Enhanced Priming of Adaptive Immunity by Mycobacterium smegmatis Mutants with High-Level Protein Secretion

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Mycobacteria have features that make them attractive as potential vaccine vectors. The nonpathogenic and rapidly growing Mycobacterium smegmatis can express both Mycobacterium tuberculosis antigens and heterologous antigens from other pathogens, and it has been used as a viable vector for the development of live vaccines. In order to further improve antigen-specific immunogenicity of M. smegmatis, we screened a random transposon mutant library for mutants displaying enhanced efficiency of protein secretion (“high secretors”) and isolated 61 mutants showing enhanced endogenic and transgenic protein secretion. Sequence analysis identified a total of 54 genes involved in optimal secretion of insert proteins, as well as multiple independent transposon insertions localized within the same genomic loci and operons. The majority of transposon insertions occurred in genes that have no known protein secretion function. These transposon mutants were shown to prime antigen-specific CD8+ T cell responses better than the parental strain. Specifically, upon introducing the simian immunodeficiency virus (SIV) gag gene into these transposon mutant strains, we observed that they primed SIV Gag-specific CD8+ T cell responses significantly better than the control prime immunization in a heterologous prime/boost regimen. Our results reveal a dependence on bacterial secretion of mycobacterial and foreign antigens for the induction of antigen-specific CD8+ T cells in vivo. The data also suggest that these M. smegmatis transposon mutants could be used as novel live attenuated vaccine strains to express foreign antigens, such as those of human immunodeficiency virus type 1 (HIV-1), and induce strong antigen-specific T cell responses.

Live bacterial vaccines are relatively cheap to produce and easy to apply. Thus, they are suitable to immunize large populations (16, 44). Induction of both cell-mediated immunity and antibody-mediated immunity is obtained by the vaccine strain’s ability to colonize and multiply in the host without causing disease. In general, live bacterial vaccines require no additional adjuvant component to evoke immune responses (4). Numerous features, such as a strong safety record, make Mycobacterium bovis bacillus Calmette-Guérin (BCG) an attractive delivery vehicle for heterologous antigens (37, 47). A range of strategies have been developed to allow controlled and stable expression of viral, bacterial, and parasite antigens in BCG (4). However, since BCG can cause a clinically significant mycobacterial infection in patients with immune deficiency (60), the nonpathogenic Mycobacterium smegmatis has instead been used to engineer stable expression of transgenes to elicit cellular and mucosal immune responses (15). Unlike other mycobacterial strains such as BCG that survive in host cells for months by inhibiting phagosome maturation, M. smegmatis is rapidly destroyed by phagolysosomal proteases in the phagosomes of infected cells (10, 27, 34, 51). Nevertheless, M. smegmatis induces cytokine production by macrophages better than pathogenic mycobacterial species and can activate and induce the maturation of major histocompatibility complex (MHC) class I and costimulatory molecules (6, 55). M. smegmatis also facilitates rapid uptake of expressed antigens and cross-presentation of antigens (12, 35). Moreover, preexisting immunity to BCG may have only a marginal effect on the immunogenicity of recombinant M. smegmatis (9). Accordingly, M. smegmatis has been used as a valuable vector for the development of live vaccines against pathogens such as human immunodeficiency virus (HIV), hepatitis B virus, and Helicobacter pylori (9, 11, 32, 33, 49, 57, 59). Recently, recombinant M. smegmatis was used successfully as a potential tuberculosis (TB) vaccine for eliciting protective immunity against Mycobacterium tuberculosis (23, 49).

Despite the success of heterologous antigen expression and, in some cases, protection induced by recombinant mycobacteria in vitro and in vivo, the stability of the recombinant vaccine is of great concern. Expression of heterologous antigens in recombinant mycobacteria necessarily imposes a metabolic burden, the extent of which will determine the degree to which the recombinant mycobacterium is compromised. Thus, the relative level of fitness of the recombinant and any derived mutants will affect the rate at which the inserted element or its expression is lost from the bacterial population (24). Another critical issue for engineering a stable and immunogenic mycobacterium-based vaccine vector is antigen localization. Membrane-anchored and secreted antigens from mycobacteria could result in earlier presentation to the immune system. In fact, it has been demonstrated that anti-HIV-1 T cell responses in splenocytes and antibody responses against HIV-1 Env were optimal in mice immunized with recombinant mycobacteria expressing HIV-1 Env engineered as secreted antigens (57). Additionally, it has been suggested that removal of foreign antigens from the mycobacterial cell by secretion may support higher levels of transgenic protein production (21).
It has been reported that mycobacteria secrete a very large number of proteins, and even more proteins are cell surface associated. Considering the importance of secreted proteins in virulence and immunogenicity, protein secretion should be tightly regulated and well synchronized by many mycobacterial gene products (1,14, 39, 43). Thus, we hypothesize that random mutations in those secretion-associated mycobacterial genes will result in various mutants displaying enhanced efficiency of secretion.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains, bacteriophages, transposons, and plasmids used in this study are listed in Table 1. *M. smegmatis* strains used in this study were grown in 7H9 or 7H10 medium supplemented with 0.5% glycerol and 0.05% tyloxapol. *E. coli* strains were grown in Luria-Bertani (LB) medium. Antibiotics were used at the following concentrations: kanamycin, 40 μg/ml for *M. smegmatis*; apramycin, 60 μg/ml for *M. smegmatis* and 40 μg/ml for *E. coli*; and hygromycin, 150 μg/ml for *M. smegmatis* and 100 μg/ml for *E. coli*. LB agar containing 60 μg/ml of BCIP (Sigma, St. Louis, MO) per ml was used for screening of PhoA-expressing clones.

**Construction of plasmids and strains.** The PhoA construct was generated as follows. The antigen 85 promoter reported signal peptide were PCR amplified from pJH154 (57) using primers XbaI-F (5'-ATCTAGAAATACGGGGCGCGTTCGGT) and NdeI-R (5'-ATCTAGAATACGAAATGAGACGACTTTGC) and generated as follows. The antigen 85 promoter reported signal peptide were PCR amplified from pJH154 (57) using primers XbaI-F (5'-ATCTAGAAATACGGGGCGCGTTCGGT) and NdeI-R (5'-ATCTAGAATACGAAATGAGACGACTTTGC) and amplified from pJH154 (57) using primers XbaI-F (5'-ATCTAGAAATACGGGGCGCGTTCGGT) and NdeI-R (5'-ATCTAGAATACGAAATGAGACGACTTTGC) and digested with NdeI and XbaI. The antigen 85 promoter was inserted into the NdeI and XbaI sites of pSL300 to generate the PhoA reporter plasmid (pSL300), the resulting product was cloned directly upstream of *phaO* of the integrating mycobacterial plasmid pSL120 containing the truncated *E. coli phoA* (8). When integrated into the *M. smegmatis* chromosome, pSL300.

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**TABLE 1 Plasmids, phages, and strains used in this study**

| Plasmid, phage, or strain | Description | Reference or source |
|---------------------------|-------------|---------------------|
| pSC347                    | Vector for cloning allelic exchange substrates to be used for specialized transduction | 26 |
| pMV361Apra                | aacC41 Phsp60 bla L5 attP int ColE1; single-copy mycobacterial shuttle plasmid | 48 |
| pMV261Km                  | aph Phsp60 oriM ColE1; multicopy mycobacterial shuttle plasmid | 48 |
| pMV261Apra                | aacC41 Phsp60 oriM ColE1; multicopy mycobacterial shuttle plasmid | This work |
| pSL120                    | hyg ‘phaO’ bla L5 attP int ColE1; a promoterless truncated *E. coli PhoA* plasmid | This work |
| pSL300                    | hyg Phsp60-LpqH-SIINFEKL oriM ColE1 pMV261Km-lpqH-SIINFEKL | This work |
| pSL301                    | aph Phsp60-fhp(Ag85B) oriM ColE1 pMV261Km-Ag85B | This work |
| pSL330                    | hyg ΔlpqM ColE1; derivative of pSL347 designed for allelic exchange of *M. smegmatis* lpqM | This work |
| pSL331                    | aacC41 Phsp60 lpqM (*M. smegmatis*) int attP ColE pMV361Apra-lpqM | This work |
| pSL7                      | aacC41 Phsp60-SIV Gag-HA oriM ColE1 pMV261Apra-SIV Gag | This work |
| Phages                    | Deletion mutant of mycobacteriophage TM4 | 26 |
| phAE159                   | Deletion mutant of mycobacteriophage TM4 | 26 |
| phAE200                   | Deletion mutant of mycobacteriophage TM4 | 26 |
| Slams, phages, and strains | Deletion mutant of mycobacteriophage TM4 | 26 |
| E. coli                   | Deletion mutant of mycobacteriophage TM4 | 26 |
| DHL50                     | Vector for cloning allelic exchange substrates to be used for specialized transduction | 26 |
| Transformax EC100D pir-116 | Vector for cloning allelic exchange substrates to be used for specialized transduction | 26 |
| HB101                     | Vector for cloning allelic exchange substrates to be used for specialized transduction | 26 |
| M. smegmatis              | Vector for cloning allelic exchange substrates to be used for specialized transduction | 26 |
| mc155                     | Vector for cloning allelic exchange substrates to be used for specialized transduction | 45 |
| SLMS300                   | Vector for cloning allelic exchange substrates to be used for specialized transduction | This work |
| SLMS301                   | Vector for cloning allelic exchange substrates to be used for specialized transduction | This work |
| SLMS302                   | Vector for cloning allelic exchange substrates to be used for specialized transduction | This work |
| SLMS330                   | Vector for cloning allelic exchange substrates to be used for specialized transduction | This work |
| SLMS331                   | Vector for cloning allelic exchange substrates to be used for specialized transduction | This work |
| SLMS332                   | Vector for cloning allelic exchange substrates to be used for specialized transduction | This work |
| SLMS333                   | Vector for cloning allelic exchange substrates to be used for specialized transduction | This work |

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**Construction of plasmids and strains.** The PhoA construct was generated as follows. The antigen 85 promoter and signal peptide were PCR amplified from pJH154 (57) using primers XbaI-F (5'-ATCTAGAAATACGGGGCGCGTTCGGT) and NdeI-R (5'-ATCTAGAATACGAAATGAGACGACTTTGC) and digested with NdeI and XbaI. The antigen 85 promoter was inserted into the NdeI and XbaI sites of pSL300 to generate the PhoA reporter plasmid (pSL300), the resulting product was cloned directly upstream of *phaO* of the integrating mycobacterial plasmid pSL120 containing the truncated *E. coli phoA* (8). When integrated into the *M. smegmatis* chromosome, pSL300.
confers a light-blue phenotype on BCP-containing medium. The resulting recombinant M. smegmatis strain is SLM300. Plasmid DNA was introduced into M. smegmatis mc^2 155 by electroporation as previously described (45). For epitope tagging of the 19-kDa lipoprotein, the 19-kDa lipoprotein gene was amplified from 190VA (22) using a primer directed at the 5’ end of the coding sequence for the 19-kDa lipoprotein gene (5’-CAGGAGGAAAGCCAGATCTGGAGCGTGACT G-A3’), and then combined with a 3’ primer containing a sequence from OVA encoding amino acids 254 to 269 (5’-AATCCGCGCTGAGCGAACAG C-G3’). The PCR product containing the 19-kDa OVA sequence was ligated into pMV261 Km, a replicating mycobacterial vector with an Hsp60 promoter. The resulting plasmid, designated pSL301 (LpqH-SINFEKL), was used to transform wild-type (WT), ΔlpqM mutant (SLMS330), and ΔlpqM complemented (SLMS331) strains of M. smegmatis, which resulted in SLM301, SLM332, and SLM333, respectively. Strain SLMS330 was generated by a specialized transduction (3) of conditionally replicating shuttle plasmid pSL300. The ΔlpqM complemented strain SLMS331 was PCR amplified using 5’-AGGTTTGGAGCCGACGTCAC CTGCTC-3’ and 5’-CTCAGACATACACGCTGGTATATCGGAGAA CC-3’. The amplicon was cloned into the HindIII and Nhel sites of pMV361Apra to generate the complementation vector pSL331. This construct was integrated into M. smegmatis:ΔlpqM (SLMS330) by electroporation. In order to generate pSL302, the full-length fbpB was amplified without the promoter region and cloned into integrating mycobacterial vector pMV361Apra.

To determine if M. smegmatis transposon mutants we identified might be effective vectors for priming CD8+ T cell responses to simian immunodeficiency virus (SIV), we transformed the M. smegmatis transposon strains with a plasmid construct containing the SIVmac239 gag open reading frame. The SIV Gag protein includes the H-2D^b-restricted immunodominant AL11 epitope, and therefore the immunogenicity of this vaccine construct could be evaluated. The SIV gag gene was cloned into a multicopy episomal E. coli-mycobacterial shuttle plasmid to generate pSL7, which expresses a fusion protein containing the 19-kDa protein secretion sequence, the full-length SIV Gag protein, and a hemagglutinin (HA) tag (Table 1).

Transposon mutagenesis and generalized transduction in M. smegmatis. In this study, Tn5372 (apranycin resistant) was used for transposon mutagenesis of M. smegmatis. Tn5372 is a Himar derivative (24), the construct of which was cloned into a temperature-sensitive mycobacteriophage, phAE159 (26). This transposon phage was used as a vehicle for transposon delivery and for random mutagenesis of M. smegmatis expressing pSL300. The isolation of mutant strains and the identification of transposon insertion sites were performed as described previously (26). Generalized transduction with mycobacteriophage Tn1 (40) was performed as described previously (29). High-titer phage lysates (10^8 PFU/ml) of phage Tn1 were prepared on the donor strain as a host, and then the phage lysates were used to transfer the marker of interest into the recipient strain, SLM301.

DNA sequence analysis. Genomic DNAs were isolated from transposon mutants by use of the cetyltrimethylammonium bromide (CTAB) protocol as described previously (28). Genomic DNA was digested with BssHII and self-ligated, producing a plasmid containing the transposon and flanking M. smegmatis genomic DNA. These plasmids were recovered by transformation into E. coli DH5a λpir and selection on LB agar with apramycin (42). Resulting plasmid DNAs were then sequenced to map the insertion sites. For DNA sequence analysis, the following bioinformatic algorithms were used: NCBI Advanced BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), the Comprehensive Microbial Resource (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi), and the Smaegmlist database (http://mycobrowser.epfl.ch/smaegmlist.html).

Western blot analysis of recombinant M. smegmatis. To monitor the expression of the M. tuberculosis ipqH transgene, individual recombinant M. smegmatis strains were grown in Middlebrook 7H9 broth in the presence of kanamycin and apramycin. The lysate of grown recombinant M. smegmatis was fractionated on 4 to 20% SDS-polyacrylamide gels and blotted onto nitrocellulose filters (Invitrogen). Mycobacterial Hsp65 protein bands were detected with monoclonal antibody Hsp65 (Santa Cruz Biotechnology) at 1 μg/ml, followed by incubation with goat anti-mouse (GAM) IgG-alkaline phosphatase (AP) antibody (GenScript). Mycobacterial DnaK protein bands were detected by incubation of the filters with monoclonal antibody IT-40 (BEI Resources) at 1 μg/ml followed by incubation with GAM IgG (Sigma, St. Louis, MO)-AP. SINFEKL epitope bands were detected with anti-SINFEKL rabbit serum (1/10,000 dilution) and goat anti-rabbit (GAR) IgG-AP (Sigma). The LpqH/Hsp65 expression ratio in scanned gels was analyzed with ImageJ.

Tetramer staining and flow cytometric analysis. Female C57BL/6 mice (Jackson Laboratory), 6 to 8 weeks of age, were used for immunogenicity studies. All animal studies were approved by the institutional animal care and use committees of Duke University. At 7 days after immunization, mice were anesthetized with isoflurane and blood was collected in RPMI 1640 containing 40 μl of heparin (American Pharmaceutical Partners, Schaumburg, IL) per ml. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphocyte-M (Cedarlane, Burlington, NC) and stained with SINFEKL-specific class I MHC tetramers (catalog no. T03000; Beckman Coulter, FL) conjugated with phycoerythrin (PE) and anti-CD8+ monoclonal antibody (Ly-2; BD Bioscience, San Jose, CA) conjugated with allophycocyanin (APC) to detect SINFEKL-specific CD8+ T cells. The cells were washed in phosphate-buffered saline (PBS) containing 2% fetal bovine serum and fixed with PBS containing 2% ultrapure methanol-free formaldehyde (58). CD8+ T cells were analyzed for tetramer staining with FlowJo software (Tree Star, OR).

IFN-γ ELISPOT assays. Enzyme-linked immunosorbent (ELISPOT) assays were performed as described previously (56). Flat-bottom 96-well plates (Millipore, MA) were coated with anti-mouse gamma interferon (IFN-γ) antibody (BD Biosciences, CA) before washing with PBS and blocked with medium. Freshly isolated spleen cells were mixed with an MHC class II antigen 85B (Ag85B) peptide (p25, QDAYNAAAGHN AVF) (53) or an MHC class I AL11 epitope (AAVKNWMTQTL) and then incubated for 24 h at 37°C in 5% CO2. Following incubation, the plates were washed, biotinylated anti-IFN-γ antibody (BD Biosciences, CA) was added, and the plates were incubated overnight at 4°C. After the plates were washed with PBS, streptavidin-horseradish peroxidase (BD Biosciences, CA) was added to each well and developed using 3-amino-9-ethyl-carbazole (AEC) substrate. To count spot-forming cells (SFCs), spots in the ELISPOT plates were scanned using an ImmunoSpot series 1 analyzer and quantified by using ImmunoSpot 2.1 software (CTL Analyzers, Cleveland, OH). Control wells included cells cultured in medium in the absence of peptide stimulation. The frequency of IFN-γ SFCs in control wells was subtracted from the frequency of IFN-γ SFCs detected in the peptide-stimulated cells to calculate antigen-specific IFN-γ responses.

Statistical analysis. Statistical significance was assessed by comparison with data from the control groups using analysis of variance (ANOVA) with Tukey’s posttest. Data are expressed as the means ± standard deviations of the means. A P value of ≤0.05 was deemed to be significant.

RESULTS
M. smegmatis transposon mutants screened for elevated levels of transgene secretion. In an effort to study mutations conferring protein export enhancement in mycobacteria, we constructed a mariner-based Himar1 transposon (Tn5372) library in a PhoA reporter strain of M. smegmatis. The schematic plan for the screening is outlined in Fig. 1A. The reporter plasmid, pSL300, was designed to express E. coli PhoA with the M. tuberculosis antigen 85 (Ag85) promoter and export a recombinant fusion of the PhoA protein with the Ag85 signal peptide (secA1-dependent signal peptide) (Fig. 1B). The M. smegmatis reporter strain, SLM300, expresses the truncated E. coli’ phoA (pSL300). The secreted fusion
Transductants were selected as apramycin-resistant (Apr) and which encodes high-affinity phosphate transporter components. smegmatis PhoA regulators (unless the transposon insertion is located within either the
no transductants should be blue on LB plates containing BCIP
Since the recipient strain, SLMS301, does not express
culture supernatant in a
antigen precursor that is transported to membrane, cell wall, and
tagged with an SIINFEKL epitope. LpqH is a 19-kDa lipoprotein
contains plasmid pSL301, encoding the
generalized transduction mycobacteriophage, I3. Strain SLMS301
Microorganism. As expected, several mutants showed transposon inser-
tion into one of the phosphate transporters or regulators of the
expression of PhoA protein. In order to exclude these
constitutive expressers of M. smegmatis PhoA protein. In order to exclude these
constitutive expressers of M. smegmatis PhoA protein and confirm the
phenotype of enhanced secretion of E. coli PhoA, we transferred
each of 72 transposon insertions from the intense-blue mutants to
M. smegmatis strain SLMS301 (M. smegmatis:lpqH) by use of a
generalized transduction mycobacteriophage, I3. Strain SLMS301 contains plasmid pSL301, encoding the M. tuberculosis LpqH
encoded by an SIINFEKL epitope. LpqH is a 19-kDa lipoprotein
antigen precursor that is transported to membrane, cell wall, and
culture supernatant in a secA1-dependent manner (5, 13, 19, 54).
Since the recipient strain, SLMS301, does not express E. coli PhoA, no
transductants should be blue on LB plates containing BCIP unless the transposon insertion is located within either the M. smegmatis PhoA regulators (senX3-regX3) or the pst operon, which encodes high-affinity phosphate transporter components. Transductants were selected as apramycin-resistant (Ap') and kanamycin-resistant (Km') colonies, as Tn5372 carries the apramycin resistance gene and the recipient strain SLMS301 has the kanamycin resistance gene in the vector. Seventy-two Ap' Km' transductants were evaluated for alkaline phosphatase (PhoA+) activity by plating on BCIP medium. Eleven Ap' Km' transduc-
tants still exhibited the elevated PhoA+ phenotype (intense-blue colonies) in the recipient SLM301 strain, which lacks E. coli PhoA; however, 61 transductants appeared as white colonies on BCIP medium (Ap' Km' PhoA−). Subsequent sequence analysis confirmed that the transposons of 11 Ap' Km' PhoA− transductants were inserted into the M. smegmatis pst operon (data not shown).
Thus, our screening yielded a total of 61 transposon mutants that
are able to enhance secretion of the PhoA protein in SecA1-dependent
manner. 

**Mutations in several independent functional groups are responsible for the enhanced secretion.** Because Tn5332 carries the oriR6K origin of replication and the apramycin 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. DNA sequence analysis of the transposon insertion sites in these mutants revealed that the mutants can be categorized into eight functional groups: (i) cell envelope (11 transposon mutants), (ii) cellular process (3 transposon mutants), (iii) energy metabolism (6 transposon mutants), (iv) fatty acid metabolism and phospholipid (2 transposon mutants), (v) protein folding and stabilization (9 transposon mutants), (vi) regulation (12 transposon mutants), (vii) transport and binding (9 transposon mutants), and (viii) hypothetical protein (9 transposon mutants). A complete list of the transposon insertion sites is in Table S1 in the supplemental material. As expected, several mutants showed transposon insertion within the same gene (Table 2). Six independent transposon mutations were mapped at different locations of the lpqH gene, encoding lipoprotein peptidase. Two transposon insertions were located in the MSMEG_6136 gene, which encodes TerC family
proteins containing a number of integral membranes. Similarly, two independent transposons were found in MSMEG_1254, which encodes DEAD/DEAH box helicase. In addition, there are several mutants with multiple transposon insertions in the same operon (Table 3). In sum, 54 $M.\!$ smegmatis genes likely have important roles in $M.\!$ smegmatis protein expression and secretion. At least one transposon mutant of a gene or operon with multiple insertions was tested for its immunogenicity and compared to wild-type $M.\!$ smegmatis. The bolded transposon identification in Tables 2 and 3 represents the mutants used for immunogenicity studies (Fig. 3).

**Immunoblot analysis reveals that cell culture supernatants of the transposon mutant candidates contain larger amounts of transgene products.** In order to further characterize the mutations responsible for the enhanced secretion of the transgene products, the cell-associated pellet (PPT) fraction and the short-term culture filtrate (CF) fraction of transposon mutant growth cultures were separated by SDS-PAGE and analyzed by Western blotting. Transposon mutants expressing $M.\!$ tuberculosis antigen LpqH tagged with the immunodominant H2-Kb-presented MHC class I peptide SIINFEKL (pSL301) (20) were used to determine the level of transgene product secretion in the transposon mutant backgrounds. Western blot analysis was performed using antibodies against SIINFEKL to detect LpqH and against Hsp65 as a loading control. Since each mutant is potentially unique in its respective secretion of transgene product, as well as endogenous protein, no loading control for culture filtrates was used. Instead we used the LpqH/Hsp65 ratio in cell lysates as an indicator of similar loading of the proteins in the culture filtrates, since the same volume and growth stage of the cultures were processed equally for the Western blot. The expression of LpqH in PPT fractions of the transposon mutants was comparable to that in PPT fractions of the parental strain (Fig. 2). As expected, the mutant strains demonstrated significantly better secretion of $M.\!$ tuberculosis LpqH than the parental strain (Fig. 2). Based on the similar amounts of Hsp65 and LpqH proteins in cell pellets, we conclude that the mutants expressed comparable quantities of endogenous and transgenic proteins. To rule out autolysis as a source of the extracellular LpqH, Hsp65 (a commonly used marker for autolysis) (31, 46) was also measured in these short-term culture filtrates. The presence of LpqH in culture filtrates was not due to leakage or shedding of the protein, since no detectable Hsp65 (a cytoplasmic protein) was found in these short-term CFs (data not shown). In order to determine if the mutations affect endogenous protein secretion, protein extracts representing whole-cell lysates and culture supernatant fractions were analyzed using DnaK antibody. DnaK is a heat shock protein that is known to be associated with the bacterial surface (20) and secreted to the culture supernatant (38). Although similar amounts of DnaK protein were detected in the PPTs of the mutant strains and the wild-type $M.\!$ smegmatis, the amount of DnaK protein in the culture supernatants of the mutants was generally higher than that in the wild-type culture. Some mutants (bold numbers in Fig. 2) used for the Western analysis were further tested for their immunogenicity (Fig. 3).

**Improved T cell response in high secretor mutants.** To facilitate analysis of T cell responses, we took advantage of recombinant transposon mutants expressing pSL301 (LpqH-SIINFEKL)

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**TABLE 2** Summary of sequence analysis of high secretor mutants having multiple transposon insertions in the same gene

| Transposon mutations | $M.\!$ smegmatis gene | Protein function |
|----------------------|-----------------------|-----------------|
| 6, 15, H92, TH46, TH63, TH65 | MSMEG_4913 | Lipoprotein peptidase, LpqM |
| H17, H24 | MSMEG_6136 | TerC family protein, membrane protein |
| TH66, TH24 | MSMEG_1254 | DEAD/DEAH box helicase |

* Bold indicates mutants used for immunogenicity studies (Fig. 2).

**TABLE 3** Summary of sequence analysis of high secretor mutants having the transposon insertion in the same operon

| Transposon mutation | $M.\!$ smegmatis gene | Protein function |
|---------------------|-----------------------|-----------------|
| TH27 30 | MSMEG_3166 | Na+/solute symporter |
| | MSMEG_3167 | Major facilitator superfamily protein MFS_1 |
| H10 | MSMEG_1253 | Hypothetical protein |
| | MSMEG_1254 | DEAD/DEAH box helicase |
| H34 | MSMEG_0136 | Virulence factor Mce family protein, putative |
| | MSMEG_0137 | Probable conserved Mce-associated membrane protein |
| TH43 20 | MSMEG_6756 | Glycerol kinase, GlpK |
| | MSMEG_6758 | Glycerol kinase, GlpK |
| 22 | MSMEG_6750 | Hypothetical protein |
| 16 | MSMEG_6752 | Endoglucanase A |

* Bold indicates mutants used for immunogenicity studies (Fig. 3).
for CD8⁺ T cell responses or expressing pSL302 (Ag85B) for CD4⁺ T cell responses. The recombinant strains were generated by generalized transduction as described in Fig. 1. Mice were immunized with 1 × 10⁷ CFU of wild-type M. smegmatis, and transposon mutant expressing pSL301 and immunogenicity were assessed by SIINFEKL-specific tetramer responses at 1 week postinfection. All of the tested transposon mutants showed significantly improved CD8⁺ T cell responses compared to those of the parental strain expressing SIINFEKL (P < 0.05). On average, 2.8-times-higher SIINFEKL-specific tetramer positive responses were detected in animals immunized with recombinant transposon mutants (Fig. 3A). The magnitude of induced CD4⁺ T cell responses was also determined by the IFN-γ ELISPOT assay against the secretion and immunogenicity of transgene products. Screening and expression vector construction.

Mutation in lpqM confers enhanced secretion of transgene products in M. smegmatis. We successfully generated a deletion mutant (SLMS330, ΔlpqM) by specialized transduction for the purpose of further studying LpqM protein function with respect to the secretion and immunogenicity of transgene products. M. smegmatis ΔlpqM contains a disrupted lpqM gene; a 1,093-bp DNA fragment in the gene’s coding region was deleted and was replaced with a hygromycin cassette (Fig. 5A). The mutation was confirmed by PCR using three sets of primers, LL/RR, LL/Hyg1, and RR/Hyg2 (Fig. 5B). The complemented ΔlpqM strain (SLMS331) was generated by expressing the deleted gene in the integrating mycobacterial vector (pMV361/Apr). The recombinant lpqM deletion mutant (SLMS332, ΔlpqM-SIINFEKL) secreted the transgenic M. tuberculosis LpqH-SIINFEKL protein better than the parental strain (SLMS301) and the complemented strain (SLMS333) (Fig. 6A). The complemented strain showed a low level of protein secretion to culture filtrates, similar to the wild-type level. We observed a slight decrease in the LpqH protein secretion by the complemented strain compared to the wild-type strain, probably due to its constitutive lpqM expression by the Hsp60 promoter. Reflecting the enhanced protein secretion, the SIINFEKL-specific tetramer response was significantly higher in the mice immunized with the new recombinant ΔlpqM mutant expressing the M. tuberculosis LpqH-SIINFEKL protein (SLMS332, ΔlpqM-SIINFEKL) than in those

FIG 3 Enhanced immunogenicity of transposon mutants with higher secretion of the transgene product. C57BL/6 mice were immunized intravenously with approximately 1 × 10⁷ CFU of either parental or transposon mutant strains expressing LpqH-SIINFEKL or Ag85B. (A) The mean (± standard error of the mean) percentages of SIINFEKL-specific tetramer positive CD8⁺ T cells from PBMCs collected at week 1 are shown (n = 5 per group). (B) MHC II-specific T cell responses in spleens were assessed by using IFN-γ ELISPOT assays to measure the recall response of CD4 T cells specific for p25 in spleen suspensions; mean (± standard error of the mean) results are shown. The experiments were repeated two times, and results of a representative experiment are shown. Statistical significance was assessed by comparing immunogenicity results for the parental strain group (MS-LpqH-SIINFEKL or MS-Ag85B) to those for recombinant transposon mutants expressing the transgenes (n = 5 per group) using ANOVA one-way analysis. *P < 0.05; **P < 0.01; ***P < 0.001 (compared to results for MS-SIINFEKL).

FIG 4 Improved SIV Gag-specific CD8⁺ T cell responses of M. smegmatis transposon mutants. Mice were immunized via the intravenous route with 1 × 10⁷ CFU of actively growing log-phase M. smegmatis transposon mutants, H10 and H24, expressing pSL7 (SIV Gag). Heterologous boost was accomplished by injecting 10⁶ PFU rAd5-SIV Gag via the intramuscular route. Recombinant transposon mutants, H10-pSL7 and H24-pSL7, were primed for higher Gag-specific CD8⁺ T cell responses following suboptimal rAd5-Gag (1 × 10⁵ PFU) boosting than the parental M. smegmatis strain expressing SIV Gag. The P values were determined by ANOVA. * and **, P < 0.05 and P < 0.01, respectively.
immunized with both the recombinant complemented (SLMS333, ΔlpqM-C-SIINFEKL) and wild-type (SLMS301, MS-SIINFEKL) strains (Fig. 6B). However, no statistical difference was measured for the CD4 T cell response, as determined by the IFN-γ ELISPOT assay (Fig. 6C).

**DISCUSSION**

Attenuated strains of bacteria have been developed as potential live vectors to express homologous or heterologous antigens of many pathogens to induce protective immune responses. Non-pathogenic *M. smegmatis* can effectively express pathogenic antigens and has been used as an important vector for the development of live vaccines (9, 11, 32, 33, 49, 57, 59). In this study, genome-wide screens were employed to identify *M. smegmatis* genes affecting transgene product expression, secretion, and immunogenicity. We have isolated 61 mutations in 54 *M. smegmatis* genes that can enhance stable secretion/expression of foreign antigens (Fig. 1) and further enhance cellular immune responses (Fig. 3). We mapped mutations to different categories: cell envelope, cellular processes, energy metabolism, fatty acid and phospholipid metabolism, protein fate, regulatory functions, and transport and binding proteins (see Table S1 in the supplemental material). These gene loci may not represent the entire list of genes involved in *M. smegmatis* protein expression and secretion due to

**FIG 5** Construction of the lpqM mutant. (A) Maps of the lpqM genomic regions of wild-type *M. smegmatis* (Msmeg) and a representative lpqM mutant. The primer sites (LL, RR, Hgy1, and Hgy2) used for PCR confirmation of mutant clones are indicated as black boxes. (B) PCR products of genomic DNAs from wild-type *M. smegmatis* (lane 1), an allelic exchange plasmid construct (pSL330) for lpqM mutagenesis (lane 2), and two independent lpqM mutant clones from *M. smegmatis* (lanes 3 and 4). Primers LL/Hgy1, RR/Hgy2, and LL/RR were used, and the expected sizes of the PCR products are indicated. A molecular size marker is shown on the right.

**FIG 6** Improved transgene secretion and CD8⁺ T cell responses by the *M. smegmatis* ΔlpqM mutant. (A) The cell-associated pellet (PPT) fraction and the short-term culture filtrate (CF) fraction of the gene deletion mutant (ΔlpqM-lpqH) and the complemented strain (ΔlpqM-C-lpqH) expressing LpqH-SIINFEKL were analyzed by Western blotting for the indicated proteins. As a control, wild-type *M. smegmatis* (MS) and *M. smegmatis* expressing LpqH-SIINFEKL (MS-lpqH) were included. (B and C) SIINFEKL-specific tetramer-positive CD8⁺ T cells from PBMCs (B) and the Ag85B-specific CD4⁺ T cell response determined by IFN-γ ELISPOT assay in splenocytes (C) of mice immunized with the indicated recombinant strains (1 × 10⁷ CFU). *, P < 0.05.
the limitation of the screening method. For example, our screening used a Sec-dependent signal peptide fused with PhoA as a reporter, but it is likely that transposon mutagenesis using report-ers fused with different secretion signals will identify other novel secretion mutants.

Three genomic loci and five additional operons were found more than once during our screening, which strongly suggest that these genes are important to enhance the expression and secretion of the transgene products (Tables 2 and 3). However, how these genomic loci contribute to enhanced secretion of the transgene products remains to be determined. Six independent transposon insertions in IpqM were responsible for higher levels of secretion of transgene products (PhoA and LpqH). LpqM, a putative lipo-protein-metalloproteasease, has been shown to be essential for effi-cient DNA transfer by conjugation (36). LpqM is a membrane-associated protein and requires an intact signal and lipobox for membrane localization. It has been proposed that LpqM probably does not contribute directly to DNA transfer but instead may generate extracellular signal molecules through the proteolysis of pro-teins released by the Ess-1 or Tad system (2). Similarly, a possible role for LpqM in protein secretion would be to process extracel-lular proteins into a form able to inactivate secretion. The en-hanced secretion of the transgene product shown in the LpqM deletion mutant and the reversion of the phenotype to wild type suggest that LpqM is involved in the protein secretion pathway of Mycobacterium smegmatis (Fig. 6). Further investigation is needed to assess potential functions of the LpqM protein in protein secretion. Possible roles of the TerC family protein or DEAD/DEAH box heli-case in enhanced protein secretion phenotype are less clear. Both genomic loci have not been shown to be important players in protein secretion and regulation. Since the TerC family protein is hypothesized to catalyze efflux of tellurium ions (25), it may also have an extra activity of secreting the target proteins directly or indirectly. We also isolated two transposon mutants (TH66 and TH24) with mutations in MSMEG_1254, encoding DEAD/DEAH box helicases, and another (H10) with a mutation upstream of MSMEG_1254. The DEAD/DEAH box helicases are a family of proteins whose purpose is to unwind nucleic acids (30, 41). RNA helicases of the DEAD box, and related DE/ex/H proteins, consti-tute a very large superfamily of proteins conserved from bacteria and viruses to humans. They are associated with all processes in-volving RNA molecules, including transcription, editing, splicing, ribosome biogenesis, RNA export, translation, RNA turnover, and organelle gene expression (17, 30, 50). In the context of pro-tein secretion, disruption of DEAD/DEAH box helicase protein may affect transcription of a rate-limiting factor(s) of the secre-tion machinery and thus cause decreased regulation in protein secretion. Interestingly, there are 6 additional annotated DEAD/ DEAH box helicases in the Mycobacterium smegmatis genome, but the precise molecular role of these proteins remains obscure.

In a similar manner, the majority of the disrupted genes in the same operons (Table 3) encode either unknown functions or known functions that are, at this time, difficult to associate with protein secretion. For example, a gene we identified, an endoglu-canase A gene homolog, encodes a functional cellulase that is also encoded in the genome of M. tuberculosis and in other mycobac-terial genomes. Considering that the function of this protein is to utilize plant cell wall material as a nutrient, it may reflect the an-cestral soil origin of Mycobacterium or hint at a novel environ-mental niche (52). It is unclear how this protein may play a role in protein secretion. Thus, further studies, including complemen-tation analysis, are needed to dissect the functions of this protein, as well as the other protein candidates. For some categories of genes involved in protein fate, transport and binding, and regulatory functions, their respective roles in protein expression and secre-tion can be speculated on easily. For others, it is not so straight-forward. We speculate with caution that cell envelope, fatty acid biosynthesis, and phospholipid metabolism may influence cell membrane integrity and thus assist protein transport. Mutations in cellular processes and energy metabolism may affect the growth of the bacteria and protein expression. For the purpose of priori-tyzing further studies to determine if the enhanced secretion by these mutants affects immunogenicity, we chose high secretor mutants displaying multiple insertions in the same gene or operon, since they likely have a more significant role in protein secretion than the mutants with a single transposon insertion.

It has been shown that higher transgene product expression and secretion are directly related to improved immunogenicity (18, 56, 58). As we expected, significantly higher immunogenicity was observed in mice infected with the transposon mutant candidates associated with higher secretion of the transgene products. Analysis by use of the SIINFEKL-specific CD8” tetramer response and the IFN-γ ELISPOT assay of responses against p25 (Fig. 3) revealed uniformly strong antigen-specific CD8+ responses in animals that received transposon mutants expressing the transgenes (Fig. 3A). However, no significant differences in CD4+ T cell responses were detected among the immunized mice (Fig. 3B). We speculate that the lack of enhanced CD4+ T cell responses in the secreted mutants may be due to a stronger innate capacity of MHC class II-restricted antigen presentation than of MHC class I-restricted antigen presentation in mycobacteria. Thus, even though there is a slight increase in antigen 85 presentation with the mu-tant strains, the difference may not be statistically significant. Re-cently it has been shown that hyperexpression of Ag85B in wild-type Mycobacterium smegmatis led to autophagy that increased its MHC class II-dependent presentation to T cells, which again may lessen the possible CD4+ T cell response difference that can be observed from the immunization with the high secretor mutants. Alter-na-tively, highly secreted antigens from the phagosomes of trans-poson mutants would have easy access to the MHC class I antigen-processing pathway to activate CD8” T cells. Since a major goal of this work is to develop novel vectors for use as priming immuno-gens, we also examined whether the transposon mutants might be effective vectors for priming CD8” T cell responses to a potential vaccine antigen, SIV Gag. Two mutants, H10 and H24, expressing SIV Gag showed enhanced immunogenicity compared to the parental strain in prime/boost vaccination (Fig. 4). These results support the view that mycobacteria possess mechanisms to regu-late protein secretion, which in turn affect stimulation of CD8+ T cell responses. The manipulation of such mechanisms can con-trIBUTE significantly to protective immunity against the pathogen.

In sum, we were able to isolate over 60 transposon mutants that enhance both secretion of transgene products and transgene-spe-cific immunogenicity. The ability of the targeted genes to alter the immunogenicity of the vaccine construct was verified through several approaches. First, we identified more than one strain with independent transposon disruptions giving rise to the same immuno-logic phenotype. Second, we employed site-directed muta-genesis to delete targeted genes in the wild-type Mycobacterium smegmatis construct; the resulting mutant strains displayed the phenotype of
the transposon-disruption mutant strains. Third, we complemented one of the transposon-disrupted genes with a wild-type copy of the gene and observed a reversion to the wild-type phenotype. Thus, we formally confirmed the contributions of the genes identified in the transposon mutant library screen of *M. smegmatis* strains.

A problem facing those attempting to generate live vaccine vectors has been the instability of the inserts due to either high or insufficient expression/secretion of transgene products. Our study shows that genetic modification of a live vector can overcome those issues without sacrificing high-level transgene product expression/secretion or immunogenicity. This novel and effective approach, of enhancing CD8+ T cell responses by deregulating secretion pathways of key antigens, may also be applied to and utilized with vaccine development strategies for numerous other significant diseases.

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