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Simple and reliable in situ CRISPR-Cas9 nuclease visualization tool is ensuring efficient editing in *Streptomyces* species

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ABSTRACT

CRISPR-Cas9 technology has emerged as a promising tool for genetic engineering of *Streptomyces* strains. However, in practice, numerous technical hurdles have yet to be overcome when developing robust editing procedures. Here, we developed an extension of the CRISPR-Cas toolbox, a simple and reliable *cas9* monitoring tool with transcriptional fusion of *cas9* nuclease to a beta glucuronidase (*gusA*) visual reporter gene. The Cas9-SD-GusA tool enables in situ identification of cells expressing Cas9 nuclease following the introduction of the plasmid carrying the CRISPR-Cas9 machinery. Remarkably, when the Cas9-SD-GusA system was applied under optimal conditions, 100% of the colonies displaying GusA activity carried the target genotype. In contrast, it was shown that the *cas9* sequence had undergone major recombination events in the colonies that did not exhibit GusA activity, giving rise to "escaper colonies" carrying unedited genotype. Our approach allows a simple detection of "escaper" phenotype and serves as an efficient CRISPR-Cas9 optimisation tool.

1. Introduction

Streptomyces species and taxonomically closely related bacteria have been one of the most abundant sources of biologically active drugs for over 70 years (Myronovskyi and Luzhetskyy, 2016; Horbal et al., 2018; Tong et al., 2020). To further exploit this potential, it is of key importance to develop advanced gene tools, since *Streptomyces* species are known to be difficult to engineer (Paradkar et al., 2003; Mitousis et al., 2020). Despite their morphological and physiological diversity, CRISPR-Cas technology has emerged as a promising tool for the genetic engineering of *Streptomyces* strains (Lee et al., 2019). The first CRISPR tools for *Streptomyces* were reported in 2015 by four independent research groups (Cobb et al., 2015; Huang et al., 2015; Tong et al., 2015; Zeng et al., 2015; Zhao et al., 2020). Since then, the use of CRISPR-Cas tools in *Streptomyces* species has grown significantly (Alberti and Corre, 2019). However, in practice, a number of technical hurdles must still be overcome when attempting to develop efficient and robust CRISPR-Cas procedures for any bacterial species (Ye et al., 2020). The CRISPR system is preferentially delivered to *Streptomyces* species via high copy-number plasmids (Wang et al., 2020; Wlodek et al., 2017), and considering that Cas9 nuclease can represent a serious burden to the host, the reproducibility of CRISPR-Cas9 editing methods is often very low. For this reason, when developing a CRISPR-Cas tool for any *Streptomyces* species, it is important to evaluate the Cas9 expression level from the selected plasmid/promoter, thus ensuring efficient editing without excessive toxicity to the host. Classically, a set of transformations with control plasmid constructs is performed when the CRISPR plasmid is introduced into *Streptomyces*. The toxicity of active Cas9 nuclease to the host can be evaluated indirectly based on the substantial difference that exists between the number of viable transformants that are obtained when transforming the culture with the control plasmid (usually empty plasmid vector or vector lacking gRNAs) and those with an active Cas9-gRNA plasmid vector (Huang et al., 2015; Ye et al., 2020; Zhang et al., 2020; Jiang et al., 2017). However, the indirect monitoring of Cas9 activity based on a reduced number of viable bacterial colonies is a work-intensive and time-consuming approach.

Recently, the use of CRISPR-Cas technology has also been reported in *Streptomyces rimosus* (Jia et al., 2017), which has been used for oxytetracycline (OTC) production for over 70 years (Petković et al., 2006). We aimed to introduce this technology in our laboratory, however, we observed low efficacy of CRISPR-Cas9 in *S. rimosus*. We also observed frequent recombination events when pIJ101-based high copy-number

Abbreviations: SD, Shine-Dalgarno sequence; X-Gluc, 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid; OTC, oxytetracycline; *StAp*, secretory tripeptidyl aminopeptidase gene; CRISPR, clustered regularly interspaced short palindromic repeats.

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plasmid was used as delivery vector for CRISPR-Cas9 in *S. rimosus*. Therefore, to better understand the underlying cause of the low reproducibility of the CRISPR-Cas9-based editing method in *S. rimosus* and to improve its robustness, we assessed the expression of the *cas9* gene by applying the GusA reporter system, a visually quantifiable marker encoding beta-glucuronidase, which is commonly used in *Streptomyces* species (Lee et al., 2019; Wang et al., 2020; Jefferson, 1989; Myronovskyi et al., 2011; Siegl et al., 2013). We constructed a plasmid composed of the *cas9* gene that is transcriptionally coupled to a promotorless *gusA* gene, thereby ensuring the correlation of the colour signal with *cas9* transcription. As a model system and to evaluate the efficacy and robustness of the developed method, we carried out the deletion of the *stAp* gene, encoding a secretory tripeptidyl aminopeptidase (StAp), which was identified in our laboratory as one of the most abundant extracellular proteins in *S. rimosus*. Remarkably, when the Cas9-SD-GusA system was applied, 100% of the colonies displaying GusA activity showed the correct genotype, thus demonstrating the high reproducibility of the developed method. Therefore, we have proven that this approach can serve as an efficient CRISPR-Cas9 optimisation tool.

2. Materials and methods

2.1. Bacterial strains, plasmids and cultivation methods

The bacterial strains and plasmids used in the present study are listed in Table S1. *E. coli* DH10β cells were used for cloning. *E. coli* ET12567/pUB307 strain was used for conjugal transfer of plasmids to *S. rimosus* strain. We used *Streptomyces rimosus* strain ATTC 10970 (NRRL 2234) from the American Type Culture Collection (R7 in most publications) (Pethick et al., 2013) with a deletion of the *otc* gene cluster, designated as ATCCΔ*otc* (Pikl et al., 2021). *S. rimosus* ATCCΔ*otc* colonies appeared completely white on MS agar plates, which simplifies the observation of the GusA phenotype. *E. coli* strains were grown in 2TY (yeast extract-tryptone) medium and cultivated at 28 °C. *S. rimosus* was cultivated at 28 °C on MS agar medium or in liquid TSB (Kieser et al., 2000) for total genomic DNA isolation. *E. coli* cultures were supplemented with 100 μg/mL apramycin (apr) and 50 μg/mL kanamycin (kan) when required. 30 μg/mL of thiostrepton (tio) was used for the selection of *S. rimosus*. After conjugation, *E. coli* was eliminated using 35 μg/mL nalidixic acid (nal). 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc), used for visualization of GusA activity, was supplied by X-gluc Direct (Spain). A stock solution (0,5 M) of X-gluc was prepared by diluting the appropriate amount of X-gluc in DMSO. When adding X-gluc directly into MS agar plates, we used a final concentration of 4 mM. Alternatively, to enhance the intensity of GusA visualization, MS agar plates were overlaid with 4 mL of 30 mM X-gluc solution.

2.2. Cas9-SD-GusA screening system construction

Primers (Table S2) were supplied by Integrated DNA Technologies (IDT, USA) and contained 22–45 bp overlapping regions to enable homology-based cloning procedures. We used the repliQa HiFi ToughMix DNA polymerase mix (Quantabio, USA) for all PCR amplifications. Genomic DNA was isolated using the peqGOLD Bacterial DNA Isolation Kit (VWR, USA). Plasmid isolation was performed using an EZNA Plasmid DNA Mini Kit (Omega Bio-tek, USA). Plasmid constructs and graphics used in this study were designed using Geneious R10 and R11.1.5 software (https://www.geneious.com).

To allow efficient selection of the plasmid in *S. rimosus*, we first introduced a thiostrepton resistance cassette (*tsr*) into the pREP_P1_cas9 plasmid (Fig. 1A), which was kindly provided by Novartis/Lek (Mrak et al., 2018). The tsr cassette was amplified from the plasmid pAB04 (Carrillo Rincón et al., 2018) and inserted, using a T4 DNA ligase (Thermo Fischer Scintific, USA), into a pREP_P1_cas9 plasmid digested with *BspOI* and *ClaI* (FastDigest, Thermo Fischer Scintific, USA) (Fig. 1A), thus generating the plasmid construct pREP_P1_cas9_tsr (Fig. 1B). pREP_P1_cas9_tsr was linearised with *SpeI* (FastDigest, Thermo Fischer Scintific, USA) (Fig. 1B) to allow for further insertions. The region containing the *gusA* sequence was amplified from plasmid pSET-GUS (Myronovskyi et al., 2011). The Shine-Dalgarno sequence (SD), identical to the SD sequence at the *cas9* transcription start site, was introduced upstream of the *gusA* start codon using the SD-GusA-Fw primer. Due to the small size of the *stAp* gene deletion (1422 bp), we designed a single sgRNA that targeted the 5′ side of *stAp* coding sequence using Geneious R10 software (Fig. 3C). The gRNA cassette was synthesised by ATG Biosynthetics GmbH (Germany). Then, a 20 bp-gRNA region targeting *stAp* was introduced into the *NcoI*-digested (FastDigest, Thermo Fischer Scintific, USA) gRNA cassette as ssDNA with the NEBuilder reaction (New England Biolabs, USA), similarly to what has been described by Tong et al. (Tong et al., 2019), and PCR amplified (Fig. 1D). This approach allows for fast replacement of the targeted sequence. Upstream (UP, 1121 bp) and downstream (DOWN, 1094 bp) homology regions that begin in close proximity to the *stAp* gene (Fig. 3C)

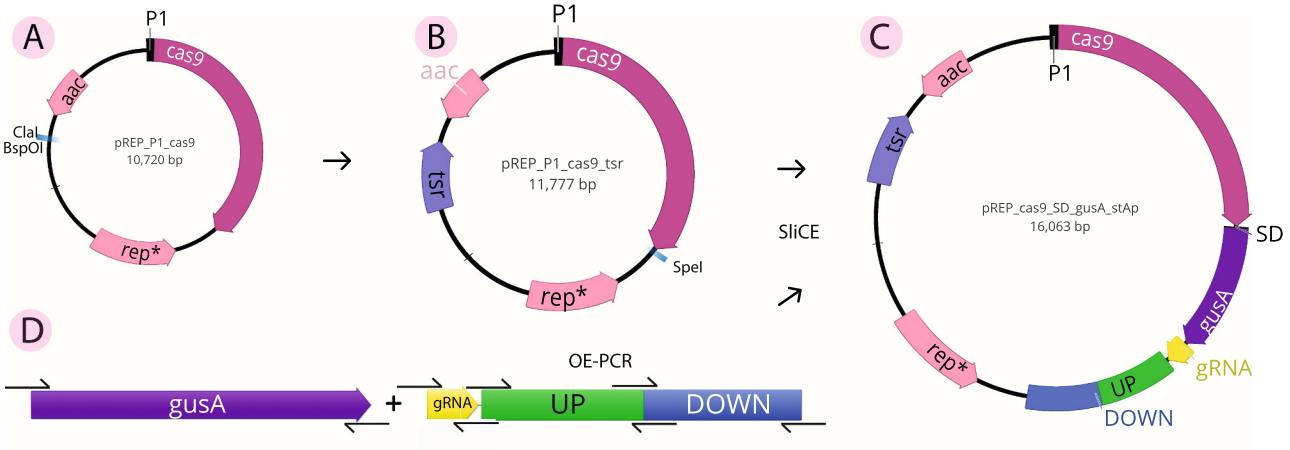

Fig. 1. A. pREP_P1_cas9 plasmid (Mrak et al., 2018) with marked *ClaI* and *BspOI* restriction sites. **B.** Plasmid construct pREP_P1_cas9_tsr with an inserted thiostrepton resistance cassette (*tsr*) and marked *SpeI* restriction site. **C.** Final plasmid construct pREP_cas9_SD_gusA_stAp with gusA, gRNA part and homology regions stAp_UP (UP) - stAP_DOWN (DOWN) inserted into a *SpeI* restriction site. **D.** Homology based cloning strategy used in pREP_cas9_SD_gusA_stAp plasmid assembly; gRNA, stAp_UP and stAp DOWN parts were first joined with OE-PCR. Then, the OE-PCR amplicon was joined with a gusA PCR fragment and *SpeI* digested pREP_P1_cas9_tsr plasmid to construct pREP_cas9_SD_gusA_stAp.

were amplified from the genomic DNA of *S. rimosus* ATCC 10970Δ*otc*. Because assembling many fragments is always a challenge, we sought to minimise the number of fragments that need to be assembled in a single reaction. This was achieved by combining the PCR method of overlap extension (OE-PCR) (Nelson and Fitch, 2011), followed by a final assembly using a homology-based cloning technique, SLiCE cloning (Zhang et al., 2012). The stAp_UP homology, stAp_DOWN homology, and stAp_gRNA were first joined into an OE-PCR fragment (2625 bp) (Fig. 1D). The OE PCR reaction consisted of equimolar amounts of each part, initially run for 10 cycles without primers. After the first step, the external primers (stAp_gRNA_Fw and stAp DOWN_Rw) were added to the reaction and run for another 30 cycles (Fig. 1D). The stAp OE-PCR fragment was then joined to the gusA PCR fragment and *SpeI*-linearized pREP_P1_cas9_tsr in a SLiCE reaction to obtain the final plasmid construct pREP_cas9_SD_gusA_stAp (Fig. 1C).

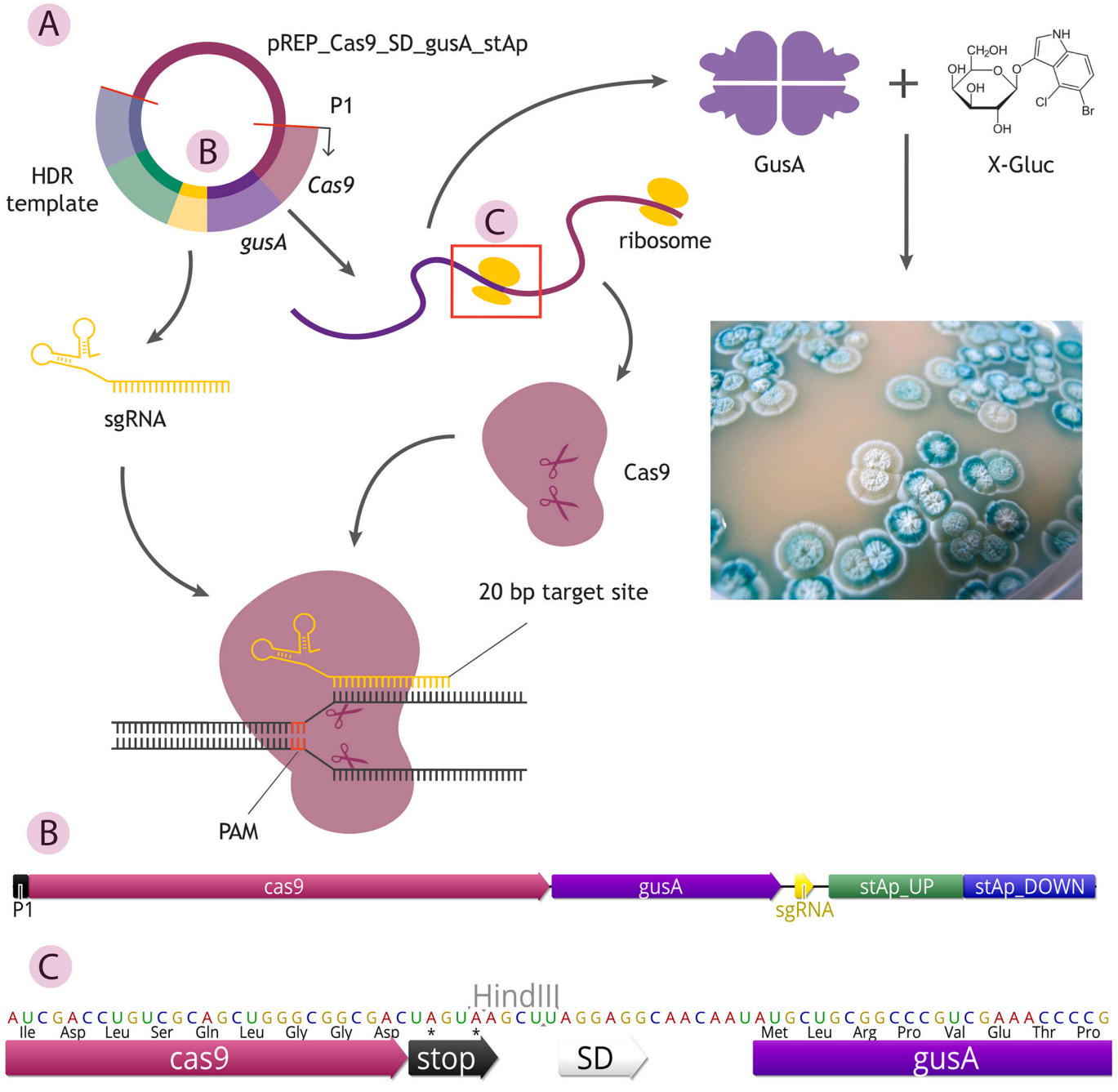

Fig. 2. A. Design of the Cas9-SD-GusA system for the co-transcriptional evaluation of Cas9 expression with GusA. Following transcription from the P1 promoter, Cas9 and GusA enzymes are simultaneously translated from a single mRNA. After translation and subsequent protein folding Cas9-sgRNA complex binds to the target sequence and creates a double-stranded break inside the target gene (*stAp* gene). The simultaneously translated GusA enzyme catalyses the conversion of the X-gluc chromogenic substrate (separately added to medium), yielding blue-coloured colonies. The double-stranded break is then repaired via homology directed repair (HDR), facilitated by the HDR template, also supplied by the plasmid. **B.** Relevant part of the pREP_cas9_SD_gusA_stAp plasmid; the c*as9-SD-gusA* transcription unit is followed by a sgRNA unit and a HDR template – stAp_UP and stAp DOWN homology regions. **C.** *cas9-SD-gusA* junction on mRNA; the *gusA* gene is out of the reading frame in relation to the upstream gene *cas9*. These two genes are also separated by two stop codons, a *HindIII* restriction site and a Shine-Dalgarno sequence (SD) – identical to SD, constituting the *P1* promoter. HDR template: homology directed repair template; PAM: protospacer adjacent motif; sgRNA: single guide RNA.

2.3. Microbiological methods

2.3.1. Intergeneric conjugation and screening of exconjugants

Conjugation was performed to introduce CRISPR-Cas9 carrier plasmids into the recipient *S. rimosus* ATCC Δ*otc*. pREP_cas9_SD_gusA_stAp was first electroporated into *E. coli* ET12567/pUB307 (Flett et al., 1997). The plasmids were then transferred to *S. rimosus* using the classical *E. coli* - *Streptomyces* conjugation method (Kieser et al., 2000; Flett et al., 1997). The conjugation mixture was spread onto MS agar plates supplemented with 10 mM $MgCl_2$ and 10 mM $CaCl_2$. After 8 h of incubation at 28 °C, plates were overlaid with 2 mL aqueous solution of tio + nal at a final concentration of 30 μg/mL. After 6 days of incubation at 28 °C, emerging exconjugants (Fig. S1) were patched onto fresh MS + tio + nal plates.

2.3.2. Identification of colonies with successful deletion of stAp gene

Visual detection of GusA activity was carried out by adding X-gluc directly to MS plates to a final concentration of 4 mM or by overlaying cultures after a 6-day incubation period with 4 mL of a 30 mM X-gluc solution (Fig. 3A). Eight exconjugants that displayed blue colouration were selected for verification of *stAp* gene deletion. We selected 10 exconjugants that did not display blue colouration after growth on MS + tio + X-gluc plates for further investigation. Genomic DNA was isolated from both groups of exconjugants, and PCR was performed with primers ΔstAp_Fw and ΔstAp_Rw to confirm *stAp* deletion (Fig. 3). The plasmid construct pREP_cas9_SD_gusA_stAp was not cleared from *S. rimosus* cultures before PCR was performed. Therefore, both primers for the conformation of *stAp* deletion were designed to anneal outside the homology regions (Fig. 3C).

2.3.3. *S. rimosus* – *E. coli* plasmid rescue experiment

We analysed the integrity of the plasmid constructs obtained directly from the exconjugants. Genomic DNA was isolated from *S. rimosus* colonies with confirmed *stAp* deletion. These colonies showed GusA activity and were termed "blue colonies". Genomic DNA was also isolated from colonies in which *stAp* deletion could not be confirmed by PCR. These colonies showed no GusA activity and where thus termed "white colonies" (Fig. 3A). Electrocompetent *E. coli* DH10β were transformed with 1 μl of each total DNA isolate from "blue" and "white" colonies and spread onto 2TY + apr agar plates. Single *E. coli* colonies that appeared after 24 h of incubation at 28 °C were inoculated into 2TY + apr liquid medium and incubated for 16 h before plasmid isolation.

3. Results

3.1. Construction and use of the pREP_cas9_SD_gusA_stAp plasmid for co-transcriptional screening

Our CRISPR system (Fig. 2A) is based on the plasmid pREP_P1_WT_cas9 (Mrak et al., 2018), containing a pTJU412 (pIJ101-derived) origin of replication (Sun et al., 2009), a codon-optimised version of the *Streptococcus pyogenes* Cas9 coding sequence, and the theophylline inducible-ErmEp1-based promoter P1 (Myronovskyi et al., 2016). The pREP_P1_cas9 plasmid contained TraJ and oriT for transfer functions and an apramycin resistance marker. sgRNA expression is under the control of a synthetic P21 promoter (Myronovskyi et al., 2016) and the native *S. pyogenes* trans-activating CRISPR RNA terminator (Mrak et al., 2018). For direct chromogenic monitoring of *cas9* transcription, we constructed the genetic element Cas9-SD-GusA (Fig. 2C), where the *cas9* gene is followed by two stop codons, an inserted *HindIII* restriction site enabling further modifications, and a 13 bp part of the P1 promoter containing the Shine-Dalgarno (SD) sequence. To prevent the translation of a fused Cas9 - GusA dual protein, the start codon of *gusA* (ATG) is transcribed in a reading frame different from that of the *cas9* gene. The transcription of Cas9-SD-GusA creates a single mRNA that is translated into two proteins via two ribosome-binding sites (Fig. 2C). According to this strategy, translation should occur simultaneously, but independently, for Cas9 and GusA. Therefore, when the GusA substrate X-gluc is added directly to MS plates, colonies expressing Cas9 nuclease should also produce a blue colour (Fig. 3A). In our model system, *stAp* gene was targeted (*S. rimosus* ATCC 10970 genome: 6064838 bp–6066259 bp; Slemc et al., 2022), which encodes one of the extracellular peptidases responsible for degrading peptides involved in cell wall rearrangements in *Streptomyces* (Butler et al., *1995*).

3.2. In situ monitoring of Cas9 expression and confirmation of the stAp deletion

After completion of the *E. coli* ET/pUB307 - *S. rimosus* ATCC 10970Δ*otc* conjugation procedure (Fig. S1), exconjugants which

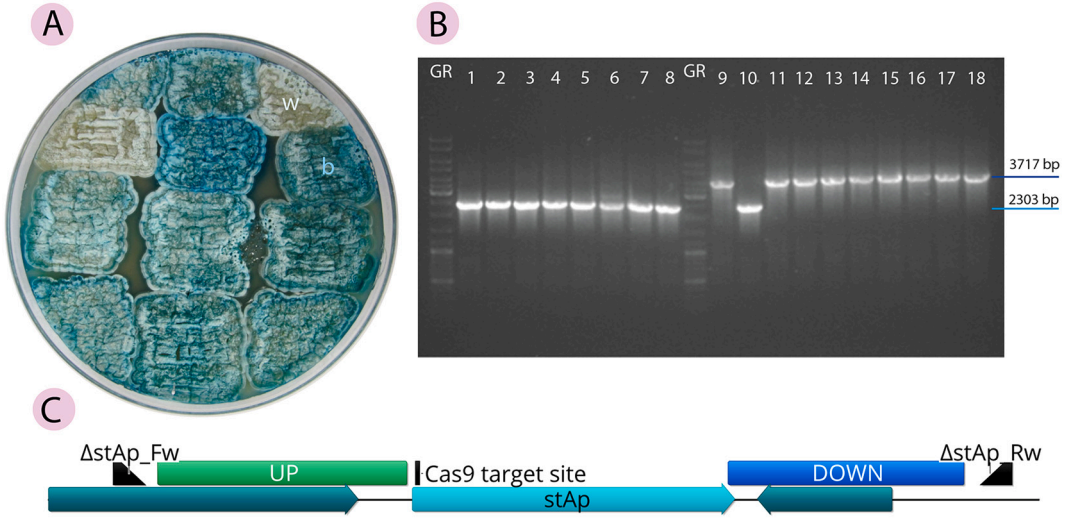

Fig. 3. A. *S. rimosus* ATCC 10970Δ*otc*/pRep_P1_cas9_SD_gusA_stAp exconjugant patches on MS agar plates overlaid with a X-gluc solution. Most of the patches displayed blue colour (b), except two that were not coloured – "white" phenotype (w). **B.** Gel electrophoresis of PCR-amplified stAp region from genomic DNA of "blue" (lanes 1–8) and "white" (lanes 9–18) *S. rimosus* colonies transformed with pREP_cas9_ SD _gusA_stAp plasmid. All tested "blue" colonies of *S. rimosus* transformed with pREP_cas9_ SD _gusA_stAp plasmid displayed the Δ*stAp* genotype (Samples 1–8) with expected band size of 2303 bp. In contrast, and with one exception, all "white" colonies displayed a WT genotype with a band size of 3717 bp. **C.** Targeted genomic region containing the *stAp* gene; the PCR reaction was performed by the set of primers cPCR_stAp_FW and cPCR_stAp_RW.

appeared on MS plates were patched onto fresh MS + tio + nal agar plates. When overlaid with X-gluc, numerous exconjugants developed blue colouring and could be clearly distinguished from those which remained uncoloured (Fig. 3A). Colonies with both blue and white phenotype were selected for further evaluation and therefore patched to selective SM medium, supplemented with X-gluc. All patched *S. rimosus* ATCCΔ*otc*/pRep_P1_cas9_SD_gusA_stAp exconjugants (Fig. 3A) were analysed for targeted deletion of the *stAp* gene via PCR with DNA isolated from individual patches. As shown in Fig. 3B, all the *S. rimosus*/pRep_P1_cas9_SD_gusA_stAp colonies displaying a blue-coloured phenotype also showed the expected PCR amplicon (2303 bp), thus confirming the deletion of the *stAp* gene. In contrast, only a single uncolored *S. rimosus*/pRep_P1_cas9_SD_gusA_stAp colony appeared to have the desired Δ*stAp* genotype, whereas the other nine colonies displayed a WT genotype (3717 bp).

3.3. Identification of plasmid recombination events by the Cas9-SD-GusA system

As spontaneous resistance to thiostrepton never occurs in *S. rimosus*, "white colonies" were subjected to further analysis. Using a plasmid rescue approach (Methods; 2.3.3), plasmid DNA was isolated and subsequently digested with the *PstI* restriction enzyme, which cuts the plasmid into DNA fragments of distinguishable sizes (Fig. 4B). As shown in the restriction analysis (Fig. 4A), the DNA fragment of the plasmid DNA encoding most of Cas9, GusA, gRNA, and the entire DNA sequence

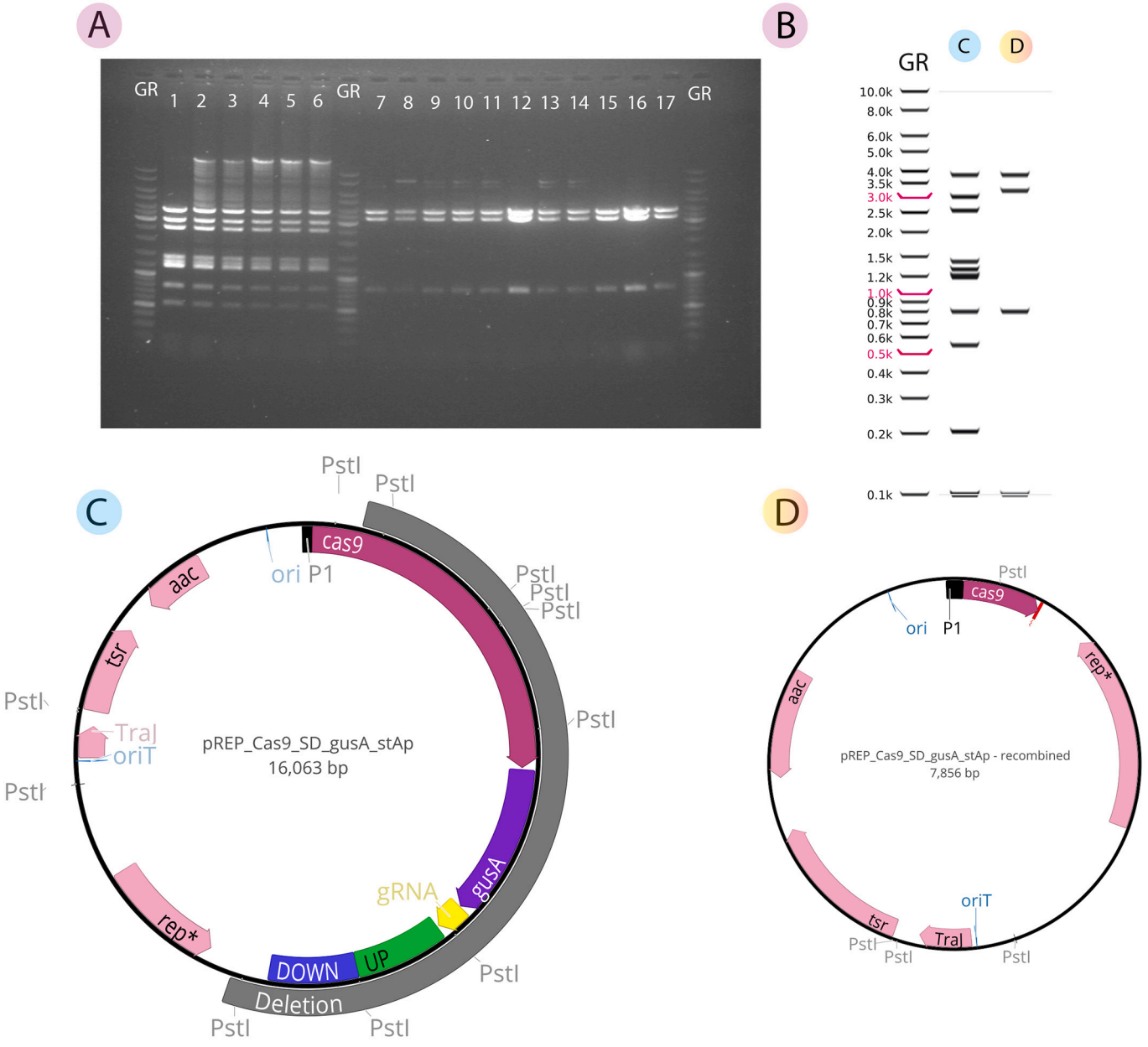

Fig. 4. A. Restriction analysis of plasmid DNA isolated via a plasmid rescue approach from *S. rimosus* exconjugants transformed with pREP_cas9_SD_gusA_stAp. Lanes 1–6: *PstI* restriction of pDNA isolated from 6 independent "blue" colonies exhibiting GusA activity and with confirmed deletion of the *stAp* gene. Lanes 7–17: *PstI* digest of pDNA isolated from 10 "white" colonies not exhibiting GusA activity and without *stAp* deletion – WT genotype. **B.** Predicted restriction pattern after *PstI* digestion of plasmid pREP_cas9_SD_gusA_stAp (C) and rearranged plasmid pREP_cas9_SD_gusA_stAp containing a large deletion (D). **C.** Plasmid map of pREP_cas9_SD_gusA_stAp, showing marked region of spontaneous deletion containing Cas9, GusA and gRNA coding sequences, together with UP and DOWN homology regions that occurred in tested *S. rimosus* "white" colonies containing rearranged plasmid (D). **D.** Plasmid map of rearranged pREP_cas9_SD_gusA_stAp with deleted CRISPR-specific regions. *PstI* restriction sites are marked on both plasmids. GR = GeneRuler DNA Ladder Mix (Thermo Fischer Scientific) (k = kbp).

containing the upper and lower sides of the homology region was deleted in the "white" colonies. Therefore, an 8,2 kbp fragment within the plasmid DNA was deleted, resulting in a much smaller plasmid (7856 bp) that only contained essential functions, that is, to ensure replication, conjugal transfer, and resistance to thiostrepton. The exact DNA sequence of the plasmids with large deletions was obtained from Sanger sequencing data (Fig. S3). Surprisingly, all analysed plasmids obtained from "white" colonies carried an identical deletion. This could indicate that the recombination event occurred at an early stage of the conjugation procedure in the *E. coli* donor. We evaluated the state of the pREP_cas9_SD_gusA_stAp plasmid isolated directly from the stored *E. coli* ET12567/pUB307 stocks which were used for conjugal transfer of the plasmid from *E. coli* into *S. rimosus*. According to restriction analysis with the *PstI* multicutter enzyme, all *E. coli* ET12567/pUB307 clones carried the intact plasmid pREP_cas9_SD_gusA_stAp. Therefore, we can conclude that the plasmid pREP_cas9_SD_gusA_stAp was stably maintained in the *E. coli* ET12567/pUB307 (Fig. S2).

4. Discussion

The difficulties associated with the use of CRISPR-Cas tools in *Streptomyces* species often stem from their potential toxicity to the host cell (Zhao et al., 2020; Ye et al., 2020; Wang et al., 2020; Alberti et al., 2018; Vento et al., 2019). Editing efficiency can vary greatly when CRISPR-Cas tools are applied in *Streptomyces* strains (Cobb et al., 2015; Huang et al., 2015; Zhang et al., 2020). Viable colonies containing either an unintended edit or the wild-type sequence are often observed after the transformation of CRISPR-Cas-bearing plasmids into the host culture. These cells, referred to as "escaper colonies", often complicate the identification of target clones that contain the correct edit (Vento et al., 2019). In various instances, their characterisation suggests that deactivation of the *cas* nuclease gene or the spacer region is a common escape mechanism, as seen by the deactivation of *cas9* gene in *Corynebacterium glutamicum* (Fischer et al., *2012*) and type I *cas* genes in the archaea *Haloferax volcanii*, where 77% of escaper colonies had deletions or mutations in the *cas* gene cluster (Liu et al., 2017). Thus, Cas9 toxicity not only results in a large proportion of non-viable cells but also exerts selection pressure for recombination events that preferentially abolish Cas9 activity (Liu et al., 2017). Although overexpression of CRISPR components often leads to severe cytotoxicity, a very weak level of expression can result in inefficient genome editing (Ye et al., 2020). To establish the optimal level of expression for the CRISPR system, the most important factors are the choice of the plasmid replicon and promoter sequences. We focused on a pIJ101 replicon-based vector, which, together with pSG5, is one of the two most commonly used CRISPR-associated replicons in *Streptomyces* species (Cobb et al., 2015; Huang et al., 2015; Tong et al., 2015; Zeng et al., 2015; Zhao et al., 2020). In addition to the drawback posed by the pSG5 replicon, which is reported to be problematic when editing highly repetitive genomic DNA regions (Wang et al., 2020; Wlodek et al., 2017), the advantage of the pIJ101-based replicon is the rapid loss of the plasmid during subcultivation procedures carried out in the absence of antibiotic pressure following CRISPR-mediated editing.

However, we observed that the pIJ101-based plasmid vectors displayed a relatively high degree of instability in *S. rimosus* (Carrillo Rincón et al., *2018*). These observations led us to believe that the selection pressure of an active Cas9-gRNA complex expressed from a pIJ101-based high-copy plasmid could also lead to recombination events, thus reducing the efficacy of CRISPR tools. To reduce the putative selection pressure caused by high-copy vectors, we used the pREP_P1_Cas9 plasmid (Mrak et al., 2018), where the *cas9* gene is placed under the control of a weak P1 promoter with an additional riboswitch (Mrak et al., 2018). The use of strong promoters for gRNA expression is seemingly non-toxic to cells, with stronger promoters showing increased editing efficiency (Zhang et al., 2020). For this reason, we focused only on monitoring Cas9 nuclease expression. To gain visual insight into the performance of the combination of the P1 promoter, the pIJ101-based replicon pTJU412, and the Cas9 nuclease plasmid in *S. rimosus*, we transcriptionally fused *gusA* (Myronovskyi et al., *2011*) to *cas9*. In this way, we not only monitored *cas9* gene expression, but also ensured the selection of transformants containing the *cas9* gene. To avoid low levels of the reporter protein, GusA, which could reduce the functionality of our system, the riboswitch region was not included upstream of the GusA coding sequence. Therefore, we aimed to achieve "full strength" translation of the *gusA* gene. In contrast, potential toxicity from the translation of the *cas9* gene is controlled by the riboswitch, thus reducing the translation efficiency of the *cas9* gene.

Importantly, we observed that deletion of the *stAp* gene occurred at a high frequency when no theophylline was added to induce the P1-riboswitch, therefore at low Cas9 expression levels. The addition of theophylline only resulted in reduced growth of *S. rimosus* cultures. A negative effect of theophylline addition in inducing Cas9 translation was recently reported in *S. coelicolor* M145 and *S.lividans* TK24 (Ye et al., 2020). Therefore, we conclude that in an *S. rimosus* background, the theophylline riboswitch is sufficiently leaky even without the addition of its inducer, ensuring sufficient Cas9 expression when combined with the P1 promoter and a high-copy pIJ101 replicon. These results also suggest that the high toxicity of the Cas9 protein is the main factor affecting the efficacy and reproducibility of CRISPR-Cas9-based tools in *S. rimosus*.

Altogether, we observed that the expression of the *gusA* reporter gene coincided entirely with the occurrence of the target CRISPR-Cas9 editing event in independent transformants, thereby confirming the efficacy of our visual exconjugant chromogenic screening approach. Interestingly, we also observed a relatively large proportion of *S. rimosus* exconjugants that did not display GusA activity ("white" colonies) and, without exception, contained a rearranged CRISPR-Cas9 plasmid. Independent plasmid isolates from the so-called "escaper colonies" in an *S. rimosus* background underwent extensive recombination events, in which most CRISPR-specific parts of the carrier plasmid were deleted.

5. Conclusion

The Cas9-SD-GusA system, where the GusA reporter system is transcriptionally fused to a Cas9 nuclease that we have developed, is a very efficient system contained within a single plasmid. We demonstrated that in *S. rimosus*, GusA activity directly correlates with the efficacy of CRISPR-Cas9-mediated editing. Our results demonstrate that this method can greatly simplify the optimisation of CRISPR-Cas tools and procedures in *Streptomyces* species, including the optimisation of cultivation conditions, selection of suitable vectors, and other gene tools such as promoters of appropriate strength. Importantly, the method we have developed allows for simple and rapid visual selection of single *Streptomyces* colonies that express Cas9, while simultaneously enabling the rapid identification of undesired "escaper colonies". With a certain re-adjustments, such as a selection of suitable vector, resistance gene and G + C DNA content readjustment, Cas9-SD-GusA system that we developed can be applied to a diverse range of microbial hosts for the purpose of CRISPR-Cas tools efficacy optimization.

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CRediT authorship contribution statement

Alen Pšeničnik: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Roman Reberšek:** Methodology, Investigation, Writing – original draft, Writing – review & editing. **Lucija Slemc:** Methodology. **Tim Godec:** Visualization. **Luka Kranjc:** Methodology, Supervision, Writing –

review & editing. **Hrvoje Petković:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors have no conflicts of interest to disclose.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2022.106545.

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