Genome-Wide Identification of *Mycobacterium tuberculosis* Exported Proteins with Roles in Intracellular Growth[∇][†]

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The exported proteins of *Mycobacterium tuberculosis* that are localized at the bacterial cell surface or secreted into the environment are ideally situated to interact with host factors and to function in virulence. In this study, we constructed a novel β-lactamase reporter transposon and used it directly in *M. tuberculosis* for genome-wide identification of exported proteins. From 177 β-lactam-resistant transposon mutants, we identified 111 different exported proteins. The majority of these proteins have no known function, and for nearly half of the proteins, our demonstration that they are exported when fused to a β-lactamase reporter is the first experimental proof of their extracytoplasmic localization. The transposon mutants in our banked library were of further value as a collection of mutants lacking individual exported proteins. By individually testing each of 111 mutants for growth in macrophages, six attenuated mutants with insertions in *mce1A*, *mce1B*, *mce2F*, *rv0199*, *ctaC*, and *lppX* were identified. Given that much of the *M. tuberculosis* genome encodes proteins of unknown function, our library of mapped transposon mutants is a valuable resource for efforts in functional genomics. This work also demonstrates the power of a β-lactamase reporter transposon that could be applied similarly to other bacterial pathogens.

Mycobacterium tuberculosis is the causative agent of tuberculosis, a devastating disease that kills nearly 2 million people each year (57). Following inhalation, *M. tuberculosis* survives and replicates within phagosomal compartments of macrophages. The phagosome acidification and fusion with lysosomes that normally occur following phagocytosis are blocked by *M. tuberculosis* (44, 56). Bacterial exported proteins, defined here as both surface-exposed and secreted proteins, are ideally positioned to interact with the host and promote this *M. tuberculosis* growth in macrophages.

M. tuberculosis has the conserved Sec and twin-arginine translocation (Tat) pathways for exporting proteins with N-terminal signal sequences beyond the cytoplasmic membrane. *M. tuberculosis* also possesses specialized secretion systems, namely, the SecA2 and ESX systems (35). These specialized systems are not well understood, but at least some of the proteins exported by these pathways lack identifiable export signals. Despite long-standing interest in the exported proteins of *M. tuberculosis*, many exported proteins have yet to be identified experimentally as such, and few have a demonstrated function.

Mutagenesis with a reporter transposon is a classic approach to first identify exported proteins and then use the resulting transposon mutants to test the importance of these proteins in virulence. Tn*phoA* (30) is a reporter transposon used extensively for this purpose. The toxin-coregulated pilus of *Vibrio cholerae* (54) and several other virulence factors (5, 16, 39) were discovered by screening Tn*phoA* mutants. However, Tn*phoA* cannot be used directly in *M. tuberculosis* because of endogenous enzymes that cleave the PhoA substrate (28).

In this study, we used the TEM-1 β-lactamase (BlaTEM-1) as an alternate reporter to identify exported proteins of *M. tuberculosis*. The BlaTEM-1 reporter exploits the fact that β-lactamases must be exported beyond the cytoplasm in order to cleave β-lactam antibiotics before they target the cell wall (53). A truncated ′BlaTEM-1 enzyme that lacks the endogenous signal for export remains cytoplasmic and fails to protect against β-lactams. If a Sec or Tat signal sequence is fused to ′BlaTEM-1, the reporter is exported and protects against β-lactams (34). Because *M. tuberculosis* is naturally resistant to β-lactams, a β-lactam-sensitive *blaC* mutant of *M. tuberculosis* is used with this reporter (17). A significant advantage of a β-lactamase reporter system is that it employs selection for exported fusions, as opposed to the more labor-intensive screen required with PhoA reporters.

By incorporating the ′BlaTEM-1 reporter coding sequence into a *mariner*-based *Himar1* transposon (43), we created Tn′*bla*$_{TEM-1}$, the first reporter transposon that can be used directly in *M. tuberculosis* for genome-wide identification of exported proteins. This reporter transposon allowed us to select in-frame insertions of the transposon in genes encoding exported proteins by simply plating transposition reaction mixtures onto β-lactam-containing agar. Among 177 β-lactam-resistant mutants, 111 different exported *M. tuberculosis* proteins were identified. For nearly half of these proteins,

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
Escherichia coli		
DH5α	K-12 DH5α F⁻ φ80d∆*lacZ*M15 ∆(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17 glnV44 thi-1 gyrA96 relA1*	Gibco-BRL
DH5α λ*pir*	K-12 DH5α F⁻ φ80d∆*lacZ*M15 ∆ (*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17 glnV44 thi-1 gyrA96 relA* λ*pir*	W. R. Jacobs, Jr.
Mycobacterium		
mc²155	*M. smegmatis ept-1*	48
H37Rv	Virulent *M. tuberculosis*	3
PM638	H37Rv ∆*blaC*	17
MBTB126	PM638 carrying integrating vector pJES137	This study
MBTB204	PM638 with a Tn′*bla*_TEM-1_ insertion in *mce1A* (Rv0169) at amino acid 449 of 455	This study
MBTB190	PM638 with a Tn′*bla*_TEM-1_ insertion in *mce1B* (Rv0170) at amino acid 223 of 337	This study
MBTB183	PM638 with a Tn′*bla*_TEM-1_ insertion in *rv0199* at amino acid 74 of 220	This study
MBTB156	PM638 with a Tn′*bla*_TEM-1_ insertion in *mce2F* (Rv0594) at amino acid 476 of 517	This study
MBTB381	PM638 with a Tn′*bla*_TEM-1_ insertion in *ctaC* (Rv2200c) at amino acid 351 of 364	This study
MBTB301	PM638 with a Tn′*bla*_TEM-1_ insertion in *lppX* (Rv2945c) at amino acid 82 of 234	This study
Plasmids		
pCR2.1	*bla aph* ColE1; TA cloning vector	Invitrogen
pMV261.kan	*aph* P_hsp60_ *oriM* ColE1; multicopy mycobacterial shuttle plasmid	51
pJES102	*aph* P_hsp60_-′*bla*_TEM-1_ (*E. coli*) *oriM* ColE1; ′*bla*_TEM-1_ from pCR2.1 cloned into pMV261.kan	34
pSH1	Tn*Himar1 oriR6K*g Hygʳ; shuttle vector carrying the *Himar1* transposon	27
pJES123	*hygR oriR6K* ′*bla*_TEM-1_; intermediate cloning vector	This study
pJES124	*hygR oriR6K* ColE1 *aph* Tn′*bla*_TEM-1_ Tnpmar λ*cos*; shuttle vector carrying Tn′*bla*_TEM-1_	This study
pJES137	*hyg int attP* ColE1 P_hsp60_ ′*bla*_TEM-1_; integrating hygromycin-marked vector with constitutively expressed ′BlaTEM-1	This study
pJES153	*aph* P_hsp60_-*mce2F oriE oriM*; multicopy kanamycin-marked vector with constitutively expressed *mce2F*	This study
pJES178	*aph* P_hsp60_-*rv0199 oriE oriM*; multicopy kanamycin-marked vector with constitutively expressed Rv0199	This study

the demonstration that they promoted export of a fused ′BlaTEM-1 protein was the first experimental proof of their subcellular location or topology. At the same time the reporter transposon identified an exported protein, it provided an insertion mutant in the respective open reading frame (ORF). Therefore, our library of transposon mutants also represents a valuable resource for assigning function to unknown exported proteins. In this study, we screened each of 111 unique Tn′*bla*_TEM-1_ insertion mutants for intracellular growth phenotypes in bone marrow-derived macrophages. Six mutants defective for growth in macrophages were obtained. Thus, our library of mapped Tn′*bla*_TEM-1_ mutants not only defined the subcellular location of 111 unique proteins but also provided a useful mutant collection for evaluation in functional assays.

MATERIALS AND METHODS

Bacterial strains and culture. Cultivation of *M. tuberculosis* strains was performed as described previously (8). Where needed, the growth medium was supplemented with 50 μg/ml hygromycin (Roche), 20 μg/ml kanamycin (Acros Chemicals), and 20 μg/ml carbenicillin (Sigma). Tween was not included in carbenicillin-containing Middlebrook 7H10 agar. *E. coli* strains were grown in Luria-Bertani medium (LB; Fisher) with 150 μg/ml hygromycin, 40 μg/ml kanamycin, and 100 μg/ml carbenicillin. *M. tuberculosis* strains used in this study were derivatives of PM638 (17). Strains and plasmids used in this study, including the attenuated mutants identified, are listed in Table 1. The complete list of transposon mutants collected is provided in Table S1 in the supplemental material.

Construction of Tn′*bla*_TEM-1_ and complementation vectors. All PCRs were done with an Expand Hi-Fidelity PCR kit (Roche) and 2 to 5% dimethyl sulfoxide (DMSO). The transposon Tn′*bla*_TEM-1_ was constructed as follows. The *bla*_TEM-1_ gene was PCR amplified without its signal sequence (′*bla*_TEM-1_) from pJES102 (34), using primers XbaTem-1F (5′-ATCTAGACCAGAAACGCCTG GTGAA-3′) and SpeITemRev (5′-GACTAGTGCTGGATCCGCAATTGTCT TGG-3′). The resulting product was cloned into pCR2.1 (Invitrogen), creating pJES122. To insert ′*bla*_TEM-1_ into the *Himar1* transposon, we used plasmid pSH1 (27). pSH1 carries a modified *Himar1* transposon that contains within its inverted repeats an *oriR6K* origin of replication and a hygromycin resistance gene (Fig. 1). Outside the transposon, pSH1 contains the *mariner* transposase gene (Tnpmar). pSH1 additionally contains a PacI site and a λcos sequence to facilitate cloning of the transposon to mycobacteriophages. pSH1 was digested with XbaI, resulting in a 2.1-kb band and a 3.1-kb band. The 2.1-kb band was ligated to a SpeI and XbaI ′*bla*_TEM-1_ fragment of pJES122. The resulting plasmid was pJES123. This ligation abolished the XbaI site at the 3′ end of the ′*bla*_TEM-1_ fragment. pJES123 was then digested with XbaI and ligated to the 3.1-kb XbaI fragment of pSH1, containing
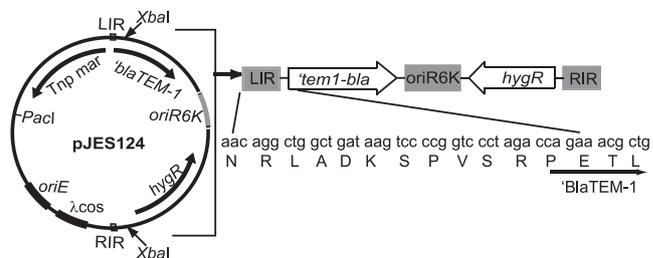

FIG. 1. Map of Tn′*bla*_TEM-1_. Plasmid pJES124 contains the signal sequence-less ′*bla*_TEM-1_ gene cloned downstream of the left inverted repeat (LIR) of a *Himar1*-based mariner transposon derived from pSH1. The codon sequence of the inverted repeat is shown in the same reading frame as the starting codons of ′BlaTEM-1. Tnpmar, mariner transposase; *hygR*, hygromycin resistance gene.

Tnpmar. In this manner, 'bla$_{TEM-1}$ was cloned directly downstream of the left inverted repeat of the transposon in the final vector, pJES124 (Fig. 1).

Construction of Tn'bla$_{TEM-1}$ delivery phage. Plasmid pJES124 was cloned into a nonessential region of the conditionally replicating mycobacteriophage phAE159 as described previously (8), with minor modifications. phAE159 DNA was digested with PacI and ligated to PacI-linearized pJES124. The ligated phasmid was then packaged into λ phage heads via the λcos sites present on pJES124, using a Max Plax kit (Epicentre). The λ packaging step allowed recovery of the newly ligated phasmid following transduction into *E. coli*. Phasmid DNA phAE159::Tn'bla$_{TEM-1}$ was isolated from hygromycin-resistant *E. coli* and electroporated into *Mycobacterium smegmatis* mc²155. Electroporated cells were mixed with late-log-phase *M. smegmatis* in top agar, poured onto solid agar, and incubated at the permissive temperature of 30°C. A single plaque was isolated, tested for temperature sensitivity, and expanded by permissive growth in *M. smegmatis*. A high-titer lysate of phAE159::Tn'bla$_{TEM-1}$ (>10^{10} PFU per ml) was prepared.

Transposon mutagenesis in *M. tuberculosis*. Transposon mutagenesis of *M. tuberculosis* strain PM638 was done via phage infection as described previously (8). A small aliquot of each transposition reaction mix was plated on 7H10 agar containing 50 μg/ml hygromycin to estimate the total number of transposition events for each reaction. The remainder of each transposition reaction mix was plated on 7H10 agar containing 50 μg/ml hygromycin and 20 μg/ml carbenicillin. Individual carbenicillin-resistant colonies were either patched or replated onto 7H10-hygromycin, with or without carbenicillin, to confirm β-lactam resistance.

We calculated the number of transposon mutants needed to evaluate every gene in the genome by using the following equation: $N = [\ln(1 - P)/\ln(1 - f)] \times 6$ reading frames, where $N =$ the number of transposon insertion mutants required to achieve a probability of P that every 1-kb ORF is represented and $f =$ the fraction of the genome represented by each 1-kb gene (45). We determined the likelihood that additional ORFs could still be identified by using the following Poisson-derived equation: $f[0] = e^{-m}$, where $f[0]$ represents the still-mutable genes that may potentially be recovered and m is the mean number of hits already recovered per gene (18).

Identification of transposon insertion sites. Genomic DNAs were isolated from transposon mutants by use of a guanidinium protocol as described previously (38). Genomic DNA was digested with BssHII and self-ligated, producing a plasmid containing the transposon and flanking *M. tuberculosis* genomic DNA. These plasmids were recovered by transformation into *E. coli* DH5α λpir and selection on LB agar with hygromycin. Resulting plasmid DNAs were then sequenced to map the insertion sites. The following bioinformatic algorithms were used to predict export signals: TMHMM (49), TopPred (13), TMPRED (19), SignalP (6), Psort (37), LipoP (22), and TatP (7).

Complementation of selected *M. tuberculosis* Tn'bla$_{TEM-1}$ mutants. The gene encoding Rv0199 was PCR amplified from *M. tuberculosis* H37Rv genomic DNA by use of primers EcoRV199F (5'-AGATATCCAATGCCTGACGGGGAGCA GAGC-3') and Rv0199Rev (5'-ACGTTCGAAACCCACCACAG-3'). The amplified product was cloned into pCR2.1 (Invitrogen), digested with EcoRV and HindIII, and then ligated into MscI-HindIII-digested pMV261.kan. The resulting plasmid, carrying Rv0199 on a multicopy expression vector, was named pJES178 (Table 1). The gene encoding Mce2F was PCR amplified from *M. tuberculosis* H37Rv genomic DNA by use of the following primers: mce2FecoRVfor1 (5'-CGATATCACATGCTGACTCGCGCTATCG-3') and mce2FnheRev1 (5'-CGCTAGCTCAGCCGGTTGGTGCCAGCATC-3'). The amplified product was cloned into pCR2.1 and digested with EcoRV and NheI. The resulting mce2F-containing fragment was ligated into pMV261.kan that had been digested with MscI and NheI, generating pJES153. pJES178 and pJES153 DNAs were electroporated into the *M. tuberculosis* Rv0199::Tn'bla$_{TEM-1}$ and mce2F::Tn'bla$_{TEM-1}$ transposon mutant strains to generate complemented strains (8).

Macrophage infections. Murine bone marrow-derived macrophage infections were completed as described previously (25). Briefly, bone marrow-derived macrophages were obtained from 6- to 24-week-old female C57BL/6 mice and seeded into 8-well chamber slides at 2×10^5 cells/well 24 h prior to infection. The indicated *M. tuberculosis* strains were inoculated from freezer stocks grown to mid-exponential phase, washed once in phosphate-buffered saline (PBS)–0.05% Tween 80, resuspended in Dulbecco's modified Eagle's medium (DMEM), and added to macrophages at a multiplicity of infection (MOI) of 1. Following a 4-h uptake period, infected cells were washed with warm DMEM three times. A subset of wells were then lysed, diluted, and plated on 7H10 agar to determine uptake. Remaining infected wells were lysed, diluted, and plated at 5 days postinfection to determine intracellular bacterial growth.

TABLE 2. Description of *M. tuberculosis* Tn'bla$_{TEM-1}$ transposon library

Parameter	Value
Total no. of transposon mutants[a]	81,000
No. of confirmed carbenicillin-resistant insertion mutants	177
No. of in-frame insertions in ORFs	172
No. of unique ORFs identified	111
No. of ORFs with standard Sec signal sequence	24
No. of ORFs with lipoprotein-type Sec signal sequence	15
No. of ORFs with Tat signal sequence	3
No. of ORFs with transmembrane domains[b]	69
No. of ORFs with homologues only in mycobacteria	41
No. of ORFs with homologues only in pathogenic mycobacteria[c]	16
No. of ORFs with no known or predicted function[d]	66

[a] Estimated by plating a fraction of each transposition reaction mix on agar containing hygromycin only.

[b] Includes proteins with at least one predicted transmembrane domain. Proteins with transmembrane domains plus a Sec, Tat, or lipoprotein-type signal sequence are most likely integral membrane proteins, and they are counted in this category.

[c] Pathogenic mycobacteria searched included *M. leprae*, *M. bovis*, *M. marinum*, *M. ulcerans*, and *M. avium*.

[d] Functions of individual ORFs are provided in Table S1 in the supplemental material.

RESULTS AND DISCUSSION

Mutagenesis with the Tn'bla$_{TEM-1}$ reporter transposon. Our objective was to develop a reporter transposon that could be used for large-scale identification of exported proteins in *M. tuberculosis*. We selected the β-lactamase 'bla$_{TEM-1}$ reporter for this purpose, as we previously established it to work directly in *M. tuberculosis* to report on proteins exported by either Sec or Tat pathways (34). For the transposon, we chose the *mariner*-based *Himar1* transposon because of its low degree of site specificity—requiring only a TA dinucleotide sequence for insertion—and its prior successful use in *M. tuberculosis* (46). To construct the reporter transposon, we inserted the truncated 'bla$_{TEM-1}$ reporter directly downstream of the naturally existing open reading frame running through the left inverted repeat of a modified *Himar1* transposon carried on plasmid pSH1 (27) (Fig. 1). Important features of this *Himar1* transposon are the presence of a hygromycin resistance gene and an *oriR6K* origin of replication, which later enabled recovery of the transposon insertion site on a plasmid. A recombinant mycobacteriophage, phAE159::Tn'bla$_{TEM-1}$, was then constructed and used to deliver the reporter transposon into the β-lactam-sensitive *blaC* mutant of *M. tuberculosis* PM638 (17). The average transposition frequency per phage infection was 1.2×10^{-6} transposon mutant/input bacillus, which is similar to the frequency reported for other phage-delivered *Himar* transposons in *M. tuberculosis* (4). Transposition events that yielded in-frame insertions of Tn'bla$_{TEM-1}$ in an ORF for an exported protein produced an exported β-lactamase fusion protein. We selected exported 'BlaTEM-1 fusions by directly plating the transposition reaction mix on agar containing hygromycin plus the β-lactam antibiotic carbenicillin. The average frequency of recovering β-lactam-resistant colonies per phage infection was 1×10^{-8}.

Collection of Tn'bla$_{TEM-1}$ library in *M. tuberculosis*. From an estimated 81,000 transposon mutants, we selected 177 mutants and confirmed their carbenicillin resistance (Table 2; see Table

S1 in the supplemental material). Because Tn'*bla*$_{TEM-1}$ carries the *oriR6K* origin of replication and the hygromycin resistance gene, the transposon insertion site of each mutant was rescued as a plasmid recovered from genomic DNA by restriction endonuclease digestion, self-ligation, and transformation into *E. coli*. The transposon insertion site on the recovered plasmids was then identified by DNA sequencing. Of the 177 carbenicillin-resistant mutants, only 5 had insertions that were out of frame or not in an ORF, and these transposon mutants are not discussed further. The remaining 172 mutants had Tn'*bla*$_{TEM-1}$ inserted in frame in an ORF. Some ORFs were identified more than once, resulting in a total of 111 different ORFs identified. Each mutant was stocked to create a sequence-defined library of *M. tuberculosis* transposon mutants. Domain prediction algorithms revealed each of the 111 ORFs to possess some type of predicted export signal: a cleavable standard Sec signal sequence, a cleavable lipoprotein-type Sec signal sequence, a cleavable Tat signal sequence, and/or a transmembrane (TM) domain (Table 2; see Table S1) (see Materials and Methods for the algorithms used). The fact that nearly every β-lactam-resistant mutant possessed an in-frame insertion in an ORF with features consistent with export demonstrated the specificity and selection power of this reporter.

For 43% of the ORFs we identified with Tn'*bla*$_{TEM-1}$, this was the first experimental evidence that the corresponding protein is exported by *M. tuberculosis* (see Table S1 in the supplemental material). Of the exported ORFs identified, 37% had homologues only in other mycobacteria and 14% had homologues only in pathogenic *Mycobacterium* spp. (Table 2; see Table S1). Emphasizing how little is known about *M. tuberculosis* exported proteins, 59% of the ORFs we identified had no known or predicted function (see Table S1). The demonstration of export, especially for these unknown proteins, is significant. Establishing the location of a protein is an important step in determining its biological function and molecular interactions.

For integral membrane proteins, the site of an active Tn'*bla*$_{TEM-1}$ insertion provides additional information about protein topology, since active insertions must be fused to domains localized on the extracytoplasmic side of the membrane. Prediction programs often disagree on the number and location of TM domains. Thus, experimental evidence is critical to establishing the topology of integral membrane proteins. For the 69 membrane proteins identified in this study, the insertion site mapped an extracytosolic domain. While the degree of saturation in our transposon library was insufficient to map the full topology of membrane proteins, there were some integral membrane proteins with multiple independent Tn'*bla*$_{TEM-1}$ insertions. One such protein was MmpL4, a protein with an undefined role in *M. tuberculosis* pathogenesis (15, 26, 46). By combining the sites of active Tn'*bla*$_{TEM-1}$ insertions with earlier data obtained using *M. smegmatis* to identify exported protein domains of MmpL4 (9), a total of seven extracytoplasmic sites could be delineated in this protein (see Fig. S1 in the supplemental material). These experimental data help to build a relatively refined topology map for this virulence factor of unknown function.

At the start, we calculated that evaluation of 80,000 transposon insertion mutants would give a 95% probability that all genes of an average size of 1 kb in the 4.4-Mb *M. tuberculosis* genome would be represented by at least one transposon insertion in the proper reading frame (see Materials and Methods). After selecting 177 β-lactam-resistant transposon insertions from 81,000 transposon mutants, a Poisson calculation on the number of times we repeatedly identified the same ORF predicted that new ORFs could still be identified among future carbenicillin-resistant mutants. Thus, even more exported *M. tuberculosis* proteins could be identified in the future. However, as with any experimental approach, there will always be some exported proteins that we cannot identify with this method. Proteins that are essential or not expressed during *in vitro* growth at a level sufficient for a fusion protein to promote growth on selective media will be missed. While our reporter transposon identifies proteins with N-terminal signal sequences, it misses proteins with C-terminal export signals. This is because the '*bla*$_{TEM-1}$ stop codon on the transposon truncates the C-terminal domains of proteins. Finally, we will miss exported proteins that are incompatible with the 'BlaTEM-1 reporter itself. In fact, the unconventional exported proteins Esat-6 and SodA, which are exported by the *M. tuberculosis* ESX-1 (1) and SecA2 (41) systems, respectively, appear to fall into this category. We did not identify these proteins with the reporter transposon, and when N-terminal or C-terminal fusions between 'BlaTEM-1 and Esat-6 or SodA were tested directly, they failed to produce β-lactam resistance, despite the fusions being produced at high levels (as determined by immunoblotting [data not shown]). A possible explanation for this incompatibility is that 'BlaTEM-1 is not exported or enzymatically active when fused to these unconventional exported proteins.

Screening the Tn'*bla*$_{TEM-1}$ library for mutants with growth defects in macrophages. In addition to defining the subcellular locations of proteins, our mutant collection is a valuable resource for efforts to assign functions to ORFs. To demonstrate this utility, we took an unbiased approach and screened our library of mutants, each with a transposon insertion in a gene encoding an exported protein, for growth in macrophages. A representative transposon mutant for each of the 111 exported ORFs identified was tested in duplicate for growth in resting murine bone marrow-derived macrophages. For ORFs that received multiple hits, a representative transposon mutant with the most 5′ insertion site was tested. The growth of each transposon mutant was compared to that of *M. tuberculosis* MBTB126, which is the *blaC M. tuberculosis* parent engineered to express the nonexported 'BlaTEM-1 reporter. Among 111 transposon mutants tested, 6 mutants had reproducible and statistically significant intracellular growth defects. These mutants had Tn'*bla*$_{TEM-1}$ insertions in one of the following genes: *mce1A*, *mce1B*, *mce2F*, *rv0199*, *ctaC*, and *lppX* (see Fig. 2 to 5). All six of these attenuated mutants were tested for growth in broth culture, and all grew at the same rate as the MBTB126 parent strain (data not shown). As discussed below, the macrophage phenotypes of two of these mutants were complemented, proving that the transposon insertions were the cause of the phenotypes. For the remaining four mutants, complementation experiments are pending. Therefore, we cannot yet say with certainty that the disrupted ORF was the cause of the intracellular growth defect.

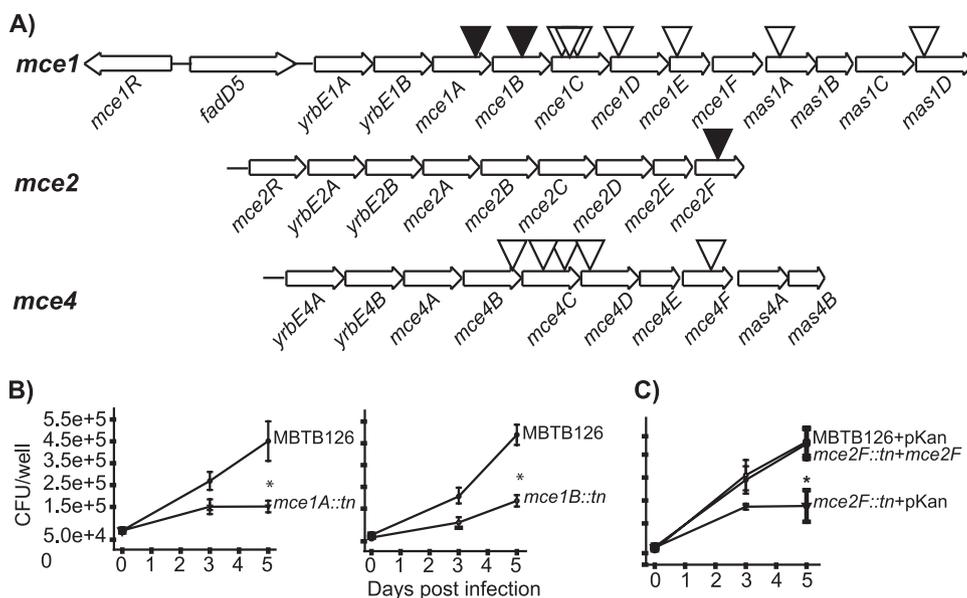For some of these mutants, our analysis was the first suggestion that the ORF promotes growth in macrophages. For

FIG. 2. Evaluation of *M. tuberculosis* mce::Tn'*bla*$_{TEM-1}$ mutants in macrophages. (A) Representation of *mce1*, *mce2*, and *mce4* operons in *M. tuberculosis* H37Rv. Black triangles indicate Tn'*bla*$_{TEM-1}$ insertion mutants with macrophage growth defects. White triangles mark Tn'*bla*$_{TEM-1}$ insertion mutants that were not attenuated. The *mce1* operon has two possible start sites and spans from either *fadD5* or *yrbE1A* to *mas1D* (12, 20). The *mce2* and *mce4* operons are transcribed in a single transcript comprised of the genes shown (11, 24). (B) Murine bone marrow-derived macrophages were infected with *M. tuberculosis* MBTB126, the *mce1A*::Tn'*bla*$_{TEM-1}$ mutant, or the *mce1B*::Tn'*bla*$_{TEM-1}$ mutant. At 4 h (day 0) and 3 and 5 days postinfection, macrophages were lysed and plated to enumerate intracellular bacteria. Error bars indicate standard deviations (SD) of the means. (C) MBTB126 carrying pMV261.kan, the *mce2F*::Tn'*bla*$_{TEM-1}$ mutant carrying pMV261.kan, and the *mce2F*::Tn'*bla*$_{TEM-1}$ mutant carrying a multicopy complementing vector expressing *mce2F* from a constitutive promoter (pJES153) were used to infect macrophages as described for panel B. ∗, $P \leq 0.05$. Each graph shows representative data from one of at least three independent replicate experiments.

other mutants, the ORF was predicted to have a role in intracellular growth by transposon site hybridization (TraSH) with a saturating pool of *M. tuberculosis* mutants (40) or a pool of 2,500 *Mycobacterium bovis* BCG transposon mutants (50). For mutants that fell into the second category, our direct testing provided necessary validation of genes predicted earlier as being important in macrophages.

Mce1A, Mce1B, and Mce2F. Each of the four *mce* operons (*mce1* to -4) in the *M. tuberculosis* genome contains six genes encoding Mce protein family members (MceA to MceF) and two YrbE homologues (12). In our Tn'*bla*$_{TEM-1}$ library, we obtained 15 unique transposon insertions in 13 genes in *mce* operons (Fig. 2A). For six of these ORFs, our identification with the reporter transposon is the first demonstration that the protein is exported (see Table S1 in the supplemental material). The function of Mce family members is still unclear, but data suggest that they function as transporters, which is consistent with an extracytoplasmic localization (21, 36).

When tested in macrophages, our Tn'*bla*$_{TEM-1}$ insertion mutants in *mce1A* and *mce1B* were attenuated for intracellular growth (Fig. 2B). In published studies, there is debate about the phenotype of *mce1* mutants. An allelic exchange mutant in which expression of the entire *mce1* operon was defective was reported to grow better than wild-type *M. tuberculosis* in macrophages (47). However, TraSH analysis of pools of *M. tuberculosis* mutants predicts that transposon mutants with insertions in genes spanning *mce1B* to *mce1F* are defective in macrophages (40). Similarly, hybridization analysis of pooled *M. bovis* BCG transposon mutants predicts that the majority of *mce1* genes contribute to intracellular growth (50). Our results

agree with the latter predictions. However, unlike the predictions obtained with pooled mutant screens, when we tested Tn'*bla*$_{TEM-1}$ insertions in genes downstream of *mce1B*, we did not observe macrophage growth defects. We speculate that mutations downstream in the *mce1* operon have more subtle defects that are not evident when tested individually but are revealed when tested in competition in pools of mutants. Another possibility is that the site of these *mce1* downstream insertions failed to disrupt function; however, several insertions in *mce1* were positioned early in an ORF, making this an unlikely explanation for all cases (Fig. 2A; see Table S1).

We have yet to complement the *mce1A* and *mce1B* transposon mutant phenotypes to rule out the possibility of polar effects on downstream genes in the *mce1* operon (spanning genes *fadD5* to *mas1D*) (12) (Fig. 2A). While not giving direct proof, the lack of macrophage phenotypes with transposon insertions in downstream *mce1* genes suggests that the impact of polar effects in our experiments is minimal. Published attempts to complement individually disrupted *mce1* genes were unsuccessful, suggesting that complementation of our *mce1* mutants may be a challenge (47).

A Tn'*bla*$_{TEM-1}$ insertion mutation in *mce2F* also led to a growth defect in macrophages (Fig. 2C). A complementation experiment in which a wild-type copy of *mce2F* was expressed from the *hsp60* promoter in the *mce2F*::Tn'*bla*$_{TEM-1}$ mutant restored intracellular growth, demonstrating that the mutant phenotype was due to the lack of Mce2F. This is the first demonstration of Mce2F being important in macrophage growth. The *mce2F* gene was not predicted to be important in macrophages among pooled *M. bovis* BCG strains (50) or by
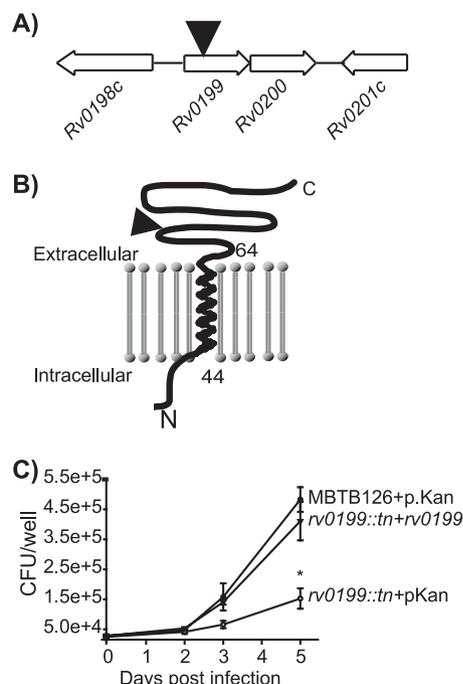
FIG. 3. *M. tuberculosis rv0199*::Tn′*bla*$_{TEM-1}$ has a growth defect in macrophages. (A) Organization of genes surrounding Tn′*bla*$_{TEM-1}$ insertion (triangle) in *rv0199*. (B) Membrane topology of Rv0199 predicted by the TMHMM algorithm (49). The site of ′BlaTEM-1 fusion is indicated with a triangle. (C) Macrophages were infected with MBTB126 carrying the empty vector pMV261.kan, the *rv0199*::Tn′*bla*$_{TEM-1}$ mutant carrying pMV261.kan, or the *rv0199*::Tn′*bla*$_{TEM-1}$ mutant carrying a multicopy complementing vector expressing *rv0199* from a constitutive promoter (pJES178). Error bars indicate SD of the means. ∗, $P \le 0.05$. The graph shows representative data from one of at least three independent replicate experiments.

FIG. 4. *M. tuberculosis ctaC*::Tn′*bla*$_{TEM-1}$ has a growth defect in macrophages and a colony phenotype on agar plates. (A) Relative location of *ctaC* in the *M. tuberculosis* genome. The black triangle indicates the site of Tn′*bla*$_{TEM-1}$ insertion. (B) Membrane topology of CtaC predicted by the TMHMM algorithm (49). The site of ′BlaTEM-1 fusion is indicated with a triangle. (C) Macrophage infection as described in the legend for Fig. 3. Error bars indicate SD of the means. ∗, $P \le 0.05$. The graph shows representative data from one of at least three independent replicate experiments. (D) *M. tuberculosis* MBTB126 and the *ctaC*::Tn′*bla*$_{TEM-1}$ mutant were plated on 7H10 agar plates supplemented with Tween 80. Colonies were examined after 25 days at 37°C.

TraSH analysis of *M. tuberculosis* (40). Furthermore, an *mce2* mutant of H37Rv was recently reported to grow normally in the RAW cell line, although the mutant was attenuated in mice (31).

We also recovered Tn′*bla*$_{TEM-1}$ insertions in five different genes in the *mce4* operon (Fig. 2A; see Table S1 in the supplemental material), but none of the resulting mutants was attenuated for growth in macrophages. While Mce4 components are important for bacterial growth in the mouse model of infection (21, 46), our results agree with the TraSH analysis predicting that *mce4* is not important for growth in macrophages (40).

Rv0199. Rv0199 is a protein with limited homology to some Mce-associated proteins but with no predicted function. However, Rv0199 is conserved in all mycobacteria examined to date (32). The Tn′*bla*$_{TEM-1}$ insertion positioned the ′BlaTEM-1 reporter after a single predicted TM domain at the N terminus in Rv0199 (Fig. 3). This indicates that the majority of Rv0199 is located on the extracytoplasmic side of the membrane. The *rv0199*::Tn′*bla*$_{TEM-1}$ mutant was defective for growth in macrophages, and this intracellular growth defect was complemented by addition of a plasmid expressing *rv0199* from the *hsp60* promoter (Fig. 3C). Rv0199 was not predicted to be important in macrophages by TraSH analysis (40), although it was predicted by another TraSH analysis to be important in a
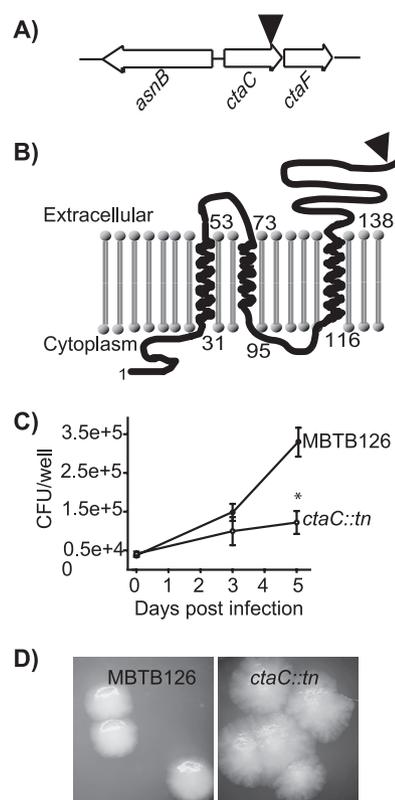
mouse infection model (46). In contrast, Rv0199 was predicted on the basis of pooled infections to be needed for *M. bovis* BCG to grow in macrophages (50).

CtaC. Our *ctaC*::Tn′*bla*$_{TEM-1}$ mutant had a growth defect in macrophages (Fig. 4C). The *ctaC* gene encodes subunit II of the cytochrome *c* oxidase, which is important for growth under aerobic conditions (23). Along with TM and signal sequence predictions (6, 49), the site of our active Tn′*bla*$_{TEM-1}$ insertion indicates that the C terminus of CtaC, which contains the cytochrome oxidase domain, is on the extracytoplasmic face of the membrane.

CtaC is predicted to be essential in *M. tuberculosis* H37Rv, and an attempt to delete the gene in *M. tuberculosis* was unsuccessful (33). However, we collected two identical *ctaC*::Tn′*bla*$_{TEM-1}$ mutants, and they did not have growth defects in liquid (data not shown). This is likely due to the ′BlaTEM-1 reporter truncating only the final 13 amino acids of the C terminus. Colonies of the *ctaC*::Tn′*bla*$_{TEM-1}$ mutant were phenotypically rough and spread out in comparison to parental MBTB126 colonies (Fig. 4D).
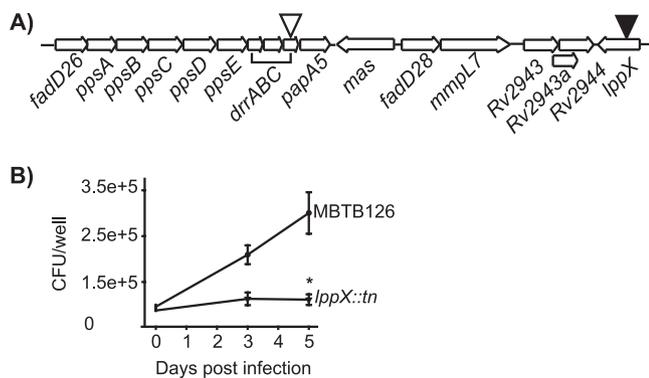
FIG. 5. *M. tuberculosis lppX*::Tn'*bla*_TEM-1 has a growth defect in macrophages. (A) Representation of insertions in the PDIM locus of *M. tuberculosis*. The black arrowhead represents a Tn'*bla*_TEM-1 insertion that resulted in attenuation in macrophages. The white arrowhead indicates a Tn'*bla*_TEM-1 insertion that did not lead to attenuation. (B) Murine bone marrow-derived macrophages were infected with *M. tuberculosis* MBTB126 and the *lppX*::Tn'*bla*_TEM-1 mutant as described in the legend to Fig. 3. Error bars indicate SD of the means. *, $P \leq$ 0.05. Each graph shows representative data from one of at least three independent replicate experiments.

Until complementation analysis is conducted, we can only speculate on the basis of attenuated mutant phenotypes in macrophages. For the *ctaC*::Tn'*bla*_TEM-1 mutant, perhaps the mutant has modestly compromised CtaC activity that is problematic to intracellular growth but not to *in vitro* growth. Another possibility is that the intracellular growth defect of the *ctaC*::Tn'*bla*_TEM-1 mutant is not caused by the insertion in *ctaC* but is due to polar effects of the transposon (Fig. 4A). The *ctaF* gene is directly downstream of *ctaC*. CtaF shares homology with predicted cytochrome *c* subunit proteins in other mycobacteria and *Rhodococcus* (2). It is possible that CtaF works in concert with CtaC in a multisubunit cytochrome *c* oxidase in mycobacteria, or it may function in another capacity.

LppX. We identified active Tn'*bla*_TEM-1 insertions in *drrC* and *lppX*. These genes are near each other in the genome and encode proteins required for translocation of the mycobacterium-specific lipid phthiocerol dimycocerosate (PDIM) (10, 14, 55) to the bacterial surface (10, 14, 52) (Fig. 5A).

Our *lppX*::Tn'*bla*_TEM-1 mutant was defective for growth in macrophages (Fig. 5B). A macrophage phenotype was not, however, observed with our *drrC*::Tn'*bla*_TEM-1 mutant, even though this mutant had an insertion truncating the protein at amino acid 57 (Fig. 5A). To our knowledge, our results are the first to report a macrophage phenotype for an *lppX* mutant. While PDIM-deficient mutants are attenuated in virulence in a mouse model of infection, a *fadD26* mutant completely devoid of PDIM was not reported to have a defect in resting macrophages (42). Furthermore, TraSH does not predict *lppX* to be important for growth in macrophages (40).

Conclusions. In this work, we utilized a new reporter transposon for large-scale identification of exported proteins of *M. tuberculosis*. We established the subcellular locations of 111 *M. tuberculosis* proteins and simultaneously collected a library of mapped mutants, each with an insertion in an exported ORF, for subsequent analyses. An important feature of our transposon reporter strategy is that it operates as a selection, not a

screen. Screening for an exported fusion phenotype is required with other reporters of protein export, such as PhoA (9), and is a labor-intensive approach that limits the number of colonies that can be surveyed. A positive-selection process made it possible for us to collect in-frame reporter fusions to exported proteins from 81,000 random transposon mutants. Given the remarkable specificity and selection exhibited by our β-lactamase reporter transposon, similar reporters should be considered for comprehensive analyses of exported proteins in other pathogens.

We found that 59% of the exported proteins we identified have no defined or predicted function (see Table S1 in the supplemental material), whereas only 27% of ORFs in the *M. tuberculosis* genome are annotated as having an unknown function (http://tuberculist.epfl.ch/). This discrepancy emphasizes how little we know about *M. tuberculosis* exported proteins. The library of mapped transposon mutants reported here is a valuable resource for assigning functions to these unknown exported proteins and for identifying new proteins important to pathogenesis. Furthermore, the mutant collection is of a size where it is feasible to rigorously screen mutants individually for phenotypes. For some of the mutants we identified as attenuated in macrophages (*ctaC*, *mce2F*, and *lppX* mutants), this was the first time that such a phenotype was observed. For other mutants, our results provide necessary validation of predictions from hybridization-based assessment of mutant pools or address conflicting reports (for *mce1A*, *mce1B*, and *rv0199*) (21, 29, 40, 46, 47, 50). Importantly, complementation analysis of the *mce2F* and *rv0199* mutants provides a foundation for unraveling the functions of two new *M. tuberculosis* exported proteins with roles in macrophage growth.

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