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

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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 smegmatis donor strains used were derivatives of strains mc2155 (24) and MKD158 (resistant to hygromycin [Hyg]! (7) and mc2155 Δesx-1. The mc2155 Δesx-1 strain contains a replacement of the esx-1 genes Msme_0056 to Msme_0082 with a gene encoding Hyg', which was constructed by J. Wang and J. Flint by allele replacement. Briefly, 2-kb segments of DNA upstream of msme_0056 and downstream of msme_0082 were PCR amplified and cloned into the mycobacterial plasmid pPR23, along with a cassette encoding hygromycin resistance; the two amplified segments were cloned in an appropriate orientation for allele replacement. pPR23 is temperature sensitive for replication, and it encodes the counter-selectable marker, Gm r. Hyg r allele replacements were selected for at the nonpermissive temperature in the presence of sucrose. Sucrose-resistant Hyg' Gm r recombinants were purified, and the precise replacement of the esx-1 region with the Hyg' gene was confirmed by Southern analysis and PCR. The recipient strain used was MKD8, which is resistant to streptomycin (Sm') (15). M. smegmatis was grown at 37°C in Trypticase soy broth or Sauton’s medium (0.5 g/liter KH₂PO₄, 0.5 g/liter MgSO₄, 4.0 g/liter l-asparagine, 6% glycerol, 0.05 g/liter ferric ammonium citrate, 2.0
gliter citric acid, and 100 μl 1% ZnSO₄). 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 mc2155 Δexs-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 mc2155 Δexs-1 strain into MKD158, using the generalized transducing phage Bzβ1 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 exs-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 sensitive, and thus, all crosses are carried out at 30°C (15).

**Subcellular fractionation.** Crude cell wall, membrane, and cytosolic fractions were prepared by differential centrifugation as described previously (8, 9, 19). mc2155 was transformed with pUAB200–lpqM–c-Myc. Six hundred milliliters of were prepared by differential centrifugation as described previously (8, 9, 19). Individual insertions were mapped as previously described (7).

**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 exs-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 exs-1 mutants. A defined deletion of the genes

<table>
<thead>
<tr>
<th>Cross</th>
<th>Relevant genotype</th>
<th>No. of transconjugants per donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Δexs-1</td>
<td>1.4 (± 0.1) x 10⁻⁴</td>
</tr>
<tr>
<td>2</td>
<td>Δexs-1 lpqM::Tn</td>
<td>8.8 (± 0.8) x 10⁻⁷</td>
</tr>
<tr>
<td>3</td>
<td>Δexs-1 ΔdinG</td>
<td>8.9 (± 3.4) x 10⁻⁷</td>
</tr>
<tr>
<td>4</td>
<td>Δexs-1 lpqM::Tn</td>
<td>17.0 (± 0.7) x 10⁻⁵</td>
</tr>
<tr>
<td>5</td>
<td>Δexs-1 ΔlpqM</td>
<td>8.5 (± 0.8) x 10⁻⁷</td>
</tr>
<tr>
<td>6</td>
<td>Δexs-1 ΔlpqM pPR23 MsqM</td>
<td>7.5 (± 1.5) x 10⁻⁵</td>
</tr>
<tr>
<td>7</td>
<td>MKD158 (wild-type)</td>
<td>5.1 (± 0.3) x 10⁻⁵</td>
</tr>
<tr>
<td>8</td>
<td>lpqM::Tn</td>
<td>1.3 (± 0.7) x 10⁻⁸</td>
</tr>
<tr>
<td>9</td>
<td>ΔlpqM</td>
<td>1.9 (± 0.8) x 10⁻⁶</td>
</tr>
<tr>
<td>10</td>
<td>ΔlpqM pPR23 MsqM</td>
<td>8.4 (± 1.4) x 10⁻⁶</td>
</tr>
<tr>
<td>11</td>
<td>ΔlpqM pPR23 Mq DrpMp</td>
<td>5.2 (± 0.7) x 10⁻⁷</td>
</tr>
<tr>
<td>12</td>
<td>ΔlpqM pPR23 Mq DrpMp</td>
<td>8.0 (± 0.5) x 10⁻⁷</td>
</tr>
<tr>
<td>13</td>
<td>ΔlpqM pPR23 22q lpqM</td>
<td>7.8 (± 0.2) x 10⁻⁸</td>
</tr>
<tr>
<td>14</td>
<td>ΔlpqM pPR23 22a lpqM</td>
<td>3.2 (± 0.5) x 10⁻⁵</td>
</tr>
<tr>
<td>15</td>
<td>ΔlpqM pPR23 2Y lpqM</td>
<td>6.0 (± 0.6) x 10⁻⁴</td>
</tr>
</tbody>
</table>

Each frequency is the average of at least three crosses, using approximately equal numbers of donor and recipient cells (2 x 10⁸).

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 exs-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 exs-1 mutants. A defined deletion of the genes

**LpqM**

...2722 NGUYEN ET AL. J. BACTERIOL. on October 1, 2017 by guest http://jb.asm.org/ Downloaded from
and is the third gene in a 3-gene operon (Fig. 1). We note that although the gene preceding \textit{lpqM} encodes a putative DNA helicase, DinG, this helicase is not required for transfer. A precise deletion of \textit{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 \textit{lpqM} gene was amplified and cloned into the multicopy, nonintegrating plasmid pPR23, creating pPR23\textit{MspqM}. This plasmid complemented the transfer defect, although only to 10% of wild-type levels (Table 1, row 4). A precise \textit{lpqM} deletion was also generated by allele exchange, resulting in a defect in transfer similar to the defect caused by the transposon insertion in \textit{lpqM}. Again, the transfer defect was rescued by complementation with pPR23\textit{MspqM} (Table 1, rows 5 and 6). The effect of the deletion establishes the requirement for \textit{lpqM} in DNA transfer and rules out potential dominant-negative effects created by the transposon insertion.

\textbf{LpqM affects transfer independently of ESX-1 function and is donor specific.} The \textit{lpqM} mutants described above were isolated in a \textit{Δesx-1} hyperconjugative background. Possibly, the requirement for \textit{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 \textit{Kmr} transposon insertions into the wild-type donor strain, MKD158. In addition, a precise deletion of \textit{lpqM} was generated in MKD158, using the same allele exchange vector as had been used for the \textit{mariner}:\textit{kem} transposon. The segment of DNA cloned for complementation studies included the 150-bp region upstream of \textit{lpqM}, which encompasses the 92-bp intergenic region between \textit{dinG} and \textit{lpqM}.

\textbf{LpqM IS REQUIRED FOR DNA TRANSFER IN \textit{M. SMEGMATIS}}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Schematic map of the \textit{lpqM} region of the \textit{M. smegmatis} genome. The map is based on the genome sequence available from TIGR (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=gm). The vertical arrows indicate the sites of insertion of the \textit{mariner}:\textit{kem} transposon. The segment of DNA cloned for complementation studies included the 150-bp region upstream of \textit{lpqM}, which encompasses the 92-bp intergenic region between \textit{dinG} and \textit{lpqM}.}
\end{figure}

\textbf{LpqM is functionally conserved across species.} Bioinformatic analyses of \textit{LpqM} indicate that it has homologues in other mycobacteria, including \textit{M. tuberculosis} (RV0419 [48.3\% amino acid identity]) and \textit{M. avium} (MAV4736 [47.2\% amino acid identity]) (Fig. 2). There is also an \textit{LpqM} paralogue within \textit{M. smegmatis} (MSMEG4893 [50\% amino acid identity]), but its inability to complement any \textit{lpqM} mutation suggests that it encodes a protein that performs different functions or is a pseudogene. The \textit{M. avium} and \textit{M. tuberculosis} \textit{lpqM} homologues were individually cloned into pPR23 and were then tested for their ability to complement the \textit{ΔlpqM} strain. In each case, transfer activity was partially restored, indicating that functional conservation of \textit{LpqM} activity exists among these mycobacterial species (Table 1, compare rows 9 to 12). Complementation does not restore \textit{LpqM} activity to the wild-type levels, and we believe this is a result of low expression levels of \textit{lpqM} from the complementing vector. The cloned DNA included a 92-bp putative promoter region immediately upstream of \textit{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 \textit{ΔlpqM} mutant.

\textbf{Transfer activity requires the conserved signal peptide motif and membrane targeting.} Computational analyses of the \textit{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 (LAAAC) 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\textit{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 \textit{ΔlpqM} donor strain, and then each mutant derivative was assessed for its ability to complement the deletion of \textit{lpqM}. Both deletion of \textit{LpqM} residues 2 to 22 (\textit{LpqMΔ22}) and substitution of Cys22 by Ala [\textit{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).
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).
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 H9004 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 \textit{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 \textit{M. tuberculosis} protein to complement the \textit{M. smegmatis} \textit{lpqM} mutant suggests that at least some of the proteins processed by the \textit{M. tuberculosis} \textit{LpqM} protein are of mycobacterial origin and not from the cell it has infected. Again, the identification of \textit{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 antitycobacterial drugs.

Lipoproteins constitute a major component of the cell envelope in mycobacteria; bioinformatic studies project that the \textit{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 lipoprotein substrates have been located in the membrane and had their putative activities confirmed. A role for lipoproteins in virulence has been implicated by showing that \textit{lspA} mutants of \textit{M. tuberculosis} are attenuated (21). \textit{lspA} is the lipoprotein signal peptidase responsible for removal of the signal peptide following transacylation and translocation. The peptidase cleavage generates the mature form of the lipoprotein that is anchored in the membrane. This \textit{lspA} mutant illustrates that lipoprotein processing is important in \textit{M. tuberculosis} virulence. A lipoprotein encoded by the \textit{M. tuberculosis} \textit{Rv2224} gene \textit{Rv2224c} modulates innate immune responses. Proc. Natl. Acad. Sci. USA 101: 12598–12603.

REFERENCES


