CRISPR-Cas strategies for natural product discovery and engineering in actinomycetes

Elena, Heng[a,c], Lee Ling, Tan[a,c], Mingzi M., Zhang[b], Fong Tian, Wong[a]

[a] Molecular Engineering Lab, Institute of Bioengineering and Nanotechnology, A*STAR, 138669, Singapore

[b] Institute of Molecular and Genomic Medicine, National Health Research Institutes, Zhunan, Miaoli, 35053, Taiwan

[c] These authors contributed equally

To whom correspondence may be addressed:

zhangmz@nhri.edu.tw, 35 Keyan Road, Zhunan, Miaoli, 35053, Taiwan

wongft@ibn.a-star.edu.sg, 31 Biopolis Way, Nanos, 138669, Singapore

Abstract

Fuelled by CRISPR-Cas technology, a synergistic combination of advances in genomics, synthetic biology and bioinformatics ushered in an era of genome-guided natural product discovery and engineering. As a versatile programmable DNA targeting tool, CRISPR-Cas is increasingly employed to accelerate host and pathway engineering of actinomycetes, which are prolific producers of bioactive natural products. Here we discuss the current state of the art, challenges and opportunities of CRISPR-Cas strategies to discover, diversify and improve the production of natural products from actinomycetes. Special emphasis is placed on the rapidly expanding CRISPR-Cas toolboxes for genome editing of non-*Streptomyces* actinomycetes.

Keywords: Genome editing; Actinobacteria; *Streptomyces*; *Corynebacterium*; *Mycobacterium; Saccharopolyspora*

Introduction

Microorganisms of the Actinomycetales order (e.g. *Streptomyces, Corynebacterium, Micromonospora, Nocardia*) are prolific producers of structurally complex secondary metabolites, which are known as natural products (NPs) [1, 2]. Throughout our history, NPs have been invaluable to human health and a sizable proportion of current antibiotics and drugs are natural products or natural product derived [3]. In 2002, the genome of extensively studied *Streptomyces coelicolor* A3(2) unveiled many more biosynthetic gene clusters (BGCs) than the number of observed NPs [4-6]. Subsequent analyses of different microbial genomes [7-9] and metagenomes [10] revealed a vast number of unexplored BGCs that can potentially encode novel NPs to tackle emerging diseases and multi-drug resistant infections [11]. With continued advancement of genomic technologies (Illumina [12], PacBio [12], Nanopore [13]), as well as bioinformatics resources to identify and prioritize BGCs [14-18], the biosynthetic potential of organisms and even microbiomes can now be rapidly interrogated *in silico*. However, translating genomic information to structural and activity characterization of the encoded NPs remains a major bottleneck in the NP discovery pipeline.

Genetic manipulation of biosynthetic pathways is increasingly used to generate structural diversity and improve production of target NPs for drug discovery and lead development. Comparatively, total synthesis of some structurally complex natural products is considered heroic efforts even among chemists [19]. For certain NP classes, extensive biochemical and structural studies of the relevant biosynthetic enzymes have enabled efficient rational engineering and production of bioactive NPs *in vitro* [20], in heterologous [21] and native hosts [22, 23]. Nonetheless, the genetic intractability of non-model actinomycetes as well as challenges in manipulating BGCs, which are often large and GC-rich, present formidable barriers to large-scale, high-throughput engineering efforts.

At the same time, powered by synthetic biology, important advances have emerged to accelerate DNA synthesis and assembly [24]. These included Gibson assembly [25], Golden Gate assembly [26] and yeast recombination [27]. Complementing these technologies are DNA cutters for precise genome editing, ranging from the meganuclease I-SceI [28], zinc-finger nucleases [29], transcription activator-like effector nucleases [30], to RNA-guided CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-associated nucleases [31]. Initially discovered as an adaptive defense mechanism in bacteria [32], the easily programmable CRISPR-Cas (CRISPR-associated proteins) systems were rapidly adapted for genome editing and other applications in a wide range of organisms [33-35].

The first CRISPR-Cas systems introduced for rapid and efficient genome editing of *Streptomyces* genomes unanimously employed the codon-optimized *Streptococcus pyogenes* Cas9 nuclease (SpCas9). Detailed comparisons of the pCRISPomyces [36], pKCcas9dO [37], pCRISPR-Cas9 [38] and pWHU2653 [39] systems can be found in the review by Alberti *et al.* [40]. Subsequent development of CRISPR systems using different Cas proteins, including *Streptococcus thermophilus* CRISPR1 (Sth1Cas9), *Staphylococcus aureus* Cas9, and *Francisella tularensis* subsp. *novicida* U112 Cpf1 (FnCpf1) for genome editing and transcriptional regulation offer opportunities to circumvent context-specific limitations of SpCas9 systems in *Streptomyces* [41, 42]. Notably, Cpf1 has the added advantage of being able to process its own pre-crRNA (CRISPR RNA), allowing for multiple targets to be transcribed on a single RNA array for multiplex genome editing [42-45]. Since their introduction in 2015, the original CRISPR-SpCas9 systems have been modified to alleviate Cas9 toxicity, improve on-target editing and facilitate screening of desired clones [46].

In this review, we provide an overview of CRISPR-Cas technological advances and strategies that are useful for NP discovery and engineering in actinomycetes. Special emphasis is placed on the adaptation and application of CRISPR-Cas systems for genome editing of rare actinomycetes (Table 1), which constitute an important source of novel NPs [47-49]. Last but not least, we discuss the challenges and opportunities for widespread adoption of CRISPR-Cas technologies in actinomycetes for NP discovery and engineering (Figure 1).

Application of CRISPR-Cas for host engineering

CRISPR-Cas systems use easily programmable mechanisms to generate targeted double-strand breaks (DSBs) in DNA [32]. With CRISPR-Cas mediated genome editing, facile and efficient gene disruptions, insertions, site-directed mutations as well as deletion of entire BGCs can be achieved in actinomycetes, accelerating genomics-guided NP discovery. Straightforward applications of CRISPR-Cas include BGC engineering and activation in native producers. To activate or improve NP production, constitutive promoters can be inserted to drive expression of main biosynthetic operons or pathway-specific activators [50, 51]. Additionally, the production and distribution of NPs and their intermediates or by-products may be manipulated genetically. Deletion of pathway-specific repressor *papR3* in *Streptomyces pristinaespiralis* yielded sole production of pristinamycin I, facilitating downstream purification while avoiding the undesirable synergistic cytotoxic effects of pristinamycins I and II [52]. Deleting an in-cluster acyltransferase improved dalbavancin yields by 25% in *Nonomuraea gerenzanensis sp.* ATCC 39727 by eliminating an inhibitory acetylated product [53]. Besides targeting specific BGCs, genetic manipulation of global regulatory genes can modulate NP yields. In *Streptomyces coelicolor* M145, deleting the nitrogen metabolism regulator *GlnR* increased actinorhodin production but decreased undecylprodigiosin production [54-56]. Inactivation of widely distributed streptothricin and streptomycin BGCs

aided the discovery of novel bioactive NPs of low abundance [57]. Culp and coworkers showed that guide RNAs designed to target conserved gene arrangements within BGCs would work in different strains without prior knowledge of target sequences, thus allowing a general NP discovery strategy for strain collections. Last but not least, genetic manipulation or engineering of enzymes in NP biosynthetic pathways are increasingly used to diversify and generate NP analogs [58-60].

Beyond generating targeted DSBs for genome editing by homology-directed repair (HDR) or error-prone non-homologous end joining (NHEJ), CRISPR-Cas variations involving deactivated nucleases or nickases have been adapted to modulate NP production in actinomycetes. Stable integrative multiplex CRISPR-interference systems using catalytically inactive Cas9 and Cpf1 have been established for reversible control of gene expression. CRISPR-interference systems have been used for the simultaneous repression of yellow-pigmented cryptic polyketone, actinorhodin and prodiginine BGCs in *S. coelicolor* [38, 42, 61]. Notably in these studies, CRISPR interference efficiencies drop significantly after the third interference target. A deactivated Sth1Cas9 has been used for gene repression in *Rhodococcus opacus* and mycobacteria [62, 63]. For targeted genome modifications, base editing involving Cas9 nickases fused to cytidine or adenine deaminases offers an attractive alternative to HDR-based genome editing for the multiplexed gene inactivation [64-66]. By passing the requirement for long homologous repair templates, base editing facilitates multiplex and high throughput genome editing. By harnessing Csy4-based sgRNA (single guide RNA) multiplexing, Tong and colleagues could simultaneously inactivate three enzymes in different BGCs in *Streptomyces* [66]. In *Corynebacterium glutamicum*, Ma's group demonstrated multiplex base editing of three different enzymes and applied the method to inactivate 94 transcription factors within 9 days at 100% efficiencies [64].

Application of CRISPR-Cas for heterologous NP production

Heterologous expression of BGCs will enable production and engineering of NPs, especially when the native host strain is inaccessible or genetically intractable. The availability of substrates, post-translational modification enzymes and innate tolerance of *Streptomyces* towards bioactive NPs make them preferred chassis for NP biosynthesis [67]. CRISPR-Cas mediated strategies can facilitate pathway and host optimization for heterologous expression of BGCs from actinomycetes [68-71]. Fazal *et al.* coupled CRISPR-Cas genome editing with conventional marker exchange mutagenesis and PCR-targeted recombineering techniques to disrupt five endogenous BGCs producing antimycin, candicidin, albaflavenone, surugamide and fredericamycin in *Streptomyces albus* S4, removing its antifungal and antibacterial properties [68]. They subsequently used this genome-reduced chassis host for the production of target NPs such as actinorhodin, cinnamycin and prunustatin. CRISPR-Cas technologies are expected to accelerate the typically tedious construction and optimization of genome-reduced specialized chassis.

Successful heterologous BGC expression requires cloning and refactoring of the required biosynthetic pathways. In actinomycetes, more often than not, large GC-rich and repetitive gene sequences would be central to NP biosynthesis [72, 73]. Traditional methods rely on unique restriction sites and enzymes to excise target BGCs for subsequent DNA capturing and refactoring strategies involving plasmid rescue [74], Gibson assembly [75] and transformation-associated recombination (TAR) [76]. Relying on naturally occurring restriction sites, Truman's group replaced promoters, deleted genes and introduced targeted mutations in one step by combining TAR cloning and yeast-mediated homologous recombination [77]. This approach is unhindered by high-GC content and enables multiple

modifications to the BGCs in a single step, but is limited by the availability of restriction sites. The *in vitro* application of CRISPR-Cas9 as a programmable DNA cutter circumvents this limitation. For example, CATCH (Cas9-assisted targeting of chromosome segments) has been used to excise up to 100 kb of DNA from bacterial chromosomes *in vitro* for subsequent capture via Gibson assembly [78]. To enhance *in vitro* BGC capture and refactoring, Liu *et al.* significantly reduced off-target effects and increased efficiencies by introducing an end repair step with T4 DNA polymerase with CRISPR-Cas [79]. mCRISTAR (multiplex CRISPR and TAR) [80] and mpCRISTAR (multiple plasmid approach CRISPR-Cas9 and TAR) [81] combined CRISPR-Cas9 and TAR for multiplex promoter refactoring of BGCs in yeast. With two rounds of mCRISTAR, the silent Tam cluster was refactored with eight characterized synthetic promoters for heterologous production of tetarimycin A in *S. albus* [80]. Instead of a single CRISPR array, spacer sequences in mpCRISTAR were distributed across four plasmids to achieve stable introduction of spacer sequences and higher multiplex efficiencies [81]. Alternatively, multiplex *in vitro* Cas9-TAR (*mi*CASTAR) circumvents the need for multiple plasmids in the CRISTAR systems and enabled combinatorial systematic refactoring of the atolypene BGC from *Amycolatopsis tolypomycina* NRRL B-24205 [82].

Challenges and opportunities in the application of CRISPR-Cas strategies in actinomycetes

Notwithstanding advances in CRISPR-Cas based engineering methods, it remains challenging to genetically manipulate actinomycetes, especially so for the less characterized and non-*Streptomyces* strains. Here we highlight the challenges, opportunities and technical considerations in adapting currently available CRISPR-Cas systems for successful genome editing of actinomycetes, with an emphasis on rare actinomycetes (Table 1). While parts of this

176 section may apply to other genetic tools including site-specific recombinases and transposons,
177 we will discuss them in the context of CRISPR-Cas engineering in actinomycetes.
178
179 There is a paucity of genetic parts to fully harness the potential of the CRISPR
180 technology in actinomycetes. CRISPR-Cas based genome or transcriptome engineering
181 methods require simultaneous introduction of multiple elements such as Cas effector proteins,
182 guide RNAs, repair templates and sometimes recombinases or reporter genes into target cells.
183 Notably, direct adoption of the original *Streptomyces* CRISPR-Cas systems without additional
184 modifications have proven successful in *Actinoplanes* and *Micromonospora* strains [83-87],
185 proving that these genetic parts can work in some rare actinomycetes. For other actinomycetes
186 or genomic contexts, however, identifying suitable vectors, promoters and selection markers is
187 a key first step for successful genome editing [88]. For example, replacing commonly used
188 temperature-sensitive pSG5 replicon with the segregationally unstable pIJ101 replicon has
189 been shown to overcome undesirable recombination events and enable editing of challenging
190 DNA regions with high sequence identities in *Saccharopolyspora erythraea* and *Streptomyces*
191 sp. AL2110 [89]. The counter-selectable CodA(sm) marker have proven useful for isolating
192 double crossover mutants and allow recycling of CRISPR plasmids after genome editing as
193 well as plasmid curing, but its use is limited to host strains that are resistant to 5-fluorocytosine
194 while sensitive to 5-fluorouracil [39, 89, 90]. In situations requiring sequential or co-
195 transformation of multiple DNA constructs, as in the case for *Corynebacterium*,
196 *Mycobacterium* and *Rhodococcus* CRISPR-Cas systems [64, 65, 91-97], compatible
197 integrative and replicative vectors with orthogonal selection or counter-selection markers are
198 indispensable but not always available depending on the target strain. The ability to fine-tune
199 or temporally restrict Cas function can minimize unwanted toxicity and off-target effects
200 associated with constitutive Cas expression (vide infra). While there has been significant

progress in identifying constitutive promoters of varying strengths that function in multiple strains, the choice of minimal synthetic constitutive or inducible promoters that can be used in different actinomycete strains is relatively limited [98-100]. Besides facilitating CRISPR-Cas activity, new genetic parts can be used to accelerate screening protocols. To expedite screening of edited strains and plasmid curing, chromogenic reporter systems were included in an updated CRISPR-Cas system for *S. coelicolor* and *Verrucosispora* sp. [101], where deletion efficiencies of 100% were achieved. An expanded catalog of well-characterized genetic parts, including promoters, selection/counterselection markers and replicons, will stimulate the adoption of CRISPR technologies in the genetic engineering of rare actinomycetes.

Another major bottleneck is the challenge of efficiently introducing recombinant DNA into cells. For actinomycetes, recombinant DNA can be typically introduced into cells by protoplast transformation, electroporation or conjugation. Compared to conjugative methods, protoplast transformation and electroporation are more amendable to multiplexing and allow the introduction of linear or single-stranded donor DNA. However, transformation efficiencies inversely correlate with the size of the DNA constructs [102]. This has to be considered when designing CRISPR constructs for transformation or electroporation since all-in-one plasmids routinely exceed 10 kb. These all-in-one plasmids will have to contain expression cassettes for Cas protein(s), sgRNAs, selectable/counter-selectable markers as well as templates for donor DNA [40]. By using PEG3350, Liu and coworkers managed to increase protoplast transformation efficiency of large all-in-one CRISPR-Cas plasmids that are needed for successful genome editing in *S. erythraea* [50, 103]. When efficient introduction of large plasmids cannot be achieved, CRISPR-Cas elements can be introduced on separate constructs by multiplex or sequential transformation depending on the overall transformation or electroporation efficiencies as demonstrated for *Corynebacterium* and *Rhodococcus* CRISPR-

226 Cas systems [91-95, 97, 104]. On the other hand, intergeneric conjugation is the only way to
227 deliver very large (>40 kb) bacterial artificial chromosomes or phage P1-
228 derived artificial chromosomes carrying entire BGCs into heterologous hosts [105-107].
229 Besides optimizing physical parameters such as media and buffer composition as well as
230 culture age and conditions, identifying strategies to interfere or bypass the restriction systems
231 of non-model actinomycetes can also be critical for boosting the efficiencies of getting foreign
232 DNA into cells [88, 97]. In general, working with unexplored actinomycete strains often
233 requires systematic screening and optimization to efficiently and reproducibly deliver foreign
234 DNA into cells, which may or may not succeed. Heterologous BGC expression can circumvent
235 these limitations, especially since heterologous host systems are more genetically amendable
236 with more matured genetic engineering tools including the CRISPR-Cas toolbox [67, 108,
237 109].
238
239 Despite successful examples of genome editing employing the well-characterized
240 SpCas9 in actinomycetes, SpCas9 might not always work due to toxicity or unwanted off-target
241 effects. Depending on the strain or target genome sequence, constitutive SpCas9 expression
242 has been observed to considerably reduce the number of recovered exconjugants or
243 transformants [38, 39, 41, 97, 110]. Besides applying sgRNA design algorithms to minimize
244 off-targets [111-113], the toxicity problem may also be alleviated by temporal modulation
245 and/or fine-tuning of SpCas9 activity at the transcriptional, translational or protein levels to
246 balance nuclease-induced toxicity and genome editing efficiencies [54, 89, 91, 110]. The use
247 of alternative Cas proteins with different PAM (protospacer adjacent motif) requirements can
248 also circumvent SpCas9 toxicity and improve the probability of recovering mutants with the
249 desired genomic edits [41, 91]. This may be due to the different levels of SpCas9 and other Cas
250 proteins such as Cpf1 that is needed for genome editing [42, 114]. Notably, only Sth1Cas9,

and not SpCas9 or Cpf1, works for genome editing in *Mycobacterium tuberculosis* [96]. Genome instability, resulting from DSBs, remains a challenge for CRISPR-Cas-based editing of actinomycete genomes [115, 116]. For example, unrepaired DSBs can cause large-scale genome rearrangements and deletions leading to cell death. To repair Cas-induced DSBs while introducing indels for gene inactivation, exogenous expression of ATP-dependent ligase LigD rescued cell damage by complementing incomplete NHEJ repair pathway in *Streptomyces* [38, 42]. Concurrent overexpression of NHEJ machinery and repression of homologous recombination enabled efficient CRISPR-assisted NHEJ genome editing in *Mycobacterium smegmatis*, *M. tuberculosis* and *C. glutamicum* [96, 117, 118]. On the other hand, to generate precise mutations, overexpression of RecET homologs Che9c60 and Che9c61 in mycobacteria and *Rhodococcus* drastically improved HDR-mediated genome engineering [98]. Similarly, introduction of the bacterial recombinase RecA enhances HDR-mediated editing efficiency in *N. gerenzanensis* [53]. Interestingly, recombinase overexpression to enhance HDR of DSBs may not be a general strategy as demonstrated by failed attempts in *S. coelicolor* [110]. Overall, the efficiency and success of CRISPR-Cas-mediated genome editing strategies are highly dependent on the context such as host strain, target genome sequence and desired edits. For challenging edits, testing multiple sgRNAs and Cas proteins will increase the probability of success. Lastly, the off-target effects of CRISPR-Cas mediated genome editing in actinomycetes will need to be systematically examined. On one hand, whole-genome sequencing did not reveal significant off-target effects of SpCas9 in *S. coelicolor* [38, 39], *Actinoplanes* sp. SE50/110 [83], and Sth1Cas9 in mycobacteria [96]. On the other hand, whole-genome sequencing of base edited *C. glutamicum* strains revealed 22 single nucleotide variants that include likely off-targets of D10A Cas9 nickase [64].

The relatively advanced CRISPR technologies in *Escherichia coli*, yeast and mammalian systems provide a glimpse of the opportunities that CRISPR-Cas can offer to tap into the enormous biosynthetic potential of actinomycetes. One of these opportunities is multiplex genome editing. Currently, a combination of Cas toxicity, low transformation and recombination efficiencies in actinomycetes has impeded multiplex genome editing. CRISPR-Cas mediated editing of multiple gene loci by HDR is further hindered by the requirement for long homology templates (typically ≥2 kb for each locus) that has to be simultaneously introduced into cells [40]. To date, a maximum of two deletions by Cas-mediated HDR has been reported in model *Streptomyces* species [36, 37, 42], while multiplex genome engineering using base editing can simultaneously target up to three genomic sites effectively in *Streptomyces* [66] and *C. glutamicum* [64]. Given the long doubling times of some actinomycetes, the ability to perform multiple genome edits in a single round will greatly increase experimental throughput for combinatorial biosynthesis and chassis optimization for heterologous NP production. In addition to the various optimization strategies mentioned above, the DNA/RNA complexity, targeting and stability of sgRNAs are also key considerations for efficient multiplex genome editing. Using biophysical modelling, a specially designed non-repetitive extra-long sgRNA array was shown to express up to 22 functional sgRNAs in *E. coli* [119]. By relieving the requirements for long homology repair templates and dependence on inefficient DNA repair mechanisms, DSB-free CRISPR editing methods, such as base editing, will likely pave the way to multiplex genome editing in actinomycetes. Advances, such as prime editing, also promise DSB-free genomic edits without the need for donor DNA and with potentially significantly lower off-target effects compared to conventional CRISPR-Cas strategies [120].

Conclusion

Recent revolutions in genomics and molecular engineering as well as the development of disruptive genome engineering technologies have reinvigorated NP discovery efforts in actinomycetes. Widely distributed cryptic or silent clusters are unchartered and valuable territory for NP discovery. Propelling genome-guided NP discovery are CRISPR-Cas technologies and applications that help accelerate the translation of genetic information into molecules. However, fundamental challenges, such as the genetic intractability of uncharacterized strains and Cas toxicity, hinder the widespread adoption of CRISPR-Cas for actinomycetes. Solutions to these bottlenecks, combined with next generation CRISPR-Cas technologies, will be the next great leap forward in our ability to genetically engineer these industrially important microorganisms and accelerate NP discovery, diversification and production.

Acknowledgements

We would like to specially acknowledge the late Sydney Brenner for his support and guidance.

Author contributions

Mingzi M., Zhang, Fong Tian Wong: Conceptualization, writing – original draft, review and editing, visualization, **Elena Heng, Lee Ling Tan:** Writing – original draft, review and editing, visualization.

Funding

F.T.W, **E.H.** and **L.L.T.** are supported by National Research Foundation, Singapore [NRF-CRP19-2017-05].

Declaration of interest

None

References

[1] Barka, E.A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.P., Clément, C., Ouhdouch, Y., van Wezel, G.P., 2016. "Taxonomy, physiology, and natural products of actinobacteria." *Microbiology and Molecular Biology Reviews*, 80.1, 1–43.

[2] Genilloud, O., 2017. "Actinomycetes: Still a source of novel antibiotics." *Natural Product Reports*, 34.10, 1203–32.

[3] Newman, D.J., Cragg, G.M., 2016. "Natural products as sources of new drugs from 1981 to 2014." *Journal of Natural Products*, 79.3, 629–61.

[4] Bentley, S.D., Chater, K.F., Cerdeño-Tárraga, A.M., Challis, G.L., Thomson, N.R., James, K.D., Harris, D.E., Quail, M.A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C.W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C.H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M.A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B.G., Parkhill, J., Hopwood, D.A., 2002. "Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2)." *Nature*, 417.6885, 141–47.

[5] Challis, G.L., Ravel, J., 2000. "Coelichelin, a new peptide siderophore encoded by the *Streptomyces coelicolor* genome: Structure prediction from the sequence of its non-ribosomal peptide synthetase." *FEMS Microbiology Letters*, 187.2, 111–14.

[6] Challis, G.L., 2008. "Genome mining for novel natural product discovery." *Journal of Medicinal Chemistry*, 51.9, 2618–28

[7] Omura, S., Ikeda, H., Ishikawa, J., Hanamoto, A., Takahashi, C., Shinose, M., Takahashi, Y., Horikawa, H., Nakazawa, H., Osonoe, T., Kikuchi, H., Shiba, T., Sakaki, Y., Hattori, M., 2001. "Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deducing

the ability of producing secondary metabolites." *Proceedings of the National Academy of Sciences*, 98.21, 12215–20.

[8] Xu, M., Wang, Y., Zhao, Z., Gao, G., Huang, S.X., Kang, Q., He, X., Lin, S., Pang, X., Deng, Z., Tao, M., 2016. "Functional genome mining for metabolites encoded by large gene clusters through heterologous expression of a whole-genome bacterial artificial chromosome library in *Streptomyces* spp." *Applied and Environmental Microbiology*, 82.19, 5795–805.

[9] Peng, Q., Gao, G., Lü, J., Long, Q., Chen, X., Zhang, F., Xu, M., Liu, K., Wang, Y., Deng, Z., Li, Z., Tao, M., 2018. "Engineered *Streptomyces lividans* strains for optimal identification and expression of cryptic biosynthetic gene clusters." *Frontiers in Microbiology*, 9, 3042.

[10] Libis, V., Antonovsky, N., Zhang, M., Shang, Z., Montiel, D., Maniko, J., Ternei, M.A., Calle, P.Y., Lemetre, C., Owen, J.G., Brady, S.F., 2019. "Uncovering the biosynthetic potential of rare metagenomic DNA using co-occurrence network analysis of targeted sequences." *Nature Communications*, 10.1, 1-9.

[11] Kemung, H.M., Tan, L.T.H., Khan, T.M., Chan, K.G., Pusparajah, P., Goh, B.H., Lee, L.H., 2018. "*Streptomyces* as a prominent resource of future anti-MRSA drugs." *Frontiers in Microbiology*, 9, 2221.

[12] Gomez-Escribano, J.P., Alt, S., Bibb, M.J., 2016. "Next generation sequencing of actinobacteria for the discovery of novel natural products." *Marine Drugs*, 14.4, 78.

[13] Walker, T., Stapleton, P., Gibbons, S., 2019. "Identification of novel antimicrobial-producing bacteria from an ancient water source by Oxford Nanopore Whole Genome Sequencing and Natural Product Chemistry." *Access Microbiology*, 1.1A.

[14] Tran, P.N., Yen, M.R., Chiang, C.Y., Lin, H.C., Chen, P.Y., 2019. "Detecting and prioritizing biosynthetic gene clusters for bioactive compounds in bacteria and fungi." *Applied Microbiology and Biotechnology*, 103.8, 3277-3287.

[15] Medema, M.H., Fischbach, M.A., 2015. “Computational approaches to natural product discovery.” *Nature Chemical Biology*, 11.9, 639.

[16] Blin, K., Shaw, S., Steinke, K., Villebro, R., Ziemert, N., Lee, S.Y., Medema, M.H., Weber, T., 2019. “AntiSMASH 5.0: Updates to the secondary metabolite genome mining pipeline.” *Nucleic Acids Research*, 47. W1, W81-W87

[17] Skinnider, M.A., Merwin, N.J., Johnston, C.W., Magarvey, N.A., 2017. “PRISM 3: Expanded prediction of natural product chemical structures from microbial genomes.” *Nucleic Acids Research*, 45. W1, W49-W54.

[18] Kautsar, S.A., Blin, K., Shaw, S., Navarro-Muñoz, J.C., Terlouw, B.R., Van Der Hooft, J.J.J., Van Santen, J.A., Tracanna, V., Suarez Duran, H.G., Pascal Andreu, V., Selem-Mojica, N., Alanjary, M., Robinson, S.L., Lund, G., Epstein, S.C., Sisto, A.C., Charkoudian, L.K., Collemare, J., Linington, R.G., Weber, T., Medema, M.H., 2020. “MIBiG 2.0: A repository for biosynthetic gene clusters of known function.” *Nucleic Acids Research*, 48.D1, D454-D458.

[19] Baran, P.S., 2018. “Natural product total synthesis: As exciting as ever and here to stay.” *Journal of the American Chemical Society*, 140.14, 4751–55.

[20] Miyazawa, T., Hirsch, M., Zhang, Z., Keatinge-Clay, A.T., 2020. “An *in vitro* platform for engineering and harnessing modular polyketide synthases.” *Nature Communications*, 11.1, 1-7.

[21] Awakawa, T., Fujioka, T., Zhang, L., Hoshino, S., Hu, Z., Hashimoto, J., Kozone, I., Ikeda, H., Shin-Ya, K., Liu, W., Abe, I., 2018. “Reprogramming of the antimycin NRPS-PKS assembly lines inspired by gene evolution.” *Nature Communications*, 9.1, 1-10.

[22] Shen, J.J., Chen, F., Wang, X.X., Liu, X.F., Chen, X.A., Mao, X.M., Li, Y.Q., 2018. “Substrate specificity of acyltransferase domains for efficient transfer of acyl groups.” *Frontiers in Microbiology*, 9, 1840.

[23] Peng, H., Ishida, K., Hertweck, C., 2019. “Loss of single-domain function in a modular assembly line alters the size and shape of a complex polyketide.” *Angewandte Chemie International Edition*, 131.50, 18420-18424.

[24] Hughes, R.A., Ellington A.D., 2017. “Synthetic DNA synthesis and assembly: Putting the synthetic in synthetic biology.” *Cold Spring Harbor Perspectives in Biology*, 9.1, a023812.

[25] Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., Smith, H.O., 2009. “Enzymatic assembly of DNA molecules up to several hundred kilobases.” *Nature Methods*, 6.5, 343-345

[26] Engler, C., Kandzia, R., Marillonnet, S., 2008. “A one pot, one step, precision cloning method with high throughput capability.” *PLoS ONE*, 3.11, E3647.

[27] Gibson, D.G., Benders, G.A., Axelrod, K.C., Zaveri, J., Algire, M.A., Moodie, M., Montague, M.G., Venter, J.C., Smith, H.O., Hutchison, C.A., 2008. “One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic *Mycoplasma genitalium* genome.” *Proceedings of the National Academy of Sciences*, 105.51, 20404-20409.

[28] Fernandez-Martinez, L.T., Bibb, M.J., 2014. “Use of the meganuclease I-SceI of *Saccharomyces cerevisiae* to select for gene deletions in actinomycetes.” *Scientific Reports*, 4, 7100.

[29] Bulyk, M.L., Huang, X., Choo, Y., Church, G.M., 2001. “Exploring the DNA-binding specificities of zinc fingers with DNA microarrays.” *Proceedings of the National Academy of Sciences*, 98.13, 7158-7163.

[30] Zhang, F., Cong, L., Lodato, S., Kosuri, S., Church, G.M., Arlotta, P., 2011. “Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription.” *Nature Biotechnology*, 29.2, 149.

[31] Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., Zhang, F., 2013. "Multiplex genome engineering using CRISPR/Cas systems." *Science*, 339.6121, 819-823.

[32] Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity." *Science*, 3376096, 816-821.

[33] Adli, M., 2018. "The CRISPR tool kit for genome editing and beyond." *Nature Communications*, 9.1, 1911.

[34] Yao, R., Liu, D., Jia, X., Zheng, Y., Liu, W., Xiao, Y., 2018. "CRISPR-Cas9/Cas12a biotechnology and application in bacteria." *Sythetic and Systems Biotechnology*, 3.3, 135-149.

[35] Tong, Y., Weber, T., Lee, S.Y., 2019. "CRISPR/Cas-based genome engineering in natural product discovery." *Natural Product Reports*, 36, 9, 1262-1280.

[36] Cobb, R.E., Wang, Y., Zhao, H., 2015. "High-efficiency multiplex genome editing of *Streptomyces* species using an engineered CRISPR/Cas System." *ACS Synthetic Biology*, 4.6, 723–728.

[37] Huang, H., Zheng, G., Jiang, W., Hu, H., Lu, Y., 2015. "One-step high-efficiency CRISPR/Cas9-mediated genome editing in *Streptomyces*." *Acta Biochimica et Biophysica Sinica*, 47.4, 231-243.

[38] Tong, Y., Charusanti, P., Zhang, L., Weber, T., Lee, S.Y., 2015. "CRISPR-Cas9 based engineering of actinomycetal genomes." *ACS Synthetic Biology*, 4.9, 1020-1029.

[39] Zeng, H., Wen, S., Xu, W., He, Z., Zhai, G., Liu, Y., Deng, Z., Sun, Y., 2015. "Highly efficient editing of the actinorhodin polyketide chain length factor gene in *Streptomyces coelicolor* M145 using CRISPR/Cas9-CodA(sm) combined system." *Applied Microbiology and Biotechnology*, 99.24, 10575-10585.

[40] Alberti, F., Corre, C., 2019. "Editing streptomycete genomes in the CRISPR/Cas9 age." *Natural Product Reports*, 36.9, 1237-1248.

[41] Yeo, W.L., Heng, E., Tan, L.L., Lim, Y.W., Lim, Y.H., Hoon, S., Zhao, H., Zhang, M., Wong, F., 2019. "Characterization of Cas proteins for CRISPR-Cas editing in streptomycetes." *Biotechnology and Bioengineering*, 116.9, 2330-2338.

[42] Li, L., Wei, K., Zheng, G., Liu, X., Chen, S., Jiang, W., Lu, Y., 2018. "CRISPR-Cpf1-assisted multiplex genome editing and transcriptional repression in *Streptomyces*." *Applied and Environmental Microbiology*, 84.18, e00827-18

[43] Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E., Joung, J., Van Der Oost, J., Regev, A., Koonin, E. V., Zhang, F., 2015. "Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system." *Cell*, 163.3, 759-771.

[44] Zetsche, B., Heidenreich, M., Mohanraju, P., Fedorova, I., Kneppers, J., DeGennaro, E.M., Winblad, N., Choudhury, S.R., Abudayyeh, O.O., Gootenberg, J.S., Wu, W.Y., Scott, D.A., Severinov, K., van der Oost, J., Zhang, F., 2017. "Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array." *Nature Biotechnology*, 35.2, 31-34.

[45] Zhang, J., Zhang, D., Zhu, J., Liu, H., Laing, S., Luo, Y., 2020. " Efficient multiplex genome editing in *Streptomyces* via engineered CRISPR-Cas12a systems." *Frontiers in Bioengineering and Biotechnology*, 8, 726.

[46] Zhao, Y., Li, G., Chen, Y., Lu, Y., 2020. "Challenges and advances in genome editing technologies in *Streptomyces*." *Biomolecules*, 10, 5, 734.

[47] Ding, T., Yang, L.J., Zhang, W.D., Shen, Y.H., 2019. "The secondary metabolites of rare actinomycetes: chemistry and bioactivity." *RCS Advances*, 9, 21964-21988.

[48] Tiwari, K., Gupta, R.K., 2012. "Rare actinomycetes: a potential storehouse for novel antibiotics." *Critical Reviews in Biotechnology*, 32.2, 108-132.

[49] Subramani, R., Sipkema, D., 2019. “Marine rare actinomycetes: A promising source of structurally diverse and unique novel natural products.” *Marine drugs*, 17.5, 249.

[50] Liu, Y., Ren, C.Y., Wei, W.P., You, D., Yin, B.C., Ye, B.C., 2019. “A CRISPR-Cas9 strategy for activating the *Saccharopolyspora erythraea* erythromycin biosynthetic gene cluster with knock-in bidirectional promoters.” *ACS Synthetic Biology*, 8.5, 1134–1143.

[51] Zhang, M.M., Wong, F.T., Wang, Y., Luo, S., Lim, Y.H., Heng, E., Yeo, W.L., Cobb, R.E., Enghiad, B., Ang, E.L., Zhao, H., 2017. “CRISPR-Cas9 strategy for activation of silent *Streptomyces* biosynthetic gene clusters.” *Nature Chemical Biology*, 13.6, 607-609.

[52] Meng, J., Feng, R., Zheng, G., Ge, M., Mast, Y., Wohlleben, W., Gao, J., Jiang, W., Lu, Y., 2017. “Improvement of pristinamycin I (PI) production in *Streptomyces pristinaespiralis* by metabolic engineering approaches.” *Synthetic and Systems Biotechnology*, 2.2, 130-136.

[53] Yue, X., Xia, T., Wang, S., Dong, H., Li, Y., 2020. “Highly efficient genome editing in *N. gerenzanensis* using an inducible CRISPR/Cas9–RecA system.” *Biotechnology Letters*, 42, 1699–1706.

[54] Tiffert, Y., Supra, P., Wurm, R., Wohlleben, W., Wagner, R., Reuther, J., 2008. “The *Streptomyces coelicolor* GlnR regulon: Identification of new GlnR targets and evidence for a central role of GlnR in nitrogen metabolism in actinomycetes.” *Molecular Microbiology*, 67.4, 861–880.

[55] Cen, X.F., Wang, J.Z., Zhao, G.P., Wang, Y., Wang, J., 2016. “Molecular evidence for the coordination of nitrogen and carbon metabolisms, revealed by a study on the transcriptional regulation of the agl3EFG operon that encodes a putative carbohydrate transporter in *Streptomyces coelicolor*.” *Biochemical and Biophysical Research Communications*, 471.4, 510–14

494 [56] He, J.M., Zhu, H., Zheng, G.S., Liu, P.P., Wang, J., Zhao, G.P., Zhu, G.Q., Jiang, W.H.,
495 Lu, Y.H., 2016. “Direct involvement of the master nitrogen metabolism regulator GlnR in
496 antibiotic biosynthesis in *Streptomyces*.” *Journal of Biological Chemistry*, 291.51, 26443-
497 26454.
498 [57] Culp, E.J., Yim, G., Waglechner, N., Wang, W., Pawlowski, A.C., Wright, G.D., 2019.
499 “Hidden antibiotics in actinomycetes can be identified by inactivation of gene clusters for
500 common antibiotics.” *Nature Biotechnology*, 37.10, 1149–54.
501 [58] Qin, Z., Munnoch, J.T., Devine, R., Holmes, N.A., Seipke, R.F., Wilkinson, K.A.,
502 Wilkinson, B., Hutchings, M.I., 2017. “Formicamycins, antibacterial polyketides produced by
503 *Streptomyces formicae* isolated from African *Tetraponera* plant-ants.” *Chemical Science*, 8.4,
504 3218-3227
505 [59] Cohen, D.R., Townsend, C.A., 2018. “Characterization of an anthracene intermediate in
506 dynemicin biosynthesis.” *Angewandte Chemie International Edition*, 57.20, 5650-5654.
507 [60] Yeo, W.L., Heng, E., Tan, L.L., Lim, Y.W., Ching, K.C., Tsai, D.-J., Jhang, Y.W.,
508 Lauderdale, T.-L., Shia, K.-S., Zhao, H., Ang, E.L., Zhang, M.M., Lim, Y.H., Wong, F.T.,
509 2020. “Biosynthetic engineering of the antifungal, anti-MRSA auroramycin.” *Microbial Cell*
510 *Factories*, 19.1, 1-11.
511 [61] Zhao, Y., Li, L., Zheng, G., Jiang, W., Deng, Z., Wang, Z., Lu, Y., 2018. “CRISPR/dCas9-
512 mediated multiplex gene repression in *Streptomyces*.” *Biotechnology Journal*, 13.9, 1800121.
513 [62] DeLorenzo, D.M., Rottinghaus, A.G., Henson, W.R., Moon, T.S., 2018. “Molecular
514 toolkit for gene expression control and genome modification in *Rhodococcus opacus*
515 PD630.” *ACS Synthetic Biology*, 7.2, 727-738.
516 [63] Rock, J.M., Hopkins, F.F., Chavez, A., Diallo, M., Chase, M.R., Gerrick, E.R., Pritchard,
517 J.R., Church, G.M., Rubin, E.J., Sassetti, C.M., Schnappinger, D., Fortune, S.M., 2017.

518 "Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR
519 interference platform." *Nature Microbiology*, 2.4, 1-9.
520 [64] Wang, Y., Liu, Y., Liu, J., Guo, Y., Fan, L., Ni, X., Zheng, X., Wang, M., Zheng, P., Sun,
521 J., Ma, Y., 2018. "MACBETH: Multiplex automated *Corynebacterium glutamicum* base
522 editing method." *Metabolic Engineering*, 44, 200–210.
523 [65] Wang, Y., Liu, Y., Li, J., Yang, Y., Ni, X., Cheng, H., Huang, T., Guo, Y., Ma, H., Zheng,
524 P., Wang, M., Sun, J., Ma, Y., 2019. "Expanding targeting scope, editing window, and base
525 transition capability of base editing in *Corynebacterium glutamicum*." *Biotechnology and*
526 *Bioengineering*, 116.11, 3016-3029.
527 [66] Tong, Y., Whitford, C.M., Robertsen, H.L., Blin, K., Jørgensen, T.S., Klitgaard, A.K.,
528 Gren, T., Jiang, X., Weber, T., Lee, S.Y., 2019. "Highly efficient DSB-free base editing for
529 streptomycetes with CRISPR-BEST." *Proceedings of the National Academy of Sciences*,
530 116.41, 20366-20375.
531 [67] Liu, R., Deng, Z., Liu, T., 2018. "*Streptomyces* species: Ideal chassis for natural product
532 discovery and overproduction." *Metabolic Engineering*, 50, 74-84.
533 [68] Fazal, A., Thankachan, D., Harris, E., Seipke, R.F., 2020. "A chromatogram-simplified
534 *Streptomyces albus* host for heterologous production of natural products." *Antonie van*
535 *Leeuwenhoek,* 1-10.
536 [69] Myronovskyi, M., Rosenkränzer, B., Nadmid, S., Pujic, P., Normand, P., Luzhetskyy, A.,
537 2018. "Generation of a cluster-free *Streptomyces albus* chassis strains for improved
538 heterologous expression of secondary metabolite clusters." *Metabolic Engineering*, 49, 316-
539 324.
540 [70] Komatsu, M., Uchiyama, T., Omura, S., Cane, D.E., Ikeda, H., 2010. "Genome-minimized
541 *Streptomyces* host for the heterologous expression of secondary metabolism." *Proceedings of*
542 *the National Academy of Sciences*, 107.6, 2646-2651.

[71] Ke, J., Yoshikuni, Y., 2020. "Multi-chassis engineering for heterologous production of microbial natural products." *Current Opinion in Biotechnology*, 62, 88-97.

[72] Nah, H.J., Pyeon, H.R., Kang, S.H., Choi, S.S., Kim, E.S., 2017. "Cloning and heterologous expression of a large-sized natural product biosynthetic gene cluster in *Streptomyces* species." *Frontiers in Microbiology*, 8, 394.

[73] Harrison, J., Studholme, D.J., 2014. "Recently published *Streptomyces* genome sequences." *Microbial Biotechnology*, 7.5, 373-380.

[74] Nah, H.J., Woo, M.W., Choi, S.S., Kim, E.S., 2015. "Precise cloning and tandem integration of large polyketide biosynthetic gene cluster using *Streptomyces* artificial chromosome system." *Microbial Cell Factories*, 14.1, 140.

[75] Zhou, Y., Murphy, A.C., Samborskyy, M., Prediger, P., Dias, L.C., Leadlay, P.F., 2015. "Iterative mechanism of macrodiolide formation in the anticancer compound conglobatin." *Chemistry & Biology*, 22.6, 745-754.

[76] Yamanaka, K., Reynolds, K.A., Kersten, R.D., Ryan, K.S., Gonzalez, D.J., Nizet, V., Dorrestein, P.C., Moore, B.S., 2014. "Direct cloning and refactoring of a silent lipopeptide biosynthetic gene cluster yields the antibiotic taromycin A." *Proceedings of the National Academy of Sciences*, 111.5, 1957-1962.

[77] Eyles, T.H., Vior, N.M., Truman, A.W., 2018. "Rapid and robust yeast-mediated pathway refactoring generates multiple new bottromycin-related metabolites." *ACS Synthetic Biology*, 7.5, 1211-1218.

[78] Jiang, W., Zhu, T.F., 2016. "Targeted isolation and cloning of 100-kb microbial genomic sequences by Cas9-assisted targeting of chromosome segments." *Nature Protocols*, 11.5, 960.

[79] Liu, Y., Tao, W., Wen, S., Li, Z., Yang, A., Deng, Z., Sun, Y., 2015. "*In vitro* CRISPR/Cas9 system for efficient targeted DNA editing." *mBio*, 6.6, e01714-15.

567 [80] Kang, H.S., Charlop-Powers, Z., Brady, S.F., 2016. “Multiplexed CRISPR/Cas9- and
568 TAR-mediated promoter engineering of natural product biosynthetic gene clusters in
569 yeast.” *ACS Synthetic Biology*, 5.9, 1002-1010.
570 [81] Kim, H., Ji, C.H., Je, H.W., Kim, J.P., Kang, H.S., 2020. “mpCRISTAR: Multiple plasmid
571 approach for CRISPR/Cas9 and TAR-mediated multiplexed refactoring of natural product
572 biosynthetic gene clusters.” *ACS Synthetic Biology*, 9.1, 175–80.
573 [82] Kim, S.H., Lu, W., Ahmadi, M.K., Montiel, D., Ternei, M.A., Brady, S.F., 2018.
574 “Atolypenes, tricyclic bacterial sesterterpenes discovered using a multiplexed *in vitro* Cas9-
575 TAR gene cluster refactoring approach.” *ACS Synthetic Biology*, 8.1, 109–118.
576 [83] Wolf, T., Gren, T., Thieme, E., Wibberg, D., Zemke, T., Pühler, A., Kalinowski, J., 2016.
577 “Targeted genome editing in the rare actinomycete *Actinoplanes* sp. SE50/110 by using the
578 CRIPSR/Cas9 system.” *Journal of Biotechnology*, 231, 122-128.
579 [84] Schaffert, L., Schneiker-Bekel, S., Gierhake, J., Droste, J., Persicke, M., Rosen, W., Pühler
580 A., Kalinowski, J., 2020. “Absence of the highly expressed small carbohydrate-binding protein
581 Cgt improves the acarbose formation in *Actinoplanes* sp. SE50/110.” *Applied Microbiology*
582 *and Biotechnology*, 104.12, 5395-5408.
583 [85] Cohen, D.R., Townsend, C.A., 2018. “A dual role for a polyketide synthase in dynemicin
584 enediyne and anthraquinone biosynthesis.” *Nature Chemistry*, 10.2, 231-236.
585 [86] Chang, Y., Chai, B., Ding, Y., He, M., Zheng, L.,Teng, Y., Deng, Z., Yu, Y., Liu, T.,
586 2019. “Overproduction of gentamicin B in industrial strain *Micromonospora echinospora*
587 CCTCC M 2018898 by cloning of the missing genes *genR* and *genS*.” *Metabolic Engineering*
588 *Communications*, 9, e00096.
589 [87] Braesel, J., Lee, J.H., Arnould, B., Murphy, B.T., Eustáquio, A.S., 2019.
590 “Diazaquinomycin biosynthetic gene clusters from marine and freshwater actinomycetes.”
591 *Journal of Natural Product*, 82.4, 937–946.

592 [88] Musiol-Kroll, E.M., Tocchetti, A., Sosio, M., Stegmann, E., 2019. "Challenges and
593 advances in genetic manipulation of filamentous actinomycetes-the remarkable producers of
594 specialized metabolites." *Natural Product Reports*, 36.9, 1351-1369.
595 [89] Mo, J., Wang, S., Zhang, W., Li, C., Deng, Z., Zhang, L., Qu, X., 2019. "Efficient editing
596 DNA regions with high sequence identity in actinomycetal genomes by a CRISPR-Cas9
597 system." *Synthetic and Systems Biotechnology*, 4.2, 86–91.
598 [90] Dubeau, M.P., Ghinet, M.G., Jacques, P.É., Clermont, N., Beaulieu, C., Brzezinski, R.,
599 2009. "Cytosine deaminase as a negative selection marker for gene disruption and replacement
600 in the genus *Streptomyces* and other actinobacteria." *Applied and Environmental Microbiology*,
601 75.4, 1211–14.
602 [91] Jiang, Y., Qian, F., Yang, J., Liu, Y., Dong, F., Xu, C., Sun, B., Chen, B., Xu, X., Li,. Y.,
603 Wang, R., Yang, S., 2017. "CRISPR-Cpf1 assisted genome editing of *Corynebacterium*
604 *glutamicum*." *Nature Communications*, 2017.8, 15179.
605 [92] Zhang, J., Yang, F., Yang, Y., Jiang, Y., Huo, Y.X., 2019. "Optimizing a CRISPR-Cpf1-
606 based genome engineering system for *Corynebacterium glutamicum*." *Microbial Cell*
607 *Factories*, 18, 60.
608 [93] Cho, J.S., Choi, K.R., Prabowo, C.P.S., Shin, J.H., Yang, D., Jang, J., Lee, S.Y., 2017.
609 "CRISPR/Cas9-coupled recombineering for metabolic engineering of *Corynebacterium*
610 *glutamicum*." *Metabolic Engineering*, 42, 157–167.
611 [94] Peng, F., Wang, X., Sun, Y., Dong, G., Yang, Y., Liu, X., Bai, Z., 2017. "Efficient gene
612 editing in *Corynebacterium glutamicum* using the CRISPR/Cas9 system." *Microbial Cell*
613 *Factories*, 16, 201.
614 [95] Liu, J., Wang, Y., Lu, Y., Zheng, P., Sun, J., Ma, Y., 2017. "Development of a
615 CRISPR/Cas9 genome editing toolbox for *Corynebacterium glutamicum*." *Microbial Cell*
616 *Factories*, 16, 205.

617 [96] Yan, M.Y., Li, S.S., Ding, X.Y., Guo, X.P., Jin, Q., Sun, Y.C., 2020. "A CRISPR-assisted
618 nonhomologous end-joining strategy for efficient genome editing in *Mycobacterium*
619 *tuberculosis*." *mBio*, 11.1, e02364-19.
620 [97] Liang, Y., Jiao, S., Wang, M., Yu, H., Shen, Z., 2020. "A CRISPR/Cas9-based genome
621 editing system for *Rhodococcus ruber* TH." *Metabolic Engineering*, 57, 13-22.
622 [98] Wagner, N., Osswald, C., Biener, R., Schwartz, D., 2009. "Comparative analysis of
623 transcriptional activities of heterologous promoters in the rare actinomycete *Actinoplanes*
624 *friuliensis*." *Journal of Biotechnology*, 142. 3-4, 200-204.
625 [99] Myronovskyi, M., Luzhetskyy, A., 2016. "Native and engineered promoters in natural
626 product discovery." *Natural Product Reports*, 33.8, 1006-1019.
627 [100] Ji, C.H., Kim, J.P., Kang, H.S., 2018. "Library of synthetic *Streptomyces* regulatory
628 sequences for use in promoter engineering of natural product biosynthetic gene clusters." *ACS*
629 *Synthetic Biology*, 7.8, 1946-1955.
630 [101] Wang, Q., Xie, F., Tong, Y., Habisch, R., Yang, B., Zhang, L., Müller, R., Fu, C., 2020.
631 "Dual-function chromogenic screening-based CRISPR/Cas9 genome editing system for
632 actinomycetes." *Applied Microbiology and Biotechnology*, 104, 225–239.
633 [102] Hanahan, D., 1983 "Studies on transformation of *Escherichia coli* with
634 plasmids." *Journal of Molecular Biology*, 166.4, 557-580.
635 [103] Liu, Y., Wei, W.P., Ye, B.C., 2018. "High GC content Cas9-mediated genome-editing
636 and biosynthetic gene cluster activation in *Saccharopolyspora erythraea*." *ACS Synthetic*
637 *Biology*, 7.5, 1338-1348.
638 [104] Yan, M.Y., Yan, H.Q., Ren, G.X., Zhao, J.P., Guo, X.P., Sun, Y.C., 2017. "CRISPR-
639 Cas12a-assisted recombineering in bacteria." *Applied and Environmental Microbiology*, 83.17,
640 e00947-17

641 [105] Jones, A.C., Gust, B., Kulik, A., Heide, L., Buttner, M.J., Bibb, M.J., 2013. "Phage P1-
642 derived artificial chromosomes facilitate heterologous expression of the FK506 gene cluster."
643 *PLoS ONE*, 8.7, e69319.
644 [106] Zhang, J.J., Moore, B.S., Tang, X., 2018. "Engineering *Salinispora tropica* for
645 heterologous expression of natural product biosynthetic gene clusters." *Applied Microbiology*
646 *and Biotechnology*, 102.19, 8437-8446.
647 [107] Zhang, J.J., Yamanaka, K., Tang, X., Moore, B.S., 2019. "Direct cloning and
648 heterologous expression of natural product biosynthetic gene clusters by transformation-
649 associated recombination." *Methods in Enzymology*, 621, 87.
650 [108] Zhang, J.J., Tang, X., Moore, B.S., 2019. "Genetic platforms for heterologous expression
651 of microbial natural products." *Natural Product Reports*, 36.9, 1313-1332.
652 [109] Zhang, M.M., Wang, Y., Ang, E.L., Zhao, H., 2016. "Engineering microbial hosts for
653 production of bacterial natural products." *Natural Product Reports*, 33.8, 963-987.
654 [110] Wang, K., Zhao, Q.W., Liu, Y.F., Sun, C.F., Chen, X.A., Burchmore, R., Burgess, K.,
655 Li, Y.Q., Mao, X.M., 2019. "Multi-layer controls of Cas9 activity coupled with ATP synthase
656 over-expression for efficient genome editing in *Streptomyces*." *Frontiers in Bioengineering*
657 *and Biotechnology*, 7, 304.
658 [111] Doench, J.G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E.W., Donovan, K.F.,
659 Smith, I., Tothova, Z., Wilen, C., Orchard, R., Virgin, H.W., Listgarten, J., Root, D.E., 2016.
660 "Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-
661 Cas9." *Nature Biotechnology*, 34.2, 184.
662 [112] Chuai, G. hui, Wang, Q.L., Liu, Q., 2017. "*In silico* meets *in vivo*: towards computational
663 CRISPR-based sgRNA design." *Trends in Biotechnology*, 35.1, 12-21.

664 [113] Liu, G., Zhang, Y., Zhang, T., 2020. “Computational approaches for effective CRISPR
665 guide RNA design and evaluation.” *Computational and Structural Biotechnology Journal*, 18,
666 35–44.
667 [114] Ye, S., Enghiad, B., Zhao, H., Takano, E., 2020. “Fine-tuning the regulation of Cas9
668 expression levels for efficient CRISPR-Cas9 mediated recombination in *Streptomyces*.”
669 *Journal of Industrial Microbiology & Biotechnology*, 47.4-5, 413-423.
670 [115] Hoff, G., Bertrand, C., Piotrowski, E., Thibessard, A., Leblond, P., 2018. “Genome
671 plasticity is governed by double strand break DNA repair in *Streptomyces*.” *Scientific*
672 *Reports*, 8.1, 1-11.
673 [116] Volff, J.N., Altenbuchner, J., 1998. “Genetic instability of the *Streptomyces*
674 chromosome.” *Molecular Microbiology*, 27.2, 239-246.
675 [117] Sun, B., Yang, J., Yang, S., Ye, R.D., Chen, D., Jiang, Y., 2018. “A CRISPR-Cpf1-
676 assisted non-homologous end joining genome editing system of *Mycobacterium smegmatis*.”
677 *Biotechnology Journal*, 13.9, e1700588.
678 [118] Wang, B., Hu, Q., Zhang, Y., Shi, R., Chai, X., Liu, Z., Shang, X., Zhang, Y., Wen, T.,
679 2018. “A RecET-assisted CRISPR-Cas9 genome editing in *Corynebacterium glutamicum*.”
680 *Microbial Cell Factories*, 17, 63.
681 [119] Reis, A.C., Halper, S.M., Vezeau, G.E., Cetnar, D.P., Hossain, A., Clauer, P.R., Salis,
682 H.M., 2019. “Simultaneous repression of multiple bacterial genes using nonrepetitive extra-
683 long sgRNA arrays.” *Nature Biotechnology*, 37.11, 1294-1301.
684 [120] Anzalone, A. V., Randolph, P.B., Davis, J.R., Sousa, A.A., Koblan, L.W., Levy, J.M.,
685 Chen, P.J., Wilson, C., Newby, G.A., Raguram, A., Liu, D.R., 2019. “Search-and-replace
686 genome editing without double-strand breaks or donor DNA.” *Nature*, 576.7785, 149-157.

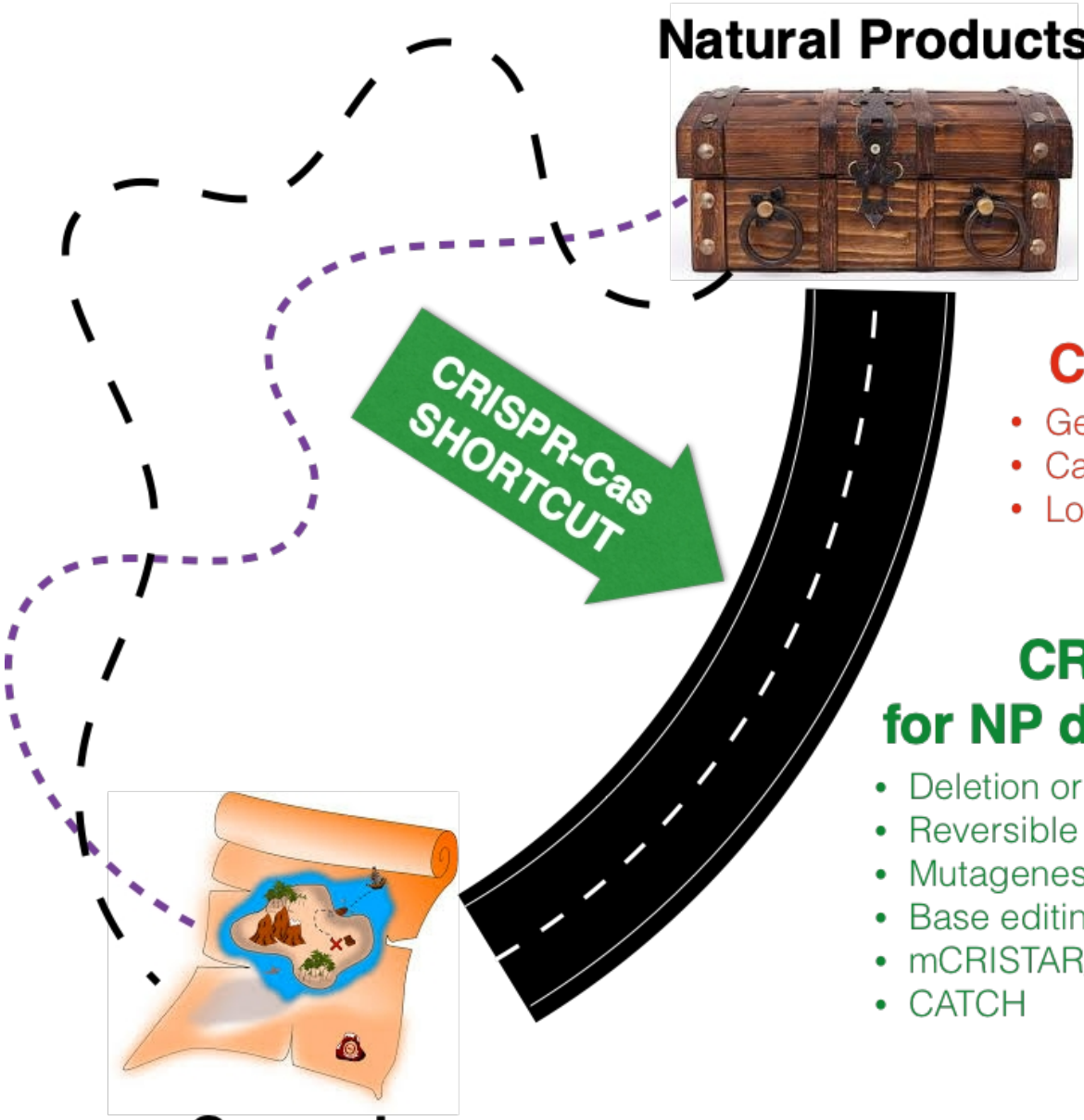
687 **Figure Legends**

688 Figure 1. CRISPR-Cas mediated strategies in host and pathway engineering have accelerated
689 genome-guided natural product discovery and engineering in actinomycetes but challenges
690 remain. BGC: biosynthetic gene cluster, CRISPR-BEST: CRISPR-Base editing system [66],
691 mCRISTAR: multiplexed-CRISPR-transformation-associated recombination (TAR) [80],
692 mpCRISTAR: multiple plasmid-based CRISTAR [81], CATCH: Cas9-assisted targeting of
693 chromosome segments) [78], *mi*CASTAR: multiplex *in vitro* Cas9-TAR [82].

694

695 **Tables**

696 Table 1. CRISPR-Cas systems and applications in rare actinomycetes. This table summarizes
697 the CRISPR-Cas systems, applications, transformation methods and editing efficiencies in
698 non-*Streptomyces* actinomycetes (up to April 2020). ssDNA: single-stranded DNA, HRT:
699 homology repair template, SD: Shine-Dalgarno, dsDNA: double-stranded DNA.



Challenges

- Genetically intractable
- Cas toxicity
- Low efficiencies in multiplex editing

CRISPR-Cas strategies for NP discovery and engineering

- Deletion or insertion of BGCs, genes and promoters
- Reversible control of gene expression
- Mutagenesis
- Base editing/CRISPR-BEST
- mCRISTAR/mpCRISTAR/*mi*CASTAR
- CATCH

Table 1. CRISPR-Cas systems and applications in rare actinomycetes.

Host	CRISPR-Cas system	Application	Description of system	Transformation method	Editing efficiencies	References
Actinoplanes sp. SE50/110	pCRISPomyces -2 [35]	Deletion	pCRISPomyces-2 system worked without additional modifications	Conjugation	80%	[83]
Actinoplanes sp. SE50/110 (ATCC 31044)	pCRISPomyces -2 [35]	Deletion	pCRISPomyces-2 system worked without additional modifications	Conjugation	Not mentioned	[84]
Corynebacterium glutamicum ATCC 13032	Two plasmid-based CRISPR-Cpf1 and RecT system	ssDNA recombineering	RecT expression and ssDNA template	Co-electroporation	ssDNA recombineering: 10-100%	[91]
	All-in-one CRISPR-Cpf1 plasmid	Deletion, insertion	All-in-one plasmid contains Cpf1, crRNA and HRT No RecT	Electroporation	Deletion: 10-15 % Insertion: 5 %	
Corynebacterium glutamicum ATCC 13032	CRISPR-Cpf1	Deletion	Optimised PAM sequence (5'-TTTA/C-3') Optimized spacer length (21 bp) Different methods of introducing repair template (all-in-one plasmid, separate plasmid, linearized dsDNA)	Co-electroporation	10-32%	[92]
Corynebacterium glutamicum ATCC 13032	Two plasmid-based CRISPR-Cas9 [38] and RecT system	Multiplex ssDNA recombineering (up to 2 loci)	RecT expression and ssDNA template Optimized transformation protocol for efficient co-electroporation of CRISPR-Cas plasmid and ssDNA Orthogonal curable plasmids	2-step sequential electroporation	0-100%	[93]
Corynebacterium glutamicum ATCC 13032 *Corynebacterium glutamicum* CGMCC1.15647	Two plasmid-based CRISPR-Cas9	Deletion, insertion, point mutation	IPTG-inducible SpCas9 with the indispensible SD sequence IPTG-inducible sgRNA with HRT on a second temperature-sensitive plasmid for fast curing Tested different lengths of HRT on editing efficiencies Recommend sgRNA with GC content <60%	Co-electroporation	Deletion: 16 - 100% Point mutation: 100% Insertion: 25 - 67%	[94]
Corynebacterium glutamicum ATCC 13032 *Corynebacterium glutamicum* ATCC 13869	Two plasmid-based CRISPR-Cas9	Deletion, insertion, multiplex ssDNA recombineering (up to 2 loci)	IPTG-inducible Cas9 For recombineering: Propionate-inducible RecT and 90-mer ssDNA targetting lagging strand	2-step sequential electroporation	Deletion: 36 - 60% Insertion: 16 - 33% ssDNA recombineering: > 70%	[95]
Corynebacterium glutamicum ATCC 13032 *Corynebacterium pekinense* CGMCC 1.563	Chromosome-borne Cas9-RecET system	Deletion (includes large deletions up to 20 kb), insertion, promoter replacement, site-directed mutation	Chromosomal integration of Cas9 and RecET Ribosome binding sequences were introduced to improve translation efficiencies of Cas9 and RecET Longer HRT have higher editing efficiencies	Electroporation	Deletion: 30 - 60% (without RecET) 78 - 92% (with RecET) Large deletion of 10 - 20 kb: 27 - 36% Insertion: 2 - 69% Site directed mutagenesis: 55 - 95%	[118]
Corynebacterium glutamicum ATCC 13032	Two plasmid-based system: nCas9(D10A)-AID and sgRNA-ccdB	Multiplex base editing (up to 3 loci)	IPTG-inducible fusion of Cas9(D10A) nickase with activation-induced cytidine deaminase (AID) Temperature-sensitive nCas9(D10A) and segregational instable sgRNA plasmids for plasmid curing Longer editing time by continuous passage increased base editing efficiency Automated CRISPR/nCas9(D10A)-AID base editing	Co-electroporation	Base editing: 62 - 100% Multiplex base editing: 41 - 85% (2 loci) and up to 23% (3 loci)	[64]
Corynebacterium glutamicum ATCC 13032	All-in-one system: pnCas9 (D10A)-AID [64] TS and pgRNA-ccdB NoPer	Base editing	Expand genome-targeting scope of base editor using Cas9 variants with altered PAM specificities Expanded editing window with different guide RNA lengths Adenine base editing with engineered adenosine deaminase and Cas9 nickase User-friendly online tool gBIG for designing base editing sgRNAs	Co-electroporation	Cytosine base editing: >50% Adenine base editing: up to 66%	[65]
Micromonospora chersina NRRL B-24756	CRISPR - Cas9 [38]	Deletion	CRISPR-Cas9 system worked without additional modifications	Conjugation	Not mentioned	[85]
Micromonospora echinospora CCTCC M 2018898	CRISPR - Cas9 [38]	Deletion, promoter replacement	CRISPR-Cas9 system worked without additional modifications	Conjugation	Not mentioned	[86]
Micromonospora sp. B006	pCRISPomyces -2 [35]	Deletion	pCRISPomyces-2 system worked without additional modifications	Conjugation	80%	[87]
Mycobacterium smegmatis	pJV53-Cpf1	Mutation, Deletion	Codon-optimized FnCpf1, with recombination proteins, gp60 and gp61 on a temperature sensitive replicon plasmid crRNA on another plasmid with temperature sensitive replicon Sequential ssDNA recombineering using crRNA plasmids with different resistances	Co-electroporation	Mutation: 60 - 87.5% Deletion: 70 - 80% (10 - 50 bp) 8 - 17% (418, 1000 bp)	[104]
Mycobacterium tuberculosis *Mycobacterium smegmatis*	iCRISPR-Sth1Cas9	CRISPRi-mediated gene repression	Single sgRNA Codon-optimized inactive Sth1Cas9-mediated gene knockdown	Not mentioned	up to 214 fold repression 7 out 8 sgRNA are successful to > 97% repression	[63]
	iCRISPR-SpCas9	CRISPRi-mediated gene repression	Single sgRNA Codon-optimized inactive SpCas9-mediated gene knockdown	Not mentioned	3 - 4 fold repression 1 out 8 sgRNA are successful to > 97% repression	
Mycobacterium smegmatis mc2155	All-in-one pSBY3_FnCpf1cg	NHEJ-mediated genome editing	FnCpf1 was codon-optimized for *C. glutamicum* did not affect *M. smegmatis* growth	Electroporation	up to 70%	[117]
Mycobacterium smegmatis mc2155 *Mycobacterium marinum* strain M *Mycobacterium bovis* BCG *Mycobacterium tuberculosis* H37Rv *Mycobacterium tuberculosis* H37Ra	CRISPR-Cas9Sth1 [104] pJV53-Cas12a	NHEJ-mediated genome editing	Overexpression of *M. marinum* NHEJ machinery Disruption of RecA function	Sequential or concurrent electroporation of CRISPR and NHEJ/RecA plasmids	80 - 90%	[96]
Nonomuraea gerenzanensis sp. ATCC 39727	All-in-one CRISPR/SpCas9–RecA	Deletion	Inducible *tipA* promoter and theophylline-inducible riboswitch for controlled SpCas9 expression Temperature-sensitive pSG5 replicon Introdution of RecA enhances editing efficiency	Conjugation	Without RecA: 69% With RecA: 100%	[53]
Rhodococcus opacus PD630	iCRISPR-Sth1Cas9 [104]	CRISPRi-mediated gene repression	Catalytically dead Sth1Cas9 under pBAD promoter Single sgRNA	Electroporation	4 - 58% repression	[62]
Rhodococcus ruber TH	Three-plasmid CRISPR/Cas9 recombineering	Insertion, deletion, site-directed mutation	Optimization of transformation protocol included methylation of sgRNA plasmids to bypass host restriction-modification system Codon-optimized SpCas9 Basal expression of SpCas9 from urea-inducible promoter Expression of Che9c60 and Che9c61 phage recombinases increases editing efficiency Linear dsDNA repair template	3-step sequential electroporation	Gene deletion: 3 - 75% Single point mutation: 60% Gene insertion: 25%	[97]
Saccharopolyspora erythraea NRRL23338 *Saccharopolyspora erythraea* Ab	pKECas9 system with *Streptomyces* codon-optimized SpCas9 from pCRISPomyces-2 [35]	Deletions and insertions	Use of PEG-3350 increased transformation efficiency of large plasmids (~14 kb)	Protoplast transformation	Deletion: 29 - 65% Insertion: < 40%	[103]
Saccharopolyspora erythraea NRRL23338 *Saccharopolyspora erythraea* Ab	pKECas9 system with *Streptomyces* codon-optimized SpCas9 from pCRISPomyces-2 [35]	Promoter insertion	Dual sgRNA expression Use of PEG-3350 increased transformation efficiency of large plasmids (~14 kb)	Protoplast transformation	Not mentioned	[50]
Saccharopolyspora erythraea HL3168 E3	pMWCas9 system based on pIJ101 replicon of pWHU2653 [39]	Deletion	Inducible tipA promoter for Cas9 expression Switching from pSG5 to the segregationally unstable pIJ101 replicon allowed deletion of highly repetitive sequences Use of CodA for counterselection	Conjugation	Not mentioned	[89]
Verrucosispora sp. MS100137	pQS system based on pWHU2653 [39]	Deletion	Chromogenic screening for accelerated plasmid curing and elimination of false positives Inducible *tipA* promoter for Cas9 expression Segregationally unstable pIJ101replicon	Conjugation	100%	[101]
Amycolatopsis tolypomycina NRRL B-24205	*mi*CASTAR (multiplex *in vitro* Cas9-TAR)	Cluster refactoring	*In vitro* Cas9 gene cluster disassembly coupled with *in vivo* TAR DNA assembly for BGC refactoring	Not applicable	Not mentioned	[82]