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LpqM, a Mycobacterial Lipoprotein-Metalloproteinase, Is Required for Conjugal DNA Transfer in *Mycobacterium smegmatis*▽

Kiet T. Nguyen,[1]§ Kristina Piastro,[1]† and Keith M. Derbyshire[1,2]*

Division of Genetics, Wadsworth Center, New York State Department of Health,[1] and Department of Biomedical Sciences, School of Public Health, University at Albany,[2] Albany, New York 12201

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We have previously described a novel conjugal DNA transfer process that occurs in *Mycobacterium smegmatis*. To identify donor genes required for transfer, we have performed a transposon mutagenesis screen; we report here that LpqM, a putative lipoprotein-metalloproteinase, is essential for efficient DNA transfer. Bioinformatic analyses predict that LpqM contains a signal peptide necessary for the protein's targeting to the cell envelope and a metal ion binding motif, the likely catalytic site for protease activity. Using targeted mutagenesis, we demonstrate that each of these motifs is necessary for DNA transfer and that LpqM is located in the cell envelope. The requirement for transfer is specific to the donor strain; an *lpqM* knockout mutant in the recipient is still proficient in transfer assays. The activity of LpqM is conserved among mycobacteria; homologues from both *Mycobacterium tuberculosis* and *Mycobacterium avium* can complement *lpqM* donor mutants, suggesting that the homologues recognize and process similar proteins. Lipoproteins constitute a significant proportion of the mycobacterial cell wall, but despite their abundance, very few have been assigned an activity. We discuss the potential role of LpqM in DNA transfer and the implications of the conservation of LpqM activity in *M. tuberculosis*.

In previous work, we have described a novel conjugation system in *Mycobacterium smegmatis* (15). Although this process meets the criteria of conjugation (successful transfer requires prolonged cell-cell contact and is DNase resistant), the mechanism of transfer is unique (27–29). Transfer is chromosomally encoded, and despite exhaustive bioinformatics searches, we have yet to identify any genes encoding obvious transfer functions. A comprehensive transposon mutagenesis screen of the donor strain failed to identify transfer-defective mutants. Instead, the screen identified hyperconjugative mutants that mapped to a large, 30-kb locus, *esx-1* (7). *esx-1* encodes a secretory apparatus, ESX-1, and we hypothesized that the secretion of proteins by ESX-1 negatively regulates transfer, either because the secreted proteins physically block transfer or because they act as intercellular quorum sensors. In a more recent study of the *M. smegmatis* recipient strain, we showed that a functional ESX-1 apparatus is essential for recipient activity (4). Recipient activity required secretion of at least two *esx-1*-encoded proteins, EsxA and EsxB. We have therefore proposed that in the recipient, as in the donor, ESX-1 is secreting proteins that regulate, rather than mediate, DNA transfer. The ESX-1 apparatus is highly conserved, and *esx-1*-encoded mutants of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium marinum* are attenuated (reviewed in references 1 and 6). Proteins known to be secreted by ESX-1 from *M. tuberculosis* include EsxA and EsxB (formerly known as ESAT-6 and CFP10, respectively), EspA, and Rv3881 (10, 12, 17, 25). Further underscoring the functional involvement of ESX-1 in both conjugation and virulence, our screen for transfer-defective recipient mutants also identified homologues of EspA (MSMEG5168a) and Rv3881 (MSMEG0076) (4). However, the mechanisms by which these proteins function, both in *M. tuberculosis* virulence and in regulation of *M. smegmatis* conjugation, are unknown.

In this study, we have reexamined donor contributions to DNA transfer by exploiting a hyperconjugative donor mutant strain. This strategy, which increased the sensitivity of the assay, has allowed us to isolate donor-defective insertions for the first time. One insertion mapped to a gene encoding a putative metallo-lipoprotein, LpqM. We describe the genetic characterization of this protein and discuss its potential role as an extracellular protease in DNA transfer.

MATERIALS AND METHODS

Bacterial strains and media. The *M. smegmatis* donor strains used were derivatives of strains mc^{2}155 (24) and MKD158 (resistant to hygromycin [Hyg^r]) (7) and mc^{2}155 Δ*esx-1*. The mc^{2}155 Δ*esx-1* strain contains a replacement of the *esx-1* genes Msmeg_0056 to Msmeg_0082 with a gene encoding Hyg^r, which was constructed by J. Wang and J. Flint by allele replacement. Briefly, 2-kb segments of DNA upstream of msmeg_0056 and downstream of msmeg_0082 were PCR amplified and cloned into the mycobacterial plasmid pPR23, along with a cassette encoding hygromycin resistance; the two amplified segments were cloned such that they flanked the Hyg^r gene in an appropriate orientation for allele replacement. pPR23 is temperature sensitive for replication, and it encodes the counter-selectable marker, *sacB*, and gentamicin resistance (Gm^r) (16). Hyg^r allele replacements were selected for at the nonpermissive temperature in the presence of sucrose. Sucrose-resistant Hyg^r Gm^s recombinants were purified, and the precise replacement of the *esx-1* region with the Hyg^r gene was confirmed by Southern analysis and PCR. The recipient strain used was MKD8, which is resistant to streptomycin (Sm^r) (15). *M. smegmatis* was grown at 37°C in Trypticase soy broth or Sauton's medium (0.5 g/liter KH_2PO_4, 0.5 g/liter $MgSO_4$, 4.0 g/liter L-asparagine, 6% glycerol, 0.05 g/liter ferric ammonium citrate, 2.0

* Corresponding author. Mailing address: Wadsworth Center, NYS Department of Health, P.O. Box 509, Albany, NY 12201. Phone: (518) 473-6079. Fax: (518) 474-3181. E-mail: keith.derbyshire@wadsworth.org.

§ Present address: National Center for Toxicological Research, 3900 NCTR, Jefferson, AR 72079.

† Present address: University at Buffalo School of Medicine and Biomedical Sciences, 155 Biomedical Education Building, Buffalo, NY 14214.

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g/liter citric acid, and 100 μl 1% $ZnSO_4$). The media were supplemented with antibiotics at the following concentrations: 100 μg/ml hygromycin, 10 μg/ml kanamycin, and 200 μg/ml streptomycin.

Escherichia coli DH5α was used throughout the study for routine molecular genetic techniques. Cell cultures were grown in LB medium at 37°C. When appropriate, the medium was supplemented with antibiotics at the following concentrations: 75 μg/ml hygromycin, 50 μg/ml kanamycin, and 15 μg/ml gentamicin.

Transposon mutagenesis. A mutant transposon insertion library was made in the mc^2155 Δ*esx-1* strain, using a temperature-sensitive phage to deliver a *mariner* transposon encoding kanamycin resistance (Km^r) (2, 22). Individual insertions were mapped as previously described (7). Briefly, chromosomal BamHI fragments encoding Km^r were cloned, and the site of insertion was determined by DNA sequence analysis using primers that annealed to the Km^r gene. The chromosomal locations of the insertions and the gene annotations are based on the April 2007 version of the *M. smegmatis* chromosomal sequence in The Institute for Genomic Research database (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=gms). Transposon insertions were transduced from the mc^2155 Δ*esx-1* strain into MKD158, using the generalized transducing phage Bxz1 as described previously (13).

Microtiter mating screen. A screen for donor mutants that are defective in conjugation was carried out as described previously (7). The Hyg^r marker replacing the *esx-1* locus was used to monitor transfer from the donor strain into the recipient (MKD8). Transconjugants were selected on Trypticase soy agar medium containing hygromycin and streptomycin. DNA transfer is temperature sensitive, and thus, all crosses are carried out at 30°C (15).

Mating assay. The effect of insertion mutations on donor activity was determined using a quantitative mating assay to measure the conjugation frequency (the number of transconjugants per donor) (15). In all crosses, transconjugants were selected on medium containing hygromycin and streptomycin. Transfer frequencies are the averages of at least three crosses, which were performed in parallel with wild-type donor and recipient controls.

Site-directed mutagenesis. The *lpqM* gene was amplified by PCR from genomic DNA and cloned into pUC19. Mutations were created by PCR, using *Pfu* polymerase (Stratagene). Primer sequences used for mutagenesis are available on request. Mutations were confirmed by DNA sequence analysis. The *lpqM* gene knockout mutant, MKD158 Δ*lpqM*, was generated by allele exchange. Briefly, 800-bp segments of DNA upstream and downstream of *lpqM* were amplified and cloned on either side of a cassette encoding kanamycin resistance in pPR23 (16). Allele replacements were then selected at the nonpermissive temperature in the presence of sucrose. Km^r Gm^s recombinants were confirmed by PCR.

Complementation studies. The *M. smegmatis lpqM* gene with 150 bp of upstream sequence was amplified from mc^2155 genomic DNA and cloned into pPR23 (16). This multicopy, nonintegrating plasmid (pPR23*MslpqM*) was transformed into mc^2155 Δ*lpqM*, and complementation was determined in a mating-out assay. All strains harboring pPR23 derivatives for *lpqM* complementation were grown at 30°C. The *lpqM* homologues from *M. tuberculosis* and *Mycobacterium avium* were constructed in a similar manner, except that each gene was expressed from the *M. smegmatis lpqM* promoter region. An epitope-tagged *M. smegmatis lpqM* derivative was constructed in pUAB200 (23). A DNA duplex encoding the c-Myc epitope (amino acid sequence, EEQKLISEDL) was ligated in-frame with the C terminus of *lpqM* to create an *lpqM*–c-Myc gene fusion. All constructs were confirmed by DNA sequencing.

Subcellular fractionation. Crude cell wall, membrane, and cytosolic fractions were prepared by differential centrifugation as described previously (8, 9, 19). mc^2155 was transformed with pUAB200–lqpM–c-Myc. Six hundred milliliters of overnight cultures grown at 37°C were pelleted by centrifugation at 7,000 rpm for 10 min at 4°C. Cells were resuspended in phosphate-buffered saline containing a protease inhibitor cocktail (Roche) and were then lysed by sonication (five 15-s pulses with a Branson microtip) to generate a total cell lysate. Insoluble material from the total cell lysate, including unbroken cells, was removed by centrifugation (15,000 rpm for 10 min at 4°C). The clarified supernatant fraction was further separated by ultracentrifugation at 200,000 × *g* for 2.5 h at 4°C (Beckman NVT90 rotor) to separate the membrane fractions from the soluble cytoplasmic fraction. The membrane fraction and insoluble pellets were resuspended in phosphate-buffered saline containing 1% Triton X-100 before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. GroEL was used to monitor the fractionation procedure. GroEL is an abundant cytoplasmic protein that should be enriched into the soluble, cytosolic fractions during the procedure.

Western blotting. Protein fractions were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to a polyvinylidene difluoride membrane (Amersham Hybond-P; GE Healthcare) by electrophoresis at 35 eV overnight at 4°C. Unless otherwise stated, all the remaining procedures were carried out at room temperature. The membrane was blocked for 1 h with 5% nonfat milk in TBS-T buffer (50 mM Tris [pH 7.5], 150 mM NaCl, and 0.1% Tween 20). c-Myc antibody (Invitrogen) was used at a 1:5,000 dilution, and GroEL antibody (Stressgen) was used at a dilution of 1:5,000 in TBS-T buffer. Secondary antibody and detection reagents were from the Amersham ECL Western Breeze kit (GE Healthcare) and were used according to the manufacturer's instructions.

TABLE 1. DNA transfer frequencies[a]

Cross	Relevant genotype	No. of transconjugants per donor
1	Δ*esx-1*	$1.4 (\pm 0.1) \times 10^{-4}$
2	Δ*esx-1 lpqM*::Tn	$8.8 (\pm 0.8) \times 10^{-7}$
3	Δ*esx-1* Δ*dinG*	$8.9 (\pm 3.4) \times 10^{-5}$
4	Δ*esx-1 lpqM*::Tn pPR23*MslpqM*	$1.7 (\pm 0.7) \times 10^{-5}$
5	Δ*esx-1* Δ*lpqM*	$8.5 (\pm 0.8) \times 10^{-7}$
6	Δ*esx-1* Δ*lpqM* pPR23*MslpqM*	$7.5 (\pm 1.5) \times 10^{-5}$
7	MKD158 (wild-type)	$5.1 (\pm 0.3) \times 10^{-5}$
8	*lpqM*::Tn	$1.3 (\pm 0.7) \times 10^{-8}$
9	Δ*lpqM*	$1.9 (\pm 0.8) \times 10^{-8}$
10	Δ*lpqM* pPR23*MslpqM*	$8.4 (\pm 1.4) \times 10^{-6}$
11	Δ*lpqM* pPR23*MtblpqM*	$5.2 (\pm 0.7) \times 10^{-7}$
12	Δ*lpqM* pPR23*MalpqM*	$8.0 (\pm 0.5) \times 10^{-7}$
13	Δ*lpqM* pPR23Δ22-*lpqM*	$7.8 (\pm 0.2) \times 10^{-8}$
14	Δ*lpqM* pPR23C22A-*lpqM*	$3.2 (\pm 0.5) \times 10^{-8}$
15	Δ*lpqM* pPR23YT-*lpqM*	$6.0 (\pm 0.6) \times 10^{-8}$

[a] Each frequency is the average of at least three crosses, using approximately equal numbers of donor and recipient cells (2×10^8).

RESULTS

LpqM is required for conjugation in *M. smegmatis*. In a previous genetic screen for conjugative donor mutants, only hyperconjugative mutants were isolated, despite the fact that we had expected to isolate both hyper- and hypoconjugative mutant strains (7). The majority of the hyperconjugative mutations mapped to the *esx-1* locus of *M. smegmatis*; we showed that this locus negatively regulates DNA transfer. A likely explanation for our inability to identify transfer-defective mutants was that the assay was suboptimal such that the background level of false-positive mutations was too high. To circumvent this issue, we took advantage of the hyperconjugative phenotype of *esx-1* mutants. A defined deletion of the genes msmeg_0056 to msmeg_0082 was created within *esx-1* by allele exchange. We chose to generate a deletion, as this could not revert and would also avoid the isolation of insertions in the *esx-1* region, resulting in a hyperconjugative phenotype. As expected, the deletion elevated the DNA transfer frequency (>10-fold), resulting in confluent growth of transconjugant mating spots in the microtiter mating assay and thereby increasing the sensitivity of the screen. The Δ*esx-1* strain was subjected to *mariner*::*km* transposon mutagenesis as previously described (7). Nine transfer-defective mutants were isolated from a screen of 18,000 mutants, and these had insertions in msmeg_0033, msmeg_1435, msmeg_1455, msmeg_3080, msmeg_4913, and msmeg_6128. Notably, four independent insertions were isolated in msmeg_4913, and quantitative transfer assays indicated that these were the most deleterious mutations, reducing transfer by over 160-fold and often down to undetectable levels (Table 1, rows 1 and 2).

msmeg_4913 encodes a putative lipoprotein protease, LpqM,

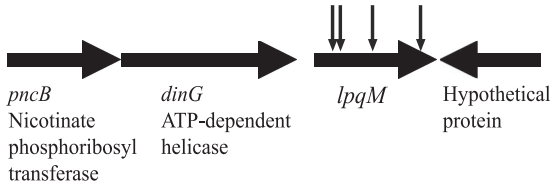

FIG. 1. Schematic map of the *lpqM* region of the *M. smegmatis* genome. The map is based on the genome sequence available from TIGR (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=gms). The vertical arrows indicate the sites of insertion of the *mariner*::*km* transposon. The segment of DNA cloned for complementation studies included the 150-bp region upstream of *lpqM*, which encompasses the 92-bp intergenic region between *dinG* and *lpqM*.

and is the third gene in a 3-gene operon (Fig. 1). We note that although the gene preceding *lpqM* encodes a putative DNA helicase, DinG, this helicase is not required for transfer. A precise deletion of *dinG* had no effect on DNA transfer frequencies (Table 1, row 3). To confirm that the transfer defect was a result of the transposon insertion, the *lpqM* gene was amplified and cloned into the multicopy, nonintegrating plasmid pPR23, creating pPR23*MslpqM*. This plasmid complemented the transfer defect, although only to 10% of wild-type levels (Table 1, row 4). A precise *lpqM* deletion was also generated by allele exchange, resulting in a defect in transfer similar to the defect caused by the transposon insertion in *lpqM*. Again, the transfer defect was rescued by complementation with pPR23*MslpqM* (Table 1, rows 5 and 6). The effect of the deletion establishes the requirement for LpqM in DNA transfer and rules out potential dominant-negative effects created by the transposon insertion.

LpqM affects transfer independently of ESX-1 function and is donor specific. The *lpqM* mutants described above were isolated in a Δ*esx-1* hyperconjugative background. Possibly, the requirement for LpqM in transfer was dependent on the lack of a functional ESX-1 apparatus. To rule out this scenario, we transduced one of the original Km^r transposon insertions into the wild-type donor strain, MKD158. In addition, a precise deletion of *lpqM* was generated in MKD158, using the same allele exchange vector as had been used for the Δ*esx-1* strain. Both of these *lpqM* mutant strains were found to be defective in DNA transfer compared to the parent MKD158 (Table 1, compare rows 7 to 9). As expected, we observed lower frequencies of transfer in the wild-type background than in the Δ*esx-1* strain. The transfer frequencies of both MKD158 *lpqM* mutant derivatives were elevated >100-fold when *lpqM* was expressed in *trans* (Table 1, compare rows 9 and 10). These data confirm that the LpqM requirement is independent of ESX-1 function. For further confirmation, we have determined that secretion of EsxB by ESX-1 occurs at wild-type levels in an *lpqM* mutant (data not shown).

Using the same allele exchange vector, we generated a deletion of *lpqM* in the recipient strain, MKD8 (15). DNA transfer from a wild-type donor into the Δ*lpqM* recipient derivative was unaffected, thereby establishing that the essential role of LpqM in transfer is specific to the donor strain.

LpqM is functionally conserved across species. Bioinformatic analyses of LpqM indicate that it has homologues in other mycobacteria, including *M. tuberculosis* (Rv0419 [48.3% amino acid identity]) and *M. avium* (MAV4736 [47.2% amino acid identity]) (Fig. 2). There is also an LpqM paralogue within *M. smegmatis* (MSMEG4893 [50% amino acid identity]), but its inability to complement any *lpqM* mutation suggests that it encodes a protein that performs different functions or is a pseudogene. The *M. avium* and *M. tuberculosis lpqM* homologues were individually cloned into pPR23 and were then tested for their ability to complement the Δ*lpqM* strain. In each case, transfer activity was partially restored, indicating that functional conservation of LpqM activity exists among these mycobacterial species (Table 1, compare rows 9 to 12). Complementation does not restore LpqM activity to the wild-type levels, and we believe this is a result of low expression levels of *lpqM* from the complementing vector. The cloned DNA included a 92-bp putative promoter region immediately upstream of *lpqM*, but it is possible that in vivo transcription is normally initiated from a stronger promoter transcribing the entire operon (Fig. 1). The important finding is that in each case the overall level of transfer is elevated above that of the Δ*lpqM* mutant.

Transfer activity requires the conserved signal peptide motif and membrane targeting. Computational analyses of the LpqM protein predict it to contain an N-terminal signal peptide motif (amino acids 1 to 22) (Fig. 2 and 3A). The signal peptide is followed by an absolutely conserved cysteine residue, embedded within a larger "lipobox" motif (LAACS) that is known to be important for outer membrane localization of lipoproteins (30, 31). Briefly, the signal peptide is hypothesized to target the protein to the cell wall. Following translocation across the cytoplasmic membrane, the lipobox cysteine is covalently linked to a diacylglycerol moiety by a membrane-bound diacylglycerol transferase. A signal peptidase then cleaves off the signal peptide, and the diacylglycerol moiety is used to anchor the lipoprotein in the cell wall. We constructed a series of pPR23*lpqM* mutant plasmid derivatives in order to confirm the significance of these conserved amino acids and to examine their impact on transfer. The plasmids were introduced into the Δ*lpqM* donor strain, and then each mutant derivative was assessed for its ability to complement the deletion of *lpqM*. Both deletion of LpqM residues 2 to 22 (LpqMΔ22) and substitution of Cys22 by Ala [LpqM(C22A)] reduced the ability of the plasmid to complement the defect in DNA transfer, indicating that the signal peptide and membrane anchor are necessary for efficient DNA transfer (Table 1, compare rows 10, 13, and 14).

The cellular location of each mutant protein was assessed to determine the effect of deleting the signaling functions. To facilitate these studies, a c-Myc epitope tag was fused to the C terminus of LpqM. This derivative had wild-type activity, as evidenced by its ability to complement the Δ*lpqM* mutation (data not shown). Importantly, the c-Myc tag allowed the use of epitope-specific antibodies to determine the location of the fusion protein. Cells expressing the fusion protein were harvested, and the cellular components were fractionated and then subjected to Western blot analysis. As predicted, wild-type LpqM is primarily associated with the membrane fraction and is absent from the cytosolic fraction (Fig. 3B). In contrast, c-Myc-tagged versions of LpqM(C22A) and LpqMΔ22 are absent from the membrane fraction and are retained in the cytosolic fraction, confirming the function of the predicted signal peptide (Fig. 3C). The overall levels of both signal peptide

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MSMEG_4913      MSRRHLLGA-----------------VIATCVVVPLAACSTIVEGSAVSVFADPFSVAGM 43
Rv0419          MHGRGRYRPLVRCVRPRRVAASVRTPIACLAAVVVIAGCTTVVDGRALSILNDPFRVGGL 60
Mb0427          MHGRGRYRPLVRCVRPRRVAASVRTPIACLAAVVVIAGCTTVVDGRALSILNDPFRVGGL 60
MAV_4736        MS--------------------------AAAVLLVAGCSTFVEGRALSMLNDPFLVGGL 33
                *                             ..*: :*.*:*.*:* *:*:: *** *.*:

MSMEG_4913      PATDGPSGLRPDAAAPTREVTDSDGGKIDELAASAVSDIEEFWSATYGEVFEGDFTPVRE 103
Rv0419          PATNGPSGARPDAPAASGTVINTNNGAIDKLSLLSVNDIEDYWMAVYSESLKGTFRPVGK 120
Mb0427          PATNGPSGARPDAPAASGTVINTNNGAIDKLSLLSVNDIEDYWMAVYSESLKGTFRPVGK 120
MAV_4736        PATNGPSGIRSNAPAPTGKVLNTDNGSIDSLSLLSINDIEDYWRSVYGQSLKGKFVPISK 93
                ***:**** *.:*.*.:  * :::.* **.*:  ::.***::* :.*.: ::* * *: :

MSMEG_4913      LISWDSESFDG-EFCGLNTFALVNAAYCKPDRTIGWDRGVLLPSLRKQNGDMGVVMVLAH 162
Rv0419          LVSYDSNDPSSPIVCHIDTYQLVNAFFSSRCNLIAWDRGVFMAVAQEYFGDMSVNGVLAH 180
Mb0427          LVSYDSNDPSSPIVCHIDTYQLVNAFFSSRCNLIAWDRGVFMAVAQEYFGDMSVNGVLAH 180
MAV_4736        IVSYNSTDPSSPIVCHNDTYQLVNAFYTSRCNLIAWDRGVFMPVAQKYFGDMSVTGVLAH 153
                ::*::* . ..  .*  :*: **** : .  . *.*****::.  ::  ***.*  ****

MSMEG_4913      EYGHAIQQQAKLVRRNTPTLVSEQQADCLAGTYMRWVAEGNSPRFTLGTGDGLNSLLAAV 222
Rv0419          EFGHALQVMANLVTRKDPTIVREQQADCFAGVYLWWVAEGKSTRFTLSTADGLDHVLAGI 240
Mb0427          EFGHALQVMANLVTRKDPTIVREQQADCFAGVYLWWVAEGKSTRFTLSTADGLDHVLAGI 240
MAV_4736        EFGHALQQMAALVTKRDATIVREQQADCFAGVYLFWVADGKSPRFTLSTADGLDHVLAGI 213
                *:***:*  * ** :. .*:* ******:**.*: ***:*:*.****.*.***: :**.:

MSMEG_4913      IAFRDPLLTESDMYLGVDEHGSAFERVSAFQFGFTDGAAACKAIDVKEISQRRGDLPVLL 282
Rv0419          ITTRDPVM-EADA-ENDDEHGSALDRVSAFQLGFINGTPACAAIDEDEVERRRGDLPTAL 298
Mb0427          ITTRDPVM-EADA-ENDDEHGSALDRVSAFQLGFINGTPACAAIDEDEVERRRGDLPTTL 298
MAV_4736        ITTRDPVM-DSET-ANDDAHGSALDRISAFQMGFLNGASACASINRKEIEQRRGDLPYAL 271
                *: ***:: :::   . * ****::*:****:** :*:.** :*: .*:.:******  *

MSMEG_4913      PED-----QTGELPITEESVRAMVDALNVVFKPKNPPRLSFAARDADDCPDARPSPPTSY 337
Rv0419          RVDASGNPETGEVGINEETLSTLMELMGKIFSPKNPPTLSYQP---AGCPDAKPSPPAAY 355
Mb0427          RVDASGNPETGEVGINEETLSTLMELMGKIFSPKNPPTLSYQP---AGCPDAKPSPPAAY 355
MAV_4736        RVDTSGRPETGEVPINQDTLSTLMELLGKIFAPANPPALSYQP---ATCPDAKPSPPASY 328
                  *     :***: *.:::: :::: :. :* * *** **: .     ****:****::*

MSMEG_4913      CPATNTIVVDLDELETVGFKPVDGQVGALALGDNSAYSLLISRYMVARQHERGLVLDNAQ 397
Rv0419          CPATNTIVVDLPALARMG-KVASAAEHSLPQGDDTSLSIVMSRYALAVQHERGLPMQSPW 414
Mb0427          CPATNTIVVDLPALARMG-KVASAAEHSLPQGDDTSLSIVMSRYALAVQHERGLPMQSPW 414
MAV_4736        CPATNTIAVDLPKLAALG-RVADENEHTLPQGDDTALSVVMSRYALAVQHERGLAMQSPW 387
                *******.***  *  :* : ..    :*. **::: *:::*** :* ****** ::..

MSMEG_4913      AALRTACLTGVATAKLSQPVTTPDGNTIVLTAGDVDEAVSGILTTGLVAGDVNGDSVPSG 457
Rv0419          TALRTACLTGVAHRKMAVPIDLPSGQQLVLTAGDLDEAVSGLLTNRMVASDADGVSVPAG 474
Mb0427          TALRTACLTGVAHRKMAVPIDLPSGQQLVLTAGDLDEAVSGLLTNRMVASDADGVSVPAG 474
MAV_4736        TALRTACLTGVVHRRMAEPIELPSHNQLLLTAGDLDEAVAGLLTNHLVASDADGTSVPAG 447
                :**********.  ::: *:  *. : ::*****:****:*:**. :**.*.:* ***:*

MSMEG_4913      FSRIDAFRVGILGDEARCLKRF-S 480
Rv0419          FTRIAAFRAGVGGDMDACYARYPG 498
Mb0427          FTRIAAFRAGVGGDMDACYARYPG 498
MAV_4736        FTRIAAFRGGVTGNADACYSRYPG 471
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FIG. 2. The *M. smegmatis* LpqM protein has homologues in other mycobacteria. The predicted amino acid sequences of LpqM from *M. tuberculosis* (Rv0419), *M. bovis* (Mb0427), and *M. avium* (Mav_4736) are aligned with the sequence from *M. smegmatis* (MSMEG913). Although the correct initiation codon for *M. tuberculosis* LpqM and *M. bovis* LpqM has not been experimentally determined, it has been assigned to the first methionine (M) codon. However, it is possible that the translation of these two homologues begins at valine (V) 19; in that case, the alignment with *M. smegmatis* LpqM and *M. avium* LpqM would be even better. The black bar over the first 23 amino acids of the *M. smegmatis* LpqM protein sequence is the predicted signal peptide and lipobox (LAACS). Residues 162 to 166 are also indicated by a black bar; they represent the conserved zinc metal binding site (HEXXH).

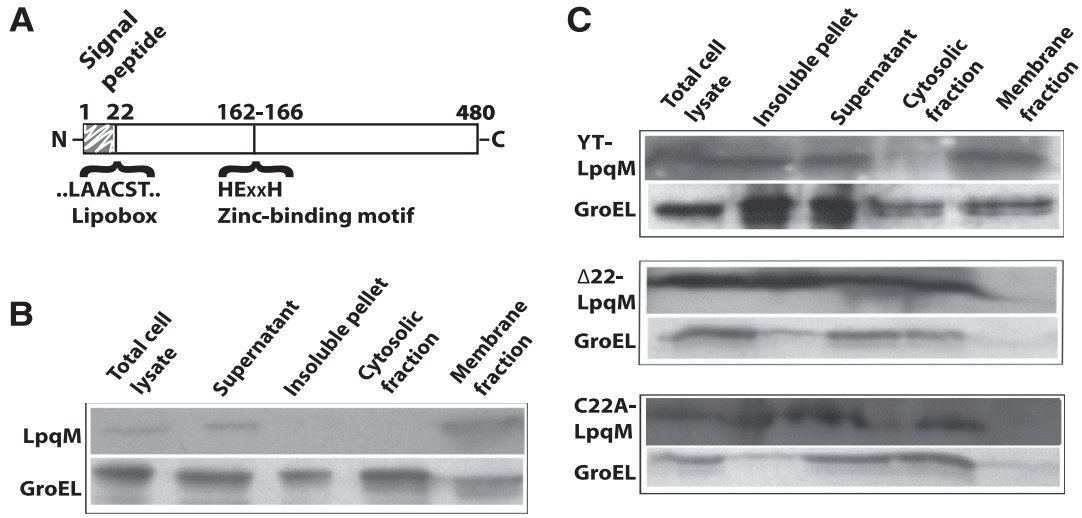

FIG. 3. LpqM is a membrane-associated protein and requires an intact signal peptide and lipobox for membrane localization. (A) Schematic representation of the LpqM protein indicating the locations of the key amino acid motifs that have been subjected to mutagenesis. (B) The wild-type LpqM protein was present in the membrane fraction. The cellular fractionation of LpqM, which used an LpqM–c-Myc derivative, was followed by Western blot analysis employing c-Myc antibodies (see Materials and Methods). Following sonication, the total cell lysate was clarified by centrifugation into the insoluble pellet and supernatant fractions. The supernatant was further fractionated by ultracentrifugation into cytosolic and membrane fractions. As predicted, LpqM was present in the total cell lysate, the lysate supernatant, and the membrane fractions, but not the cytosolic fraction. The soluble GroEL protein is found predominantly in the cytoplasm. Note that the insoluble pellet is from the initial, clarifying spin of the total cell lysate and includes both insoluble proteins and unbroken cells; therefore, this fraction is often contaminated with both LpqM and GroEL. (C) Removal of the signal peptide, i.e., deletion of residues 2 to 22 (Δ22), or a C22A substitution of the essential lipobox cysteine prevents LpqM targeting to the membrane fraction, and instead, it accumulates in the cytosol. However, mutation of the zinc binding catalytic site in the LpqM(YT) derivative does not disrupt membrane targeting.

mutant proteins are similar to wild-type LpqM levels, indicating that the absence of the mutant proteins from the membrane fraction is not due to protein turnover, but rather to the mutation.

LpqM contains a zinc ion binding site essential for DNA transfer. Consistent with its predicted role as a metalloproteinase, LpqM contains a conserved zinc ion binding motif (HEXXH) (Fig. 2 and 3A). The side chains of the two histidine residues in the motif coordinate the binding of the zinc metal ion, while the glutamate residue is thought to play a catalytic role (14). To address whether the predicted HEXXH motif is indeed an important component of the catalytic site and necessary for transfer, we mutated the motif to YTXXH, so as to disrupt both the zinc coordination and catalysis. The LpqM(YT) derivative could not rescue DNA transfer from the Δ*lpqM* donor strain, indicating that zinc binding, and therefore protease activities, is important for transfer (Table 1, row 15). Importantly, LpqM(YT) was expressed at wild-type levels and was found to be associated with the membrane, indicating that the transfer defect was not a consequence of the misfolding or improper targeting of the protein (Fig. 3C).

DISCUSSION

LpqM is the first mycobacterial protein that has been shown to be essential for donor conjugal transfer activity. The requirement for protease catalytic activity was demonstrated by disruption of the zinc metal binding motif, which abolished transfer, despite appropriate expression of LpqM and its localization to the membrane. The requirement of a membrane-associated protease in DNA transfer is consistent with previous models that we have proposed concerning the regulation of conjugation (4, 7). Briefly, we have observed that proteins secreted from both the donor and recipient by the ESX-1 secretory apparatus regulate transfer, and we have suggested that the secreted proteins act as quorum sensors, or as sex pheromones. Thus, through the secretion of different sets of proteins, the donor and recipient cells can respond, if conditions for genetic exchange are appropriate. A possible role for LpqM in conjugation would be to process extracellular proteins into a form able to activate or repress transfer. In this scenario, we envisage LpqM processing either donor or recipient proteins or proteins from both cell types. LpqM would process donor proteins translocated across the cytoplasmic membrane before their release into the surrounding milieu, while recipient proteins would be translocated from the exterior across the outer membrane, before being processed and imported into the donor cell. If recipient proteins were substrates of LpqM, the recipient ESX-1 apparatus could secrete some of these. However, if LpqM processes donor proteins, these would have to be secreted via an apparatus other than ESX-1, given that we have demonstrated that the LpqM requirement is independent of ESX-1 function. The identification of LpqM substrates in future studies will help to refine this hypothesis.

The ability of LpqM homologues to complement the *M. smegmatis lpqM* mutant indicates that LpqM protease activity is conserved across several mycobacterial species. However, the complementation does not necessarily indicate that conjugation occurs in *M. tuberculosis* or *M. avium*; it indicates only that the LpqM protein recognizes and processes similar sub-

strates in each species. It is well established that *M. tuberculosis* secretes proteins that are essential for its virulence, but the targets of these proteins are unknown (3, 20, 26). The ability of the *M. tuberculosis* protein to complement the *M. smegmatis lpqM* mutant suggests that at least some of the proteins processed by the *M. tuberculosis* LpqM protein are of mycobacterial origin and not from the cell it has infected. Again, the identification of LpqM substrates will allow us to test this model further.

The cell envelope of mycobacteria is a thick, lipid-rich, complex structure that forms an important permeability barrier around the cell; it is thought to play a critical role in the biology and pathogenicity of mycobacteria (5). Although the subject of much research, only recently has the envelope been recognized to be composed of multilayered structures that include an outer membrane (11). Thus, the cell envelope is not dissimilar to those of gram-negative bacteria, with two membranes sandwiching a periplasmic space. Determination of the composition of these distinct layers, the functions of the layers, and the process by which molecules (including DNA) are translocated through them is obviously an important goal in the ongoing development of antimycobacterial drugs.

Lipoproteins constitute a major component of the cell envelope in mycobacteria; bioinformatic studies project that the *M. tuberculosis* genome encodes 99 lipoproteins representing 2.5% of the proteome (26). These lipoproteins are thought to be translocated across the cytoplasmic membrane and anchored to structures within the periplasm, although some could instead be anchored to the outer membrane. They are predicted to participate in diverse cellular functions, including transport, cell wall metabolism, cell adhesion, signaling, and protein degradation, and thus, some lipoproteins will play a significant role in virulence. However, only a few of the potential lipoproteins have been shown to be located in the membrane and had their putative activities confirmed. A role for lipoproteins in virulence has been implicated by showing that *lspA* mutants of *M. tuberculosis* are attenuated (21). *lspA* is the lipoprotein signal peptidase responsible for removal of the signal peptide following transacetylation and translocation. The peptidase cleavage generates the mature form of the lipoprotein that is anchored in the membrane. This *lspA* mutant illustrates that lipoprotein processing is important in *M. tuberculosis* virulence. A lipoprotein encoded by the *M. tuberculosis* gene Rv2224 is also essential for virulence, and it is a protease, as our data suggest for LpqM (18). Rv2224 cleaves GroEL2 and releases it into the culture medium. The directed release of proteins processed by Rv2224 into the culture supernatant is similar to the one that we have proposed for the processing and release of donor proteins by LpqM. Regardless of the mechanism, our data now add conjugation to the list of functional roles attributed to mycobacterial lipoproteins.

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