

**Supplementary Material**  
**CRISPR interference-mediated silencing of the *mmpL3* gene in *Mycobacterium smegmatis* and its impact on antibiotic susceptibility**

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**Supplementary Data**

**Materials and methods**

**Biofilm and pellicle formation**

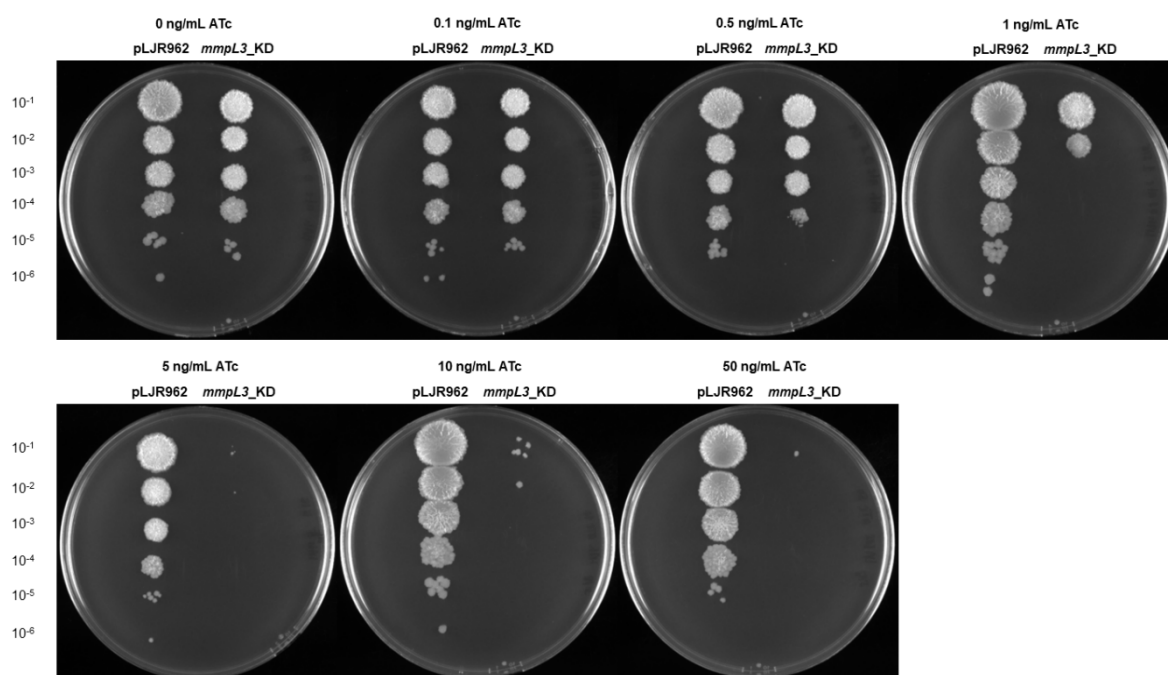
*M. smegmatis* MC<sup>2</sup>155 derivative strains were cultured on Middlebrook 7H10 solid medium (BD Difco, Sparks, MD, USA) supplemented with 0.2% v/v glycerol (HiMedia, Maharashtra, India), 10% ADC, and 25 µg/mL kanamycin (GoldBio, St. Louis, MO, USA) at 37°C until visible colonies were observed. A single colony from each strain was then inoculated into Middlebrook 7H9 broth (BD Difco, Sparks, MD, USA) supplemented with 10% ADC and 0.05% Tween 80 (Ajax Finechem, NSW, Australia), along with 25 µg/mL kanamycin, and incubated for 24 hours with shaking at 200 rpm and 37°C. Biofilm and pellicle formation were assessed as previously described [30] with slight modifications. Log-phase cultures of *M. smegmatis* were adjusted to an OD<sub>600</sub> of approximately 0.1 in Sauton medium (HiMedia, Maharashtra, India) containing varying concentrations of ATc (0 to 50 ng/mL) and 25 µg/mL kanamycin. For pellicle formation assays, 2 mL aliquots of the diluted culture were incubated in glass tubes without shaking at 37°C for 3 days. For biofilm formation assays, 200 µL aliquots of the diluted cultures were inoculated into 96-well polystyrene plates (Jet Bio-filtration, Guangzhou, China) in replicates of six and incubated at 37°C for 5 days.

After incubation, the 96-well plates were washed twice with water, stained with 0.1% crystal violet for 30 minutes, and then washed twice with water. Bound crystal violet was solubilized with 30% acetic acid, and the absorbance was measured at 595 nm.

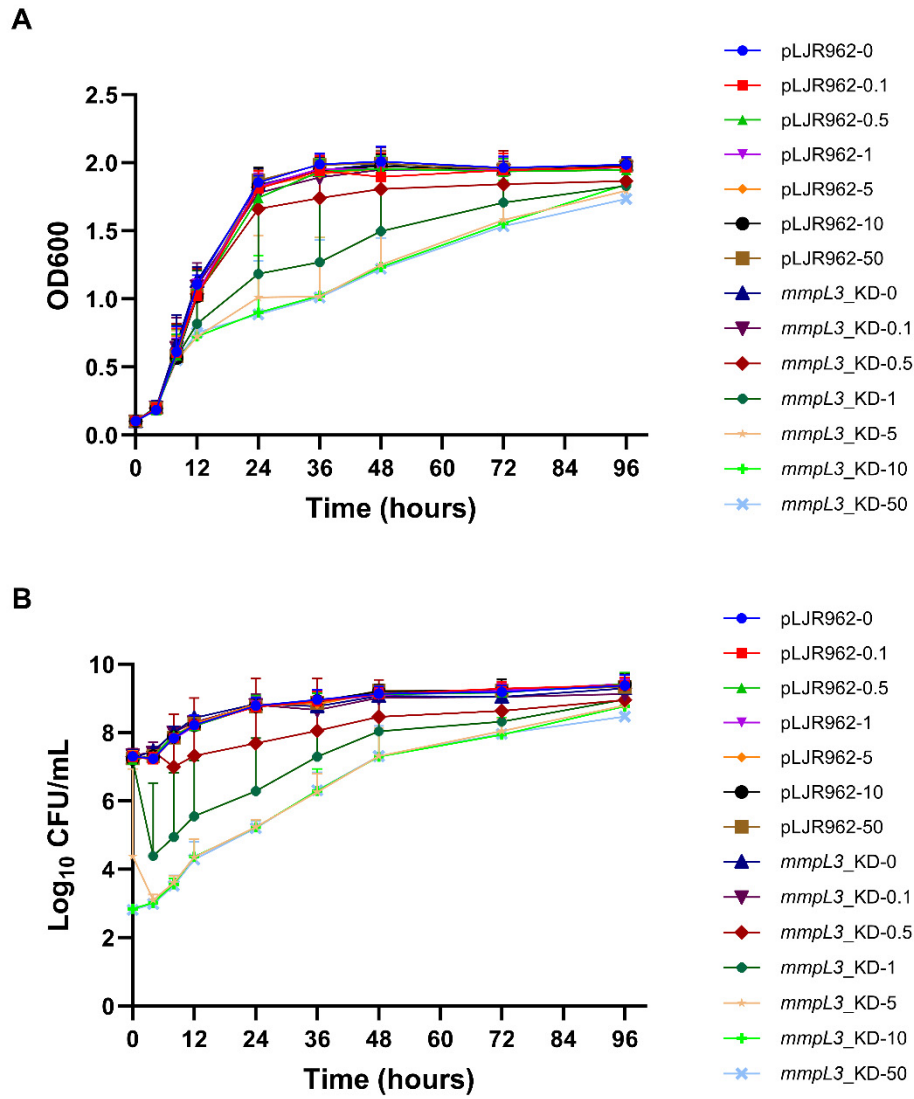
**Statistical analysis**

The statistical significance of biofilm formation under different ATc concentrations was assessed using two-way ANOVA. A *p* value less than 0.05 was considered to indicate statistical significance. Statistical analysis and graph were generated using GraphPad Prism version 10.0.0 for Windows.

## Supplementary Figures



**Figure S1. Essentiality of the *mmpL3* gene in the *mmpL3\_KD* and pLJR962 control *M. smegmatis* strains.** Serial dilutions (10<sup>-1</sup> to 10<sup>-6</sup>) of log-phase cultures of the *mmpL3\_KD* and pLJR962 control *M. smegmatis* strains were plated on 7H10 agar in both the absence and presence of varying concentrations of ATc (0, 0.1, 0.5, 1, 5, 10, or 50 ng/mL).



**Figure S2. Growth curve and viability of the *mmpL3\_KD* and pLJR962 control *M. smegmatis* strains at different time points.** (A) The optical density at 600 nm (OD<sub>600</sub>) of the *mmpL3\_KD* and pLJR962 control *M. smegmatis* strains at different ATc concentrations (0, 0.1, 0.5, 1, 5, 10, or 50 ng/mL). (B) The viability (log<sub>10</sub> CFU/mL) of the *mmpL3\_KD* and pLJR962 control *M. smegmatis* strains at different ATc concentrations (0, 0.1, 0.5, 1, 5, 10, or 50 ng/mL). The experiments were conducted in biological and technical triplicates. The error bars represent the SDs of the means.

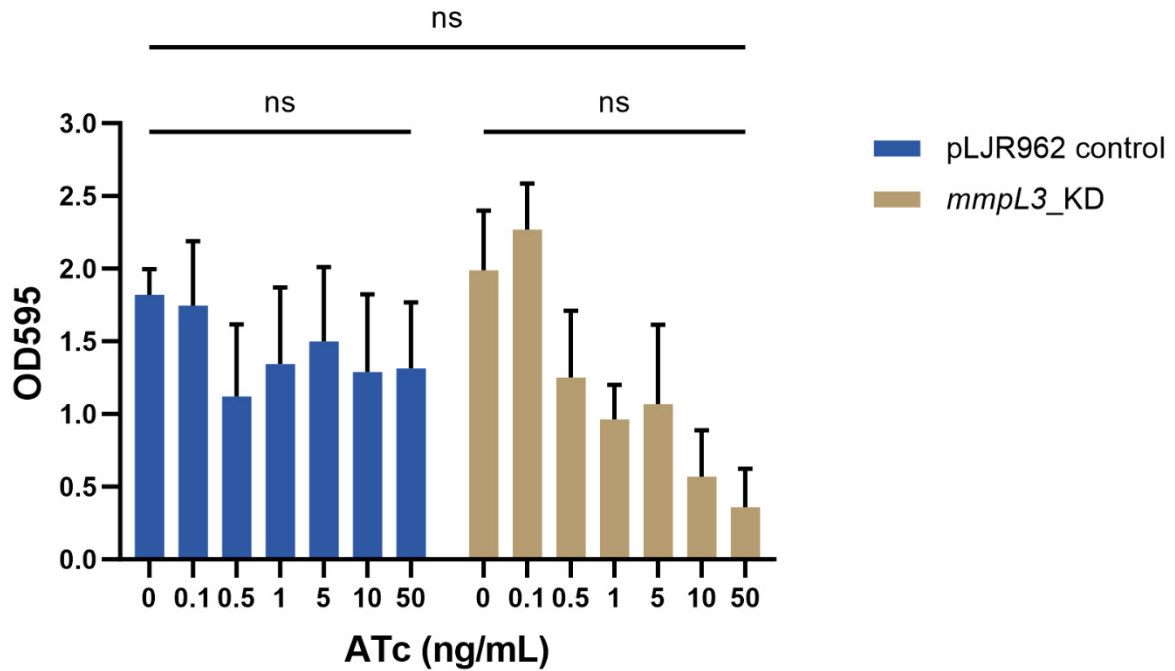


Figure S3. Biofilm formation of the *mmpL3\_KD* and pLJR962 control *M. smegmatis* strains assessed by a quantitative crystal violet assay. A decrease in the biofilm mass of the *mmpL3\_KD* *M. smegmatis* strain was observed after induction with 0.5 ng/mL to 50 ng/mL ATc ( $p > 0.05$ ). The experiments were conducted with six replicates. “ns” indicates not statistically significant. The error bars represent the SDs of the means.

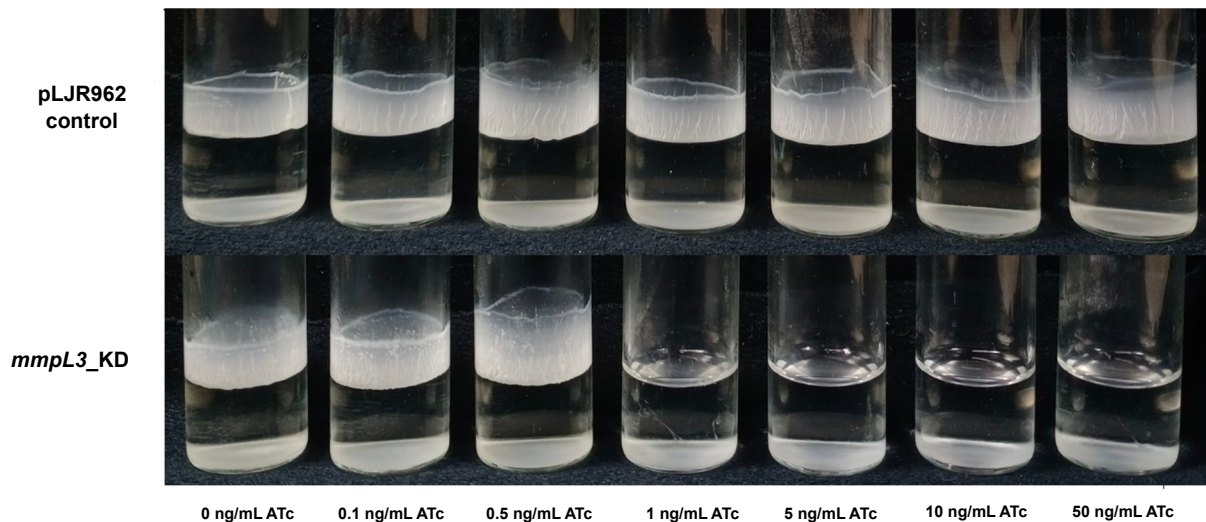


Figure S4. Pellicle formation of the *mmpL3\_KD* and pLJR962 control *M. smegmatis* strains observed on day 3 of incubation. Pellicle formation of the *mmpL3\_KD* *M. smegmatis* strain decreased in the presence of 1 ng/mL to 50 ng/mL ATc compared to the pLJR962 control strain.