Specialized Transduction Designed for Precise High-Throughput Unmarked Deletions in *Mycobacterium tuberculosis*

Paras Jain,[a,d] Tsungda Hsu,[a*] Masayoshi Arai,[a*] Karolin Biermann,[a*] David S. Thaler,[a,d*] Andrew Nguyen,[a] Pablo A. González,[a,d*] Joann M. Tufariello,[a] Jordan Kriakov,[a] Bing Chen,[a] Michelle H. Larsen,[a,b,d] William R. Jacobs, Jr.[a,c,d]

Departments of Microbiology and Immunology,[a] Medicine,[b] and Genetics[c] and Howard Hughes Medical Institute,[d] Albert Einstein College of Medicine, Bronx, New York, USA

* Present address: Tsungda Hsu, Department of Microbiology, University of Massachusetts, Amherst, Massachusetts, USA; Masayoshi Arai, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; Karolin Beirmann, Bayer AG, Leverkusen, Germany; David S. Thaler, Department of Earth and Environmental Engineering, Department of Pathology & Cell Biology, Columbia University Medical Center, New York, New York, USA; Pablo A. González, Millennium Institute on Immunology and Immunotherapy, Department of Molecular Genetics and Microbiology, Pontificia Universidad Católica de Chile, Santiago, Chile.

P.J. and T.H. contributed equally to this article.

ABSTRACT **Specialized transduction has proven to be useful for generating deletion mutants in most mycobacteria, including virulent *Mycobacterium tuberculosis*. We have improved this system by developing (i) a single-step strategy for the construction of allelic exchange substrates (AES), (ii) a temperature-sensitive shuttle phasmid with a greater cloning capacity than phAE87, and (iii) bacteriophage-mediated transient expression of site-specific recombinase to precisely excise antibiotic markers. The methods ameliorate rate-limiting steps in strain construction in these difficult-to-manipulate bacteria. The new methods for strain construction were demonstrated to generalize to all classes of genes and chromosomal loci by generating more than 100 targeted single- or multiple-deletion substitutions. These improved methods pave the way for the generation of a complete ordered library of *M. tuberculosis* null strains, where each strain is deleted for a single defined open reading frame in *M. tuberculosis*.**

IMPORTANCE **This work reports major advances in the methods of genetics applicable to all mycobacteria, including but not limited to virulent *M. tuberculosis*, which would facilitate comparative genomics to identify drug targets, genetic validation of proposed pathways, and development of an effective vaccine. This study presents all the new methods developed and the improvements to existing methods in an integrated way. The work presented in this study could increase the pace of mycobacterial genetics significantly and will immediately be of wide use. These new methods are transformative and allow for the undertaking of construction of what has been one of the most fruitful resources in model systems: a comprehensive, ordered library set of the strains, each of which is deleted for a single defined open reading frame.**

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Address correspondence to William R. Jacobs, Jr., jacobsw@hhmi.org.

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Tuberculosis (TB) is a major global health challenge, with approximately 8 million new cases and almost 2 million deaths per year (1). Genetic analysis of the causative organism *Mycobacterium tuberculosis* is essential to furthering the prevention and treatment of TB. A powerful genetic approach to characterize gene function is to construct defined null alleles in an organism, so that isogenic strains, differing at the defined locus, can be compared (2, 3). *M. tuberculosis* has a doubling time of approximately 24 h, and colony formation from a single cell takes 3 to 4 weeks. Slow growth means that it takes at least a month for each step of multistep genetic manipulations. Thus, there is particular value in methods that decrease the number of steps that require the growth of a single cell into a colony. The approaches taken with this challenging system may also interest those working with other "noncanonical" organisms whose genetic manipulation and laboratory domestication will require new approaches.

In this study, we identified and improved upon several rate-limiting steps for the construction of deletion-substitution and unmarked deletion alleles in mycobacteria. First, the multistep process of engineering allelic exchange substrates (AES) for homologous recombination (HR) has been shortened to a single step. The new AES vector also contains both a hygromycin resistance gene as a selection and a *sacB* gene as a counterselection flanked by gamma delta resolvase sites for simplified unmarking. Second, an improved shuttle, phAE159, with an increased cloning capacity and efficient DNA delivery in comparison to phAE87, was constructed. Third, we have developed a phage-based transient expression of γδ resolvase methodology that allows recovery of an antibiotic-sensitive deletion strain in a single step.

We have used these methods to construct a fully drug-sensitive double auxotroph of *M. tuberculosis* H_{37}Rv (mc^2 6206) which displays less virulence in mouse studies than the current human vac-

cine strain *Bacillus* Calmette-Guérin (BCG). The mc^2 6206 strain is an excellent substrate for further genetic manipulation, as this *M. tuberculosis* derivative has been reclassified for biosafety level 2 (BSL2) containment. These methods have also been used to generate over 100 additional strains with defined deletion-substitution in virulent *M. tuberculosis* H_{37}Rv to demonstrate that the methods apply to all classes of genes and at all chromosomal locations.

RESULTS

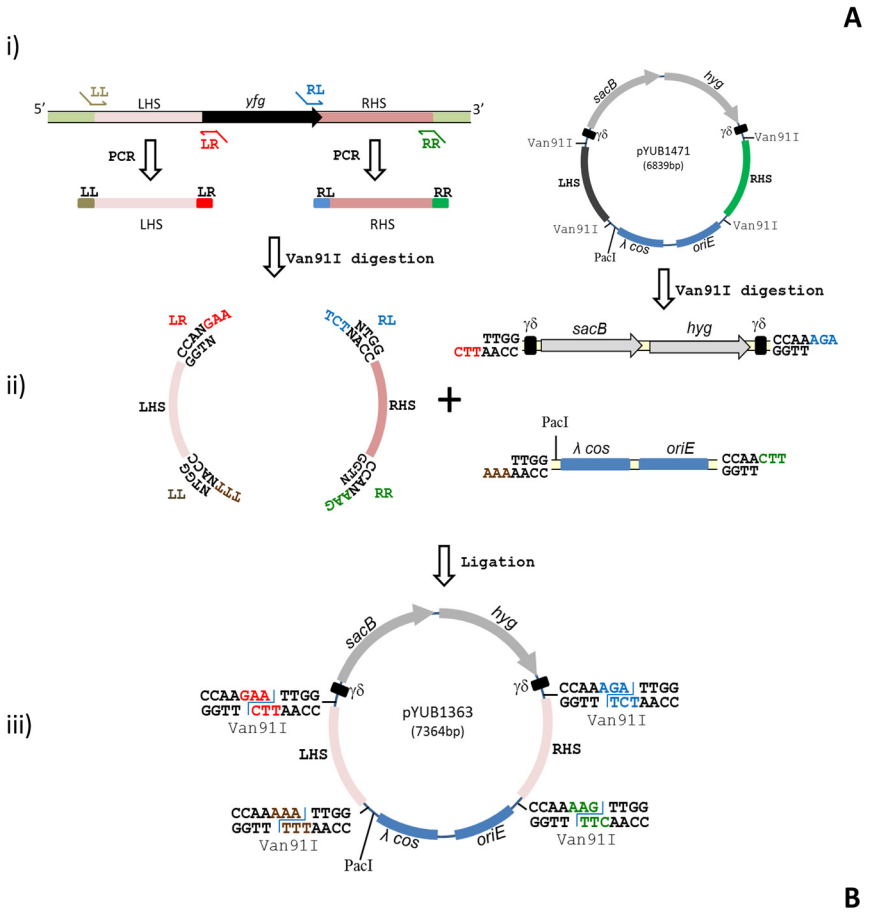
One-step construction of AES. The left homology sequence (LHS) and right homology sequence (RHS) flanking the region targeted for deletion-substitution need to be cloned on either side of a selectable/counterselectable cassette [γδ(*sacB-hyg*)γδ] to generate an AES for HR (Fig. 1A). Current directional cloning approaches to construct AES require different restriction enzymes at both the ends of each flanking sequence (4, 5). Besides primer design complexity and the number of consecutive steps, this approach is often confounded by the presence of restriction sites, needed for cloning, within the genomic region to be cloned. We have exploited the property a type IIP restriction enzyme Van91I (isoschizomers PflmI, AccB7I, and BasI) (6), which recognizes a discontinuous palindrome interrupted by a segment of 5 bases of unspecified sequence (CCAN_NNN^NTGG), to accomplish a 4-fragment oriented ligation. Using this approach, we were able to construct the AES in a single reaction (Fig. 1A). Van91I cleaves within the degenerate sequence, allowing us to dictate the sequence of sticky ends. This methodology to generate one-step AES is applicable to all the genes in *M. tuberculosis* and other mycobacterial species. DraIII, BstAp1, and AlwNI restriction enzymes can also be used in place of Van91I to construct AES in one step using the same methodology (Fig. 1B).

Construction of shuttle phasmid phAE159 and specialized transducing phages (STPs). Mycobacteriophage vector phAE159 was constructed by deletion of an ~6-kb region encompassing genes *gp48* to *gp64* from a temperature-sensitive mutant of TM4, PH101 (7, 8) (see Fig. S1 in the supplemental material). Similar to parent PH101, shuttle phasmid phAE159 propagates as a phage at 30°C but only injects its DNA and does not propagate in the infected mycobacterial cell at 37°C. The shuttle phasmid phAE159 delivers up to 10 kb of recombinant DNA into mycobacterial cells.

Mycobacteriophage-based STPs harboring AES at the nonessential region in phAE159 were used to disrupt a specific gene in the mycobacterial chromosome via genomic deletion-substitution (4, 9, 10). For each STP construction, the carbenicillin cassette between the two PacI sites in phAE159 phasmid DNA was replaced by the desired AES targeting a specific region of the bacterial genome (Fig. 2). All the AES were hygromycin resistant, and the process of STP construction was simplified by the selection for an antibiotic marker (hygromycin) different from the original phAE159 marker (carbenicillin) and the use of *in vitro* lambda packaging to size select phasmid DNA (Fig. 2). Phasmid DNA was electroporated into *Mycobacterium smegmatis* mc^2 155 to obtain STP plaques at the permissive temperature of 30°C. STPs were picked and amplified at 30°C to obtain high-titer allele-specific STPs.

Specialized transduction. Specialized transduction and subsequent incubations were performed at 37°C, a temperature nonpermissive for the amplification of these STPs. At this temperature, STPs inject their DNA into mycobacterial cells but do not propagate and thus provide AES for HR. The transduced *M. tuberculosis* cells were selected on hygromycin plates to obtain colonies from *M. tuberculosis* cells that had undergone HR (Fig. 2). On average, we obtained 10 to 30 colonies on hygromycin plates after transduction, and >10 or no colonies appeared only in few cases. In these latter cases, transductions were repeated after retitration of the STP. Three colonies per transduction were analyzed either by PCR or Southern blotting to differentiate HR from illegitimate recombination. In most transductions, either all three or two-thirds of the colonies were the result of HR. Transductions for *Rv0404* and *Rv3801c*, however, yielded no colonies despite repeated attempts ($n = 5$) and could be deleted only when transductions ($n = 1$) were performed over merodiploid strains expressing *Rv0404* (mc^2 7212) or *Rv3801c* (mc^2 7241), suggesting their essentiality (see Fig. S2 in the supplemental material). To show the ability of STPs to provide substrate for HR at different genomic locations, we have generated more than 100 Southern blotting/PCR-confirmed deletion-substitution mutants in *M. tuberculosis* H_{37}Rv by specialized transduction (Fig. 3A). The alteration of a desired locus by specialized transduction is not restricted to genes belonging to one functional group. The majority of the genes deleted in this study have been annotated as lipid metabolism (34%) and cell wall and cell processes (21%), with the rest of the genes showing a diverse distribution of functional classes (Fig. 3B).

Phage-based transient expression system for gamma-delta resolvase. There are practical and scientific needs to remove the antibiotic resistance marker from the mutant strains. The practical need is that there is only one other class of antibiotic resistance marker (kanamycin) available for mycobacteria, which has an unacceptable spontaneous resistance rate (11). Therefore, the hygromycin cassette needs to be excised out of the first mutant strain so that the hygromycin-marked AES can again be used for the iterative construction of multiple mutant strains. The scientific need is that insertion of a selectable marker cassette could also be like a transposon insertion in terms of polarity, so being able to specifically remove the selection cassette to generate an in-frame deletion would reduce the polar effects of the manipulation carried out to make a deletion. Removal of the antibiotic resistance cassette in a deletion-substitution strain requires the expression of a site-specific resolvase (4, 12). We hypothesized that the temperature-sensitive mycobacteriophage vector phAE159 at 37°C would provide a platform for the transient expression of cloned genes into the transduced mycobacterial cells and could be used for the excision of γδ(*sacB-hyg*)γδ cassette from deletion-substitution mutants generated as described above. Because the phAE159 is a temperature-sensitive phage, it is readily lost at 37°C and thus does not require curing after unmaking, contrarily to the resolvase-expressing plasmids (4, 12). To test our hypothesis, the *tnpR* gene (γδ resolvase) from the γδ transposon was cloned into phAE159 to generate phAE280 (Fig. 4A). Mycobacterial strains harboring the γδ(*sacB-hyg*) γδ cassette were incubated with phAE280 and plated on sucrose-containing plates to counterselect for the loss of the *sacB-hyg* region between two γδ sites (Fig. 4B). Colonies obtained on sucrose plates were scored for hygromycin sensitivity, and resolution of γδ(*sacB-hyg*)γδ by the γδ resolvase were confirmed by PCR and sequencing. Figure 4C shows one such example of this analysis from *M. tuberculosis*. The results for this particular strain to excise the *sacB-hyg* cassette by γδ resolvase were similar to other unmarked knockouts in different classes of genes (data not shown). The residual sequence after recombination between the two γδ sequences flanking *sacB-hyg* is shown in Fig. 4D. The primers can be designed to generate an in-frame



	Van91I	DraIII
LL	TTTTTTTTCCAT**AAA**TTGG-N_{18-20}	TTTTTTTTCAC**AAA**GTG-N_{18-20}
LR	TTTTTTTTCCAT**TTC**TTGG-N_{18-20}	TTTTTTTTCAC**TTC**GTG-N_{18-20}
RL	TTTTTTTTCCAT**AGA**TTGG-N_{18-20}	TTTTTTTTCAC**AGA**GTG-N_{18-20}
RR	TTTTTTTTCCAT**CTT**TTGG-N_{18-20}	TTTTTTTTCAC**CTT**GTG-N_{18-20}
	BstAPI	**AlwNI**
LL	TTTTTTTTGCAT**AAA**TTGC-N_{18-20}	TTTTTTTTCAG**AAA**CTG-N_{18-20}
LR	TTTTTTTTGCAT**TTC**TTGC-N_{18-20}	TTTTTTTTCAG**TTC**CTG-N_{18-20}
RL	TTTTTTTTGCAT**AGA**TTGC-N_{18-20}	TTTTTTTTCAG**AGA**CTG-N_{18-20}
RR	TTTTTTTTGCAT**CTT**TTGC-N_{18-20}	TTTTTTTTCAG**CTT**CTG-N_{18-20}

FIG 1 High-throughput construction of AES via four-fragment oriented ligation. (A) Regions of 650 to 1,000 bases that flank the region to be deleted (Your Favorite Gene [yfg], *M. tuberculosis recD* in this case) were amplified with primers harboring the Van91I site at the 5′ end to obtain left homology sequence (LHS) and right homology sequence (RHS). (i) Van91I recognizes a 6-bp symmetric bipartite sequence bisected with 5 bp of degenerate sequence (CCAN_NNN^NTGG). Van91I digestion results in 3′ overhangs of three bases, which are shown at each end of the two PCR products. The sequences of the overhangs depend on the intervening degenerate sequences and are specified in the primer shown below. (ii) Complementary 3′ overhangs at the end of the 3.6-kb linear fragment [contains γδ*(sacB-hyg)*γδ cassette] and a 1.6-kb linear fragment (contains the lambda *cos* site, ColE1 origin of replication [oriE], and an 8-bp recognition site for PacI) were obtained after Van91I digestion of pYUB1471 to specify the site and orientation for the four fragments. (iii) Ligation of four different DNA fragments, Van91I-digested LHS, RHS, and 3.6-kb and 1.6-kb linear fragments, results in AES (pYUB1363, AES for *recD*). All the AES are approximately 6- to 7.5-kb plasmids which are propagated as plasmids in *E. coli* and cloned into mycobacteriophage vectors via their unique PacI site for generating STPs. (B) The 5′ sequence for primers depends upon the choice of restriction enzyme. If the sequence within the PCR amplicon contains a Van91I recognition site, BstAPI, AlwNI, or DraIII is used instead. The overhangs used in each case (LL, LR, RL, RR) are shown for all four restriction enzymes. The gene-specific sequence is indicated by N_{18-20} at the ends of primers. Any combination of LL/LR and RL/RR primers (i.e., LL/RR with Van91I overhangs and RL/RR primers with DraIII overhangs) can be used to generate AES.

deletion irrespective of the restriction enzyme used to generate the AES. The amino acid sequence of the residues for an in-frame deletion is indicated below the nucleotide sequence (Fig. 4D). These methods to generate unmarked deletion mutants are also applicable to *M. smegmatis*, *Mycobacterium bovis*, and various *M. tuberculosis* strains. More than 80% of colonies recovered from

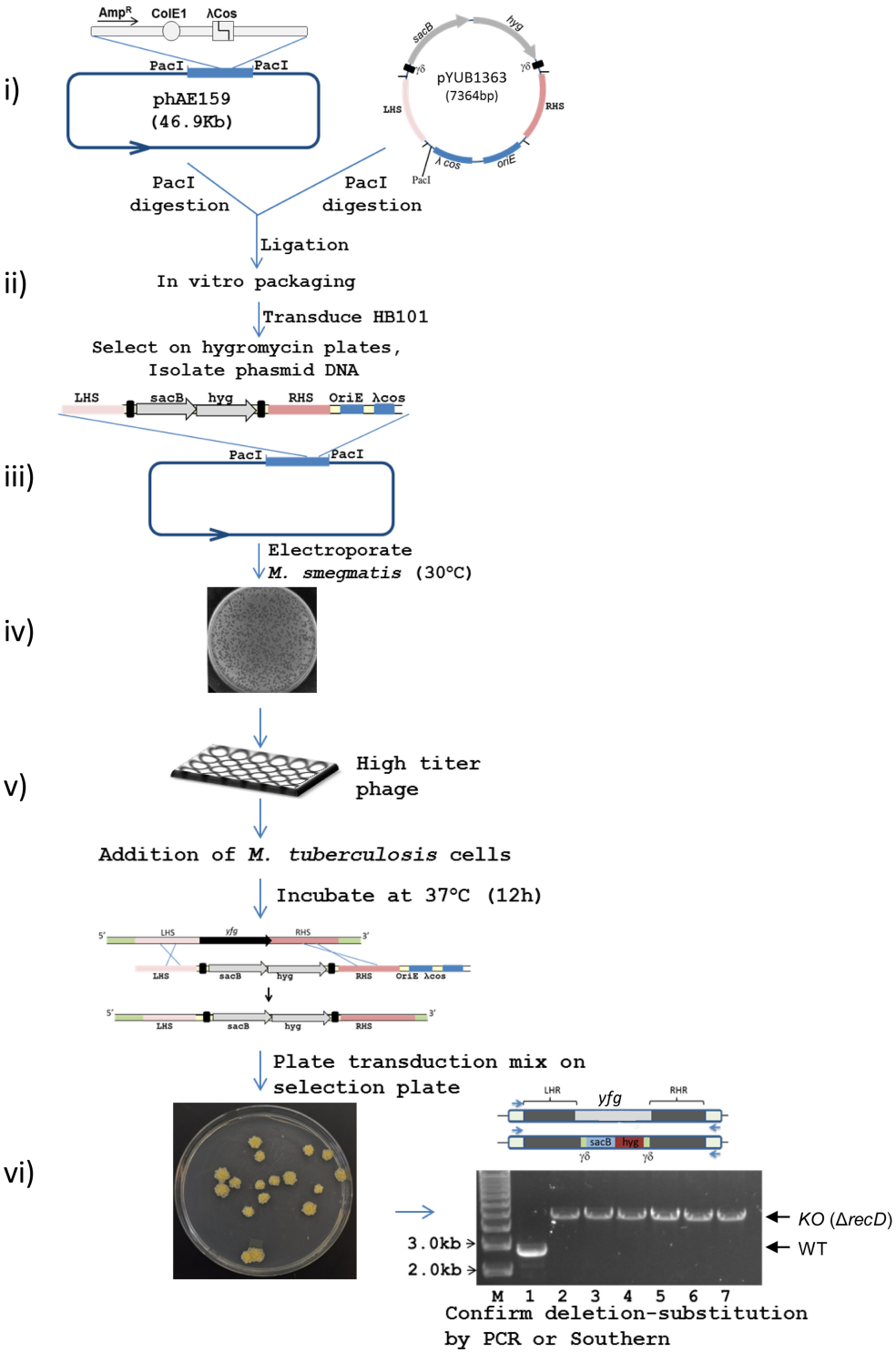


FIG 2 Construction of gene-specific STPs and high-throughput amplification of phage stocks to generate deletion-substitution mutants of *M. tuberculosis* by specialized transduction. (i) phAE159 DNA is digested with PacI to release the ampicillin resistance insert and was ligated to PacI-digested AES (pYUB1363). The resulting ligation product is hygromycin resistant. (ii) The ligated products are packaged *in vitro* via the lambda *cos* site present in the AES. (iii) *E. coli* HB101 was transduced with lambda-packaged STP and selected for hygromycin resistance. (iv) STP phasmid DNA was isolated from HB101 as plasmid, transfected into mc^2155 at 30°C to obtain mycobacterial phage plaques for STP. (v) High-titer phage stocks of each STP were grown at 30°C in a 24-well high-throughput format on a lawn of mc^2155. Phage stocks were grown on an agar pad of 1 ml, essentially a miniaturized plate stock as described before (21). Transduction-competent *M. tuberculosis* or other recipient cells were incubated with the high-titer STP stock and incubated for 24 h at 37°C. (vi) Transduced *M. tuberculosis* recipients were plated on 7H10 supplemented with hygromycin (75 μg/ml) and incubated 3 to 4 weeks at 37°C to obtain deletion-substitution mutants. The colonies obtained were confirmed for HR using PCR primers upstream of LHS and downstream of RHS (shown as arrow). Expected-size products were obtained for WT (~2.9 kb) and knockout (~5.0 kb). The Invitrogen 1Kb Plus DNA ladder was used for the agarose gel, and all the bands differ by 1 kb.

A

Rv0007	Rv0008c	Rv0010c	Rv0011c	Rv0023	Rv0024	Rv0025	Rv0026	Rv0028	Rv0030
Rv0031	Rv0033	Rv0036c	Rv0037c	Rv0038	Rv0039c	Rv0040c	Rv0042c	Rv0043c	Rv0047c
Rv0049	Rv0051	Rv0056	Rv0057	Rv0059	Rv0061	Rv0063	Rv0068	Rv0069	Rv0070c
Rv0071	Rv0072	Rv0073	Rv0074	Rv0075	Rv0076c	Rv0077c	Rv0078	Rv0081	Rv0082
Rv0083	Rv0084	Rv0085	Rv0086	Rv0089	Rv0090	Rv0091	Rv0092	Rv0093c	Rv0096
Rv0098	Rv0099	Rv0100	Rv0103c	Rv0105c	Rv0106	Rv0109	Rv0110	Rv0112	Rv0113
Rv0114	Rv0115	Rv0116c	Rv0117	Rv0118c	Rv0119	Rv0125	Rv0126	Rv0128	Rv0129c
Rv0131c	Rv0133	Rv0136	Rv0166	Rv0214	Rv0270	Rv0275c	Rv0551c	Rv0629c	Rv0852
Rv1058	Rv1185	Rv1193	Rv1206	Rv1345	Rv1427	Rv1521	Rv1529	Rv1550	Rv1683
Rv1750c	Rv1925	Rv2187	Rv2505c	Rv2590	Rv2930	Rv2941	Rv2948c	Rv2950c	Rv3089
Rv3506	Rv3515c	Rv3561	Rv3826						

B

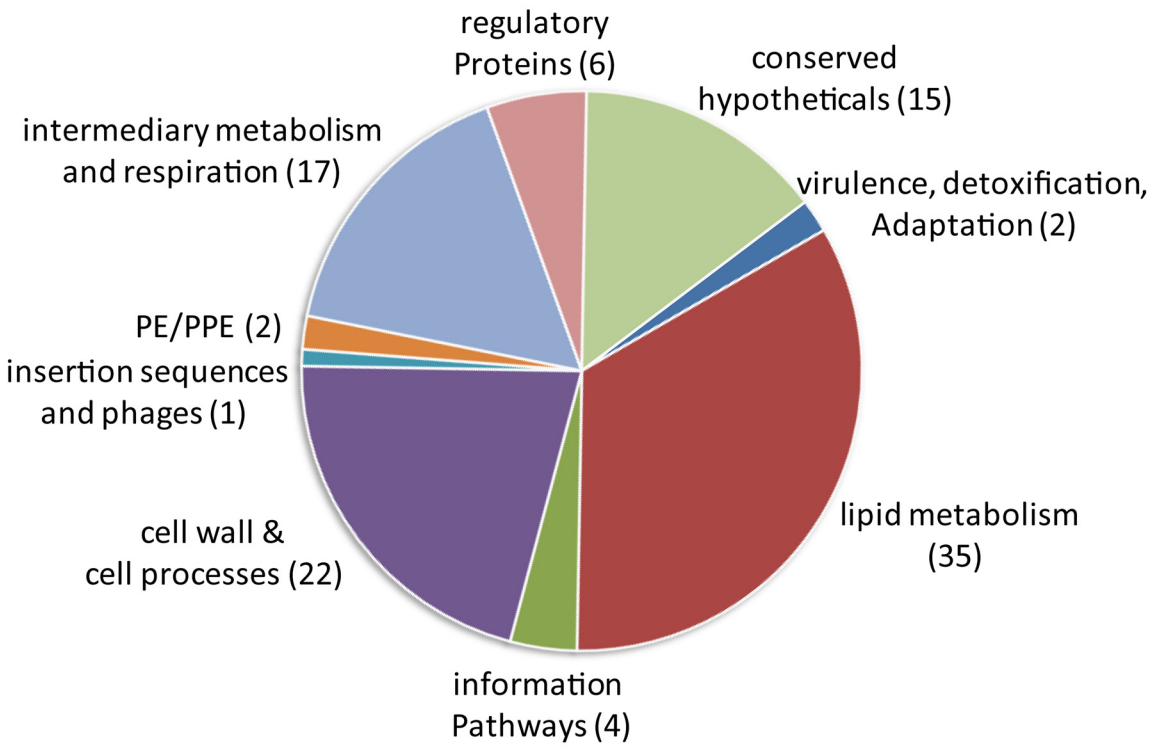


FIG 3 High-throughput generation of gene deletion in mycobacteria. (A) List of 104 mutants generated by specialized transduction in *M. tuberculosis* H_{37}Rv. (B) Functional categories of genes in virulent *M. tuberculosis* H_{37}Rv, which were subjected to deletion-substitution via specialized transduction. List of primers to generate the AES are listed in Table S1 in the supplemental material.

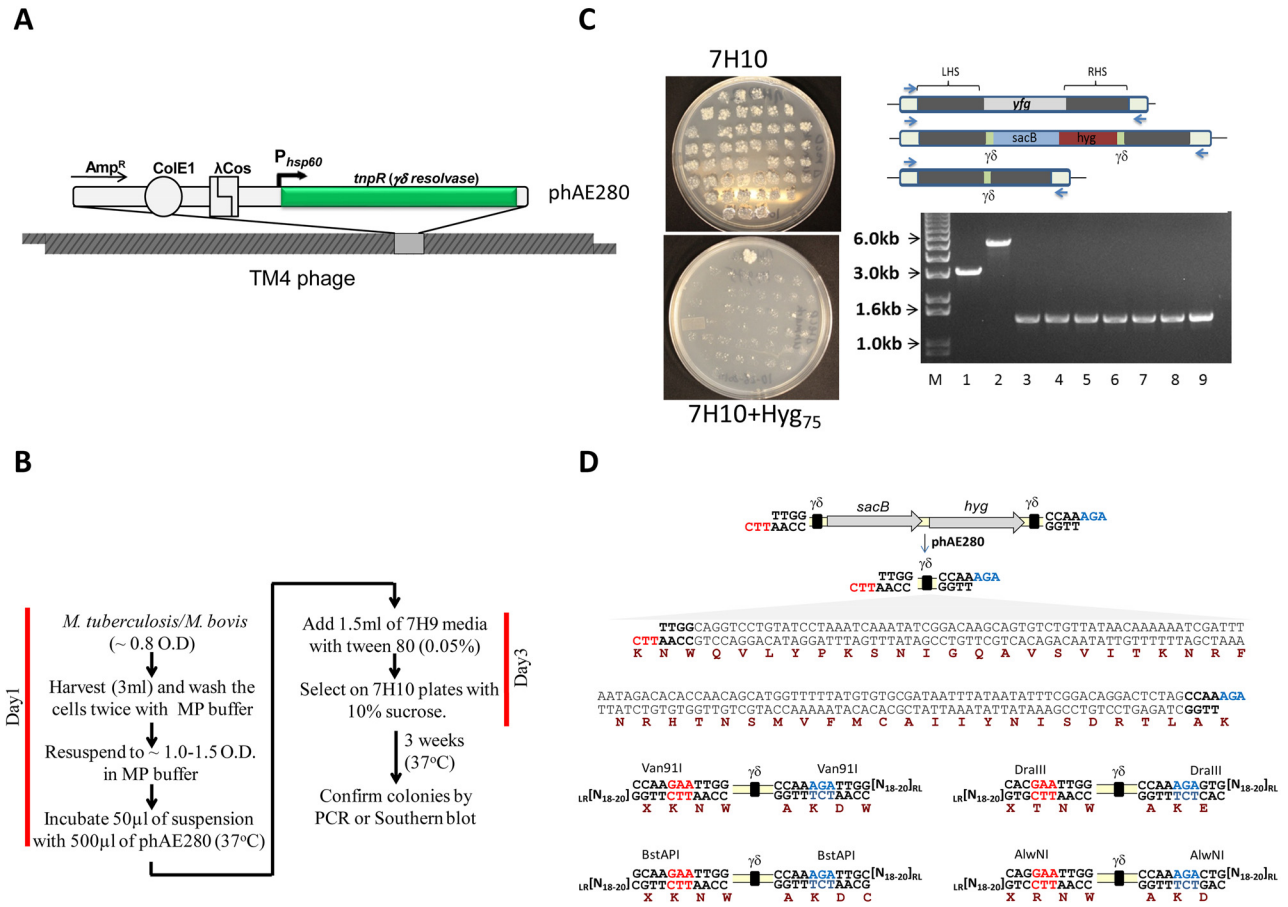


FIG 4 Phage-mediated unmarking. (A) Schematic of the mycobacteriophage expressing γδ resolvase (phAE280); (B) flow chart description of single-step excision of the γδ(*sacB-hyg*)γδ cassette using phAE280. (C) mc²7204 [M. *tuberculosis* H_{37}Rv Δ*recD*::γδ(*sacB-hyg*)γδ] was transduced with phAE280, using the protocol listed above, to excise the selection/counterselection cassette and yield mc²7205 (Δ*recD*::γδ). Hygromycin sensitivity was confirmed by picking and patching (left); deletion was confirmed by PCR with primers that flank the targeted region (right, blue arrows). Lane 1, PCR on *M. tuberculosis* cells (wild type [WT]); lane 2, PCR on mc²7204 [Δ*recD*::γδ(*sacB-hyg*)γδ; hygromycin resistant]; lanes 3 to 9, PCR on hygromycin-sensitive colonies from pick and patch plate, unmarked Δ*recD* mutant (mc²7205 [Δ*recD*::γδ]). The excision of the *sacB-hyg* cassette was also confirmed by sequencing of PCR products. (D) Generation of the in-frame deletions. The two γδ recombine to excise the *sacB-hyg* cassette after the expression of γδ resolvase from phAE280. The residual nucleotide sequence after the excision is shown. The encoded amino acids are shown below the nucleotide sequence. The amino acid at the junction depends upon the restriction enzyme used as shown. The primer sequence of LR and RL should match the coding frame to maintain the reading frame to generate in-frame deletion. The amino acid at the RL-restriction enzyme junction depends upon the gene-specific primer sequence (N_{18-20}) and is indicated by X.

sucrose plates after transduction by phAE280, in more than 40 independent experiments, were sensitive to hygromycin and had the expected residual excision sequence in all the cases, regardless of the targeted genomic locus. The remaining 20% were bypass mutants (i.e., *sacB* mutants or sucrose resistance mutants).

An exemplar for a multigenic deletion mutant: a BSL2-safe *M. tuberculosis* H_{37}Rv derivative. WHO guidelines mandate that laboratory work with live *M. tuberculosis* can be carried outside BSL3 containment only with strains that harbor two unlinked nonreverting attenuating mutations (13). We have previously shown that the deletion of *panCD*, which results in pantothenate auxotroph, or deletion of *leuD*, which results in a leucine auxotroph in *M. tuberculosis*, leads to bacterial attenuation in mice (5, 14) and that immunization with these strains of *M. tuberculosis* elicits some protective immunity against virulent *M. tuberculosis*. First, we deleted the *panCD* gene in the *M. tuberculosis* genome using specialized transduction. The resulting strain was unmarked using phAE280 as described above. The antibiotic-sensitive *M. tuberculosis* Δ*panCD* strain was then used as a substrate for the deletion of the *leuCD* locus. The *sacB-hyg* cassette inserted at the Δ*leuCD* locus was excised again using phAE280, and the *M. tuberculosis* strain was designated mc²6206. This strain is auxotrophic for both leucine and pantothenate and is fully antibiotic sensitive. The kinetics of persistence of this attenuated strain in C57BL/6 mice was similar to that of the currently used human vaccine BCG (Fig. 5A). The safety of mc²6206 was further assessed by infecting severe combined immune-deficient (SCID) mice intravenously with a high dose (10^5 CFU) of bacteria. SCID mice succumbed to virulent *M. tuberculosis* H_{37}Rv infection 25 days after infection, while mice challenged with BCG died by day 230. Those challenged with mc²6206 had a survival rate of 100% by day 250 (Fig. 5B), showing that the mc²6206 is safer than *M. bovis* BCG in SCID mice. These observations have led to the approval from the Albert Einstein College of Medicine Institutional Biosafety Committee for reclassification of this strain for BSL2 containment. This strain is being used as a base strain to develop hybrid vaccines

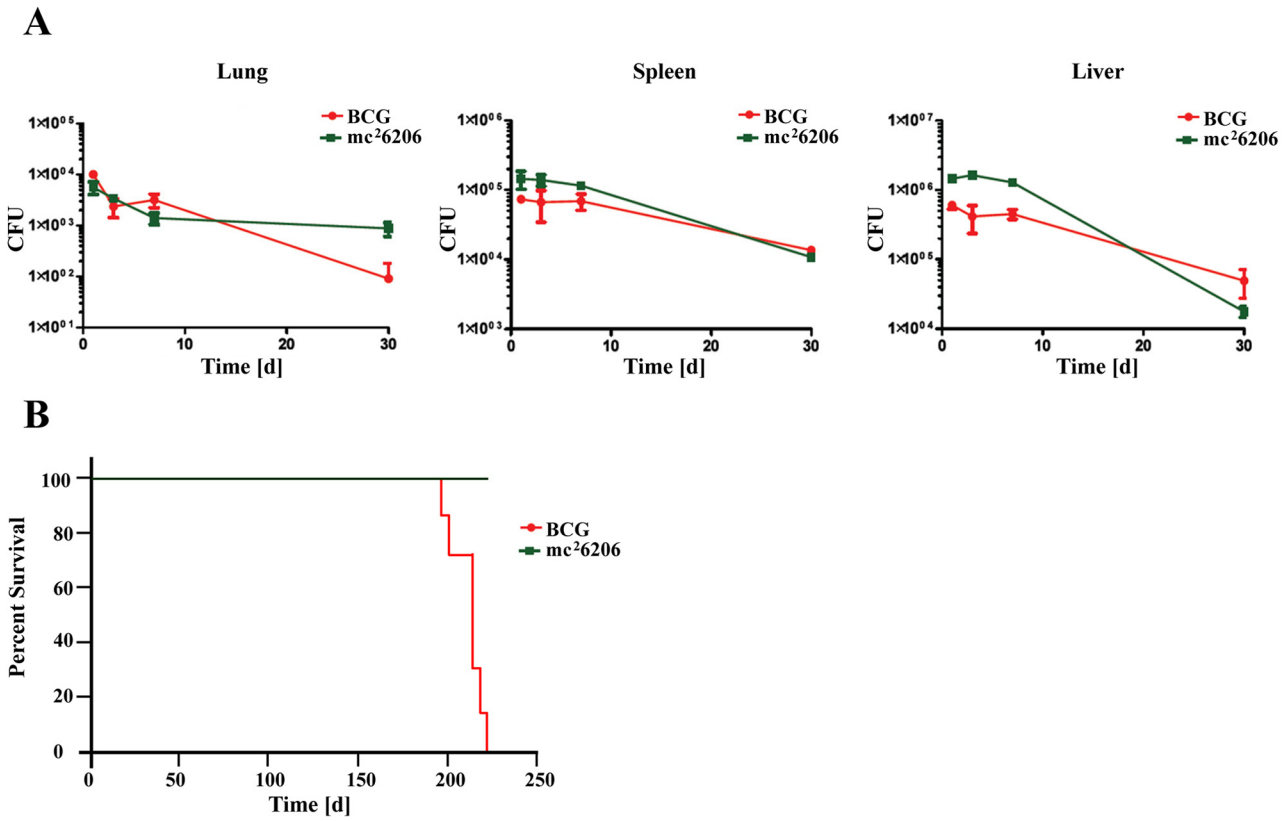


FIG 5 Δ*leuCD* Δ*panCD* deletions attenuate virulence of *M. tuberculosis* H_{37}Rv. (A) Kinetics of persistence of *M. bovis* BCG SSI Danish and the *M. tuberculosis* H_{37}Rv Δ*leuCD* Δ*panCD* strain (mc²6206) in immunocompetent mice. C57BL/6 mice were intravenously (i.v.) infected with approximately 10^6 CFU of *M. bovis* BCG or mc²6206. Organs were harvested at different time points as indicated. (B) Ten mice were i.v. infected with approximately 10^5 CFU *M. bovis* BCG SSI or mc²6206 and their survival times were recorded. Results are representative of three independent experiments.

against TB and human immunodeficiency virus (HIV) or to study *M. tuberculosis* physiology (see Discussion).

DISCUSSION

Rate-limiting steps for strain construction in mycobacteria, including virulent *M. tuberculosis*, have been identified and partially ameliorated. More than a hundred distinct loci at diverse chromosomal locations have been engineered in virulent H_{37}Rv. The methods described are not restricted to any particular region of the chromosome or any functional category of genes and appear broadly applicable to different species and strains of mycobacteria. *M. tuberculosis* H_{37}Rv, *M. tuberculosis* CDC1551, *M. tuberculosis* clinical isolates, and several *M. bovis* and *M. smegmatis* strains have all proven amenable to these methods. Multiple, up to 14, adjacent reading frames, which span 16.5 Mb and two operons, have been deleted in a single step (C. Vilchèze, unpublished data). The same AES generated by one-step cloning can be used for recombineering (15) or cloned into phAE159 to obtain STP. The STPs can be amplified by growing a phage stock. In most cases, the same STP can be used in multiple strains or species of mycobacteria which often harbor 95% or more sequence similarity (e.g., between *M. bovis* and various *M. tuberculosis* strains). The ability to use the same STP in different species will facilitate study of the subtle variations in *M. bovis* and BCG and *M. tuberculosis* physiology (16).

Transposon mutagenesis has been conducted at a large scale appropriate for saturation for various *M. tuberculosis* strains (17). Tn*Himar1* insertion mutants are available for many genes from the Target website (http://webhost.nts.jhu.edu/target/) and are valuable tools for mycobacterial genetics. As informative as transposon insertions are, construction of defined null deletion by the methods described in this work has advantages. (i) The endpoints of these deletion-substitutions are precisely engineered and do not include the pseudo-random aspect of transposon insertions. (ii) Transposons must be mapped after the fact, whereas AES used in specialized transduction are mapped before by the choice of sequences for replacement and are verified by a simple PCR (Fig. 2). (iii) The antibiotic resistance cassette can be removed in a single step (Fig. 4); there is no easy way to remove the drug marker from transposon insertions. (iv) About half the genes in *M. tuberculosis* are in operons (18), and transposon insertions are known to often have polar effects (19), which can be easily avoided by generating in-frame deletions (Fig. 4D).

In addition to the larger cloning capacity of 10 kb, phAE159 is also deleted for TM4 gene 49, which has been implicated in superinfection exclusion (20). Expression studies with green fluorescent protein (GFP) are consistent with the interpretation that phAE159 is superior to parental TM4 in allowing independent phage to infect and express in the same host cell (21). The ability to transiently express heterologous genes is a very effective tool to generate in-frame unmarked deletion mutants in mycobacteria and implies that phAE159 is a general and effective vector for transient gene expression in mycobacteria.

TABLE 1 List of primers used to generate pYUB1471

Primer name	Primer sequence[a]
pJSC347HL	TTTTTTTTCCAT**GAA**TTGGCAGGTCCTGTATCCTAAATC
pJSC347HR	TTTTTTTTCCAT**TCT**TTGGCTAGAGTCCTGTCCGAAATA.
pJSC34OL	TTTTTTTTCCAT**AAG**TTGGCAGGTTTGACAGCTTATCCAT
pJSC34OR	TTTTTTTTCCAT**TTT**TTGGAGTGAGTCGTATTACGATCCT
0004LL	TTTTTTT CCAT**AAA**TTGGCCGCACCGTGACGACCTAA
0004LR	TTTTTTTTCCAT**TTC**TTGGAGCGGTTGTGGATCACGAATG
0004RL	TTTTTTTTCCAT**AGA**TTGGCGCGACACCTACGGATAACA
0004RR	TTTTTTTT CCAT**CTT**TTGGGCGTTCACTTGCCGACTT
sacBL	TTTTTTTTCACGCGGTGCTTTTTAACCCATCACATATAACC
sacBR	TTTTTTTTCAGCGCCTGAGATCGGCATTTTCTTTTGCGTT

[a] The variable overhangs in the Van91I recognition sequences at primer ends are in bold.

Vaccination protection results obtained with the fully antibiotic-sensitive double-deletion strain mc^{2}6206 are consistent with the recently published results of a hygromycin-resistant Δ*leuD* and Δ*panCD* deletion strain (22). Thus, mc^{2}6206 appears to be significantly safer than *M. bovis* BCG (a BSL2 organism) (Fig. 5B). This strain has been cleared for BSL2 work by the biosafety committee of Albert Einstein College of Medicine and is available for further biochemical and genetic studies. The mc^{2}6206 strain is fully antibiotic sensitive and amenable to further genomic manipulations. *M. tuberculosis* mc^{2}6206 has been used as a base strain to delete *secA2* (mc^{2}6208) and has been unmarked to generate the antibiotic-sensitive triple-deletion Δ*panCD* Δ*leuCD* Δ*secA2* mutant (mc^{2}6209). It has been observed that the deletion of *secA2* in mc^{2}6206 makes the strain proapoptotic (U. D. Ranganathan, M. H. Larsen, W. R. Jacobs, Jr., G. J. Fennelly, unpublished data). Another mc^{2}6206 derivative, *M. tuberculosis* mc^{2}6435 (Δ*panCD* Δ*leuCD* Δ*secA2*, expressing simian immunodeficiency virus [SIV] Gag), was found to be safe for oral or intradermal administration to non-SIV-infected and SIV-infected infant macaques (23), suggesting mc^{2}6206 as a platform for a hybrid TB and HIV vaccine.

Strain construction in *M. tuberculosis* remains inherently difficult because of the organism's slow growth and pathogenicity. The apparently low rate of homologous recombination in mycobacteria means that even these new methods do not bring mycobacterial genetics to the level of the most facile microbial systems, such as *Escherichia coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*. Nonetheless, these new methods allow undertaking construction of what has been one of the most fruitful resources in model systems: a comprehensive set of strains, each of which is deleted for a single defined open reading frame.

MATERIALS AND METHODS

Generation of pYUB1471. The AES cloning vector, pYUB1471, was generated by directional cloning of four PCR fragments via primers with a unique variable region at the 5′ end of their Van911 site. Initially, hygromycin and ColEI origin of replication (*oriE*) fragments were PCR amplified using primer pairs pJSC347HL/pJSC347HR and pJSC34OL/pJSC34OR, respectively, from the pJSC347 (5) template. LHS and RHS fragments of *Rv0004* were PCR amplified with primer pairs 0004LL/0004LR and 0004RL/0004RR, respectively, from H_{37}Rv genomic DNA. The four fragments, hygromycin, *oriE*, LHS, and RHS, were Van91I digested and ligated directionally to construct pYUB1165. pYUB1471 was generated by ligating the Van91I digested pYUB1165 to DraIII and AlwNI digested PCR product obtained using primer pair sacBL/sacBR and pYUB870 as template (4). The sequence of pYUB1471 is provided in Text S1 in the supplemental material. Sequences of all the primers are listed in Table 1.

Construction of phAE280. Plasmid carrying resolvase enzyme was constructed by digestion of plasmid pYUB870 (4) with XbaI and SpeI, and a 5.7-kb fragment carrying *tnpR* (γδ resolvase), a kanamycin resistance gene, and the *sacB* gene was ligated to a 1.9-kb XbaI and SpeI fragment from plasmid pYUB854 (4), which has *oriE*, a lambda *cos* site, and PacI recognition sequences. The resulting plasmid, pYUB1672, was cloned into phAE159 and electroporated into mc^{2}155 to obtain temperature-sensitive phage phAE280.

Construction of AES. Amplicons between 650 and 1,000 bases flanking the gene were PCR generated with primer sets (LL/LR and RL/RR) to generate gene-specific LHS and RHS. Plasmid pYUB1471 was digested with Van91I to release four fragments. Two vector fragments (3.6 kb and 1.6 kb) which correspond to the *sacB-hyg* cassette and *oriE-cos* fragment, respectively, were cut out of the agarose gel and were prepared in bulk for all AES constructions. These two fragments were ligated in one step to Van91I-digested LHS and RHS fragments corresponding to the gene of interest (Fig. 1A). If a Van91I site is present in LHS or RHS, DraIII, BstAPI, or AlwNI restriction sites (Fig. 1B) can be used for the generation of AES. The ligation mix was transformed in *E. coli* DH5α, and the clones were confirmed by Van91I digestion. It is important that the *E. coli* strain used for plasmid and phasmid propagation does not express γδ resolvase. All the primers used to generate the AES are listed in Table S1 in the supplemental material. The sequence of the plasmid pYUB1471 is provided in Text S1 the supplemental material.

Construction of temperature-sensitive TM4 shuttle phasmid phAE159 is described in Text S2 the supplemental material. The sequence of the phasmid phAE159 is provided in Text S1 in the supplemental material.

High-throughput generation of STPs and transduction. A stepwise protocol for the generation of deletion-substitution mutants by transduction is shown in Fig. 2. A detailed protocol is given in the supplemental material.

Unmarking of the γδ(*sacB-hyg*)γδ cassette from deletion-substitution mutants. The protocol is summarized in Fig. 4B. Briefly, the log-phase *M. tuberculosis* culture, obtained by subculturing at least once at an optical density at 600 nm (OD_{600}) of 0.6 to 0.8, was grown to an OD_{600} of 0.6 to 0.8. Three milliliters of culture was centrifuged at 4,000 relative centrifugal force (RCF) at room temperature (RT). The pellet was washed twice by resuspending in 10 ml MP buffer followed by centrifugation at 4,000 RCF at RT. The pellet was suspended in MP buffer (100 to 200 μl) to obtain an OD_{600} of 1.0 to 1.5. A total of 50 μl of washed cells was incubated with 500 μl of phAE280 (titer of ~10^{10}) at 37°C for 3 days. A total of 100 μl of sample was plated directly on 10% sucrose, and the remaining 450 μl of sample was diluted with 1.5 ml of 7H9 medium with 0.05% Tween. A total of 20 μl, 100 μl, 300 μl, and the remaining sample (after centrifugation) were plated on 10% sucrose. The plates were incubated at 37°C for 3 weeks. Colonies obtained were analyzed for the excision of *sacB-hyg*, after recombination between the two γδ sites in the cassette γδ(*sacB-hyg*)γδ, by both colony PCR and replica patching on plates with and without hygromycin (75 μg/ml) to determine the frequency of unmarking.

Colony PCR and Southern analysis of *M. tuberculosis* mutants. Approximately half the colony was mixed with 100 μl of Bio-Rad DNA matrix with Triton X-100 (90 μl matrix [catalog number 7326030] and 10 μl of 0.1% Triton X-100) and incubated at 98°C for 40 min in a PCR machine. PCR tubes were briefly centrifuged at ~3,000 rpm. A total of 10 μl of the sample was used to set up a PCR. Southern blot analysis was performed as described previously (4).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01245-14/-/DCSupplemental.

Text S1, DOC file, 0.1 MB.
Text S2, DOC file, 0.2 MB.
Figure S1, PDF file, 0.1 MB.
Figure S2, TIF file, 8.7 MB.
Table S1, DOC file, 0.2 MB.

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