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Note

Gene knock-out in *Mycobacterium abscessus* using *Streptococcus thermophilus* CRISPR/Cas

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ABSTRACT

The CRISPRi system using dCas9$_{Sth1}$ from *Streptococcus thermophilus* developed for *Mycobacterium tuberculosis* and *M. smegmatis* was modified to allow gene knock-out in *M. abscessus*. Efficacy of the knock-out system was evaluated by applying deletions and insertions to the *mps1* gene. A comparative genomic analysis of mutants and wild type validated the target specificity.

M. abscessus has the potential to induce severe lung infections in individuals with preexisting lung conditions, including Cystic Fibrosis. Its remarkable resistance to antibiotics necessitates prolonged multidrug therapy, typically extending for at least 12 months after culture conversion (Weng et al., 2020). Despite these extensive treatment efforts, complete bacterial eradication is often not achieved (Hughes et al., 2021). Hence, there is an urgent need for a deeper understanding of the resistance and virulence mechanisms of this pathogen. However, the application of mutagenesis procedures in *M. abscessus* has been hampered by several difficulties such as low transformation efficiencies, the high frequency of spontaneous mutations to frequently used selection markers and the low allelic exchange efficiency obtained by homologous recombineering (Cortes et al., 2011; Medjahed and Reyrat, 2009). Rock and colleagues (Rock et al., 2017) have previously established a CRISPR interference system (CRISPRi) for the mycobacterial species *M. tuberculosis* and *M. smegmatis*. This interference system was successfully adapted for gene silencing in *M. abscessus* (Nguyen et al., 2023). In contrast to knock-out systems, gene silencing systems have the advantage to also work with essential genes, but the disadvantages of only incomplete loss of gene function, polar effects and continuous requirement for induction of the system by addition of inducer substances during the experiments. To enhance the utility of CRISPR for gene function analysis in *M. abscessus*, we modified the original (Rock et al., 2017) system to be able to generate stable and permanent gene knock-out mutations in *M. abscessus* resulting in complete loss of gene function and allowing to perform experiments without continuous induction of the CRISPR system. CRISPR gene knock-out systems require a functioning Non-Homologous-End-Joining (NHEJ) system for repair of the double-strand breaks introduced by the nucleases being part of the CRISPR system. The NHEJ repair requires homodimers of the protein Ku, which bind to the DNA ends and LigD, an ATP-dependent DNA ligase, which processes and ligates the two DNA ends (Bertrand et al., 2019). In addition to LigD, LigC1 may support the NHEJ repair (Yan et al., 2020). It is an error-prone mechanism causing deletions and insertions of variable sizes. (Yan et al., 2020) observed deletions in the range of 1 to >8.000 bp in *M. smegmatis* when using Cas9$_{Sth1}$ for knock-out mutagenesis. Here we describe the generation and use of the CRISPR/Cas9$_{Sth1}$ system for generating knock-out mutations in *M. abscessus* and the evaluating of its efficiency and specificity.

To assess the efficacy of the CRISPR system, we selected the gene *mps1* from *M. abscessus*, which plays a vital role in the synthesis of Glycopeptidolipids (GPL) found in the cell wall of smooth *M. abscessus* colony morphotypes. When the Mps1 protein is nonfunctional, it leads to a deficiency in GPL production, resulting in the formation of rough colony morphotypes (Li et al., 2020). This distinct colony morphology change allowed for straightforward visual identification of potential mutants. To ascertain the gene-specificity of our mutagenesis system, we sequenced and compared the genomes of the mutants with those of the

Abbreviations: ATc, Anhydrotetracycline; Cas, CRISPR-associated proteins; CRISPR, clustered regularly interspaced short palindromic repeats; GPL, glycopeptidolipids; PAM, protospacer adjacent motif; TLC, Thin Layer Chromatography..

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wild type.

The vector pLJR965 (Rock et al., 2017) encodes a nuclease-inactive dCas9$_{Sth1}$. To establish a knock-out system providing a catalytically active Cas9, the Q5 site-directed mutagenesis kit (New England Biolabs, Ipswich, MA, USA) was used according to the manufacturer's protocol to exchange in pLJR965 the codon (GCC) for alanine at position 9 to aspartic acid (GAC) and the codon (GCC) for alanine at position 599 to histidine (CAC). The plasmid containing the gene for the nuclease-active Cas9 was called pSAK14 (see Table 1).

To design the sgRNA sequence, the protocol by Rock (2017) was followed and the highly effective PAM site AGAAG was chosen. The oligos sgRNA_mps1_a and sgRNA_mps1_b (Table 1) were annealed and inserted into the *Bsm*BI sites of pSAK14 to allow expression of a sgRNA complementary to the *mps1* gene binding at position 6885 to 6904 of *mps1*.

This plasmid (pSAK33) was introduced into *M. abscessus* strain 09–13-3 (Lewin et al., 2021) by electroporation with addition of Kanaymcin (100 μg/ml) for selection of recombinant colonies and 50 or 100 ng/ml Anhydrotetracycline (ATc) for induction of Cas9 and sgRNA expression to the agar plates [BBL Seven H11 Agar Base plates (Becton Dickinson) supplemented with 10% glycerol and 10% modified ADC (2 g Glucose, 5 g BSA Fract. V, 0.85 g NaCl in 100 ml of water)].

Rough colonies (Fig. 1 b) were picked and PCR (primers FW-msp1/RV-msp1 and FW-msp1–2/RV-msp1–2, Table 1) followed by Sanger sequencing was performed to confirm mutation of *mps1*. Although only relatively few rough colonies were obtained (6 rough colonies when selecting with ATc 50 ng/ml and 9 with ATc 100 ng/ml), a high proportion of rough colonies (83% with ATc 50 ng/ml and 100% with ATc 100 ng/ml) exhibited mutations in *mps1*.

Confirmation that rough colonies were indeed affected in their GPL synthesis was achieved by GPL isolation followed by Thin Layer Chromatography (TLC). The GPL was extracted from the dried bacterial pellets from 50 ml cultures by adding 10 ml of chloroform-methanol (2:1, V: V) followed by sonification for 1 min (100% power, Branson Sonifier-450 D). The liquid phase was hydrolyzed with 3 ml of 0.2 N sodium hydroxide in methanol to remove the alkali-labile lipids. After neutralizing the pH to pH 7, 6 ml of chloroform and 5 ml of water were added, and the mixture was centrifuged. The organic solvent-containing bottom phase was dried, and the resulting pellet dissolved in 1 ml of chloroform/methanol (2:1, V: V). The lipid fractions were separated on TLC silica gel 60 TLC plates (Merck, Germany) using chloroform/methanol (9:1, V: V) as mobile phase. As shown in Fig. 1 c, the rough colonies lacked GPL.

To confirm the specificity of the CRISPR system, the genomes of 14 *mps1* mutants and of the wild type were sequenced by Illumina sequencing (Paired-end 2x150bp with Illumina NextSeq2000; Illumina NexteraXT Library Preparation Kit using the Hamilton Microlab STAR Liquid Handler). Software used for sequencing and reporting were IDeFIX v1.5.1, bcl2fastq v2.19.1.403, QCumber-2 v2.1.1, Trimmomatic v0.36, FastQC v0.11.5, and Kraken v0.10.6-unreleased and Snakemake

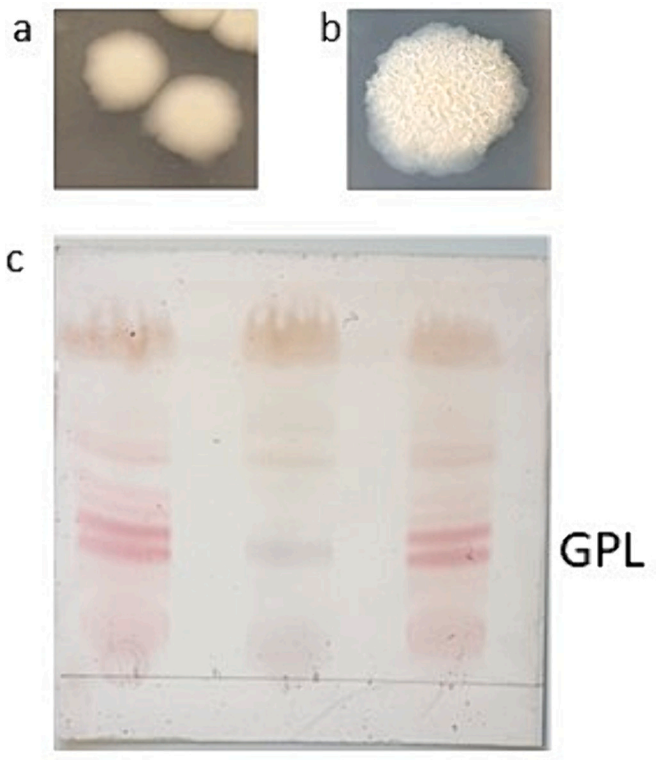

Fig. 1. Effect of the mutation of *mps1* on colony morphology and Glycopeptidolipid (GPL) content. The wild type *M. abscessus* 09–13-3 (Lewin et al., 2021) and an *mps1* mutant were grown on BBL Seven H11 Agar Base plates (Becton Dickinson) supplemented with 10% glycerol and 10% modified ADC for 5–7 days. a) shows the smooth colony morphotype of the wild type 09–13-3 and b) shows the rough colony morphotype of an *mps1* mutant. c) shows a Thin Layer Chromatography of GLP isolated from the wildtype (1st lane), *mps1* mutant (2nd lane) and as control the strain carrying pSAK14 without sgRNA sequence (3rd lane). In comparison to the other two isolates, the mutant is lacking GPL.

Table 1
Primers/oligonucleotides and plasmids used or generated in the study.

Primers/ Oligo-nucleotides		
Name	**Sequence (5′ - > 3′)**	**Application**
sgRNA_mps1_a	GGGAGGAGCCAACCGTCGAGCAC	Cloning of sgRNA for *mps1*
sgRNA_mps1_b	AAACGTGCTCGACGGTTGGCTCC	Cloning of sgRNA for *mps1*
FW-msp1	GTTGTCCGCTCACCAGAAGA	Confirmation of mutation in *mps1*
RV-msp1	CGGCAAAGCATCCAAAACCA	Confirmation of mutation in *mps1*
FW-msp1–2	GAACACGACCGGTTGGACAA	Confirmation of mutation in *mps1*
RV-msp1–2	AGTGTGGCATGCCTATCCAG	Confirmation of mutation in *mps1*

Plasmids		
Name	**Properties/generation**	**Reference**
pLJR965	Integrative CRISPR/dCas9 interference backbone vector (8631 bp) for gene knockdown in *M. tuberculosis* and *M. smegmatis* coding for a dead Cas9$_{Sth1}$ endonuclease gene and *Bsm*BI cloning sites for insertion of a sgRNA sequence under the control of anhydrotetracycline-inducible promoters; as selection marker a Kanamycin resistance gene is present.	(Rock et al., 2017); (Rock, 2017) https://media.addgene.org/data/plasmids/115/115163/115163-attachment_vIKvu61hzB2c.pdf
pSAK14	Derivative of pLJR965 encoding a nuclease-active Cas9.	This study
pSAK33	Derivative of pSAK14 containing the annealing product of oligonucleotides sgRNA_mps1_a and sgRNA_mps1_b inserted into the BsmBI cloning sites from pSAK14	This study

Table 2
Genes mutated and types of mutation in *mps1* CRISPR mutants in comparison to the wild type 09–13-3 genome sequence.

Isolate name	Mutated genes	Type and size of mutation in *mps1*
Wild type	none	none
Mutant 308	*mps1*	862 bp deletion
Mutant 309	*mps1*	2164 bp deletion
Mutant 311	*mps1*	12 bp deletion
Mutant 312	*mps1*	4 bp deletion
Mutant 313	*mps1* and downstream region	3151 bp deletion
Mutant 314	*mps1*	1 bp insertion
Mutant 318	*mps1*	55 bp deletion
Mutant 319	*mps1*	9 bp deletion
Mutant 320	*mps1*	21 bp deletion
Mutant 321	*mps1*	9 bp deletion
Mutant 322	*mps1*, MAB_3167c	1 bp deletion
Mutant 323	*mps1*	1 bp insertion
Mutant 324	*mps1*	29 bp deletion
Mutant 325	*mps1*	4757 bp deletion

v3.12.0. SNPs, Indels, and larger deletions were identified using Geneious Prime v11.1.5 (Biomatters) (workflow "Map reads then discover variations/SNPs", settings "coverage 20" and "variation frequency 0.9").

Thirteen out of 14 mutants that were sequenced displayed deletions and insertions only in the *mps1* gene or its surrounding region, indicating the specificity of the CRISPR system (Table 2). Only one mutant showed an additional mutation in a non-target gene (MAB_3167c). The size of deletions varied, with most being below 100 base pairs, but some reached up to 4757 base pairs.

In addition to the *mps1* gene, the study was extended to the application of the CRISPR system to four other genes with various PAM sequences. While the porin gene MAB_1080 (PAM site AGAAG), and *ppiA* (PAM site GGAAG) were successfully mutated, attempts to target *erm (41)* (PAM sites GGAAA and AGCAG), and *whiB7* (PAM site AGAAT) were unsuccessful suggesting a crucial role of choice of efficient PAM sites.

In conclusion, this study demonstrates that the CRISPR/Cas9$_{Sth1}$ in pSAK14 with its enzymatically active Cas9 is an efficient tool for generating gene mutations in *M. abscessus*. During the review process for this manuscript, a publication by (Neo et al., 2024) on the generation of a dual-plasmid CRISPR/Cas9-based method appeared that confirms the usefulness of Cas9 from *S. thermophilus* for knock-out mutagenesis of *M. abscessus*. Our system exhibits high specificity for the target region, resulting in mutations in the desired gene region without affecting non-target sequences. This approach allows for the generation of mutations of various sizes, thus expanding the possibilities for genetic studies in *M. abscessus*. Selecting an efficient PAM site is essential for the successful generation of mutants. This advancement in CRISPR-mediated gene mutation holds great promise for improving the understanding of mycobacterial virulence and resistance, which will facilitate the identification of novel drug targets to treat *M. abscessus* infections and the development of vaccines to protect vulnerable populations.

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CRediT authorship contribution statement

Suriya Akter: Conceptualization, Investigation, Methodology, Writing – original draft. **Elisabeth Kamal:** Methodology. **Carsten Schwarz:** Resources. **Astrid Lewin:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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