nature portfolio

Peer Review File

Engineered CRISPR-Cas9 for Streptomyces sp. genome editing to improve specialized metabolite production

Corresponding Author: Professor Min-Kyu Oh

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors implemented modification to Cas9, for addressing challenges encountered during gene manipulation using Cas9 within strains possessing high GC content genome. The Cas9-BD, a modified Cas9 with the addition of polyaspartate to its N- and C-termini, was developed with decreased off-target binding and cytotoxicity compared with wild-type Cas9. Cas9-BD and similarly modified dCas9-BD have been successfully employed for simultaneous biosynthetic gene cluster (BGC) refactoring, multiple BGC deletions, or multiplexed gene expression modulations in Streptomyces. They also demonstrated improved secondary metabolite production using multiplexed genome editing with multiple single guide RNA libraries in several Streptomyces strains. Cas9-BD was also used to capture large BGCs using a newly developed in vivo cloning method. The modified CRISPR-Cas9 system was successfully applied to many Streptomyces sp., providing versatile and efficient genome editing tools for strain engineering of actinomycetes with high GC content genome. I really want to appreciate the amount of works done in this study, but on the other hand, I feel a general lack of innovation among these works. As a potential publication in the leading comprehensive journal - Nature Communications, the manuscript does not sufficiently depth and innovation.
Major comments:
1. The authors claim that Cas9-BD has decreased off-target binding and cytotoxicity. It would be beneficial to provide quantitative data to support this claim.
The off-target activity of cas9-CD at the NGC site is significantly higher than that of the wild type, can you explain the reason? Comparative analysis between the off-target effects of wild-type Cas9 and Cas9-BD should be included, possibly through whole-genome sequencing or other high-throughput methods to assess off-target mutations. The possible off-target sites during single or multiple editing should also be predicted, and the off-target activity of Cas9-BD should be further demonstrated in subsequent experiments.
2. The addition of polyaspartate to Cas9 may affect its secondary structure and, consequently, its function. The authors should consider conducting and presenting a structural analysis, such as circular dichroism spectroscopy, to assess any conformational changes in the modified Cas9 proteins.
3. The study focuses on Streptomyces, but the implications of this work could extend to other organisms with high GC content genomes. The authors should discuss the potential broader applicability of their modified Cas9 system.
4. For all the editing events with systems, I only see the data from T0 generation. The readers have no idea whether the editing events in T0 plants would be faithfully inherited to T1, T2 generation? Or whether there will be some new type of editing events generated in the T1 generation? The genetic analysis of progeny from genome editing is an essential step for the evaluation of the target genes' function and the precision of the editing tools.
5. For the testing of editing efficiency, I suggest the authors to test the editing efficiency using high-throughput deep sequencing analysis.
6. It would have been more convincing if the authors could have demonstrated more conformational change or binding sites changes in Cas9-CD, Cas9-ND and Cas9-BD compared to wild type.
7. The author claimed that Cas9-BD showed substantially lower toxicity than wild-type Cas9, possibly attributable to reduced off-target cleavage while maintaining high on-target cleavage efficiency. But the results showed that the on-target DNA cleavage efficiency of the modified Cas9s was decreased by less than 20% compared to wild-type Cas9. Could it be reduced toxicity due to reduced Cas9-BD cleavage activity?
8. The authors should describe in detail the editing efficiency when using Cas-BD for genome editing to increase the yield of secondary metabolites of Streptomyces sp.
9. In a silent BGC for the production of oviedomycin using Cas9-BD, it would be more interesting if wild-type Cas9 could be

a control. In addition, there are many reports confirming the use of the CRISPR/Cas9 system to mediate the synthesis of BGCs to produce novel or interesting compounds. The use of wild-type Cas9 as a control might be a better way to demonstrate alterations in on- or off-target activity.

Minor issues:
1. The authors should provide more details of off-target assay and explain why Cas9-CD, Cas9-ND and Cas9-BD is judged using this method.
2. The design of sgRNAs should be described in detail.
3. In addition, the possible off-target sites of sgRNA should be predicted when performing multiple edits.
4. Line 72, page 4: C-to-T or A-to-G switches should change as A-to-G or C-to-T mutation;
5. Line 76, page4: To achieve this Cas9, I think this should be “To achieve this, Cas9”;
6. Line 87, page5: “various species whose genomes have a high GC content” should be “various species genomes with high GC content”

Reviewer #2

(Remarks to the Author)
This manuscript addresses the issue of off-target mutations/toxicity associated with existing Cas9-based tools used in the Streptomyces bacteria. Cas9 and dCas9 variants where poly-aspartate fragments have been inserted (at N-terminal, at C-terminal or at both ends of Cas9) were constructed and assessed in an extensive range of previously reported applications both in vitro and in vivo (in diverse bacterial species). The efficiency of new variants, in particular Cas9-BD and dCas9-BD is convincingly demonstrated and will be useful to a broad range of scientists working with Streptomyces bacteria or scientists aiming to capture (any) large DNA fragments of DNA in vitro.
Importantly, the significantly reduced toxicity of Cas9-BD and dCas9-BD now opens the way to the efficient implementation of technology such as multiplexing CRISPRi.
Overall, the authors tested their systems to: (i) edit genomes and increase production of specialised metabolites in Streptomyces antibioticus and Streptomyces rapamycinicus; (ii) to delete multiple biosynthetic gene clusters (BGC) at once in Streptomyces coelicolor; (iii) to capture large (>100 kb) BGCs in vitro; (iv) to silence multiple genes at once using dCas9DB in Streptomyces coelicolor.

Additional comments:
1. The authors could consider replacing “secondary metabolite” with “specialized metabolite” in the manuscript title
2. The authors should add a comment in the discussion to explicitly state that there may still be a number of off-target mutations in the engineered strains they generated (using Cas9-DB, not dCas9-DB) and that it is important to work with multiple exconjugants. Ideally, the authors would have sequenced the genomes of some of the exconjugants but they convincingly demonstrated that off-target mutations (if any) cause no or reduced toxicity.

Minor comments:
1. line 34: replace “gram” with “Gram”
2. line 36: the word “most” should be remove as this is incorrect
3. line 43: replace “high genome GC” with “high GC genome”
4. line 60: replace “if” with “of”
5. line 100: unclear how Suppl. Fig. 1b and 1c relate to the text. “Modified Cas9 with polyaspartate at the N- and/or C 100 termini, or both ends of the polypeptide, was developed (Fig. 1b, Supplementary Fig. 1b and c)…” The Supp. Fig. 1 does not show any polyaspartate modification but only the relative position of Cas9 C-terminal end with recognized DNA (5’ end) and that of Cas9 N-terminal end with DNA (3’ end) respectively.

In summary, the work carried out is very convincing and represents a very significant advance that will facilitate genetic manipulation of Streptomyces bacteria. I would recommend this work to be published in Nature Communications without hesitations.

Reviewer #3

(Remarks to the Author)
In this study, the authors designed a novel modified Cas9 (Cas9-BD) with the addition of polyaspartate to its N- and C-termini, which showed decreased off-target binding and cytotoxicity compared with the wild-type Cas9. Based on Cas9-BD, the authors achieved simultaneous biosynthetic gene cluster (BGC) refactoring, multiple BGC deletions as well as multiplex gene repression in the tested Streptomyces strains. Particularly, they also developed a novel in vivo BGC cloning method using Cas9-BD, which will facilitate BGC cloning as well as novel drug discovery by BGC heterologous expression. Overall, the research content of this study is innovative, especially the development of in vivo BGC cloning method. In my opinion, the research results obtained in this study will have great reference value for readers in this field. So, I think, this work is suitable for publication. However, the research work design is not rigorous enough, and the experimental results are mainly qualitative analysis, lacking quantitative analysis data, which need to be improved. The following comments and suggestion should also be addressed.
1. The authors reasoned that the addition of acidic residues to the Cas9 protein could reduce the off-target cleavage, thereby reducing the in vivo host toxicity. The aspartate residue was tested in this study. My question is: why did the authors only test aspartate? Did they test another acidic amino acid, glutamate?

2. Line 131-133, in conjugation assay, how many spores were used? Qualitative analysis should be replaced by quantitative analysis? How much does the conjugation efficiency increase for Cas9-BD with respect to Cas9?
3. Line 153-156, It is recommended to draw a detailed workflow for the construction of the plasmid library to facilitate reader understanding.
4. Line 158-159, the authors showed that only five exconjugants appeared on the agar plates using the modified Cas9-BD. How many spores were used? Did the authors use Cas9 as a control? Only by comparing these two different versions can the advantages of Cas9-BD be demonstrated. Similarly for Line 175-177. In addition, how many times are these experiments repeated?
5. Line 207, change “identify” to “increase”.
6. Line 213-218, What are the efficiencies of single-gene and multi-gene deletions?
7. The development of this novel Cas9-BD-based in vivo BGC capturing method will greatly facilitate BGC cloning (Line 231-246). It is necessary to briefly introduce the design concept and novelty of this method. Line 244, Four exconjugants were obtained, here, I think, the detailed conjugation condition should be provided in the materials and methods. In addition, I wonder what happened to the genome? whether the target BGC in the genome has been deleted? If that’s the case, how did cells survive? I strongly recommend to sequence the genome of the host after BGC capturing. Did the authors test in vivo BGC capturing using the wild-type Cas9?
8. Line 284-285, transcriptional analysis should be provided for the dCas9 and dCas9-BD-based CRISPRi.
9. Line 311-314, similarly, suggest drawing a detailed workflow for the construction of the plasmid library to facilitate reader understanding.
10. Line 319-325, it is recommended to test the levels of the Acetyl-CoA and malonyl-CoA in these mutant with significantly enhanced production of three different metabolites.
11. Supplementary Fig.5. The electrophoresis image is too blurry, please replace them.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have thoroughly addressed all my questions, significantly improving the clarity and completeness of the manuscript. The revisions have successfully incorporated my suggestions, resulting in a much stronger and more impactful paper. I have only minor formatting suggestions. The authors should revise the spelling of some words throughout the manuscript; for example, "exoconjugats" (lines 178, 189, 267, 295, etc.) and "transcritomic" (line 348). With these minor corrections, I believe the manuscript is ready for publication.

Reviewer #3

(Remarks to the Author)
The authors have addressed most of my concerns raised previously. The following comments should be further addressed.
1. For the verification of the correctness of the captured BGCs, PCR analysis is not enough. I strongly recommend that the authors should conduct restriction enzyme digestion and DNA sequencing of the captured BGCs.
2. Fig.2, in the picture, M1152 should be M1146.
3. In response to Reviewers’ Comments: the author mentioned that "there have been no previous reports of genome editing in those strains". To my knowledge, in S.rapamycincius, CRISPR/Cas12a has been employed for genome editing.The authors can check it.
4. In supplementary Fig.9, the results of PCR analysis is not convincing.I don't think they are specific PCR products.
5. I have some doubts about the data of the multiplexed promoter replacement. According to my experience, the authors obtained just about 4 exconjugants for single promoter replacement after conjugation (Supplementary Figure 5)；Under the same conditions, it is difficult to obtain exconjugants for multiplexed promoter replacement. Please provide the numbers of the exconjugants for multiplexed promoter replacement.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have addressed all of my comments and concerns. I have no other comments.

Response to Reviewers' Comments

NCOMMS-24-11378

Engineered CRISPR-Cas9 for Streptomyces sp. genome editing to improve specialized metabolite production

Duck Gyun Kim, Boncheol Gu, Yujin Cha, Jeonghan Ha, Yongjae Lee, Gahyeon Kim, Byung-Kwan Cho, & Min-Kyu Oh

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We have responded to the reviewer's comments as the followings:

Reviewer #1: In this manuscript, the authors implemented modification to Cas9, for addressing challenges encountered during gene manipulation using Cas9 within strains possessing high GC content genome. The Cas9-BD, a modified Cas9 with the addition of polyaspartate to its N- and C-termini, was developed with decreased off-target binding and cytotoxicity compared with wild-type Cas9. Cas9-BD and similarly modified dCas9-BD have been successfully employed for simultaneous biosynthetic gene cluster (BGC) refactoring, multiple BGC deletions, or multiplexed gene expression modulations in Streptomyces. They also demonstrated improved secondary metabolite production using multiplexed genome editing with multiple single guide RNA libraries in several Streptomyces strains. Cas9-BD was also used to capture large BGCs using a newly developed in vivo cloning method. The modified CRISPR-Cas9 system was successfully applied to many Streptomyces sp., providing versatile and efficient genome editing tools for strain engineering of actinomycetes with high GC content genome. I really want to appreciate the amount of works done in this study, but on the other hand, I feel a general lack of innovation among these works. As a potential publication in the leading comprehensive journal - Nature Communications, the manuscript does not sufficiently depth and innovation.

Major comments:

1. The authors claim that Cas9-BD has decreased off-target binding and cytotoxicity. It would be beneficial to provide quantitative data to support this claim. The off-target activity of cas9-CD at the NGC site is significantly higher than that of the wild type, can you explain the reason? Comparative analysis between the off-target effects of wild-type Cas9 and Cas9-BD should be included, possibly through whole-genome sequencing or other high-throughput methods to assess off-target mutations. The possible off-target sites during single or multiple editing should also be predicted, and the off-target activity of Cas9-BD should be further demonstrated in subsequent experiments.

Response: Thank you for the reviewer's comments.

1) For quantitative analysis of off-target activities of wild-type Cas9 and Cas9-BD in *Streptomyces* sp., we designed a genome editing experiment of *matAB* gene deletion in the genome of *S. coelicolor* M1146 (Supplementary Fig. 4a). This experiment was chosen because this editing experiment produced exoconjugates with wild-type Cas9 expressed under a strong promoter, allowing a direct comparison between Cas9 and Cas9-BD. Most other editing experiments with wild-type Cas9 failed to produce any exoconjugate. The numbers of exoconjugates with plasmids expressing Cas9 proteins as well as Cas9-sgRNA-HSR (homologous sequence regions for

recombination) were quantitatively compared to those obtained with Cas9-BD (Supplementary Fig. 4b). In both experiment with and witout sgRNA-HSR, Cas9-BD produced 7.7- and 73-fold more exoconjugates, respectively, than wild-type Cas9.

We further analyzed the whole-genome sequences of two exoconjugates from each editing experiment (*matAB* deleted strain using Cas9 and Cas9-BD). A higher frequency of off-target modifications was observed in one exoconjugate edited with the wild-type Cas9 (Supplementary Fig. 4e, f). Interestingly, none of the mutations occurred at predicted off-target sites identified using RGEN OFFinder[1] and BLAST searches with the NCBI database (Fig. R1). When examined with PCR and sequencing, most exoconjugates contained the *matAB* deletion in their genome (Supplementary Fig. 4c, d), showing an editing efficiency of nearly 100%.

The results were added as supplementary Fig. 4 and briefly explained in the revised manuscript, line 151-158. The methods including prediction tools of possible off-target sites and genome sequencing were included in the manuscript, line 569-570 and 743-773.

Site	Sequence (5` to 3`)	Location	Gene (Protein)
matAB on-target site	GCGGGATCGGTGACGTCCGC CGG	3,036,850 – 3,036,872	SCO2782 (Pyridoxal-dependent decarboxylase)
matAB off-target site #1	GCGCGTTCACCGCCATCAGA TGG	116,105 – 116,127	SCO0127 (Beta keto-acyl synthase)
matAB off-target site #2	GCAGGTGGCTGGCCATCAGA TGC	121,275 – 121,297	SCO0130 (Beta-lactamase)
matAB off-target site #3	TCGTGCCCGCCGCCATCAGA CGC	2,731,100 – 2,731,122	SCO2532 (PhoH-like portein)
matAB off-target site #4	GCATGCCGAGGGCCATCAGA CGC	3,189,679 – 3,189,701	SCO2937 (Transmembrane transport protein)
matAB off-target site #5	AGCCGGACTTGGCCATCAGA GGG	3,576,728 – 3,576,750	SCO3232 (CDA peptide synthetase III)
matAB off-target site #6	AGGTGTACTGGGCCATCAGA AGG	4,497,192 – 4,497,214	SCO4097 (Amino acid / metabolite permease)
matAB off-target site #7	TGGTGGTGGTAGCCATCAGA TGT	5,133,790 – 5,133,812	SCO4714 (50s ribosomal protein L5)
matAB off-target site #8	GTAGACATGCCGCCATCAGA TGT	8,225,807 – 8,225,829	SCO7410 (Multiple sugar transport protein)
matAB off-target site #9	TCTGGAAGGACGCCATCAGA CGG	8,375,427 – 8,375,449	SCO7553 (Oxidoreductase)

Fig. R1. Prediction of possible off-target sites with sgRNA. The possible off-target sites with sgRNA for *matAB* gene deletion were predicted by using OFFinder or BLAST searches using the NCBI database.

2) Off-target mutations were also evaluated for strains with multiple BGC deletions in *S. coelicolor* M1146, as shown in Supplementary Fig. 7. Potential off-target sites for these deletion experiments were predicted using BLAST searches with the NCBI database and RGEN OFFinder, based on the sgRNA sequences as described earlier. PCR amplification and sequence analysis were then used to examine mutations at the predicted sites in the genomes. No mutations were detected at the predicted off-target sites following the deletions using Cas9-BD in *S. coelicolor* M1146. With wild-type Cas9, no exoconjugates were formed during the deletion experiment, preventing a direct comparison between Cas9 and Cas9-BD.

   The results were added in Supplementary Fig. 7 and briefly mentioned in the revised manuscript, line 245-247. Methods for confirmation of mutations were included in the revised manuscript, line 612-615.

3) A relatively high error bar led to skewed results in the *in vitro* experiments on –NGC site with Cas9-CD in the previously submitted manuscript. The reaction was repeated (Fig. R2) and the results were recalculated.

   The data in Fig. 1d and Supplementary Fig. 3h were updated accordingly.

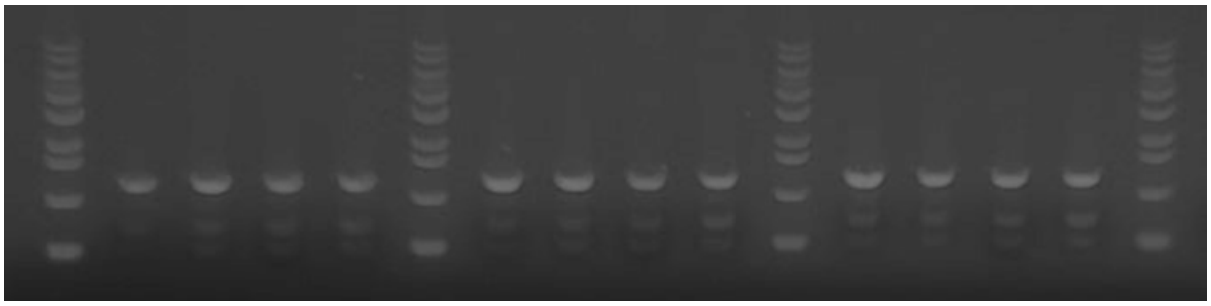

Figure R2. Re-performed *in vitro* reactions of Cas9-CD with a off-target sequence. *In vitro* reaction of Cas9-CD with linear DNA containing target sequence and -NGC PAM was performed. Samples were harvested at 10-, 30-, 60-, and 120-min. Reaction products were dissolved in 0.8% agarose gel and visualized.

2. The addition of polyaspartate to Cas9 may affect its secondary structure and, consequently, its function. The authors should consider conducting and presenting a structural analysis, such as circular dichroism spectroscopy, to assess any conformational changes in the modified Cas9 proteins.

Response: In response to the reviewer's comments, structural prediction and circular dichroism spectroscopy analyses were conducted. Protein structures of wild-type Cas9 and Cas9-BD were predicted using AlphaFold 3[2], with the results shown in Supplementary Fig. 1a, b. Additionally, circular dichroism analysis of each engineered protein (Cas9-CD, Cas9-ND, and Cas9-BD) was performed, and the results were compared to wild-type Cas9 in Supplementary Fig. 1c. These analyses suggest that the polyaspartate linked to Cas9 does not interfere with the folding of the Cas9 protein structure, indicating no major changes in its function are expected.

The results and experimental methods were added in the revised manuscript, line 108-110 and 520-533, respectively.

3. The study focuses on Streptomyces, but the implications of this work could extend to other organisms with high GC content genomes. The authors should discuss the potential broader applicability of their modified Cas9 system.

Response: Thank you for highlighting the general effects of modified Cas9 in organisms with high GC content genomes. In response, additional experiments were conducted with strains that have high GC content genomes (Supplementary Fig. 16a) in our laboratory. As anticipated, Cas9-BD exhibited lower toxicity when introduced into various high GC content organisms, resulting in higher numbers of exoconjugates across five different strains. The editing experiment, applied for gene deletion in the *A. hankyoungi* I2-34 strain, was successful in most exoconjugates (Supplementary Fig. 16b). These findings support that Cas9-BD can be effectively used for genome editing in various high GC content organisms due to its reduced toxicity.

These results were briefly mentioned in the revised manuscript, line 460-461.

4. For all the editing events with systems, I only see the data from T0 generation. The readers have no

idea whether the editing events in T0 plants would be faithfully inherited to T1, T2 generation? Or whether there will be some new type of editing events generated in the T1 generation? The genetic analysis of progeny from genome editing is an essential step for the evaluation of the target genes' function and the precision of the editing tools.

Response: Thank you for your feedback on the paper. To verify the inheritance of mutations from editing and assess the potential generation of unexpected mutations in the T1 and T2 generations, whole genome sequencing was performed on T1 and T2 generation strains of *matAB*-deleted *S. coelicolor*. After confirming the successful *matAB* deletion and then plasmid curing using an antibiotic marker, the strain was cultured on an agar plate to produce the T1 generation. A colony from this plate was then streaked onto another agar plate to generate the T2 generation. In each generation, a colony was randomly selected for sequencing. The results showed no additional mutations in the T1 and T2 generations compared to the T0 generation. The genome sequences have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB79564.

5. For the testing of editing efficiency, I suggest the authors to test the editing efficiency using high-throughput deep sequencing analysis.

Response: Since non-homologous end joining (NHEJ) is not highly active in most prokaryotes, a double-strand break (DSB) in the genome must be repaired through homologous recombination, which requires the addition of homologous sequence regions (HSR). In *Streptomyces* sp., the genome sequence near the DSB induced by the Cas9-sgRNA complex needs to be replaced by homologous DNA through recombination for the cell to survive. If the editing is unsuccessful, the strains cannot survive due to the unrepaired DSB. This makes it difficult for non-edited strains to survive, resulting in high editing efficiency in prokaryotic genome editing experiments.

In this experiment, PCR amplification using genomic DNA from all exoconjugants in the *matAB* deletion region was performed to confirm the editing efficiency of Cas9 and Cas9-BD (Supplementary Fig. 4c). Additionally, genomic sequences at the edited site were analyzed for further validation (Supplementary Fig. 4d). The results confirmed that genome editing with both Cas9 and Cas9-BD achieved near 100% efficiency. Notably, the number of colonies obtained using Cas9-BD and the Cas9-BD-sgRNA-HSR was significantly higher than those obtained with wild-type Cas9 and Cas9-sgRNA-HSR (Supplementary Fig. 4b).

6. It would have been more convincing if the authors could have demonstrated more conformational change or binding sites changes in Cas9-CD, Cas9-ND and Cas9-BD compared to wild type.

Response: As requested by the reviewer, the structures of Cas9 proteins bound to sgRNA were analyzed using circular dichroism spectroscopy. The results, presented in Supplementary Fig. 1d, showed no significant structural changes between wild-type and engineered Cas9 when bound to sgRNA. These findings have been included in the revised manuscript, along with the response to the reviewer's comment #2.

7. The author claimed that Cas9-BD showed substantially lower toxicity than wild-type Cas9, possibly attributable to reduced off-target cleavage while maintaining high on-target cleavage efficiency. But the results showed that the on-target DNA cleavage efficiency of the modified Cas9s was decreased by less than 20% compared to wild-type Cas9. Could it be reduced toxicity due to reduced Cas9-BD cleavage activity?

Response: Thank you for sharing your opinion. Based on the *in vitro* assay, the on-target cleavage efficiency was reduced by less than 20% when using Cas9-BD compared to Cas9, while off-target site efficiencies were reduced by up to 80%. We believe that the reduction in off-target activity by Cas9-BD is the main factor contributing to the decrease in toxicity. Unlike on-target binding, double-strand breaks (DSBs) caused by off-target binding cannot be repaired by homologous recombination due to the absence of appropriate homologous sequence regions (HSRs). As a result, increased off-target binding leads to a higher likelihood of cell death.

The toxicity caused by Cas9 alone, without sgRNA-HSR, can be attributed to off-target effects with small RNA fragments present in the cell. The high GC content of the genome produces more RNA fragments that contain the PAM sequence (AGG) of Cas9, thereby increasing the likelihood of off-target binding and cellular toxicity.

In the case of on-target binding, DSBs can be repaired through homologous recombination. If high on-target binding were to result in significant cytotoxicity, it would likely be due to improper recombination during the repair process. However, both Cas9 and Cas9-BD showed nearly 100% editing efficiency, indicating that homologous recombination was generally successful at on-target sites (Supplementary Fig. 4c, d). This suggests that the main cause of Cas9-induced toxicity in high GC-content organisms is off-target binding, and the reduction of off-target activity by Cas9-BD is the primary reason for the observed decrease in cytotoxicity.

8. The authors should describe in detail the editing efficiency when using Cas-BD for genome editing to increase the yield of secondary metabolites of Streptomyces sp.

Response: As requested by the reviewers, a quantitative analysis of editing efficiency to enhance secondary metabolite production was conducted in *Streptomyces* sp. For this purpose, the promoters of *ovmOI* and *rapH* were refactored to stronger versions. These promoters are located in the oviedomycin biosynthetic gene cluster (BGC) of *S. antibioticus* NRRL 3238 and the rapamycin BGC of *S. rapamycinicus* NRRL 5491, respectively. Editing efficiency was confirmed through PCR amplification and sequence analysis. Although the number of exoconjugates was lower than in the case of *S. coelicolor*, the editing efficiency in the obtained exoconjugates was 100% (Supplementary Fig. 5c-f). Furthermore, the refactored strains demonstrated improved production of oviedomycin and rapamycin (Supplementary Fig. 5g, h).

The results were presented in Supplementary Fig. 5 and the results were described in the revised manuscript, line 163–168 and 193–197. The method of this experiment was added in the revised manuscript, line 601-603.

9. In a silent BGC for the production of oviedomycin using Cas9-BD, it would be more interesting if wild-type Cas9 could be a control. In addition, there are many reports confirming the use of the

CRISPR/Cas9 system to mediate the synthesis of BGCs to produce novel or interesting compounds. The use of wild-type Cas9 as a control might be a better way to demonstrate alterations in on- or off-target activity.

Response: As requested by the reviewer, additional experiments were conducted to assess the editing efficiency of Cas9. However, no colonies were obtained after conjugation of pCRISPomyec-2 (Cas9) with *S. antibioticus* NRRL 3238 and *S. rapamycinicus* NRRL 5491, both with and without sgRNA-HSR. To our knowledge, no studies have reported *in vivo* application of CRISPR/Cas9, including nCas9 or dCas9, in *S. antibioticus* or *S. rapamycinicus*.

Additionally, a few reports have used Cas9 for genome editing under inducible or weak promoters in other *Streptomyces* sp., such as *tipA*p[3]. Although using those promoters significantly reduce the editing efficiency, we followed the protocols. No colonies were obtained in these strains (data not shown), making a comparison impossible.

Minor comments：

1. The authors should provide more details of off-target assay and explain why Cas9-CD, Cas9-ND and Cas9-BD is judged using this method.

Response: Thank you for your comment. The detailed procedure for the *in vitro* reaction was already described in the manuscript, lines 491-518. The method for measuring cleavage efficiency using linear DNA *in vitro* has been widely used in previous studies involving various Cas proteins[4,5,6,7], and we have applied a similar approach in this research.

2. The design of sgRNAs should be described in detail.

Response: Thank you for your comment. sgRNAs used in this study were designed by using CRIPSR RGEN tool (http://www.rgenome.net/)[8].

The sentence describing sgRNA design was added in the manuscript, line 551-552.

3. In addition, the possible off-target sites of sgRNA should be predicted when performing multiple edits.

Response: Thank you for your comments. The relevant details were provided in response to reviewer's comment #1. BLAST search with NCBI database and RGEN OFFinder[1] were used for prediction of possible off-target.

The sentence about prediction of possible off-target sites was added in the revised manuscript, line 568-569.

4. Line 72, page 4: C-to-T or A-to-G switches should change as A-to-G or C-to-T mutation;

Response: Thank you for your comment. “C-to-T or A-to-G” was revised as “A-to-G or C-to-T” in manuscript.

5. Line 76, page4: To achieve this Cas9, I think this should be “To achieve this, Cas9”;

Response: Thank you for your comment. Manuscript was revised at line 82.

5. Line 87, page5: “various species whose genomes have a high GC content” should be “various species genomes with high GC content”

Response: Thank you for your comment. Manuscript was revised at line 93-94.

Reviewer #2: This manuscript addresses the issue of off-target mutations/toxicity associated with existing Cas9-based tools used in the Streptomyces bacteria. Cas9 and dCas9 variants where poly-aspartate fragments have been inserted (at N-terminal, at C-terminal or at both ends of Cas9) were constructed and assessed in an extensive range of previously reported applications both in vitro and in vivo (in diverse bacterial species). The efficiency of new variants, in particular Cas9-BD and dCas9-BD is convincingly demonstrated and will be useful to a broad range of scientists working with Streptomyces bacteria or scientists aiming to capture (any) large DNA fragments of DNA in vitro.

Importantly, the significantly reduced toxicity of Cas9-BD and dCas9-BD now opens the way to the efficient implementation of technology such as multiplexing CRISPRi.

Overall, the authors tested their systems to: (i) edit genomes and increase production of specialised metabolites in Streptomyces antibioticus and Streptomyces rapamycinicus; (ii) to delete multiple biosynthetic gene clusters (BGC) at once in Streptomyces coelicolor; (iii) to capture large (>100 kb) BGCs in vitro; (iv) to silence multiple genes at once using dCas9DB in Streptomyces coelicolor.

Major comments:

1. The authors could consider replacing “secondary metabolite” with “specialized metabolite” in the manuscript title

Response: Thank you for your opinion. In the title of the manuscript, “secondary metabolite” was replaced by “specialized metabolite”.

2. The authors should add a comment in the discussion to explicitly state that there may still be a number of off-target mutations in the engineered strains they generated (using Cas9-DB, not dCas9-DB) and that it is important to work with multiple exconjugants. Ideally, the authors would have sequenced the genomes of some of the exconjugants but they convincingly demonstrated that off-target mutations (if any) cause no or reduced toxicity.

Response: Thank you for your comment. Whole genome sequencing was indeed conducted to compare the off-target effects between Cas9 and Cas9-BD, with the results shown in Supplementary Fig. 5. While a few off-target edits led to missense mutations in some proteins, no significant phenotypic differences were observed.

We have added sentences explaining these results and the importance of working with multiple exoconjugants in the manuscript, lines 452-454.

Minor comments：

1. line 34: replace “gram” with “Gram”

Response: Thank you for comment. It was fixed accordingly at line 40.

2. line 36: the word “most” should be remove as this is incorrect

Response: Thank you for comment. The “most of the” commented word was replaced by “many of the” at line 42.

3. line 43: replace “high genome GC” with “high GC genome”

Response: Thank you for opinion about the paper. The sentence was revised at line 49.

4. line 60: replace “if” with “of”

Response: Thank you for comment. Manuscript was fixed accordingly at line 66.

5. line 100: unclear how Suppl. Fig. 1b and 1c relate to the text. “Modified Cas9 with polyaspartate at the N- and/or C 100 termini, or both ends of the polypeptide, was developed (Fig. 1b, Supplementary Fig. 1b and c)…” The Supp. Fig. 1 does not show any polyaspartate modification but only the relative position of Cas9 C-terminal end with recognized DNA (5’ end) and that of Cas9 N-terminal end with DNA (3’ end) respectively.

Response: Thank you for comment. The structures of the modified Cas9 added to both ends of the protein, were predicted by AlphaFold 3[2].

The predicted structure with polyaspartate regions highlighted in green, is provided in Supplementary Fig. 1b.

Reviewer #3: In this study, the authors designed a novel modified Cas9 (Cas9-BD) with the addition of polyaspartate to its N- and C-termini, which showed decreased off-target binding and cytotoxicity compared with the wild-type Cas9. Based on Cas9-BD, the authors achieved simultaneous biosynthetic gene cluster (BGC) refactoring, multiple BGC deletions as well as multiplex gene repression in the tested Streptomyces strains. Particularly, they also developed a novel in vivo BGC cloning method using Cas9-BD, which will facilitate BGC cloning as well as novel drug discovery by BGC heterologous expression. Overall, the research content of this study is innovative, especially the development of in vivo BGC cloning method. In my opinion, the research results obtained in this study will have great reference value for readers in this field. So, I think, this work is suitable for publication. However, the research work design is not rigorous enough, and the experimental results are mainly qualitative analysis, lacking quantitative analysis data, which need to be improved. The following comments and suggestion should also be addressed.

1. The authors reasoned that the addition of acidic residues to the Cas9 protein could reduce the off-target cleavage, thereby reducing the in vivo host toxicity. The aspartate residue was tested in this study. My question is: why did the authors only test aspartate? Did they test another acidic amino acid, glutamate?

Response: Thank you for your comments. The selection of polyaspartate was based on several factors: (1) aspartate carries a strong negative charge, enabling robust binding with the positively charged residues in the Cas9 active site; (2) its short functional group enhances accessibility to the protein's active site; and (3) it is a major component of the anti-CRISPR protein ACRII4, which has been shown to effectively inhibit Cas9 activity[9]. These points are already explained in the manuscript, lines 391-398. While glutamate is another potential candidate for introducing negative charges to reduce off-target cleavage efficiency, our attempts to construct a polyglutamate version have been unsuccessful so far. We agree that various constructs are possible and that further optimization is needed to develop the best-performing Cas9 variant in *Streptomyces* species.

2. Line 131-133, in conjugation assay, how many spores were used? Qualitative analysis should be replaced by quantitative analysis? How much does the conjugation efficiency increase for Cas9-BD with respect to Cas9?

Response: Thank you for the reviewer's comments. For conjugation, 10 µL of spore stock was used, usually containing 1.0×10^8 CFU/mL. The calculated conjugation efficiencies, based on the number of exoconjuates for the pCRISPomyces-2 plasmid (expressing Cas9) and pCRISPomyces-2BD plasmid (Cas9-BD) in *S. coelicolor* M1146, were 1.3×10^{-5} ($\pm 8.3 \times 10^{-6}$) and 1.0×10^{-4} ($\pm 1.0 \times 10^{-5}$), respectively. Also, with the inclusion of sgRNA and homologous sequence regions (HSR) for *matAB*

gene deletion, conjugation efficiencies using Cas9 and Cas9-BD were 9.4×10^{-7} ($\pm 6.7 \times 10^{-7}$) and 4.9×10^{-5} ($\pm 1.9 \times 10^{-5}$). In Summary, the conjugation efficiency with Cas9-BD was 7.7 times higher than that with Cas9 without sgRNA-HSR and 73 times higher with sgRNA-HSR.

These results were briefly mentioned at line 151-158 in manuscript and presented in Supplementary Fig. 4b.

3. Line 153-156, It is recommended to draw a detailed workflow for the construction of the plasmid library to facilitate reader understanding.

Response: As reviewer's request, the figure describing detailed workflow for construction of the plasmid library for multiple promoter replacement of genes coded in BGC was added in Supplementary Fig. 6.

4. Line 158-159, the authors showed that only five exconjugants appeared on the agar plates using the modified Cas9-BD. How many spores were used? Did the authors use Cas9 as a control? Only by comparing these two different versions can the advantages of Cas9-BD be demonstrated. Similarly for Line 175-177. In addition, how many times are these experiments repeated?

Response: Thank you for the reviewer's comments. For the editing experiment with *S. antibioticus* NRRL 3238 with Cas9-BD, 10 µL of spore stock, concentrated to 1.0×10^{8} CFU/mL, was used. Five exoconjuates were appeared, so that the conjugation efficiency was 1.7×10^{-7} ($\pm 4.7 \times 10^{-8}$). In case of *S. rapamycinicus* NRRL 5491, 30 µL of spore stock was used due to the strain's low conjugation efficacy. After repeating the experiments three times, a total of four exoconjugates were obtained leading to the conjugation efficiency was 1.5×10^{-8} ($\pm 1.4 \times 10^{-8}$). The same experiments with Cas9, but no exoconjuate was obtained (Supplementary Fig. 5a, b). Notably, there have been no previous reports of genome editing in those strains.

As the reviewer 1 raised similar concerns (major comments 8 and 9), we conducted the experiments for single promoter editing in *S. antibioticus* and *S. rapamycinicus* with Cas9-BD. Those results were included in Supplementary Fig. 5.

5. Line 207, change "identify" to "increase".

Response: Thank you for your opinion about the paper. Manuscript was revised in line 233.

6. Line 213-218, What are the efficiencies of single-gene and multi-gene deletions?

Response: Thank you for your comments. The conjugation efficiency for the deletion of the desferrioxamine B BGC, SapB BGC, and SCO-2138 BGC based on the number of exoconjugates and spores were 2.0×10^{-5}, 1.8×10^{-5}, and 1.2×10^{-5}, respectively, with nearly 100% editing efficiency in all

three cases. For the simultaneous deletion of both dessferrioxamine B and SapB BGCs, as well as the triple BGCs, (dessferrioxamine B, SapB, and SCO-2138) conjugation efficiencies were 6.0×10^{-6} and 1.0×10^{-6}, respectively, also achieving a 100% success rate. (Fig. 3b-f).

These explanations were added in the revised manuscript, line 240-245.

7. The development of this novel Cas9-BD-based in vivo BGC capturing method will greatly facilitate BGC cloning (Line 231-246). It is necessary to briefly introduce the design concept and novelty of this method. Line 244, Four exconjugants were obtained, here, I think, the detailed conjugation condition should be provided in the materials and methods. In addition, I wonder what happened to the genome? whether the target BGC in the genome has been deleted? If that's the case, how did cells survive? I strongly recommend to sequence the genome of the host after BGC capturing. Did the authors test in vivo BGC capturing using the wild-type Cas9?

Response: Thank you for the reviewer's comments. The *in vivo* capturing method developed in this study offers a more convenient and efficient alternative to the *in vitro* BGC capturing approach. The existing *in vitro* Cas9 method relied on high-purity, long genomic DNA, which requires specialized DNA purification techniques, such as phenol-chloroform extraction, ethanol precipitation, isolation using pulse field gel electrophoresis, etc[10,11]. After cleavage of the BGC by Cas9-sgRNAs complexes, Gibson assembly should be optimized to integrate the fragments into capturing vector, followed by a screening of successfully captured BGC due to low efficiency.

In contrast, the developed *in vivo* capturing method uses conjugation of a capturing plasmid. Once conjugated, *in vivo* capturing occurs by Cas9 editing and endogenous homologous recombination system. After acquiring exoconjugates, the BGC-captured plasmid is transformed into *E. coli* without the need for extensive purification, relying on antibiotic selection markers in the plasmid. This process was highly successful, as all four exoconjugates contained intact FK506 BGCs.

We appreciate the reviewer pointing out the genomic DNA issue in the exoconjugates after capturing. To verify the presence of the BGC region in the genomic DNA of exoconjugates following *in vivo* capturing, the following experiment was conducted. The exoconjugant was cultured and subjected to a curing process to remove the pCap-00 plasmid. After confirming plasmid curing, the genomic DNA of the strain was extracted and used as a template for PCR amplification of the BGC region. The results confirmed that all exoconjugates retained the FK506 BGC region in their genomic DNA (Fig. R3a, b). It is known that during mycelial growth in *Streptomyces* sp., individual cells may contain multiple copies of the genome[12]. Our hypothesis is that the genome involved in capturing was removed, but other genome copies replicated to sustain cell growth. As explained in the manuscript, the sgRNA-encoding region is removed from the pCap-00 plasmid during the capturing process, preventing further cleavage of genomic DNA once the capturing is complete.

The concept and novelty of *in vivo* BGC capturing were described in original manuscript. We added a few sentences to stress them in the revised manuscript, line 269-271. Detailed conjugation method was already described in the manuscript, line 694-714.

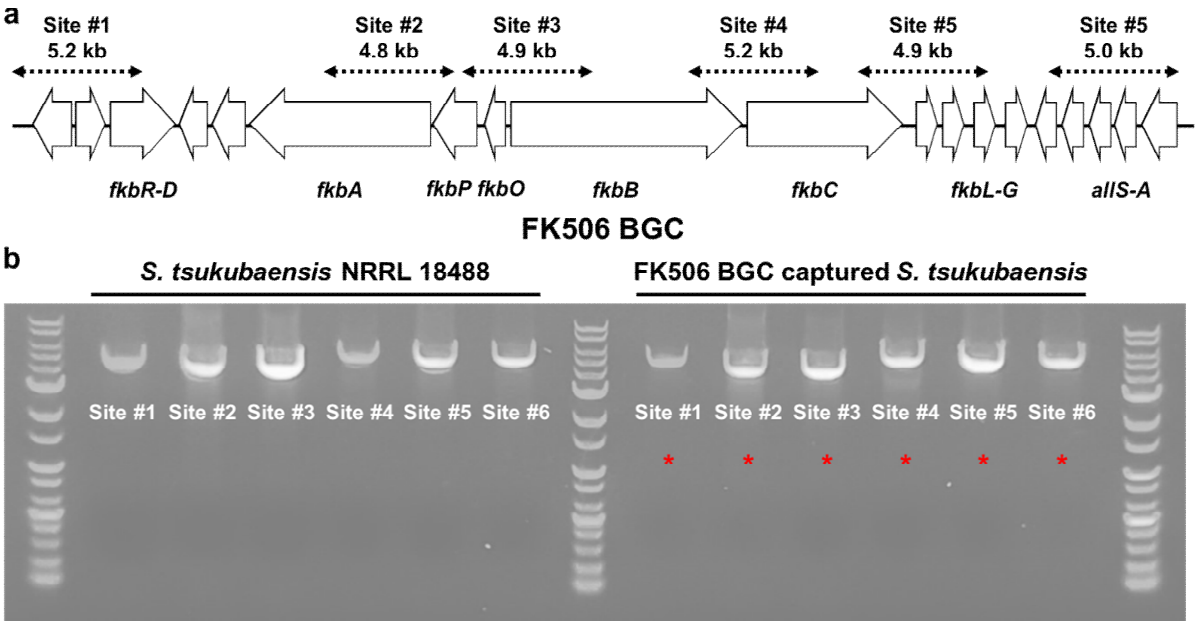

Figure R3. Confirmation of the FK506 BGC in *S. tsukubaensis* NRRL 14844 after *in vivo* BGC capturing. a FK506 BGC region in the genome of *S. tsukubaensis* NRRL 14844 was presented with six loci for PCR amplification to confirm its presence. **b** FK506 BGC sequence was confirmed via PCR amplification and subsequent sequence analysis.

8. Line 284-285, transcriptional analysis should be provided for the dCas9 and dCas9-BD-based CRISPRi.

Response: As requested by the reviewer, transcriptomic analysis was conducted using *S. coelicolor* M1146 strains containing dCas9 or dCas9-BD with and without three sgRNAs targeting *gusA* (Supplementary Fig. 11). Differentially expressed genes (DEGs) were calculated by comparison with the control strain (SCOXG). Both dCas9 and dCas9-BD effectively inhibited of *gusA* expression via sgRNAs (Supplementary Fig. 11d, e). However, no significant differences in transcriptomic patterns were observed between the strains expressing dCas9 and dCas9-BD (SCO0G vs SCO0G*), In the strains with sgRNAs (SCO3G and SCO3G*), genes up- or down- regulation were less pronounced compared to those without sgRNAs (SCO0G and SCO0G*) likely due to the slow growth rates, especially that of the SCO3G strain (Supplementary Fig. 10j).

These results were explained in revised manuscript, line 320-324.

9. Line 311-314, similarly, suggest drawing a detailed workflow for the construction of the plasmid library to facilitate reader understanding.

Response: Thank you for your opinion. The figure describing detailed workflow for construction of the plasmid library for multiple inhibition about expression of various genes was newly added in Supplementary Fig. 12.

10. Line 319-325, it is recommended to test the levels of the Acetyl-CoA and malonyl-CoA in these mutant with significantly enhanced production of three different metabolites.

Response: Thank you for the reviewer's comments. The concentrations of acetyl-CoA and malonyl-CoA were measured using reverse phase HPLC with a C18 column. The results were presented in Supplementary Fig. 14, showing that acetyl-CoA and malonyl-CoA were generally higher in strains harboring dCas9-BD with multiple sgRNAs.

These results were explained in revised manuscript, line 355-356. Also, the method for measuring acetyl-CoA and malonyl-CoA was added in the manuscript, line 681-692.

11. Supplementary Fig.5. The electrophoresis image is too blurry, please replace them.

Response: Thank you for your comments. The figure was generated with DNA from PCR amplification using boiled colonies as template without further purification, which resulted in unclear PCR product bands. Unfortunately, replicating the experiment is not feasible at this time, so that the figure has been revised by enhancing the color contrast and resolution for clarity.

The updated figure was presented in Supplementary Fig. 9.

Additional Revision

1. New authors who involved in the genome and transcriptome analysis have been added to the revised manuscript.
2. Genome sequencing results revealed that the *S. coelicolor* strain used in this experiment was M1146, not M1152. We have updated the strain name accordingly throughout the manuscript.
3. The references added during the revision have been included in the list and marked in red.

References

1. Sangsu Bae, Jeongbin Park, Jin-Soo Kim, Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases, *Bioinformatics,* **10**, 1473-1475 (2014).
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12. Celler K., Koning RI., Willemse J., *et al*., Cross-membranes orchestrate compartmentalization and morphogenesis in *Streptomyces*, *Nat. Commun.,* **7**, 1 (2016).

Response to Reviewers' Comments

NCOMMS-24-11378

Engineered CRISPR-Cas9 for Streptomyces sp. genome editing to improve specialized metabolite production

Duck Gyun Kim, Boncheol Gu, Yujin Cha, Jeonghan Ha, Yongjae Lee, Gahyeon Kim, Byung-Kwan Cho, & Min-Kyu Oh

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We have responded to the reviewer's comments as the followings:

Reviewer #1: The authors have thoroughly addressed all my questions, significantly improving the clarity and completeness of the manuscript. The revisions have successfully incorporated my suggestions, resulting in a much stronger and more impactful paper. I have only minor formatting suggestions. The authors should revise the spelling of some words throughout the manuscript; for example, "exoconjugats" (lines 178, 189, 267, 295, etc.) and "transcritomic" (line 348). With these minor corrections, I believe the manuscript is ready for publication.

1. I have only minor formatting suggestions. The authors should revise the spelling of some words throughout the manuscript; for example, "exoconjugats" (lines 178, 189, 267, 295, etc.) and "transcritomic" (line 348).

Response: Thank you for your comment. The manuscript has been revised accordingly.

Reviewer #3: The authors have addressed most of my concerns raised previously. The following comments should be further addressed. 1. For the verification of the correctness of the captured BGCs, PCR analysis is not enough. I strongly recommend that the authors should conduct restriction enzyme digestion and DNA sequencing of the captured BGCs. 2. Fig.2, in the picture, M1152 should be M1146. 3. In response to Reviewers' Comments: the author mentioned that "there have been no previous reports of genome editing in those strains". To my knowledge, in S. rapamycinicus, CRISPR/Cas12a has been employed for genome editing. The authors can check it. 4. In supplementary Fig.9, the results of PCR analysis is not convincing. I don't think they are specific PCR products. 5. I have some doubts about the data of the multiplexed promoter replacement. According to my experience, the authors obtained just about 4 exconjugants for single promoter replacement after conjugation (Supplementary Figure 5)； Under the same conditions, it is difficult to obtain exconjugants for multiplexed promoter replacement. Please provide the numbers of the exconjugants for multiplexed promoter replacement.

1. For the verification of the correctness of the captured BGCs, PCR analysis is not enough. I strongly recommend that the authors should conduct restriction enzyme digestion and DNA sequencing of the captured BGCs.

Response: Thank you for your comment. To ensure accurate capture of the FK506 BGC, the plasmid containing the BGC region was amplified via PCR in 8 fragments and sequenced using a long-range sequencing method. Details of the sequencing experiment design are presented in Supplementary Fig.

10, while the sequencing results are included as a separate Excel file in the Supplementary Data.

A brief description of this experiment has been added to the revised manuscript (lines 280–282), along with the newly provided Supplementary Fig. 10 and Supplementary Data.

2. 2. Fig.2, in the picture, M1152 should be M1146.

Response: Thank you for the reviewer's comments. The figure and two other mistakes were fixed accordingly.

3. 3. In response to Reviewers' Comments: the author mentioned that "there have been no previous reports of genome editing in those strains". To my knowledge, in S.rapamycincius, CRISPR/Cas12a has been employed for genome editing. The authors can check it.

Response: Thank you for the reviewer's comments. We have found the previously published paper on *S, rapamycinicus* genome editing in the rapamycin BGC using CRISPR/Cas12a[1]. While our work represents the first application of CRISPR/Cas9 for multiplexed editing in *S. rapamycinicus*, the statement in our previous response to Comment 9 of Reviewer #1 (page 6), which claimed, "To our knowledge, no studies have reported *in vivo* application of CRISPR/Cas9, including nCas9 or dCas9, in *S. antibioticus* or *S. rapamycinicus*," was partially incorrect.

4. 4. In supplementary Fig.9, the results of PCR analysis is not convincing.I don't think they are specific PCR products.

Response: Thank you for your comments. To confirm the presence of the BGC-harboring plasmid, a two-step verification process was conducted. First, a negative confirmation step was performed to exclude colonies containing the plasmid without the BGC. This was followed by a positive confirmation step to identify colonies with the plasmid containing the target BGC. During this process, PCR amplification was initially performed using boiled colonies as templates, resulting in unclear images of the PCR products in the figure. To provide more reliable results, the plasmids were extracted and used as templates for PCR amplification at this time. Additionally, the figure images from the negative confirmation step were excluded, as they were not critical for identifying plasmids with the desired BGC.

These results have been revised in the Supplementary Fig. 9.

5. 5. I have some doubts about the data of the multiplexed promoter replacement. According to my experience, the authors obtained just about 4 exconjugants for single promoter replacement after conjugation (Supplementary Figure 5)；Under the same conditions, it is difficult to obtain exconjugants for multiplexed promoter replacement. Please provide the numbers of the exconjugants for multiplexed promoter replacement.

Response: Thank you for your comment. As described in lines 554–556 and 603–604 of the manuscript, 10 μL of spore stock, concentrated to 1.0×10^8 CFU/mL, was used for most editing experiments. However, due to the challenges associated with multiplexed promoter replacement in *S. rapamycinicus* NRRL 5491, 30 μL of spore stock was used exclusively for this experiment. As a result, triplicated experiments yielded a total of 12 exconjugants for single promoter replacement (Supplementary Fig. 5) and 3 exconjugants for multiplexed promoter replacement, even with a threefold higher spore quantity (Fig. 2). While multiplexed promoter replacement proved more challenging than single replacement, increasing the spore stock for conjugation enabled the successful generation of exconjugants.

To clarify this point, additional explanations were added in line 181, 202, and 604 of the revised manuscript.

References

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---

We have responded to the reviewer's comments as the followings:

Reviewer #3: The authors have addressed all of my comments and concerns. I have no other comments.

Response: Thank you for your comment and opinion about the paper.