RND proteins (resistance, nodulation, and cell division proteins) are a family of multidrug resistance pumps that recognize and mediate the transport of a great diversity of cationic, anionic, or neutral compounds, including various drugs, heavy metals, aliphatic and aromatic solvents, bile salts, fatty acids, detergents, and dyes (30, 33). They mediate the transport of these substances across the cytoplasmic membrane driven by the proton motive force of the transmembrane electrochemical proton gradient (16, 53). RND pumps are large proteins ranging from 800 to 1,100 amino acids that are characterized by the presence of 12 transmembrane domains (TMD) and 2 extracytoplasmic loops of approximately 300 amino acids located between the first and second TMD and the seventh and eighth TMD (16, 40). Both the N-terminal and C-terminal halves share sequence similarities, suggesting that these proteins may have evolved by gene duplication (39). Both halves have been shown to be required for transport activity (12). The substrate specificity of the RND proteins has been found to be dictated by the large periplasmic loops (13, 23, 50), while transmembrane domains 4 and 10 present amino acid residues that are algal in time-to-death studies. These studies support the concept that MmpL-mediated lipid secretion both contributes to the innate ability of the pathogen to survive intracellularly and also contributes directly to the host-pathogen dialogue that determines the ultimate outcome of infection.

The genome sequence of Mycobacterium tuberculosis revealed the presence of 12 membrane proteins proposed to have a function in the transport of lipids. Insertional inactivation of 11 of these has revealed that only 1 (MmpL3) is apparently essential for viability. Five of these proteins are conserved within the genome of Mycobacterium leprae. The drug susceptibilities of these 11 mutants to a broad spectrum of agents are unaltered, suggesting that unlike their function in other organisms, these proteins do not play a significant role in intrinsic drug resistance. Each of these mutants was assessed for growth kinetics and lethality in a murine low-dose aerosol model of tuberculosis, and four were found to be impaired in one or both measures of virulence. Two of these, with mutations of MmpL4 and the previously characterized MmpL7, which transports phthiocerol dimylocerosate, were found to have both impaired growth kinetics and impaired lethality. Mutants with inactivation of MmpL8, which transports a precursor of the sulfatides, or MmpL11, which transports an unknown substrate, were found to establish infection normally but to be significantly attenuated for lethality in time-to-death studies. These studies support the concept that MmpL-mediated lipid secretion both contributes to the innate ability of the pathogen to survive intracellularly and also contributes directly to the host-pathogen dialogue that determines the ultimate outcome of infection.
Materials and Methods

Bacterial strains, culture conditions, and plasmids. The ElectroMAX DH5α Escherichia coli strain (Invitrogen) used for cloning was grown in Luria-Bertani medium with hygromycin (200 μg/ml) (Invitrogen) or gentamicin (Gibco BRL) (5 μg/ml) where indicated. M. tuberculosis strains were grown either in Middlebrook 7H9 broth (Difco) supplemented with ADC (NaCl, 8.1 g/liter; bovine albumin fraction V [Calbiochem], 50 g/liter; tr-glyceraldehyde, 20 g/liter), 0.02% glycerol, and 0.05% Tween 80 (Sigma) or on Middlebrook 7H11 agar (Difco) with OADC enrichment (the same components as ADC, with the addition of 0.6 ml/liter oleic acid [ICN Biochemicals] and 3.6 mM sodium hydroxide). Where indicated, hygromycin (50 μg/ml) or 2% sucrose was added (31). M. tuberculosis H37Rv (Pasteur) was used as the parental strain for generation of the mmpL::hyg mutants.

Computer analysis. M. tuberculosis H37Rv, M. bovis, and M. leprae DNA sequences were obtained from the Tubercule List, BosList, and Lepromina servers of the Institut Pasteur (www.pasteur.fr). The M. tuberculosis CDC1551 DNA sequence is available at the TIGR Microbial Database (www.tigr.org). Searches for MmpL orthologs in M. avium subsp. paratuberculosis strain K10 and M. smegmatis were performed by using the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Alignment of the MmpL protein sequences was carried out using the ClustalW algorithm (49), and the phylogenetic tree was built using the neighboring-joining algorithm of Saitou and Nei (41) included in the Vector NTI Suite program (InforMax Inc.).

Nucleic acid techniques. E. coli transformations, cloning, and PCR were based on standard conditions (42). Southern blotting and hybridization procedures were also performed as previously described (39). MGGCA DNA was isolated using the protocol of Pelicic et al. (31). Transformation of M. tuberculosis was carried out as previously described (47).

Construction of MmpL-disrupted mutants. Generation of the M. tuberculosis disrupted mutants (mmpL1::hyg, mmpL2::hyg, mmpL3::hyg, mmpL4::hyg, mmpL5::hyg, mmpL6::hyg, mmpL7::hyg, mmpL8::hyg, mmpL9::hyg, mmpL10::hyg, mmpL11::hyg, mmpL12::hyg) was accomplished by homologous recombination using the system developed by Pelicic et al. (31). Each mmpL::hyg construct was introduced in M. tuberculosis in three steps. In the first step, an M. tuberculosis PCR fragment of approximately 2 kb was amplified and cloned (pUC18 or pcDNA2.1). The oligonucleotides used in the PCR were designed with an appropriate restriction enzyme site in order to facilitate the cloning process (Table 1). In the second step, disruption of the gene was accomplished by inserting a 1.6-kbp fragment harboring the hyg resistance cassette into the cloned mmpL gene. Finally, the 3.6-kbp fragment harboring the mmpL::hyg construct was excised and cloned into the NotI site of pUC18. Each mmpL gene was then cloned into the NotI site (position 1280). For mmpL1, the 2.033-bp PCR fragment from −992 to 1041 was cloned into the NotI and SpeI sites of vector pcDNA2.1 (Invitrogen). A 1.6-kbp fragment carrying the hyg gene was then cloned into the mmpL1 gene at the Smal site (position 532). For mmpL2::hyg, the 2.051-bp PCR fragment from −699 to 1352 was cloned into the XbaI site of pUC18 (United States Biochemical). Insertion of the hyg fragment into mmpL2 was at the Stul site (position 519). For mmpL3::hyg, the 2.073-bp PCR fragment from +106 to +2179 was cloned into the NotI and SpeI sites of pcDNA2.1. The hyg gene was then cloned into mmpL3 at the Hpal site (position 1167). For mmpL4::hyg, a 1.961-bp PCR fragment spanning +243 to +2204 was cloned into the XbaI site of pUC18 and the hyg gene was inserted at the NotI site (position 1280). For mmpL5::hyg, a 2.159-bp PCR fragment from +318 to +2477 was cloned into the XbaI site of pUC18 and the hyg gene was cloned at the EcoRV site (position 1453). For mmpL6::hyg, the 2.085-bp PCR fragment from −935 to +1150 was cloned into the NotI and SpeI sites of pcDNA2.1 and the hyg gene was cloned into the HindIII site (position 144). For mmpL7::hyg, a 2.373-bp PCR fragment from −40 to 2333 was cloned into the NotI and SpeI sites of pcDNA2.1 and the hyg cassette was inserted into an Smal site (position 952). For mmpL8::hyg, a 2.147-bp PCR fragment from +538 to +2086 was cloned into the SpeI site of pcDNA2.1 and the hyg gene was cloned into an Hpal site (position 1656). For mmpL9::hyg, the 2.260-bp PCR fragment from +13 to +2173 was cloned into the pUC18 Xbal site and replacement of a 249-bp fragment by the hyg gene was at the two internal NotI sites (positions 1026 and 1277). For mmpL10::hyg, a 2.667-bp PCR fragment from +302 to +2969 was cloned into the NotI and SpeI sites of pcDNA2.1 and a 585-bp fragment of mmpL10 was replaced by inserting the 1.6-kbp hyg gene between the Smal sites (positions 1381 and 1966). For mmpL11::hyg, the 2.383-bp fragment from −42 to +2341 was cloned into the SpeI site of pcDNA2.1 and the hyg cassette was cloned at the Bpl site (position 1423). For mmpL12::hyg, a 1.826-bp PCR fragment from −524 to +2350 was cloned into the NotI and SpeI sites of pcDNA2.1 and the hyg gene was inserted into the PshAI site (position 1459).

M. tuberculosis H37Rv transfectants were plated on 7H11 medium with 50 μg/ml hygromycin at 32°C for 5 weeks. The resulting colonies were grown at 32°C in 10 ml of 7H9 medium containing 50 μg/ml hygromycin and were subsequently plated on 7H11 medium with 50 μg/ml hygromycin and 2% sucrose at 39°C. DNA from Hg' Su' colonies was digested with the indicated restriction enzymes, transferred to Hybond-N nylon membranes (Amersham Pharmacia), and hybridized with a probe containing the 2-kbp PCR fragment of the corresponding mmpL gene amplified from M. tuberculosis H37Rv Pasteur.

Mic assays. Susceptibilities of the mmpL::hyg mutants to different antituberculosis drugs were tested by a twofold serial broth dilution method as follows. Cultures of each mutant and the parental strain were grown in 7H9 medium to an optical density at 650 nm of 0.2. These cultures were used as inocula after a 1/100 dilution. The antibiotics used were amikacin, cycloserine, ethambutol, ethionamide, isoniazid (INH), kanamycin, ofloxacin, rifampin, streptomycin, tetracycline, and vancomycin, all purchased from Sigma. The assay was performed in 96-well round-bottom plates (Nunc). The plates were read after 7 and 14 days.
of incubation, and the MIC was considered the lowest concentration that completely inhibited visible growth. The experiments were performed in duplicate and repeated three times.

**Mouse experiments.** Prior to infection, well-dispersed liquid cultures were adjusted to an optical density at 650 nm of 0.5 and were stored at −70°C as 20% glycerol stocks. Inocula were prepared by diluting these stocks to 4×10^6 CFU/ml in phosphate-buffered saline–Tween 0.05%. Eight-week-old DBA2 (Taconic) were infected using a BioAerosol nebulizing generator (CH Technologies Inc., NJ) for 10 min. Bacterial numbers were enumerated at 1, 14, 49, 98, and 182 days postinfection (4 mice/time point) by homogenizing the lungs and spleens of infected mice in 1 ml of 7H9 medium and plating 10-fold serial dilutions on 7H11 medium. An additional 12 mice/group were used in survival studies. Survival fractions were calculated using the Kaplan-Meier method (20), and the log rank test was used to determine the statistical significance of observed differences in survival (GraphPad Prism v3.0; GraphPad Software, CA).

**RESULTS**

MmpL proteins occur in both slow- and fast-growing mycobacterial species. Genes encoding MmpL proteins were first identified in the genome of *M. tuberculosis* H37Rv (5). The genome of this strain encodes 13 putative MmpL proteins, 11 of which are predicted to range from 100 kDa (MmpL3) to 122 kDa (MmpL12) and to possess 12 TMD and 2 extracytoplasmic loops. The MmpL13 gene consists of two putative open reading frames, mmpL13a and mmpL13b, that separately encode proteins of 32 and 50 kDa, containing four and seven TMD, respectively. MmpL6, a 42-kDa protein with five TMD, shows 70% homology to the C-terminal half of MmpL2. The gene encoding MmpL6 is truncated in H37Rv and is located in the TbD1 region, a region absent in “modern” *M. tuberculosis* strains (1). Searches of the available mycobacterial genome databases revealed that the MmpL protein family is present not only in other members of the *M. tuberculosis* complex but also in other slow-growing mycobacteria such as *Mycobacterium avium* subsp. paratuberculosis strain K10, as well as in fast-growing species such as *Mycobacterium smegmatis* (Fig. 1A). *M. tuberculosis* strain CDC1551 contains an additional mmpL gene not encoded by strain H37Rv, designated mmpL14, which is contained within the RvD2 deleted region of *M. tuberculosis* H37Rv (17).

*Mycobacterium bovis* encodes 14 putative mmpL genes; however, like the mmpL13 gene of *M. tuberculosis* H37Rv, the *M. bovis* homolog of mmpL1 is split into two contiguous genes, Mb0409c and Mb0408c, whose gene products of 64 and 40 kDa likely do not produce a functional transporter. Similarly, the putative *M. bovis* homolog of the *M. tuberculosis* MmpL9 protein is encoded by two split open reading frames, Mb2367 and Mb2368. Only five intact mmpL genes are present in the reduced *Mycobacterium leprae* genome (corresponding to mmpL3, mmpL4, mmpL7, mmpL10, and mmpL11), and all except mmpL7 are highly conserved in each of the mycobacterial genomes analyzed, suggesting the possibility that the four remaining proteins perform a function conserved at the genus level. Close homologs of MmpL1, MmpL8, and MmpL9 are also absent from *M. avium* K10 and *M. smegmatis*, although in these two species, additional mmpL genes (mmpLN) are present. Six of the 10 predicted MmpLN proteins encoded by *M. avium* subsp. paratuberculosis strain K10 show greater than 70% homology with *M. tuberculosis* MmpL4, including the TmtpB and TmtpC proteins.

An alignment of the sequences of the MmpL proteins present in these different mycobacterial species showed that the regions of highest homology lie within the TMDs and revealed the presence of conserved positively and negatively charged residues located in TMD 4 and 10 (e.g., Asp285, Arg294, His927, Asp 944, and Arg 952 of MmpL8 of *M. tuberculosis* H37Rv) (data not shown). By analogy with AcrA, these amino acids are possible candidates for the proton translocation pathway (26).

**Genomic context and phylogeny suggest a role in lipid secretion.** Four of the genes encoding MmpL proteins (mmpL1, mmpL2, mmpL4, and mmpL5) are associated with smaller genes designated mmpS that are predicted to encode proteins...
with one TMD in the N terminus. These MmpS proteins may be the equivalent of the MFPs that are frequently associated with RND proteins in gram-negative organisms.

Five mmpL genes (mmpL1, mmpL7, mmpL8, mmpL10, and mmpL12) are colocated in the genome with pks genes, suggesting a role in transport of the products of the proteins encoded by these pks genes (Fig. 1B). Interestingly, the genes encoding the TmtpB and TmtpC proteins in M. avium and M. smegmatis are located in the glycopeptidolipid biosynthetic gene cluster (11, 19), and M. smegmatis mutants with the tmtpC gene interrupted are deficient in glycopeptidolipid and are unable to form biofilms on polyvinyl chloride (34).

A phylogenetic analysis of the MmpL proteins from M. tuberculosis, M. avium, and M. leprae revealed several discrete groups of these proteins (Fig. 2). MmpL3 and MmpL11, for example, which are present in M. leprae, are closely related, and the encoding genes occur within the same apparent gene cluster. This cluster is conserved in all the mycobacterial genomes sequenced so far, indicating a possible fundamental role of this cluster in mycobacterial biology. In another large cluster are the genes encoding MmpL8, MmpL10, and MmpL12. These three genes are colocated with pks genes, and the genetic contexts containing mmpL8 and mmpL10 are similar: both are located in a cluster that includes three genes, pks2-papA1-mmpL8 and pks3/4-papA3-mmpL10. Pks2 is a polyketide synthase involved in the synthesis of hepta- and octamethyl branched fatty acids present in the major sulfatide of M. tuberculosis, SL-1 (46). As mentioned above, MmpL8 is involved in the transport of SL-N, a precursor of SL-1 (6, 9). Pks3 is a polyketide synthase involved in the synthesis of mycolipanolic and mycolipenic acids, the trimethyl branched fatty acids present in the polycyctrehalose of M. tuberculosissis (10). Unfortunately, the H37Rv strain used for creating the mmpL::hyg mutants contains a C→A mutation at position 1467 of the pks3 gene, resulting in a stop codon that is also present in the H37Rv strain sequenced by Cole and colleagues (5). In other strains, such as M. tuberculosis CDC1551 and ATCC 2729, a tyrosine codon is present. The absence of polycyctrehalose in our M. tuberculosis parental strain limits our ability to test the hypothesis that MmpL10 transports this molecule or a...
precursor. The PapA proteins are acyltransferases, suggesting that PapA1 and PapA3 may be involved in the esterification of trehalose with the methyl-branched fatty acids during the biosynthesis of polyacyltrehalose and SL-1 (29).

**MmpL proteins of M. tuberculosis do not play a critical role in antituberculosis drug resistance.** In order to begin to understand the range of efflux substrates for the MmpL proteins and to assess any potential role in drug resistance in *M. tuberculosis*, we prepared inactivation constructs for all 12 mycobacterial proteins. These were prepared by PCR amplification of a genomic fragment within each *mmpL* gene, insertion of a hygromycin resistance marker, and incorporation of these constructs into a standard thermosensitive plasmid with a SacB counterselectable marker. Homologous recombinants were selected by screening *M. tuberculosis* H37Rv colonies resulting from transformants following a temperature shift in the presence of sucrose and hygromycin. Figure 3 shows Southern blots of DNA prepared from the resulting insertion mutants. In 11 out of 12 *mmpL* genes, mutants were successfully produced (*mmpL1*: ::hyg, *mmpL2*: ::hyg, *mmpL4*: ::hyg to *mmpL12*: ::hyg). Several attempts at generating the *mmpL3*: ::hyg strain, however, were unsuccessful. This result suggests that *mmpL3* may be an essential gene in *M. tuberculosis*, a suggestion supported by the fact that this gene is one of the four *mmpL* genes conserved in all the mycobacterial genome sequences available. No differences in growth were observed in vitro between any of the *mmpL*: ::hyg mutants and the wild-type strain (data not shown).

MIC assays were performed by broth microdilution in liquid medium with all the *mmpL*: ::hyg mutants as well as the parental strain. The antibiotics tested included the most widely used antituberculosis drugs, isoniazid, ethambutol, rifampin, pyrazinamide, and streptomycin, as well as other drugs known to be efflux substrates for RND proteins from other organisms (Table 2). None of the *M. tuberculosis* *mmpL*: ::hyg mutants displayed large alterations in susceptibility to the antibiotics tested in multiple independent experiments. Some minor differences were noted, however, including a two- to fourfold increase in the ethambutol MIC for the *mmpL8*: ::hyg strain and a twofold increase in the cycloserine MIC for the *mmpL1*: ::hyg strain. These results suggest that the MmpL proteins do not play a large role in innate *M. tuberculosis* drug resistance, although compensatory effects among the different MmpL proteins cannot be ruled out at this stage.

**Role of MmpL proteins during acute low-dose aerosol infection of mice.** To begin to study the potential role of MmpL efflux substrates during acute murine infections, we aerogenically infected C57BL/6 mice with 50 to 200 bacteria of each *mmpL*: ::hyg mutant or the parental strain. Growth kinetics were monitored for 182 days. During the first 2 weeks of infection, the doubling time for the parental strain ranged between 24 and 27 h, while among the 11 *mmpL*: ::hyg mutants tested, only 2 were impaired for growth during this time in both the lung (Fig. 4A) and spleen (Fig. 4B). By 7 weeks postinfection, a 1- to 2-log$_{10}$-unit reduction in the number of bacteria in the lungs and spleen was observed for the *mmpL4*: ::hyg and *mmpL7*: ::hyg mutants, respectively. Hence, both MmpL4 and MmpL7 appear to be required for normal *M. tuberculosis* growth early in the infection in the mouse model, in agreement with previous reports (4, 7). At later time points, when the infection was contained (98 to 182 days), both of these mutants appeared to reach a plateau at significantly lower total bacterial loads. In addition, a slight reduction (0.5 log$_{10}$) unit) in bacterial numbers with the *mmpL11*: ::hyg mutant was also noted, which may reflect a specific impairment in survival of this strain during the chronic phase of infection. The remainder of the *mmpL*: ::hyg mutants did not show significant differences in growth from the parental strain at any stage during infection (data not shown).

**Effect of mmpL inactivation on murine survival in chronic infection.** The mycobacterial growth rate in mice is not the only indicator of virulence, and several studies have shown that
strains with little or no difference in the growth rate or total bacterial numbers still yield infections with large differences in the total survival time (21, 22, 28). In order to assess the effect of loss of specific MmpL proteins, we carried out survival experiments with C57/BL6 mice (12 mice/group) infected aero-
genically with a low dose of each mutant. Bacterial loads in the lungs were also assessed at 1 and 49 days postinfection to confirm infection and to reproduce the differences in total bacterial burden observed previously. Consistent with the results of the previous experiment at 49 days postinfection, only the mmpL4::hyg and mmpL7::hyg mutants (approximately 10^4 CFU/lung) were significantly impaired for growth compared to the parental H37Rv strain (Fig. 5A). Except for those infected with the mmpL4::hyg or mmpL7::hyg mutant, by 430 days after infection, the majority of mice in each group had died. With the two growth-impaired mutants, none of the mice in either group had died when the experiment was terminated. Kaplan-Meier analysis revealed that the median survival time of mice infected with the wild-type H37Rv strain was 265 days (Fig. 5B and Table 3). Among the mice infected with mutants other than the mmpL4::hyg and mmpL7::hyg strains, only those infected with the mmpL8::hyg and mmpL11::hyg strains showed median survival times that were significantly greater than those of wild-type-infected mice. The median survival times for the mmpL8::hyg and mmpL11::hyg groups were 328 (P < 0.0006) and 398 (P < 0.0001) days, respectively (Table 3). Aside from the slight reduction in CFU at a very late stage of infection noted above for mmpL11::hyg, these two mutants did not show any disadvantage for in vivo growth by 49 days in either experiment. This result suggests that the MmpL8 and MmpL11 proteins may be involved in the transport of molecules that are involved in the host-pathogen interaction, thereby impacting on the outcome of the infection. To confirm the attenuated phenotype of the mmpL4::hyg and mmpL11::hyg mutants, the infections were repeated with a second strain of mice which is slightly more susceptible to tuberculosis [(C57/BL6 × DBA2)F1 hybrid mice]. The Kaplan-Meier curves from this experiment are shown in Fig. 6A. By 400 days postinfection, only a single mouse infected with the mmpL4::hyg mutant had died. The median survival of mice infected with H37Rv was 173 days, while for those infected with the M. tuberculosis mmpL11::hyg mutant, median survival was 333 days (P < 0.0001). We have recently published the confirmation of the attenuated phenotype of the mmpL8::hyg mutant elsewhere (9).

![Graph A](https://example.com/graphA.png)

**Fig. 4.** MmpL4 and MmpL7 are required for normal growth of *M. tuberculosis* in mice. C57BL/6 mice were infected aerogenically with the mmpL4::hyg (MmpL4 knockout [L4 KO]) (diamonds), mmpL7::hyg (L7 KO) (triangles), mmpL8::hyg (L8 KO) (circles), or mmpL11::hyg (L11 KO) (stars) strain or with the parental strain H37Rv (squares). Bacterial numbers were monitored at the indicated times postinfection by harvesting lungs (A) and spleens (B) of infected mice. Results are expressed as the average log_{10} CFU (+ standard deviation) obtained from four mice at each time point.

### Table 2. MICs of various antibiotics for the parental (H37Rv) and mmpL::hyg strains

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*Values are expressed as the MIC obtained for each mmpL::hyg strain divided by the MIC for the parental strain (Lx/Rv). Values were determined from three independent experiments, each performed in duplicate.*

*ETA, ethionamide.*
parental strain (H37Rv).

Fig. 5. Inactivation of MmpL4, MmpL7, MmpL8, or MmpL11 increases mouse survival. C57BL/6 mice were infected aerogenically with each of the mmpL::hyg mutants or the parental strain H37Rv. (A) Bacterial numbers were monitored at days 1 and 49 postinfection by harvesting lungs of infected mice. Results are expressed as the average log_{10} CFU (± standard deviation) obtained from four mice at each time point. From left to right, the columns correspond to mice infected with the M. tuberculosis H37Rv, mmpL1::hyg, mmpL2::hyg, mmpL4::hyg, mmpL5::hyg, mmpL6::hyg, mmpL7::hyg, mmpL8::hyg, mmpL9::hyg, mmpL10::hyg, mmpL11::hyg strain. (B) Survival curves of mice infected with the mmpL::hyg (MmpL4 knockout [L4 KO]), mmpL7::hyg (L7 KO), mmpL8::hyg (L8 KO), or mmpL11::hyg (L11 KO) mutant or the parental strain H37Rv. Analysis of these data was carried out using the Kaplan-Meier method, and a log rank test was used to determine the statistical significance of observed differences in survival (GraphPad Prism, v3.0; GraphPad Software, CA).

Table 3. Median survival and P values (by the log rank test) of different groups of mice

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<th>Strain with which mice were infected</th>
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<th>P value</th>
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</tr>
<tr>
<td>M. tuberculosis mmpL1::hyg</td>
<td>214</td>
<td>0.0005</td>
</tr>
<tr>
<td>M. tuberculosis mmpL2::hyg</td>
<td>177</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>M. tuberculosis mmpL5::hyg</td>
<td>Undef.</td>
<td>0.9081</td>
</tr>
<tr>
<td>M. tuberculosis mmpL6::hyg</td>
<td>235</td>
<td>0.0433</td>
</tr>
<tr>
<td>M. tuberculosis mmpL7::hyg</td>
<td>Undef.</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>M. tuberculosis mmpL8::hyg</td>
<td>328</td>
<td>&lt;0.0006</td>
</tr>
<tr>
<td>M. tuberculosis mmpL9::hyg</td>
<td>302</td>
<td>0.2482</td>
</tr>
<tr>
<td>M. tuberculosis mmpL10::hyg</td>
<td>252</td>
<td>0.5444</td>
</tr>
<tr>
<td>M. tuberculosis mmpL11::hyg</td>
<td>398</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>M. tuberculosis mmpL12::hyg</td>
<td>217</td>
<td>0.0029</td>
</tr>
</tbody>
</table>

* Data were analyzed as described in the legend to Fig. 5B. Undef., undefined.

In the first survival experiment, mice infected with the mmpL1::hyg, mmpL2::hyg, and mmpL12::hyg mutants showed somewhat less significant shorter survival times than wild-type-infected mice (Table 3). In this experiment, however, there was as much as a two- to fourfold difference in the initial inoculum delivered to mice (mmpL1::hyg, 90 CFU; mmpL2::hyg, 242 CFU; mmpL12::hyg, 193 CFU; wild-type H37Rv, 62 CFU). To confirm this apparent “hypervirulent” phenotype, we infected another group of mice with more closely matched inocula of the same strains. This time there were no significant differences in survival between any of the three strains and the wild type (data not shown). In fact, when we plotted the initial inoculum of each strain of M. tuberculosis against the median survival time for the first survival experiment, we noted an almost perfect correlation (Pearson’s correlation value [R^2] = 0.8693) between survival and the initial inoculum, the only exceptions being the four attenuated strains mmpL4::hyg, mmpL7::hyg, mmpL8::hyg, and mmpL11::hyg (Fig. 6B). Thus, even slight differences in bacterial numbers observed at the time of infection can have profound effects on the time to death observed in murine tuberculosis infections.
In order to rule out the possibility that a polar effect caused by the insertion of the hyg resistance cassette was responsible for the attenuated phenotype found in the *M. tuberculosis* mmpL4::hyg, mmpL7::hyg, and mmpL11::hyg strains, we analyzed by reverse transcription-PCR the expression of the genes located around mmpL4, mmpL7, and mmpL11 in the wild-type strain as well as in the mmpL4::hyg, mmpL7::hyg, and mmpL11::hyg mutants. No significant changes were observed in the expression patterns of the mmpL4::hyg mutant, the Rv0201c and Rv0203 genes in the mmpL11::hyg mutant, the Rv0449c and Rv0451c genes in the mmpL7::hyg mutant, or the Rv2941 and Rv2943 genes in the mmpL7::hyg mutant relative to the wild-type strain (data not shown).

**DISCUSSION**

In this paper we have examined the potential role of the MmpL family of proteins in virulence by generating a set of 11 *M. tuberculosis* strains in which each *mmpL* gene had been insertionally inactivated with a hygromycin resistance cassette. To date, our results have shown that unlike in other bacteria, these proteins do not appear to play important roles individually in transport of, or resistance to, antituberculosis drugs.

We have also shown that among the 12 MmpL proteins present in *M. tuberculosis*, 4 (MmpL4, MmpL7, MmpL8, and MmpL1) are necessary to fully maintain the virulence of the tubercle bacilli in mice. Two of these proteins, MmpL4 and MmpL7, are essential for normal replication during the active-growth phase of the murine tuberculosis model. Attenuation of *MmpL7*, are essential for normal replication during the active-growth phase, these mutants showed growth patterns similar to that of the wild-type during this active-growth phase, these mutants are attenuated for survival in the chronic stages of infection. 

The *M. tuberculosis* mmpL8::hyg mutant is deficient in SL-1 because the MmpL8 protein is involved in the transport of SL-N (2,3-diacyl-β-D-trehalose-2'-sulfate), a precursor of SL-1 (9). It has recently been demonstrated that SL-N stimulates human CD1b-restricted T cells (15); if this molecule has a related effect on murine CD1d-restricted T cells, it may help to explain the attenuated phenotype observed in mice. An alternative explanation is that the lack of SL-1 or some non-immune consequence of the accumulation of SL-N is responsible for the attenuation of the MmpL8-deficient strain. These hypotheses are currently being tested.

The putative secreted molecules and the molecular mechanisms by which the mmpL4::hyg and mmpL11::hyg mutants are attenuated remain unclear. The possibility that these strains were more sensitive to reactive oxygen intermediates and reactive nitrogen intermediates in vitro has been tested through the use of various reagents including H2O2, cumene hydroperoxide, menadione, diethylylamine nitric oxide, and S-nitrosothiathione. Differential sensitivities to these compounds have not been observed for either of these strains thus far (data not shown).

Among the wide range of putative MmpL substrates are the distinct lipids and mycolic acids present in the unique mycobacterial envelope, some of which have been shown to be released from the mycobacterial envelope within the phagosomes of infected macrophages (14, 36). Indeed, two of the MmpLs, MmpL7 and MmpL8, are involved in transport of methyl-branched lipids containing carbohydrates or polycyclites present in the *M. tuberculosis* cell wall. MmpL7, interestingly, appears to transport both PDIM and a related but structurally distinct phenolic glycolipid expressed only in a subset of highly virulent *M. tuberculosis* strains (35). The presence of at least three sugars on the phenolic glycolipid distinguishes it from PDIM, suggesting that MmpL7 has a relatively broad substrate range.

Analysis of [1-14C]propionic acid-labeled lipids from the rest of the mutant strains in which the *mmpL* genes are associated with pks3/4 genes—*mmpL1::hyg*, *mmpL10::hyg*, and *mmpL12::hyg*—did not reveal any differences in lipids containing methyl-branched fatty acids within these mutants, either in bacteria-associated lipids or in lipids secreted into the medium. Aside from MmpL7 and MmpL8, the substrates for the MmpL proteins are unknown. As described above, based on its genomic location, it is likely that MmpL10 is involved in the transport of diacyl trehalose and/or pentaacyl trehalose. However, because of the parental H37Rv (Pasteur) strain used in these studies, this hypothesis remains to be tested. It should be noted that disruption of the intact pks3/4 gene cluster present in H37Rv (ATCC 2729) has no effect on virulence (our laboratory, unpublished observations). Because the mmpL4 gene of *M. tuberculosis* is repressed by iron in an IdeR-dependent manner (37), we have also examined the Fe2+ growth requirements of the *M. tuberculosis* mmpL4::hyg strain, although, again, no differences were observed with respect to the parental strain (data not shown). Other possible substrates of the MmpL proteins are the antimicrobial peptides produced by the host. Antimycobacterial activity has been found in several defensins, such as human and rabbit neutrophil peptide-1 or porcine protegrin (25, 44, 45). The MtrD protein of *Neisseria gonorrhoeae*, which is also a member of the RND family, modulates susceptibility to antibacterial peptides (43). The possibility that one or more of the mycobacterial MmpL proteins are involved in resistance to defensins remains to be tested.

It is hoped that the mutants described here will provide the basis for further studies aimed at characterizing the nature of the transport processes mediated by this family of *M. tuberculosis* proteins. In the future, we will also begin to combine multiple MmpL mutations within the same strain in order to explore the possibility of overlapping functions of related MmpL proteins. The ability to identify inhibitors of important transport processes such as those mediated by MmpL3, MmpL4, or MmpL11, for example, may provide novel drug targets at some point in the future.

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