophila*. It is a 38-kilodalton Zn^{2+} metalloprotease with activity on a variety of substrates including casein (9). Several lines of evidence suggest that Msp may be an important virulence determinant in the pathogenesis of Legionnaires disease. It has been reported that treatment of guinea pig lungs with Msp causes lung lesions morphologically indistinguishable from those seen in the lungs of Legionnaires disease victims (7). Although infection with *L. pneumophila* stimulates both humoral and cellular immunity, it is the cell-mediated responses which play the dominant role in countering disease. It has been determined that vaccination with purified Msp induces protective cell-mediated immunity (3). In addition, Msp is a dominant antigen recognized by guinea pig T cells following either sublethal infection with virulent *L. pneumophila* (3, 4) or vaccination with an avirulent mutant (S. Blander and M. A. Horwitz, personal communication). Recently, a gene encoding a 38-kilodalton metalloprotease similar to Msp was isolated from a genomic library of *L. pneumophila* in *Escherichia coli* (30). *E. coli* containing this plasmid appear to express proteolytic, cytopathic, and hemolytic activities (30). In another study, a mutant strain of *L. pneumophila* which exhibited decreased caseinolytic, cytopathic, and hemolytic activities was isolated (19). The ability of this mutant to grow within or kill macrophages was not determined.

In this study, the gene for Msp was isolated from an *L. pneumophila* genomic library in *E. coli*. The expression of the *mspA* gene in *E. coli* was examined, and the cellular location of its gene product was determined. The gene and surrounding sequences were disrupted by transposon mutagenesis. Mutations either abolishing or increasing Msp expression were introduced into the *L. pneumophila* genome, and the phenotype conferred by the regulatory insertions was determined. In the *mspA*::Tn*9* *L. pneumophila*, residual activities not due to Msp, such as proteolysis and cytopathic effect, were measured. Finally, the ability of *mspA*::Tn*9* *L. pneumophila* to grow in human macrophages was determined.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. All strains and plasmids and the bacteriophage used in these studies are described in Table 1. High-level expression of Msp in *E. coli* from pLS2 resulted in an unhealthy phenotype. In order to decrease the copy number of pLS2, we constructed a host strain containing a *pcnB* mutation. This mutation decreases the copy number of a variety of plasmids. Expression of Msp by pLS2 in a *pcnB* host did not result in any detectable adverse effect. LS1443 was constructed by selecting a tetracycline-resistant derivative of MC1061 following P1 transduction with a lysate grown on a strain with *pcnB* linked to Tn*10*.

Media. All *E. coli* bacterial strains were grown in Luria-Bertani (LB) liquid and solid media (23). Where indicated, drugs were included at the following concentrations: tetracycline (20 μg/ml), carbenicillin (100 μg/ml), chloramphenicol (25 μg/ml), and kanamycin (50 μg/ml). All *L. pneumophila* bacterial strains were grown in albumin yeast extract (AYE) liquid medium or on buffered charcoal-yeast extract (BCYE) solid medium (18). When indicated, the semidefined medium used was Casamino Acids (CAA) (Difco Laboratories) medium (26). The solid medium for detecting protease activity was similar to BCYE, but the charcoal was replaced with 5 g of corn starch per liter, buffered starch-yeast extract (BSYE), and 10 g of casein per liter. Where indicated, drugs were included at the following concentrations: chloramphenicol (5 μg/ml) and streptomycin (50 μg/ml). All other drugs were used at the same concentrations indicated for *E. coli*.

Construction of a *L. pneumophila* genomic library. Genomic DNA was isolated from hen egg-cultivated *L. pneumophila* that had been passaged one time on BCYE medium. A genomic library of strain Philadelphia-1 was constructed in the cosmid vector pLAFR1, as described previously (14).

TABLE 1. Strains, plasmids, and bacteriophage used in this study

Strain, plasmid, or bacteriophage	Genotype	Reference or source
Escherichia coli		
HB101	F⁻ *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Sm^r) *xyl-5 mtl-1 supE44 leuB6*	23
MC1061	Δ(*lacIPOZYA*)*X74 galKU strA araD139* Δ(*araOIBA leu*)*7697 hsdR*	31
LS1443	MC106 *pcnB*	(22, this study)
SM10(λpir)	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu	33
CC118	*araD139* Δ(*ara leu*)*7697* Δ(*lac*)*X74 phoA galEK thi rpsE*(Spec^r) *rpoB* (Rif^r) *argE*(Am) *recA1*	C. Lee via C. Manoil
Legionella pneumophila		
LS2029	Philadelphia-1 Sm^r Rif^r *lpnR*	24
LS2040	Philadelphia-1 Sm^r Rif^r *lpnR* avirulent	17
LS2102	LS2029 *mspA1*::Tn*9*	This study
Plasmid		
pLAFR1	IncP *oriT cos tet*(Tc^r)	14
pLS1	pLAFR1 *mspA*	This study
pLS10	pLAFR1 *mspA1*::Tn*9*	This study
pLTJS53	ColE1 *bla*(Cb^r) *oriT*	29
pLS2	pTJS53 *mspA*	This study
pJM703.1	R6K *oriV oriT bla*(Cb^r)	33
pGP81	R6K *oriV oriT bla*(Cb^r) *rpsL*$^+$(Sm^s)	G. Pearson
pLS12	pJM703.1::Tn*9*	This study
pLS11	pGP81 *mspA1*::Tn*9*	This study
pMMB33	IncQ *oriT, cos neo*(Km^r)	13
pLS13	pMMB33 *mspA*	This study
Bacteriophage		
λ577	*c*I857 *O*am29 Tn*9* *b2 b522*	J. Way via S. Brown

Southern blot hybridization. Probe DNA was purified according to the protocol of the manufacturer on pZ523 columns (5 Prime→3 Prime, Inc.). DNA was radioactively labeled with [^{32}P]dCTP by the random primer extension technique (10). Southern blotting was performed as described previously by using Zetaprobe nylon filters (23).

Cell fractionation. *E. coli* cultures were grown overnight in LB plus tetracycline or LB plus carbenicillin for strains containing pLAFR1 or pTJS53 plasmid derivatives, respectively. To prepare samples of extracellular media, cultures were centrifuged and the supernatants were filtered through 0.2-μm-pore-size filter membranes to remove all bacteria. The protein in the supernatant was purified and concentrated by binding to DE52 cellulose (10 g/liter) for 1.5 h at 37°C. The DE52 cellulose and bound material were separated by centrifugation for 10 min at 6,000 rpm in the SS-34 rotor in the Sorvall RC-5B centrifuge and washed once with 50 mM Tris hydrochloride, pH 7.5, containing 10 mM EDTA. The proteins were eluted in 0.5 M NaCl–50 mM Tris hydrochloride, pH 7.5, containing 10 mM EDTA. To prepare periplasmic fractions, the bacterial pellet was washed and the cells were converted to spheroplasts, as described previously (36). Cells were suspended in 100 mM Tris hydrochloride (pH 7.5)–250 mM sucrose–10 mM EDTA–50 μg of lysozyme per ml for 30 min. To remove any DNA from cells that had lysed prematurely, the spheroplasts were brought to 10 mM $MgSO_4$ and DNase I was added to 25 μg/ml for 10 min on ice. The spheroplasts were separated from the periplasmic proteins by centrifugation for 10 min at 6,000 rpm. The supernatant was then filtered through a 0.2-μm-pore-size filter membrane. To prepare cytoplasmic extracts, the spheroplasts were suspended in 50 mM Tris hydrochloride (pH 7.5)–5 mM EDTA and diluted 10-fold in distilled H_2O. Lysis was completed by sonication three times for 20 s each time on ice with the medium probe of a W-220 Heat Systems ultrasonicator at maximun output. Unbroken cells were removed by centrifugation for 10 min at 6,000 rpm. Cytoplasmic proteins were separated from membrane components by ultracentrifugation at 50,000 rpm for 1 h at 5°C in a Ti65 rotor. The supernatant was removed, kept overnight on ice, and recentrifuged. The membranes were washed with 50 mM Tris hydrochloride, pH 7.5, containing 10 mM $MgSO_4$ and suspended in 1/100 the original culture volume. The extracellular, periplasmic, and cytoplasmic fractions were further concentrated by an Amicon microconcentrator (10-kilodalton cutoff size) to a final volume approximately 1/100 the original culture volume. All fractions were stored at −20°C.

To prepare cell fractions from *L. pneumophila* strains, cultures for all extracts were grown in AYE medium without bovine serum albumin. Fractions were prepared as described above for *E. coli*, by substituting 100 μg of lysozyme per ml to prepare spheroplasts. To prepare unpurified extracellular extracts, cultures were centrifuged and the supernatants were filtered through 0.2-μm-pore-size filter membranes to remove all bacteria. The proteins were concentrated approximately 20-fold by using the Amicon microconcentrator.

Immune detection of Msp in cell fractions. Samples of the cell fractions were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein bands were transferred to nitrocellulose by electroblotting for 1 h at 200 mA. Immunoblotting was performed as described previously (16). Rabbit anti-Msp antibodies were generously provided by Marcus A. Horwitz at the UCLA School of Medicine, Los Angeles, Calif. Msp expressed in *E. coli* was detected with rabbit anti-Msp antibodies adsorbed with *E. coli* to remove contaminating antibodies. Msp from *L. pneumophila*

was visualized by using affinity-purified (25) rabbit anti-Msp antibodies. In all cases, bound antibody was visualized with sheep anti-rabbit immunoglobulin labeled with alkaline phosphatase. The alkaline phosphatase activity was detected with indoxyl phosphate and Nitro Blue Tetrazolium under conditions specified by the supplier (Sigma Chemical Co., St. Louis, Mo.).

Protease assays. The proteolytic activities of *L. pneumophila* and *E. coli* bacterial colonies were evaluated on BSYE plates containing casein. Proteolytic activities in cellular extracts were assayed by using casein-fluorescein isothiocyanate (FITC-casein) by a modified procedure (35). Immediately prior to the assay, the extracts were drop dialyzed against 50 mM Tris hydrochloride (pH 7.0)–1 mM $ZnCl_2$ for 1 h on ice. The assay reaction contained 20 μl of 50 mM Tris hydrochloride (pH 7.0), 20 μl of 1% (wt/vol) FITC-casein in 50 mM Tris hydrochloride (pH 7.0), and 10 μl of cell extract. Reactions were incubated at 37°C. The reaction was stopped, and the cleaved FITC was quantitated by adding 120 μl of 5% trichloroacetic acid and incubating the mixture for 1 h at room temperature. The mixture was centrifuged at 14,000 rpm for 10 min in a Microfuge (Brinkmann Instruments, Inc.), and 60 μl of supernatant was added to 240 μl of 0.5 M Tris hydrochloride, pH 8.5, in a microtiter well. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm on a Titertek Fluoroskan II microplate fluorescence reader. The assay is linear up to 500 fluorescence units/h.

RESULTS

Isolation of the structural gene for *L. pneumophila* Msp protease. *L. pneumophila* hydrolyzed casein in solid BCYE medium and made visible rings showing proteolysis in 3 days, the same amount of time required to make a visible colony. Colonies from a genomic library of *L. pneumophila* Philadelphia-1 in the cosmid vector pLAFR1 in *E. coli* were screened for the ability to make rings showing proteolysis on casein medium. Four of these colonies were purified, and the plasmid (pLS1) from one of these clones was used in these studies.

In order to further localize the *mspA* gene on this large fragment of DNA, a 6-kilobase (kb) *Eco*RV fragment containing DNA from pLAFR1 and one end of the inserted *L. pneumophila* genomic sequences from pLS1 was ligated into the *Sma*I site of the high-copy-number plasmid pTJS53. The insert in this plasmid, pLS2, was sufficient to confer caseinolytic activity to *E. coli*. pLS2 was maintained in *E. coli* LS1443 *pcnB* because expression of the high-copy-number wild type was lethal.

To be certain that the *mspA* gene originated from *L. pneumophila*, digests of *L. pneumophila* and *E. coli* genomic DNAs as well as pLS1 plasmid DNA were probed with radioactively labeled pLS2. The probe did not hybridize with any *E. coli* fragments but did hybridize with fragments from *L. pneumophila* and pLS1, indicating that the cloned gene originates from *L. pneumophila* (data not shown).

Cellular localization of Msp expressed in *E. coli*. Because HB101(pLS1) made rings of casein hydrolysis in 7 to 10 days, it was determined whether the cause for this delay was due to improper cellular localization of Msp in *E. coli*. Cells were fractionated into extracellular, periplasmic, cytoplasmic, and membrane components. Immunoblot analysis of fractions from HB101(pLS1) and LS1443(pLS2) by using anti-Msp antibodies confirmed that Msp could be found predominantly in the periplasm and cytoplasm (Fig. 1). Lesser amounts were detectable in the extracellular compartment.

The proteolytic activity of the cellular fractions was measured by using FITC-casein as a substrate. Table 2 shows that the periplasmic fractions from HB101(pLS1) and LS1443(pLS2) had the highest specific activities. The specific activities for the cytoplasmic fractions were approximately half those of the corresponding periplasmic extracts. Neither the extracellular nor membrane proteins showed significant caseinolytic activity. While there was a small amount of proteolysis due to endogenous *E. coli* periplasmic and cytoplasmic proteins, the specific activity attributable to these proteases did not exceed 16% that attributable to Msp.

Transposon mutagenesis of pLS1. Random mutagenesis of the inserted genomic fragment in pLS1 was carried out in order to (i) further localize the *mspA* gene, (ii) locate other genes which might affect the regulation or localization of Msp, and (iii) generate mutations which can be used to interrupt *mspA* on the *L. pneumophila* genome. Transposon Tn*9* was introduced into *E. coli* CC118 containing pLS1 by using λ577 as described previously (32). Three classes of insertions resulted, those which continued to express normal amounts of Msp, those which no longer expressed detectable Msp (pLS10), and those which expressed more Msp than the clone from which the mutants were derived. All insertions which resulted in loss of Msp expression were located in either the 1.1- or 4.3-kb *Eco*RI fragments of pLS1. All of the insertions which resulted in increased expression of Msp were located in the 4.3-kb *Eco*RI fragment. Two of these were determined to be at close but distinct positions 800 to 1,000 base pairs to the right of the central *Eco*RI site shown in Fig. 3B.

The higher levels of Msp in some of the Tn*9* insertions suggested that *mspA* expression might be regulated in *E. coli* and/or in *L. pneumophila*. We evaluated the level of Msp expression in *L. pneumophila* grown in rich (AYE) or semidefined (CAA) liquid medium. Figure 2 shows that wild-type *L. pneumophila* LS2029 grown in rich medium expresses Msp (and some other proteins) at a much higher level than bacteria grown in semidefined CAA medium. The expression of Msp was not regulated in *E. coli* (data not shown). To determine the phenotype that the Tn*9* insertions which increase expression of Msp in *E. coli* would have in an *L. pneumophila* background, the mutations were transferred to the *L. pneumophila* genome by allelic exchange (as described below). There was no detectable difference from wild type in the expression of Msp in these mutants grown in either AYE or CAA medium (Fig. 2). An explanation for these results is that the Tn*9* insertions may be upstream of *mspA* and an IS1 promoter is leading to increased expression in *E. coli* (note that at the present time we do not know the direction of *mspA* transcription). In *L. pneumophila*, however, the expression from the *mspA* promoter is much more efficient and the effects of the IS1 promoter are masked.

Construction of an *mspA*::Tn*9* *L. pneumophila*. To determine the role that Msp plays in the ability of *L. pneumophila* to grow in and kill phagocytic cells, a mutant was constructed, thus abolishing Msp activity. The *mspA*::Tn*9* insertion present on pLS10 was transferred on a 6-kb *Eco*RV fragment to plasmid pGP81 (pLS11). This plasmid cannot replicate in *L. pneumophila* and therefore integrates into the *L. pneumophila* chromosome by homologous recombination at the *msp* locus, (data not shown). The insertion was transferred to the *L. pneumophila* genome by allelic exchange, as described previously (LS2102) (6). To confirm that Tn*9* was in the expected location and that pLS11 was no

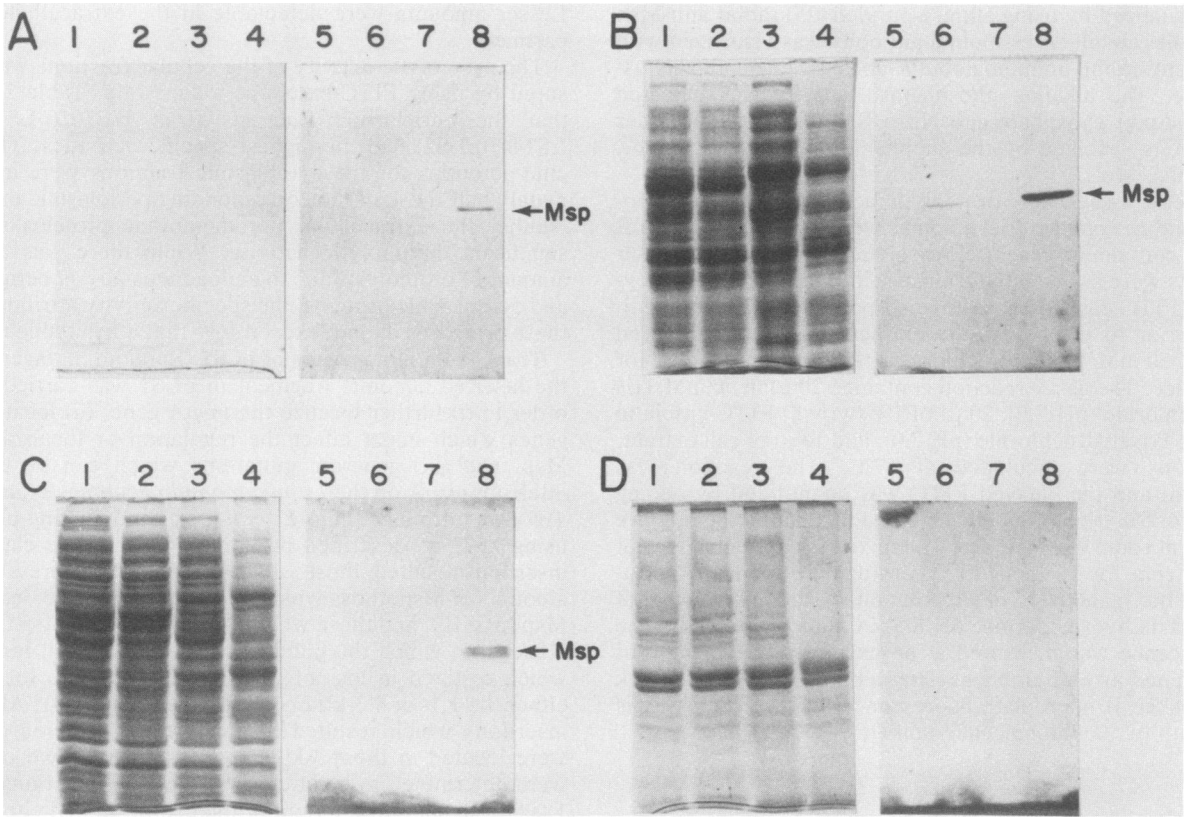


FIG. 1. Immunoblot analysis of Msp expressed in *E. coli*. Cellular extracts were separated by polyacrylamide gel electrophoresis on 12% gels. Proteins were visualized by Coomassie blue staining. Msp was visualized by immunoblot analysis. Proteins were transferred to nitrocellulose filters by electrotransfer and detected with anti-Msp antibodies. (A) Extracellular proteins; (B) periplasmic proteins; (C) cytoplasmic proteins; (D) membrane proteins. Lanes: 1 to 4, Coomassie blue-stained gels; 5 to 8, immunoblots; 1 and 5, HB101(pLAFR1); 2 and 6, HB101(pLS1); 3 and 7, LS1443(pTJS53); 4 and 8, LS1443(pLS2).

longer present, Southern hybridization analysis of LS2102 genomic DNA was performed. Genomic DNA was cleaved with *Eco*RI and hybridized with three probes: pJM703.1, pJM703.1::Tn*9*, and pLS2. When pJM703.1 (which contains the same backbone as pGP81) was used as a probe to detect plasmid sequences, no fragments hybridized, confirming that the plasmid sequences were no longer present. Two fragments (C and D) hybridized with the pJM703.1::Tn*9* probe as expected (Tn*9* contains one *Eco*RI site), indicating that there was only one transposon insertion site in the genome of the mutant. Two fragments (A and D) were visible with the pLS2 probe. Three fragments were expected, but because the location of Tn*9* in the 4.3-kb *Eco*RI fragment is so close to one end of the fragment (C), there is not enough homologous DNA remaining in this new *Eco*RI band to detect hybridization with the pLS2 probe. The nucleotide sequence of this

TABLE 2. Specific activity of fractions by using the FITC-casein assay

Strain	Sp act of fraction[a]				
	Extracellular		Periplasm	Cytoplasm	Membranes
	Amicon	DE52			
HB101(pLAFR1)		ND	7.4	2.4	0.4
HB101(pLS1)		2.0	54.5	22.1	0.3
LS1443(pTJS53)		ND	2.0	1.4	ND
LS1443(pLS2)		21.5	2,607.6	1,614.8	3.5
LS2029	7,918.3	841.7	4.6	0.3	ND
LS2102	16.9	0.8	ND	0.2	ND

[a] Sp act (specific activity) = fluorescence units per minute per milligram of total protein; ND, not detectable. Fractions are described in Materials and Methods.

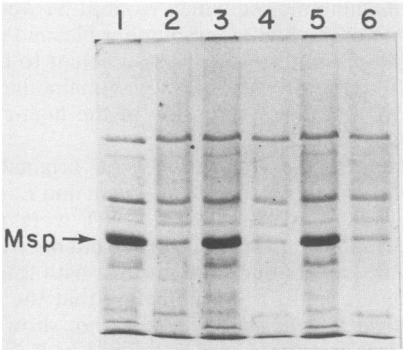


FIG. 2. Extracellular proteins from wild-type and potential regulatory mutants of *L. pneumophila*. Strains were grown in rich AYE and semidefined CAA media, and extracellular proteins were concentrated by precipitation with 5% trichloroacetic acid. The proteins were separated by polyacrylamide gel electrophoresis on a 10% acrylamide gel and stained with Coomassie blue. Lanes: 1 and 2, LS2029; 3 to 6, potential regulatory mutants; 1, 3, and 5, proteins from bacteria grown in AYE medium; 2, 4, and 6, proteins from bacteria grown in CAA medium.

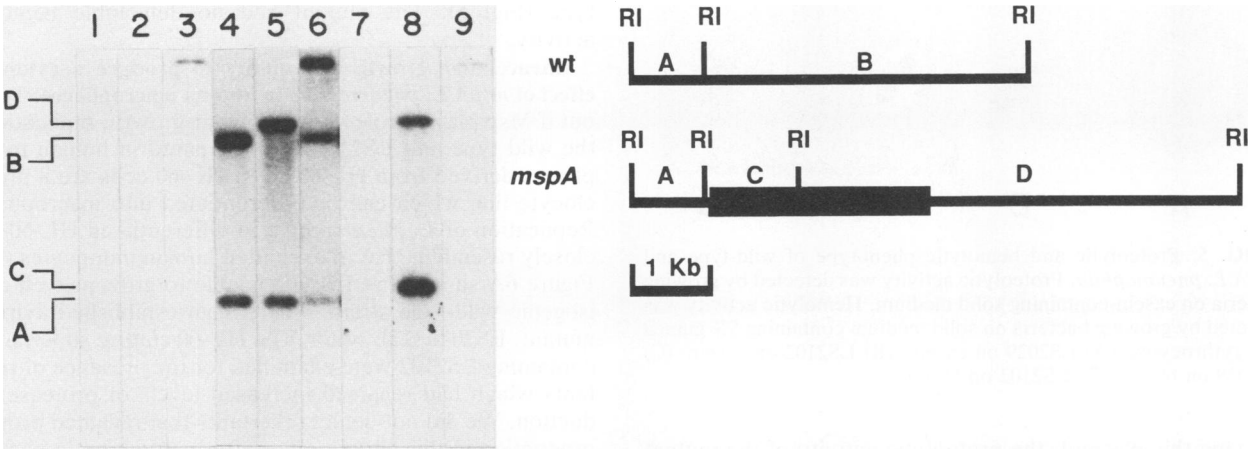


FIG. 3. Southern analysis of genomic DNA from wild-type and *mspA L. pneumophila*. (Left) Genomic and plasmid DNAs were digested to completion with *Eco*RI, and fragments were separated on a 0.8% agarose gel. Fragments were transferred to nylon filters and hybridized with linearized, radiolabeled probe DNA. Lanes: 1 to 3, probed with pJM703.1; 4 to 6, probed with pLS2; 7 to 9, probed with pJM703.1::Tn*9*; 1, 4, and 7, LS2029 genomic DNA; 2, 5, and 8, LS2102 genomic DNA; 3, 6, and 9, pLS1 DNA. (Right) Diagram of *Eco*RI restriction fragments from wild-type (wt) and *mspA*::Tn*9* genomic DNAs. Thin lines represent genomic DNA, and the thick line represents Tn*9*. A, B, C, and D refer to restriction fragments.

region indicates that the site of insertion is 131 base pairs from the *Eco*RI site (data not shown). The new fragment (C) is present, however, and does hybridize as expected to the pJM703.1::Tn*9* probe. The 4.3-kb *Eco*RI fragment (B) hybridizing with pLS2 in wild-type *L. pneumophila* is not present in LS2102, confirming that the insertion was transferred to the expected location (Fig. 3).

Immunoblot analysis of *mspA L. pneumophila*. To find out if Msp protein or truncated fragments of Msp were produced in LS2102, extracellular, periplasmic, cytoplasmic, and membrane fractions were prepared and analyzed by immunoblotting with affinity-purified anti-Msp antibodies. The color reaction was intentionally overexposed to maximize detection of low-abundance cross-reacting proteins. Although no full-size Msp was found in LS2102, two new, smaller cross-reacting fragments appeared in the extracellular compartment (Fig. 4). These smaller proteins may either be truncated forms of Msp or crossreactive proteins normally undetectable when Msp is present.

Proteolytic phenotype of *mspA L. pneumophila*. The availability of LS2102 made it possible to evaluate which extracellular activities were attributable to Msp and which were due to other proteins. LS2102 and LS2029 were grown on casein plates to determine whether caseinolytic proteases other than Msp were present in *L. pneumophila*. As can be seen in Fig. 5A and B, LS2102 is still able to hydrolyze casein, although much less than LS2029. The amount of residual caseinolytic activity was determined quantitatively by using the FITC-casein assay. Table 2 shows that in wild-type *L. pneumophila*, 99% of all Msp activity is found in the extracellular compartment. In addition, the specific activity of the extracellular proteins for the *mspA* mutant is only 0.2% that of the wild type for Amicon microconcentrator extracts and 0.1% that of the wild type for DE52 concentrated extracts. Finally, caseinolytic activity is not detectable in any other cellular compartment of the mutant.

To confirm that the phenotype observed in the *mspA* mutant was due to the Tn*9* insertion, the defect was complemented *in trans* by the *mspA* (1.1- and 4.3-kb *Eco*RI fragments) gene carried on pMMB33 (pLS13), an IncQ plasmid which can replicate in *L. pneumophila* (24). When

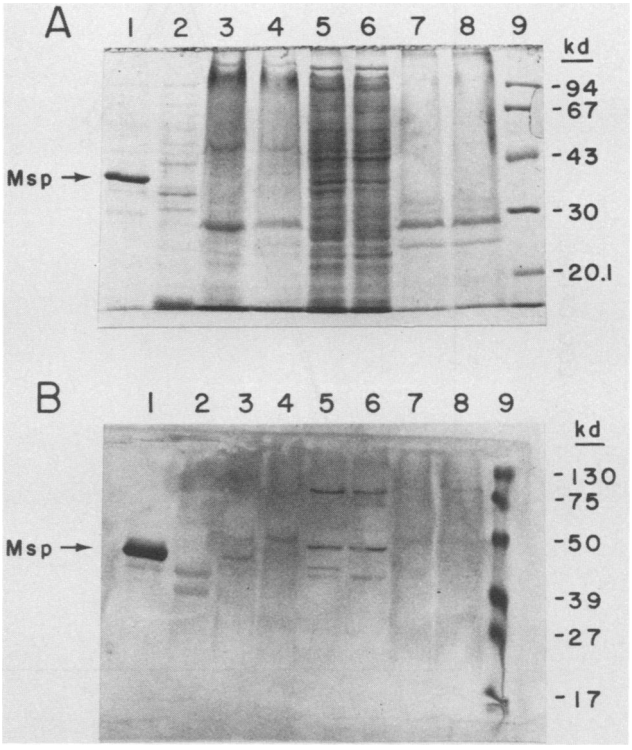


FIG. 4. Immunoblot analysis of Msp expressed from wild-type and *mspA L. pneumophila*. Cellular extracts were separated by polyacrylamide gel electrophoresis on 12% gels. Proteins were visualized by Coomassie blue staining. Msp was visualized by immunoblot analysis. Proteins were transferred to nitrocellulose filters by electrotransfer and hybridized with anti-Msp antibodies. (A) Coomassie blue-stained gel; (B) immunoblot. Lanes: 1 and 2, extracellular proteins; 3 and 4, periplasmic proteins; 5 and 6, cytoplasmic proteins; 7 and 8, membrane proteins; 9, molecular weight markers; 1, 3, 5, 7, LS2029; 2, 4, 6, and 8, LS2102. kd, Kilodaltons.

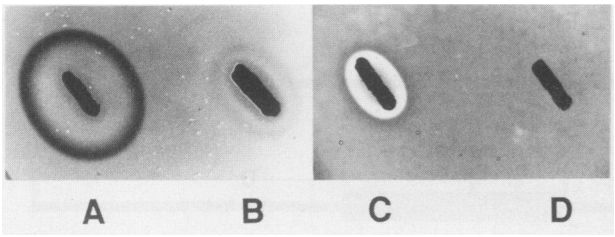
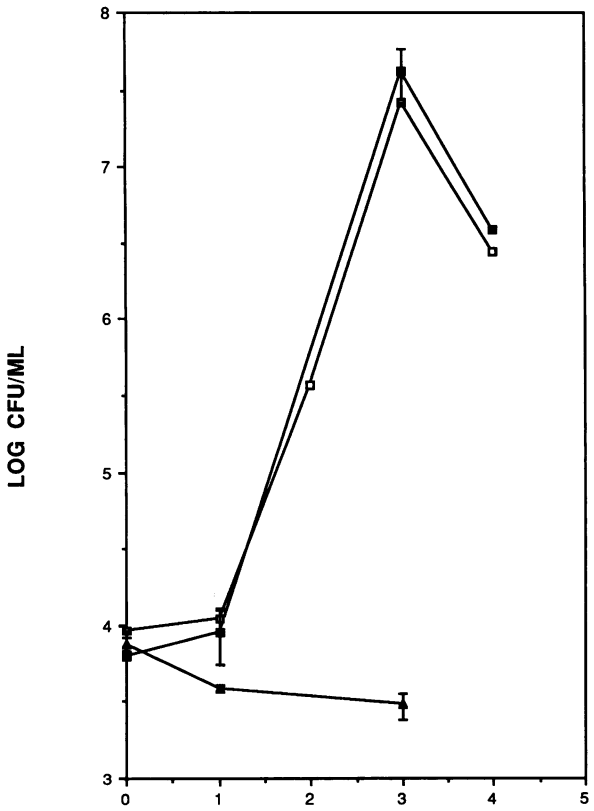


FIG. 5. Proteolytic and hemolytic phenotype of wild-type and *mspA L. pneumophila*. Proteolytic activity was detected by growing bacteria on casein-containing solid medium. Hemolytic activity was detected by growing bacteria on solid medium containing 5% guinea pig erythrocytes. (A) LS2029 on casein; (B) LS2102 on casein; (C) LS2029 on blood; (D) LS2102 on blood.

carrying this plasmid, the proteolytic activity of the mutant was indistinguishable from that of the wild type (data not shown). Because Msp has been reported to have hemolytic activity on guinea pig erythrocytes, the hemolytic phenotype of *mspA L. pneumophila* was compared with that of the wild type (Fig. 5). The mutant had no detectable hemolytic activity.

Intracellular growth and ability to produce a cytopathic effect of *mspA L. pneumophila* in human macrophages. To find out if Msp plays a role in intracellular growth, replication of the wild type and LS2102 was compared in human macrophages derived from HL-60 cells. HL-60 cells are a promyelocyte line which can be differentiated into macrophages. Replication of *L. pneumophila* in differentiated HL-60 cells closely resembles that in explanted human monocytes (23a). Figure 6A shows that LS2102 is able to grow as well as an isogenic wild-type strain, whereas an established avirulent mutant, LS2040 (17), is not. The HL-60 culture supernatants containing LS2102 were examined for the presence of revertants which had regained increased levels of protease production. We did not detect revertants that regained protease production in this situation nor after cultivation in bacterial culture media.

The ability of strains LS2029 and LS2102 to kill macrophages was also compared. Different numbers of bacteria were used to infect wells containing phorbol myristate



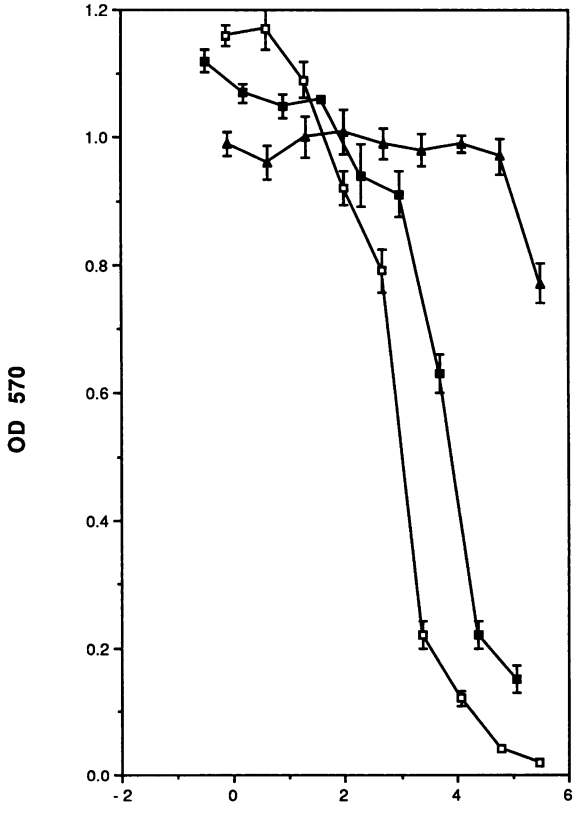


FIG. 6. Intracellular growth and cytopathic effect of wild-type and *mspA L. pneumophila* strains in human macrophages. (Left) HL-60 cells were differentiated in 24-well plates (1.5×10^6 per well in RPMI 1640 supplemented with 1% L-glutamine–20% normal human serum–10 ng of phorbol myristate acetate per ml for 24 h. After washing with RPMI 1640 and replacement of medium lacking phorbol myristate acetate, bacteria were added to the wells. The titer of bacteria in the wells was determined over a 4-day period by plating dilutions of the culture supernatants on BCYE plates. Each point is the average of two different wells. Error bars indicate the standard errors of the means. (Right) Increasing numbers of bacteria were added to human macrophages derived from 4×10^5 differentiated HL-60 cells in 96-well microtiter wells. After 5 days, the number of viable macrophages remaining was determined by measuring the reduction of MTT to a blue formazan by the living adherent macrophages. Symbols: ■, LS2029; □, LS2102; ▲, LS2040. OD 570, optical density at 570 nm.

acetate-differentiated HL-60 cells. The number of surviving macrophages was determined by using the vital stain MTT (23a, 28) (Fig. 6B). The mutant strain was able to kill these cells at least as well as wild-type cells.

DISCUSSION

Extracellular proteases are produced by many bacteria. The roles of some of these enzymes in pathogens have been examined. *Pseudomonas aeruginosa* alkaline protease and elastase have been demonstrated to inhibit neutrophil chemotaxis, oxidative metabolism, and phagocytosis (20, 21). Along with Msp, these proteases have been suggested to be an important component in the pathogenesis of pulmonary lesions (7, 15). Type II protease from *Vibrio cholerae* activates cholera toxin as well as proteolyzing mucus and immunoglobulins (37). *Shigella flexneri* contains a plasmid-encoded hemolytic protease, the presence of which has been correlated with the induction of phagocytosis of macrophages and escape from the phagosomal vacuole (5). Because of these results, it was not unreasonable to suspect that Msp was also involved in pathogenesis. Mutants in *P. aeruginosa* which no longer express elastase are less virulent than the wild type (2); however, mutants in the *Neisseria gonorrhea* immunoglobulin A protease are still able to attach to and invade fallopian tube mucosa as efficiently as the wild type (8). These examples and the results of this study indicate the utility of defined mutants in evaluating the contribution of specific proteins to virulence.

To determine if Msp is required for infection of its host, intracellular growth of the wild type and an *mspA*::Tn*9* mutant were compared by using a macrophage-like cell line. In addition, the abilities of the two strains to exert a cytopathic effect were compared. When differentiated into macrophages, HL-60 cells have been demonstrated to be an excellent tissue culture model for the interaction of *L. pneumophila* with human monocytes (A. Marra et al., in press). The *mspA*::Tn*9* *L. pneumophila* strain was just as capable of intracellular growth as the wild type. This shows that a 1,000-fold decrease in Msp activity does not interfere with successful replication in macrophages. In addition, it strongly suggests that Msp may play no role in this process because the number of progeny released during the course of the infection is just as high as for the wild type. These results alone do not preclude a role for Msp in pathogenesis. To determine if Msp has a role in causing Legionnaires disease, the ability of *mspA*::Tn*9* *L. pneumophila* to cause Legionnaires disease in a guinea pig animal model was determined. Results show that these mutants are just as capable of causing lethal pneumonia as the wild type (S. Blander, L. Szeto, H. A. Shuman, and M. A. Horwitz, J. Clin. Invest., in press). It has been suggested recently that the presence of the *L. pneumophila mspA*$^+$ gene confers increased cytotoxic activity to *E. coli* (30). These cytotoxicity assays were performed by using CHO cells, which are not a physiologically relevant target in Legionnaires disease. The results of the MTT assay for cytopathic effects in HL-60 cells clearly demonstrate that a 1,000-fold decrease in caseinolytic activity does not decrease the ability of *L. pneumophila* to kill phagocytic cells. There must be other *L. pneumophila* components responsible for ability to produce a cytopathic effect.

Although it has been clearly demonstrated in this study that Msp is not required for the ability of *L. pneumophila* to grow in or kill macrophages, it is possible that Msp is necessary for growth in the environment. *L. pneumophila* is found in fresh water associated with blue-green algae (cyanobacteria) (34). In addition, *L. pneumophila* can grow intracellularly in single-celled protozoa such as *Acanthamoebae*, *Naegleria*, *Tetrahymena*, and *Hartmanella* species in the laboratory (1, 11, 12, 27). Preliminary results to determine whether Msp may play some role in exploiting these host organisms indicate that the *mspA*::Tn*9* *L. pneumophila* mutant is as capable of intracellular growth in *Hartmannella vermiformis* and *Tetrahymena pyriformis* as the wild type (B. Fields, personal communication).

There are two explanations for the residual caseinolytic activity of the *mspA*::Tn*9* *L. pneumophila* mutant. Either the residual activity is due to another protease or the activity is due to a small amount of truncated Msp. We feel the latter is unlikely because five other *mspA*::Tn*9* *L. pneumophila* mutants with insertions more than 300 base pairs away from that of the mutant described in this study also had the same level of residual caseinolytic activity. If another protease is responsible and proteolytic activity is required for virulence, this other protease may suffice in the absence of Msp. Isolation of derivatives of LS2029 which no longer have any caseinolytic activity would address this point.

It has been shown in this study that the levels of Msp and other proteins are controlled by components of the growth medium. It remains to be determined whether the level of Msp expression depends on amino acids in the semidefined medium or complex proteins in the rich medium. Alternatively, other components of the medium could regulate Msp expression. Identification of this factor could help to elucidate the role of this abundant secreted protein.

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