Sodani *et al. Journal of Biological Engineering* (2025) 19:21
https://doi.org/10.1186/s13036-025-00490-3

Journal of Biological Engineering

RESEARCH Open Access

CRISPR/Cas12a-mediated gene silencing across diverse functional genes demonstrates single gene-specific spacer efficacy in *Mycobacterium smegmatis*

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Abstract

Background Tuberculosis, a persistent global health threat, necessitates a comprehensive understanding of the genes and pathways crucial for the survival and virulence of the causative pathogen, *Mycobacterium tuberculosis*. Working with *M. tuberculosis* (M.tb) presents significant challenges; therefore, the use of *M. smegmatis* as a surrogate system for conducting genetic studies of M.tb has proven to be highly valuable. Development of novel genetic tools to probe cellular processes accelerates the progress in the field of drug development and also helps in understanding the basic physiology of the bacterium.

Results This study reports the successful implementation and evaluation of the CRISPR-Cas12a system for gene repression in *Mycobacterium smegmatis*, a surrogate for *M. tuberculosis*. We engineered a Cas12a-based CRISPR interference (CRISPRi) system and assessed its functionality. Targeting 45 genes with a single sgRNA per gene, we achieved efficient gene repression, leading to marked phenotypic changes. Each knockdown strain was evaluated individually for growth phenotypes, and a comparison of the results with the reported essential gene library probed with dCas9 demonstrated congruous results across diverse gene categories. The study shows that CRISPR/Cas12a system can be effectively utilised with a single gene specific target for efficient silencing of the gene and highlights the importance of subsequent growth assays required to evaluate the vulnerability of targeted gene silencing.

Conclusion Our findings reveal the robustness and versatility of the dCas12a-CRISPRi system in *M. smegmatis*, providing a valuable tool for functional genomics research. This work showcases the potential of the dCas12a-CRISPRi system in investigating essential genes, enabling a deeper understanding of the biology and potential therapeutic targets in mycobacterium species.

Keywords CRISPRi, Cas12a, Mycobacterium, Essential gene, Gene silencing

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Introduction

Tuberculosis is a disease with a long history, dating back over a century, and its causative agent was discovered by Robert Koch in 1882. Despite its long-standing presence, effective vaccines for the disease remain elusive, and the emergence of drug-resistant strains has made treatment challenging. To develop a vaccine or drug, it is crucial to identify effective vaccine or druggable targets. Investigating genes as potential vaccine or drug targets often requires efficient genetic tools to study the pathogen's physiology and pin-point these targets. However, working with mycobacteria has proven to be particularly challenging. These challenges stem from the bacterium's slow growth rate, its ability to spread via aerosol transmission, and its resistance to genetic modifications. The genetic intractability of mycobacteria is a primary reason behind the significant number of uncharacterized genes in these pathogens.

The introduction of targeted genetic changes are essential for uncovering the functions of genes and the physiology of pathways. Creating recombinant strains, knockouts, and knockdowns is pivotal for gene functional studies. However, the construction of recombinant strains posed challenges in mycobacteria for a long time due to their limited ability to take up exogeneous DNA [1]. Nevertheless, significant progress has been made using techniques such as transduction and standardized electroporation protocols [2, 3], enabling the development of competence in this species. Constructing genetic knockouts was particularly problematic due to the lower rate of homologous recombination (HR) in mycobacterium [4, 5]. This challenge has been well addressed by introducing recombineering to enhance allele exchange frequency [6]. However, creating knockout strains using this method involved a laborious and time-consuming process of vector construction. Furthermore, knockout methods are unsuitable for the study of essential genes. Therefore, a knockdown system becomes crucial for investigating essential core life processes. Such a system also accelerates vaccine and drug development, and provides insights into central life process pathways. In this context, promoter replacement through allele exchanges has been utilized to obtain conditional knockdowns, a development that has facilitated the study of numerous genes and pathways in mycobacteria [7–9]. However, this method still involves multiple rounds of vector construction.

CRISPR interference (CRISPRi) coupled with high-throughput sequencing has emerged as a promising and powerful approach for conducting functional genomics research in bacteria [10–12]. The Cas9 systems derived from *S. pyogenes* and *S. thermophilus* have found extensive applications in mycobacteria [13–15]. Notably, the dCas9 system from *S. thermophilus* has demonstrated superior performance compared to its *S. pyogenes* counterpart [14]. Based on these, some significant and robust CRISPR screens have been conducted in mycobacteria, involving high-throughput pooled and arrayed gene library studies [16–18]. In a pooled CRISPRi library, multiple guide RNAs targeting different genes are mixed together in a single pool which is then introduced into the target cells as a mixture. This proves cost-effective for screening large numbers of genes compared to individually arrayed libraries. However, the complexity of these pooled libraries increases the risk of false positives and negatives. Experimental noise and off-target effects may complicate the interpretation of results further. A detailed analysis of all genes with respect to their essential character has been carried out by Bosch et al. using dCas9 from *S. thermophilus* in a pooled library format [17] and also by de Wet et al. using pooled and arrayed library format [16, 18].

Recently, CRISPRi based on a CRISPR-Cas12a system has been reported in mycobacteria [19], where non-native genes and a few native genes (*trpD* and *proC*) have been targeted to demonstrate the functionality of the system. However, despite offering advantages such as absence of toxicity and independence from trans-activating CRISPR RNA (tracrRNA), this system has not been tested and adopted widely in mycobacteria [14, 19, 20]. In this study, we established a dead Cas12a (dCas12a) based CRISPRi system and confirmed that it is functional in mycobacteria. Furthermore, we conducted a comprehensive assessment of the system's robustness and functionality by targeting 45 genes using a single sgRNA per gene of interest. We were able to achieve efficient gene repression and observed marked phenotypic changes using the optimized dCas12a-CRISPRi system.

Results

Establishment and testing of CRISPR/Cas12a based CRISPRi in *M. smegmatis*

The CRISPR-Cas12a system from *Francisella novicida,* codon optimised for *Corynebacterium glutamicum* was successfully adapted in mycobacteria [21]. To make Cas12a amenable for CRISPRi, the D917A mutation was introduced in the RuvC DNAse domain which rendered it "dead" or inactive [22]. This CRISPRi system was developed as a two-plasmid system (Fig. 1A). In this setup, the dCas12a gene was cloned under an inducible Tet promoter in an integrative plasmid (pSTKiT) [23] thereby generating pSTKiT-dCas12 for stable integration into the host cell. The sgRNA (single guide RNA), on the other hand, was provided through an easy oligo-based Golden Gate cloning method on a replicative vector (pST-HT) producing pST-HT-cr. Importantly, unlike

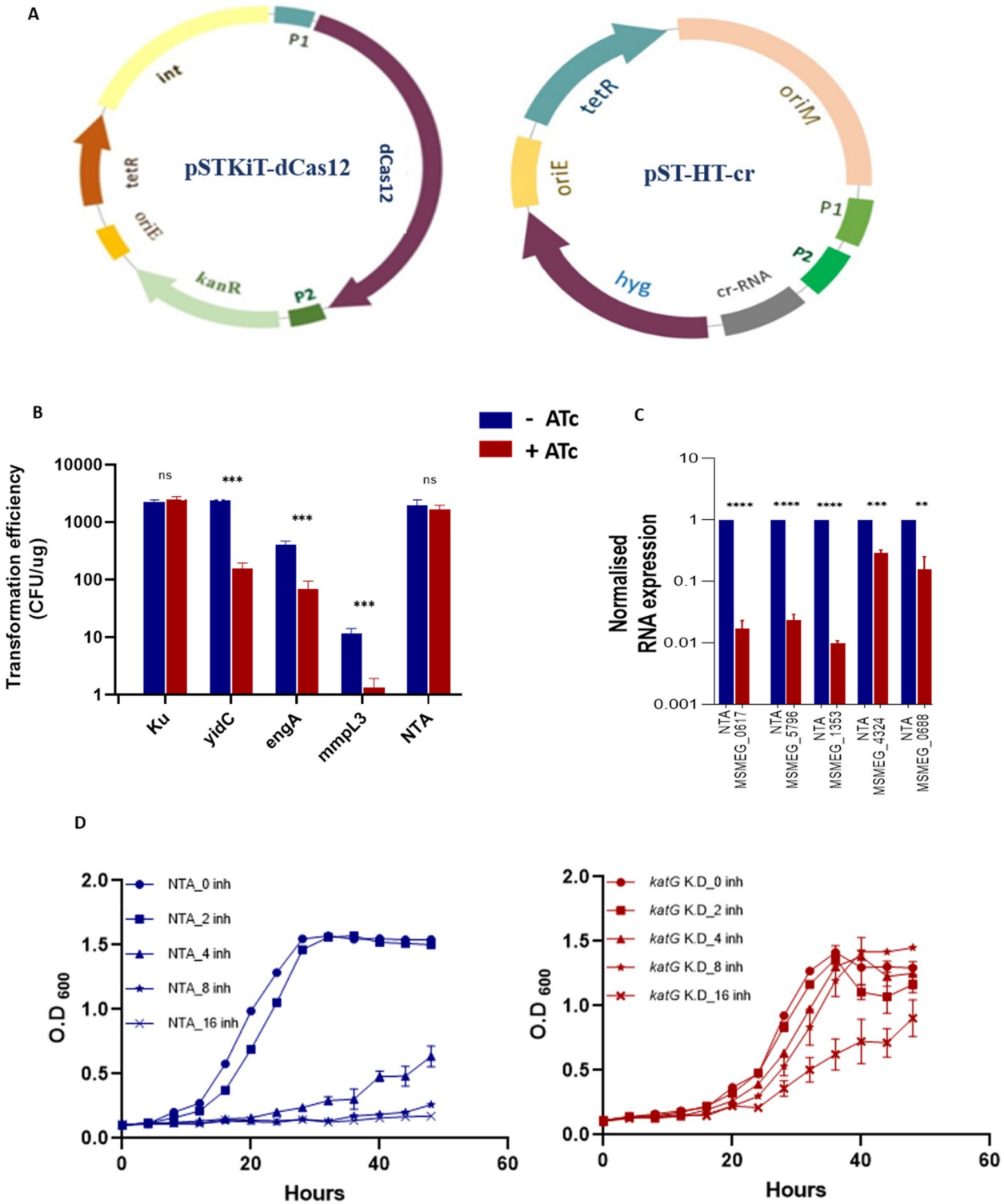

Fig. 1 **A**) Plasmids constituting the dCas12a-CRISPRi system; pSTKiT-dCas12 is an integrative plasmid carrying dCas12a under inducible promoter and pST-HT-cr is the replicative plasmid for cloning of desired spacer for CRISPRi targeting. **B**) Transformation efficiency of pST-HT-cr with spacers targeting indicated genes in *M. smegmatis* strain bearing dCas12a. (Statistical significance was calculated as *p*-value with unpaired t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) **C**) qPCR result showing relative quantification of few targeted genes (Statistical significance was calculated as p-value with unpaired t-test. *, $p < 0.05$; **, $p <$;0.01; ***, $p < 0.001$; ****, $p < 0.0001$). **D**) Growth curve of NTA (blue) and *katG* knockdown strain (red) grown in presence of varied isoniazid (inh) concentrations (0–16 µg/ml) as indicated

the reported toxicity associated with certain Cas systems [24], dCas12a was found to be non-toxic to mycobacterium, even at high anhydrotetracycline (ATc- the inducer for expression of Tet regulated dCas12a) concentrations of 200 ng/ml (Fig. S1). The expression of dCas12a was also confirmed using western blot analysis (Fig. S2).

To validate the functionality of the system, known essential and non-essential genes were targeted, and their survival was studied to confirm gene repression (Fig. 1B). For example, genes such as *yidC, engA, mmpL3* which are reported to be essential [14] had reduced survival in presence of ATc, while Ku, which is known to be non-essential, and a non-targeted array (NTA -a scrambled spacer not matching with mycobacterial genomic region) had no significant effect in presence of the inducer. These results showed the functioning of the system as an effective gene silencing tool. Also, for a few of the targeted loci- MSMEG_0617, MSMEG_5796, MSMEG_1353, MSMEG_4324 and MSMEG_0688, reduction in expression was quantified with qPCR (Fig. 1C). The gene repression achieved was ~ 50-fold for three of the five genes tested while the other two were repressed two to three-fold, thereby showing efficient gene repression.

Additionally, a functional assay was designed to assess the system's effectiveness. The *katG* gene was targeted, and the bacteria were allowed to grow in the presence of a drug, isoniazid (INH). *katG* is responsible for converting the pro-drug form of INH into its active form, and any deficiency in *katG* would render the drug ineffective. As expected, the *katG* gene knockdown strain (*katG* K.D) was able to grow in the presence of INH, indicating its decreased ability to activate the drug due to repression of *katG*. In contrast, the control strain with NTA spacer was susceptible to isoniazid at varied concentrations (Fig. 1D). The protective phenotype of the *katG* knockdown strain at different concentrations of INH, further confirms the functionality of the dCas12a in causing efficient repression.

Transformation efficiency of dCas12a targeted gene knockdowns

The essential gene database of *Mycobacterium tuberculosis* (MTB) which enlists 687 essential genes of MTB was referred to [25] and an attempt was made to target 50 of those gene homologs in *M. smegmatis*. In order to target genes performing different kind of functions, genes from six different functional categories were targeted (Fig. 2) – cell wall processes (8 genes), conserved proteins (10 genes), secretion system (8 genes), intermediary metabolism (9 genes) information pathway (4 genes), and replication, transcription and translation (6 genes). We also targeted four hypothetical genes (MSMEG_0311, MSMEG_0315, MSMEG_0317 and MSMEG_0319) and one pseudogene (MSMEG_0310), however, the analysis of these results were reported in a recent publication [26] and hence, not discussed here.

Spacers designed to target the beginning of open reading frame (with 5'-TTN-3' as PAM) of the genes of interest from diverse functional categories were cloned into the pST-HT-cr plasmid (S. Table 1). These constructs, engineered to produce crRNA against the target genes, were introduced into the strain bearing an integrative plasmid containing dCas12a under Tet promoter (pST-KiT-dCas12a). Transformants were selected on agar plates in presence or absence of the inducer, ATc. The relative transformation efficiency (normalised against the control -without ATc condition) was calculated, and the results showed that some of the constructs produced fewer colonies in presence of the inducer, while others displayed nearly identical transformation efficiency in both presence and absence of the inducer (Fig. 2). The genes were categorized as non-vulnerable when there was no significant difference in transformation efficiency of constructs (expressing crRNAs targeting the genes) in presence or absence of inducer. Conversely, genes were recognized as vulnerable when constructs expressing targeted crRNAs exhibited decreased transformation efficiency in the presence of the inducer (ATc).

In the cell wall category, 4 of the 8 targeted genes were found to be vulnerable. For genes encoding conserved proteins, 9 out of the 10 targeted were non-essential, while MSMEG_6928 was observed to be essential. Further, in this category, MSMEG_1353, despite having comparable transformation efficiency in presence or absence of the inducer, displayed tinier colonies in presence of ATc (Fig. S3). Among secretory proteins, none of the 8 proteins targeted were essential in *M. smegmatis*, in contrast to what was observed with *M. tuberculosis* [27]. Interestingly, MSMEG_0617 targeted cells took longer (~ 7 days) to yield colonies on plates containing ATc. Of the 9 intermediary metabolism genes targeted, only MSMEG_0384 and MSMEG_6917 showed vulnerability in presence of ATc. In case of the 4 genes targeted in information pathway category, all were found to have comparable transformation efficiencies in presence/absence of inducer, hence all were annotated as non-vulnerable. However, MSMEG_6933 showed smaller colonies on ATc plates from this category (Fig. S3). Next, on targeting the 6 genes in replication transcription and translation machinery, 4 were observed as essential, while MSMEG_0252 displayed equal numbers but smaller sized colonies. Overall, in a group of 45 genes, we found 11 genes whose knockdown displayed reduced survival (Fig. 2) and 4 genes which showed smaller colony size upon induction of CRISPRi (Fig. S3).

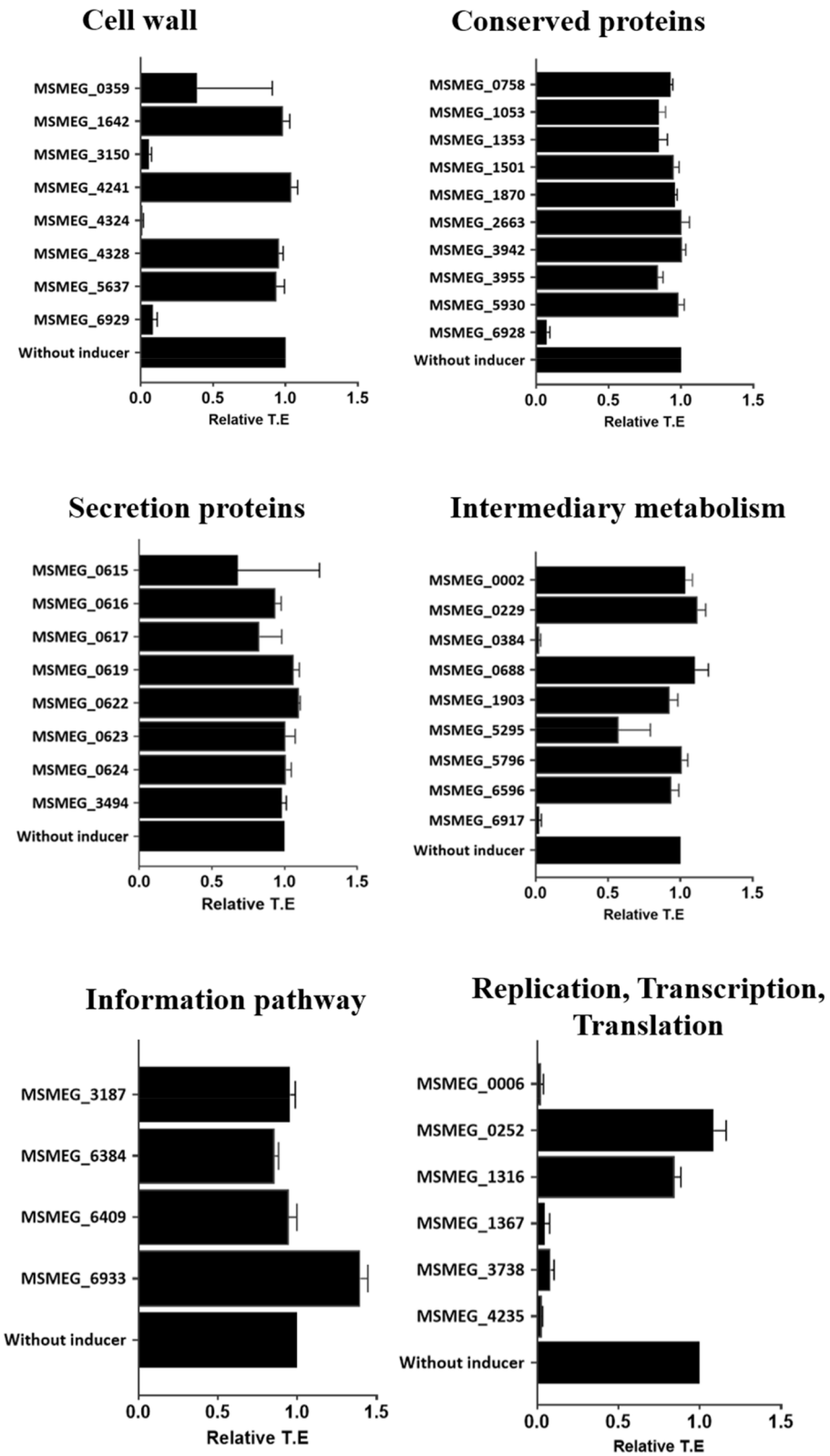

Fig. 2 Relative transformation efficiency of pST-HT-cr plasmids carrying spacers targeting indicated genes in *M. smegmatis* bearing dCas12a grown in presence/absence of inducer, ATc

Evaluation of growth fitness in solid agar and broth conditions

The knockdown strains were checked for their fitness in spot dilution assay to further assess the impact of gene knockdown on growth. The strains transformed with constructs expressing crRNA targeting the genes of interest were sub-cultured in absence of the inducer (ATc). Subsequently, they were serially diluted and spotted onto plates with or without ATc. The cells were allowed to grow for three days, and the resulting spots were observed and documented. The analysis showed that of the 45 genes, 30 displayed nearly equal spot density in both presence and absence of ATc, indicating no or minimal impact of these genes on growth while remaining 15 genes exhibited reduced growth only in presence of ATc, thereby pointing towards their essentiality in cell survival (Fig. 3). A comparison with the transformation efficiency data discussed in previous section showed that among these 15 genes, 11 had shown reduced transformation efficiency (Fig. 2). The remaining four (MSMEG_0252, MSMEG_0617, MSMEG_1353, MSMEG_6933), interestingly, were the ones that showed tinier colonies on ATc plates after transformation, indicating an associated growth defect (Fig. S3). The discrepancy between the lack of a drop in survival in transformation assay and the observed defects in growth assays for some essential genes can be attributed to the residual activity of the essential genes and the sensitivity of the assays. Even when essential genes are knocked down or disrupted, they may retain partial functionality, allowing the bacteria to survive during the transformation process. While transformation efficiency assays primarily measure overall survival, phenotype assays are sensitive to subtle functional disruptions that may not significantly affect survival but can lead to defects in specific cellular processes that affect growth. This distinction underscores the value of phenotype assays in capturing nuanced defects in essential genes that might be overlooked by survival-based metrics.

The effect of knockdown on fitness was also assessed in broth conditions to investigate the kinetics throughout the growth phase. The strains which showed growth defect in presence of ATc are shown in Fig. 4 while the growth curves of the remaining strains are shown in Fig. S4. The results of growth on solid medium largely mirrored those of broth conditions and could be used to determine gene vulnerability. Interestingly, two gene knockdowns (MSMEG_0688 and MSMEG_3494), which did not display growth defect on solid medium and also were classified as non-essential by the transformation efficiency results were surprisingly found to have a clear growth defect in broth conditions (Fig. 4). Of these, MSMEG_0688 was also found as essential in a transposon study conducted in *M. smegmatis* [28]. This highlights the importance of growth kinetics in evaluating the fitness defect which might not show up in pooled library assays. For some genes where the growth defect is not pronounced such as MSMEG_0384 and MSMEG_0252, the knockdown strain may appear to "catch up" with the control at a later time, especially as the control cells enter the stationary phase.

Discussion

A major goal of genetics is to pinpoint genes crucial for the survival of cells or organisms. Contemporary molecular genetics techniques, such as transposon mutagenesis, gene trapping, homologous recombination, and the relatively recent CRISPR gene editing, achieve this by initially disabling these genes and subsequently assessing whether cells or organisms with these mutations can still thrive. Since these methods rely on negative selection, there is no assurance and frequently, no concrete evidence exist that the specific gene of interest has indeed been disrupted or deactivated in the non-viable cells. Further, such methods have limited application when an investigation is warranted into the functional aspect of essential genes. CRISPR interference approaches can be used to fill this gap as these do not lead to alteration of the target gene. Additionally, when coupled with high-throughput sequencing, CRISPRi has emerged as a promising and powerful tool for conducting functional genomics research in bacteria [29].

One of the primary objectives of our study was to evaluate the utility of dCas12-based CRISPRi in mycobacteria vis-à-vis the already established dCas9 based CRISPRi. For this reason, we targeted a number of genes (45) so that a representative data set could be generated to compare our results with the previous studies using dCas9 system. Moreover, we used assays such as transformation, growth on solid medium (spot assay) and growth in liquid medium (growth kinetics) to thoroughly study the growth pattern as consolidated in Table 1. We reasoned that pooled CRISPRi screening is likely to show most essential genes or highly vulnerable genes but may miss out on some vulnerable genes which can still be good potential candidates for drugs. Of the 45 genes that were targeted, we identified 17 genes showing growth defect (Table 1). Our results show that transformation efficiency per se is a good indicator of gene essentiality using CRISPRi.

The essentiality of genes across almost the entire mycobacterial genome was assessed by Bosch et al. and De Wet et al. [16–18], who employed pooled and arrayed libraries, to identify essential genes in *M. smegmatis*. The groups used the dCas9 system from *S. thermophilus* to target almost all essential gene homologs

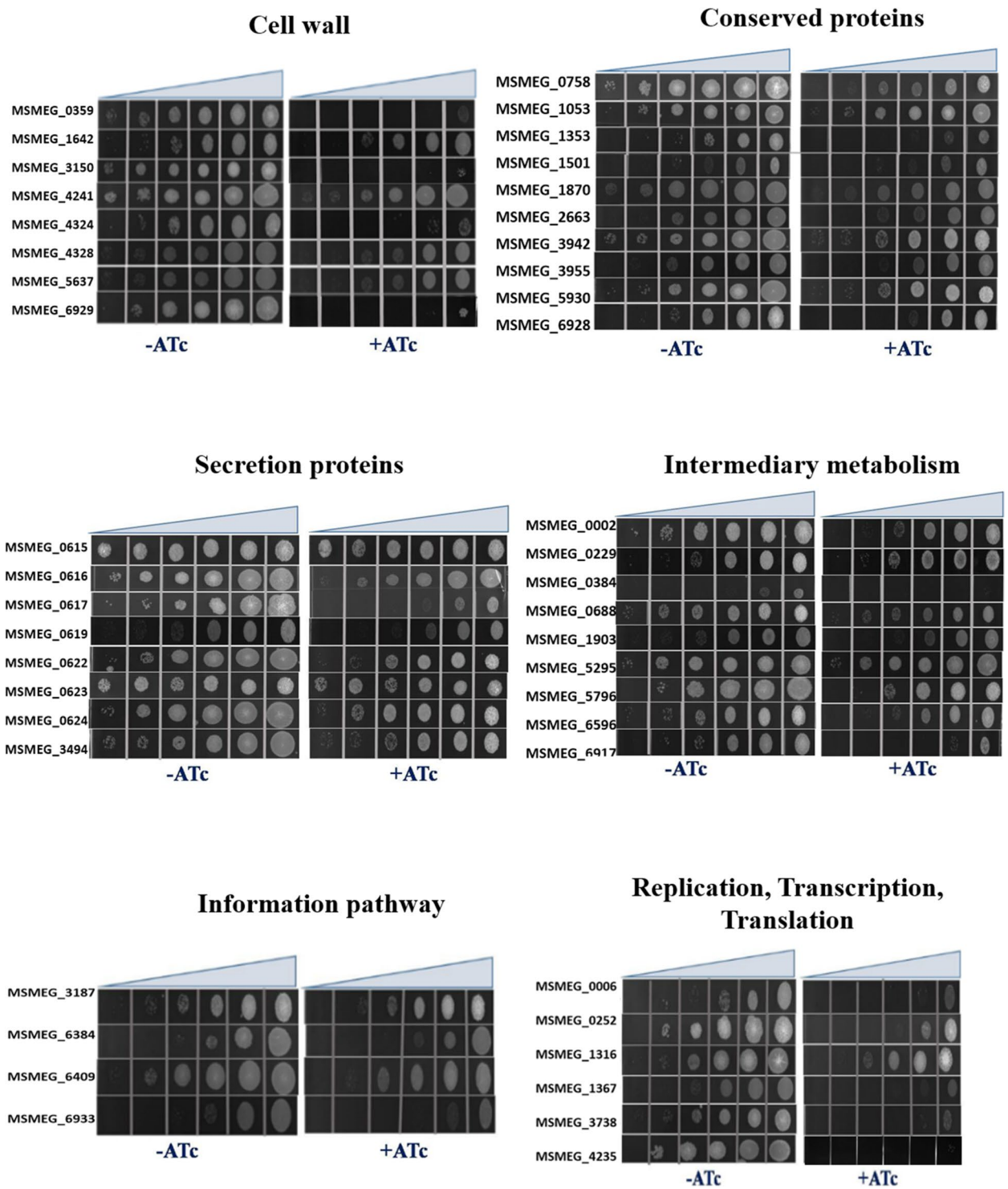

Fig. 3 Spot assays showing growth of pST-HT-cr plasmids (carrying spacers targeting indicated genes) transformants on agar plates with/without ATc. (The cultures are spotted in increasing concentrations starting from right to left)

of *M. tuberculosis* in *M. smegmatis*. The genes targeted in this study were scored in the heat map in a range of 0–3 (Fig. 5). Genes which showed vulnerability across all three assays were given a score of 3, while genes which showed growth defect in 2 or 1 assays were scored 2 and 1, respectively. Genes which did not show any defect in

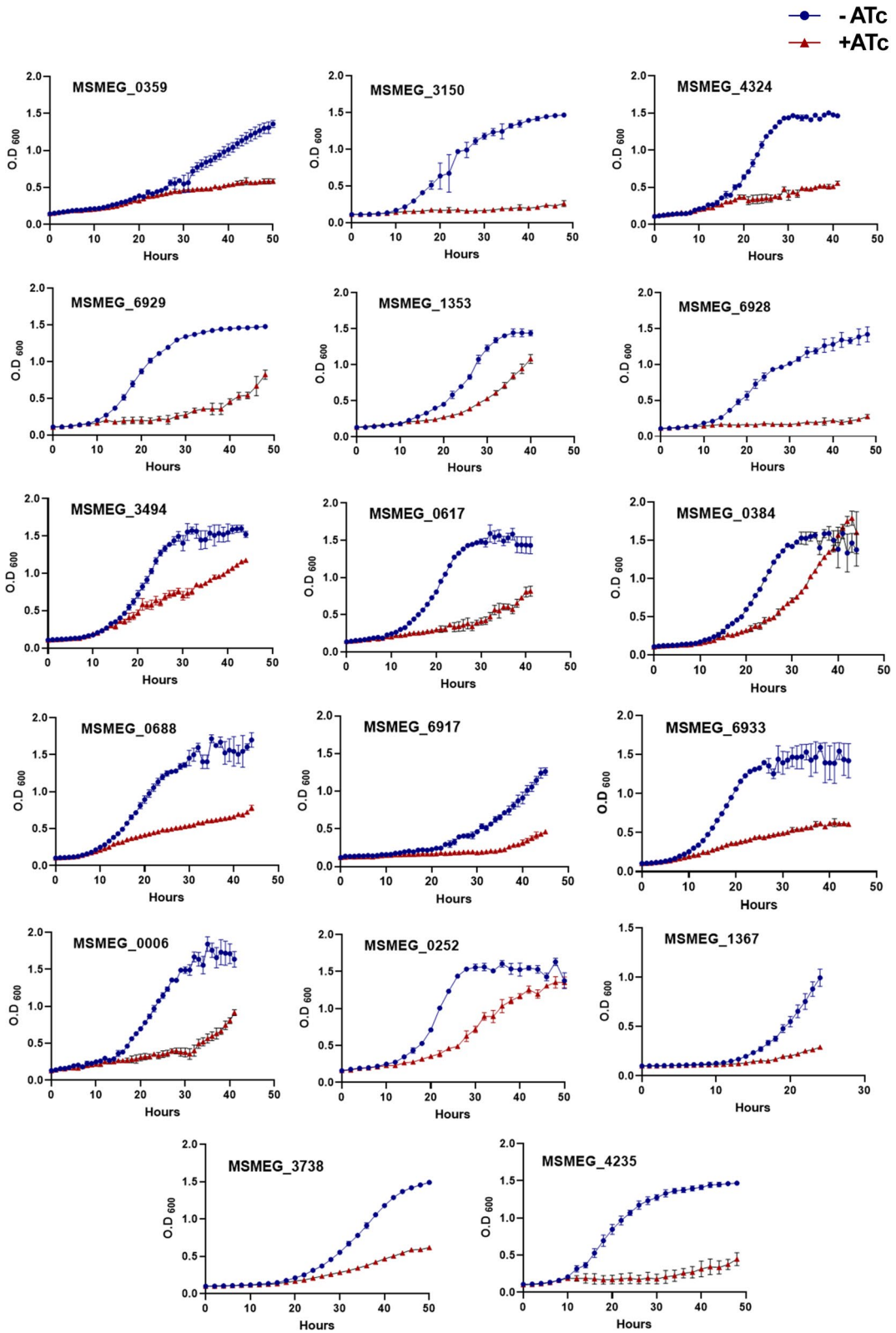

Fig. 4 Growth curve of pST-HT-cr plasmids (carrying spacers targeting indicated genes) transformants in broth in presence (red) /absence (blue) of ATc

Table 1 List of the CRISPRi phenotypic outcome of each gene in the three assays as indicated (NV- Non-vulnerable genes highlighted in green, GD- Growth defect genes highlighted in pink)

Functional Category	MSMEG_Gene ID	Transformation Efficiency	Spot Assay	Growth Curve
Cell wall	0359	GD	GD	GD
	1642	NV	NV	NV
	3150	GD	GD	GD
	4241	NV	NV	NV
	4324	GD	GD	GD
	4328	NV	NV	NV
	5637	NV	NV	NV
	6929	GD	GD	GD
Conserved proteins	0758	NV	NV	NV
	1053	NV	NV	NV
	1353	NV	GD	GD
	1501	NV	NV	NV
	1870	NV	NV	NV
	2663	NV	NV	NV
	3942	NV	NV	NV
	3955	NV	NV	NV
	5930	NV	NV	NV
	6928	GD	GD	GD
Secretory proteins	0615	NV	NV	NV
	0616	NV	NV	NV
	0617	NV	GD	GD
	0619	NV	NV	NV
	0622	NV	NV	NV
	0623	NV	NV	NV
	0624	NV	NV	NV
	3494	NV	NV	GD
Intermediary metabolism	0002	NV	NV	NV
	0229	NV	NV	NV
	0384	GD	GD	GD
	0688	NV	NV	GD
	1903	NV	NV	NV
	5295	NV	NV	NV
	5796	NV	NV	NV
	6596	NV	NV	NV
	6917	GD	GD	GD
Information Pathway	3187	NV	NV	NV
	6384	NV	NV	NV
	6409	NV	NV	NV
	6933	NV	GD	GD
ReplicationTranscription Translation	0006	GD	GD	GD
	0252	NV	GD	GD
	1316	NV	NV	NV
	1367	GD	GD	GD
	3738	GD	GD	GD
	4235	GD	GD	GD

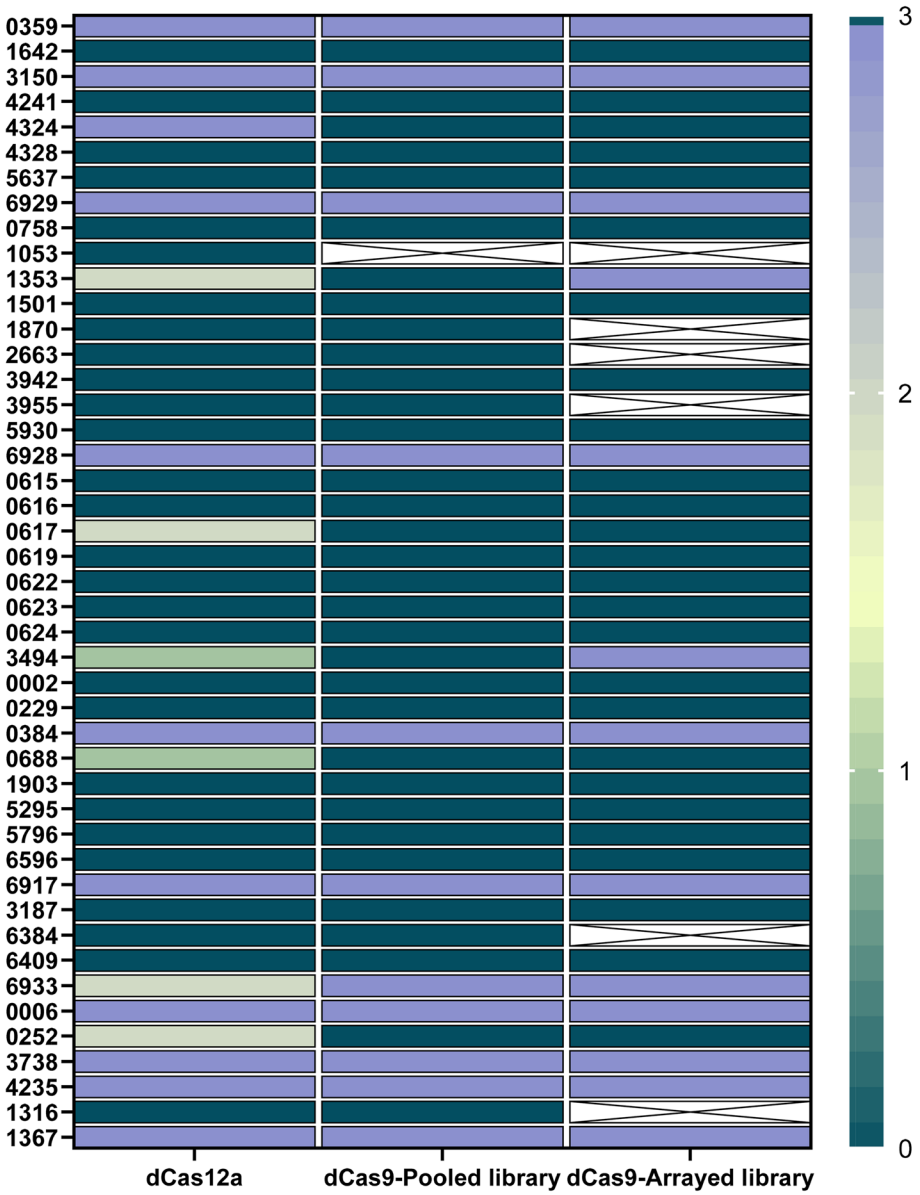

Fig. 5 Heat map showing the gene vulnerability calls. Each gene in the dCas12a CRISPRi study is given a score (0, 1, 2 or 3) based on the growth defect seen in the assays conducted. As the other two studies did not employ multiple growth assays, the scores are either 0 (non-vulnerable) or 3 (vulnerable). The white boxes with crossed symbol indicate that the given gene's final call was not available in that study

any of the assays were given a score of 0, representing non-essential genes. Out of the 45 genes tested using the dCas12a system, we could compare 44 genes that were common across one of the two groups and found that the CRISPRi/Cas12a based outcomes were largely in agreement with the calls obtained using the pooled CRISPRi screen with *S. thermophilus* dCas9 system [17] (Fig. 5). However, there were some interesting differences in a few genes. For MSMEG_4324, there was a severe drop in survival in transformation assay followed by the growth defect observed in the phenotype-based assays. However, MSMEG_4324 is annotated as non-essential by the dCas9 study. This could be owing to the difference in efficiencies of gene knockdowns between CRISPR-based systems like Cas12 and dCas9. If one system achieves only partial knockdown, sufficient residual gene activity may remain to support viability, categorizing the gene as "non-essential," whereas more efficient knockdown in the other system could render the gene "essential". MSMEG_4324, which is a *mabR* homologue

regulating the FASII operon of mycolic acid biosynthesis, is also annotated as essential in a separate study where the group could generate a knockout only in a mero-diploid background [30]. Conversely, MSMEG_6933 which is annotated as essential in the dCas9 study appeared as non-essential as per our dCas12a transformation study, but did show growth defect in subsequent spot and growth curve study (Table 1). This discrepancy could be attributed to the protein's half-life. Proteins with longer half-lives may persist and retain functionality even after gene disruption, allowing the cells to survive during the T.E. assay. Despite this, a growth defect was still evident in terms of colony size on the T.E. plate, highlighting that residual protein activity can sustain survival but not completely mitigate functional impairments, which become apparent in growth-related observations. Additionally, there were five genes that behaved differently in transformation survival vs growth kinetics. Three of these genes (MSMEG_0252, MSMEG_0617, and MSMEG_1353) which were annotated as non-essential by Bosch et al. [17] yielded fitness defect using dCas12a system in growth curves and spot assays. Among these, MSMEG_1353 is annotated as essential by De Wet et al. (Fig. 5). On the other hand, the remaining two genes (MSMEG_0688 and MSMEG_3494) showed distinct growth defects upon knocking down only in broth conditions. MSMEG_3494 is annotated as essential by the dCas9 arrayed library [18] and MSMEG_0688 is annotated as essential in a separate transposon study [28]. While for the rest of the genes, essentiality was unambiguous with distinct growth defect discernible on solid medium as well as in broth conditions occurring in transformants recovered at lower efficiencies.

The already established *S. thermophiles* (Sth) dCas9 which utilizes an identified PAM consensus sequence of NNAGAAW, provides only limited targeting opportunities, especially in GC-rich mycobacterial genomes. However, recent research has shown that $Cas9_{Sth1}$ can tolerate variations from this consensus sequence [31]. Further, an extensive study elucidating the full spectrum of PAMs that can be recognized by $dCas9_{Sth1}$ in mycobacteria has been reported [14]. The dCas12a system from *F. novocida*, developed in this study, offers some advantages over the dCas9 *S. thermophilus* system. *F. novocida* dCas12a uses a smaller PAM ("TTN") as compared to the *S. thermophilus* dCas9 (NNAGAAW). The T-rich PAM sequence is believed to mitigate any associated toxicity to the cell [32]. *F. novocida* dCas12a has a smaller protein size, making it more suitable for cloning and expression. Also, its lack of requirement for a tracrRNA simplifies cloning while the ability to process the sgRNA array facilitates targeting of multiple genes. Additionally, this study demonstrates that CRISPRi with the dCas12a system is a robust and a versatile tool for identifying essential genes as well as studying them further for functional studies. Compared to the established dCas9 system where different PAMs show varying CRISPRi efficiency thus requiring standardisation of PAMs, dCas12a offers easier spacer design as all the PAMs showing efficacy similar efficiencies for CRISPRi, simplifying the process of targeting multiple genes.

Conclusion

Our results showed that, even with a subset of just 45 genes, there were interesting differences between dCas12a-CRISPRi and dCas9-based CRISPRi screens. Therefore, this study underscores the significance of probing growth in mutants and knockdowns, individually, to assess fitness defects, as high-throughput pooled sequencing studies that quantify spacer depletion may miss essential genes for reasons that are not immediately clear. Compared to the already established *S. thermophilus* based dCas9 silencing, dCas12a offers hassle-free designing of single spacer with a single PAM. Overall, we show that CRISPRi with dCas12a system is robust, versatile and an easy-to-use tool in identifying essential genes as well as carry out further functional studies.

Materials and methods

Strain construction

The dead Cas12a (dCas12a) system for gene repression using CRISPR technology in *Mycobacterium smegmatis* was constructed as follows: The Cas12a gene from *Francisella novicida* codon optimised for *Corynebacterium glutamicum* and with a D917A mutation was synthesized by GenScript and then cloned into the integrative vector pSTKiT at KpnI and HindIII restriction sites. The plasmid was electroporated (using standard protocol as described in [20]) in *M. smegmatis* which was incorporated in the genome at L5 *attB* site. The integration was confirmed by antibiotic selection. For construction of pST-HT-cr, pSTKT [23] was used as the backbone plasmid and a synthesised cassette comprising of hygromycin resistant gene and crRNA under Tet promoter was cloned at KpnI and SpeI sites. Depending on the target gene, a 20-nucleotide spacer sequence was introduced into pST-HT-cr using Golden Gate BsaI cloning. Spacers were carefully designed to target 20 nucleotide region immediately following the start codon of non-template strand. The spacer sequences targeting each gene were incorporated with complementary oligonucleotides. These oligonucleotides were designed to generate TAGA overhang at 5' end (top strand) and CA overhang at 3' end (bottom strand) upon annealing. The synthesized spacers were then annealed in a 20 µl reaction containing TRIS buffer pH-8 (10 mMTris HCL, 50 mM NaCl and 1 mM EDTA)

by gradual cooling from 94 °C for 4 min, 75 °C for 5 min, 65 °C for 15 min to 25 °C for 20 min. The annealed oligos were diluted 10 times and ligated into pST-HT using BsaI Golden Gate assembly facilitating digestion and ligation in the same tube. An aliquot (4 µL) of the spacer and pST-HT-cr mixture was used to transform *E. coli* DH5alpha. The resulting constructs were sequenced to verify their correct assembly before being transformed into *Mycobacterium smegmatis* strains already possessing the pSTKiT-dCas12. Finally, these transformed strains were selected on plates containing hygromycin (50 µg/ml) and kanamycin (20 µg/ml), and also ATc (anhydrotetracycline- 100 ng/ml) as required for induction of CRISPRi.

qPCR

Mycobacterium strains grown to OD_{600nm} of 3–5 were harvested by centrifugation at 2000 g for 10 min. The resulting pellet was suspended in 1 ml of TRIzol and transferred to screw-capped tubes containing lysing matrix for homogenization. These tubes, containing the cell lysate, underwent pulsed agitation in an MP Biomed bead beater (FastPrep-25 5G) at 6 m/sec for 45 s, repeated for 2 cycles with a 2-min ice-cooling step between cycles. Subsequently, the lysate with matrix beads was centrifuged at 8000 g, and the supernatant was carefully transferred to a fresh tube. RNA isolation from the lysate was performed using the Zymo Research RNA zol kit, following the manufacturer's instructions. The eluted RNA was treated with TURBO™ DNase for 1 h at 37 °C to eliminate residual DNA, and then column purified using the NEB Monarch DNA clean-up kit. The purified RNA (1 µg) was used to synthesize cDNA using random hexamers with the Verso cDNA Kit (Thermo Fisher Scientific), following the manufacturer's protocol. This cDNA was used as template for qPCR with RNA quantification carried out using SYBR Green chemistry. To normalize gene expression, it was referenced to *sigA* (MSMEG_2758) and quantified utilizing the ΔΔCt algorithm [33]. Each experiment included appropriate controls, such as reverse transcriptase and template-negative controls, as well as genomic DNA-positive controls. Primers were designed using the OligoAnalyzer™ Tool (IDT) and are listed in Table S1.

Western blot

Mycobacterium cells cultured in the presence of ATc were harvested during the logarithmic growth phase (OD_{600nm}). The cells were then washed with phosphate buffered saline (PBS, 0.1 M) and lysed using PBS containing 1% SDS in an MB Biomed Fast Prep homogenizer. After a brief centrifugation at 10,000 g, the lysate was separated from the matrix. Protein quantification was performed using the Bradford assay, and 60 µg of protein was loaded onto 4–12% acrylamide gels for SDS-PAGE. Following electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membrane was equilibrated in transfer buffer (25 mM Tris, 193 mM glycine, 20% ethanol) and placed in a transfer cassette. Proteins were electrophoretically transferred at a constant voltage of 60 V for 45 min using a Bio-Rad Transfer System. Subsequently, the membrane was blocked overnight at 4 °C in 5% dry milk in tris-buffered saline containing Tween 20 (TBST, pH 7.6; 50 mM Tris, 0.5 M NaCl, 0.02% Tween 20). Anti-Cas12a antibody from Genscript was used at a 1:1000 dilution in 2% dry milk in TBST and incubated with the membrane overnight at 4 °C. The membrane was washed three times for 15 min each with TBST, followed by treatment with an ECL based chemiluminescent reagent (Thermo Fisher Scientific). Chemiluminescence was detected using a ChemiDoc Imaging system (Bio-Rad).

Growth curve

Cells from colonies obtained upon transformation using plasmids targeting different genes, were cultured by growing them in Middlebrook broth supplemented with hygromycin (50 µg/ml) and kanamycin (20 µg/ml) for a period of two days until they reached saturation. Subsequently, these strains were sub-cultured at an optical density (OD_{600nm}) of 0.02 in 1 mL of Middlebrook broth containing antibiotics alone (hygromycin and kanamycin) in one batch, and both antibiotics and inducer (hygromycin, kanamycin and anhydrotetracycline) in another batch. 200 µL of each strain was dispensed in triplicate into a 96-well plate. The plate was then placed in a Tecan Max Pro plate reader, and absorbance measurements were taken at approximately 2-h intervals over a period of around 48 h or until the culture reached saturation.

Spot assay

The control and knockdown strains were cultivated in Middlebrook broth supplemented with hygromycin (50 µg/ml) and kanamycin (20 µg/ml) for two days until the cultures reached saturating optical density. Subsequently, the cultures were diluted in Middlebrook medium to a final concentration of 1,000,000 cells per millilitre. To assess their growth characteristics, these cultures were serially diluted and 5 µL aliquots were spotted onto Middlebrook agar plates containing hygromycin and kanamycin (H/K) with (H/K/ATc) or without anhydrotetracycline.

These plates were then incubated for a duration of 2 to 3 days at 37 °C, allowing the bacterial colonies to grow before recording images on a ChemiDoc imaging system (Bio-Rad).

Isoniazid resistance assay

A knockdown strain targeting the *katG* was successfully constructed using the protocol described above. To evaluate its response to different concentrations of isoniazid (INH), the growth of the knockdown strain was monitored in presence of various INH concentrations. The growth media contained the antibiotics- hygromycin and kanamycin and the inducer- ATc. The samples were prepared in triplicate and dispensed into a 96-well plate. The growth of the knockdown strain was monitored by measuring absorbance at regular intervals using a plate reader. The resulting data was plotted as optical density (OD_{600nm}) against time.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13036-025-00490-3.

Supplementary Material 1.

Acknowledgements
Authors thank Snehalata Desai for the technical help in conducting experiments. pSTKiT and pST-KT was a kind gift from Dr. Vinay K. Nandicoori.

Authors' contributions
MS- Methodology, Data Analysis, and Manuscript Writing. CSM- Data Analysis, Manuscript Editing. SPK- Data analysis, Supervision. DR- Conceptualisation, Data Analysis, and Manuscript Finalisation. All authors have read and approved the final manuscript.

Funding
Present study was supported by institutional funding from Bhabha Atomic Research Centre, Department of Atomic Energy (DAE), Mumbai, India.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 9 August 2024 Accepted: 14 February 2025
Published online: 28 February 2025

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