ORIGINAL ARTICLE

Transposon mutagenesis in *Mycobacterium kansasii* links a small RNA gene to colony morphology and biofilm formation and identifies 9,885 intragenic insertions that do not compromise colony outgrowth

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Abstract

Mycobacterium kansasii (*Mk*) is a resilient opportunistic human pathogen that causes tuberculosis-like chronic pulmonary disease and mortality stemming from comorbidities and treatment failure. The standard treatment of *Mk* infections requires costly, long-term, multidrug courses with adverse side effects. The emergence of drug-resistant isolates further complicates the already challenging drug therapy regimens and threatens to compromise the future control of *Mk* infections. Despite the increasingly recognized global burden of *Mk* infections, the biology of this opportunistic pathogen remains essentially unexplored. In particular, studies reporting gene function or generation of defined mutants are scarce. Moreover, no transposon (Tn) mutagenesis tool has been validated for use in *Mk*, a situation limiting the repertoire of genetic approaches available to accelerate the dissection of gene function and the generation of gene knockout mutants in this poorly characterized pathogen. In this study, we validated the functionality of a powerful Tn mutagenesis tool in *Mk* and used this tool in conjunction with a forward genetic screen to establish a previously unrecognized role of a conserved mycobacterial small RNA gene of unknown function in colony morphology features and biofilm formation. We also combined Tn mutagenesis with next-generation sequencing to identify 12,071 Tn insertions that do not compromise viability in vitro. Finally, we demonstrated the susceptibility of the *Galleria mellonella* larva to *Mk*, setting the stage for further exploration of this simple and economical infection model system to the study of this pathogen.

KEYWORDS
biofilm, *Galleria mellonella*, gene essentiality, *Mycobacterium kansasii*, nontuberculous mycobacteria, small noncoding RNA

1 | INTRODUCTION

Chronic pulmonary disease (CPD) due to opportunistic infections of nontuberculous mycobacterial (NTM) pathogens from environmental sources has become an increasingly recognized global public health problem (Kendall & Winthrop, 2013; Prevots & Marras, 2015). At least forty NTM spp. are associated with CPD (Daley & Griffith, 2010). The ubiquitous slow-growing *Mycobacterium kansasii* (*Mk*) is among the six most common NTM spp. associated with CPD in the United States and many other areas of the world, where it is often surpassed only by the *Mycobacterium avium* complex (Davies, Roberts, Kaul, Klein, & Milburn, 2012; Hoefsloot et al., 2013; Mirsaeidi, Farshidpour, Allen, Ebrahimi, & Falkinham, 2014; Prevots & Marras, 2015). The burden of *Mk* infections is not surprising given that the risk factors include HIV infection, pharmacological immunosuppression, chronic obstructive pulmonary disease (COPD), malignancy, bronchiectasis, and previous mycobacterial disease (Maliwan & Zvetina, 2005; Prevots & Marras, 2015). *Mk* is often present in human-made water systems, where it forms resilient biofilms (Chakraborty & Kumar, 2019; Kwenda et al., 2015; Vaerewijck, Huys, Palomino, Swings, & Portaels, 2005). Infection generally originates following inhalation, aspiration, ingestion, or local inoculation (Griffith et al., 2007; Vaerewijck et al., 2005). Although person-to-person transmission has not been reported, some studies suggest that it might be possible, and the potential emergence of strains with an epidemiologically meaningful person-to-person transmission capacity is a concern (Ricketts, O'Shaughnessy, & van Ingen, 2014).

Mycobacterium kansasii has seven subtypes, with subtype 1 being by far the most prevalent in human isolates, followed by subtype 2. The remaining subtypes are recovered from environmental samples almost exclusively (Bakula, Safianowska, Nowacka-Mazurek, Bielecki, & Jagielski, 2013; Picardeau, Prod'Hom, Raskine, LePennec, & Vincent, 1997; Taillard et al., 2003; Tortoli, 2003). *Mk* has a relatively close phylogeny to the *Mycobacterium tuberculosis* (*Mtb*) complex and its related *Mycobacterium canettii* clade, and an *Mk*-like opportunistic pathogen is believed to be an *Mtb* complex ancestor (Minnikin et al., 2015; Veyrier, Pletzer, Turenne, & Behr, 2009; Wang et al., 2015). Moreover, *Mk* has been considered as a possible model to explore the evolutionary transition from the environmental opportunistic pathogen lifestyle to the obligate pathogen lifestyle seen in the genus, and as a surrogate of *Mtb* for tuberculosis (TB) pathogenesis studies (Minnikin et al., 2015; Wang et al., 2015).

The CPD caused by *Mk* resembles TB and its mortality is associated with comorbidities and treatment failure (Ehsani, Reddy, Mosunjac, Kraft, & Guarner, 2015; Evans et al., 1996; Hirashima et al., 2014; Maliwan & Zvetina, 2005). Relapses occur often (≈10%) and cure rates are only 80%–90% (Davies et al., 2012; Mirsaeidi et al., 2014; Santin et al., 2009). The prevalence of *Mk* CPD remains under-recognized, particularly in high TB-HIV burden areas, due to overlapping features with TB, lack of proper diagnosis, and absence of compulsory reporting policy. Nevertheless, it is clear that *Mk* CPD continues to rise with the enduring HIV epidemic and the increase

of other predisposing factors in the population (e.g., immunosuppression and COPD). In addition, infection of skin and subcutaneous tissue is not uncommon, and, like primary lung infection, may lead to disseminated disease in people with a compromised immune system or other vulnerabilities (Brown-Elliott, Nash, & Wallace, 2012; Flor, Capdevila, Martin, Gavalda, & Pahissa, 1996; Henkle & Winthrop, 2015; Johnston & Elwood, 2011).

The complex drug treatment of *Mk* CPD exacerbates the public health challenge this pathogen presents. Costly, long-term, multidrug courses with adverse side effects often reduce compliance and positive treatment outcomes (Brown-Elliott et al., 2012; Griffith et al., 2007). A main first-line treatment in the Unites States has been a regimen that includes three anti-TB drugs (typically rifampin or rifabutin, ethambutol, and isoniazid) daily until the patient has at least 12 months of negative sputum cultures while on therapy (Griffith et al., 2007; Philley & Griffith, 2015). *Mk* isolates resistant to TB drugs have been reported (Brown-Elliott et al., 2012; Lyons et al., 2014; Wang et al., 2010; Wu et al., 2009; Yamada et al., 1997). In particular, resistance to rifampin, a drug which has a key role in the multidrug treatment of *Mk* infections, correlates with higher rates of treatment failure and relapse (Brown-Elliott et al., 2012; Griffith et al., 2007; Wu et al., 2009). For patients with rifampin-resistant infections, a usual guideline is a regimen based on in vitro-determined isolate susceptibilities to secondary agents (e.g., fluoroquinolones, tetracyclines, amikacin, linezolid, trimethoprim-sulfamethoxazole, and rifabutin) and including at least three drugs (Griffith et al., 2007; Philley & Griffith, 2015).

Despite the burden of *Mk* infections, the biology of this pathogen remains poorly explored. Encouragingly, the completion of the *Mk* (subtype 1 strain) genome sequence was recently reported (Wang et al., 2015), and various aspects of *Mk* biology have been inferred by bioinformatics and extrapolation from insights gained for *Mtb* (Veyrier et al., 2009; Wang et al., 2015). However, the 6.57 Mb of genetic material carried by *Mk* represents 2.16 Mb more than that present in *Mtb* (Cole et al., 1998), and thus in silico approaches do not provide a full picture and may even lead to flawed conclusions. In all, it is clear that experimentation focused on *Mk* is needed to deepen our knowledge of its biology, further understand its ability to cause disease, and illuminate paths to better therapeutics. In particular, the scarcity of gene function studies and the lack of defined mutants are major barriers to progress in the field. In fact, to our knowledge, there are only four reported *Mk* gene function studies (Klein, Brown, & French, 2001; Nataraj et al., 2015; Onwueme, Vos, Zurita, Soll, & Quadri, 2005; Sun & Zhang, 1999). Moreover, no transposon (Tn) mutagenesis tool has been validated for use in *Mk*, a situation limiting the repertoire of genetic approaches to accelerate gene function dissection in this pathogen.

In this study, we explored the use of genome-wide Tn mutagenesis-based approaches to interrogate gene function in *Mk*. We provide proof-of-principle for the application of these approaches to *Mk* by using them to (a) link a conserved small RNA (sRNA) gene to colony morphology and biofilm formation and to (b) identify ≈12,000 Tn insertions that do not impede colony outgrowth. Lastly,

we present the first demonstration of the susceptibility of *Galleria mellonella* (GM) larvae to *Mk*, thus setting the stage for further exploration of the GM infection model system to study this opportunistic pathogen.

2 | MATERIALS AND METHODS

2.1 | Routine culturing conditions, molecular biology techniques, and reagents

Mycobacteria were cultured under standard conditions in Middlebrook 7H9, 7H10, or 7H11 media (Difco, Becton-Dickinson and Co.) supplemented as reported (Chavadi, Edupuganti, et al., 2011; Chavadi, Stirrett, et al., 2011). *Mk* cultures were agitated at 170 rpm with a few sterile glass beads (3 mm diameter) to reduce aggregation. *Mk* growth curve experiments were carried out using a 96-well plate-based growth assay we reported previously (Chavadi, Edupuganti, et al., 2011), except that the plates were incubated at 37°C. Where appropriate, kanamycin (Km, 30 µg/ml) and/or hygromycin (Hyg, 150 µg/ml) were added to the Middlebrook media. Culturing of *Escherichia coli* strains was done under standard conditions in Luria–Bertani broth and agar media (Sambrook & Russell, 2001). When required, ampicillin (Amp, 100 µg/ml), Km (30–50 µg/ml), Hyg (150 µg/ml), and/or 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal, 56 µg/ml) were added to the Luria–Bertani media. DNA manipulations were carried out using established protocols and *E. coli* as the primary cloning host (Sambrook & Russell, 2001). PCR-generated DNA fragments used in plasmid constructions were sequenced by the Sanger method to verify fidelity (Genewiz, Inc.). Sequence alignments were carried out using DNASTAR's Lasergene sequence analysis software (DNASTAR, Inc.). Predictions of sRNA structures and minimum free energies were obtained with mFold software using default parameters (Zuker, 2003). Isolation of *Mk* genomic DNA, plasmid electroporation into *Mk*, and selection of *Mk* transformants were carried out following standard methodologies (Chavadi, Edupuganti, et al., 2011; Chavadi, Stirrett, et al., 2011). Unless otherwise indicated, reagents were purchased from Sigma-Aldrich Inc., Thermo Fisher Scientific Inc., New England Biolabs Inc., Qiagen LLC , or VWR International, LLC. The strains and oligonucleotides used in this study are listed in Tables A1 and A2, respectively (Appendix 1).

2.2 | Phage stock preparation, transductions, and transposon mutant selection

High-titer stocks (≈10^{11} PFU/ml) of the φMycoMarT7 phage were generated and titrated using *Mycobacterium smegmatis* (*Msm*) as reported (Siegrist & Rubin, 2009). For *Mk* transductions, bacteria from actively growing cultures were harvested by centrifugation, washed twice with 1 culture volume of phage buffer (PB:

supplemented 7H9 medium lacking Tween-80), and concentrated 10 times in PB. *Mk* suspension aliquots were combined with phage stock aliquots to obtain transduction mixtures with desired PFU-to-CFU multiplicity of infection (MOI) values. MOIs of 0.3:1 and 1:1 (average) were used in the transductions for generation of the mutant libraries used in the genetic screen and the transposon insertion sequencing experiment, respectively. Mock transduction controls were routinely prepared by adding equivalent volumes of PB to the cell suspensions. Transduction mixtures and mock controls were incubated at 37°C without shaking for 4 hr. After incubation, aliquots of transduction mixtures and mock controls were used to enumerate Km resistant (Km[R]) CFU and total CFU on agar plates with and without Km, respectively. The remainder of each transduction mixture was made 10% glycerol, aliquoted, and stored at −80°C until use.

2.3 | Arrayed library preparation

Transduction mixtures were plated on supplemented Middlebrook 7H10 medium with Km to obtain 100 Tn mutant colonies per plate. The plates were incubated at 37°C for three weeks for Km[R] colony growth. The Km[R] colonies were picked and individually arrayed in 96-well plates (Falcon, Corning) preloaded with supplemented Middlebrook 7H9 medium with Km (200 µl/well). The 96-well plates were incubated at 37°C with orbital shaking (170 rpm) under a humidified atmosphere for 8 days to grow the microcultures. After incubation, the microcultures were homogenized with a multichannel pipette and replicate inoculated (1.5 µl transfer) with a 96-pin inoculator (Clonemaster™; Immusine Laboratories, Inc.) into new 96-well culture plates preloaded with fresh medium. The inoculated plates were then incubated as described above for microculture growth. After incubation, the microcultures were made 4% glycerol and the 96-well library plates were sealed with sterile adhesive foil film sheets and stored at −80°C until use.

2.4 | Morphology screen

The microcultures in the 96-well library plates were defrosted and homogenized with a multichannel pipette. The microcultures of each library plate were then subcultured into a pair of 96-well plates (5 µl inoculum into a well preloaded with 195 µl of medium) using an every-other-well redistribution pattern. The inoculated 96-well plates were incubated to grow the microcultures to late-log phase. Following the incubation, the microcultures were homogenized as above, and 1.5 µl of each microculture was spotted onto supplemented 7H10 plates using a 96-pin inoculator. The inoculated screen plates were incubated at 37°C until most macrocolonies reached 0.5–1.0 cm in diameter (≈18 days). After incubation, the screen plates were scrutinized for macrocolonies with mutant phenotypes by naked eye and using an Olympus SZX7 stereo microscope (Olympus Corp.).

2.5 | Southern blot hybridization analysis

Samples of genomic DNA (0.5–1.0 µg) digested with SacII (an enzyme that does not cut within the Tn) were separated alongside a prestained DNA molecular weight marker (F. Hoffmann-La Roche, Ltd.) by electrophoresis in a 0.7% agarose gel. After electrophoresis, the DNA on the gel was denatured and transferred onto a nylon membrane (Amersham Hybond N+; GE Healthcare Life Sciences) as reported (Quadri, Sailer, Roy, Vederas, & Stiles, 1994). The Tn-specific 512-bp DIG-labeled DNA hybridization probe was generated by PCR amplification using primers GG1 and GG2 and a PCR DIG-labeled probe synthesis kit (F. Hoffmann-La Roche, Ltd.) according to manufacturer's instructions. The probe was used for the hybridization analysis following reported protocols (Quadri et al., 1994; Sambrook & Russell, 2001) and visualized by chromogenic detection using a DIG-labeled DNA detection kit (F. Hoffmann-La Roche, Ltd.) according to the DIG Application manual (F. Hoffmann-La Roche, Ltd.).

2.6 | Identification of the transposon insertion site in *Mycobacterium kansasii* 13D6

Cloning of the Tn-containing genomic fragment and Tn-genome junction sequencing were carried out as reported (Siegrist & Rubin, 2009). Briefly, genomic DNA digested with SacII was self-ligated and transformed into *E. coli* DH5α λpir for rescue cloning of Tn-containing genomic fragments. The cloned plasmids were purified, and the Tn-genome junctions were sequenced using primers CB2 and CB2b. The genomic sequences identified were mapped onto the *Mk* ATCC 12478 genome (chromosome: GenBank NC_022663.1; plasmid pMK12478: GenBank NC_022654.1).

2.7 | Construction of plasmids pML-B11 and pML1335$^{\Delta xylE}$

To generate pML-B11, vector pML1335 (Huff, Czyz, Landick, & Niederweis, 2010) was digested with BspHI and NotI to excise the *xylE* marker segment. The resulting linearized vector backbone was ligated to a BspHI-NotI fragment encompassing the *Mk B11* gene along with its adjacent extragenic regions (chromosomal coordinates 2809862–2810381, 520 bp). The fragment was generated by PCR amplification from genomic DNA using primers CB3 and CB7, cloned into pCR2.1-TOPO (TOPO TA cloning kit; Invitrogen), sequenced for fidelity verification, and then subcloned into the pML1335 backbone as noted above to create pML-B11. The sequence of *B11* determined for the ATCC® 12478™ strain used in this study was found to have a hepta-C string instead of the hexa-C string in the annotated chromosome (GenBank: NC_022663.1) that maps to the loop of the middle stem-loop of the sRNA. Plasmid pML1335$^{\Delta xylE}$ (empty vector control) was generated by removal of the *xylE* marker segment of pML1335 and subsequent vector backbone religation.

2.8 | RNA isolation

Pellets containing 1–5×10^7 CFU of *Mk* grown in supplemented Middlebrook 7H9 medium to log phase or scrapings of equivalent mass taken from macrocolonies grown at 37°C for 23 days on supplemented Middlebrook 7H10 plates were resuspended in 1 ml TRIzol™ reagent (Invitrogen™). The resuspended cells were lysed with zirconia/silica beads (Bio Spec Products Inc.) in a bead-beater homogenizer (Mini-BeadBeater 16, Bio Spec Products Inc.) using two bead-beating cycles of 50 s each separated by a 10-min chilling step on ice. The lysates were spun (22,000 *g*, 2 min, 4°C) to pellet beads and cellular debris. The supernatants were transferred to siliconized microcentrifuge tubes, combined with 300 µl of chloroform:isoamyl alcohol (24:1), shaken vigorously for 15 s, and incubated at room temperature for 3 min. After incubation, the samples were centrifuged (12,000 *g*, 15 min, 4°C), and the RNA was isolated from the supernatant using PureLink™ RNA Mini and miRNA Isolation Kits (Invitrogen™) following the manufacturer's instructions.

2.9 | Northern blot analysis

Total RNA (1 µg, originated from 2×10^6 CFU) was separated on a 6% denaturing polyacrylamide gel (Novex precast 6% TBE-Urea polyacrylamide gel, Invitrogen™) run in a XCell SureLock™ Mini-Cell (Invitrogen™) according to manufacturer's protocols. Prestained molecular weight markers (DynaMarker DM270; BioDynamics Laboratory Inc.) were separated alongside *Mk* total RNA and the 100-bp hybridization probe fragment (see below), which was used as reference for maker size calibration. After electrophoresis, the gel was electroblotted onto a nylon membrane (Amersham Hybond N+; GE Healthcare Life Sciences) using a XCell SureLock™ Mini-Cell Blot Module (Invitrogen™) in the XCell SureLock™ Mini-Cell according to manufacturer's instructions and subsequently baked for 2 hr at 80°C. An *Mk B11*-specific DIG-labeled hybridization probe (100 bp; genome coordinates 2810109–2810208) was generated by PCR amplification using primers CB9c3 and CB8c and a PCR DIG-labeled probe synthesis kit (F. Hoffmann-La Roche, Ltd.) according to manufacturer's instructions. The probe was used for the hybridization analysis following reported protocols (Quadri et al., 1994; Sambrook & Russell, 2001) and visualized by chromogenic detection using a DIG-labeled nucleic acid detection kit (F. Hoffmann-La Roche, Ltd.) according to the DIG Application manual (F. Hoffmann-La Roche, Ltd.).

2.10 | RT-qPCR analysis

The experiments were done using transcript target-specific primer pairs (Table A2 in Appendix 1) and the QuantiNova® SYBR® Green RT-PCR Kit as recommended by the manufacturer (Qiagen, LLC). All primer pairs were optimized to have >90% amplification efficiency (as judged by standard curves generated from genomic DNA) and to produce a unique amplicon (as judged by melt curve analysis) of

the expected size (as judged by agarose gel electrophoresis). RNA samples used for RT-qPCR were DNase-treated and verified to be free of DNA contamination by PCR. One-step RT-qPCR reactions (10 µl, 30 pg of RNA template) were run in a Rotor-Gene Q light cycler (Qiagen, LLC). Two-step PCR cycling was performed as follows: 95°C for 5 s and 60°C for 10 s for 40 cycles. For each transcript target, the threshold cycle (C_t) was normalized to the C_t of the standard mycobacterial internal calibrator *sigA* transcript to generate transcript relative abundance data as reported (Dubnau, Fontan, Manganelli, Soares-Appel, & Smith, 2002; Livak & Schmittgen, 2001).

2.11 | Biofilm formation

The biofilm formation assay was carried out using the MBEC™ device (Innovotech Inc.) and a protocol comparable to one we recently reported (Mohandas et al., 2016). Briefly, *Mk* cells from actively growing cultures were harvested by centrifugation and resuspended to an OD_{600} of 0.1 in Middlebrook 7H9 lacking ADN supplementation (Chavadi, Stirrett, et al., 2011) and Tween-80. The cell suspensions were loaded (150 µl/well) into the 96-well plates, and the plates were incubated with 100 rpm agitation at 37°C for 6 weeks under a humidified atmosphere. After incubation, *Mk* culture-exposed pegs and sterile broth-exposed control pegs were washed twice with distilled water, briefly dried at room temperature, stained for 15 min with 0.5% crystal violet (CV), rinsed thrice with distilled water, and dried overnight. The CV on the pegs was then extracted in 95% ethanol and quantified spectrophotometrically at 595 nm using a DTX 880 multimode detector microplate reader (Beckman Coulter, Inc.).

2.12 | *Galleria mellonella* larva killing assay

The assay was carried out using an infectious dose and methodologies comparable to those recently reported for other slow-growing mycobacteria (Li et al., 2018). Larvae in the sixth instar stage were purchased from Timberline Fisheries, Corp. (Marion, IL) and used the day of delivery. Only larvae displaying healthy movement, no melanization signs, and 150–250 mg body weight were used in the experiments. Larvae were restrained for injection as reported (Dalton, Uy, Swift, & Wiles, 2017), and injections were given into the last left pro-leg using a 22 s ga beveled stainless steel needle affixed to a 50-µl Hamilton syringe. For each experiment, groups of 15 larvae were injected with 10 µl of either sterile phosphate-buffered saline [PBS (Li et al., 2018)] or PBS carrying 1×10^7 CFU, or not injected. Each larval group was placed in a paper-lined 10-cm petri dish, and the dishes were incubated in the dark at 37°C or 25°C and observed daily for larva survival. Larvae were considered dead when no movement was elicited upon physical stimulation. Kaplan–Meier curves were used to plot survival data, and the Log-rank (Mantel-Cox) test embedded in Prism v6.01 (GraphPad Software, Inc.) was used for curve comparisons.

2.13 | Transposon insertion sequencing experiment

Colonies from a library of ≈14,700 Tn mutants were scraped from supplemented 7H11 plates, and genomic DNA was isolated from the mutant pool as reported (Chavadi, Stirrett, et al., 2011). A modified TraDIS-type method (Fan et al., 2018; Langridge et al., 2009) customized for the *Himar1*-based Tn in ϕMycoMarT7 was used to identify the insertion sites in the genomic DNA library pool. Briefly, genomic DNA was fragmented to a ≈350-bp average length and adaptor sequences were ligated to each end of the fragments using the KAPA HyperPlus Kit (Kapa Biosystems, Inc.) as recommended by the manufacturer. Two rounds of optimized PCR reactions (PCR-1 and PCR-2) were performed to enrich for Tn-genomic DNA junction fragments and make the final PCR product suitable for Illumina sequencing. PCR-1 was done with a Tn-specific primer (CB1) and an adapter-specific primer (IS5) for Tn-genome junction enrichment. PCR-2 (hemi-nested) was run with the adapter-specific primer IS5 and a Tn-specific primer adding the sequences needed for Illumina sequencing and barcoding of each sample (primers CB25.3_R or CB44.3_R). (See Appendix 3) The generated fragment libraries were sequenced on an Illumina NextSeq 500 System using a NextSeq500/550 Mid Output Kit (150 cycles; 2 × 75 cycles; paired-end reads; Illumina Inc.). Custom primers were spiked to initiate the index read (primer barcodeseq) and read two sequencing (primer SP2). Raw sequencing read data were processed as follows. (a) Duplicate reads (same sequences for paired-end reads, considered PCR siblings) were compressed into a single representative read. (b) Reads were filtered further based on the presence of the terminal 5-bp Tn sequence (TGTTA) adjacent to the Tn primer (CB25.3_R or CB44.3_R). (c) The primer sequences were trimmed. (d) Each filtered and trimmed read was mapped onto the *Mk* chromosome (GenBank: NC_022663.1) and plasmid pMK12478 (GenBank: NC_022654.1) sequences using BLAST (https://blast.ncbi.nlm.nih.gov/BLAST).

3 | RESULTS AND DISCUSSION

3.1 | Evaluation of the ϕMycoMarT7 transposon mutagenesis system in *Mycobacterium kansasii*

The ϕMycoMarT7 phagemid system has been the most successful Tn mutagenesis approach for mycobacteria thus far (Sassetti, Boyd, & Rubin, 2001). The system relies on specialized transduction by a temperature-sensitive mycobacteriophage to deliver a *Himar1* derivative Tn. The Tn inserts randomly at TA dinucleotide sites via a cut-and-paste mechanism dependent on a transposase encoded outside the Tn in ϕMycoMarT7. In susceptible hosts, the ϕMycoMarT7 phage typically defaults to a lytic cycle at the permissive temperature (30°C), but it does not replicate and becomes an effective Tn delivery suicide vector at the nonpermissive temperature (37°C). The system has been demonstrated to be functional in a few *Mycobacterium* spp. (*Mtb*, *Msm*, *M. bovis* BCG, *M. avium* subsp.

paratuberculosis, *M. marinum*, and *M. fortuitum*) (Driessen et al., 2010; Sassetti et al., 2001; Wang, Pritchard, Kreitmann, Montpetit, & Behr, 2014). However, it is not expected to work in all *Mycobacterium* spp. due to phage host range, Tn functionality, or other limitations (Bardarov et al., 1997; Rybniker, Kramme, & Small, 2006). Ultimately, the functionality of the system in any given *Mycobacterium* sp. needs to be experimentally determined.

Due to the advantages of the ϕMycoMarT7 system, we set out to evaluate its performance in *Mk*. To this end, we first assessed the ability of the phage to produce plaques on *Mk* lawns at the permissive and nonpermissive temperatures. As a control, we carried out the equivalent experiment using lawns of *Msm*, the standard host for ϕMycoMarT7 phage propagation and titration. These experiments revealed that the phage was able to produce plaques on *Mk* lawns only at the permissive temperature (Figure 1a) and that the plaque formation efficiency was ≈800-fold lower in the pathogen than in *Msm* (Figure 1b). The latter finding suggests that either the infection step or the lytic cycle, or both, is less efficient in *Mk*. Overall, these results demonstrate that the ϕMycoMarT7 phage infects *Mk* and its temperature sensitivity trait is maintained in this pathogen.

We next investigated whether ϕMycoMarT7 could deliver the Tn to *Mk* at the nonpermissive temperature to give rise to Tn mutants. We carried out *Mk* transductions and followed each of them with selective outgrowth and enumeration of Km^R colonies. We also carried out mock transduction control experiments (phage omitted) to determine the frequency of spontaneous Km^R mutants under our experimental conditions. In all, we found that the number of Tn mutants increased with the MOI used in the transduction mixture (Figure 1c) and observed no meaningful frequency of spontaneous Km^R colonies (less than 1 in 10^6 CFU; not shown). Lastly, the results of the transposon insertion sequencing (TIS) experiment described below confirmed that the TA site specificity of the Tn is preserved in *Mk*. In all, our results demonstrate that the ϕMycoMarT7-based Tn mutagenesis system is fully functional in *Mk*.

3.2 | A *Mycobacterium kansasii* transposon mutant library screen links a conserved sRNA gene of unknown function to colony morphology

The realization that the ϕMycoMarT7 system is functional in *Mk* opens the possibility to explore its use in forward genetic screens to unveil genotype-phenotype associations and dissect gene function. To pilot this concept, we carried out a small screen for macrocolony morphology mutants. We note that the term "macrocolony" used herein refers to the growth arising from spot inoculation (multi-CFU seeding) from a liquid culture onto solid growth media, a colonial growth often considered a biofilm community (e.g., Chapelais-Baron et al., 2018; Kempes, Okegbe, Mears-Clarke, Follows, & Dietrich, 2014).

We selected a macrocolony morphology screen for our concept exploration because we hypothesized that it might lead to identification of mutants with insertions in genes involved in defining cell

envelope surface properties with an impact on biofilm formation capacity, a trait linked to the public health problem associated with *Mk*. Environmental *Mk* biofilms are considered recalcitrant pathogen reservoirs, and biofilm formation in the host is thought to contribute to the intractability of mycobacterial infections (Aldridge, Keren, & Fortune, 2014; Chakraborty & Kumar, 2019). Thus, identification of genes involved in biofilm formation will lead to a better understanding of *Mk* biology and illuminate potential paths to new therapeutics and strategies for pathogen reservoir eradication.

For the pilot screen, we generated a library of several hundred Tn mutants arrayed in 96-well plates, spot-inoculated the library onto solid growth medium plates, incubated the screen plates for macrocolony growth, and then scrutinized the plates for mutants with macrocolony abnormalities. We identified a mutant (herein referred to as 13D6) visibly distinct from the wild type (WT) (Appendix 2, Figure A1) and selected it for further analysis. We found that the typical 13D6 macrocolony had a reduced diameter, abnormal surface topology, and a smooth and glistening appearance that contrasted the rough and dried phenotype of the WT (Figure 2a). The distinct phenotype of the mutant was even more evident on streak plates (Figure 2b). Single colonies of 13D6 were also reduced in size (Figure 2c) and displayed an atypical surface appearance (Figure 2d) compared to WT. Notably, the mutant displayed cell shape and planktonic growth comparable to that of the parental strain (Appendix 2, Figure A2).

Colony morphology is a trait influenced by multiple factors. It is well documented that mycobacterial mutants with abnormal colony morphology often carry mutations in genes implicated in metabolism of complex glycolipids of the outer membrane-like structure (i.e., the mycomembrane) of the mycobacterial cell envelope, (Ren et al., 2007; Tatham et al., 2012; Yu et al., 2012). In particular, alterations in the content of mycomembrane glycolipids known as lipooligosaccharides (LOS) have been linked occasionally to smooth-rough morphotype changes in *Mk* (Belisle & Brennan, 1989; Nataraj et al., 2015). Thus, it is tempting to speculate that the abnormal (macro)colony appearance of the 13D6 mutant suggests that the Tn insertion led to changes in mycomembrane composition and/or structure.

We verified that 13D6 had a single Tn insertion (Appendix A2, Figure A3) and identified the insertion site. We found that the Tn had inserted 6 bp upstream of what we propose is a nonannotated sRNA gene orthologous to a nonessential *Mtb* sRNA gene of unknown function named *B11* (a.k.a. *ncRv13660c*) (Arnvig, Cortes, & Young, 2014; Arnvig & Young, 2009; DeJesus et al., 2017) (Figure 3a). The two orthologs have high sequence identity (Figure 3b), and their sRNA products have essentially the same predicted secondary structure (Figure 3c). Following the new nomenclature guidelines for naming mycobacterial noncoding sRNAs (Lamichhane, Arnvig, & McDonough, 2013), we named the predicted *Mk* sRNA gene *ncMKAN_RS112130c* (herein referred to as *Mk B11* for simplicity). Our cross-species genome sequence similarity analysis revealed that the *B11* gene locus is highly conserved in the *Mycobacterium* genus (Appendix 2, Figure A4a) and in many representative members of

FIGURE 1 Functionality of the φMycoMarT7-based transposon mutagenesis system in *Mycobacterium kansasii*. (a) Images showing the plaque formation capacity of the φMycoMarT7 phage on lawns of *Mk* and *Msm* at the permissive (30°C) and nonpermissive (37°C) temperatures. The numbers to the left of the image represent a 10-fold dilution series of the phage stock. UD, undiluted phage stock. (b) Comparison of plaque formation efficiency on lawns of *Mk* and *Msm* at the permissive temperature. The data represent mean ± *SE* of at least two PFU titrations. (c) Effect of the multiplicity of infection (MOI) used in the transduction mixture on the efficiency of generation of *Mk* Tn mutants
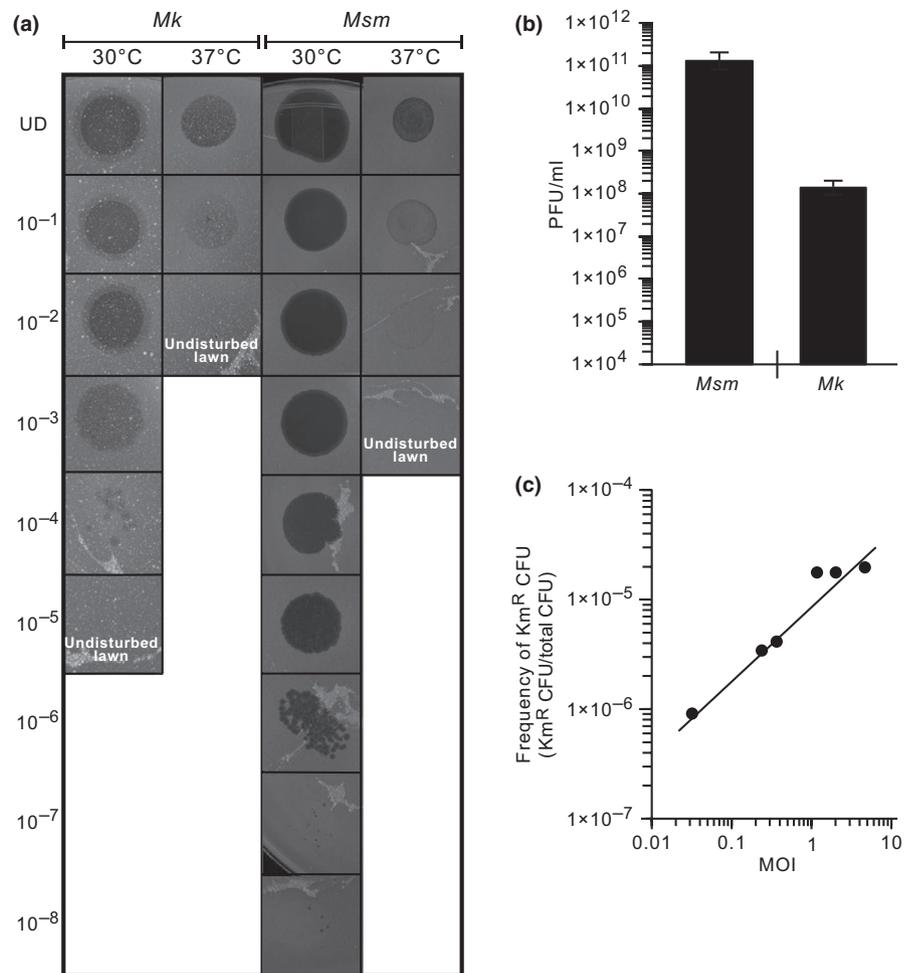

other genera of *Actinobacteria* (not shown). These findings are consistent with reported analyses (DiChiara et al., 2010; Mai et al., 2019; Weinberg et al., 2007). The conservation of this gene suggests that its sRNA product has a critical function in the biology of *Actinobacteria*.

Based on the position of the Tn in the 13D6 mutant, we hypothesized that the insertion might have abolished *B11* transcription by separating the gene from its promoter. Consistent with this idea, the *Mk B11* 5′ flanking region contains a putative SigA-dependent promoter (Figure 3b), a feature conserved in the *Mtb B11* locus (Arnvig & Young, 2009). We found the equivalent putative promoter sequence upstream of the *B11* orthologs in other *Mycobacterium* spp. (Appendix 2, Figure A4b). To investigate the hypothesized impact of the Tn insertion in 13D6 on *B11* expression, we compared *B11* transcript levels between the 13D6 and WT strains by northern blot analysis of RNA isolated from planktonic cultures and by RT-qPCR analysis of RNA isolated from macrocolonies and from planktonic cultures. We also assessed whether the transcript levels of the genes flanking *B11* (i.e., *MKAN_RS12130* and *MKAN_RS12135*; Figure 3a) had been altered in 13D6. The northern blot analysis revealed an sRNA transcript (≈80 nt) in the expected size range in the WT strain but not in the mutant (Figure 4a). In agreement with this, the RT-qPCR analysis showed that the insertion led to reductions in transcript levels of 769-fold and 833-fold in macrocolony

cells (Figure 5a) and planktonic cells (Figure 5b), respectively, relative to WT. Conversely, the insertion had no significant impact on the transcript levels of the flanking genes in either macrocolony (Figure 5c) or planktonic (Figure 5d) cells. Thus, we conclude that the Tn insertion essentially shut down *B11* transcription from its native promoter, and attribute the residual *B11* transcript detected in the mutant by the highly sensitive RT-qPCR analysis to Tn-driven trace transcription of the downstream *B11* gene, a view supported by the literature on the Tn delivered by φMycoMarT7 (Rubin et al., 1999; Zhang et al., 2012).

In light of these results, we hypothesized that the lack of WT levels of *B11* transcription in the 13D6 mutant led to the morphology phenotype. To test this idea, we engineered and analyzed the phenotype of a genetic complementation control strain (13D6-c). The strain was generated using the plasmid pML-B11 to endow 13D6 with a WT copy of the *B11* locus by plasmid integration at the mycobacteriophage L5 *attB* site of the *Mk* chromosome (coordinates 1225397–1225458; Appendix 2, Figure A5), which we identified using the *Msm attB* site as reference sequence (Lee, Pascopella, Jacobs, & Hatfull, 1991). pML-B11 carries *B11* along with its adjacent extragenic regions (Figure 3a), which were included in the cloned fragment to preserve any unrecognized cis-acting elements that might have been required for control of *B11* transcription or *B11* transcript termination or processing. We
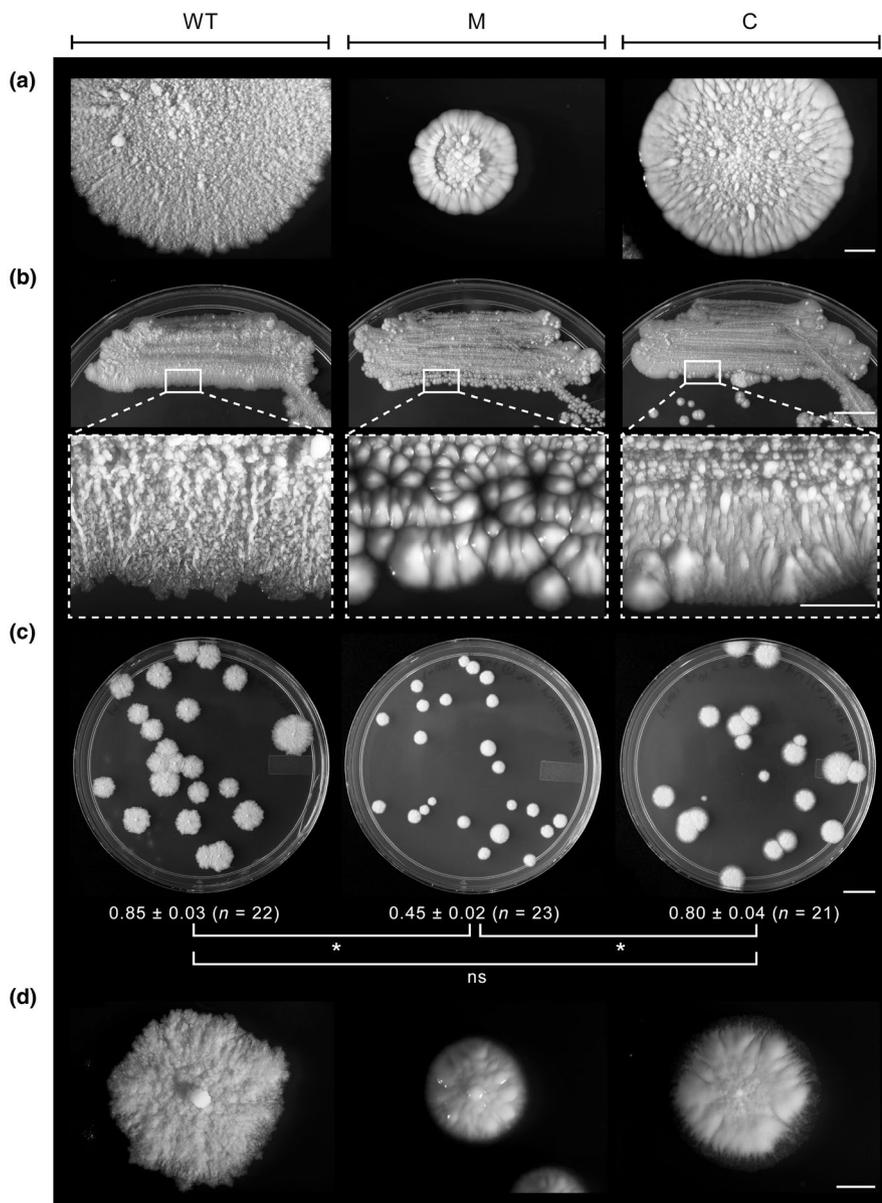
FIGURE 2 Morphology phenotypes of *Mycobacterium kansasii* strains. (a) Representative spot inoculation-derived macrocolonies showing morphological features. Scale bar = 2 mm. (b) Plates with bacterial streaks (top; scale bar = 1 cm) and enlargements (bottom; scale bar = 2 mm) of the boxed sections showing surface topology and appearance features. (c) Plates with isolated single colonies. Scale bar = 1 cm. Colony diameter (cm) information is presented below each plate. The values shown represent mean ± *SE*. *Student's *t* test *p* values <.05; ns, no significant difference (*p* ≥ .05). (d) Representative single colonies showing morphology detail. Scale bar = 2 mm. WT, wt-v; M, 13D6-v; C, 13D6-c

also constructed an empty vector (pML1335$^{\Delta xylE}$)-carrying control of 13D6 (strain 13D6-v) and one of the WT (strain wt-v), and included these strains in the complementation analysis. Evaluation of the morphology phenotype of the various strains revealed that empty vector integration had no impact on the phenotype of the mutant, whereas integration of pML-B11 led to a strong partial complementation (Figure 2).

The partial complementation seen in 13D6-c led us to assess whether the transcript level of *B11* in the strain had indeed been restored to WT levels. We found that integration of pML-B11 in 13D6 raised the *B11* transcript level 179-fold (to 23.30% of WT) in macrocolony cells (Figure 5a) and 373-fold (to 44.81% of WT) in planktonic cells (Figure 5b) as judged by RT-qPCR analysis. The results are in good agreement with those derived from the northern blot analysis, which indicates a *B11* transcript level recovery of 34.14% in the planktonic cells of 13D6-c relative to WT levels (Figure 4b). In light of the results of the *B11* expression analysis, we conclude that the partial

complementation is likely due to incomplete restoration of *B11* transcript levels. Overall, our findings establish a previously unrecognized role of *Mk B11*, or any mycobacterial sRNA gene, in the development of (macro)colony morphology features.

3.3 | Disruption of *B11* transcription leads to biofilm formation deficiency

As noted above, we theorized that our macrocolony morphology screen could lead to identification of mutants with insertions in genes involved in defining cell surface properties with an impact on biofilm formation, a trait that contributes to the pathogen's resilience in environmental reservoirs and in the host. Thus, we investigated the effect of the Tn insertion in 13D6 on biofilm formation using the MBEC™ device. Comparison of the WT and mutant strains in biofilm experiments revealed that only the WT formed biofilm
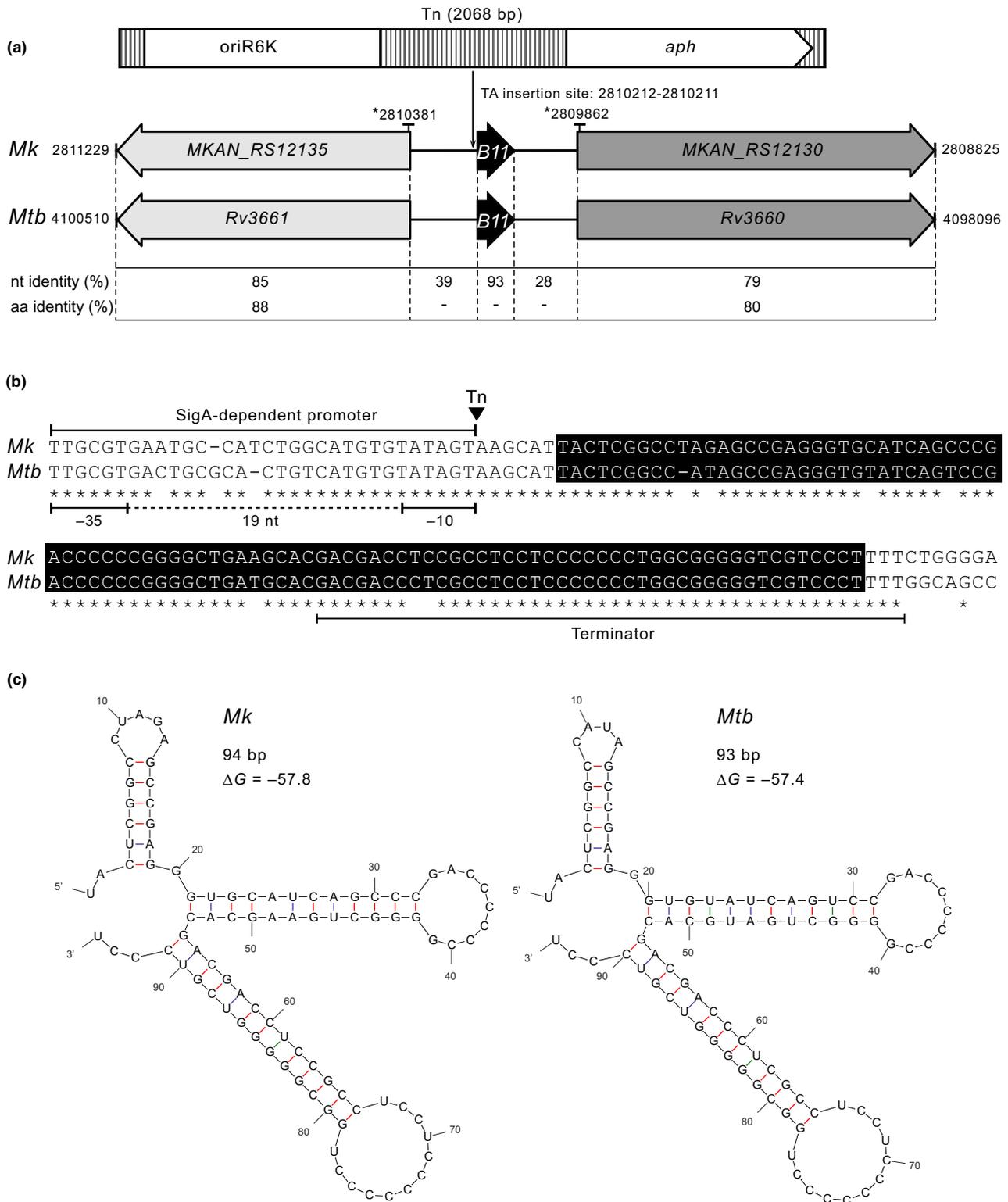
FIGURE 3 *Mycobacterium kansasii* 13D6 has a transposon insertion located 6 bp upstream of an sRNA gene (*Mk B11*) orthologous to the *Mycobacterium tuberculosis* sRNA gene *B11*. (a) Outline depicting the Tn (*Escherichia coli* replication origin oriR6k and *aph* Km^R marker are represented), the location of the TA insertion site (chromosomal coordinates are shown), and the conservation of genetic features nearby the insertion site in the *Mk* chromosome (NC_022663.1) and the orthologous segment in the *Mtb* chromosome (NC_000962.3). Chromosomal coordinates of the segments are indicated. The coordinates of the chromosomal fragment included in the complementation plasmid pML-B11 are marked by an asterisk (*). Nucleotide (nt) and amino acid (aa) identity between specific sections of the segments is provided. (b) Sequence alignment of *Mk* and *Mtb B11* loci. The boxed sequences correspond to the annotated *Mtb B11* gene. The location of the Tn (inverted triangle) and putative promoter and terminator sequences are depicted. (c) Predicted secondary structures for the sRNA products of the *B11* genes. The minimum free energy associated with each structure is shown
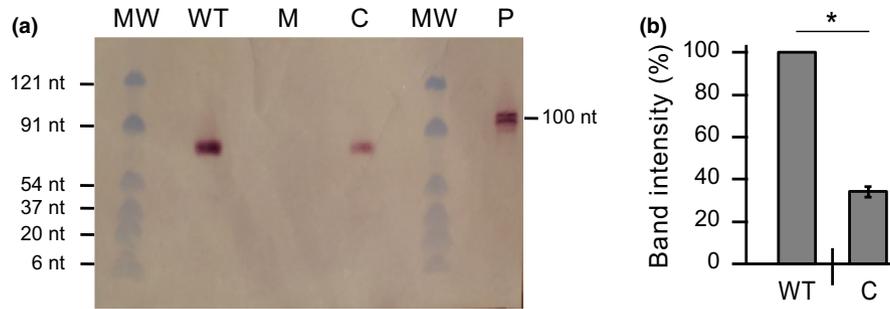
FIGURE 4 Northern blot analysis of *Mycobacterium kansasii* B11 sRNA in *M. kansasii* strains. (a) Representative northern blot demonstrating the presence of B11 sRNA in the WT strain (wt-v, lane WT) and complemented strain (13D6-c, lane C) and its absence in the mutant strain (13D6-v, lane M). Lane MW, molecular weight markers; lane P, B11 probe fragment (100 bp). (b) Northern blot-based comparison of B11 expression in the WT (WT) and complemented (C) strains. The bands on the blot were quantified by densitometry analysis using ImageJ software (NIH, available in the public domain). The data represent mean ± *SE* of quantifications from three northern blots. *Student's *t* test *p* values <.0001
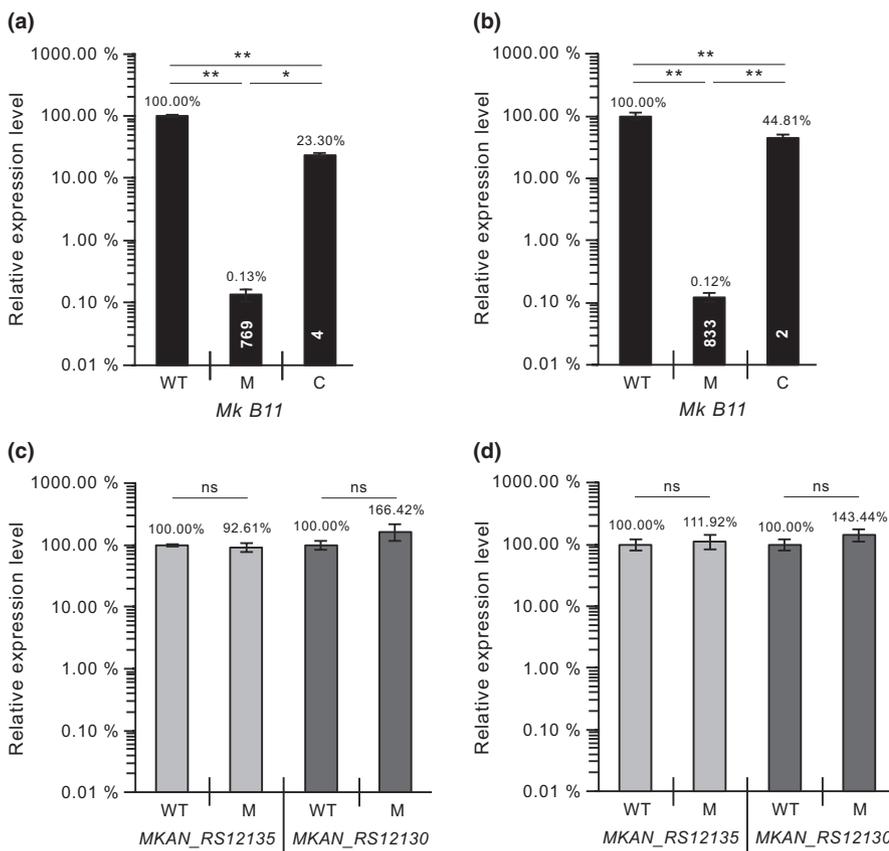

FIGURE 5 RT-qPCR expression analysis of *B11* and adjacent genes in *Mycobacterium kansasii* strains. (a) Expression of *B11* in macrocolonies. (b) Expression of *B11* in planktonic cells. (c) Expression of *B11* gene neighbors in macrocolonies. (d) Expression of *B11* gene neighbors in planktonic cells. WT, wt-v; M, 13D6-v; C, 13D6-c. In-bar values reflect fold reduction in the indicated strain relative to WT. Data represent mean ± *SE* of at least two independent experiments. Student's *t* test *p* values: *<.05; **<.01; ns, no significant difference (*p* ≥ .05)

microcolonies on the pegs of the device (Figure 6a). As noted above, the strains have comparable planktonic growth (Appendix 2, Figure A2b) and planktonic growth in MBEC™ plates were indistinguishable between both strains, ruling out the possibility that the observed biofilm formation deficiency of the mutant was due to lack of bacterial growth. The biofilm formation capacity of the *B11* locus-complemented strain 13D6-c was restored to a level comparable to WT (Figure 6a). Biofilm quantification using a CV-based colorimetric method (Figure 6b) recapitulated the results from direct peg observation. Taken together, our results demonstrate that the biofilm formation defect of 13D6 is due to a lack of *B11* expression. To our

knowledge, 13D6 is the first *Mk* biofilm deficient mutant reported, and our results provide the first example of a mycobacterial sRNA gene involved in biofilm formation.

Overall, our findings indicate that the lack of *B11* expression in 13D6 has a pleiotropic effect that impacts (macro)colony morphology features and biofilm formation. The molecular mechanism behind this phenomenon remains to be elucidated. It is tempting to speculate, however, that the sRNA regulates *Mk* genes influencing colony morphology features and biofilm formation. Interestingly, recent studies have shown that expression of *Mtb B11* in *Msm*, which carries its native *B11* ortholog (Appendix 2, Figure A4a), leads to

FIGURE 6 Biofilm formation
by *Mycobacterium kansasii* strains.
(a) Representative image of crystal
violet-stained MBEC™ device pegs.
(b) Quantification of biofilm formation
on pegs using the crystal violet-based
colorimetric assay. WT, wt-v; M, 13D6-v;
C, 13D6-c; ctrl, sterile medium control.
Data represent mean ± *SE* of three
independent experiments. *Student's
t test *p* values <.05; ns, no significant
difference (*p* ≥ .05)
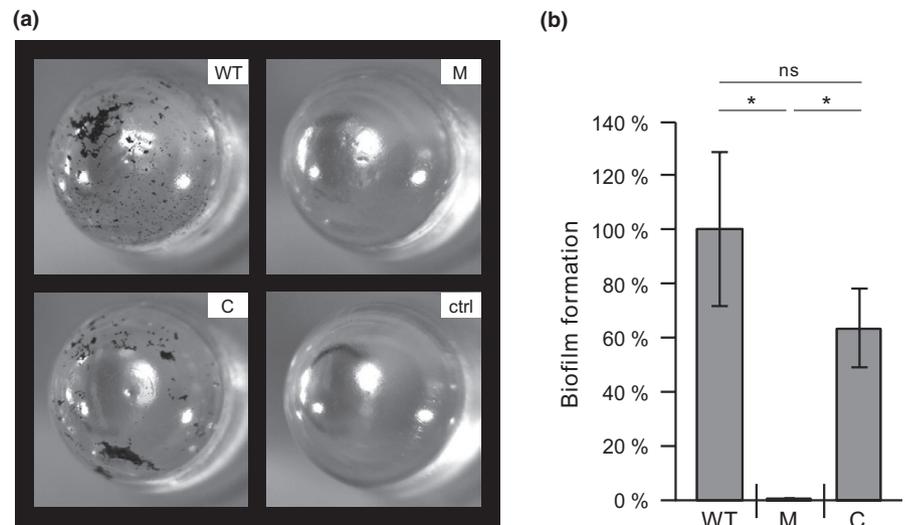
FIGURE 7 Kaplan–Meier survival
curves demonstrating temperature-
dependent killing of *Galleria mellonella*
larvae by *Mycobacterium kansasii*. WT,
WT strain; M, 13D6 mutant; BC, PBS
buffer control; NIC, noninjected control.
Statistical differences between survival
curves were assessed using the Log-rank
test. *p* values: ***<.0001; **.0053; *.0362;
ns, no significant difference (*p* ≥ .05)
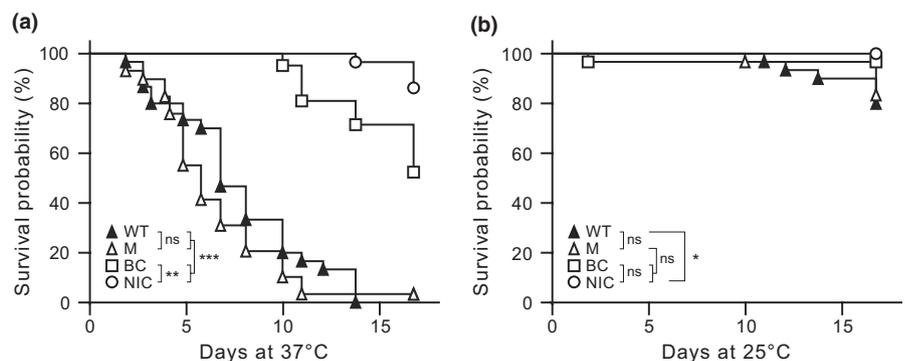
reduced growth and elongated cell shape, and support a model in which the *Mtb B11* sRNA represses *Msm* gene expression by a mechanism requiring base-pairing of C-rich loops in the sRNA to G-rich stretches in mRNA targets (Arnvig & Young, 2009; Mai et al., 2019). Despite this recent progress, the role of this family of sRNA orthologs in actinobacterial biology remains poorly understood and further studies to elucidate their regulatory functions are warranted.

3.4 | Disruption of B11 transcription does not cause attenuation in a *Galleria mellonella* larva killing assay

The GM larva has emerged as a simple and economical invertebrate model host to evaluate virulence of microbial pathogens and efficacy of antimicrobial compounds (Tsai, Loh, & Proft, 2016). Recent studies have validated the use of this model with mycobacteria and shown that larva lethality is species-dependent (Entwistle & Coote, 2018; Li et al., 2018; Meir, Grosfeld, & Barkan, 2018; Rwegasila, Mubofu, Nyandoro, Erasto, & Munissi, 2016). Given the tractability of this model, we used a GM larva killing assay to provide a first comparison of the WT and 13D6 strains in an in vivo setting. We challenged GM with these strains and monitored larval survival during incubation at either 37°C or 25°C.

Comparison of the survival curves of the infected groups with the uninfected controls clearly demonstrated *Mk*-dependent killing at 37°C (Figure 7a). However, we found no difference between the survival curves of the *Mk*-infected groups. Interestingly, we found no significant *Mk*-dependent killing at 25°C (Figure 7b), a temperature much less favorable for *Mk* replication. Overall, our infection experiments revealed that the Tn-dependent disruption of *B11* transcription in 13D6 had no substantial impact on virulence in the GM model. To our knowledge, our findings provide the first demonstration of the susceptibility of GM larva to *Mk*, thus setting the stage for further exploration of this model for *Mk* studies.

3.5 | A transposon insertion sequencing approach identifies 12,071 insertions that do not compromise *Mycobacterium kansasii* viability

TIS approaches are powerful tools for high-throughput gene function studies and systems-level analysis of microorganisms (van Opijnen & Camilli, 2013). We probed the application of TIS in *Mk* using a modified TraDIS-type method (Fan et al., 2018; Langridge et al., 2009) customized for the Tn of ϕMycoMarT7 to identify the insertion sites in a DNA library pool generated from a nonsaturated Tn mutant library of ≈14,700

	Chromosome	Plasmid
Total TA sites hit	11,696	375
Total TA sites hit relative to total TA sites	12% (of 97,702)	17% (of 2,251)
Intragenic TA sites hit	9,573	312
Intragenic TA sites hit relative to total TA sites hit	82%	83%
Intragenic TA sites hit relative to total intragenic TA sites	12% (of 78,383)	18% (of 1,723)
Intergenic TA sites hit	2,123	63
Intergenic TA sites hit relative to total TA sites hit	18%	17%
Intergenic TA sites hit relative to total intergenic TA sites	11% (of 19,319)	12% (of 528)
Total genes hit	3,474	113
Genes hit relative to total annotated genes	62%	74%

TABLE 1 Summary of mapped transposon insertion sites in the _Mycobacterium kansasii_ chromosome and plasmid

mutant colonies. The analysis mapped a total of 12,071 different TA insertion sites (Table 1, Figure A6 in Appendix 2), indicating that none of these insertions disrupted essential functions required for colony outgrowth under our experimental conditions. Of the 12,071 insertions, 9,885 were intragenic, impacting 3,474 (62%) genes of the chromosome (Table S1, available at https://doi.org/10.5281/zenodo.3561175) and 112 (74%) genes of the plasmid pMK12478 (Table S2, available at https://doi.org/10.5281/zenodo.3561175). The TA sites hit by the Tn in the chromosome and in pMK12478 represent 12% and 17% of the total TA sites in each amplicon, respectively (Table 1). The chromosome and pMK12478 have a comparable percent of coding bp (87% and 84%, respectively), and the same average frequencies of intragenic TA sites (1 TA/71 bp) and intergenic TA sites (1 TA/43 bp). Notably, however, while the percent of intergenic TA sites hit for the two amplicons relative to the respective total intergenic TA sites is essentially the same (11%–12%), the percent of intragenic TA sites hit relative to the amplicon's total intragenic TA sites is considerably depressed in the chromosome (12%) compared to that of the plasmid (18%). Since insertions disrupting essential functions required for colony outgrowth are not expected to be represented in the mutant library, the observed bias against chromosomal intragenic insertions is probably a reflection of the presence of essential genes in the chromosome. We note that the nonsaturated nature of the library analyzed (Table 1, Figure A6 in Appendix 2) precludes the statistical analysis of insertion distribution along replicons and locus-dependent transposition biases often associated with saturation Tn mutagenesis approaches used in gene essentiality analysis (DeJesus, Ambadipudi, Baker, Sassetti, & Ioerger, 2015; DeJesus et al., 2017; DeJesus & Ioerger, 2013; DeJesus et al., 2013; Griffin et al., 2011; Long et al., 2015). Nevertheless, in all, our results expand our knowledge of _Mk_ gene function, provide proof-of-principle for the use of a φMycoMarT7-based TIS approach in _Mk_, and set a methodological framework for coupling saturating Tn mutagenesis and TIS for genome-scale gene function analysis and systems-level studies in this mycobacterial pathogen.

4 | CONCLUSIONS

Gaining a comprehensive understanding of the biology of _Mk_ is important to address the public health challenges arising from this poorly studied NTM pathogen. This understanding will help advance our

preparedness against the development of multidrug resistant _Mk_ CPD, a likely collateral outcome of the pervasive use of anti-TB drugs to curb the TB epidemic. In particular, a deeper knowledge of _Mk_ gene function is needed to help illuminate avenues to new and more effective therapeutics. The establishment of the φMycoMarT7 system as an effective mutagenesis tool in _Mk_ sets the stage for further use of powerful, genome-wide scale genetic approaches to accelerate the unraveling of gene function in this pathogen. The use of the φMycoMarT7 system reported herein led to the discovery of a role of the conserved mycobacterial _B11_ sRNA gene in determination of (macro)colony morphology features and biofilm formation. It also permitted the identification of ≈10,000 intragenic Tn insertions that do not impede growth in vitro. These findings expand our knowledge of _Mk_ gene function. Finally, the demonstration of the susceptibility of GM larvae to _Mk_ opens the possibility of using this simple and economical invertebrate infection model system for virulence assessment of _Mk_ mutants and in vivo evaluation of novel antimicrobial lead compounds against _Mk_.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

William Budell: Formal analysis-Lead, Investigation-Lead, Methodology-Lead, Validation-Equal, Visualization-Equal, Writing-original draft-Equal, Writing-review & editing-Equal. Gabrielle Germain: Investigation-Supporting, Writing-review & editing-Supporting. Niklas Janisch: Investigation-Supporting, Writing-review

& editing-Supporting. Zaid McKie-Krisberg: Data curation-Equal, Methodology-Supporting, Resources-Equal. Anitha Jayaprakash: Data curation-Equal, Formal analysis-Supporting, Investigation-Supporting, Methodology-Equal, Writing original draft-Supporting, Writing-review & editing-Supporting. Andrew Resnick: Investigation-Supporting, Writing-review & editing-Supporting. Luis Quadri: Conceptualization-Lead, Formal analysis-Equal, Funding acquisition-Lead, Project administration-Lead, Supervision-Lead, Validation-Equal, Visualization-Equal, Writing-original draft-Lead, Writing-review & editing-Lead.

ETHICS STATEMENT

Protocols and procedures employed in this investigation were reviewed and approved by the appropriate institutional review committees. Experiments with the invertebrate *Galleria mellonella* are exempt from Institutional Animal Care and Use Committee oversight (U. S. Public Law 99-158, Sec. 495).

DATA AVAILABILITY STATEMENT

All data are provided in full in this paper with the exception of the data in Table S1 (Location of intragenic and intergenic transposon insertion sites in the chromosome of *M. kansasii*) and Table S2 (Location of intragenic and intergenic transposon insertion sites in the plasmid pMK12478 of *M. kansasii*), which are available in the Zenodo repository at https://doi.org/10.5281/zenodo.3561175.

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APPENDIX 1

TABLE A1 Bacterial strains

Strain	Characteristics	Source
Mycobacterium kansasii	Wild type, subtype 1, type strain (ATCC® 12478™)	American Type Culture Collection
M. kansasii wt-v	*M. kansasii* carrying empty vector pML1335$^{\Delta xylE}$ integrated at the phage L5 *attB* integration site	This study
M. kansasii 13D6	*M. kansasii* carrying a Tn insertion upstream of *B11* (nc*MKAN_RS112130c*)	This study
M. kansasii 13D6-v	*M. kansasii* 13D6 carrying empty vector pML1335$^{\Delta xylE}$ integrated at the phage L5 *attB* integration site	This study
M. kansasii 13D6-c	*M. kansasii* 13D6 carrying complementation plasmid pML-B11 integrated at the phage L5 *attB* integration site	This study
Mycobacterium smegmatis mc^{2}155	Wild-type laboratory strain (ATCC® 700084™)	American Type Culture Collection
Escherichia coli DH5α	Strain used for general cloning applications	Thermo Fisher Scientific, Inc.
E. coli DH5α λpir	Strain used for rescue cloning of Tn-containing genomic fragments. The strain containing the *pir* gene required for replication of plasmids with the R6K-derived origin of replication present in the Tn	Thermo Fisher Scientific, Inc.
E. coli K12 dam⁻/dcm⁻	Strain used for propagation of plasmids free of Dam and Dcm methylation	New England BioLabs, Inc.

TABLE A2 Oligonucleotides

Name	Sequence (5′ → 3′)	Comments
GG1	GACTGGCTGCTATTGGGCGAAG	Primers for PCR-based generation of Tn-specific probe
GG2	AGAAGGCGATAGAAGGCGATG	
CB2b	CGCTTCCTCGTGCTTTACGGTATCGC	Primers for sequencing Tn-genome junctions. CB2b and CB2 bind the Km resistance gene and the oriR6K origin of replication, respectively
CB2	GAGATAGGGTTGAGTGTTGTTCC	
CB3	AT**GCGGCCGC**TTCGAATACGTATCATCATCGCCAACACTCCGGGACCAAGGGT	Primers for PCR-based generation of the 520-bp chromosomal fragment carried by pML-B11; genome coordinates 2809862–2810381; NotI and BspHI sites used in cloning are in bold in CB3 and CB7, respectively
CB7	GA**TCATGA**GGACGGTCACCAATCAAGCATAGGT	
CB10	GGCCAGCCCCGTACTCTCGAC	RT-qPCR primers for *MKAN_RS24220* (*sigA*); 135-bp amplicon; genome coordinates 5589258–5589392
CB11	ATCCAGGTAGTCGCGCAGGACT	
CB8c	CATTACTCGGCCTAGAGCCGAGGGTGCATC	Primers used for PCR-based generation of *Mk* B11-specific probe for northern analysis and as RT-qPCR primers for *Mk B11*; 100-bp amplicon; genome coordinates 2810109–2810208
CB9c3	AAAAGGGACGACCCCCGCCAGGG	
CB49a	CGCCGACCTCGTTGTCCTGGTCT	RT-qPCR primers for *MKAN_RS12130*; 104-bp amplicon; genome coordinates 2809046–2809149
CB50a	CCAGCCCCAGGTTGGGGTTGA	
CB43a	GCGCGGTTGGCAGGTGTTGTCATT	RT-qPCR primers for *MKAN_RS12135*; 115-bp amplicon; genome coordinates 2811070–2811184
CB44a	GGCCAGCGCACTGACTCCCA	
IS5	AATGATACGGCGACCACCGA	Adapter-specific forward primer used in both PCR reaction rounds (PCR-1 and PCR-2) to generate next-generation Tn-specific deep sequencing libraries on the Illumina platform.
CB1	CGAGATAGGGTTGAGTGTTGTTC	First Tn-specific reverse primer used in PCR-1 to generate next-generation Tn-specific deep sequencing libraries on the Illumina platform
CB25.3_R	*CAAGCAGAAGACGGCATACGAGAT*<u>ATCAGT</u>ATCT**CCGGGGACTTATCAGCCAACC**	Second Tn-specific reverse primer used in PCR-2. The primer has the Illumina flow cell sequence tag (italicized), followed by a sample barcode (underlined), followed by a random filler sequence (four nucleotides), followed by a Tn-specific sequence (21 nucleotides; in bold). This will generate Tn-specific deep sequencing libraries on the Illumina platform

(Continues)

TABLE A2 (Continued)

Name	Sequence (5′ → 3′)	Comments
CB44.3_R	*CAAGCAGAAGACGGCATACGAGAT*<u>ATTATA</u>ATCT **CCGGGGACTTATCAGCCAACC**	Second Tn-specific reverse primer used in PCR-2. The primer has the Illumina flow cell sequence tag (italicized), followed by a sample barcode (underlined, different from that in CB25.3_R), followed by the filler sequence (same as in CB25.3_R), followed by the Tn-specific sequence (bold). This will generate Tn-specific deep sequencing libraries on the Illumina platform
Barcodeseq	**GGTTGGCTGATAAGTCCCCGG**AGAT	Tn-specific index primer used to sequence the sample-specific barcodes on the Illumina platform. The primer has the Tn-specific sequence (bold) followed by the filler sequence
SP2	ATCT**CCGGGGACTTATCAGCCAACC**	Tn-specific read 2 primer used to initiate the paired-end sequencing on the Illumina platform. The primer has the filler sequence followed by the Tn-specific sequence (bold)

APPENDIX 2
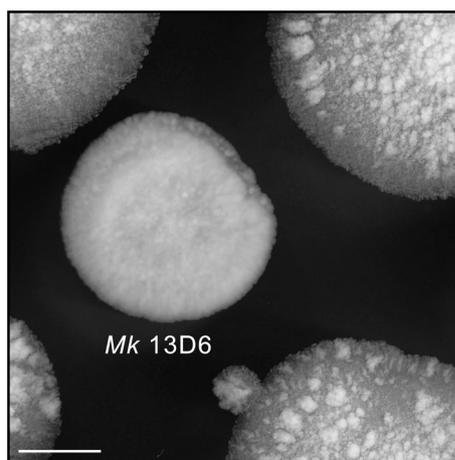
FIGURE A1 Section of the original screen plate showing the abnormal macrocolony morphology phenotype of *Mycobacterium kansasii* 13D6. Scale bar = 2 mm

(a)

FIGURE A3 Southern blot hybridization analysis of *Mycobacterium kansasii* 13D6. Agarose gel electrophoresis of SacII-digested genomic DNA (left panel) and southern blot membrane after hybridization with a Tn-specific probe (right panel). Lanes: L, DNA molecular weight ladder; WT, WT strain; M, 13D6 mutant

(a)

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Mtb  TACTCGGCCAT-AGCCGAGGGTGTATCAGTCCGACCCCCCGGGGCTGATGCACGACGACCCTCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTGGCAGCC  4099478 - 4099376
Mk   TACTCGGCCTAGAGCCGAGGGTGCATCAGCCCGACCCCCCGGGGCTGAAGCACGACGACCTCCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTCTGGGGA  2810205 - 2810102
Mb   TACTCGGCCAT-AGCCGAGGGTGTATCAGTCCGACCCCCCGGGGCTGATGCACGACGACCCTCGCCTCCTCCCCCCC-CTGGCGGGGGTCGTCC-CTTTTGGCAGCC  4041237 - 4041134
BCG  TACTCGGCCAT-AGCCGAGGGTGTATCAGTCCGACCCCCCGGGGCTGATGCACGACGACCCTCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTGGCAGCC  4072591 - 4072488
Mm   TACTCGGCCACGAGCCGAGGGTGCATCAGTCCGACCCCCCGGGGCTGAACACGACGACCTCCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTGTGGGGT  6232277 - 6232174
Mu   TACTCGGCCATGAGCCGAGGGTGCATCAGTCCGACCCGCCGGGGCTGAAGCACGACGACCTCCGCCTCCTCCCCCCCCTGGCGGGGGTCGTCC-CTTTTGTGGGGT  4706834 - 4706729
Mp   TACTCGGCCTG-AGCCGAGGGTGTATCAGCCCGACCCCCCGGGGCTGATACACGACGACCCTCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTCTCCGGT  298523 - 298421
Ma   TACTCGGCCTG-AGCCGAGGGTGTATCAGCCCGACCCCCCGGGGCTGATGCACGACGACCCTCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTCTCGGGT  459934 - 460036
Mc   TACTCGGCCTG-AGCCGAGGGTGTATCAGCCCGACCCCCCGGGGCTGATGCACGACGACCTCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTCTAGGGT  3634216 - 3634114
Mi   TACTCGGCCTG-AGCCGAGGGTGTATCAGCCCGACCCCCCGGGGCTGATGCACGACGACCCTCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTCTCGGGT  422436 - 422538
Ms   CACTCGGCTTA-TGCTGAGGGTGTATCGGCCGACCCCCCGGGGTTGATACACGACGACCTCCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCT-TTTTTAACCGGT  4191134 - 4191032
Mab  TACTCGGCT-A-TGCCGAGTGTGTCTCAGCCCGACCCCCCGGGGCTGATGCACGACGACCCTCGCCTCCTCCCCCC--CTGGCGAGGGTCGTCCTCTTTTCTGGGGA  433363 - 433465
Mt   TACCCGGGTTA-TGCCGGGTGTGCATCAGTCCGACCCCCCGGGACTGATACACGACGACCTCCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCT-CTTTTCCGCAC  4464192 - 4464090
JLS  TACCCGGGTTA-TGCCGGGTGTGCATCAGCCCGACCCCCCGGGGCTGATACACGACGACCTCCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTCGGGGT  5443228 - 5443125
Mn   CACTCGGCCTT-AGCCGAGTGTGGATCAGCCCGACCCCCCGGGGCTGATATACGACGACCTCCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTTCCGGAC  5321769 - 5321667
Msm  TACTCGGCTTA-TGCCGAG-GTGTATCAGCCCGACCCCCCGGGGCTGATACGCGACGACCTCCGCCTCCTCCCCCC--CTGGCGGGGGTCGTTCTTTTTCCAGGGA  6242833 - 6242731
Mph  TACTCGGCT-A-TGCCGAGTGTGTCTCAGCCCGACCCCCCGGGGCTGAAGCACGACGACCCTCGCCTCCTCCCCCC--CTGGCGAGGGTCGTCCTCTTTTCTGGGGA  417657 - 417759
Mr   CACTCGGCTTA-TGCTGAGTGTGCATCAGCCCGACCCCCCGGGGCTGATGCACGACGACCTCCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTGTGGGGG  2177225 - 2177327
Mg   TACTCGGCTTA-TGCCGAGTGTGCATCAGCCCGACCCCCCGGGGCTGATACACGACGACCTCCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTGTGGGGT  1438719 - 1438822
Mv   TACTCGGCTTA-TGCCGGGTGTGCATCAGCCCGACCCCCCGGGGCTGATACACGACGACCTCCGCCTCCTCCCCCC--CTGGCGGGGGTCGTCC-CTTTTCTGGGGT  5803054 - 5802952
      **  ***      **  *  ***   **  *  ******* *****   ***  *  ******** **************  ****** ******** *****
```

(b)

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Mtb  CGT-CCGG-TACTTGCGTGACTGCGCACTGTCATGTGTATAGTAAGCAT  4099525 - 4099479
Mk   GTG-CAAT-TACTTGCGTGAATGCCATCTGGCATGTGTATAGTAAGCAT  2810252 - 2810206
Mb   CGT-CCGG-TACTTGCGTGACTGCGCACTGTCATGTGTATAGTAAGCAT  4041284 - 4041238
BCG  CGT-CCGG-TACTTGCGTGACTGCGCACTGTCATGTGTATAGTAAGCAT  4072638 - 4072592
Mm   GCA-CCGA-TACTTGCGTTAATCCAGTCTGGCATGTGTATAGTAAGCAT  6232324 - 6232278
Mu   GCA-CCGA-TACTTGCGTTAATCCAGTCTGGCATGTGTATAGTAAGCAT  4706881 - 4706835
Mp   GAA-AGAG-CTATTGCGTTACCGCGGCTTGTCATGTGTATAGTAAGCAT  298570 - 298524
Ma   AAA-TGAT-CTATTGCGTTACTGCCGACTGTCATGTGTATAGTAAGCAT  459887 - 459933
Mc   AAA-TGAT-GTCTTGCGTTACTCCGGCTTGTCATGTGTATAGTAAGCAT  3634263 - 3634217
Mi   AAA-TGAT-CTATTGCGTTATTGCGGACTGTCATGTGTATAGTAAGCAT  422389 - 422435
Ms   GGTGACAA-CACTTGCGCCGGACCGGCATGTCGTGTGTATAGTG-TCAT  4191181 - 4191135
Mab  GCC-GATTGGTCTTGCCACCCTCACCCGGTTCGTGTGTATAGTGGG-GT  433316 - 433362
Mt   GAT-TCGG-GGCTTGCGCCGCGGGCCAGGGTCGTGTGTATAGTGAGCAT  4464239 - 4464193
JLS  CAT-GCGG-GACTTGCATTCCGTGGCCGTACCGTGTGTATAGTGGGCGT  5443275 - 5443229
Mn   CGT-CCGT-TACTTGCACTGCACGGCGAATCCGTGTGTATAGTGGCAAT  5321816 - 5321770
Msm  GTC-AGAT-CTCTTGCGACTTTCGGCAGTTACATGTGTATAGTGAGCAT  6242880 - 6242834
Mph  GCC-GATTGGTCTTGCCACCCTCACCTGGTTCGTGTGTATAGTGGG-GT  417610 - 417656
Mr   ACAATCAA-GTCTTGCGCCGACCCGTGCAGCCGTGTGTATAGTGAGTAT  2177177 - 2177224
Mg   AAT-TCGA-TACTTGCGCTGGGCCAGACTGGCGTGTGTATAGTGGGCAT  1438672 - 1438718
Mv   ATT-TCGA-TACTTGCGGTGGCCCTGTCTGGCGTGTGTATAGTGGGCAT  5803101 - 5803055
         ****              *  **********    *
         -35     - - - - - - - 19 bp - - - - - - -   -10
                         SigA-dependent promoter
```

FIGURE A4 Conservation of the *B11* gene locus in mycobacteria. (a) Sequence alignment of *Mycobacterium tuberculosis B11*, *Mycobacterium kansasii B11*, and predicted orthologs in other mycobacteria. (b) Sequence alignment of *B11* promoter regions. Gray shading highlights identity with the *Mtb* sequence. Asterisks below the alignment indicate 100% conservation. The bar under the alignment of *B11* orthologs marks the sequence annotated for *Mtb B11*. Abbreviations to the left and chromosomal coordinates to the right of the sequences correspond to the following bacteria and NCBI reference sequences, respectively. *Mtb*: *M. tuberculosis* strain H37Rv, NC_000962.3; *Mk*: *M. kansasii* strain ATCC 12478, NC_022663.1; *Mb*: *Mycobacterium bovis* strain AF2122/97, NC_002945; BCG: *M. bovis* BCG Pasteur 1173P2, NC_008769.1; *Mm*: *Mycobacterium marinum* strain M, NC_010612.1; *Mu*: *Mycobacterium ulcerans* strain Agy99, NC_008611.1; *Mp*: *Mycobacterium parascrofulaceum* strain ATCC BAA-614 SCAFFOLD1, NZ_GG770553.1; *Ma*: *Mycobacterium avium* strain 104, NC_008595.1; *Mc*: *Mycobacterium columbiense* strain CECT 3035, NZ_CP020821.1; *Mi*: *Mycobacterium intracellulare* strain ATCC 13950, NC_016946.1; *Ms*: *Mycobacterium sinense* strain JDM601, NC_015576.1; *Mab*: *Mycobacterium abscessus* strain ATCC 19977, NC_010397.1; *Mt*: *Mycobacterium thermoresistible* strain NCTC10409, NZ_LT906483.1; JLS: *M. sp.* JLS, NC_009077.1; *Mn*: *Mycobacterium neoaurum* strain VKM Ac-1815D, NC_023036.2; *Msm*: *Mycobacterium smegmatis* strain mc²155, NC_018289.1; *Mph*: *Mycobacterium phlei* strain NCTC8151, NZ_LR134347.1; *Mr*: *Mycobacterium rhodesiae* strain NBB3, NC_016604.1; *Mg*: *Mycobacterium gilvium* strain PYR-GCK, NC_009338.1; and *Mv*: *Mycobacterium vanbaalenii* strain PYR-1, NC_008726.1

```
Msm  4764505  CAATGGTAGAGCCCTAGTCTTCCAAACTAGCTACGCGGGTTCGATTCCCGTCGCCCGCTCGG  4764566
                       ||||||||||||||||||||||||||||||| |||||||||||||||||||||||||| |
Mk   1225397  CAATGGTAGAGCCCTAGTCTTCCAAACTAGCGACGCGGGTTCGATTCCCGTCGCCCGCTCAG  1225458
```

FIGURE A5 Mycobacteriophage L5 integration *attB* site in *Mycobacterium kansasii*. Alignment of the 62-bp *attB* site in *Mycobacterium smegmatis* strain mc²155 (*Msm*, NCBI reference sequence: NC_008596.1) and the predicted *attB* site in *M. kansasii* (*Mk*, NCBI reference sequence: NC_022663.1). Longer bar, 43-bp *attB* common core. Shorter bar, 29-bp necessary and sufficient for *attB* function. Chromosomal coordinates are indicated

(a)

(b)
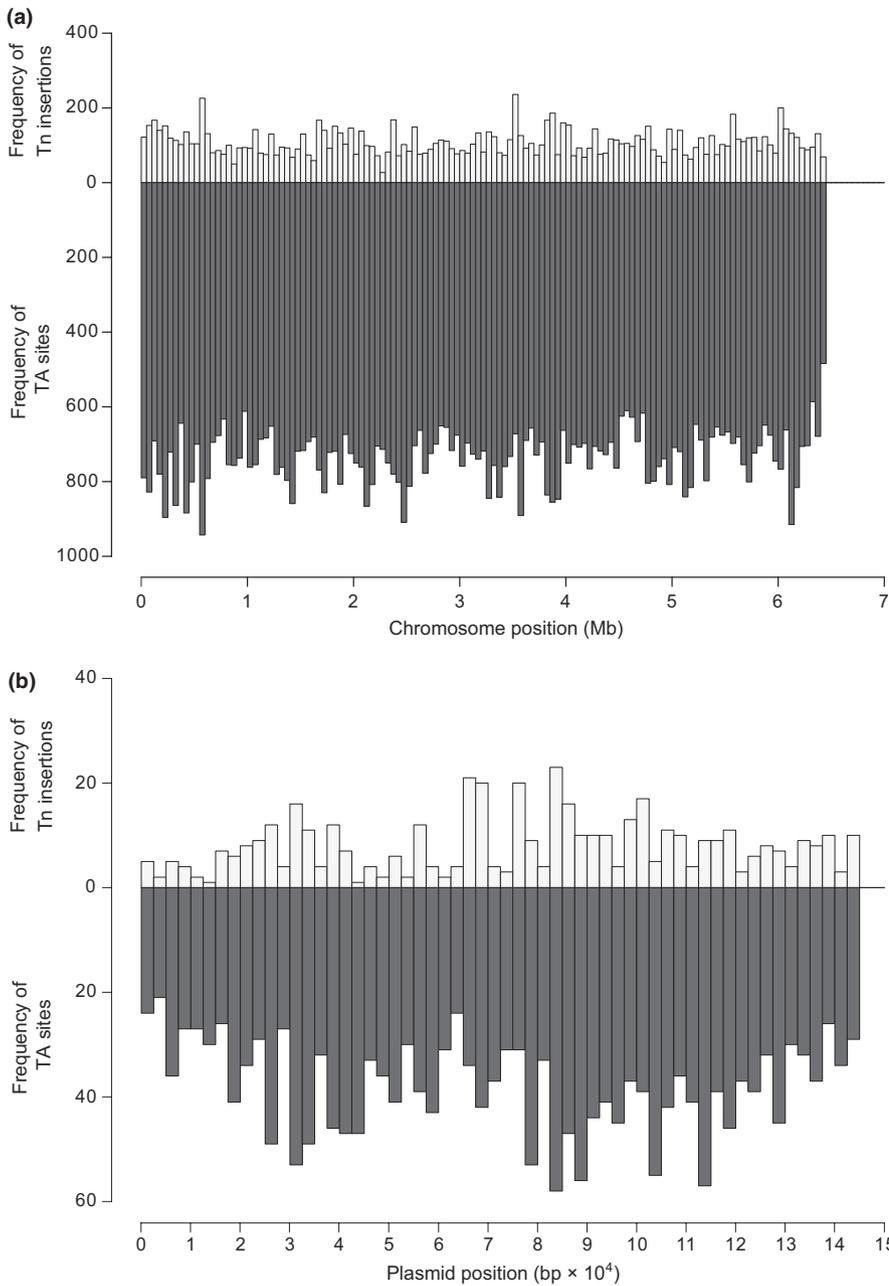
FIGURE A6 Histograms showing overviews of frequency distributions of Tn insertions and TA dinucleotide sites along the chromosome (a) and the plasmid pMK12478 (b) of *Mycobacterium kansasii*. The data of the Tn insertions for the chromosome and plasmid are derived from Tables S1 and S2, respectively. The data of TA dinucleotide sites for the chromosome and plasmid are derived from NCBI Reference Sequences NC_022663.1 and NC_022654.1, respectively. The data presented for the chromosome and plasmid are organized in bins of 50,000 and 2,500 bp, respectively.

APPENDIX 3

GENERATION OF FRAGMENT LIBRARIES FOR φMYCOMART7 TRANSPOSON INSERTION SEQUENCING USING ILLUMINA NEXT-GENERATION SEQUENCING TECHNOLOGY

A. Critical materials and reagents required

- KAPA HyperPlus Kit (Cat. no.: KR1145, Kapa Biosystems)
- Kapa Beads/SPRI (Cat. no.: KK8000, Kapa Biosystems)
- Magnetic separation rack
- OneTaq Hot Start DNA Polymerase (Cat. no.: M0481S, NEB)
- Qubit dsDNA HS Assay Kit (Cat. no.: Q32851, Thermo Fisher Scientific)
- Transposon specific primers: IS5; CB1; CB25.3_R; CCB44.3_R; barcodeseq; and SP2 (see Table A2 in Appendix 1)
- Input DNA, 100–500 ng

B. Enzymatic fragmentation

1. Add the following components in the order noted. Keep tubes on ice.

Component	Volume (µl)
Input DNA in water (if required to make the volume higher)	11.7
KAPA fragmentation buffer (10×)	1.7
KAPA fragmentation enzyme	3.3
Total volume	16.7

2. Vortex gently and spin down briefly. Return the tubes to ice. Proceed immediately to the next step.
3. Incubate the tubes in a thermocycler for 4 min at 37°C for a DNA input of 10 ng or for 15 min at 37°C for a DNA input of 500 ng. The fragmentation target size is 350 bp.

C. End repair and A-tailing

4. In the same tube in which enzymatic fragmentation was performed, assemble each end repair and A-tailing reactions. Keep tubes on ice. (Same composition for 10 and 500 ng of DNA input.)

Component	Volume (µl)
Fragmented DNA	16.7
End repair & A-tailing buffer	2.3
End repair & A-tailing enzyme mix	1
Total volume	20

5. Vortex gently and spin down briefly. Return the reaction tubes to ice.
6. Incubate in a thermocycler at 65°C for 30 min. Hold at 4°C.
7. Proceed immediately to the next step.

D. Adapter ligation

8. Take 1.8 µl of a 25-µM adapter stock tube and make the volume up with water such that the final volume is 5 µl.
9. In the same tube in which the end repair and A-tailing were performed, add the following.

Component	Volume (µl)
End repair and A-tailing reaction product	20
Adapter stock	5
PCR–grade water	1.7
Ligation buffer	10
DNA ligase	3.3
Total volume	40

10. Mix thoroughly and centrifuge briefly.
11. Incubate at 20°C for 15 min. Proceed immediately to next step.

E. Post-ligation cleanup

12. To the same tube, add the following.

Component	Volume (µl)
Adapter ligated reaction product	40
KAPA beads	32

13. Mix thoroughly by vortexing and pipetting up and down multiple times. Incubate for 5 min at room temperature.
14. Place the tube on a magnet rack and wait until the supernatant is clear. Carefully remove and discard the supernatant.
15. Keep the tube on the magnet rack and add 200 µl of 80% ethanol.
16. Carefully remove and discard the ethanol.
17. Repeat steps 15–16 for a total of 2 washes.
18. Dry the beads for 2 min to evaporate any residual ethanol.
19. Remove the tubes from the magnet rack and resuspend beads in 12 µl of nuclease-free water.
20. Incubate the tube at room temperature for 2 min to elute DNA off the beads.
21. Place the tube on a magnet rack and wait for the supernatant to be clear.
22. Transfer the clear supernatant to a fresh tube.

F. First transposon-specific PCR—PCR-1

23. Set up and run PCR-1 as follows.

Reagent	Volume (µl)
OneTaq polymerase	10
PCR-grade water	3
Primer mix: IS5 + CB1; 10 µM each	2
Library from step 22	5

Temperature	Time	Cycles
94°C	30 s	1
94°C	15 s	10
60°C	30 s	10
68°C	45 s	10
68°C	5 min	1

Temperature	Time	Cycles
4°C	Hold	—

24. Perform a post-PCR cleanup. To the 20 µl PCR 1 reaction, add 14 µl of KAPA beads and mix well. Repeat steps 13–22, but elute in 15 µl of nuclease-free water.

G. Second transposon-specific PCR—hemi-nested PCR-2

25. Set up and run PCR-2 as follows.

Reagent	Volume (µl)
OneTaq polymerase	10
PCR-grade water	3
Primer mix: IS5 + CB25.3_R or CB44.3_R; 10 µM each	2
Cleaned PCR-1 product from step 24	5

Temperature	Time	Cycles
94°C	30 s	1
94°C	15 s	15
60°C	30 s	15
68°C	45 s	15
68°C	5 min	1
4°C	Hold	—

H. Final library QC

26. Analyze 3 µl of PCR-2 reaction by agarose gel electrophoresis. When DNA of transposon mutant pools is used, a library-like smear in the transposon enriched library should be seen. The smear should not be present for controls done with wild-type DNA as input. See example image; lanes: M, GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific); 1, sample with DNA of M. kansasii transposon mutant pool as input DNA; 2, sample with DNA of M. kansasii wild type as input DNA; 3, No DNA template PCR control.
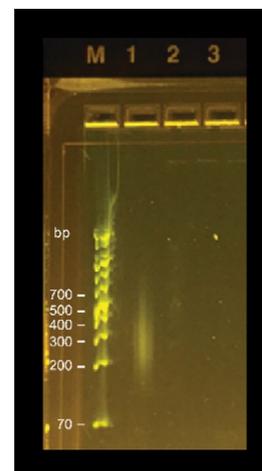
27. Clean the final PCR-2 reaction. Repeat step 24.

28. Using 1 µl from cleaned PCR-2 and Qubit dsDNA HS Assay Kit, check the concentration of the library.

29. Use another 1 µl from cleaned PCR-2 to verify for the library size on the Agilent Bioanalyzer.

30. The final library is then sequenced as described in the Materials and Methods section of the manuscript proper.