CRISPR/CAS9 based engineering of actinomycetal genomes

Weber, Tilmann; Tong, Yaojun; Lee, Sang Yup

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(71) Applicant: **DANMARKS TEKNISKE UNIVERSITET** [DK/DK]; Anker Engelunds Vej 1, Bygning 101A, 2800 Kgs. Lyngby (DK).

(72) Inventors: **WEBER, Tilmann**; Slotsgade 8F, st.tv., 3480 Fredensborg (DK). **TONG, Yaojun**; Kammerrådensvej 31, st.tv., 2970 Hørsholm (DK). **LEE, Sang Yup**; Expo Apt. 212-702, Expo-ro 448, Yuseong-gu, Daejeon 305-761 (KR).

(74) Agent: **HØIBERG A/S**; Adelgade 12, 1304 Copenhagen K (DK).

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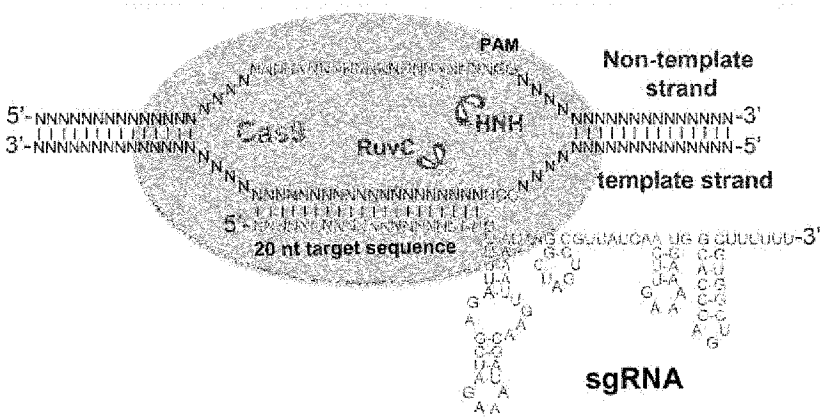
Figure 1



(57) Abstract: The present invention relates to CRISPR/Cas-based methods for generating random-sized deletions around at least one target nucleic acid sequence, or for generating precise indels around at least one target nucleic acid sequence, or for modulating transcription of at least one target nucleic acid sequence. Also disclosed is a clonal library comprising clones with random-sized deletions, as well as polynucleotides, polypeptides, cells and kits useful for performing the present methods. The present methods can be performed in organisms where gene editing is typically considered as difficult, such as actinomycetes, in particular streptomycetes.

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CRISPR/CAS9 BASED ENGINEERING OF ACTINOMYCETAL GENOMES

Field of invention

The present invention relates to CRISPR/Cas-based methods for generating random-sized deletions around at least one target nucleic acid sequence, or for generating precise indels around at least one target nucleic acid sequence, or for modulating transcription of at least one target nucleic acid sequence. Also disclosed is a clonal library comprising clones with random-sized deletions, as well as polynucleotides, polypeptides, cells and kits useful for performing the present methods. The present methods can be performed in organisms where gene editing is typically considered as difficult, such as actinomycetes, in particular streptomycetes.

Background of invention

Actinomycetes are Gram-positive bacteria with the capacity to produce a wide variety of medically and industrially relevant secondary metabolites, including many antibiotics, herbicides, parasiticides, anti-cancer agents, and immunosuppressants. It becomes harder and harder to find new bioactive compounds from actinomycetes using traditional approaches.

Recent advances in genome sequencing and genome mining have significantly accelerated the ability to identify secondary metabolism genes and gene clusters. Precise gene editing technologies are needed to enable systematic reverse engineering of causal genetic variations by allowing selective perturbation of individual genetic elements, as well as to advance synthetic biology and biotechnology. There are four major universal gene editing tools developed so far: 1) meganucleases derived from microbial mobile genetic elements, 2) zinc finger (ZF) nucleases based on eukaryotic transcription factors, 3) transcription activator-like effectors (TALEs) from *Xanthomonas* bacteria, and 4) the RNA-guided DNA endonuclease Cas9 from the type II bacterial adaptive immune system Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), called CRISPR-Cas9 system. However, each of the first three methods has its own unique limitations: the specificity of a meganuclease for a target DNA is difficult to control, the assembly of functional zinc finger proteins with the desired DNA binding

specificity remains a major challenge, and the construction of novel TALE arrays are labour intensive and costly.

The CRISPR-Cas9 system displays certain advantages. The CRISPR nuclease Cas9 can be guided by a short single guide RNA (sgRNA) that recognizes the target DNA via Watson-Crick base pairing (Figure 1A) instead of complex protein-DNA recognition, thereby easing the design and construction of targeting vectors. The sgRNAs are artificially generated chimeras of the CRISPR RNA (crRNA) and the associated trans-activating CRISPR RNA (tracrRNA) found in the native CRISPR systems, which originally corresponds to phage sequences, constituting the natural mechanism for CRISPR antiviral defense of bacteria and archaea, but can be easily replaced by a sequence of interest to reprogram the Cas9 nuclease for gene editing. Multiplexed targeting by Cas9 can now be achieved at an unprecedented scale by introducing a plurality of sgRNAs rather than a library of large, bulky proteins.

The Cas9 protein family is characterized by two signature nuclease domains, HNH and RuvC. A critical feature of recognition by CRISPR-Cas9 is the protospacer-adjacent motif (PAM), which flanks the 3' end of the DNA target site (Figure 1) and directs the DNA target recognition by the Cas9-sgRNA complex. The Cas9 and the sgRNA first form a complex, and the complex subsequently starts to scan the whole genome for the PAM sequences. Once the complex has identified the PAM, which can have on its 5’ flank a sequence complementary to the target sequence within the sgRNA in the complex, the complex binds to this position. This triggers the Cas9 nuclease activity by activating the HNH and RuvC domains.

The CRISPR/Cas9 system generates a break, such as a nick or a double-strand break (DSB) in the DNA, which is repaired by one of the two main repair pathways: non-homologous end-joining (NHEJ) or homologous recombination (HR). HR requires the presence of a homologous template DNA, which can comprise additional sequences which can thus be introduced at the site of the break. NHEJ does not require the presence of donor DNA, and usually results in small deletions. The system can thus be used for integrating new sequences into a target sequence, or for the precise generation of deletions around the target site.

Because of its modularization and easy handling, the CRISPR-Cas9 system has been successfully applied as a gene editing tool in a wide range of organisms such as *Saccharomyces cerevisiae*, some plants, *Caenorhabditis elegans*, *Drosophila*, Chinese hamster ovary (CHO) cells, frogs, mice, rats, rabbits, and human cells with high specificity. Recently, the CRISPR-Cas9 system was re-programmed to control gene expression by mutating the HNH and RuvC domains of Cas9 (D10A and H840A), resulting in a catalytically dead Cas9 (dCas9) lacking endonuclease activity. This system has so far successfully been applied in *Escherichia coli* (Qi, L. S., et, al. 2013).

As stated above, one of the challenges in the deep application of actinomycetes is to systematically engineer them for the overproduction of effective secondary metabolites and non-natural chemical compounds as well as new bioactive compounds, which corresponds to a fundamental objective of metabolic engineering. Unfortunately, genetic manipulation of actinomycetes is considered to be more difficult than model organisms, such as *Escherichia coli* and *Saccharomyces cerevisiae.* This is due in part to their more diverse genomic contents; for example, the GC content of their genomes is high.

There are to our knowledge only two very recent publications describing a CRISPR based system using homologous recombination templates to generate defined mutations in streptomycetes (Cobb et al., 2014, Huang et al., 2015). The use of CRISPR-based systems for generating random-sized, targeted deletions around a target site has not yet been reported.

Thus, rapid, efficient and convenient methods for gene editing of actinomycetes, in particular for streptomycetes, are needed.

Summary of invention

The invention is as defined in the claims.

Herein are disclosed methods useful for gene editing. These methods are based on the surprising finding that in organisms having a partly deficient non-homologous end-joining pathway (NHEJ), gene editing based on the CRISPR/Cas9 system targeting a nucleic acid sequence of interest results in the generation of clones with random-sized deletions around the target site. In order to generate precise indels (i.e. precise inser-

tions or deletions) around a target site in such organisms, the NHEJ pathway can be restored by engineering the host cell so that it has a fully functional NHEJ pathway.

The methods described herein are of particular interest for organisms where gene editing is typically considered to be labor-intensive, such as actinomycetes. The methods can be used to generate clonal libraries in order to investigate a given pathway, for example in order to optimize production of a secondary metabolite.

Also described herein is a method for modulating transcription of a nucleic acid sequence of interest by using a catalytically dead Cas9. This method can be applied to actinobacteria, e.g. streptomycetes.

Description of Drawings

Figure 1. Diagram of the Cas9 and sgRNA complex. The Cas9 HNH and RuvC-like domains each cleave one strand of the sequence targeted by the sgRNA; the trinucleotide PAM is labelled; the binding of the 20 nt target sequence to the genome is shown; the sgRNA core structure and sequence is shown.

Figure 2. Design of easily changeable sgRNA scaffold: the forward primer, labelled as "P-F", comprises a 20 nt sgRNA core sequence, a 20 nt target sequence and the NcoI sequence, while the reverse primer, labelled as "P-R", comprises a 20 nt sgRNA core sequence and the SnaBI sequence. To construct a new sgRNA, a 20 nt target sequence of interest is designed and integrated in the forward primer. The arrow represents the ermE* promoter, while the circle represents the to terminator, and the core sgRNA is shown as a box.

Figure 3. Map of pCRISPR-Cas9. Restriction endonuclease sites are available for additional elements sub-cloning, for instance, the StuI site.

Figure 4. Actinorhodin biosynthesis. A. Organization of the actinorhodin biosynthetic gene cluster; B. The steps to synthetize actinorhodin are: **I.** 1x Acetyl-CoA and 7x malonyl-CoA are condensed to form the carbon skeleton by ActI; **II.** The above carbon backbone is cyclized to form a three ring intermediate, DNPA by ActIII, ActVII, ActIV, ActVI-1 and ActVI-3; **III.** DNPA is then modified to form DHK by ActVI-2, ActVI-4 and

ActVA-6; **IV.** 2 DHK is dimerized to form the final product, actinorhodin by ActVA-5 and ActVB. The arrows mark the two selected genes.

Figure 5. Functional sgRNAs PCR screening results: the positive size is 234 bp, the negative size is 214 bp, the agrose gel concentration is 4% in TAE. A-C, 36 clones for *actIORF1* gene; D-F, 36 clones for *actVB* gene.

Figure 6. Actinorhodin biosynthetic pathway was inactivated by CRISPR-Cas9. 1-5, represent strains WT, Δ*actIorf1*-1, Mismatch, Δ*actvb*-1, and No Target, respectively; the plate in the left panel is without inducer thiostrepton, while the plate in the right panel is with inducer thiostrepton, the pH of the plates is >7. A. ISP2 plate without antibiotics. All five strains are blue. B. ISP2 plate with 1 μg/ml thiostrepton. Labels correspond to those in B. The blue from strains Δ*actIorf1*-1 and Δ*actvb*-1 disappeared. The photos were taken after 7 days incubation at 30°C.

Figure 7. Actinorhodin detection by UV-visible spectrometry. When the pH is lowered to 2, actinorhodin turns from blue to red, and has a maximum absorption at about 530 nm. From the scanning, the actinorhodin peak of Δ*actIorf1* and Δ*actvb* disappeared.

Figure 8. Analysis of the sequencing data. A. Heatmap of the 7 mapped sequencing samples to the *S. coelicolor* A3(2) reference genome. Dark colours represent a high read coverage, white represents low/no coverage. Displayed is the region spanning 5508800 to 5557230 of the *S. coelicolor* genome. The actinorhodin gene cluster is denoted by brackets; the target sites of the *actIORF1* and *actVB* sgRNAs are displayed as arrows. The deletion sizes are shown on the map. 1-7 represent strains: WT, No Target, Mismatch, Δ*actIorf1*-1, Δ*actIorf1*-2, Δ*actvb*-1, and Δ*actvb*-2, respectively. B. Alignment of the sequence traces of Δ*actIorf1*-1 with the WT. The arrow indicates the genomic target site of the sgRNA: ActIorf1-6 T. The PAM sequence is shown. C. and D. DNA sequences of 8 randomly selected clones without actinorhodin production aligned to the WT genomic sequence of *actIORF1* and *actVB*, respectively. The arrow indicates the genomic target sites of the related sgRNAs. The PAM sequences are shown. Dark shadow, light shadow with a dash and dark shadow with a box indicate insertions, deletions and substitutions, respectively.

Figure 9. Plasmid map for pCRISPR-Cas9-ScaligD. An expression cassette of *S. carneus* ligD was introduced into pCRISPR-Cas9 using Gibson Assembly in StuI site. The *S. carneus* ligD was under control by ermE* promoter, ending with a to terminator.

Figure 10. HDR pathway to repair the DNA DSBs caused by CRISPR-Cas9 system. A. and B. Diagrams of the CRISPR-Cas9 vectors with homologous recombination templates for *actIORF1* and *actVB*. C. and D. Colony PCR of 10 randomly selected clones that lost actinorhodin production to confirm deletion of *actIORF1* (C) and *actVB* (D) after use of the two vectors in A and B. I, II, and III represent the WT genome, *actIORF1* deleted and *actVB* deleted genome, respectively. 1-10 represent 10 randomly selected clones that lost actinorhodin production.

Figure 11. The plasmid map for pCRISPR-dCas9. The only difference between pCRISPR-dCas9 and pCRISPR-Cas9 is the Cas9 was a catalytically dead version without the endonuclease activity (D10A and H840A), called dCas9 in pCRISPR-dCas9.

Figure 12. CRISPRi effectively silences *actIORF1* expression in a reversible manner. A. Location of the twelve sgRNAs for CRISPRi. Half were designed to target the promoter region, while the other half were designed to target the ORF. In addition, half target the template strand and half target the non-template strand. The dashes represent sgRNAs. B. 530 nm absorbance of extracts from cultures tested with the twelve sgRNAs shown in A relative to the wild-type control. Left panel shows the sgRNAs target on promoter region, while right panel shows the sgRNAs target on ORF region. Mean values from three independent extractions are shown. Error bars represent the standard deviation from three independent extractions. C. and D. Reversibility of the CRISPRi system. Red clones become blue when the incubation temperature is increased to 37°C, indicating that the CRISPRi effect has gone away. The red color is boxed, while the blue is not. 0-12 represent sgRNAs: control (without any sgRNA), orf1p-A1 NT, orf1p-A4 NT, orf1p-A5 NT, orf1p-S1 T, orf1p-S3 T, orf1p-S5 T, Actlorf1-1 NT, Actlorf1-7 NT, Actlorf1-8 NT, Actlorf1-2 T, Actlorf1-3 T, and Actlorf1-4 T, respectively.

Detailed description of the invention

The present inventors have surprisingly found that a partial deficiency of the non-homologous end-joining (NHEJ) pathway in a host cell conferred the host cell interesting properties. For example, inducing a CRISPR-Cas9 system in said host cell results in the generation of random-sized deletions around a target site recognized by said CRISPR-Cas9 system. On the other hand, restoring full functionality of the NHEJ pathway prior to or simultaneously with induction of the CRISPR-Cas9 system results in the generation of precise indels around the target site.

In a first aspect, the invention relates to a method for generating at least one deletion around at least one target nucleic acid sequence comprised within a host cell having a non-homologous end-joining (NHEJ) pathway which is at least partly deficient,

said method comprising the steps of:

(i) optionally, restoring the full functionality of the NHEJ pathway,

(ii) inducing a CRISPR-Cas9 system in said host cell, wherein said CRISPR-Cas9 system is able to generate at least one break in said at least one target nucleic acid sequence and wherein the CRISPR-Cas9 system comprises a Cas9 nuclease and at least one guiding means,

thereby generating:

a. if the method does not comprise step (i)., at least one random-sized deletion around said at least one target nucleic acid sequence, wherein said at least one deletion is a random-sized deletion of at least 1 bp; or

b. if the method does comprise step (i), at least one indel around said at least one target nucleic acid sequence, wherein said at least one indel is a deletion or insertion of at least 1 bp.

In a second aspect, the invention relates to a polynucleotide having at least 94% identity with SEQ ID NO: 1, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity, such as 100% identity with SEQ ID NO: 1.

In yet another aspect, the invention relates to a polypeptide encoded by the polynucleotide described herein.

In yet another aspect, the invention relates to a cell comprising the polynucleotide described herein.

In yet another aspect, the invention relates to a cell comprising the polypeptide described herein.

In yet another aspect, the invention relates to a vector comprising the polynucleotide described herein.

In yet another aspect, the invention relates to a clonal library obtainable by the above method, said clonal library comprising a plurality of clones harboring at least one deletion and/or indel around at least one target nucleic acid sequence, wherein said deletion is a random-sized deletion of at least 1 bp and wherein said indel is a deletion or insertion of at least 1 bp.

In yet another aspect, the invention relates to a method for selectively modulating transcription of at least one target nucleic acid sequence in a host cell, the method comprising introducing into the host cell:

i. at least one guiding means, or a nucleic acid comprising a nucleotide sequence encoding guiding means, wherein the guiding means comprises a nucleotide sequence that is complementary to a target nucleic acid sequence in the host cell; and

ii. a variant Cas9, or a nucleic acid comprising a nucleotide sequence encoding the variant Cas9, wherein the variant Cas9 is the polypeptide described herein, or wherein the nucleotide sequence encoding the variant Cas9 is the polynucleotide described herein, and wherein the variant Cas9 has reduced endodeoxyribonuclease activity,

wherein said guiding means and said variant Cas9 form a complex in the host cell, said complex selectively modulating transcription of at least one target nucleic acid in the host cell.

In yet another aspect, the invention relates to a clonal library obtainable by the methods disclosed herein, said clonal library comprising a plurality of clones harbouring at least one deletion and/or indel around at least one target nucleic acid sequence, wherein said deletion is a random-sized deletion of at least 1 bp and wherein said indel is a deletion or insertion of at least 1 bp.

In yet another aspect, the invention relates to a kit for performing the method of the first aspect, said kit comprising a vector comprising a nucleic acid sequence encoding a Cas9 nuclease or a variant thereof, and instructions for use.

In yet another aspect, the invention relates to a kit for performing the method of the second aspect, said kit comprising a vector comprising a variant Cas9, or a nucleic acid comprising a nucleotide sequence encoding the variant Cas9, wherein the variant Cas9 is the polypeptide of claim 4 or the nucleotide sequence encoding the variant Cas9 is the polynucleotide of claim 3, and wherein the variant Cas9 has reduced endodeoxyribonuclease activity, and instructions for use.

Definitions

Break: the term 'break' shall be construed as referring to a double strand break, a single strand break or a nick in a DNA strand.

Cluster or gene cluster: these terms refer to a group of closely linked genes that are collectively responsible for a multi-step process such as the biosynthesis of a metabolite, for example a secondary metabolite.

CRISPR-Cas9 system: the terms 'CRISPR-Cas9', 'CRISPR/Cas9' and 'type II CRISPR' and systems thereof will be used interchangeably and refer to a system comprising a CRISPR-Cas9 protein and at least one guiding means, so that the CRISPR-Cas9 system is capable, when induced, of generating at least one break in at least one target nucleic acid sequence. Thus a CRISPR-Cas9 system herein comprises Cas9 and at least one guiding means. The guiding means are as defined below.

Deletion: the term 'deletion' refers to the deletion of one or more nucleotides or base pairs in a nucleic acid sequence. The term 'precise deletion' refers to smaller deletions, while the term 'random-sized deletion' refers to deletions of at least 1 bp which can

span over several kilobases, as detailed below.

Double strand break (DSB): a double strand break (DSB) as understood herein refers to a break on both strands of a nucleic acid. DSBs are particularly hazardous to the cell because they can lead to genome rearrangements. Two major mechanisms exist to repair DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR). The choice of pathway depends on parameters such as the nature of the organism and the cell cycle phase.

Enhancers: enhancers are *cis*-acting elements that can regulate transcription from nearby genes and function by acting as binding sites for transcription factors.

Gene: A gene as understood herein refers to a gene or a putative gene. The gene may code for a selection marker, a protein of interest, a peptide, a secondary metabolite, or it may be a gene resulting in the production of a miRNA, a siRNA, a tRNA, or any gene which can be transcribed and/or translated.

Guiding means: in the present context, the term refers to an element capable of guiding a nuclease such as Cas9 towards its target. Guiding means can be for example a single guide RNA (sgRNA) or a crRNA/tracrRNA set.

Homologous Recombination (HR): Homologous Recombination is one of the two major pathways for repairing DSBs. HR is a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA. HR involves copying information from a donor DNA. The terms HR and HDR (homology-directed repair) are herein used interchangeably.

Homology arm or homologous recombination (HR) template: the term covers a stretch of DNA with sequences homologous to the upstream and downstream regions of a region of interest, in particular of a cut site or a targeted endonuclease site.

Indel: an indel refers to a mutation class, resulting in an insertion and/or a deletion of nucleotides, leading to a net change in the total number of nucleotides. The change in the total number of nucleotides is typically in the range of 1 to 5 nucleotides, but may be up to 100 nucleotides or more.

Knockdown: the term refers to the process by which genes transcription levels can be reduced in an organism.

Knockin: the term refers to the process by which genes can be inserted in a genome. The inserted genes may be genes from the same organism or from other species.

Knockout: the term refers to the process by which genes can be inactivated in an organism, for example by deletion or mutation of part or all of the gene, or of part or all of the elements necessary for the gene to be expressed in a functional protein.

Multiplex editing: the term refers herein to editing nucleic acid sequences of multiple sequences, which can be performed simultaneously or serially. For example, multiplex editing may refer to serial knockins and/or serial knockouts or a combination of knockins and knockouts. It may also refer to simultaneous knockins and/or knockouts of multiple target nucleic acid sequences.

Nick: a nick is a discontinuity in a double-stranded DNA molecule where there is no phosphodiester bond between adjacent nucleotides of one strand.

Non-Homologous End Joining (NHEJ): NHEJ is one of the two major pathways for repairing DSBs. The NHEJ pathway harbours four NHEJ activities defined below, which usually involve at least one Ku protein and a ligase. The two ends at the break are joined directly. The ends at the break may be resected prior to repair, which may lead to loss of some nucleotides and improper repair. Thus NHEJ is often error-prone.

NHEJ activity: the term 'activity' as used herein may refer to a protein activity such as an enzymatic activity involved in the NHEJ pathway. In particular, the term is used to refer to a domain, a peptide or a protein capable of acting as a ligase, or as a polymerase, or as a primase, or as a protein capable of binding DNA ends around a break. The DNA binding activity is typically performed by one or more Ku proteins. The ligase and primase activities can be performed by a single protein, such as ligase D. Ligase D can however also be capable of performing only one of the primase or ligase or polymerase activities. A fully functional NHEJ pathway comprises all four activities,

while a partly functional or partly deficient NHEJ lacks at least one of these four activities.

Nuclear Localisation Sequence (NLS): a nuclear localisation signal or sequence (NLS) is an amino acid sequence which 'tags' a protein for import into the cell nucleus by nuclear transport. Typically, this signal consists of one or more short sequences of positively charged lysines or arginines exposed on the protein surface. Different nuclear localised proteins may share the same NLS. An NLS has the opposite function of a nuclear export signal, which targets proteins out of the nucleus.

Nucleic acid: the term refers herein to a sequence of nucleotides.

Parasiticide: the term is to be understood in its broadest sense as an agent capable of inactivating or killing any undesirable organism and thus comprises insecticides, anthelmintic compounds, larvacides, antiparasitic agents and antiprotozoal agents.

Polynucleotide / Oligonucleotide: the terms "polynucleotide" and “oligonucleotide” as used herein denote a nucleic acid chain. Throughout this application, nucleic acids are designated starting from the 5'-end.

Promoter: a promoter is a DNA sequence near the beginning of a gene (typically upstream) that signals the RNA polymerase where to initiate transcription. Eukaryotic promoters may comprise regulatory elements several kilobases upstream of the gene and typically bind transcription factors involved in the formation of the transcriptional complex. Promoters may be inducible, i.e. their activity may be induced by the presence or absence of a biotic or abiotic compound.

Recognition: as understood herein, the term ‘recognition’ refers to the ability of a molecule to identify a nucleotide sequence. Certain enzymes may require the presence of additional recognition means, such as guiding RNAs or DNA binding domains, to efficiently recognise their substrate sequence. For example, an enzyme or a DNA binding domain may recognise a nucleic acid sequence as a potential substrate and bind to it. Guiding means such as sgRNAs or crRNA/tracrRNA sets may recognise a specific sequence to which they are at least partly homologous.

Recombinase: as understood herein, the term 'recombinase' refers to an enzyme that can catalyse directionally sensitive DNA exchange reactions between short (30–40 nucleotides) target site sequences. These reactions enable four basic functional modules, excision/insertion, inversion, translocation and cassette exchange.

Terminator: a terminator is a DNA sequence near the end of a gene (typically downstream) that signals the RNA polymerase where to stop transcription. Eukaryotic terminators are recognized by protein factors and termination is followed by polyadenylation of the mRNA.

CRISPR-Cas9 system

The invention relates to methods for gene editing around or modulation of the transcription of at least one target nucleic acid sequence in a host cell based on the use of a CRISPR-Cas9 system. The terms 'target nucleic acid sequence' and 'target sequence' will be used interchangeably.

It will be understood that throughout this document, the term 'CRISPR-Cas9' system refers to a system comprising a CRISPR-Cas9 protein and at least one guiding means, so that the CRISPR-Cas9 system is capable of recognising at least one target nucleic acid sequence. In some embodiments, the CRISPR-Cas9 system is capable of generating a break in the target nucleic acid sequence, such as a nick on one of the two strands or a double-strand break. Thus the CRISPR-Cas9 system herein comprises Cas9 and at least one guiding means, where the guiding means is capable of directing Cas9 to its target nucleic acid sequence. The guiding means may be any guiding means known in the art and suitable for this purpose. In some embodiments, the guiding means is a single guide RNA. In other embodiments, the guiding means is a set of a crRNA and a tracrRNA. The skilled person knows how to design guiding means which direct the CRISPR-Cas9 system to a desired target nucleic acid sequence.

The nucleic acid sequence encoding Cas9 may be present in the genome of the host cell, e.g. on a chromosome of the host cell, or it may be present on a vector comprised within the host cell. Likewise, the guiding means may be present in the genome of the host cell, e.g. on a chromosome of the host cell, or it may be present on a vector comprised within the host cell. The term 'present in the genome of the host cell' means that either the Cas9 gene or the guiding means are naturally present in the genome of the

host cell or that they has been introduced e.g. by genome editing and conventional transformation.

In embodiments where the nucleic acid sequence encoding Cas9 and the guiding means are comprised within a vector, Cas9 and the guiding means may be comprised within the same vector. In embodiments where the guiding means are comprised within a vector and the guiding means is a crRNA and a tracrRNA, the nucleic acid sequences for the crRNA and the tracrRNA may be comprised within two different vectors. The nucleic acid sequence encoding Cas9 may then be comprised within one of these two vectors, within a third vector or within the genome of the host cell.

The CRISPR-Cas9 system used for the methods disclosed herein may be capable of generating a break in at least one target nucleic acid sequence, such as in at least two target nucleic acid sequences, such as in at least three target nucleic acid sequences, such as in at least four target nucleic acid sequences, such as in at least five target nucleic acid sequences. The CRISPR-Cas9 system can thus be used for multiplex editing.

The skilled person knows how to adapt the CRISPR-Cas9 system recognising more than one target nucleic acid sequence. By way of illustration, the system may comprise two different sgRNAs that each target one target nucleic acid sequence when recognition of two target nucleic acid sequences is desired, or the system may comprise one sgRNA targeting a first target nucleic acid sequence and a crRNA and tracrRNA targeting a second target nucleic acid sequence. Where editing of three target sequences is desired, three different sgRNAs can be used, or two different sgRNAs each targeting a first and a second target sequence and a crRNA and tracrRNA targeting a third sequence, or one sgRNA targeting a first sequence and two sets of crRNA and tracrRNA each targeting a second and a third sequence, or three sets of crRNA and tracrRNA each targeting a different target sequence.

The sequences of the nucleic acid(s) encoding the elements of the CRISPR-Cas9 system may be codon-optimized depending on the host cell in which gene editing is to be performed. Methods for codon optimization are known in the art.

Host cell

The methods of the present invention allow editing of at least one target nucleic acid sequence comprised within a host cell.

The present method can be performed in an archaea, in a prokaryotic cell or in a eukaryotic cell. In one embodiment, the host cell is a prokaryotic cell. The present methods are particularly advantageous for gene editing in host cells that have a high GC content and where gene editing can be difficult to perform. In some embodiments, the GC content is higher than 50% or more, such as 55% or more, such as 60% or more, such as 65% or more, such as 70% or more, such as 75% or more, such as 80% or more. In a particular embodiment, the host cell is an actinobacterium. The host cell may be selected from the group consisting of *Actinomycetales*, such as *Streptomyces sp., Amycolatopsis sp.* or *Saccharopolyspora sp.* In some embodiments, the host cell is selected from the group consisting of *Streptomyces coelicolor, Streptomyces avermitilis*, *Streptomyces aureofaciens*, *Streptomyces griseus, Streptomyces parvulus, Streptomyces albus, Streptomyces vinaceus, Streptomyces acrimycinis, Streptomyces calvuligerus, Streptomyces lividans, Streptomyces limosus, Streptomyces rubiqinosis, Streptomyces azureus, Streptomyces glaucenscens, Streptomyces rimosus, Streptomyces violaceoruber, Streptomyces kanamyceticus, Amycolatopsis orientalis, Amycolatopsis mediterranei* and *Saccharopolyspora erythraea*. In a preferred embodiment, the host cell is *Streptomyces coelicolor.*

In some embodiments, the host cell is from the order *Micromonosporales*, in particular from the family *Micromonosporaceae*. In one embodiment, the genus of the host cell is selected from *Actinocatenispora, Actinoplanes, Allocatelliglobosispora, Asanoa, Catellatospora, Catelliglobosispora, Catenuloplanes, Couchioplanes, Dactylosporangium, Hamadaea, Jishengella, Krasilnikovia, Longispora, Luedemannella, Micromonospora, Phytohabitans, Phytomonospora, Pilimelia, Planosporangium, Plantactinospora, Polymorphospora, Pseudosporangium, Rhizocola, Rugosimonospora, Salinispora, Solwaraspora, Spirilliplanes, Verrucosispora, Virgisporangium, Wangella* or *Xiangella.*

In some embodiments, the host cell is from the order *Streptomycetales*, in particular from the family *Streptomycetaceae*. In one embodiment, the genus of the host cell is selected from *Kitasatospora, Parastreptomyces, Streptacidiphilus, Streptomyces* or *Trichotomospora.*

In some embodiments, the host cell is from the order *Propionibacteriales*, in particular from the family *Nocardioidaceae*. In one embodiment, the genus of the host cell is selected from *Actinopolymorpha, Aeromicrobium, Flindersiella, Friedmanniella, Kribbella, Marmoricola, Micropruina, Mumia, Nocardioides, Pimelobacter, Propionicicella, Propionicimonas, Tenggerimyces* or *Thermasporomyces.*

In some embodiments, the host cell is from the order *Propionibacteriales*, in particular from the family *Propionibacteriaceae*. In one embodiment, the genus of the host cell is selected from *Aestuariimicrobium, Auraticoccus, Brooklawnia, Granulicoccus, Luteococcus, Mariniluteicoccus, Microlunatus, Naumannella, Ponticoccus, Propionibacterium, Propioniciclava, Propioniferax, Propionimicrobium* or *Tessaracoccus.*

In some embodiments, the host cell is from the order *Pseudonocardiales*, in particular from the family *Pseudonocardiaceae*. In one embodiment, the genus of the host cell is selected from *Actinoalloteichus, Actinokineospora, Actinomycetospora, Actinophytocola, Actinorectispora, Actinosynnema, Alloactinosynnema, Allokutzneria, Amycolatopsis, Crossiella, Goodfellowiella, Haloechinothrix, Kibdelosporangium, Kutzneria, Labedaea, Lechevalieria, Lentzea, Longimycelium, Prauserella, Prauseria, Pseudonocardia, Saccharomonospora, Saccharopolyspora, Saccharothrix, Saccharothrixopsis, Sciscionella, Streptoalloteichus, Tamaricihabitans, Thermocrispum, Thermotunica, Umezawaea* or *Yuhushiella.*

In some embodiments, the host cell is from the order *Streptosporangiales*, in particular from the family *Nocardiopsaceae*. In one embodiment, the genus of the host cell is selected from *Allosalinactinospora, Haloactinospora, Marinactinospora, Murinocardiopsis, Nocardiopsis, Salinactinospora, Spinactinospora, Streptomonospora* or *Thermobifida.*

In some embodiments, the host cell is from the order *Streptosporangiales*, in particular from the family *Streptosporangiaceae*. In one embodiment, the genus of the host cell is selected from *Acrocarpospora, Astrosporangium, Clavisporangium, Herbidospora, Microbispora, Microtetraspora, Nonomuraea, Planobispora, Planomonospora, Planotetraspora, Sinosporangium, Sphaerimonospora, Sphaerisporangium, Streptosporangium, Thermoactinospora, Thermocatellispora* or *Thermopolyspora.*

In some embodiments, the host cell is from the order *Streptosporangiales*, in particular from the family *Thermomonosporaceae*. In one embodiment, the genus of the host cell is selected from *Actinoallomurus, Actinocorallia, Actinomadura, Spirillospora* or *Thermomonospora.*

The following table lists examples of species for the host cell.

Table 1. Non-exhaustive list of suitable host cells.

Class	**Order**	**Family**	**Genus**	**Species**
Actinobacteria	*Micromonosporales*	*Micromonosporaceae*	*Actinocatenispora*	*Actinocatenispora rupis*
				Actinocatenispora sera
				Actinocatenispora thailandica
			Actinoplanes	*Actinoplanes abujensis*
				Actinoplanes consettensis
				Actinoplanes philippinensis
			Allocatelliglobosispora	*Allocatelliglobosispora scoriae*
			Asanoa	*Asanoa endophytica*
				Asanoa ferruginea
				Asanoa hainanensis
			Catellatospora	*Catellatospora bangladeshensis*
				Catellatospora chokoriensis
				Catellatospora

	citrea
Catelli-globosispora	*Catelliglobosispora koreensis*
Catenuloplanes	*Catenuloplanes atrovinosus*
	Catenuloplanes castaneus
	Catenuloplanes crispus
Couchioplanes	*Couchioplanes caeruleus*
Dactylosporangium	*Dactylosporangium darangshiense*
	Dactylosporangium fulvum
	Dactylosporangium luridum
Hamadaea	*Hamadaea flava*
	Hamadaea tsunoensis
Jishengella	*Jishengella endophytica*
Krasilnikovia	*Krasilnikovia cinnamomea*
Longispora	*Longispora albida*
	Longispora fulva
Luedemannella	*Luedemannella flava*
	Luedemannella helvata
Micromonospora	*Micromonospora aquatica*
	Micromonospora arenae
	Micromonospora

	arenincolae
Phytohabitans	*Phytohabitans flavus*
	Phytohabitans houttuyneae
	Phytohabitans rumicis
Phytomonospora	*Phytomonospora endophytica*
Pilimelia	*Pilimelia anulata*
	Pilimelia columellifera
Planosporangium	*Planosporangium flavigriseum*
	Planosporangium mesophilum
	Planosporangium thailandense
Plantactinospora	*Plantactinospora endophytica*
	Plantactinospora mayteni
	Plantactinospora siamensis
Polymorphospora	*Polymorphospora rubra*
Pseudosporangium	*Pseudosporangium ferrugineum*
Rhizocola	*Rhizocola hellebori*
Rugosimonospora	*Rugosimonospora acidiphila*
	Rugosimonospora africana
Salinispora	*alinispora arenico-*

			la
			Salinispora pacifica
			Salinispora tropica
		Solwaraspora	
		Spirilliplanes	*Spirilliplanes yamanashiensis*
		Verrucosispora	*Verrucosispora andamanensis*
			Verrucosispora fiedleri
			Verrucosispora gifhornensis
		Virgisporangium	*Virgisporangium aliadipatigenens*
			Virgisporangium aurantiacum
			Virgisporangium ochraceum
		Wangella	*Wangella harbinensis*
		Xiangella	*Xiangella phaseoli*
Streptomycetales	*Streptomycetaceae*	*Kitasatospora*	*Kitasatospora arboriphila*
			Kitasatospora viridis
			Kitasatospora cystarginea
		Parastreptomyces	*Parastreptomyces abscessus*
		Streptacidiphilus	*Streptacidiphilus albus*
			Streptacidiphilus griseus
			Streptacidiphilus

	rugosus
	Streptacidiphilus thailandensis
	Streptacidiphilus carbonis
Streptomyces	*Streptomyces albidoflavus group*
	Streptomyces acrimycinis
	Streptomyces avermitilis
	Streptomyces aureofaciens
	Streptomyces albus
	Streptomyces azureus
	Streptomyces cattleya
	Streptomyces clavuligerus
	Streptomyces collinus
	Streptomyces eurocidicus
	Streptomyces erythrogriseus
	Streptomyces filamentosus
	Streptomyces fradiae
	Streptomyces griseus group
	Streptomyces glaucenscens

Streptomyces himastatinicus
Streptomyces hygroscopicus
Streptomyces hygrospinosus
Streptomyces kanamyceticus
Streptomyces lactacystinaeus
Streptomyces lavendulae
Streptomyces levis
Streptomyces libani
Streptomyces limosus
Streptomyces lividans
Streptomyces lomondensis
Streptomyces marinus
Streptomyces melanosporofaciens group
Streptomyces mexicanus
Streptomyces mobaraensis
Streptomyces polyantibioticus
Streptomyces parvulus
Streptomyces pur-

			pureus
			Streptomyces rapamycinicus
			Streptomyces rimosus
			Streptomyces rosa
			Streptomyces rubiqinosis
			Streptomyces scabrisporus
			Streptomyces sparsogenes
			Streptomyces somaliensis
			Streptomyces venezuelae
			Streptomyces vinaceus
			Streptomyces violaceoruber
			Streptomyces viridochromogenes
		Trichotomospora	*Trichotomospora caesia*
Propionibacteriales	*Nocardioidaceae*	*Actinopolymorpha*	*Actinopolymorpha alba*
			Actinopolymorpha cephalotaxi
			Actinopolymorpha pittospori
			Actinopolymorpha rutila
			Actinopolymorpha singaporensis
		Aeromicrobium	*Aeromicrobium*

	fastidiosum
	Aeromicrobium flavum
	Aeromicrobium ginsengisoli
	Aeromicrobium halocynthiae
	Aeromicrobium kazakhstani
	Aeromicrobium kwangyangensis
	Aeromicrobium marinum
Flindersiella	*Flindersiella endophytica*
Friedmanniella	*Friedmanniella aerolata*
	Friedmanniella antarctica
	Friedmanniella capsulata
	Friedmanniella flava
	Friedmanniella lacustris
	Friedmanniella lucida
	Friedmanniella luteola
	Friedmanniella okinawensis
	Friedmanniella sagamiharensis
	Friedmanniella spumicola

Kribbella	*Kribbella alba*
	Kribbella albertanoniae
	Kribbella aluminosa
	Kribbella amoyensis
	Kribbella antibiotica
	Kribbella catacumbae
	Kribbella flavida
Marmoricola	*Marmoricola aequoreus*
	Marmoricola aquaticus
	Marmoricola aurantiacus
	Marmoricola bigeumensis
	Marmoricola ginsengisoli
	Marmoricola korecus
	Marmoricola pocheonesis
	Marmoricola scoriae
	Marmoricola soli
Micropruina	*Micropruina glycogenica*
Mumia	*Mumia flava*
Nocardioides	*Nocardioides aestuarii*
	Nocardioides

		agariphilus
		Nocardioides albertanoniae
		Nocardioides albidus
		Nocardioides albus
	Pimelobacter	*Pimelobacter simplex*
	Propionicicella	*Propionicicella superfundia*
	Propionicimonas	*Propionicimonas paludicola*
	Tenggerimyces	*Tenggerimyces flavus*
		Tenggerimyces mesophilus
	Thermasporomyces	*Thermasporomyces composti*
Propionibacteriaceae	*Aestuariimicrobium*	*Aestuariimicrobium kwangyangense*
	Auraticoccus	*Auraticoccus monumenti*
	Brooklawnia	*Brooklawnia cerclae*
		Brooklawnia massiliensis
	Granulicoccus	*Granulicoccus phenolivorans*
	Luteococcus	*Granulicoccus phenolivorans*
		Luteococcus peritonei
		Luteococcus san-

	guinis
	Luteococcus sediminum
Mariniluteicoccus	*Mariniluteicoccus endophyticus*
	Mariniluteicoccus flavus
Microlunatus	*Microlunatus aurantiacus*
	Microlunatus endophyticus
	Microlunatus ginsengisoli
	Microlunatus ginsengiterrae
	Microlunatus panaciterrae
	Microlunatus parietis
Naumannella	*Naumannella halotolerans*
Ponticoccus	*Ponticoccus gilvus*
Propionibacterium	*Propionibacterium acidifaciens*
	Propionibacterium acidipropionici
	ropionibacterium acnes
	Propionibacterium avidum
Propioniciclava	*Propioniciclava tarda*
Propioniferax	*Propioniferax innocua*
Propionimicro-	*Propionimicrobium*

		bium	*lymphophilum*
		Tessaracoccus	*Tessaracoccus bendigoensis*
			Tessaracoccus flavescens
			Tessaracoccus flavus
			Tessaracoccus lapidicaptus
			Tessaracoccus lubricantis
			Tessaracoccus oleiagri
			Tessaracoccus profundi
			Tessaracoccus rhinocerotis
Pseudonocar-diales	*Pseudonocardi-aceae*	*Actinoalloteich-us*	*Actinoalloteichus alkalophilus*
			Actinoalloteichus cyanogriseus
		Actinokineospo-ra	*Actinokineospora auranticolor*
			Actinokineospora baliensis
			Actinokineospora bangkokensis
			Actinokineospora cianjurensis
			Actinokineospora cibodasensis
			Actinokineospora diospyrosa
			Actinokineospora enzanensis

	Actinokineospora inagensis
Actinomycetospora	*Actinomycetospora chiangmaiensis*
	Actinomycetospora chibensis
	Actinomycetospora chlora
	Actinomycetospora cinnamomea
Actinophytocola	*Actinophytocola burenkhanensis*
	Actinophytocola corallina
	Actinophytocola gilvus
	Actinophytocola oryzae
	Actinophytocola sediminis
	Actinophytocola timorensis
	Actinophytocola xinjiangensis
Actinorectispora	*Actinorectispora indica*
Actinosynnema	*Actinosynnema mirum*
Alloactinosynnema	*Alloactinosynnema album*
	Alloactinosynnema iranicum
Allokutzneria	*Allokutzneria albata*
	Allokutzneria mul-

	tivorans
	Allokutzneria oryzae
Amycolatopsis	*Amycolatopsis alba*
	Amycolatopsis azurea
	Amycolatopsis coloradensis
	Amycolatopsis coloradensis
	Amycolatopsis halophila
	Amycolatopsis lurida
	Amycolatopsis mediterranei
	Amycolatopsis pigmentata
	Amycolatopsis taiwanensis
Crossiella	*Crossiella cryophila*
	Crossiella equi
Goodfellowiella	*Goodfellowiella coeruleoviolacea*
Haloechinothrix	*Haloechinothrix alba*
Kibdelosporangium	*Haloechinothrix alba*
Kutzneria	*Kutzneria albida*
Labedaea	*Labedaea rhizosphaerae*
Lechevalieria	*Lechevalieria aerocolonigenes*

	Lechevalieria atacamensis
	Lechevalieria deserti
	Lechevalieria flava
	Lechevalieria fradiae
	Lechevalieria nigeriaca
	Lechevalieria roselyniae
	Lechevalieria xinjiangensis
Lentzea	*Lentzea albida*
	Lentzea albidocapillata
	Lentzea californiensis
	Lentzea flaviverrucosa
	Lentzea jiangxiensis
	Lentzea kentuckyensis
	Lentzea violacea
	Lentzea waywayandensis
Longimycelium	*Longimycelium tulufanense*
Prauserella	*Prauserella aidingensis*
	Prauserella alba
	Prauserella coralliicola
	Prauserella flava

Prauseria	*Prauseria hordei*
Pseudonocardia	*Pseudonocardia acaciae*
	Pseudonocardia asaccharolytica
	Pseudonocardia spinosispora
	Pseudonocardia sulfidoxydans
	Pseudonocardia tetrahydrofuranoxydans
	Pseudonocardia tetrahydrofuranoxydans
Saccharomonospora	*Saccharomonospora azurea*
	Saccharomonospora cyanea
	Saccharomonospora viridis
	Saccharomonospora marina
Saccharopolyspora	*Saccharopolyspora antimicrobica*
	Saccharopolyspora cavernae
	Saccharopolyspora cebuensis
	Saccharopolyspora dendranthemae
	Saccharopolyspora emeiensis
	Saccharopolyspora endophytica

	Saccharopolyspora erythraea
	Saccharopolyspora spinosa
	Saccharopolyspora rosea
Saccharothrix	*Lentzea flavoverrucoides*
	Saccharothrix algeriensis
	Saccharothrix australiensis
	Saccharothrix carnea
	Saccharothrix coeruleofusca
	Saccharothrix espanaensis
Saccharothrixopsis	*Saccharothrixopsis albidus*
Sciscionella	*Sciscionella marina*
Streptoalloteichus	*Streptoalloteichus hindustanus*
	Streptoalloteichus tenebrarius
Tamaricihabitans	*Tamaricihabitans halophyticus*
Thermocrispum	*Thermocrispum agreste*
	Thermocrispum municipale
Thermotunica	*Thermotunica guangxiensis*
Umezawaea	*Umezawaea tan-*

			gerina
		Yuhushiella	*Yuhushiella deserti*
Streptosporangiales	*Nocardiopsaceae*	*Allosalinactinospora*	*Allosalinactinospora lopnorensis*
		Haloactinospora	*Haloactinospora alba*
		Marinactinospora	*Marinactinospora thermotolerans*
		Murinocardiopsis	*Murinocardiopsis flavida*
		Nocardiopsis	*Nocardiopsis aegyptia*
			Nocardiopsis alba
			Nocardiopsis algeriensis
			Nocardiopsis alkaliphila
			Nocardiopsis baichengensis
			Nocardiopsis chromatogenes
			Nocardiopsis ganjiahuensis
			Nocardiopsis lucentensis
			Nocardiopsis potens
			Nocardiopsis synnemataformans
			Nocardiopsis prasina
			Nocardiopsis halophila

		Salinactinospora	*Salinactinospora qingdaonensis*
			Salinactinospora qingdaonensis
		Spinactinospora	*Streptomonospora alba*
		Streptomonospora	*Streptomonospora algeriensis*
			Streptomonospora amylolytica
			Streptomonospora arabica
			Streptomonospora flavalba
			Streptomonospora halophila
			Streptomonospora nanhaiensis
			Streptomonospora salina
			Streptomonospora sediminis
		Thermobifida	*Thermobifida cellulosilytica*
			Thermobifida fusca
			Thermobifida alba
	Streptosporangiaceae	*Acrocarpospora*	*Acrocarpospora corrugata*
			Acrocarpospora macrocephala
			Acrocarpospora phusangensis
			Acrocarpospora pleiomorpha

Astrosporangium	*Astrosporangium hypotensionis*
Clavisporangium	*Clavisporangium rectum*
Herbidospora	*Herbidospora cretacea*
	Herbidospora daliensis
	Herbidospora mongoliensis
	Herbidospora sakaeratensis
	Herbidospora yilanensis
Microbispora	*Microbispora amethystogenes*
	Microbispora bryophytorum
	Microbispora camponoti
	Microbispora corallina
	Microbispora griseoalba
	Microbispora hainanensis
	Microbispora mesophila
	Microbispora rosea
Microtetraspora	*Microtetraspora fusca*
	Microtetraspora glauca
	Microtetraspora

	malaysiensis
	Microtetraspora niveoalba
Nonomuraea	*Nonomuraea aegyptia*
	Nonomuraea africana
	Nonomuraea angiospora
	Nonomuraea antimicrobica
	Nonomuraea asiatica
	Nonomuraea aurea
	Nonomuraea bangladeshensis
	Nonomuraea candida
Planobispora	*Planobispora longispora*
	Planobispora rosea
	Planobispora siamensis
	Planobispora takensis
Planomonospora	*Planomonospora alba*
	Planomonospora parontospora
Planotetraspora	*Planotetraspora kaengkrachanensis*
	Planotetraspora

	mira
	Planotetraspora phitsanulokensis
	Planotetraspora silvatica
	Planotetraspora thailandica
Sinosporangium	*Sinosporangium album*
	Sinosporangium siamense
Sphaerimonospora	*Sphaerimonospora cavernae*
Sphaerisporangium	*Sphaerisporangium album*
	Sphaerisporangium cinnabarinum
	Sphaerisporangium flaviroseum
Streptosporangium	*Sphaerisporangium album*
	Sphaerisporangium cinnabarinum
	Sphaerisporangium flaviroseum
	Sphaerisporangium krabiense
	Sphaerisporangium melleum
	Sphaerisporangium rubeum
	Sphaerisporangium rufum
	Sphaerisporangium siamense

		Sphaerisporangium viridialbum
	Thermoactinospora	*Thermoactinospora rubra*
	Thermocatellispora	*Thermocatellispora tengchongensis*
	Thermopolyspora	*Thermopolyspora flexuosa*
Thermomonosporaceae	*Actinoallomurus*	*Actinoallomurus caesius*
		Actinoallomurus coprocola
		Actinoallomurus fulvus
		Actinoallomurus iriomotensis
		Actinoallomurus acaciae
		Actinoallomurus acanthiterrae
		Actinoallomurus amamiensis
		Actinoallomurus bryophytorum
	Actinocorallia	*Actinocorallia aurantiaca*
		Actinocorallia aurea
		Actinocorallia cavernae
		Actinocorallia glomerata
		Actinocorallia herbida
		Actinocorallia liba-

	notica
	Actinocorallia longicatena
	Actinocorallia spatholoba
Actinomadura	*Actinomadura alba*
	Actinomadura amylolytica
	Actinomadura apis
	Actinomadura atramentaria
	Actinomadura bangladeshensis
	Actinomadura catellatispora
	Actinomadura cellulosilytica
	Actinomadura chibensis
Spirillospora	*Spirillospora albida*
	Spirillospora rubra
Thermomonospora	*Thermomonospora curvata*
	Thermomonospora chromogena

Method for generating random-sized deletions or indels around a target site

In a first aspect, the invention relates to a method for generating at least one deletion around at least one target nucleic acid sequence comprised within a host cell having a non-homologous end-joining (NHEJ) pathway which is at least partly deficient,

said method comprising the steps of:

(i) optionally, restoring the full functionality of the NHEJ pathway,

(ii) inducing a CRISPR-Cas9 system in said host cell, wherein said CRISPR-Cas9 system is able to generate at least one break in said at least one target nucleic acid sequence and wherein the CRISPR-Cas9 system comprises a Cas9 nuclease and at least one guiding means,

thereby generating:

a. if the method does not comprise step (i), at least one random-sized deletion around said at least one target nucleic acid sequence, wherein said at least one deletion is a random-sized deletion of at least 1 bp; or

b. if the method does comprise step (i), at least one indel around said at least one target nucleic acid sequence, wherein said at least one indel is a deletion or insertion of at least1 bp.

The methods the present disclosure thus take advantage of the fact that in host cells, wherein the NHEJ pathway is at least partly deficient, a CRISPR-Cas9 system can be induced and generates either random-sized deletions around a target site, or indels around a target site if the functionality of the NHEJ pathway is restored prior to or simultaneously with induction of the CRISPR-Cas9 system.

Method for generating random-sized deletions around a target site

In some embodiments, the method does not comprise step (i). In other words, the NHEJ pathway is maintained partly deficient. The present disclosure thus provides a method for generating at least one random-sized deletion around at least one target nucleic acid sequence comprised within a host cell having a non-homologous end-joining (NHEJ) pathway which is at least partly deficient, said method comprising the step of inducing a CRISPR-Cas9 system in a host cell, said CRISPR-Cas9 system being able to generate at least one break in said at least one target nucleic acid sequence, thereby generating at least one deletion around said at least one target nucleic acid sequence, wherein said at least one deletion is a deletion of at least 1 bp.

The method is based on the surprising finding that performing CRISPR-Cas9 directed gene editing in organisms having a partly deficient NHEJ pathway leads to the generation of random-sized deletions around a target nucleic acid sequence. This is surprising because performing CRISPR-Cas9 directed editing in organisms lacking NHEJ was

believed to be lethal (Citorik, R. J. et, al 2014, Gomaa, A. et, al 2014, Bikard, D., et, al, 2014). The gene editing is preferably performed without homology arms so that the repair of the at least one break generated by Cas9 is directed towards the NHEJ pathway. Thus in some embodiments, the method for generating at least one deletion described herein is performed with the proviso that the editing is not done with a homologous template.

In some embodiments, the guiding means comprises at least one sgRNA and/or at least one crRNA/tracrRNA set.

Also disclosed herein is a method for generating at least one deletion around at least one target nucleic acid sequence comprised within a host cell having a non-homologous end-joining (NHEJ) pathway which is at least partly deficient, said method comprising the step of inducing a CRISPR-Cas9 system in a host cell, said CRISPR-Cas9 system being able to generate at least one break in said at least one target nucleic acid sequence, thereby generating at least one deletion around said at least one target nucleic acid sequence, wherein said at least one deletion is a deletion of at least 1 bp, wherein the CRISPR-Cas9 system comprises a Cas9 nuclease encoded by a polynucleotide having at least 93% identity with SEQ ID NO: 1, such as at least 94% identity, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity, such as 100% identity with SEQ ID NO: 1. In some embodiments, the Cas9 nuclease is identical to SEQ ID NO: 2.

NHEJ

The method disclosed herein for generating random-sized deletions around at least one target nucleic acid sequence is preferably performed in a host cell wherein the NHEJ pathway is at least partly deficient.

The NHEJ pathway involves four activities dependent on two groups of proteins:

(a) the Ku proteins, which bind to DNA double-strand break ends and are required for the non-homologous end joining;

(b) the ligase, such as the ligase D ligD, which can perform the activities of ligase, polymerase and primase.

In some embodiments, the NHEJ pathway of the host cell thus lacks at least one of the four NHEJ activities defined as:

- a DNA-binding activity,
- a primase activity,
- a ligase activity,
- a polymerase activity.

The DNA-binding activity is typically performed by Ku proteins such as Ku70, Ku80, or homologues, orthologues or paralogues thereof. The primase activity can be performed by a eukaryotic-archeal DNA primase (EP) or a homologue, an orthologue or a paralogue thereof, or by a ligase D or a homologue, an orthologue or a paralogue thereof. The ligase activity is typically performed by ligase D or a homologue, an orthologue or a paralogue thereof. The polymerase activity is typically performed by a ligase D or a homologue, an orthologue or a paralogue thereof.

As understood herein, a functional NHEJ pathway comprises all four activities, e.g. it may comprise one Ku protein with a DNA-binding activity and a ligase capable of performing the activities of ligase, polymerase and primase. In some embodiments, the activities of ligase, polymerase and primase are performed by the same or by two, three or four different proteins, peptides or domains. A partly deficient NHEJ pathway lacks at least one of the four activities. In some embodiments, the NHEJ pathway of the host cell thus lacks at least one of the DNA-binding activity, of the ligase activity, of the polymerase activity and of the primase activity. In a preferred embodiment, the NHEJ pathway is partly deficient because the ligase can only perform the primase activity. For example, the Ku proteins are present and functional, but the ligase lacks the ligase activity.

The NHEJ pathway may be deficient because it is naturally deficient in the host cell, or because at least one of the four activities has been inactivated. In some embodiments, the DNA-binding activity is inactivated, e.g. by targeted deletion of the nucleic acid sequence(s) encoding the Ku protein(s). In further embodiments, the primase activity is inactivated. In other embodiments, the ligase activity is inactivated. In yet other embodiments, the polymerase activity is inactivated. Preferably, at least the ligase activity is inactivated. Other methods for inactivating at least one of the four NHEJ activities are known to the skilled person.

Host cells where the NHEJ pathway is naturally deficient can be identified by methods known in the art, such as gene mining or sequence blasting.

The activities referred to above may be performed by a domain, peptide or protein. The nucleic acid sequences encoding the domain, peptide or protein capable of performing said activities may be comprised within the genome of the host cell or may be comprised on a vector.

Target nucleic acid

The method disclosed herein is particularly useful for generating random-sized deletions around at least one target nucleic acid sequence of interest. The present method can thus be used in order to generate clonal libraries containing a plurality of cells having deletions of different sizes around at least one target nucleic acid of interest, as described below. The method can thus be useful for, but not limited to, the investigation of pathway regulations and identification of metabolite production bottlenecks, the screening of producer strains and the identification of new compounds produced by the host cell. The libraries thus generated are not completely random in that the target nucleic acid is predefined.

The target nucleic acid sequence may be comprised within any nucleic acid sequence of interest. For example, the target sequence may be comprised within or may comprise an open reading frame or a putative open reading frame, or it may be comprised within or may comprise a regulatory region or a putative regulatory region, such as an enhancer, a promoter, an insulator, a terminator.

The target nucleic acid sequence may be involved in a pathway of interest. In some embodiments, the target nucleic acid encodes an enzyme or a protein. In other embodiments, the target nucleic acid is comprised within or comprises a biosynthetic gene or a putative biosynthetic gene. In some embodiments, the biosynthetic gene is involved in the synthesis of a secondary metabolite.

In some embodiments, the target nucleic acid sequence is comprised within a gene cluster. In specific embodiments, the gene cluster is a secondary metabolite gene cluster.

There is thus disclosed herein a method for editing a target nucleic acid sequence optionally comprised within or comprising a gene cluster, where the target nucleic acid sequence is involved or is suspected of being involved in the biosynthesis of a secondary metabolite.

In some embodiments, the secondary metabolite is selected from the group consisting of antibiotics, herbicides, anti-cancer agents, immunosuppressants, flavors, parasiticides and proteins. The term 'parasiticide' is to be understood in its broadest sense as an agent capable of inactivating or killing any undesirable organism and thus comprises insecticides, anthelmintic compounds, larvacides, antiparasitic agents and antiprotozoal agents.

In some embodiments, the secondary metabolite is an antibiotic selected from the group consisting of apramycin, bacitracin, chloramphenicol cephalosporins, cycloserine, erythromycin, fosfomycin, gentamicin, kanamycin, kirromycin, lassomycin, lincomycin, lysolipin, microbisporicin, neomycin, noviobiocin, nystatin, nitrofurantoin, platensimycin, pristinamycins, rifamycin, streptomycin, teicoplanin, tetracycline, tinidazole, ribostamycin, daptomycin, vancomycin, viomycin and virginiamycin.

In other embodiments, the secondary metabolite is a herbicide selected from the group consisting of bialaphos, resormycin and phosphinothricin.

In yet other embodiments, the secondary metabolite is an anti-cancer agent selected from the group consisting of doxorubicin, salinosporamides, aclarubicin, pentostatin, peplomycin, thrazarine and neocarcinostatin.

In yet other embodiments, the secondary metabolite is an immunosuppressant selected from the group consisting of rapamycin, FK520, FK506, cyclosporine, ushikulides, pentalenolactone I and hygromycin A.

In yet other embodiments, the secondary metabolite is a flavor such as geosmin.

In yet other embodiments, the secondary metabolite is a parasiticide such as an insecticide, an anthelmintic, a larvacide, or an antiprotozoal agent such as spinsad or avermectin.

In other embodiments, the target nucleic acid codes for an enzyme selected from the group consisting of an amylase, a protease, a cellulase, a chitinase, a keratinase and a xylanase.

In some embodiments, only one target nucleic acid sequence is targeted for editing and generation of random-sized deletions. In other embodiments, more than one target nucleic acid sequence is targeted and the method is a multiplex method. Thus the method can be used for generating at least one deletion around at least one target nucleic acid sequence, such as at least two deletions around at least two target nucleic acid sequences, such as at least three deletions around at least three target nucleic acid sequences, such as at least four deletions around at least four target nucleic acid sequences, such as at least five deletions around at least five target nucleic acid sequences, or more, wherein each deletion as a deletion of at least 1 bp. The method can thus be used for generating one deletion around one target nucleic acid sequence, or two deletions around at least two target nucleic acid sequences, or three deletions around three target nucleic acid sequences, or four deletions around four target nucleic acid sequences, or five deletions around five target nucleic acid sequences, or more. As explained above, in the case of multiplex editing, a guiding means is preferably provided for each target nucleic acid sequence.

In some embodiments, the at least one deletion results in the inactivation of at least one gene. In some embodiments, the at least one gene is comprised within a gene cluster. In other embodiments, the at least one gene is not comprised within a gene cluster.

The at least one deletion generated by the present method is a deletion of at least 1 bp and may range over several thousands kilobases. In some embodiments, the deletion is a deletion of 1 to 2. 10^6 bp, such as 1 to 1. 10^6 bp, such as 1 to 500000 bp, such as 1 to 400000 bp, such as 1 to 300000 bp, such as 1 to 200000 bp, such as 1 to 100000 bp, such as 2 to 75000 bp, such as 3 to 50000 bp, such as 4 to 40000 bp, such as 5 to 30000 bp, such as 10 to 20000 bp, such as 25 to 10000 bp, such as 50 to 9000 bp,

such as 75 to 8000 bp, such as 100 to 7000 bp, such as 150 to 6000 bp, such as 200 to 5000 bp, such as 250 to 4000 bp, such as 300 to 3000 bp, such as 400 to 2000 bp, such as 500 to 1000 bp, such as 600 to 900 bp, such as 700 to 800 bp. In some embodiments, the deletion is a deletion of at least 1 bp, such as at least 2 bp, such as at least 3 bp, such as at least 4 bp, such as at least 5 bp, such as at least 10 bp, such as at least 15 bp, such as at least 20 bp, such as at least 50 bp, such as at least 100 bp, such as at least 250 bp, such as at least 500 bp. In some embodiments, the deletion is a deletion of 1 to 100 bp, such as 1 to 75 bp, such as 1 to 50 bp, such as 1 to 40 bp, such as 1 to 30 bp, such as 1 to 20 bp, such as 1 to 10 bp, such as 1 to 9 bp, such as 1 to 8 bp, such as 1 to 7 bp, such as 1 to 6 bp, such as 1 to 5 bp, such as 1 to 4 bp, such as 1 to 3 bp, such as 1 to 2 bp.

Efficiency and off-target effects

Several parameters can have an impact on the efficiency of the present method for generating random-sized deletions around at least one target sequence. Some parameters can be adjusted as known in the art. Parameters susceptible of having an impact on the efficiency include, but are not limited to: the sequence of the guiding means (sgRNA or crRNA/tracrRNA), the sequence of the target nucleic acid, the GC content of the host cell and the GC content of the target nucleic acid sequence.

The method can be performed with relatively few off-target effects. In some embodiments, the desired deletion is generated in more than 1% of the host cells, such as in more than 5% of the host cells, such as in more than 10% of the host cells, such as in more than 15% of the host cells, such as in more than 20% of the host cells, such as in more than 25% of the host cells, such as in more than 30% of the host cells, such as in more than 35% of the host cells, such as in more than 40% of the host cells, such as in more than 45% of the host cells, such as in more than 50% of the host cells, such as in more than 55% of the host cells, such as in more than 60% of the host cells, such as in more than 65% of the host cells, such as in more than 70% of the host cells, such as in more than 75% of the host cells, such as in more than 80% of the host cells, such as in more than 85% of the host cells, such as in more than 90% of the host cells, such as in more than 95% of the host cells, such as in 100% of the host cells.

Characterisation and screening

The present method can thus be used for generating random sized deletions around a target nucleic acid sequence of interest, for example a sequence encoding for a gene involved in a pathway of interest. This can result in a plurality of clones having random-sized deletions around the target sequence. These clones can then be further analysed or screened. For example, producer strains having advantageous production profiles for a desired compound can be selected.

In some embodiments, it may be of interest to determine the size of the at least one deletion for a particular clone. Thus the method may comprise a further step of determining the size of the at least one deletion. Methods for determining the size of a deletion are known in the art and include, but are not limited to, whole genome sequencing, pulsed field gel electrophoresis, nucleic acid amplification-based methods such as PCR, for example followed by restriction analysis and detection of the PCR products on a gel and determination of the size of the products using an appropriate marker. The PCR products can also be sequenced if precise determination of the size of the deletion is desired.

In some embodiments, the method further comprises a step of selection of clones having the desired characteristics. Such selection methods are known in the art and encompass screening methods, chemical analysis of the related gene products (proteins or metabolites), sequencing of the related gene regions, and/or analysis of the gene expression level.

Clonal library

In one aspect, the disclosure relates to a clonal library obtainable by the method for generating random-sized deletions around at least one target nucleic acid sequence as described herein above. Such clonal libraries comprise a plurality of clones obtained by said method, wherein each clone harbours at least one deletion around at least one target nucleic acid sequence, wherein each of said deletions is a deletion of at least 1 bp.

The clonal libraries may be generated by multiplex methods, wherein more than one deletion is generated around more than one target nucleic acid in each clone.

The clonal libraries may be libraries of archaea, prokaryotes or eukaryotes. In one embodiment, the clonal library is a prokaryotic clonal library. In some embodiments, the clones of the clonal library have a high GC content. In some embodiments, the GC content is higher than 45%, such as 50% or more, such as 55% or more, such as 60% or more, such as 65% or more, such as 70% or more, such as 75% or more, such as 80% or more. In a particular embodiment, the clonal library is a library of an actinobacterium, for example selected from the group consisting of *Actinomycetales*, such as *Streptomyces sp., Amycolatopsis sp.* or *Saccharopolyspora sp.* In some embodiments, the clonal library is a library of clones derived from *Streptomyces coelicolor, Streptomyces avermitilis*, *Streptomyces aureofaciens*, *Streptomyces griseus, Streptomyces parvulus, Streptomyces albus, Streptomyces vinaceus, Streptomyces acrimycinis, Streptomyces calvuligerus, Streptomyces lividans, Streptomyces limosus, Streptomyces rubiqinosis, Streptomyces azureus, Streptomyces glaucenscens, Streptomyces rimosus, Streptomyces violaceoruber, Streptomyces kanamyceticus, Amycolatopsis orientalis, Amycolatopsis mediterranei* or *Saccharopolyspora erythraea*. In a preferred embodiment, the clonal library is a library of *Streptomyces coelicolor* clones.

Method for generating precise indels around a target site

In some embodiments, the method comprises the step of restoring full functionality of the at least partly deficient NHEJ pathway in the host cell prior to or simultaneously with the step of inducing a CRISPR-Cas9 system. This results in generation of at least one indel around at least one target nucleic acid sequence comprised within a host cell having a non-homologous end-joining (NHEJ) pathway which is at least partly deficient, said method comprising the steps of (i) restoring the full functionality of the NHEJ pathway in said host cell; (ii) inducing a CRISPR-Cas9 system in said host cell, said CRISPR-Cas9 system being able to generate at least one break in said at least one target nucleic acid sequence, thereby generating at least one indel around said at least one target nucleic acid sequence, wherein said at least one indel is an insertion or a deletion of at least 1 bp such as at least 2 bp, such as at least 3 bp, such as at least 4 bp, such as at least 5 bp, such as at least 10 bp, such as at least 15 bp, such as at least 20 bp, such as at least 50 bp, such as at least 100 bp, such as at least 250 bp, such as at least 500 bp.

In some embodiments, the guiding means comprises at least one sgRNA and/or at least one crRNA/tracrRNA set.

In a host cell having a partly deficient NHEJ pathway, CRISPR-Cas9 gene editing results in the generation of random-sized deletions around the target sites, as disclosed in the first aspect of the invention. The deletions can, as described above and as shown in the examples, be very large. While this may be of interest in some cases, it may sometimes be desirable to generate precise deletions or insertions around target sequences instead. The terms 'precise deletion' or 'precise insertion' or 'precise indel' preferably refer herein to to insertions, deletions or indels of which the size can be determined in advance, as opposed to random-sized deletions. These can be short deletions, insertions or indels, i.e. spanning over small areas as detailed below. The second aspect of the invention describes how this can be achieved. In some embodiments, the gene editing is performed without homology arms so that the repair of the at least one break generated by Cas9 is directed towards the NHEJ pathway. In other embodiments, the gene editing is performed with homology arms so that the repair of the at least one break generated by Cas9 is directed toward the HDR pathway.

There is disclosed herein a method for generating at least one indel around at least one target nucleic acid sequence comprised within a host cell having a non-homologous end-joining (NHEJ) pathway which is at least partly deficient, said method comprising the steps of (i) restoring the full functionality of the NHEJ pathway in said host cell; (ii) inducing a CRISPR-Cas9 system in said host cell, said CRISPR-Cas9 system being able to generate at least one break in said at least one target nucleic acid sequence, thereby generating at least one indel around said at least one target nucleic acid sequence, wherein said at least one indel is an indel of at least 1 bp, wherein the CRISPR-Cas9 system comprises a Cas9 nuclease encoded by a polynucleotide having at least 93% identity with SEQ ID NO: 1, such as at least 94% identity, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity, such as 100% identity with SEQ ID NO: 1. In some embodiments, the Cas9 nuclease is identical to SEQ ID NO: 2.

Restoring NHEJ

The method disclosed herein for generating precise indels around at least one target nucleic acid sequence is preferably performed in a host cell wherein the NHEJ pathway is at least partly deficient.

Host cells where the NHEJ pathway is naturally deficient can be identified by methods known in the art, such as gene mining or sequence blasting.

The NHEJ pathway involves four activities dependent on two groups of proteins:

(a) the Ku proteins, which bind to DNA double-strand break ends and are required for the non-homologous end joining;

(b) the ligase, such as the ligase D ligD, which can perform the activities of ligase, polymerase and primase.

In some embodiments, the NHEJ pathway of the host cell thus lacks at least one of four activities defined as:

- a DNA-binding activity,
- a primase activity,
- a ligase activity
- a polymerase activity.

The DNA-binding activity is typically performed by Ku proteins such as Ku70, Ku80, or homologues, orthologues or paralogues thereof. The primase activity can be performed by a eukaryotic-archeal DNA primase (EP) or a homologue, an orthologue or a paralogue thereof, or by a ligase D or a homologue, an orthologue or a paralogue thereof. The ligase activity is typically performed ligase D or a homologue, an orthologue or a paralogue thereof. The polymerase activity is typically performed by a ligase D or a homologue, an orthologue or a paralogue thereof.

As understood herein, a functional NHEJ pathway comprises all four activities, e.g. it comprises one Ku protein with a DNA-binding activity and a ligase capable of performing the activities of ligase and primase. A partly deficient NHEJ pathway lacks at least one of the four activities. In some embodiments, the NHEJ pathway of the host cell thus lacks at least one of the DNA-binding activity, of the polymerase activity, of the ligase activity and of the primase activity. In a preferred embodiment, the NHEJ pathway is partly deficient because the ligase can only perform the primase activity. For example, the Ku proteins are present and functional, but the ligase lacks the ligase activity.

The NHEJ pathway may be deficient because it is naturally deficient in the host cell, or because at least one of the four activities has been inactivated. In some embodiments, the DNA-binding activity is inactivated, e.g. by targeted deletion of the nucleic acid sequence(s) encoding the Ku protein(s). In further embodiments, the primase activity is inactivated. In other embodiments, the ligase activity is inactivated. In yet other embodiments, the polymerase activity is inactivated. Preferably, at least the ligase activity is inactivated. Other methods for inactivating at least one of the four NHEJ activities are known to the skilled person.

The activities referred to above may be performed by a domain, peptide or protein. The nucleic acid sequences encoding the domain, peptide or protein capable of performing said activities may be comprised within the genome of the host cell or may be comprised on a vector.

In order to generate precise indels around at least one target nucleic acid sequence, the at least one NEHJ activity which is lacking in the host cell may need to be restored. This can be achieved by introducing a nucleic acid sequence comprising a sequence encoding a domain, a peptide or a protein capable of performing said lacking NHEJ activity into the host cell.

The nucleic acid sequence comprising a sequence such as an open reading frame encoding said domain, peptide or protein capable of performing said lacking activity (hereinafter also referred to as 'the nucleic acid sequence encoding said lacking activity') can be introduced into the host cell's genome, e.g. on a chromosome, or it can be comprised within a vector and the vector can be introduced within the host cell.

The nucleic acid sequence encoding the lacking NHEJ activity can be under the control of an inducible promoter and may comprise other elements besides an open reading frame encoding the activity. For example, the nucleic acid sequence may further comprise a terminator, a sequence encoding a selection marker and/or a sequence encoding a fluorescent protein.

In some embodiments, the nucleic acid sequence encoding the lacking NHEJ activity and the nucleic acid sequence encoding Cas9 may be comprised within a single nucleic acid, for example they may be on the same vector or they may be integrated at the

same location in the genome of the host cell. Likewise, the nucleic acid sequence encoding the lacking NHEJ activity and the nucleic acid sequence encoding the guiding means may be comprised within a single nucleic acid, for example they may be on the same vector or they may be integrated at the same location in the genome of the host cell. In some embodiments, the nucleic acid sequence encoding the lacking NHEJ activity, the nucleic acid sequence encoding Cas9 and the nucleic acid sequence encoding the guiding means are all comprised within a single nucleic acid. Each of these three elements may also be comprised each within one nucleic acid.

In some embodiments, the host cell is lacking more than one NHEJ activity. It may lack two NHEJ activities or it may lack three NHEJ activities or four NHEJ activities. In order to restore NHEJ, it may be necessary to restore each of the lacking activities. The nucleic acid sequences encoding each of the lacking activities can be comprised within a single nucleic acid, or they can be comprised within different nucleic acids. The guiding means and Cas9 may be comprised within the same nucleic acid as one or all of the sequences encoding the lacking activity, or they may be comprised within a different nucleic acid, as above.

In some embodiments, restoration of the lacking NHEJ activity or activities is achieved by introduction of a heterologous gene encoding a domain, protein or peptide capable of performing the lacking activity when it is expressed in the host cell. Suitable heterologous genes can be identified by methods such as blasting a genome database using a nucleic acid sequence encoding the lacking activity as a query. The query sequence is preferably the sequence of a cell naturally possessing the activity lacking in the host cell in which the method is to be performed. Preferably, the query sequence is taken from a cell which is related to the host cell, for example from a cell which is phylogenetically close to the host cell.

In embodiments where the host cell having a partly deficient NHEJ pathway is an actinobacterium, the cell from which the query sequence is derived is preferably also an actinobacterium.

Once a sequence encoding the lacking activity has been identified, the sequence (hereinafter also termed ‘heterologous sequence’) may be codon-optimised as is

known in the art, in order to increase the chances that the heterologous sequence is properly expressed after introduction in the host cell.

The below table shows examples of host cells, the NHEJ actity(ies) they lack and where suitable heterologous genes can be found for restoring the NHEJ pathway.

Table 2 – overview of suitable heterologous genes for host cells lacking various NHEJ activities.

Host cell	Lacking activity(ies)	Suitable heterologous genes can be found in (non-exhaustive list)
Streptomyces griseus, *Streptomyces acidiscabies*, *Streptomyces auratus*, *Streptomyces bottropensis*, *Streptomyces chartreusis*, *Streptomyces clavuligerus*, *Streptomyces coelicoflavus*, *Streptomyces gancidicus*, *Streptomyces ghanaensis*, *Streptomyces globisporus*, *Streptomyces griseoaurantiacus*, *Streptomyces griseoflavus*, *Streptomyces himastatinicus*, *Streptomyces ipomoeae*, *Streptomyces lividans*, *Streptomyces mobaraensis*,	DNA-binding Ligase Primase Polymerase	*Mycobacterium tuberculosis H37Rv*, *Mycobacterium canettii*, *Mycobacterium spp.*, *Rhodococcus erythropolis*, *Rhodococcus equi*, *Rhodococcus fascians*, *Rhodococcus rhodochrous*, *Rhodococcus spp.*, *Nocardia araoensis*, *Nocardia transvalensis*, *Nocardia exalbida*, *Nocardia spp.*, *Tomitella biformata*, *Amycolatopsis mediterranei*, *Amycolatopsis orientalis*, *Saccharopolyspora erythraea*, *Pseudonocardia dioxanivorans*, *Ralstonia pickettii*, *Kribbella flavida*, *Saccharothrix espanaensis*, *Sinorhizobium meliloti*, *Actinoplanes friuliensis*, *Stenotrophomonas maltophilia*, *Sinorhizobium meliloti*,

Streptomyces pristinaespiralis, Streptomyces prunicolor, Streptomyces rimosus subsp. rimosus, Streptomyces roseosporus, Streptomyces scabrisporus, Streptomyces somaliensis, Streptomyces sulphureus, Streptomyces sviceus, Streptomyces tsukubaensis, Streptomyces turgidiscabies, Streptomyces viridochromogenes, Streptomyces viridosporus, Streptomyces vitaminophilus, Streptomyces zinciresistens, Amycolatopsis azurea, Amycolatopsis decaplanina, Amycolatopsis methanolica, Saccharopolyspora spinosa, Nocardia abscessus, Nocardia aobensis, Nocardia araoensis, Rhodococcus jostii, Blastococcus saxobsidens, Beutenbergia cavernae, Streptomyces collinus, Arthrobacter phenanthrenivorans, Arthrobacter chlorophenolicus, Xanthomonas campestris pv. raphani, Xylanimonas cellulosilytica, Thermobispora bispora, Sinorhizobium medicae, Sanguibacter keddieii, Sinorhizobium meliloti, Ramlibacter tataouinensis, Intrasporangium calvum

Nocardia asiatica,
Nocardia asteroides,
Nocardia brasiliensis,
Nocardia brevicatena,
Nocardia carnea,
Nocardia cerradoensis,
Nocardia concava,
Nocardia cyriacigeorgica,
Nocardia exalbida,
Nocardia higoensis,
Nocardia jiangxiensis,
Nocardia niigatensis,
Nocardia otitidiscaviarum,
Nocardia paucivorans,
Nocardia pneumoniae,
Nocardia takedensis,
Nocardia tenerifensis,
Nocardia terpenica,
Nocardia testacea,
Nocardia thailandica,
Nocardia veterana,
Nocardia vinacea,
Rhodococcus erythropolis,
Rhodococcus imtechensis,
Rhodococcus opacus,
Rhodococcus pyridinivorans,
Rhodococcus qingshengii,
Rhodococcus rhodochrous,
Rhodococcus ruber,
Rhodococcus triatomae,
Rhodococcus wratislaviensis,

Smaragdicoccus niigatensis, Mycobacterium leprae, Mycobacterium tuberculosis Mycobacterium abscessus subsp. bolletii, Mycobacterium abscessus, Mycobacterium avium subsp. avium, Mycobacterium canettii, Mycobacterium colombiense, Mycobacterium fortuitum subsp. fortuitum, Mycobacterium hassiacum, Mycobacterium massiliense, Mycobacterium parascrofulaceum, Mycobacterium phlei, Mycobacterium rhodesiae, Mycobacterium smegmatis, Mycobacterium thermoresistibile, Mycobacterium tusciae, Mycobacterium vaccae, Mycobacterium xenopi		
Streptomyces albus, Streptomyces avermitilis, Streptomyces bingchenggensis,	Ligase	*Streptomyces carneus, Mycobacterium tuberculosis H37Rv, Mycobacterium abscessus, Mycobac-*

Streptomyces coelicolor, Streptomyces pratensis, Streptomyces rapamycinicus, Streptomyces scabiei, Streptomyces venezuelae, Streptomyces violaceusniger, Frankia symbiont of Datisca glomerata, Rhodococcus equi,		*terium canettii, Mycobacterium mageritense, Mycobacterium farcinogenes, Mycobacterium spp., Rhodococcus erythropolis, Rhodococcus equi, Rhodococcus fascians, Rhodococcus rhodochrous, Rhodococcus pyridinivorans, Rhodococcus rhodnii, Rhodococcus spp., Nocardia araoensis, Nocardia transvalensis, Nocardia exalbida, Nocardia spp., Gordonia polyisoprenivorans, Gordonia spp., Smaragdicoccus niigatensis,*
Frankia symbiont of Datisca glomerata, Rhodococcus equi,	Primase and Polymerase	*Streptomyces carneus, Mycobacterium tuberculosis H37Rv, Mycobacterium canettii, Mycobacterium orygis, Mycobacterium spp., Rhodococcus erythropolis, Rhodococcus equi, Rhodococcus ruber, Rhodococcus pyridinivorans, Rhodococcus fascians, Rhodococcus rhodochrous, Rhodococcus fascians Rhodococcus spp., Nocardia thailandica, Nocardia exalbida, Nocardia asteroides, Nocardia vinacea, Nocardia spp. Amy-*

		colicicoccus subflavus, Tomitella biformata, Smaragdicoccus niigatensis
Streptomyces scabiei	DNA-binding	*Mycobacterium tuberculosis H37Rv, Mycobacterium africanum, Mycobacterium canettii, Mycobacterium spp. Streptomyces coelicolor, Streptomyces cattleya, Streptomyces purpureus, Streptomyces varsoviensis, Streptomyces thermolilacinus, Streptomyces roseoverticillatus, Streptomyces venezuelae, Streptomyces spp. Amycolatopsis mediterranei, Amycolatopsis halophila, Amycolatopsis vancoresmycina, Amycolatopsis orientalis, Amycolicicoccus subflavus, Amycolatopsis spp., Nakamurella multipartita, Beutenbergia cavernae, Arthrobacter castelli, Saxeibacter lacteus, Rhodococcus equi, Nocardia jiangxiensis, Gordonia rubripertincta, Clavibacter michiganensis, Gordonia aichiensis, Microbacterium paraoxydans*

In one embodiment, the host cell is *S. coelicolor.* This organism lacks the ligase activity of the NHEJ pathway and only displays the DNA-binding activity via the Ku proteins and the primase and polymerase activity (SEQ ID NO: 70). In one embodiment, NHEJ is restored in *S. coelicolor* by introducing at least part of the ligD gene from *S. carneus*, wherein said part encodes the ligase activity. In other embodiments, NHEJ is restored by introducing the ligD gene from *M. tuberculosis, Nocardia spp., Smaragdicoccus niigatensis, Rhodococcus spp., Mycobacterium abscessus, Mycobacterium mageritense* or *Mycobacterium farcinogenes.*

Target nucleic acid

The method disclosed herein is particularly useful for generating precise indels around at least one target nucleic acid sequence of interest. The method is thus useful for, but not limited to, the investigation of pathway regulations and the identification of metabolite production bottlenecks, the screening of producer strains and the identification of new compounds produced by the host cell.

The target nucleic acid sequence may be comprised within any nucleic acid sequence of interest. For example, the target sequence may be comprised within or may comprise an open reading frame or a putative open reading frame, or it may be comprised within or may comprise a regulatory region or a putative regulatory region, such as an enhancer, a promoter, an insulator, a terminator.

The target nucleic acid sequence may be involved in a pathway of interest. In some embodiments, the target nucleic acid encodes an enzyme or a protein. In other embodiments, the target nucleic acid is comprised within or comprises a biosynthetic gene or a putative biosynthetic gene. In some embodiments, the biosynthetic gene is involved in the synthesis of a secondary metabolite.

In some embodiments, the target nucleic acid sequence is comprised within a gene cluster. In specific embodiments, the gene cluster is a secondary metabolite gene cluster.

There is thus disclosed herein a method for generating precise indels such at precise deletions or precise insertions around a target nucleic acid sequence optionally comprised within or comprising a gene cluster, where the target nucleic acid sequence is

involved or is suspected of being involved in the biosynthesis of a secondary metabolite.

In some embodiments, the secondary metabolite is selected from the group consisting of antibiotics, herbicides, anti-cancer agents, immunosuppressants, flavors, parasiticides and proteins. The term 'parasiticide' is to be understood in its broadest sense as an agent capable of inactivating or killing any undesirable organism and thus comprises insecticides, anthelmintic compounds, larvacides, antiparasitic agents and antiprotozoal agents.

In some embodiments, the secondary metabolite is an antibiotic selected from the group consisting of apramycin, bacitracin, chloramphenicol cephalosporins, cycloserine, erythromycin, fosfomycin, gentamicin, kanamycin, kirromycin, lassomycin, lincomycin, lysolipin, microbisporicin, neomycin, noviobiocin, nystatin, nitrofurantoin, platensimycin, pristinamycins, rifamycin, streptomycin, teicoplanin, tetracycline, tinidazole, ribostamycin, daptomycin, vancomycin, viomycin and virginiamycin.

In other embodiments, the secondary metabolite is a herbicide selected from the group consisting of bialaphos, resormycin and phosphinothricin.

In yet other embodiments, the secondary metabolite is an anti-cancer agent selected from the group consisting of doxorubicin, salinosporamides, aclarubicin, pentostatin, peplomycin, thrazarine and neocarcinostatin.

In yet other embodiments, the secondary metabolite is an immunosuppressant selected from the group consisting of rapamycin, FK520, FK506, cyclosporine, ushikulides, pentalenolactone I and hygromycin A.

In yet other embodiments, the secondary metabolite is a flavor such as geosmin.

In yet other embodiments, the secondary metabolite is a parasiticide such as an insecticide, an anthelmintic, a larvacide, or an antiprotozoal agent such as spinsad or avermectin.

In other embodiments, the target nucleic acid encodes an enzyme such as a metabolic enzyme selected from the group consisting of an amylase, a protease, a cellulase, a chitinase, a keratinase and a xylanase, a glycosyltransferase, an oxygenase, a hydroxylase, a methyltransferase, a dehydrogenase, a dehydratase.

In some embodiments, only one target nucleic acid sequence is targeted for editing and generation of precise indels. In other embodiments, more than one target nucleic acid sequence is targeted and the method is a multiplex method. Thus the method can be used for generating at least one indel around at least one target nucleic acid sequence, such as at least two indels around at least two target nucleic acid sequences, such as at least three indels around at least three target nucleic acid sequences, such as at least four indels around at least four target nucleic acid sequences, such as at least five indels around at least five target nucleic acid sequences, or more. The method can thus be used for generating one indel around one target nucleic acid sequence, or two indels around at least two target nucleic acid sequences, or three indels around three target nucleic acid sequences, or four indels around four target nucleic acid sequences, or five indels around five target nucleic acid sequences, or more. As explained above, in the case of multiplex editing, a guiding means is preferably provided for each target nucleic acid sequence.

In some embodiments, the at least one indel results in the inactivation of at least one gene. In some embodiments, the at least one gene is comprised within a gene cluster. In other embodiments, the at least one gene is not comprised within a gene cluster.

The at least one indel generated by the present method is an indel of at least 1 bp.

Efficiency and off-target effects

Several parameters can have an impact on the efficiency of the present method for generating precise indels around at least one target sequence. Some parameters can be adjusted as known in the art. Parameters susceptible of having an impact on the efficiency include, but are not limited to: the sequence of the guiding means (sgRNA or crRNA/tracrRNA), the sequence of the target nucleic acid, the GC content of the host cell and the GC content of the target nucleic acid sequence.

The method for generating precise indels around a target nucleic acid sequence described herein can be performed with high efficiency, with relatively few off-target effects. In some embodiments, the desired indel is generated in more than 65% of the host cells, such as in more than 70% of the host cells, such as in more than 75% of the host cells, such as in more than 80% of the host cells, such as in more than 85% of the host cells, such as in more than 90% of the host cells, such as in more than 95% of the host cells, such as in 100% of the host cells.

Without being bound by theory, the use of homology arms to direct the repair of the break generated by the Cas9 nuclease towards the HR pathway is believed to reduce the occurrence of off-target effects. When homology arms are used, higher efficiency can be achieved, so that the desired indel is generated in more than 90% of the host cells, such as in more than 95% of the host cells, such as in more than 96% of the host cells, such as in more than 97% of the host cells, such as in more than 98% of the host cells, such as in more than 99% of the host cells, such as in 100% of the host cells.

Characterisation and screening

The present method can thus be used for generating precise indels around a target nucleic acid sequence of interest, for example a sequence encoding for a gene involved in a pathway of interest. This can result in a plurality of clones having precise indels around the target sequence. These clones can then be further analysed or screened. For example, producer strains having advantageous production profiles for a desired compound can be selected.

In some embodiments, it may be of interest to determine the size of the at least one indel for a particular clone. Thus the method may comprise a further step of determining the size of the at least one indel. Methods for determining the size of an indel are known in the art and include, but are not limited to, whole genome sequencing, pulsed field gel electrophoresis, nucleic acid amplification-based methods such as PCR, for example followed by restriction analysis and detection of the PCR products on a gel and determination of the size of the products using an appropriate marker. The PCR products can also be sequenced if precise determination of the size of the indel is desired.

In some embodiments, the method further comprises the selection of clones having the desired characteristics. Such selection methods are known in the art and encompass screening methods, chemical analysis of the related gene products (proteins or metabolites), sequencing of the related gene regions, and/or analysis of the gene expression level.

CRISPR-Cas9 system for actinomycetes

The most studied CRISPR-Cas9 system is from *Streptococcus pyogenes*, which has a GC content of about 35%. In contrast, actinomycetes have a high GC content. *S. coelicolor* for example has a GC content of about 72%. Likewise, codon usage varies from organism to organism.

Herein is thus disclosed a codon optimised nucleic acid sequence encoding Cas9 which is codon optimised for streptomycetes (SEQ ID NO: 1). The optimisation was done based on the codon usage table of the most studied actinomycete, *Streptomyces coelicolor*, as described in example 1.

In one aspect, the invention thus relates to a polynucleotide having at least 94% identity with SEQ ID NO: 1, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity, such as 100% identity, said polynucleotide encoding a Cas9 nuclease or a variant thereof. It will be understood that sequences closely related to SEQ ID NO: 1 with mutations such as e.g. silent mutations are envisaged.

In some embodiments, the polynucleotide is non-naturally occurring.

Also within the scope of the present disclosure is a polypeptide encoded by a polynucleotide having at least 94% identity with SEQ ID NO: 1, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity, such as 100% identity with SEQ ID NO: 1. In one embodiment, the polypeptide has the sequence as set forth in SEQ ID NO: 2.

It will be understood that sequences closely related to SEQ ID NO: 2 with mutations that do not disrupt the function of Cas9 are also within the scope of the invention. In particular, mutations in non-conserved domains of Cas9 which are unlikely to affect its

function and conservative mutations in conserved or non-conserved domains of Cas9 are envisaged.

In some embodiments, the polypeptide is non-naturally occurring.

Also within the scope of the present disclosure is a cell comprising the polynucleotide disclosed herein. Such a cell may be a host cell as detailed above. In particular, the cell may be an archaea, in a prokaryotic cell or in a eukaryotic cell. In one embodiment, the host cell is a prokaryotic cell. The host cell may be a cell with a high GC content, for example a GC content of 50% or more, such as 55% or more, such as 60% or more, such as 65% or more, such as 70% or more, such as 75% or more, such as 80% or more, such as 85% or more, such as 90% or more. In a particular embodiment, the host cell is an actinobacterium. The host cell may thus be selected from the group consisting of *Actinomycetales*, such as *Streptomyces sp., Amycolatopsis sp.* or *Saccharopolyspora sp.* In some embodiments, the host cell is selected from the group consisting of *Streptomyces coelicolor, Streptomyces avermitilis*, *Streptomyces aureofaciens*, *Streptomyces griseus, Streptomyces parvulus, Streptomyces albus, Streptomyces vinaceus, Streptomyces acrimycinis, Streptomyces calvuligerus, Streptomyces lividans, Streptomyces limosus, Streptomyces rubiqinosis, Streptomyces azureus, Streptomyces glaucenscens, Streptomyces rimosus, Streptomyces violaceoruber, Streptomyces kanamyceticus, Amycolatopsis orientalis, Amycolatopsis mediterranei, Saccharopolyspora erythraea, Mycobacterium tuberculosis, Streptomyces carneus, Nocardia spp., Smaragdicoccus niigatensis, Rhodococcus spp., Mycobacterium abscessus, Mycobacterium mageritense, Mycobacterium farcinogenes*. In a preferred embodiment, the host cell is *Streptomyces coelicolor.*

The present disclosure also relates to a vector comprising the polynucleotide as described herein. Thus some embodiments relate to a vector comprising a polynucleotide having at least 94% identity with SEQ ID NO: 1, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity, such as 100% identity with SEQ ID NO: 1.

The polynucleotide, the polypeptide and/or the vector comprising the polynucleotide, as all disclosed herein, may be used for performing the methods disclosed herein. In pre-

ferred embodiments, they are used to perform the present methods in a host cell, where the host cell is a Streptomycetes.

In some embodiments, the method is a method for generating at least one deletion around at least one target nucleic acid sequence comprised within a host cell having a non-homologous end-joining (NHEJ) pathway which is at least partly deficient,

said method comprising the steps of:

(i) optionally, restoring the full functionality of the NHEJ pathway,

(ii) inducing a CRISPR-Cas9 system in said host cell, wherein said CRISPR-Cas9 system is able to generate at least one break in said at least one target nucleic acid sequence and wherein the CRISPR-Cas9 system comprises a Cas9 nuclease and at least one guiding means,

thereby generating:

a. if the method does not comprise step (i), at least one random-sized deletion around said at least one target nucleic acid sequence, wherein said at least one deletion is a random-sized deletion of at least 1 bp; or

b. if the method does comprise step (i), at least one indel around said at least one target nucleic acid sequence, wherein said at least one indel is a deletion or insertion of at least1 bp,

wherein Cas9 is a polypeptide as described above, or wherein Cas9 is encoded by a polynucleotide as described above.

Accordingly, in some embodiments, the method does not comprise step (i) of restoring the full functionality of the NHEJ pathway and results in generation of random-sized deletions, where Cas9 is a polypeptide encoded by a polynucleotide having at least 94% identity with SEQ ID NO: 1, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity, such as 100% identity with SEQ ID NO: 1. In one embodiment, the polypeptide has the sequence as set forth in SEQ ID NO: 2. In some embodiments, the polynucleotide encoding Cas9 is codon-optimised for the host cell in which the method is to be performed.

In other embodiments, the method comprises step (i) of restoring the full functionality of the NHEJ pathway and results in generation of indels, i.e. insertions of deletions of at least 1 bp, where Cas9 is a polypeptide encoded by a polynucleotide having at least 94% identity with SEQ ID NO: 1, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity, such as 100% identity with SEQ ID NO: 1. In one embodiment, the polypeptide has the sequence as set forth in SEQ ID NO: 2. In some embodiments, the polynucleotide encoding Cas9 is codon-optimised for the host cell in which the method is to be performed.

Method for selective modulation of transcription

In another aspect, a method for selectively modulating transcription of at least one target nucleic acid sequence in a host cell is disclosed, the method comprising introducing into the host cell:

i. at least one guiding means, or a nucleic acid comprising a nucleotide sequence encoding guiding means, wherein the guiding means comprises a nucleotide sequence that is complementary to a target nucleic acid sequence in the host cell; and
ii. a variant Cas9, or a nucleic acid comprising a nucleotide sequence encoding the variant Cas9, wherein the variant Cas9 has reduced endodeoxyribonuclease activity,

wherein said guiding means and said variant Cas9 form a complex in the host cell, said complex selectively modulating transcription of at least one target nucleic acid in the host cell.

In some embodiments, the method for selectively modulating transcription of at least one target nucleic acid sequence in a host cell comprises introducing into the host cell:

(i) at least one guiding means, or a nucleic acid comprising a nucleotide sequence encoding guiding means, wherein the guiding means comprises a nucleotide sequence that is complementary to a target nucleic acid sequence in the host cell; and
(ii) a variant Cas9, or a nucleic acid comprising a nucleotide sequence encoding the variant Cas9, wherein the variant Cas9 is a variant of the polypeptides disclosed herein or of a polypeptide encoded by the nucleotide sequences disclosed herein, and wherein the variant Cas9

has reduced endodeoxyribonuclease activity, with reduced endodeoxyribonuclease activity and is codon-optimised for Streptomycetes,

wherein said guiding means and said variant Cas9 form a complex in the host cell, said complex selectively modulating transcription of at least one target nucleic acid in the host cell.

In some embodiments, the guiding means comprises at least one sgRNA and/or at least one crRNA/tracrRNA set.

Modulation

This method allows selective modulation of the transcription of at least one target nucleic acid sequence comprised within a host cell.

Modulation of the transcription can be an increase of the transcription level or a decrease of the transcription level.

The method for modulation of transcription is based on the use of a CRISPR-Cas9 system comprising a variant Cas9 and at least one guiding means, wherein the variant Cas9 is capable of forming a complex with each of the at least one guiding means and is thereby capable of binding to the target nucleic acid sequence but is not capable of inducing a break therein or is not capable of leaving the target nucleic acid sequence. In other words, variant Cas9 remains on the target nucleic acid sequence, whereby it is hypothesized that transcription is prevented because of steric hindrance or lower accessibility of a polymerase such as an RNA polymerase to the DNA. In order to achieve an increase of transcription, a transcription activator can be fused to the variant Cas9, wherein the variant Cas9 is capable of forming a complex with at least one guiding means targeting e.g. the promoter of a gene of interest; the complex remains on the target nucleic acid sequence and thereby provides a transcription activator, thereby activating expression of the gene.

In some embodiments, the variant Cas9 is a variant Cas9 which can cleave one of the strands of the target nucleic acid sequence but has reduced ability to cleave the other strand of the target nucleic acid sequence. In some embodiments, the variant Cas9 is selected from the group consisting of Cas9-H840A, Cas9-D10A and Cas9-H840A,

D10A, where H840A indicates a substitution at amino acid residue 840 of SEQ ID NO: 2, and D10A indicates a substitution at amino acid residue 10 of Cas9. It will be understood that sequences having mutations that do not disrupt the function of the variant Cas9 are also within the scope of the invention. In particular, mutations in non-conserved domains of Cas9 which are unlikely to affect its function and conservative mutations in conserved or non-conserved domains of Cas9 are envisaged.

In some embodiments, the expression of the variant Cas9 is inducible, e.g. the nucleic acid sequence encoding the variant Cas9 may be under the control of an inducible promoter. Other methods of inducing expression of the variant Cas9 will be apparent to the skilled person.

In some embodiments, the nucleic acid sequence encoding the variant Cas9 is comprised within a vector to be introduced in the host cell. In other embodiments, the nucleic acid sequence encoding the variant Cas9 is comprised within the genome of the host cell, e.g. on a chromosome.

The CRISPR-Cas9 system preferably further comprises at least one guiding means allowing the variant Cas9 to bind to the at least one target nucleic acid sequence and to modulate its transcription. As detailed above, the nucleic acid sequence encoding the variant Cas9 and the at least one nucleic acid sequence encoding the at least one guiding means may be comprised within a single nucleic acid such as a vector or a chromosome comprised within the host cell.

Host cell

The present method can be performed in an archaea, in a prokaryotic cell or in a eukaryotic cell. In one embodiment, the host cell is a prokaryotic cell. The present methods are particularly advantageous for modulating transcription in host cells that have a high GC content, for example a GC content of 50% or more, such as 55% or more, such as 60% or more, such as 65% or more, such as 70% or more, such as 75% or more, such as 80% or more. In a particular embodiment, the host cell is an actinobacterium. The host cell may thus be selected from the group consisting of *Actinomycetales*, such as *Streptomyces sp., Amycolatopsis sp.* or *Saccharopolyspora sp.* In some embodiments, the host cell is selected from the group consisting of *Streptomyces coelicolor, Streptomyces avermitilis*, *Streptomyces aureofaciens*, *Streptomyces*

griseus, Streptomyces parvulus, Streptomyces albus, Streptomyces vinaceus, Streptomyces acrimycinis, Streptomyces calvuligerus, Streptomyces lividans, Streptomyces limosus, Streptomyces rubiqinosis, Streptomyces azureus, Streptomyces glaucenscens, Streptomyces rimosus, Streptomyces violaceoruber, Streptomyces kanamyceticus, Amycolatopsis orientalis, Amycolatopsis mediterranei, Saccharopolyspora erythraea, Mycobacterium tuberculosis, Streptomyces carneus, Nocardia spp., Smaragdicoccus niigatensis, Rhodococcus spp., Mycobacterium abscessus, Mycobacterium mageritense, Mycobacterium farcinogenes. In a preferred embodiment, the host cell is *Streptomyces coelicolor.*

The host cell may be any of the organisms listed herein elsewhere.

Target nucleic acid

The method disclosed herein is particularly useful for modulating transcription of least one target nucleic acid sequence of interest. The method is thus useful for, but not limited to, the investigation of pathway regulations and identification of metabolite production bottlenecks, the design of producer strains and the identification of new compounds produced by the host cell.

The target nucleic acid sequence may be comprised within any nucleic acid sequence of interest. For example, the target sequence may be comprised within or may comprise an open reading frame or a putative open reading frame, or it may be comprised within or may comprise a regulatory region or a putative regulatory region, such as an enhancer, a promoter, an insulator, a terminator.

The target nucleic acid sequence may be involved in a pathway of interest. In some embodiments, the target nucleic acid encodes an enzyme. In other embodiments, the target nucleic acid is comprised within or comprises a biosynthetic gene or a putative biosynthetic gene. In some embodiments, the biosynthetic gene is involved in the synthesis of a secondary metabolite.

In some embodiments, the target nucleic acid sequence is comprised within a gene cluster. In specific embodiments, the gene cluster is a secondary metabolite gene cluster.

There is thus disclosed herein a method for modulating transcription of at least one target nucleic acid sequence optionally comprised within or comprising a gene cluster, where the target nucleic acid sequence is involved or is suspected of being involved in the biosynthesis of a secondary metabolite.

In some embodiments, the secondary metabolite is selected from the group consisting of antibiotics, herbicides, anti-cancer agents, immunosuppressants, flavors, parasiticides, enzymes and proteins. The term 'parasiticide' is to be understood in its broadest sense as an agent capable of inactivating or killing any undesirable organism and thus comprises insecticides, anthelmintic compounds, larvacides, antiparasitic agents and antiprotozoal agents.

In some embodiments, the secondary metabolite is an antibiotic selected from the group consisting of apramycin, bacitracin, chloramphenicol cephalosporins, cycloserine, erythromycin, fosfomycin, gentamicin, kanamycin, kirromycin, lassomycin, lincomycin, lysolipin, microbisporicin, neomycin, noviobiocin, nystatin, nitrofurantoin, platensimycin, pristinamycins, rifamycin, streptomycin, teicoplanin, tetracycline, tinidazole, ribostamycin, daptomycin, vancomycin, viomycin and virginiamycin.

In other embodiments, the secondary metabolite is a herbicide selected from the group consisting of bialaphos, resormycin and phosphinothricin.

In yet other embodiments, the secondary metabolite is an anti-cancer agent selected from the group consisting of doxorubicin, salinosporamides, aclarubicin, pentostatin, peplomycin, thrazarine and neocarcinostatin.

In yet other embodiments, the secondary metabolite is an immunosuppressant selected from the group consisting of rapamycin, FK520, FK506, cyclosporine, ushikulides, pentalenolactone I and hygromycin A.

In yet other embodiments, the secondary metabolite is a flavor such as geosmin.

In yet other embodiments, the secondary metabolite is a parasiticide such as an insecticide, an anthelmintic, a larvacide, or an antiprotozoal agent such as spinsad or avermectin.

In other embodiments, the target nucleic acid encodes an enzyme such as metabolic enzyme selected from the group consisting of an amylase, a protease, a cellulase, a chitinase, a keratinase and a xylanase, a glycosyltransferase, an oxygenase, a hydroxylase, a methyltransferase, a dehydrogenase, a dehydratase.

In some embodiments, transcription of only one target nucleic acid sequence is modulated. In other embodiments, transcription of more than one target nucleic acid sequence is modulated and the method is a multiplex method. Thus the method can be used for modulating transcription of at least one target nucleic acid sequence, such as of least two target nucleic acid sequences, such as of at least three target nucleic acid sequences, such as of at least four target nucleic acid sequences, such as of at least five target nucleic acid sequences, or more. The method can thus be used for modulating transcription of one target nucleic acid sequence, of two target nucleic acid sequences, of three target nucleic acid sequences, of four target nucleic acid sequences, of five target nucleic acid sequences, or more. As explained above, in the case of multiplex modulation, a guiding means is preferably provided for each target nucleic acid sequence.

In some embodiments, the at least one nucleic acid sequence is at least one gene. The gene may be comprised within a gene cluster. In other embodiments, the at least one gene is not comprised within a gene cluster.

Kits

Kit for generating random-sized deletions and/or indels

In a further aspect, the disclosure relates to a kit for performing the methods described herein.

In some embodiments, the kit is for generating at least one random-sized deletion around at least one target nucleic acid sequence described above, said kit comprising a vector comprising a nucleic acid sequence encoding a Cas9 nuclease or a variant thereof and instructions for use.

The vector comprised within said kit can be an integrative vector for integrating the nucleic acid sequence encoding the nuclease into the genome, or it can be comprised

within a non-integrative vector, e.g. to be used as a template for amplifying the nucleic acid sequence encoding the nuclease prior to introduction into the cell, or to be transformed and maintained in the host cell.

In preferred embodiments, the nuclease is Cas9 or a variant thereof. In some embodiments, the nucleic acid sequence encoding the nuclease is a sequence encoding Cas9 such as a polynucleotide having at least 93% identity with SEQ ID NO: 1, such as at least 94% identity, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity, such as 100% identity with SEQ ID NO: 1.

The kit may further comprise at least one guiding means and/or at least one host cell having a non-homologous end-joining (NHEJ) pathway which is at least partly deficient.

In some embodiments, the kit further comprises at least one guiding means, where the guiding means is as described above. The guiding means may be comprised within the vector or it may be provided on a different vector. The at least one guiding means may be any guiding means described above, such as an sgRNA or a crRNA/tracrRNA set.

In some embodiments, the kit further comprises a host cell or a plurality of host cells. In one embodiment, the host cell is a cell having a partly deficient NHEJ pathway, i.e. lacking at least one of the four NHEJ activities defined above. The host cell may be any of the host cells described herein elsewhere. The NHEJ pathway may be partly deficient because it is naturally partly deficient in said host cell, or it may have been inactivated by the manufacturer or by the user. In one embodiment, the host cell is *S. coelicolor* and lacks the ligase activity.

In other embodiments, the host cell has a functional NHEJ pathway. The kit may then further comprise means for at least partly inactivating the NHEJ pathway in said host cell. This can be done as described above, i.e. by inactivating at least one of the four NHEJ activities (DNA binding, ligase, polymerase or primase activity). Thus in one embodiment the kit comprises means for inactivating the ligase activity of the host cell.

In some embodiments, the kit is for performing the method for generating at least one precise indel around at least one target nucleic acid sequence, said kit comprising a

first vector comprising a nucleic acid sequence encoding Cas9 or a variant thereof and instructions for use.

In some embodiments, the nucleic acid sequence encoding Cas9 is a polynucleotide having at least 93% identity with SEQ ID NO: 1, such as at least 94% identity, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity, such as 100% identity with SEQ ID NO: 1.

In some embodiments, the kit further comprises at least one guiding means, where the guiding means is as described above. The guiding means may be comprised within the first vector or it may be provided on a different vector. The at least one guiding means may be any guiding means described above, such as an sgRNA or a crRNA/tracrRNA set.

In some embodiments, the kit further comprises a host cell or a plurality of host cells. In one embodiment, the host cell is a cell having a partly deficient NHEJ pathway, i.e. lacking at least one of the four NHEJ activities defined above. The host cell may be any of the host cells described herein elsewhere. The NHEJ pathway may be partly deficient because it is naturally partly deficient in said host cell, or it may have been inactivated by the manufacturer. In one embodiment, the host cell is *S. coelicolor* and lacks the ligase activity.

In other embodiments, the host cell has a functional NHEJ pathway. The kit may then further comprise means for at least partly inactivating the NHEJ pathway in said host cell. This can be done as described above, i.e. by inactivating at least one of the four NHEJ activities (DNA binding, ligase, polymerase or primase activity). Thus in one embodiment the kit comprises means for inactivating the ligase activity of the host cell.

In some embodiments, the kit further comprises a second vector comprising a nucleic acid sequence encoding at least one of the four NHEJ activities defined above. In one embodiment, the nucleic acid thus encodes at least one of:

- a DNA-binding activity,
- a primase activity,
- a ligase activity,

- a polymerase activitiy.

In some embodiments, the nucleic acid sequence encodes two or three of the four NHEJ activities. In some embodiments, the nucleic acid sequence encodes all four NHEJ activities. In some embodiments, the nucleic acid sequence encodes the ligase D from *S. carneus* or *M. tuberculosis*. In a particular embodiment, the host cell is *S. coelicolor* and the nucleic acid sequence encoding the missing NHEJ activity comprises the ligase D gene from *S. carneus* or *M. tuberculosis.* Examples of which organisms having sequences that can be used for restoring NHEJ activity are provided above (Table 2).

In other embodiments, the nucleic acid sequence encoding at least one of the four NEHJ activities and the nucleic acid sequence encoding Cas9 are all comprised within the first vector.

Kit for modulating transcription

In yet another aspect is disclosed a kit for performing the method for modulating transcription of at least one target nucleic acid as described above, said kit comprising a vector comprising a nucleic acid sequence encoding a variant Cas9; and instructions for use. In preferred embodiments, the variant Cas9 has reduced endodeoxyribonuclease activity.

In some embodiments, the variant Cas9 is a variant Cas9 which can cleave one of the strands of the target nucleic acid sequence but has reduced ability to cleave the other strand of the target nucleic acid sequence. In some embodiments, the variant Cas9 is selected from the group consisting of Cas9-H840A, Cas9-D10A and Cas9-H840A, D10A, where H840A indicates a substitution at amino acid residue 840 of SEQ ID NO: 2, and D10A indicates a substitution at amino acid residue 10 of Cas9. It will be understood that sequences having mutations that do not disrupt the function of the variant Cas9 are also within the scope of the invention. In particular, mutations in non-conserved domains of Cas9 which are unlikely to affect its function and conservative mutations in conserved or non-conserved domains of Cas9 are envisaged.

In some embodiments, the kit further comprises at least one guiding means, where the guiding means is as described above, and/or at least one host cell or plurality of host

cells. The guiding means may be comprised within the first vector or it may be provided on a different vector. The at least one guiding means may be any guiding means described above, such as an sgRNA or a crRNA/tracrRNA set.

The host cell may be an archaea, in a prokaryotic cell or in a eukaryotic cell. In one embodiment, the host cell is a prokaryotic cell. The present methods can be used for modulating transcription in host cells that have a high GC content, for example a GC content of 50% or more, such as 55% or more, such as 60% or more, such as 65% or more, such as 70% or more, such as 75% or more, such as 80% or more. In a particular embodiment, the host cell is an actinobacterium. The host cell may thus be selected from the group consisting of *Actinomycetales*, such as *Streptomyces sp., Amycolatopsis sp.* or *Saccharopolyspora sp.* In some embodiments, the host cell is selected from the group consisting of *Streptomyces coelicolor, Streptomyces avermitilis*, *Streptomyces aureofaciens*, *Streptomyces griseus, Streptomyces parvulus, Streptomyces albus, Streptomyces vinaceus, Streptomyces acrimycinis, Streptomyces calvuligerus, Streptomyces lividans, Streptomyces limosus, Streptomyces rubiqinosis, Streptomyces azureus, Streptomyces glaucenscens, Streptomyces rimosus, Streptomyces violaceoruber, Streptomyces kanamyceticus, Amycolatopsis orientalis, Amycolatopsis mediterranei, Saccharopolyspora erythraea, Mycobacterium tuberculosis, Streptomyces carneus, Nocardia spp., Smaragdicoccus niigatensis, Rhodococcus spp., Mycobacterium abscessus, Mycobacterium mageritense, Mycobacterium farcinogenes*. In a preferred embodiment, the host cell is *Streptomyces coelicolor.*

Examples

Example 1: Materials and methods

Strains and chemicals

ISP2: Yeast Extract, 0.4%, Malt Extract, 1%, Dextrose, 0.4%, 2% agar for solidification, pH 7.2. Cullum agar, also termed SFM (soya flour mannitol) agar: 2% organic soya flour (low fat), 2 % mannitol, 2% agar, 10mM $MgCl_2$, natural pH. LB: Tryptone, 1%, Yeast Extract, 0.5%, NaCl, 0.5%, pH, 7.0. 2×YT: Tryptone, 1.6%, Yeast Extract, 1%, NaCl, 0.5%, pH 7.

Chemicals and solutions: apramycin sulfate (stock solution 100 mg/ml in ddH_2O), nalidixic acid (stock solution 50 mg/ml in ddH_2O of pH 11), thiostrepton (stock solution 50 mg/ml in DMSO), kanamycin (stock solution 50 mg/ml in ddH_2O), chloramphenicol (stock solution 50 mg/ml in ethanol), chloroform, methanol, and DMSO. The working concentrations for apramycin, nalidixic acid, thiostrepton, kanamycin, and chloramphenicol were 50 µg/ml, 50 µg/ml, 1 µg/ml, 25 µg/ml, and 25 µg/ml, respectively.

The below tables list selected target sequences (Table 3), primers (Table 4) and strains and plasmids (table 5) used in the following examples.

Table 3. Selected target sequences

sgRNA	The target Sequences	PAM	Purpose
Actlorf1-1 NT	GTGGCTCGAAGGAGGCTCGA	AGG	Gene deletion/ expression control
Actlorf1-2 T	AGCTCGATCAAGTCGATGGT	CGG	Gene deletion/ expression control
Actlorf1-3 T	GAAGCGCAGAGTCGTCATCA	CGG	Gene deletion/ expression control
Actlorf1-4 T	CCCCTCGCCCTACCGTTCAC	AGG	Gene deletion/ expression control
Actlorf1-5 T	GCGCGAGTATCTGCTGCTGT	CGG	Gene deletion
Actlorf1-6 T	CTGCAACGCGTACCACATGA	CGG	Gene deletion
Actvb-1 NT	TCGCCGCAACTGTCGAACAC	CGG	Gene deletion
Actvb-2 NT	CTGCCATCTTCGAACTCCCT	AGG	Gene deletion
Actvb-3 T	TTCCCGGTGTTCGACAGTTG	CGG	Gene deletion
Actvb-4 T	ACTGGTCTGCCTGGCTCGTA	CGG	Gene deletion
Actvb-5 NT	ATCTTCGAACTCCCTAGGCG	AGG	Gene deletion
Actvb-6 NT	GTCCCGGAGCATTCCCTGGT	CGG	Gene deletion
orf1p-S1 T	GTGTTCCCCTCCCTGCCTCG	TGG	Gene expression control
orf1p-S3 T	TCCCTCACGCGCTCAGCTTT	GGG	Gene expression control
orf1p-S5 T	CTTTGGGCGCCCGGCTCGAG	CGG	Gene expression con-

			trol
orf1p-A1 NT	CCTTCGACCGCCGCTCGAGC	CGG	Gene expression control
orf1p-A4 NT	GCCCAAAGCTGAGCGCGTGA	AGG	Gene expression control
orf1p-A5 NT	TGAGCGCGTGAGGGACCACG	AGG	Gene expression control
Actlorf1-7 NT	TGAGCAGTTCCCAGAACTGC	CGG	Gene expression control
Actlorf1-8 NT	AGGAGGCTCGAAGGCCGATA	CGG	Gene expression control

Table 4. Primer list.

Sets	Primer name	Sequence (5'-3') # *§	Purpose
1	Actlorf1-F1	CATGCCATGG**GTGGCT** **CGAAGGAGGCTCGA** GTTTTAGAGCTAGAAATAGC	sgRNAs Amplification
2	Actlorf1-F2	CATGCCATGG**AGCTCG** **ATCAAGTCGATGGT**GT TTTAGAGCTAGAAATAGC	
3	Actlorf1-F3	CATGCCATGG**GAAGCG** **CAGAGTCGTCATCA**GTT TTAGAGCTAGAAATAGC	
4	Actlorf1-F4	CATGCCATGG**CCCCTCG** **CCCTACCGTTCAC**GTTTT AGAGCTAGAAATAGC	
5	Actlorf1-F5	CATGCCATGG**GCGCGA** **GTATCTGCTGCTGTG**TT TTAGAGCTAGAAATAGC	
6	Actlorf1-F6	CATGCCATGG**CTGCAAC** **GCGTACCACATGA**GTT TTAGAGCTAGAAATAGC	
7	Actlorf1-F7	CATGCCATGG**TGAGCA** **GTTCCCAGAACTGC**GTT	

8	Actlorf1-F8	CATGCCATGG**AGGAGGCT** **CGAAGGCCGATA**GTT
9	ActVB-F1	CATGCCATGG**TCGCCG** **CAACTGTCGAACAC**GTT TTAGAGCTAGAAATAGC
10	ActVB-F2	CATGCCATGG**CTGCCAT** **CTTCGAACTCCCT**GTT TTAGAGCTAGAAATAGC
11	ActVB-F3	CATGCCATGG**TTCCCG** **GTGTTCGACAGTTG**GTT TTAGAGCTAGAAATAGC
12	ActVB-F4	CATGCCATGG**ACTGGT** **CTGCCTGGCTCGTA**GTT TTAGAGCTAGAAATAGC
13	ActVB-F5	CATGCCATGG**ATCTTCG** **AACTCCCTAGGCG**GTT TTAGAGCTAGAAATAGC
14	ActVB-F6	CATGCCATGG**GTCCCGG** **AGCATTCCCTGGT**GTT TTAGAGCTAGAAATAGC
15	orf1p-S1 T-F	CATGCCATGG**GTGTTC** **CCCTCCCTGCCTCG**GTT TTAGAGCTAGAAATAGC
16	orf1p-S3 T-F	CATGCCATGG**TCCCTCA** **CGCGCTCAGCTTT**GTT TTAGAGCTAGAAATAGC
17	orf1p-S5 T-F	CATGCCATGG**CTTTGG** **GCGCCCGGCTCGAG**GTT TTAGAGCTAGAAATAGC
18	orf1p-A1 NT-F	CATGCCATGG**CCTTCG** **ACCGCCGCTCGAGC**GTT TTAGAGCTAGAAATAGC
19	orf1p-A4 NT-F	CATGCCATGG**GCCCAAA** **GCTGAGCGCGTGA**GTT TTAGAGCTAGAAATAGC

20	orf1p-A5 NT-F	CATGCCATGG**TGAGCG** **CGTGAGGGACCACG**GTT TTAGAGCTAGAAATAGC	
21	sgRNA-R	ACGCCTACGTAAAAAAA GCACCGACTCGGTGCC	
22	gRNA check-F	ACATGTGCGGTCGATCTT	sgRNAs sequencing
23	gRNA check-R	TACGTAAAAAAAGCACCGAC	
24	orf1-5'F	*TCGTCGAAGGCACTAGAAGG* CATCCGCTGAACGAGACCC	For *actIORF1* homologous recombination template construction
25	orf1-5'R	GCTCACGTCGAAGCGGGTG ACCACGCAGGACTCCGAAGTC	
26	orf1-3'F	TCACCCGCTTCGACGTGAG	
27	orf1-3'R	*GGTCGATCCCCGCATATAGG* TTCGCCGAGCACCAGGTC	
28	VB-5'F	*TCGTCGAAGGCACTAGAAGG* CGACTCGCTCGCCCTGATG	For *actVB* homologous recombination template construction
29	VB-5'R	CACCAACCTGCTCGGGCTG CGCCGTGGAAGTGGGTGTTGAC	
30	VB-3'F	GCAGCCCGAGCAGGTTGG	
31	VB-3'R	*GGTCGATCCCCGCATATAGG* TCCGTTGCGGCGTCCATC	
32	VB-check-F	CGGCTGGTGCGTCAGCAAC	Check *actVB* deletion
33	VB-check-R	ACGTGGCGGGTCGAACGG	
34	ORF1-check-F	CCGCCTTGAGGACCTGTTTG	Check *actIORF1* deletion
35	ORF1-check-R	ACACGCTGACCGACTTGGG	
36	CAS9-check-F	TCCACGAGCACATCGCCAAC	Check *cas9* subcloning
37	CAS9-check-R	GACCTTGTAGTCGCCGTAGACG	
36	ScaligD-F	TCGTCGAAGGCACTAGAAGGG CGGTCGATCTTGACGGCTG	ScaligD expression cassette amplification
37	ScaligD-R	GGTCGATCCCCGCATATAGGT GCCGCCGGGCGTTTTTTAT	
38	orf1-6 ligD test-F	CCGCCGACACCCCGATCACC	Check NHEJ for *actIORF1* editing
39	orf1-6 ligD test-R	ACCGCAGCTTCCGCTCCCTG	
40	vb2 ligD test-F	CGAGGTGATCGACGCCAACC	Check NHEJ for *actVB* editing
41	vb2 ligD test-R	TCGCCGAGCAGGATGATGTG	

#: The restriction sites are underlined; the 20 nt target sequences are shown in bold, the pattern of the sgRNA-F primer is:
CATGCCATGG**N_{20}**GTTTTAGAGCTAGAAATAGC.

*: The overlap sequence for Gibson assembly is shown in italic.

§: The restriction sites are underlined.

Table 5. Strains and plasmids

Name	Description	Reference
WT	*Streptomyces coelicolor* A3(2)	95 SNPs and 1 deletions of (Bentley et al., 2002)
No Target	WT with pCRISPR-Cas9	This study
Mismatch	WT with sgRNA: Actlorf1-1 NT including its PAM sequence	This study
Δ*actlorf1*-1	WT with pCRISPR-Cas9 carrying sgRNA: Actlorf1-1 NT, 1 bp insertions from the DSB site	This study
Δ*actlorf1*-2	WT with pCRISPR-Cas9 carrying sgRNA: Actlorf1-6 T, 10721 bp deletion around the DSB site	This study
Δ*actvb*-1	WT with pCRISPR-Cas9 carrying sgRNA: Actvb-2 NT, 14716 bp deletion around the DSB site	This study
Δ*actvb*-2	WT with pCRISPR-Cas9 carrying sgRNA: Actvb-5 NT, 37173 bp deletion around the DSB site	This study
Δ*actlorf1*-ligD1-Δ*actlorf1*-ligD8	WT with pCRISPR-Cas9-ScaligD carrying sgRNA: Actlorf1-6 T, 8 random red clones	This study
Δ*actvb*-ligD1-Δ*actvb*-ligD8	WT with pCRISPR-Cas9-ScaligD carrying sgRNA: Actvb-2 NT, 8 random red clones	This study
orf1 deletion1-orf1 deletion10	WT with *actIORF1* recombination arm in the pCRISPR-Cas9 carrying sgRNA: Actlorf1-6 T, *actIORF1* gene was deleted, 10 random clones	This study

vb deletion1-vb deletion10	WT with *actVB* recombination arm in the pCRISPR-Cas9 carrying sgRNA: Actvb-2 NT, *actVB* gene was deleted, 10 random clones	This study
orf1 knock-down-1	WT with pCRISPR-dCas9 carrying sgRNA: orf1p-S1 T	This study
orf1 knock-down-2	WT with pCRISPR-dCas9 carrying sgRNA: orf1p-S3 T	This study
orf1 knock-down-3	WT with pCRISPR-dCas9 carrying sgRNA: orf1p-S5 T	This study
orf1 knock-down-4	WT with pCRISPR-dCas9 carrying sgRNA: orf1p-A1 NT	This study
orf1 knock-down-5	WT with pCRISPR-dCas9 carrying sgRNA: orf1p-A4 NT	This study
orf1 knock-down-6	WT with pCRISPR-dCas9 carrying sgRNA: orf1p-A5 NT	This study
orf1 knock-down-7	WT with pCRISPR-dCas9 carrying sgRNA: Ac-tlorf1-2T	This study
orf1 knock-down-8	WT with pCRISPR-dCas9 carrying sgRNA: Ac-tlorf1-3T	This study
orf1 knock-down-9	WT with pCRISPR-dCas9 carrying sgRNA: Ac-tlorf1-4T	This study
orf1 knock-down-10	WT with pCRISPR-dCas9 carrying sgRNA: Ac-tlorf1-1NT	This study
orf1 knock-down-11	WT with pCRISPR-dCas9 carrying sgRNA: Ac-tlorf1-7NT	This study
orf1 knock-down-12	WT with pCRISPR-dCas9 carrying sgRNA: Ac-tlorf1-8NT	This study
ET12567/pUZ8002	*Escherichia coli* for conjugation *dam-13::Tn9 dcm-6 hsdM* Cml^R, carrying helper plasmid pUZ8002	(2)
Mach1™-$T1^R$	*Escherichia coli* for routine cloning *lacZΔM15 hsdR lacX74 recA endA tonA*	Life Technologies
pGM1190	temperature sensitive plasmid, tsr, aac(3)IV, oriT, to terminator PtipA, RBS, fd terminator	(3)
pGM1190-	pGM1190 with sgRNA scaffold	This study

sgRNA		
pCRISPR-Cas9	pGM1190-sgRNA with *cas9*	This study
pCRISPR-dCas9	pGM1190-sgRNA with d*cas9* (D10A and H840A)	This study
pCRISPR-Cas9-ScaligD	pCRISPR-Cas9 with a ScaligD expression cassette	This study
pCRISPR-Cas9-orf1-1	pCRISPR-Cas9 carrying sgRNA: Actlorf1-1 NT	This study
pCRISPR-Cas9-orf1-2	pCRISPR-Cas9 carrying sgRNA: Actlorf1-2 T	This study
pCRISPR-Cas9-orf1-3	pCRISPR-Cas9 carrying sgRNA: Actlorf1-3 T	This study
pCRISPR-Cas9-orf1-4	pCRISPR-Cas9 carrying sgRNA: Actlorf1-4 T	This study
pCRISPR-Cas9-orf1-5	pCRISPR-Cas9 carrying sgRNA: Actlorf1-5 T	This study
pCRISPR-Cas9-orf1-6	pCRISPR-Cas9 carrying sgRNA: Actlorf1-6 T	This study
pCRISPR-Cas9-vb1	pCRISPR-Cas9 carrying sgRNA: Actvb-1 NT	This study
pCRISPR-Cas9-vb2	pCRISPR-Cas9 carrying sgRNA: Actvb-2 NT	This study
pCRISPR-Cas9-vb3	pCRISPR-Cas9 carrying sgRNA: Actvb-3 T	This study
pCRISPR-Cas9-vb4	pCRISPR-Cas9 carrying sgRNA: Actvb-4 T	This study
pCRISPR-Cas9-vb5	pCRISPR-Cas9 carrying sgRNA: Actvb-5 NT	This study
pCRISPR-Cas9-vb6	pCRISPR-Cas9 carrying sgRNA: Actvb-6 NT	This study
pCRISPR-Cas9-orf1-6-Tem	pCRISPR-Cas9-orf1-6 with *actIORF1* homologous recombination template	This study
pCRISPR-	pCRISPR-Cas9-vb2 with *actVB* homologous	This study

Cas9-vb2-Tem	recombination template	
pCRISPR-Cas9-ScaligD-orf1-6T	pCRISPR-Cas9-ScaligD carrying sgRNA: Actlorf1-6 T	This study
pCRISPR-Cas9-ScaligD-vb2	pCRISPR-Cas9-ScaligD carrying sgRNA: Actvb-2 NT	This study
pCRISPR-dCas9-1	pCRISPR-dCas9 carrying sgRNA: orf1p-S1 T	This study
pCRISPR-dCas9-2	pCRISPR-dCas9 carrying sgRNA: orf1p-S3 T	This study
pCRISPR-dCas9-3	pCRISPR-dCas9 carrying sgRNA: orf1p-S5 T	This study
pCRISPR-dCas9-4	pCRISPR-dCas9 carrying sgRNA: orf1p-A1 NT	This study
pCRISPR-dCas9-5	pCRISPR-dCas9 carrying sgRNA: orf1p-A4 NT	This study
pCRISPR-dCas9-6	pCRISPR-dCas9 carrying sgRNA: orf1p-A5 NT	This study
pCRISPR-dCas9-7	pCRISPR-dCas9 carrying sgRNA: Actlorf1-1NT	This study
pCRISPR-dCas9-8	pCRISPR-dCas9 carrying sgRNA: Actlorf1-2T	This study
pCRISPR-dCas9-9	pCRISPR-dCas9 carrying sgRNA: Actlorf1-3T	This study
pCRISPR-dCas9-10	pCRISPR-dCas9 carrying sgRNA: Actlorf1-4T	This study
pCRISPR-dCas9-11	pCRISPR-dCas9 carrying sgRNA: Actlorf1-7NT	This study
pCRISPR-dCas9-12	pCRISPR-dCas9 carrying sgRNA: Actlorf1-8NT	This study

Cas9 codon optimization for streptomycetes

The most studied CRISPR-Cas9 system is from *Streptococcus pyogenes.* As there is significant difference of GC content (35% vs. 72%) and codon usage between *S. pyogenes* and *Streptomyces coelicolor*, a codon optimization of the *S. pyogenes cas9* according to the codon usage of streptomycetes was performed. In order to make the optimized cas9 as compatible as possible for all streptomycetes, the codon usage table of the most studied actinomycete, *Streptomyces coelicolor* was used as template for codon optimization, using the *S. pyogenes cas9* sequence as starting sequence (SEQ ID NO: 3).

The codon optimization was done by GenScript inc. using the OptimumGeneTM algorithm, which optimizes a variety of parameters critical to the efficiency of gene expression, including but not limited to: codon usage bias, GC content, CpG dinucleotides content, mRNA secondary structure, cryptic splicing sites, premature PolyA sites, internal chi sites and ribosomal binding sites, negative CpG islands, RNA instability motif (ARE), repeat sequences (direct repeat, reverse repeat, and Dyad repeat) and restriction sites that may interfere with cloning.

The *S. pyogenes cas9* gene comprises tandem rare codons that can reduce the efficiency of translation or even disengage the translational machinery. The codon usage bias in *Streptomyces coelicolor* was modified by upgrading the CAI from 0.09 to 0.94. GC content (from 35.04 to 61.79) and unfavorable peaks were optimized to prolong the half-life of the mRNA. The Stem-Loop structures, which impact ribosomal binding and stability of mRNA, were broken. In addition, negative cis-acting sites were screened and successfully modified.

Design of the sgRNA scaffold

The sequence of the core guide RNA is GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT (SEQ ID NO: 67); the RNA structure is shown in Figure 1. An ermE* promoter was introduced upstream the core sequence and two unique restriction sites, NcoI and SnaBI (underlined) were introduced into the scaffoled in order to make the scaffold easy adaptable when changing the 20 nt target sequences. When constructing new functional sgRNAs, only the 20 nt target sequence of the forward primer needs be changed, while the reverse primer including the SnaBI restriction site needs not be changed.

The fragment is amplified by PCR and digested using the NcoI and SnaBI sites before cloning the functional sgRNA into the vector, under the control of the ermE* promotor (Figure 2). The final sgRNA scaffold sequence is:

GCGGTCGATCTTGACGGCTGGCGAGAGGTGCGGGGAGGATCTGACCGAC-GCGGTCCACACGTGGCACCGCGATGCTGTTGTGGGCACAATCGTGCCGGTTGG-TAGGATCGAC-GGCCATGG(N_{20})GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTACGTA (SEQ ID NO: 68), where N_{20} represents the 20 nt target sequence.

For the “one plasmid strategy”, we selected the vector pGM1190 (Muth et al., 1989) as the backbone. pGM1190 is temperature sensitive in streptomycetes and will be lost at temperatures above 34°C; the selection markers are apramycin and thiostrepton, the regulatory elements include: a thiostrepton-inducible promoter tipA, a RBS, a *to* and an *fd* terminator. This plasmid can be shuttled in *E. coli* and streptomycetes.

The sgRNA scaffold was subcloned into pGM1190 upstream of the *to* terminator using the Gibson cloning method, resulting in pGM1190-sgRNA. The *to* terminator exited in pGM1190 is used as a secondary terminator for the sgRNA scaffold. Alternatively, it can be sub-cloned into a different vector; this strategy is termed the ‘two plasmids strategy’.

Construction of one plasmid based CRISPR-Cas9 system

The codon optimized Cas9 was synthetized as set forth in SEQ ID NO: 1, flanked by the following restriction sites: CATATG in the 5’-end, where ATG is the start codon of SEQ ID NO: 1; and AAGCTTTCTAGA in the 3’-end, immediately downstream of the stop codon.

For the one plasmid strategy, the gene was sub-cloned into pGM1190-sgRNA with NdeI and XbaI sites, under the control of the thiostrepton inducible tipA promoter. The final vector was named pCRISPR-Cas9 (Figure 3). The sgRNA and *cas9* fragments were confirmed by PCR (with the primers, sgRNA check-F and sgRNA check-R) and digested by NdeI and XbaI.

Insertion of the target sequence into the guide RNA

In order to construct a functional vector for the one plasmid strategy, it is sufficient to introduce the 20 nt target sequence upstream of the sgRNA. Design software such as CRISPRy and other similar software can be used for sgRNA design. Here, we used CRISPRy for *S. coelicolor* (http://staff.biosustain.dtu.dk/laeb/crispy_scoeli/ or , or http://crispy.secondarymetabolites.org).

Based on the specificity of the target sequences with the gene, one or more target sequences were chosen. Based on the target sequences, the forward PCR primer as designed: CATGCCATGG N_{20}GTTTTAGAGCTAGAAATAGC (N_{20} is the 20 nt target sequence) (SEQ ID NO: 69), while the reverse primer remains the same: ACGCC-TACGTAAAAAAAGCACCGACTCGGTGCC (sgRNA-R; SEQ ID NO: 44) (the restriction sites are underlined). PCR as used to amplify the functional sgRNAs from the pCRISPR-Cas9 template. The PCR products were digested with NcoI and SnaBI. The pCRISPR-Cas9 was also digested with the same restriction enzymes. After agrose gel purification, the ~110 bp PCR fragment and the ~ 11 kb pCRISPR-Cas9 backbone were ligated by T4 ligase and the ligation mix was transformed into competent *E. coli*. Several positive transformants for each target sequence were picked for colony PCR screening using the primers, sgRNA check-F and sgRNA check-R. The expected sizes were 234 bp for positive clones and were confirmed by sequencing.

Example 2: generation of random-sized deletions around a target site

This example describes how to apply the present method to inactivate the actinorhdin biosynthetic genes, as well as control the target gene expression in *Streptomyces coelicolor* A3(2). *S. coelicolor* A3(2) is a well-known actinorhdin producer. Actinorhodin is a benzoisochromanequinone polyketide antibiotic with pH-dependent colors: blue color when pH>7, red color when pH<7.

Actinorhdin biosynthesis is encoded by a PKS type II gene cluster, named *act* gene cluster (Figure 4). The steps to synthetize actinorhodin are: **I.** 1x Acetyl-CoA and 7x malonyl-CoA are condensed to form the carbon skeleton by ActI; **II.** The above carbon backbone is cyclized to form a three ring intermediate, DNPA by ActIII, ActVII, ActIV, ActVI-1 and ActVI-3; **III.** DNPA is then modified to form DHK by ActVI-2, ActVI-4 and ActVA-6; **IV.** 2 DHK is dimerized to form the final product, actinorhodin, by ActVA-5 and ActVB (Figure 4). Two genes were selected as targets (marked by arrows in Figure 4):

ActORF1 is the actinorhodin ketosynthase subunit alpha (KS domain of PKS II), and ActVB is the actinorhodin polyketide dimerase. A deletion of any of these two genes results in a loss of actinorhodin production, which can be easily monitored by the disappearance of the blue pigment.

For each gene inactivation, 6 different sgRNAs were designed for each gene using CRISPRy webserver (http://staff.biosustain.dtu.dk/laeb/crispy_scoeli/), resulting in 12 sgRNAs (listed in Table 3).

PCR was used to amplify the functional sgRNAs from the pCRISPR-Cas9 template (for primers, see Table 4). The fragments and pCRISPR-Cas9 were digested using NcoI and SnaBI. After agarose gel purification, the PCR fragment (1~10 bp) and the pCRISPR-Cas9 backbone (~11 kb) were ligated, and transferred into One Shot® Mach1™-T1^R chemically competent *E. coli*. 6 positive transformants for each target sequence were picked for colony PCR screening using the primers set, sgRNA check-F and sgRNA check-R (Table 4), a set of primers resulting in products of 234 bp for positive clones and 214 bp for the negative clones. The PCR screening results are shown in Figure 10A-F (A-C for *actIORF1*, D-F for *actVB*).

2-3 positive clones for each target sequence were confirmed by sequencing and matched the results of the colony PCR 100%. Colony PCR is thus a valid way of screening the clones.

One correct clone for each target sequences was selected randomly to be transferred into the ET12567/pUZ8002 *E. coli* strain for conjugation. In addition, two negative controls were used: the first is the empty vector, pCRISPR-Cas9 (No Target), which has no target matches on the genome, and the second is a target sequence with a 3 nt PAM motif “NGG”. The inclusion of the PAM as part of the sgRNA abolishes correct recognition of the genomic target (Mismatch).

The PCR validated conjugates for each target sequence plus the two controls were inoculated into 20 ml LB broth with 25 µg/ml kanamycin, 25 µg/ml chloramphenicol and 50 µg/ml apramycin. After overnight shaking at 37°C, the *E. coli* cells were harvested by centrifuging at 5000 g for 5 minutes at room temperature; fresh LB was used without

antibiotics to wash 2 times. The donor cells then were resuspended in 0.5-2 ml LB broth and placed at room temperature. To collect *S. coelicolor,* spores from one ISP2 plate were resuspended in 0.9% saline, and filtered through a cotton pad. The spore suspension was concentrated by centrifuging at 5000 g for 5 minutes at room temperature, then the spores were resuspended in 0.5 ml-1ml 2×YT broth. To induce germination, the spore suspension was heated to 50°C for 10 minutes, and then cooled down to room temperature. 500 µl of the relevant ET12567/pUZ8002 cells were added to the heat treated pre-germinated spores and mixed by inversion. The mixture was centrifuged for 2 minutes at top speed, the supernatant was decanted and the pellet was resuspended in the remaining fluid so that the final volume was about 50 µl. The cells were then plated on Cullum agar plates and incubated for 16 h at 30°C. After 16h, the plates were overlaid with a solution containing the selection antibiotics: 20 µl of 50 mg/ml nalidixic acid, against *E. coli* cells or 10 µl of 100 mg/ml apramycin for the selection of clones with the transferred DNA, dissolved in 1 ml of sterile H_2O. The overlaid plates were further incubated for 3-7 days at 30°C, or until colonies became visible. 50-80 conjugates for each target sequence were randomly picked onto ISP2 plates with 50 µg/ml apramycin, 50 µg/ml nalidixic acid (to avoid *E. coli* contamination), and 1 µg/ml thiostrepton (to induce Cas9). In parallel, the same sets of clones were also streaked onto ISP2 plate with 50 µg/ml apramycin and 50 µg/ml nalidixic acid, but without thiostrepton. The plates were incubated for 7-10 days at 30°C.

From the red colonies, the following clones were randomly selected: one clone for each gene (Δ*actIorf1*-1 and Δ*actvb-1*), as well as one clone for each negative control (Mismatch and No Target), and one clone for the wild type (WT), resulting in 5 strains (Figure 6 and Figure 7).

Besides ISP2 agar plates, the above selected five strains (from ISP2 plates with thiostrepton) were also inoculated in 100 ml ISP2 liquid medium, and incubated with shaking for 7 days at 30°C. 30 ml cultures were used for each strain to perform actinorhodin extraction. The cultures were centrifuged at 8000 g for 10 minutes at room temperature, the supernatant was transferred to a 50 ml tube, the pH was adjusted to 2 with 1M HCl, before adding ¼ volume chloroform. The solution was intensively mixed by vortex, and then centrifuged at 8000 g for 5 minutes at room temperature. The chloroform phase was collected for drying, the dried samples were re-dissolved using 2 ml solvent (methanol: chloroform=1:1). The solutions were analyzed using the Evolution™

201/220 UV-Visible Spectrophotometers to scan from 420 nm to 720 nm (the actinorhodin in these conditions has a maximum absorption at about 530 nm). The scanning results show that the actinorhodin peaks in Δ*actIorf1*-1 and Δ*actVb*-1 disappeared (Figure 7).

Genomic DNA was extracted using 10 ml of the above cultures for each strain using Blood & Cell Culture DNA Kit (QIAGEN, Germany). The genomic libraries were generated using the TruSeq ®Nano DNA LT Sample Preparation Kit (Illumina Inc., San Diego CA). Briefly, 100 ng of genomic DNA diluted in 52.5 µl TE buffer was fragmented in Covaris Crimp Cap microtubes on a Covaris E220 ultrasonicator (Covaris, Brighton, UK) with 5% duty factor, 175 W peak incident power, 200 cycles/burst, and 50 s duration under frequency sweeping mode at 5.5 to 6°C (Illumina recommendations for a 350-bp average fragment size). The ends of fragmented DNA were repaired by T4 DNA polymerase, Klenow DNA polymerase, and T4 polynucleotide kinase. The Klenow exo minus enzyme was then used to add an 'A' base to the 3' end of the DNA fragments. After the ligation of the adapters to the ends of the DNA fragments, DNA fragments ranging from 300 - 400 bp were recovered by bead purification. Finally, the adapter-modified DNA fragments were enriched by 3 cycle-PCR. The final concentration of each library was measured by Qubit® 2.0 Florometer and Qubit DNA Broad range assay (Life Technologies, Paisley, UK). The average sizes of the dsDNA libraries were determined using the Agilent DNA 7500 kit on an Agilent 2100 Bioanalyzer. Libraries were normalised and pooled in 10 mM Tris-Cl, pH 8.0, plus 0.05% Tween 20 to the final concentration of 10 nM. After denaturation in 0.2N NaOH, a 10 pm pool of 20 libraries in 600 µl ice-cold HT1 buffer was loaded onto the flow cell provided in the MiSeq Reagent kit v2 (300 cycles) and sequenced on a MiSeq (Illumina Inc., San Diego, CA) platform with a paired-end protocol and read lengths of 151 nt.

Mapping of the sequencing reads to the *S. coelicolor* A3(2) reference genome (Genbank accession AL645882).

The reads obtained above were mapped to the *S coelicolor* A3(2) reference genome using the software BWA (Li et al., 2009) using the BWA-mem algorithm. The data was inspected and visualized using readXplorer (Hilker et al., 2014) and Artemis (Rutherford et al., 2000). Comparison of the refererence *S. coelicolor* A3(2) wild type strain used in this study with the *S. coelicolor* A3(2) reference sequence deposited as AL645882 in Genbank resulted in 95 SNPs and fragment (5797650− 5818686) dele-

tion. For the following, *S. coelicolor* A3(2) WT refers to the sequences obtained in this study. The detailed mapping results are shown Table 6.

Table 6. List of mutations detected from whole genome sequencing (the results shown are after subtracted from the WT)

Name	Position	Mutation	Annotation	Gene	Description
Mismatch	2,474,084	A→C	T8P (ACC→CCC)	SCO2305→	putative ABC transporter ATP-binding subunit
	4,477,934	2 bp→TC	coding (195-196/609 nt)	SCO4084→	hypothetical protein SCD25.20
	8,265,166	G→C	intergenic (+76/-125)	SCO7449→/→SCO7450	putative membrane protein. /putative secreted protein
	8,267,257	G→C	intergenic (+13/+26)	SCO7451→/←SCO7452	conserved hypothetical protein SC5C11.08/putative O-methyltransferase.
No Target	1,645,577	+G	intergenic (-554/+422)	SCO1536←/←SCO1537	conserved hypothetical protein SCL2.26c/putative transport system membrane protein
	1,645,634	A→G	intergenic (-611/+365)	SCO1536←/←SCO1537	conserved hypothetical protein SCL2.26c/putative transport system membrane protein

	2,462,898	(G)12→13	intergenic (-386/+324)	SCO2292←/←SCO2293	secreted endo-1,4-beta-xylanase B (xylanase B)/putative integral membrane protein
	5,093,984	G→C	P550A (CCC→GCC)	SCO4664←	putative integral membrane protein
	6,442,710	(G)9→10	intergenic (-96/+43)	SCO5885←/←SCO5886	putative membrane protein/3-oxoacyl-[acyl-carrier-protein] synthase II
	8,163,408	T→C	T129T (ACA→ACG)	SCO7350←	putative membrane efflux protein.
	2,311,509	(TGA)4→5	coding (176/1638 nt)	SCO2148←	cytochrome B subunit
***Δac-tlorf1*-1**	2,440,703	A→G	L173P (CTC→CCC)	SCO2271←	hypothetical protein SCC75A.17c.
	7,846,245	A→G	S10P (TCC→CCC)	SCO7056←	putative gntR-family transcriptional regulator
	5,529,858	.→A	coding (58/1494 nt)	SCO5087←	actinorhodin polyketide beta-ketoacyl synthase alpha subunit
	7,846,250	T→G	D8A (GAC→GCC)	SCO7056←	putative gntR-family transcriptional regulator
Δac-	2,462,898	(G)12→11	intergenic (-	SCO2292←/	secreted endo-

***tlorf1*-2**			386/+324)	←SCO2293	1,4-beta-xylanase B (xylanase B)/putative integral membrane protein
	7,846,245	A→G	S10P (TCC→CCC)	SCO7056←	putative gntR-family transcriptional regulator
	8,267,257	G→C	intergenic (+13/+26)	SCO7451→/←SCO7452	conserved hypothetical protein SC5C11.08/putative O-methyltransferase.
	5,527,269	Δ10721		[SCO5084]–[SCO5096]	11 genes lost, SCO5087 included
***Δactvb*-2**	4,501,350	T→G	T39P (ACC→CCC)	SCO4102←	putative MerR family transcriptional regulator
	5,500,560	G→C	intergenic (-152/-34)	SCO5060←/→SCO5061	putative integral membrane protein/putative ATP/GTP binding protein
	5,500,565	T→C	intergenic (-157/-29)	SCO5060←/→SCO5061	putative integral membrane protein/putative ATP/GTP binding protein
	7,557,356	G→C	intergenic (+35/-82)	SCO6794→/→SCO6795	putative membrane protein./conserved hypothetical protein SC1A2.04.

	7,557,360	G→C	intergenic (+39/-78)	SCO6794→/→SCO6795	putative membrane protein/conserved hypothetical protein SC1A2.04.
	7,959,767	T→C	T571A (ACC→GCC)	SCO7164←	hypothetical protein SC9A4.26c
Δ*actvb*-1	2,440,703	A→G	L173P (CTC→CCC)	SCO2271←	hypothetical protein SCC75A.17c.
	3,180,456	A→C	intergenic (+74/+48)	SCO2928→/←SCO2929	putative asnC-family transcriptional regulator/putative transposase
	5,513,345	Δ37,173 bp		[SCO5070]–[SCO5107]	38 genes lost, SCO5092 included
sgRNA: Actvb-5 NT	5,818,673	Δ1 bp	intergenic (+125/-)	SCO5350→/–	hypothetical protein SCBAC5H2.19/–
	7,186,210	Δ9 bp	coding (1379-1387/1998 nt)	SCO6492→	hypothetical protein
	5,532,664	Δ14,716 bp		[SCO5089]–[SCO5105]	17 genes lost, SCO5092 included

Interestingly, the inactivation of the genes were caused by rearrangement events including 1 bp insertions and deletions between 1 bp and more than 30000 bps around the DSB site (Figure 8A and B). In other words, the deletion can be both very precise

and random sized around the DSB site. It appears this is effect is due to partially deficient NHEJ in *S. coelicolor.*

It was also tested whether deletions could be generated in other organisms. Deletions were successfully generated in *Streptomyces collinus* Tü365, in *Streptomyces avermitilis*, *Streptomyces pristinaespiralis* and *Verrucosispora spp*.

Streptomyces collinus Tü365 and in *Verrucosispora spp*. were investigated further , and random-sized deletions ranging from a few kilobase pairs to more than 1 kb were observed.

Species tested	**Deletion size (kb)**	**Numbers of tested genes (gene clusters)**
Streptomyces collinus Tü365	23-1200	6
Verrucosispora spp.	5-80	3

This example shows that the present method can be used to obtain a set of random sized deletions around a precisely defined site from a target sequence in different microorganisms using the present CRISPR-Cas9 system.

Example 3: generation of precise deletions around a target site by introduction of a functional NHEJ pathway

Genome mining indicated that the NHEJ pathway of some streptomycetes is not complete because one core component called DNA ligase D is missing. In order to reconstitute the NHEJ pathway of *S. coelicolor*, homologues of *ligD* were identified by blasting, using the mycobacterial *ligD* amino acid sequence as a query. A homologue of *ligD* was found in *S. carneus*.

An *S. carneus ligD* expression cassette was designed, where the *S. carneus* ligD (ScaligD; SEQ ID NO: 70) was cloned under control of an ermE* promoter, and a *to* terminator introduced downstream of *ligD*. This expression cassette was subcloned into the StuI site of pCRISPR-Cas9 by Gibson assembly. The construction was called pCRISPR-Cas9-ligD (Figure 9).

One sgRNA was selected for each of the two targeted genes (sgRNA: Actlorf1-6 T for actlORF1, and sgRNA: Actvb-2 NT for *actVB*) to test whether the natively deficient NHEJ pathway was fixed.

Comparison to the non-ScaligD CRISPR-Cas9 system (example 2) showed that the inactivation efficiency increased from 45% to 77%, and 37% to 69% for sgRNA: Actlorf1-6 T and sgRNA: Actvb-2 NT, respectively, after the ScaligD was introduced into the system (Table 7).

Table 7 The inactivation efficiency of different sgRNAs with different DSB repair pathways.

Ways of DSB repair	sgRNAs	Colony Count[a]				Efficiency (%)
		No growth	Red[b]	Blue	Total	Red/Total
Incomplete NHEJ	Actlorf1-1 NT	20	31	30	81	38
	Actlorf1-2 T	3	1	7	11	9
	Actlorf1-3 T	7	18	49	74	24
	Actlorf1-4 T	43	10	1	54	19
	Actlorf1-5 T	8	18	8	34	53
	Actvb-1 NT	10	20	22	52	38
	Actvb-3 T	17	6	40	63	10
	Actvb-4 T	30	6	5	41	15
	Actvb-5 NT	7	20	10	37	54
	Actvb-6 NT	1	1	30	32	3
	Actlorf1-6 T	10	18	12	40	45
	Actvb-2 NT	20	13	2	35	37
Reconstituted NHEJ	Actlorf1-6 T	0	24	7	31	77
	Actvb-2 NT	0	18	8	26	69
HDR (with homology templates)	Actlorf1-6 T	0	52	0	52	100
	Actvb-2 NT	0	35	1	36	97

[a] Denotes the number of colonies with the indicated phenotype after induction with thiostrepton. [b] Actinorhodin is blue. Upon loss of actinorhodin production, the red color of the 2nd pigmented antibiotic, undecylprodigiosin, becomes visible.

To further validate this observation, primers were designed to detect the ~ 600 bp fragment containing the theoretical cleavage sites of the used sgRNAs. Eight red clones for each gene were randomly selected for colony PCR, and the PCR products were sequenced. No long fragment deletions were found in any of the 16 sequencing clones; instead, most of them just had 1 to 3 bp deletion, substitution, or insertion (Figure 8C and D). In contrast, without the ScaligD, long fragment deletions were found in 3 of the 4 red clones for which whole genome sequencing was performed (Figure 8A).

These results indicated the natively deficient incomplete NHEJ pathway was successfully fixed by complementary its missing component, DNA ligase D.

Example 4: HDR -directed gene editing

In this example, in order to bypass the NHEJ pathway, a template for homologous recombination was introduced into the CRISPR-Cas9 system to let the organism use HDR to repair the DSBs. Again the genes *ActIORF1* and *ActVB* were selected for testing, only one sgRNA (sgRNA: Actlorf1-6 T, and sgRNA: Actvb-2NT) was designed for each gene. PCR was used to amplify the ~1 kb fragments of the 5' and the 3' regions out of the targeted genes with the primers orf1-5'F, orf1-5'R, orf1-3'F, orf1-3'R, and VB-5'F, VB-5'R, VB-3'F, VB-3'R, for *actORF1* and *actVB*, respectively. The orf1-5'F and VB-5'F primers contain a 20 bp overlap region of the 5' of the StuI site from the pCRISPR-Cas9 plasmid, and the orf1-3'R and VB-3'R primers contain a 20 bp overlap region of the 3' of the StuI site from the pCRISPR-Cas9 plasmid, while the orf1-5'R and VB-5'R primers contain a 20 bp overlap region of the orf1-3' fragment and VB-3' fragment, respectively. After gel purification of the fragments, orf1-5', orf1-3', and the StuI digested pCRISPR-Cas9 plasmid, and VB-5', VB-3', and the StuI digested pCRISPR-Cas9 plasmid were assembled by Gibson assembly (New England Biolabs). The transformants were screened by PCR using orf1-check-F, orf1-check-R and VB-check-F, VB-check-R for the homologous recombination templates of *actIORF1* and *actVB*, respectively, and finally confirmed by sequencing. All 52 clones picked randomly for *actIORF1*, and 35 out of 36 clones picked randomly for *actVB* were red after induction (Table 7).

In order to find out whether the deletion was a precise deletion, we designed primers around the target cleavage site. For both genes, 10 red clones were randomly selected for colony PCR validation. The colony PCR was performed as follows: mycelia of the selected colonies were scraped from the plates using a sterile toothpick into 10 µl pure DMSO in PCR tubes. The tubes were shaken vigorously for 10 min at 100°C in a heating block. After this step, the solution was centrifuged at top speed for 10 seconds, 1 µl of the supernatant were used for PCR template in a 20 µl PCR reaction.

The sizes of all 20 PCR products corresponded to the predicted sizes of the gene deletion (Figure 10). Importantly, the CRISPR-Cas9 system with the homologous recombination template showed even higher efficiency and precision in gene editing in comparison to the gene deletion system relying on functional NHEJ described in example 3 (Table 7).

This example shows that gene editing can be performed in actinomycetes using the CRISPR/Cas9 system with homologous recombination with high precision and efficiency.

Example 5: modulation of gene expression

This example describes how gene expression in Actinomycetes can be modulated. The *actIORF1* gene was selected for these experiments.

The codon-optimised Cas9 (SEQ ID NO: 1) was mutated to a catalytically dead version, which was done by point mutation of D10A and H840A. This version of Cas9 was called dCas9 and is lacking endonuclease activity (Figure 11).

Three sgRNAs targeting the non-template strand DNA and three sgRNAs targeting the template strand DNA of the coding region of *actIORF1* gene were selected. Another set of three sgRNAs targeting the template / non-template strand of the promoter region of *actIORF1* gene (total 12) were chosen (Table 3). In this example, a catalytically dead Cas9 (dCas9) having both mutations D10A and H840A was used.

The cloning strategy for sgRNA was the same as for the CRISPR-Cas9 system for deletion described above. The conjugates were streaked on the ISP2 agar containing 1

µg /ml thiostrepton (the inducer for dCas9), 50 µg /ml apramycin, and 50 µg /ml nalidixic acid and incubated for 7 days at 30 °C.

Actinorhodin production was abolished or dramatically reduced (Figure 12) in clones encoding sgRNAs targeted on the promoter region of *actIORF1* gene, independently of which of the template strand DNA or non-template strand DNA was targeted. In contrast, loss or decrease of actinorhodin production in clones carrying sgRNAs that target the coding region, was only observed in the clones with sgRNAs directed to the non-template strand (Figure 12).

To provoke the loss of the pCRISPR-Cas9 plasmid, the temperature of the incubaton was raised to 37°C for 24 h, before transferring the cultures to fresh ISP2 plates without antibiotics and incubating for another 5 days at 37°C. The previously red clones began to turn blue (Figure 12), indicating that the repression of actinorhodin biosynthesis by the CRISPR-dCas9 system was abrogated and the related gene started to express.

This example shows that gene expression can be modulated in actinomycetes by using the present system.

Sequences

SEQ ID NO	Name	Description
1	Codon-optimised Cas9	DNA sequence, codon-optimised for *Streptomyces coelicolor*
2	Cas9 protein	Translation of SEQ ID NO: 1
3	*cas9*	DNA from *S. pyogenes*
4	Actlorf1-1 NT	Table 3
5	Actlorf1-2 T	Table 3
6	Actlorf1-3 T	Table 3
7	Actlorf1-4 T	Table 3
8	Actlorf1-5 T	Table 3
9	Actlorf1-6 T	Table 3
10	Actvb-1 NT	Table 3

11	Actvb-2 NT	Table 3
12	Actvb-3 T	Table 3
13	Actvb-4 T	Table 3
14	Actvb-5 NT	Table 3
15	Actvb-6 NT	Table 3
16	orf1p-S1 T	Table 3
17	orf1p-S3 T	Table 3
18	orf1p-S5 T	Table 3
19	orf1p-A1 NT	Table 3
20	orf1p-A4 NT	Table 3
21	orf1p-A5 NT	Table 3
22	Actlorf1-7 NT	Table 3
23	Actlorf1-8 NT	Table 3
24	Actlorf1-F1	Table 4
25	Actlorf1-F2	Table 4
26	Actlorf1-F3	Table 4
27	Actlorf1-F4	Table 4
28	Actlorf1-F5	Table 4
29	Actlorf1-F6	Table 4
30	Actlorf1-F7	Table 4
31	Actlorf1-F8	Table 4
32	ActVB-F1	Table 4
33	ActVB-F2	Table 4
34	ActVB-F3	Table 4
35	ActVB-F4	Table 4
36	ActVB-F5	Table 4
37	ActVB-F6	Table 4
38	orf1p-S1 T-F	Table 4
39	orf1p-S3 T-F	Table 4
40	orf1p-S5 T-F	Table 4
41	orf1p-A1 NT-F	Table 4
42	orf1p-A4 NT-F	Table 4
43	orf1p-A5 NT-F	Table 4
44	sgRNA-R	Table 4
45	gRNA check-F	Table 4

46	gRNA check-R	Table 4
47	orf1-5‘F	Table 4
48	orf1-5‘R	Table 4
49	orf1-3‘F	Table 4
50	orf1-3‘R	Table 4
51	VB-5‘F	Table 4
52	VB-5‘R	Table 4
53	VB-3‘F	Table 4
54	VB-3‘R	Table 4
55	VB-check-F	Table 4
56	VB-check-R	Table 4
57	ORF1-check-F	Table 4
58	ORF1-check-R	Table 4
59	CAS9-check-F	Table 4
60	CAS9-check-R	Table 4
61	ScaligD-F	Table 4
62	ScaligD-R	Table 4
63	orf1-6 ligD test-F	Table 4
64	orf1-6 ligD test-R	Table 4
65	vb2 ligD test-F	Table 4
66	vb2 ligD test-R	Table 4
67	core guide RNA	Example 1
68	sgRNA scaffold	Example 1
69	Target-specific Fw primer	Table 3
70	Translation of SEQ ID NO: 3	
71	*S. carneus ligD* DNA	
72	Translation of SEQ ID NO: 71	

SEQ ID NO: 1 Codon-optimised Cas9

ATGGACAAGAAGTACTCCATCGGCCTCGACATCGGCACCAACTCCGTGGGCTGG

GCGGTCATCACCGACGAGTACAAGGTCCCCTCCAAGAAGTTCAAGGTCCTGGGC

AACACCGACCGGCACTCGATCAAGAAGAACCTGATCGGCGCCCTGCTCTTCGAC

AGCGGCGAGACCGCCGAGGCGACCCGCCTGAAGCGGACCGCGCGTCGCCGCTA
CACCCGGCGCAAGAACCGCATCTGCTACCTGCAGGAAATCTTCTCCAACGAGATG
GCCAAGGTGGACGACTCGTTCTTCCACCGCCTGGAGGAGAGCTTCCTGGTGGAG
GAGGACAAGAAGCACGAGCGCCACCCGATCTTCGGCAACATCGTGGACGAGGTG
GCCTACCACGAGAAGTACCCCACCATCTACCACCTCCGCAAGAAGCTGGTGGACT
CGACCGACAAGGCGGACCTGCGGCTCATCTACCTGGCCCTCGCGCACATGATCA
AGTTCCGCGGCCACTTCCTCATCGAGGGCGACCTGAACCCGGACAACTCCGACG
TGGACAAGCTCTTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTCGAGGAGAA
CCCCATCAACGCCAGCGGCGTGGACGCCAAGGCGATCCTCTCCGCGCGCCTGA
GCAAGTCCCGGCGCCTGGAGAACCTCATCGCCCAGCTGCCGGGCGAGAAGAAG
AACGGCCTCTTCGGCAACCTGATCGCGCTGTCGCTCGGCCTGACCCCCAACTTC
AAGAGCAACTTCGACCTGGCCGAGGACGCGAAGCTCCAGCTGTCCAAGGACACC
TACGACGACGACCTGGACAACCTGCTCGCCCAGATCGGCGACCAGTACGCGGAC
CTCTTCCTGGCCGCGAAGAACCTCTCGGACGCCATCCTGCTCAGCGACATCCTG
CGGGTCAACACCGAGATCACCAAGGCCCCGCTGTCGGCGAGCATGATCAAGCGG
TACGACGAGCACCACCAGGACCTGACCCTGCTCAAGGCCCTCGTGCGCCAGCAG
CTGCCCGAGAAGTACAAGGAAATCTTCTTCGACCAGTCCAAGAACGGCTACGCCG
GCTACATCGACGGCGGCGCGTCGCAGGAGGAGTTCTACAAGTTCATCAAGCCGA
TCCTGGAGAAGATGGACGGCACCGAGGAGCTGCTCGTCAAGCTGAACCGCGAGG
ACCTGCTCCGCAAGCAGCGGACCTTCGACAACGGCTCCATCCCGCACCAGATCC
ACCTGGGCGAGCTCCACGCCATCCTCCGGCGCCAGGAGGACTTCTACCCCTTCC
TGAAGGACAACCGCGAGAAGATCGAGAAGATCCTGACCTTCCGCATCCCGTACTA
CGTCGGCCCCCTGGCCCGCGGCAACTCCCGGTTCGCGTGGATGACCCGGAAGT
CGGAGGAGACCATCACCCCGTGGAACTTCGAGGAGGTCGTGGACAAGGGCGCG
TCCGCGCAGTCGTTCATCGAGCGCATGACCAACTTCGACAAGAACCTCCCGAACG
AGAAGGTCCTGCCCAAGCACTCCCTGCTCTACGAGTACTTCACCGTGTACAACGA
GCTGACCAAGGTCAAGTACGTGACCGAGGGCATGCGGAAGCCGGCCTTCCTGTC
GGGCGAGCAGAAGAAGGCGATCGTGGACCTGCTCTTCAAGACCAACCGCAAGGT
CACCGTGAAGCAGCTGAAGGAGGACTACTTCAAGAAGATCGAGTGCTTCGACTCC
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GACCTGCTCAAGATCATCAAGGACAAGGACTTCCTCGACAACGAGGAGAACGAG
GACATCCTGGAGGACATCGTCCTCACCCTGACCCTCTTCGAGGACCGCGAGATG
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CAGCTGAAGCGTCGCCGCTACACCGGCTGGGGCCGCCTCTCCCGGAAGCTGATC
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GACGGCTTCGCCAACCGCAACTTCATGCAGCTCATCCACGACGACAGCCTGACCT
TCAAGGAGGACATCCAGAAGGCCCAGGTCTCGGGCCAGGGCGACAGCCTCCAC
GAGCACATCGCCAACCTGGCGGGCTCCCCGGCGATCAAGAAGGGCATCCTCCAG
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CGTCGTGGGCACCGCGCTGATCAAGAAGTACCCGAAGCTGGAGTCCGAGTTCGT
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TTCAAGACCGAGATCACCCTGGCCAACGGCGAGATCCGCAAGCGGCCCCTGATC
GAGACCAACGGCGAGACCGGCGAGATCGTCTGGGACAAGGGCCGCGACTTCGC
CACCGTCCGGAAGGTGCTGTCGATGCCGCAGGTCAACATCGTGAAGAAGACCGA
GGTGCAGACCGGCGGCTTCAGCAAGGAGTCCATCCTCCCCAAGCGCAACAGCGA
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CTCCTCGTTCGAGAAGAACCCGATCGACTTCCTGGAGGCCAAGGGCTACAAGGA
GGTCAAGAAGGACCTCATCATCAAGCTGCCCAAGTACAGCCTGTTCGAGCTGGAG
AACGGCCGCAAGCGGATGCTCGCCTCCGCGGGCGAGCTGCAGAAGGGCAACGA
GCTGGCCCTCCCGTCGAAGTACGTCAACTTCCTGTACCTCGCGTCCCACTACGAG
AAGCTGAAGGGCTCGCCCGAGGACAACGAGCAGAAGCAGCTCTTCGTGGAGCAG
CACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAGTTCAGCAAGCGC
GTCATCCTGGCCGACGCGAACCTCGACAAGGTGCTGTCCGCCTACAACAAGCAC
CGCGACAAGCCGATCCGGGAGCAGGCGGAGAACATCATCCACCTGTTCACCCTC

ACCAACCTGGGCGCCCCCGCCGCGTTCAAGTACTTCGACACCACCATCGACCGC
AAGCGGTACACCTCCACCAAGGAGGTCCTCGACGCGACCCTGATCCACCAGAGC
ATCACCGGCCTGTACGAGACCCGCATCGACCTGTCCCAGCTCGGCGGCGACTGA

SEQ ID NO: 2 - Protein sequence for codon-optimised Cas9:
MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETA
EATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHP
IFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDN
SDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLF
GNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLS
DAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKN
GYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHL
GELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITP
WNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEG
MRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLG
TYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKR
RRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKA
QVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQT
TQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQ
ELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYW
RQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTK
YDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKY
PKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRP
LIETNGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLI
ARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNP
IDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYL
ASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKH
RDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYET
RIDLSQLGGD.

SEQ ID NO: 3 *S. pyogenes cas9*
ATGGATAAGAAATACTCAATAGGCTTAGATATCGGCACAAATAGCGTCGGATGGG
CGGTGATCACTGATGAATATAAGGTTCCGTCTAAAAAGTTCAAGGTTCTGGGAAAT
ACAGACCGCCACAGTATCAAAAAAAATCTTATAGGGGCTCTTTTATTTGACAGTGG
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SEQ ID NO: 71. *S. carneus ligD*

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Items

1. A method for generating at least one deletion around at least one target nucleic acid sequence comprised within a host cell having a non-homologous end-joining (NHEJ) pathway which is at least partly deficient,
said method comprising the step of inducing a CRISPR-Cas9 system in a host cell, wherein said CRISPR-Cas9 system is able to generate at least one break in said at least one target nucleic acid sequence and wherein the

CRISPR-Cas9 system comprises a Cas9 nuclease and at least one guiding means,
thereby generating at least one deletion around said at least one target nucleic acid sequence,
wherein said at least one deletion is a deletion of at least 1 bp.

2. The method of item 1, further comprising the step of determining the size of the deletion.

3. The method of any one of the preceding items, wherein said at least one deletion is one deletion.

4. The method of any one of the preceding items, wherein said at least one target nucleic acid sequence is one target nucleic acid sequence.

5. The method of any one of the preceding items, wherein the guiding means comprises at least one sgRNA and/or at least one crRNA/tracrRNA set.

6. The method of any one of the preceding items, wherein the host cell is an archae, a prokaryotic cell or a eukaryotic cell.

7. The method of any one of the preceding items, wherein the NHEJ pathway of said host cell comprises at least one of four activities defined as:

- a DNA-binding activity,
- a primase activity,
- a ligase activity.
- a polymerase activity.

8. The method of item 7, wherein at least one is two or three.

9. The method of any one of items 7 or 8, wherein said host cell is naturally lacking at least one said four activities or wherein at least one of said four activities has been inactivated.

10. The method of any one of the preceding items, wherein the host cell is selected from the group consisting of actinobacteria.

11. The method of any one of the preceding items, wherein the host cell is selected from the group consisting of *Actinomycetales*, such as *Streptomyces sp., Amycolatopsis sp.* or *Saccharopolyspora sp.*

12. The method of any one of the preceding items, wherein the host cell is selected from the group consisting of *Streptomyces coelicolor, Streptomyces avermitilis*, *Streptomyces aureofaciens*, *Streptomyces griseus, Streptomyces parvulus, Streptomyces albus, Streptomyces vinaceus, Streptomyces acrimycinis, Streptomyces calvuligerus, Streptomyces lividans, Streptomyces limosus, Streptomyces rubiqinosis, Streptomyces azureus, Streptomyces glaucenscens, Streptomyces rimosus, Streptomyces violaceoruber, Streptomyces kanamyceticus, Amycolatopsis orientalis, Amycolatopsis mediterranei* and *Saccharopolyspora erythraea.*

13. The method of any one of the preceding items, wherein the at least one target nucleic acid sequence is comprised within a secondary metabolite biosynthetic gene.

14. The method of any one of the preceding items, wherein the at least one target nucleic acid sequence is comprised within a gene cluster such as a secondary metabolite gene cluster.

15. The method of any one of items 13 to 14, wherein the secondary metabolite is selected from the group consisting of antibiotics, herbicides, anti-cancer agents, immunosuppressants, flavors, parasiticides, enzymes and proteins.

16. The method of any one of items 13 to 15, wherein the secondary metabolite is an antibiotic selected from the group consisting of apramycin, bacitracin, chloramphenicol cephalosporins, cycloserine, erythromycin, fosfomycin, gentamicin, kanamycin, kirromycin, lassomycin, lincomycin, lysolipin, microbisporicin, neomycin, noviobiocin, nystatin, nitrofurantoin, platensimycin,

pristinamycins, rifamycin, streptomycin, teicoplanin, tetracycline, tinidazole, ribostamycin, daptomycin, vancomycin, viomycin and virginiamycin.

17. The method of any one of items 13 to 15, wherein the secondary metabolite is a herbicide selected from the group consisting of bialaphos, resormycin and phosphinothricin.

18. The method of any one of items 13 to 15, wherein the secondary metabolite is an anti-cancer agent selected from the group consisting of doxorubicin, salinosporamides, aclarubicin, pentostatin, peplomycin, thrazarine and neocarcinostatin.

19. The method of any one of items 13 to 15, wherein the secondary metabolite is an immunosuppressant selected from the group consisting of rapamycin, FK520, FK506, cyclosporine, ushikulides, pentalenolactone I and hygromycin A.

20. The method of any one of items 13 to 15, wherein the secondary metabolite is a flavor such as geosmin.

21. The method of any one of items 13 to 15, wherein the secondary metabolite is a parasiticide such as an insecticide, an anthelmintic, a larvacide, or an antiprotozoal agent such as spinsad or avermectin.

22. The method of any one of items 1 to 12, wherein the at least one nucleic acid encodes an enzyme such as a metabolic enzyme selected from the group consisting of an amylase, a protease, a cellulase, a chitinase, a keratinase and a xylanase, a glycosyltransferase, an oxygenase, a hydroxylase, a methyltransferase, a dehydrogenase, a dehydratase.

23. The method of any one of the preceding items, wherein the generation of at least one deletion results in the inactivation of at least one gene.

24. The method of any one of the preceding items, wherein said deletion is a deletion of 1 to 1 500 000 bp, such as 1 to 1200000 bp, such as 1 to

1000000 bp, such as 1 to 500000 bp, such as 1 to 400000 bp, such as 1 to 300000 bp, such as 1 to 200000 bp, such as 1 to 100000 bp, such as 2 to 75000 bp, such as 3 to 50000 bp, such as 4 to 40000 bp, such as 5 to 30000 bp, such as 10 to 20000 bp, such as 25 to 10000 bp, such as 50 to 9000 bp, such as 75 to 8000 bp, such as 100 to 7000 bp, such as 150 to 6000 bp, such as 200 to 5000 bp, such as 250 to 4000 bp, such as 300 to 3000 bp, such as 400 to 2000 bp, such as 500 to 1000 bp, such as 600 to 900 bp, such as 700 to 800 bp.

25. The method of any one of the preceding items, wherein said deletion is a deletion of at least 1 bp, such as at least 2 bp, such as at least 3 bp, such as at least 4 bp, such as at least 5 bp, such as at least 10 bp, such as at least 15 bp, such as at least 20 bp, such as at least 50 bp, such as at least 100 bp, such as at least 250 bp, such as at least 500 bp.

26. The method of any one of the preceding items, wherein said deletion is a deletion of 1 to 100 bp, such as 1 to 75 bp, such as 1 to 50 bp, such as 1 to 40 bp, such as 1 to 30 bp, such as 1 to 20 bp, such as 1 to 10 bp, such as 1 to 9 bp, such as 1 to 8 bp, such as 1 to 7 bp, such as 1 to 6 bp, such as 1 to 5 bp, such as 1 to 4 bp, such as 1 to 3 bp, such as 1 to 2 bp.

27. A method for generating at least one indel around at least one target nucleic acid sequence comprised within a host cell having a non-homologous end-joining (NHEJ) pathway which is at least partly deficient,
said method comprising the steps of:
i. restoring the full functionality of the NHEJ pathway in said host cell;
ii. inducing a CRISPR-Cas9 system in said host cell, wherein said CRISPR-Cas9 system is able to generate at least one break in said at least one target nucleic acid sequence and wherein the CRISPR-Cas9 system comprises a Cas9 nuclease and at least one guiding means,
thereby generating at least one indel around said at least one target nucleic acid sequence,
wherein said at least one indel is a deletion or insertion of at least1 bp.

28. The method of item 27, further comprising the step of determining the size of the indel.

29. The method of any one of items 27 to 28, wherein said at least one indel is one indel.

30. The method of any one of items 27 to 29, wherein said at least one target nucleic acid sequence is one target nucleic acid sequence.

31. The method of item 30, wherein the guiding means is a single guide RNA (sgRNA).

32. The method of any one of items 27 to 31, wherein the host cell is an archaea, a prokaryotic cell or a eukaryotic cell.

33. The method of any one of items 27 to 32, wherein the NHEJ pathway of said host cell comprises at least one of four activities defined as:
- a DNA-binding activity,
- a primase activity,
- a ligase activity
- a polymerase activity.

34. The method of any one of items 27 to 33, wherein the NHEJ pathway of said host cell lacks the ligase activity.

35. The method of item 34, wherein the ligase activity is restored by expression of a functional ligase such as a heterologous ligase.

36. The method of item 35, wherein the heterologous ligase is derived from an organism selected from the group consisting of: *Streptomyces carneus, Mycobacter tuberculosis, Nocardia spp.*, *Smaragdicoccus niigatensis*, *Rhodococcus spp.*, *Mycobacterium abscessus, Mycobacterium mageritense* and *Mycobacterium farcinogenes*.

37. The method of any one of items 27 to 36, wherein the host cell is selected from the group consisting of actinobacteria.

38. The method of any one of items 27 to 37, wherein the host cell is selected from the group consisting of *Actinomycetales*, such as *Streptomyces sp., Amycolatopsis sp.* or *Saccharopolyspora sp..*

39. The method of any one of items 27 to 38, wherein the host cell is selected from the group consisting of *Streptomyces coelicolor, Streptomyces avermitilis*, *Streptomyces aureofaciens*, *Streptomyces griseus, Streptomyces parvulus, Streptomyces albus, Streptomyces vinaceus, Streptomyces acrimycinis, Streptomyces calvuligerus, Streptomyces lividans, Streptomyces limosus, Streptomyces rubiqinosis, Streptomyces azureus, Streptomyces glaucenscens, Streptomyces rimosus, Streptomyces violaceoruber, Streptomyces kanamyceticus, Amycolatopsis orientalis, Amycolatopsis mediterranei* and *Saccharopolyspora erythraea.*

40. The method of any one of items 27 to 39, wherein the at least one target nucleic acid sequence is comprised within a secondary metabolite biosynthetic gene.

41. The method of any one of items 27 to 40, wherein the at least one target nucleic acid sequence is comprised within a gene cluster such as a secondary metabolite gene cluster.

42. The method of any one of items 40 to 41, wherein the secondary metabolite is selected from the group consisting of antibiotics, herbicides, anti-cancer agents, immunosuppressants, flavors, parasiticides, enzymes and proteins.

43. The method of any one of items 40 to 42, wherein the secondary metabolite is an antibiotic selected from the group consisting of apramycin, bacitracin, chloramphenicol cephalosporins, cycloserine, erythromycin, fosfomycin, gentamicin, kanamycin, kirromycin, lassomycin, lincomycin, lysolipin, microbisporicin, neomycin, noviobiocin, nystatin, nitrofurantoin, platensimycin,

pristinamycins, rifamycin, streptomycin, teicoplanin, tetracycline, tinidazole, ribostamycin, daptomycin, vancomycin, viomycin, virginiamycin.

44. The method of any one of items 40 to 42, wherein the secondary metabolite is a herbicide selected from the group consisting of bialaphos, resormycin and phosphinothricin.

45. The method of any one of items 40 to 42, wherein the secondary metabolite is an anti-cancer agent selected from the group consisting of doxorubicin, salinosporamides, aclarubicin, pentostatin, peplomycin, thrazarine and neocarcinostatin.

46. The method of any one of items 40 to 42, wherein the secondary metabolite is an immunosuppressant selected from the group consisting of rapamycin, FK520, FK506, cyclosporine, ushikulides, pentalenolactone I and hygromycin A.

47. The method of any one of items 40 to 42, wherein the secondary metabolite is a flavor such as geosmin.

48. The method of any one of items 40 to 42, wherein the secondary metabolite is a parasiticide such as an insecticide, an anthelmintic, a larvacide, or an antiprotozoal agent such as spinsad or avermectin.

49. The method of any one of items 27 to 39, wherein the at least one nucleic acid encodes an enzyme such as a metabolic enzyme selected from the group consisting of an amylase, a protease, a cellulase, a chitinase, a keratinase and a xylanase, a glycosyltransferase, an oxygenase, a hydroxylase, a methyltransferase, a dehydrogenase, a dehydratase.

50. The method of any one of items 27 to 49, wherein the generation of at least one indel results in the inactivation of at least one gene.

51. A method for selectively modulating transcription of at least one target nucleic acid sequence in a host cell, the method comprising introducing into the host cell:
    i. at least one guiding means, or a nucleic acid comprising a nucleotide sequence encoding guiding means, wherein the guiding means comprises a nucleotide sequence that is complementary to a target nucleic acid sequence in the host cell; and
    ii. a variant Cas9, or a nucleic acid comprising a nucleotide sequence encoding the variant Cas9, wherein the variant Cas9 has reduced endodeoxyribonuclease activity,

    wherein said guiding means and said variant Cas9 form a complex in the host cell, said complex selectively modulating transcription of at least one target nucleic acid in the host cell.

52. The method of item 51, wherein the guiding means comprises at least one sgRNA and/or at least one crRNA/tracrRNA set.

53. The method of item 52, wherein the variant Cas9 can cleave one of the strands of the target nucleic acid sequence but has reduced ability to cleave the other strand of the target nucleic acid sequence.

54. The method of any one of items 51 to 53, wherein the variant Cas9 is selected from the group consisting of Cas9-H840A, Cas9-D10A and Cas9-H840A,D10A.

55. The method of any one of items 51 to 54, wherein the host cell is a prokaryotic cell selected from the group consisting of actinobacteria.

56. The method of any one of items 51 to 55, wherein the host cell is selected from the group consisting of *Actinomycetales*, such as *Streptomyces sp., Amycolatopsis sp.* or *Saccharopolyspora sp.*

57. The method of any one of items 51 to 56, wherein the host cell is selected from the group consisting of *Streptomyces coelicolor, Streptomyces avermitilis*, *Streptomyces aureofaciens*, *Streptomyces griseus, Streptomyces*

parvulus, Streptomyces albus, Streptomyces vinaceus, Streptomyces acrimycinis, Streptomyces calvuligerus, Streptomyces lividans, Streptomyces limosus, Streptomyces rubiqinosis, Streptomyces azureus, Streptomyces glaucenscens, Streptomyces rimosus, Streptomyces violaceoruber, Streptomyces kanamyceticus, Amycolatopsis orientalis, Amycolatopsis mediterranei and *Saccharopolyspora erythraea.*

58. The method of any one items 51 to 57, wherein the at least one target nucleic acid sequence is comprised within a secondary metabolite biosynthetic gene.

59. The method of any one items 51 to 58, wherein the at least one target nucleic acid sequence is comprised within a gene cluster such as a secondary metabolite gene cluster.

60. The method of any one items 58 to 59, wherein the secondary metabolite is selected from the group consisting of antibiotics, herbicides, anti-cancer agents, immunosuppressants, flavors, parasiticides, enzymes and proteins.

61. The method of any one items 58 to 60, wherein the secondary metabolite is an antibiotic selected from the group consisting of apramycin, bacitracin, chloramphenicol cephalosporins, cycloserine, erythromycin, fosfomycin, gentamicin, kanamycin, kirromycin, lassomycin, lincomycin, lysolipin, microbisporicin, neomycin, noviobiocin, nystatin, nitrofurantoin, platensimycin, pristinamycins, rifamycin, streptomycin, teicoplanin, tetracycline, tinidazole, ribostamycin, daptomycin, vancomycin, viomycin, virginiamycin.

62. The method of any one items 58 to 60, wherein the secondary metabolite is a herbicide selected from the group consisting of bialaphos, resormycin and phosphinothricin.

63. The method of any one items 58 to 60, wherein the secondary metabolite is an anti-cancer agent selected from the group consisting of doxorubicin, salinosporamides, aclarubicin, pentostatin, peplomycin, thrazarine and neocarcinostatin.

64. The method of any one items 58 to 60, wherein the secondary metabolite is an immunosuppressant selected from the group consisting of rapamycin, FK520, FK506, cyclosporine, ushikulides, pentalenolactone I and hygromycin A.

65. The method of any one items 58 to 60, wherein the secondary metabolite is a flavor such as geosmin.

66. The method of any one items 58 to 60, wherein the secondary metabolite is a parasiticide such as an insecticide, an anthelmintic, a larvacide, or an antiprotozoal agent such as spinsad or avermectin.

67. The method of any one items 51 to 57, wherein the at least one nucleic acid encodes an enzyme such as a metabolic enzyme selected from the group consisting of an amylase, a protease, a cellulase, a chitinase, a keratinase and a xylanase, a glycosyltransferase, an oxygenase, a hydroxylase, a methyltransferase, a dehydrogenase, a dehydratase.

68. The method of any one of items 51 to 67, wherein:
    i. the transcription of the guiding means is under the control of an inducible promoter; or
    ii. the expression of the variant Cas9 is inducible.

69. A polynucleotide having at least 93% identity with SEQ ID NO: 1, such as at least 94% identity, such as at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity, such as 100% identity.

70. The polynucleotide of item 69, wherein the polynucleotide is non-naturally occurring.

71. A polypeptide encoded by the polynucleotide of any of items 69 to 70.

72. The polypeptide of any item 71, wherein the polypeptide is non-naturally occurring.

73. A cell comprising the polynucleotide of any of items 69 to 70.

74. A cell comprising the polypeptide of any of items 71 to 72.

75. A vector comprising the polynucleotide of any of items 69 to 70.

76. A clonal library obtainable by the method of any of items 1 to 26, said clonal library comprising a plurality of clones, each clone harbouring at least one deletion around at least one target nucleic acid sequence, wherein each of said deletion is a deletion of at least 1 bp.

77. A kit for performing the method of any of items 1 to 26, said kit comprising:
- a vector comprising a nucleic acid sequence encoding a Cas9 nuclease or variant thereof; and
- instructions for use.

78. The kit of item 77, wherein the nucleic acid sequence is the polynucleotide of items 69 to 70.

79. The kit of any one of items 77 to 78, further comprising at least one guiding means and/or at least one host cell.

80. The kit of any one of items 77 to 79, wherein the host cell has a non-homologous end-joining (NHEJ) pathway which is at least partly deficient.

81. The kit of any one of items 77 to 80, further comprising means for partly inactivating NHEJ in the host cell.

82. A kit for performing the method of any of items 27 to 50, said kit comprising:
- a first vector comprising a nucleic acid sequence encoding Cas9 or a variant thereof; and
- instructions for use.

83. The kit of item 82, further comprising a second vector comprising at least one nucleic acid encoding at least one of the NHEJ activities defined in item 33.

84. The kit of item 83, wherein the at least one nucleic acid encodes a ligase derived from *S. carneus*.

85. A kit for performing the method of any of items 51 to 68, said kit comprising:
- a vector comprising a nucleic acid sequence encoding a variant Cas9; and
- instructions for use.

86. The kit of item 85, wherein the variant Cas9 is Cas9-H840A, Cas9-D10A or Cas9-H840A,D10A.

87. The kit of any of items 85 to 86, further comprising at least one guiding means and/or at least one host cell.

Claims

1. A method for generating at least one deletion around at least one target nucleic acid sequence comprised within a host cell having a non-homologous end-joining (NHEJ) pathway which is at least partly deficient,
said method comprising the steps of:
(i) optionally, restoring the full functionality of the NHEJ pathway,
(ii) inducing a CRISPR-Cas9 system in said host cell, wherein said CRISPR-Cas9 system is able to generate at least one break in said at least one target nucleic acid sequence and wherein the CRISPR-Cas9 system comprises a Cas9 nuclease and at least one guiding means,
thereby generating:
a. if the method does not comprise step (i)., at least one random-sized deletion around said at least one target nucleic acid sequence, wherein said at least one deletion is a random-sized deletion of at least 1 bp; or
b. if the method does comprise step (i), at least one indel around said at least one target nucleic acid sequence, wherein said at least one indel is a deletion or insertion of at least1 bp.

2. The method of claim 1, wherein the host cell is an actinobacterium, such as an *Actinomycetales*, such as *Streptomyces sp., Amycolatopsis sp.* or *Saccharopolyspora sp,* such as wherein the host cell is selected from the group consisting of *Streptomyces coelicolor, Streptomyces avermitilis*, *Streptomyces aureofaciens*, *Streptomyces griseus, Streptomyces parvulus, Streptomyces albus, Streptomyces vinaceus, Streptomyces acrimycinis, Streptomyces calvuligerus, Streptomyces lividans, Streptomyces limosus, Streptomyces rubiqinosis, Streptomyces azureus, Streptomyces glaucenscens, Streptomyces rimosus, Streptomyces violaceoruber, Streptomyces kanamyceticus, Amycolatopsis orientalis, Amycolatopsis mediterranei* and *Saccharopolyspora erythraea.*

3. A polynucleotide having at least 94% identity with SEQ ID NO: 1, such as at least 95% identity, such as at least 96% identity, such as at least 97% iden-

tity, such as at least 98% identity, such as at least 99% identity, such as 100% identity, said polynucleotide encoding a Cas9 nuclease or a variant thereof.

4. The polynucleotide of claim 3, wherein the Cas9 nuclease or variant thereof is codon-optimised for Streptomycetes.

5. A polypeptide encoded by the polynucleotide of any one of claims 3 to 4.

6. A cell comprising the polynucleotide of claims 3 or 4.

7. A cell comprising the polypeptide of claim 5.

8. A vector comprising the polynucleotide of claims 3 or 4.

9. A clonal library obtainable by the method of any of claims 1 to 2, said clonal library comprising a plurality of clones harbouring at least one deletion and/or indel around at least one target nucleic acid sequence, wherein said deletion is a random-sized deletion of at least 1 bp and wherein said indel is a deletion or insertion of at least 1 bp.

10. A method for selectively modulating transcription of at least one target nucleic acid sequence in a host cell, the method comprising introducing into the host cell:

(i) at least one guiding means, or a nucleic acid comprising a nucleotide sequence encoding guiding means, wherein the guiding means comprises a nucleotide sequence that is complementary to a target nucleic acid sequence in the host cell; and

(ii) a variant Cas9, or a nucleic acid comprising a nucleotide sequence encoding the variant Cas9, wherein the variant Cas9 is a variant of the polypeptide of claim 5 or of a polypeptide encoded by the nucleotide sequence encoding the variant Cas9 of claims 3 or 4, with reduced endodeoxyribonuclease activity and is codon-optimised for Streptomycetes,

wherein said guiding means and said variant Cas9 form a complex in the host cell, said complex selectively modulating transcription of at least one target nucleic acid in the host cell.

11. The method of claim 10, wherein the host cell is an actinobacterium, preferably the host cell is selected from the group consisting of *Actinomycetales*, *Streptomyces sp., Amycolatopsis sp.* and *Saccharopolyspora sp,* even more preferably the host cell is selected from the group consisting of *Streptomyces coelicolor, Streptomyces avermitilis*, *Streptomyces aureofaciens*, *Streptomyces griseus, Streptomyces parvulus, Streptomyces albus, Streptomyces vinaceus, Streptomyces acrimycinis, Streptomyces calvuligerus, Streptomyces lividans, Streptomyces limosus, Streptomyces rubiqinosis, Streptomyces azureus, Streptomyces glaucenscens, Streptomyces rimosus, Streptomyces violaceoruber, Streptomyces kanamyceticus, Amycolatopsis orientalis, Amycolatopsis mediterranei* and *Saccharopolyspora erythraea.*

12. A kit for performing the method of any of claims 1 to 2, said kit comprising:
    - a vector comprising a nucleic acid sequence encoding a Cas9 nuclease or variant thereof; and
    - instructions for use.

13. A kit for performing the method of any of claims 10 to 11, said kit comprising:
    - a vector comprising a variant Cas9, or a nucleic acid comprising a nucleotide sequence encoding the variant Cas9, wherein the variant Cas9 is the polypeptide of claim 5 or the nucleotide sequence encoding the variant Cas9 is the polynucleotide of claims 3 or 4, and wherein the variant Cas9 has reduced endodeoxyribonuclease activity; and
    - instructions for use.

Figure 1

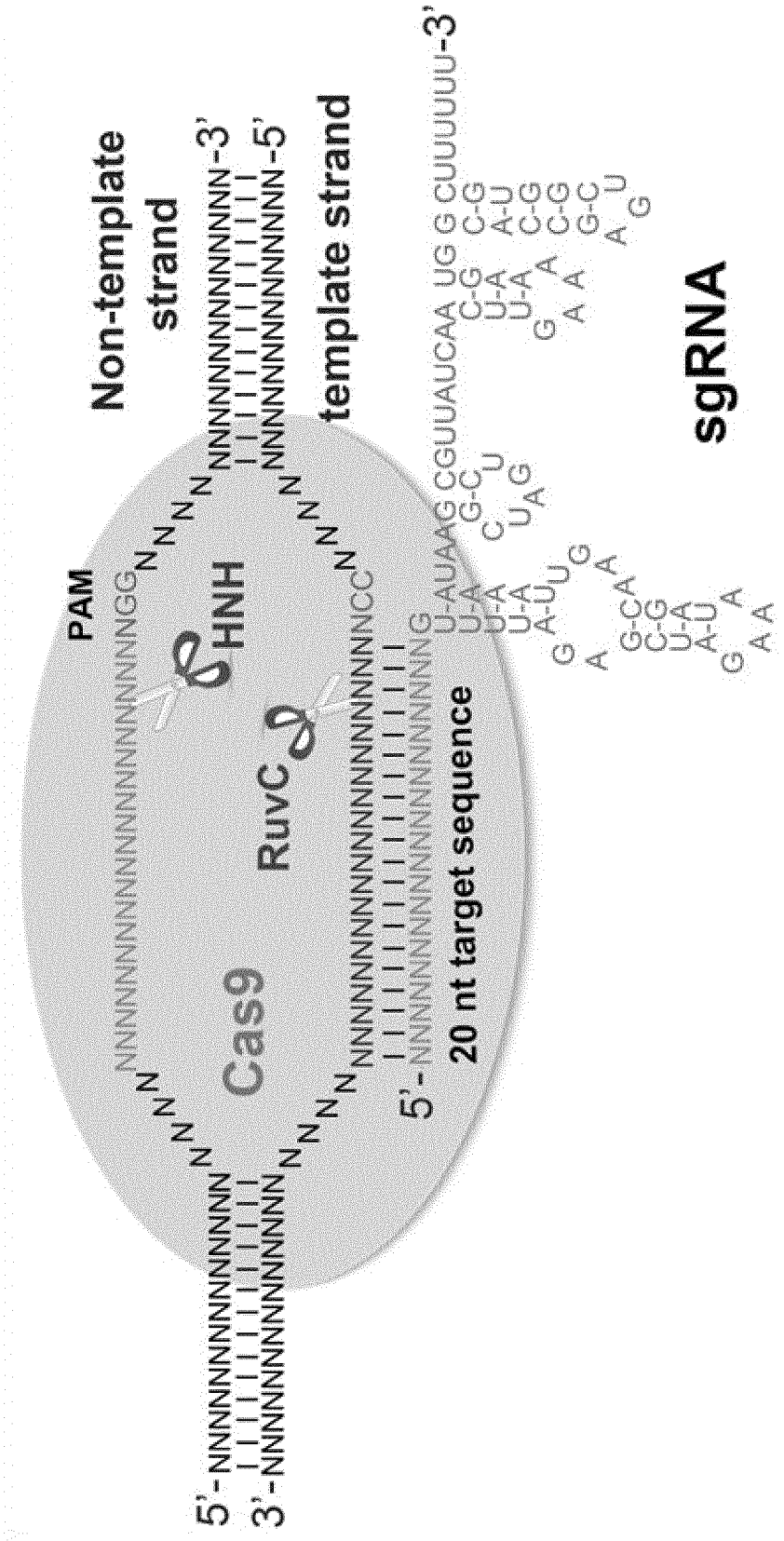

Non-template strand
template strand
PAM
HNH
RuvC
Cas9
sgRNA
20 nt target sequence
5'-
3'-
-3'
-5'

Figure 2

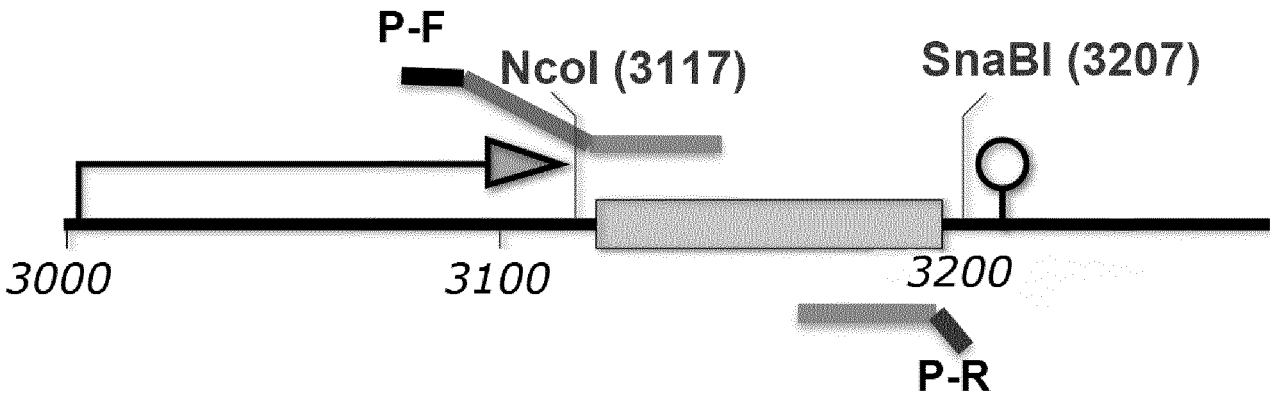


Figure 3

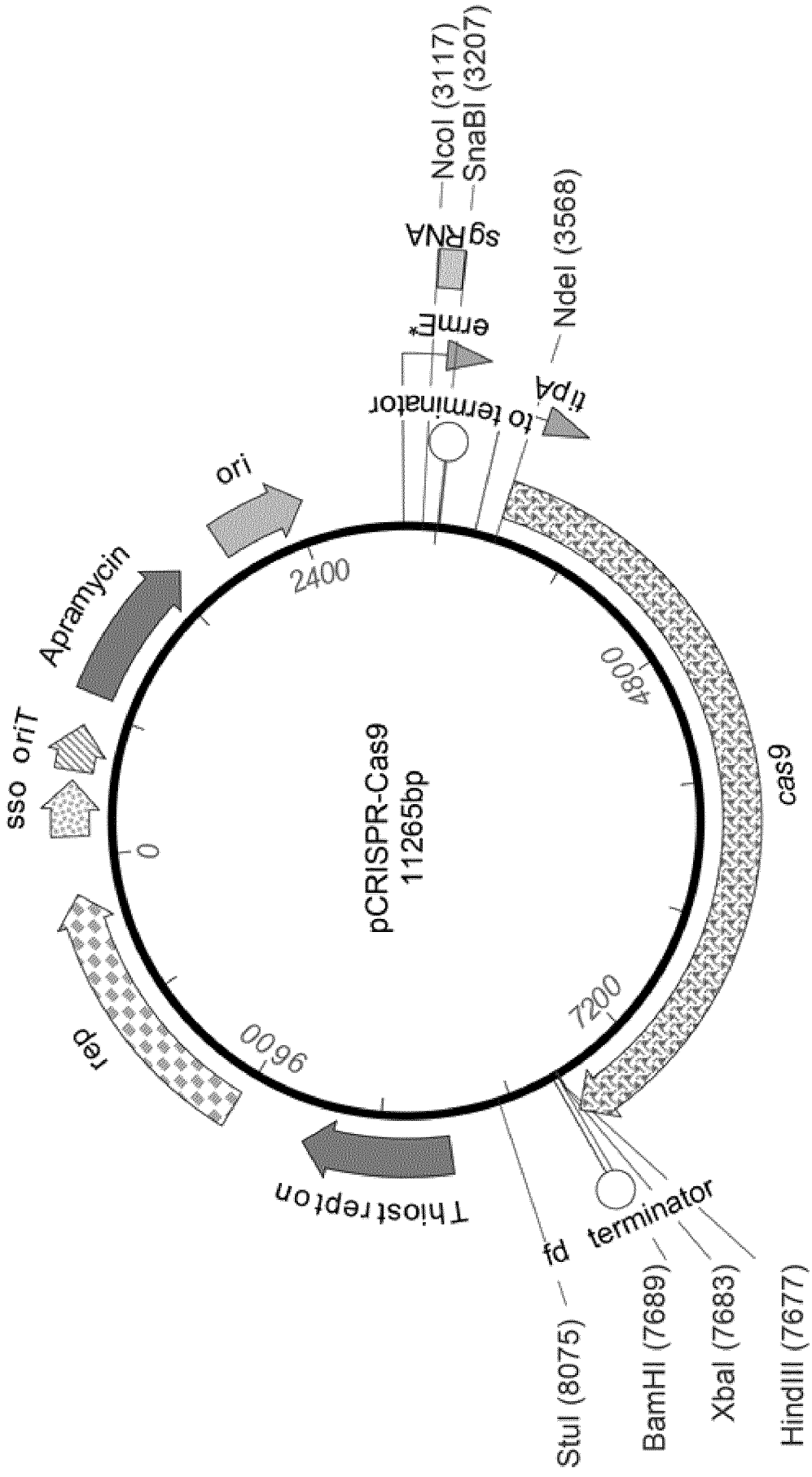

pCRISPR-Cas9
11265bp
0
2400
4800
7200
9600
cas9
NdeI (3568)
tipA
to terminator
ermE*
sgRNA
NcoI (3117)
SnaBI (3207)
ori
Apramycin
sso oriT
rep
Thiostrepton
fd terminator
StuI (8075)
BamHI (7689)
XbaI (7683)
HindIII (7677)

Figure 4

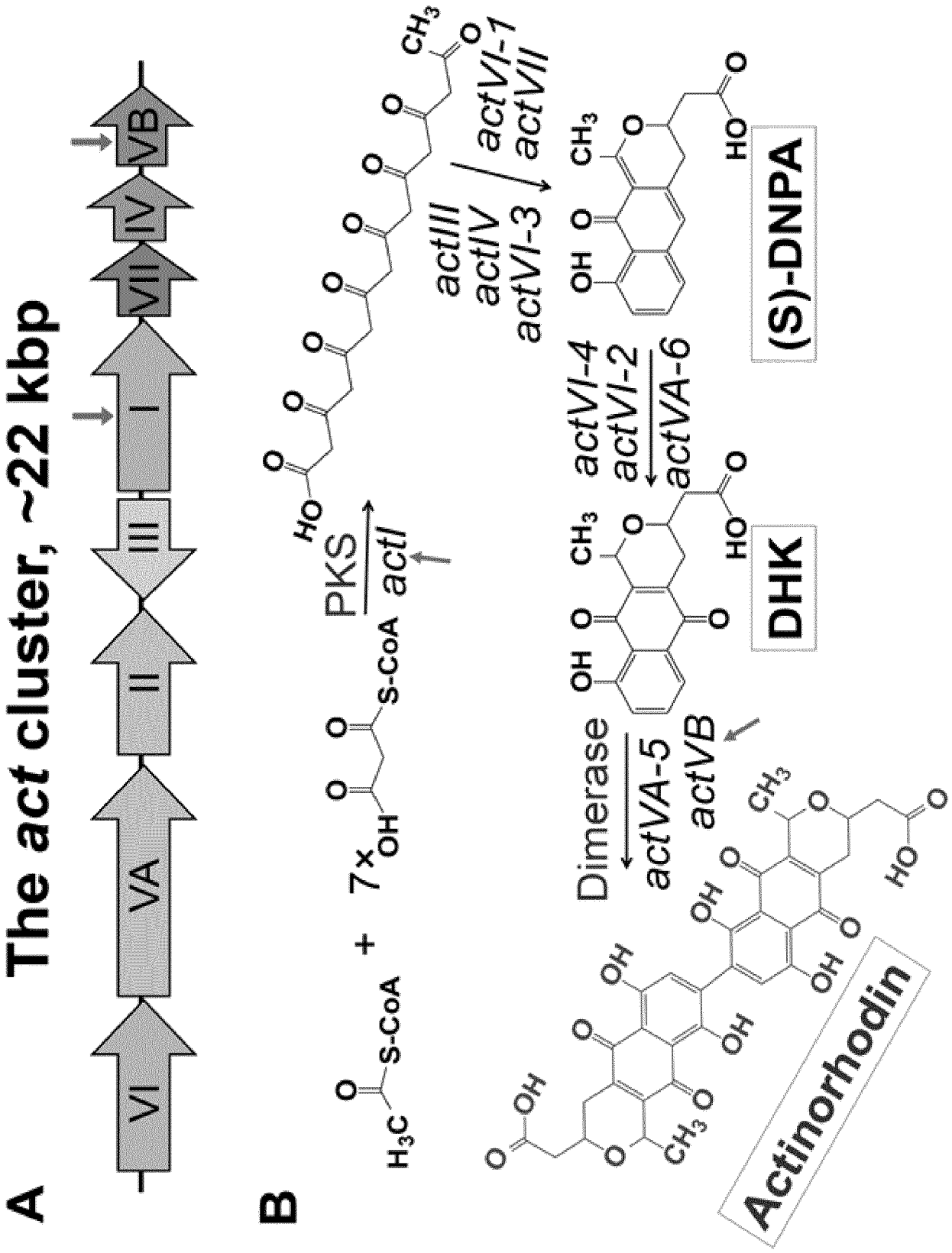

A
The act cluster, ~22 kbp
VI
VA
II
III
I
VII
IV
VB
B
PKS
actI
7x
S-CoA
actIII
actIV
actVI-3
actVI-1
actVII
actVI-4
actVI-2
actVA-6
(S)-DNPA
DHK
Dimerase
actVA-5
actVB
Actinorhodin

Figure 5

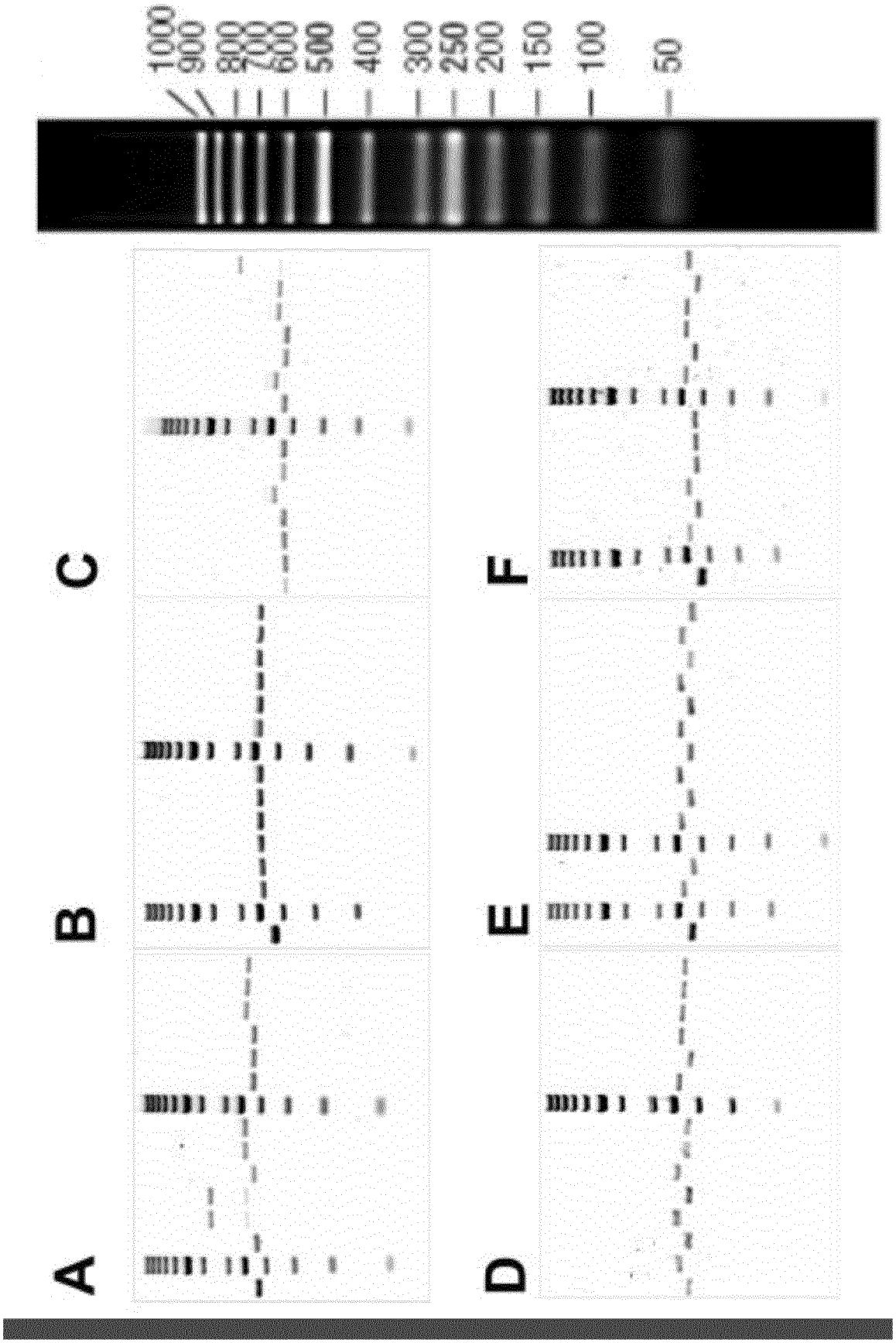


Figure 6

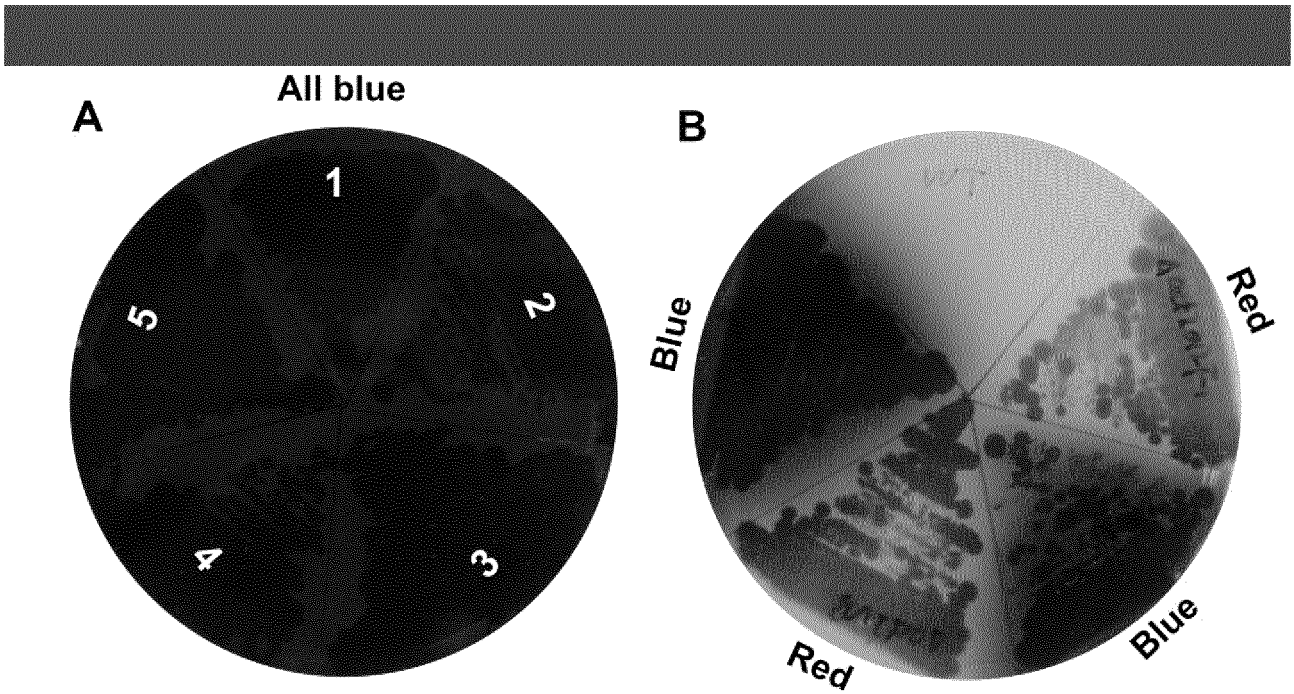


Figure 7

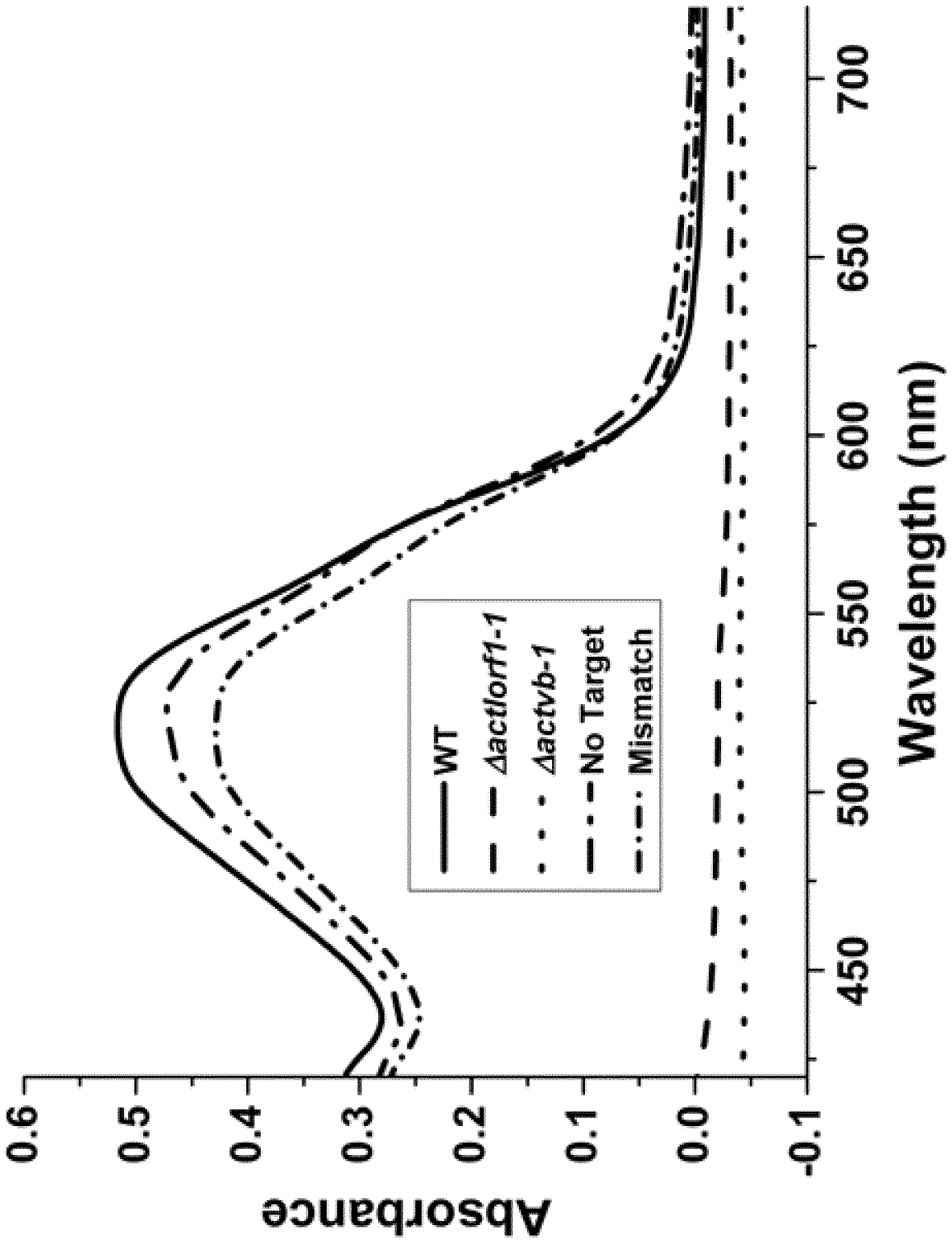

Absorbance
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
450
500
550
600
650
700
Wavelength (nm)
WT
Δactlorf1-1
Δactvb-1
No Target
Mismatch

Figure 8

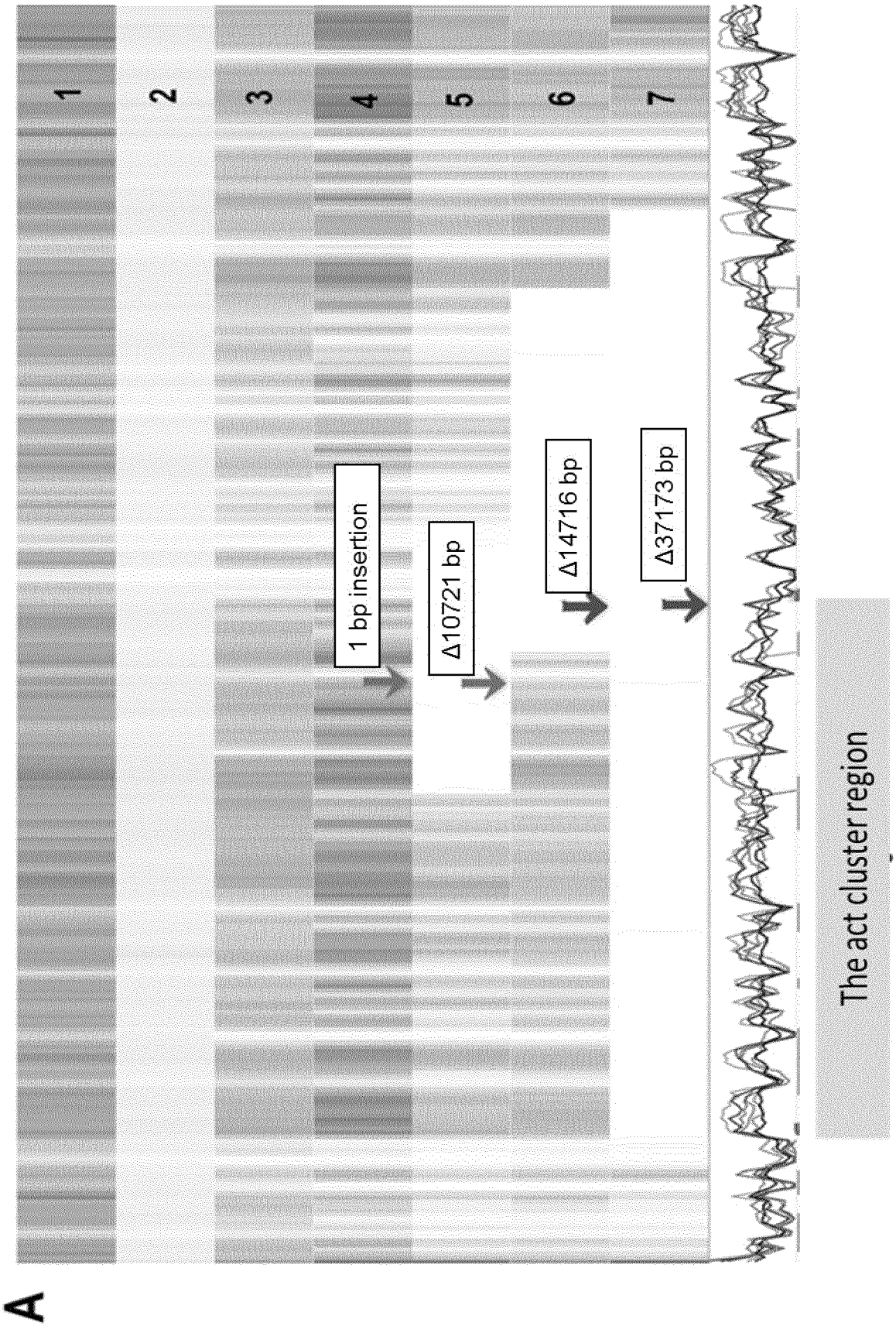

A
1
2
3
4
5
6
7
1 bp insertion
Δ10721 bp
Δ14716 bp
Δ37173 bp
The act cluster region

Figure 8 (CONT.)

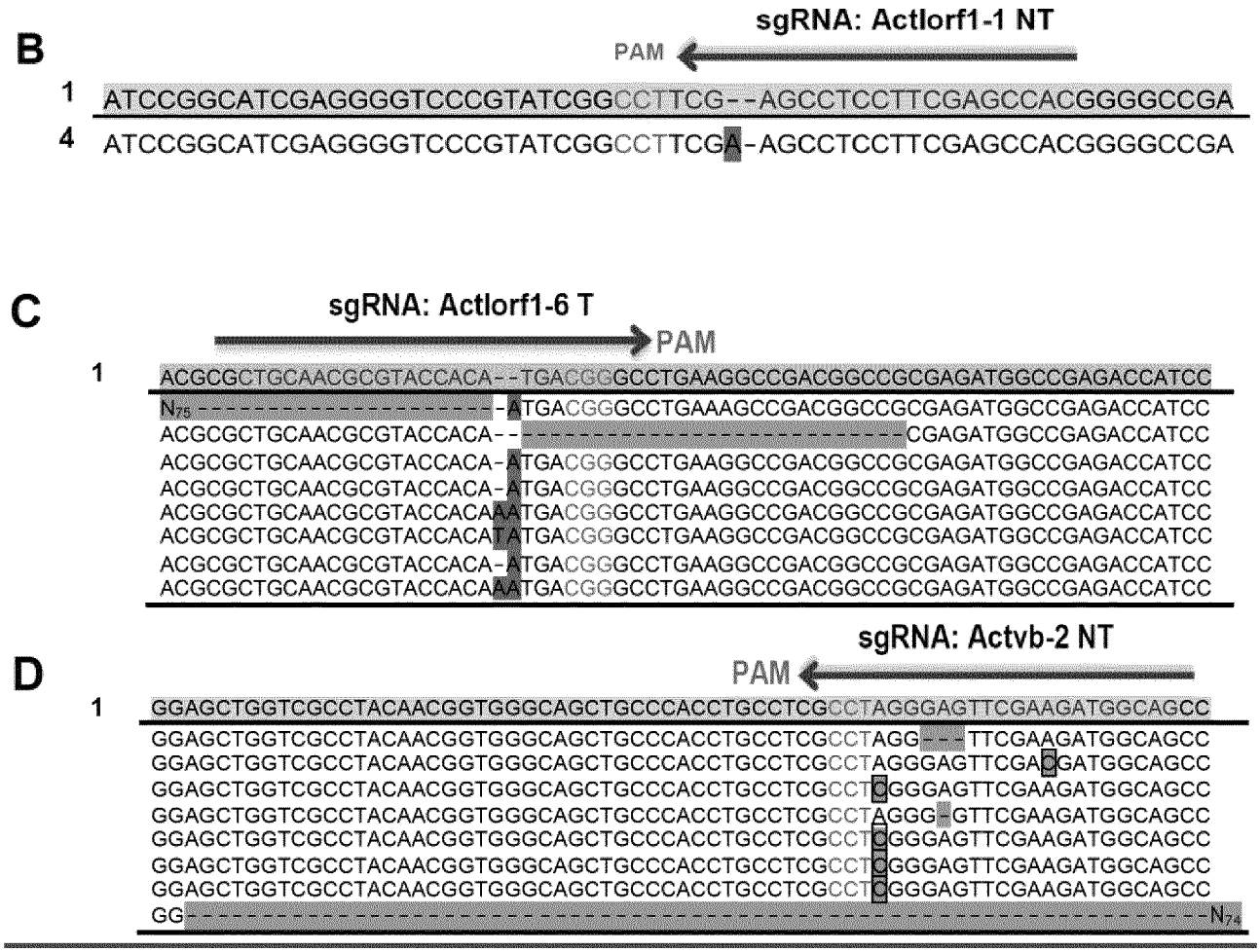


Figure 9

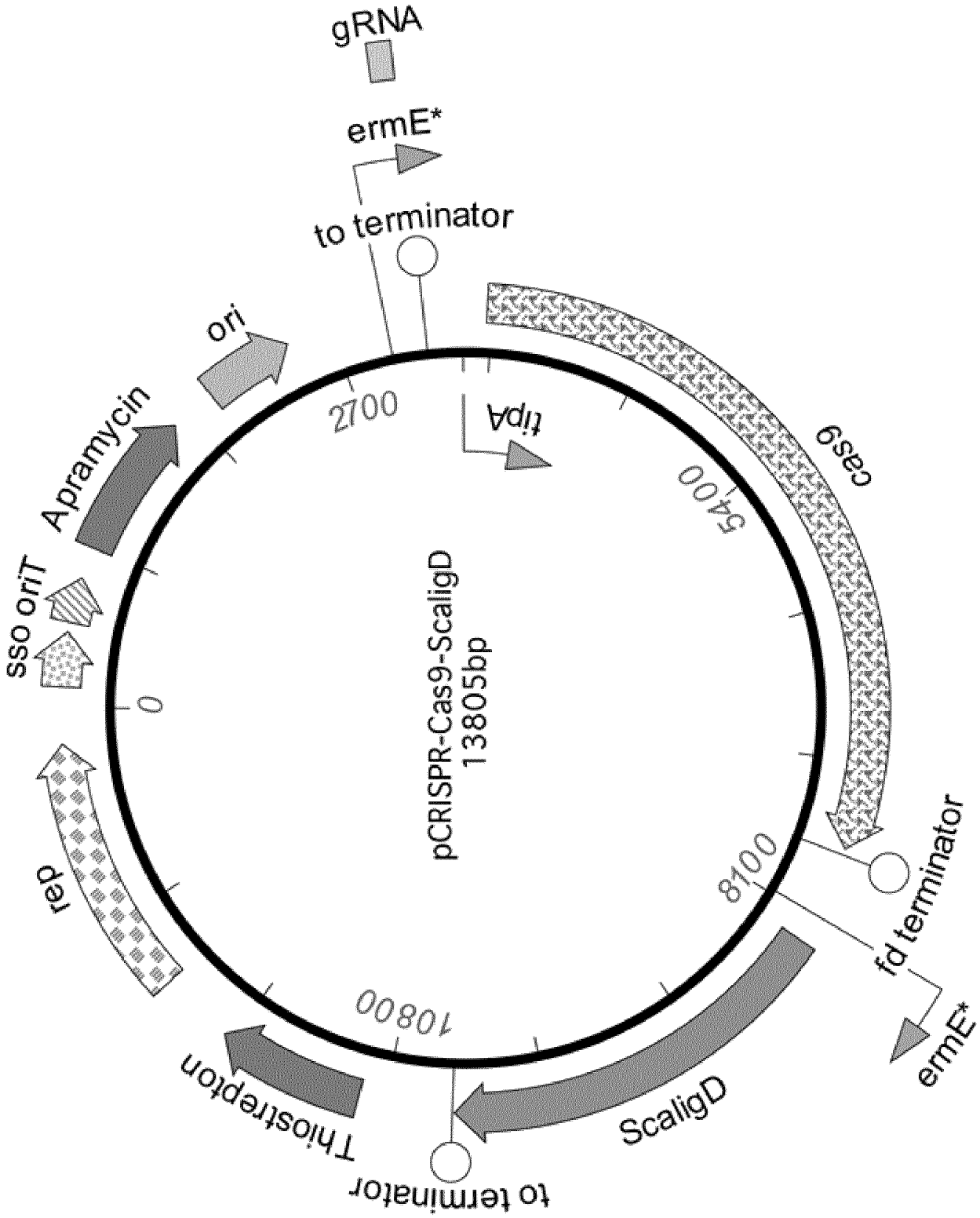

gRNA
ermE*
to terminator
ori
Apramycin
sso oriT
rep
Thiostreptom
to terminator
ScaligD
fd terminator
ermE*
cas9
tipA
pCRISPR-Cas9-ScaligD
13805bp
0
2700
5400
8100
10800

Figure 10

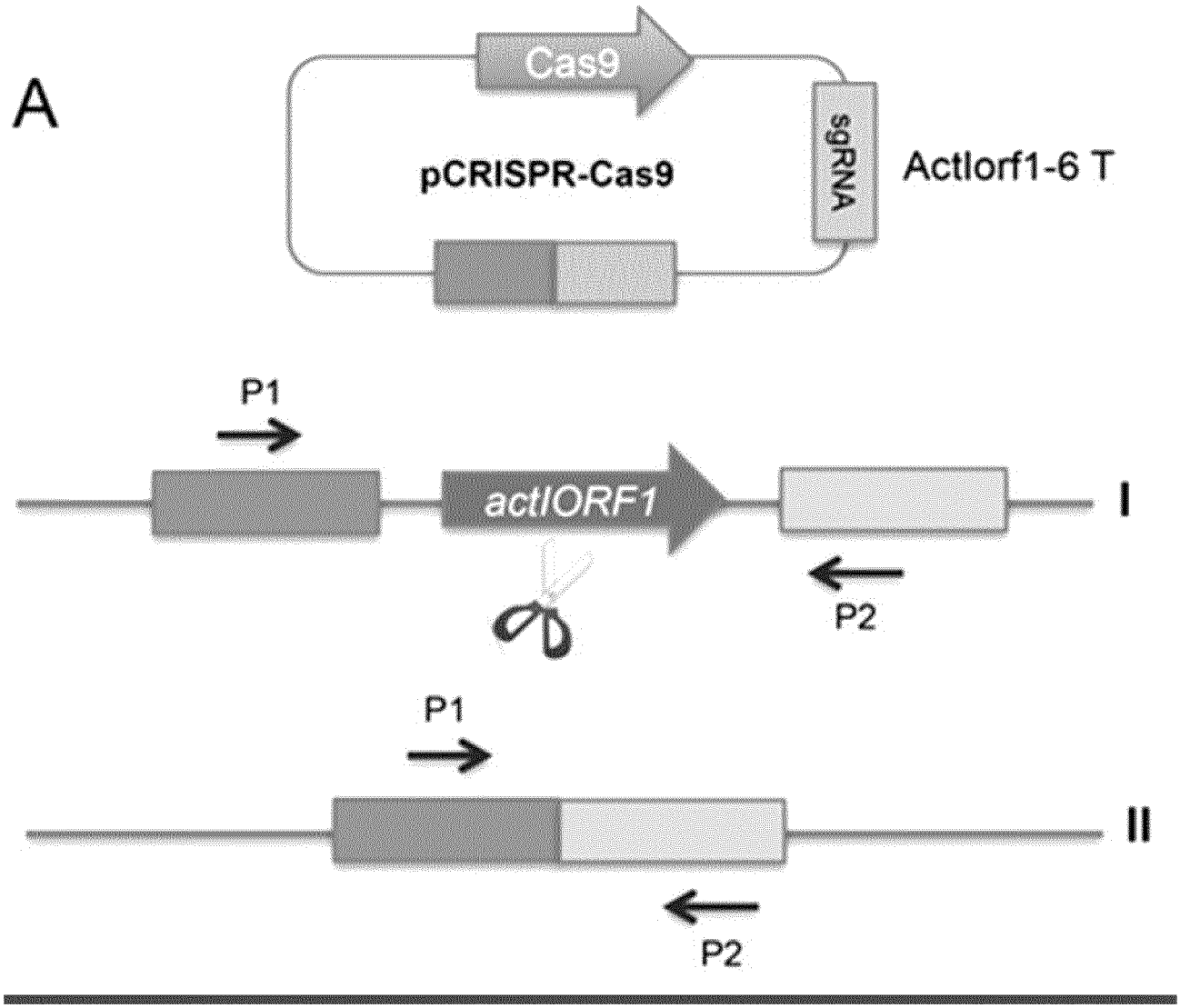

A
Cas9
pCRISPR-Cas9
sgRNA
Actlorf1-6 T
P1
actIORF1
I
P2
P1
II
P2

Figure 10 (CONT.)

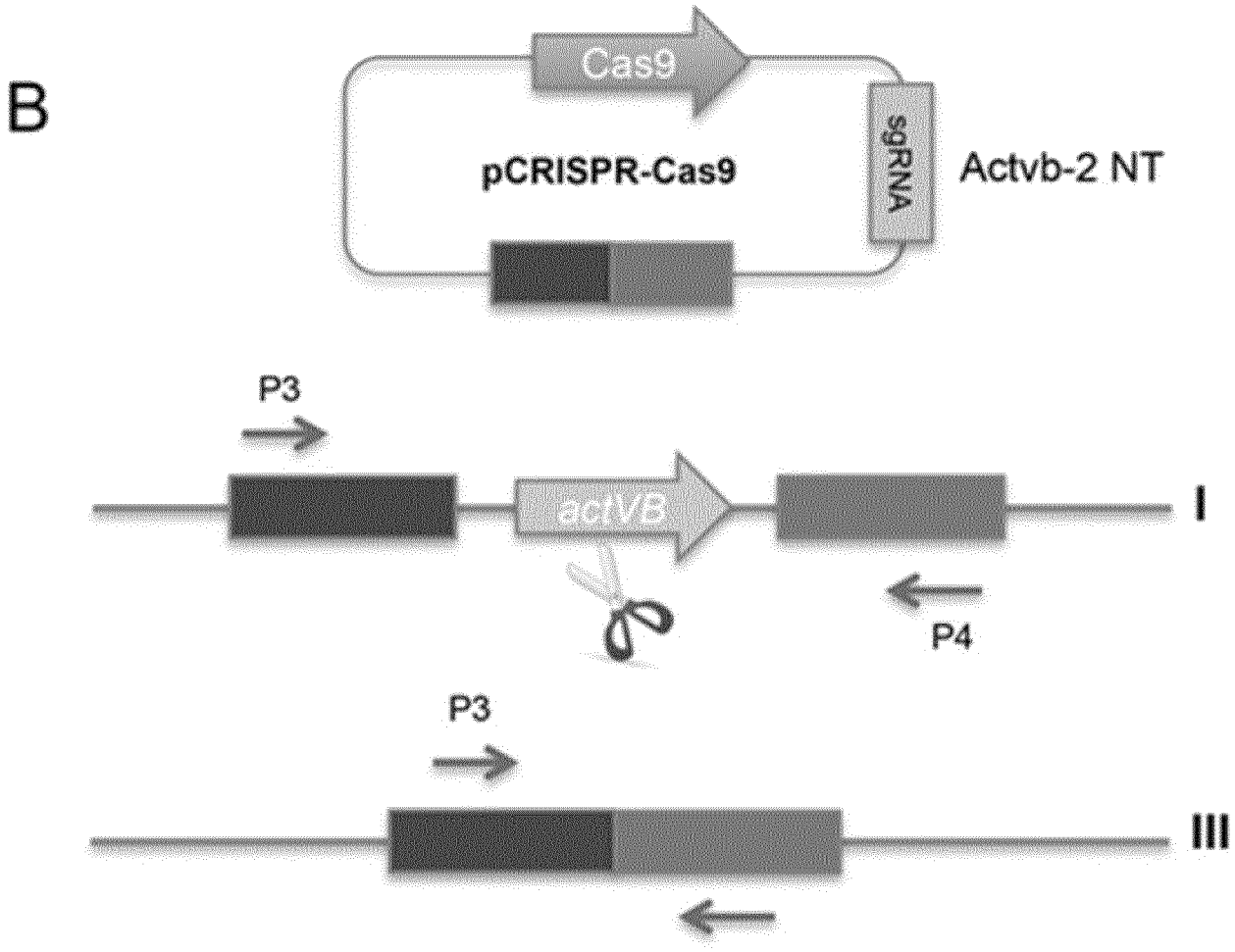


Figure 10 (CONT.)

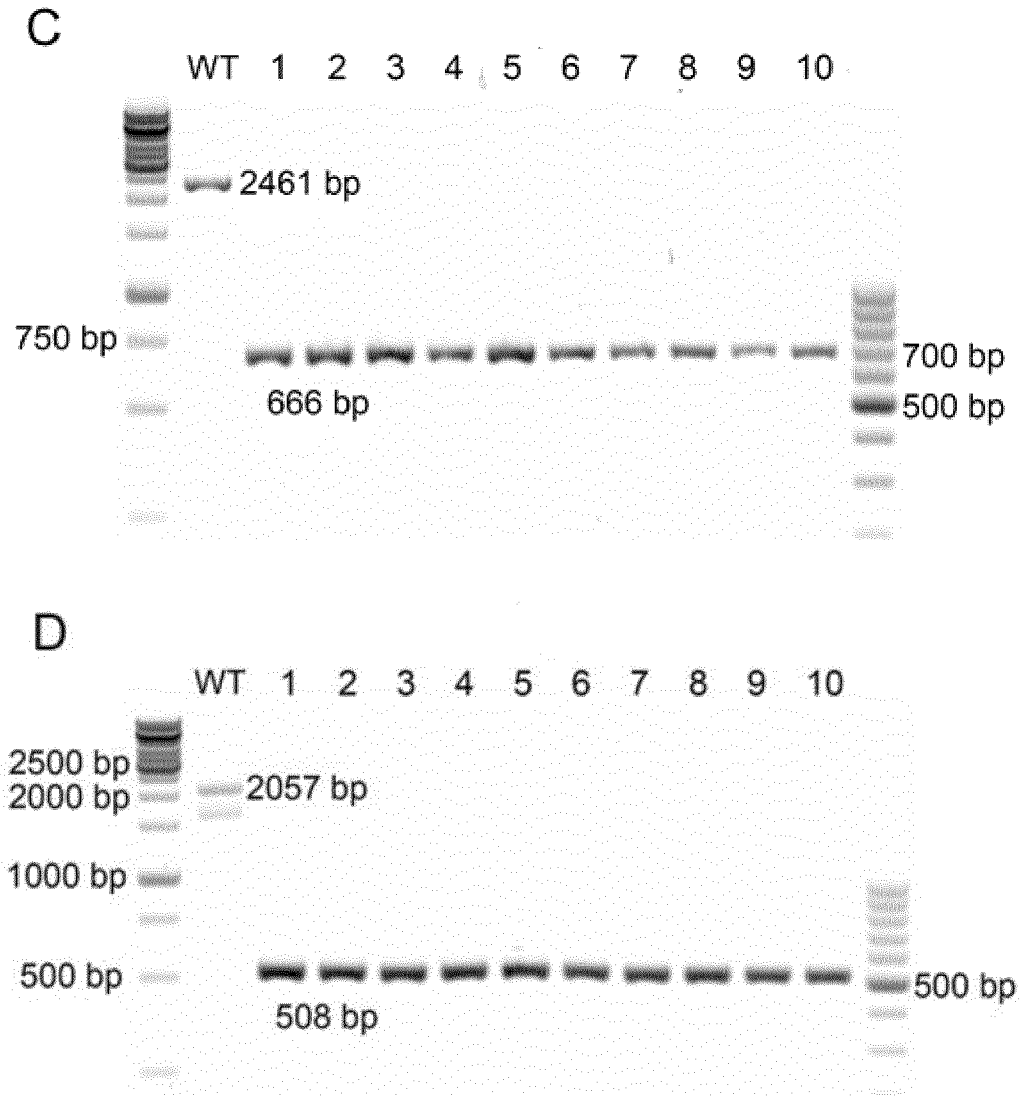


Figure 11

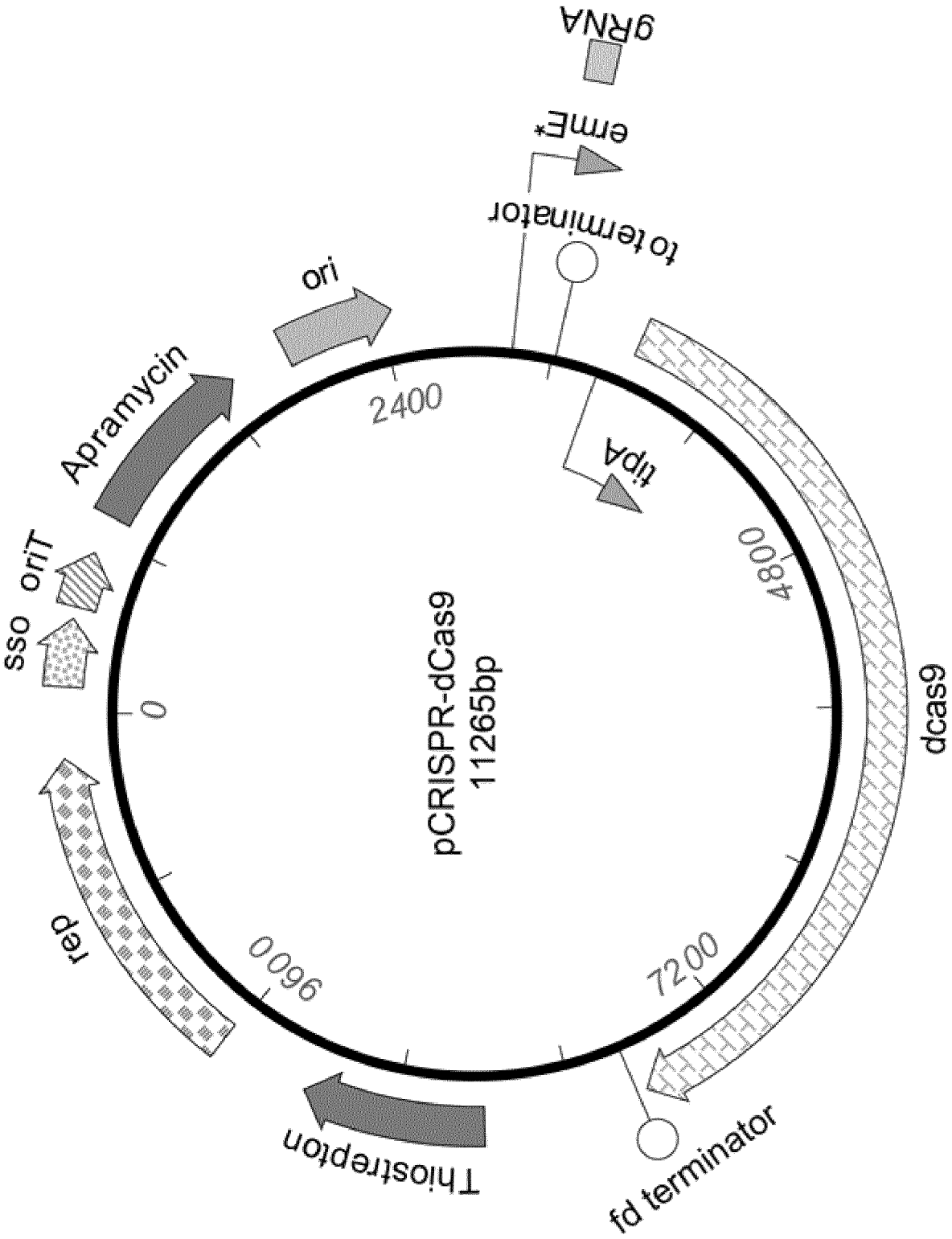

gRNA
ermE*
to terminator
ori
Apramycin
2400
tipA
4800
oriT
sso
pCRISPR-dCas9
11265bp
0
dcas9
rep
9600
7200
Thiostrepton
fd terminator

Figure 12

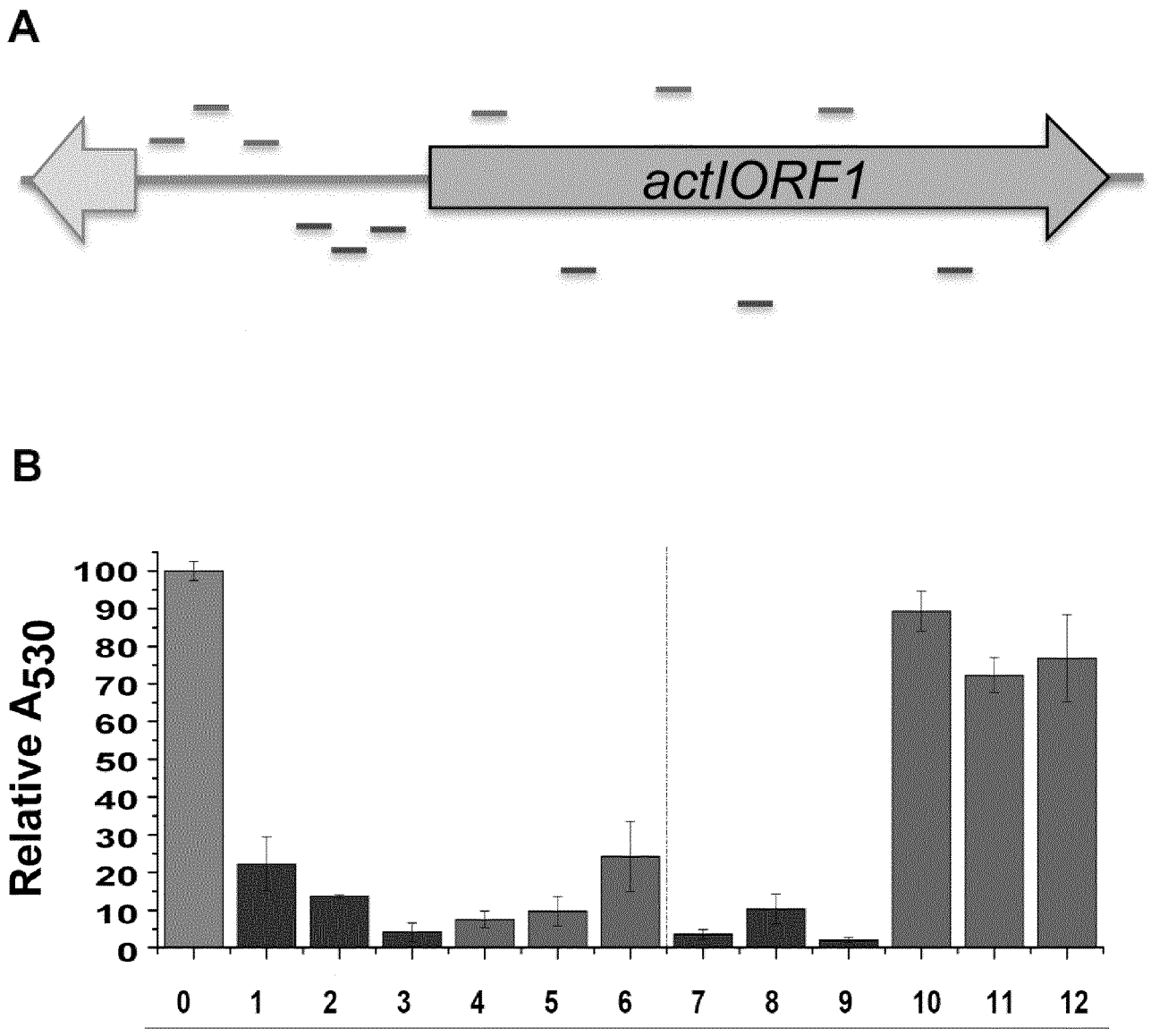
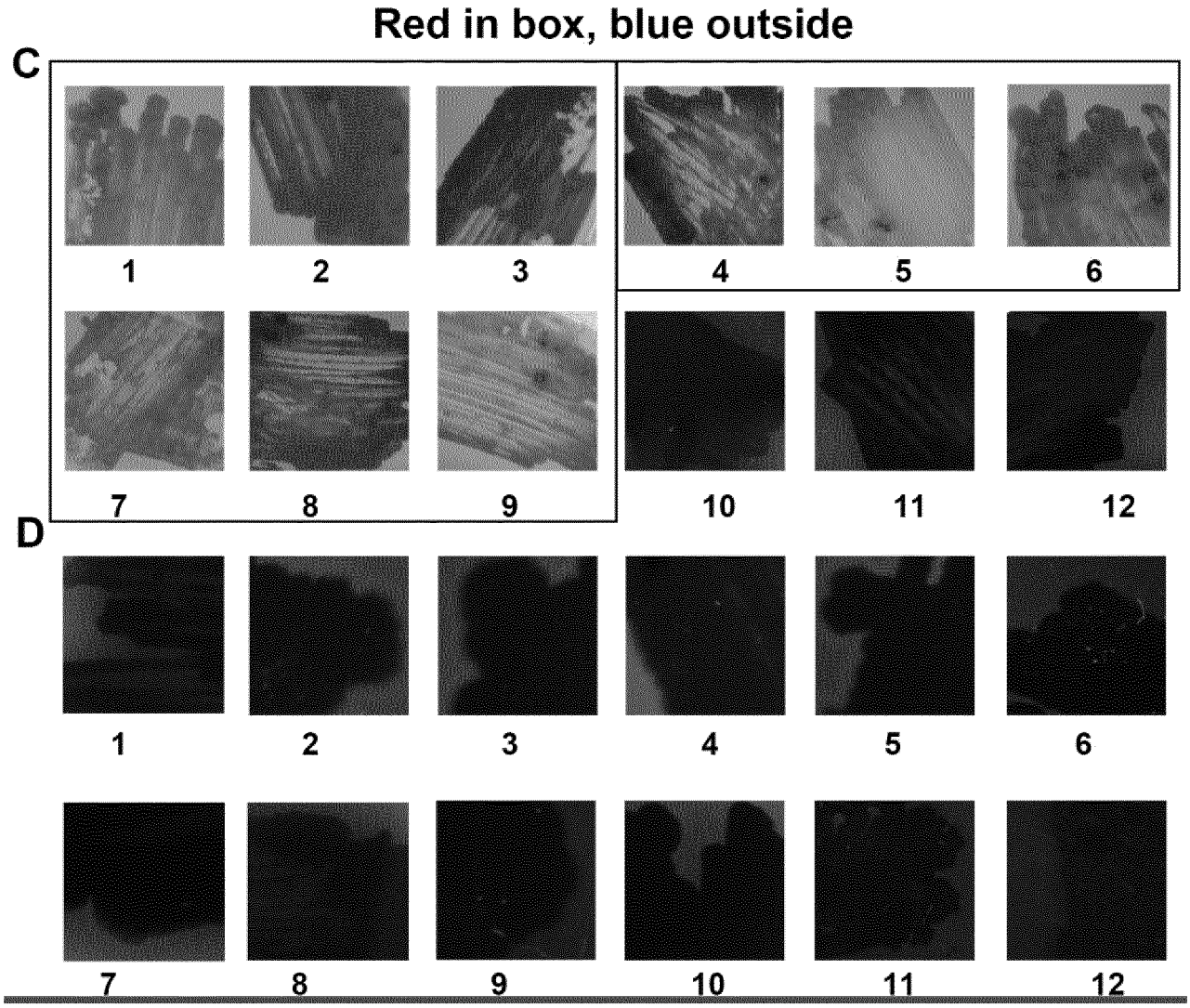

A
actIORF1
B
Relative A530
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
11
12

Figure 12 (CONT.)



INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/055967

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 2, 9, 12(all partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/055967

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/63 C12N15/90
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	H. HUANG ET AL: "One-step high-efficiency CRISPR/Cas9-mediated genome editing in Streptomyces", ACTA BIOCHIMICA ET BIOPHYSICA SINICA, vol. 47, no. 4, 3 March 2015 (2015-03-03), pages 231-243, XP055204421, ISSN: 1672-9145, DOI: 10.1093/abbs/gmv007 page 235, column 1, paragraph 1 - page 238, column 1, paragraph 1 ----- -/--	1,2,9,12

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 May 2016	03/08/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Burkhardt, Peter

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	XIAOJUAN ZHANG ET AL: "Deletion of homologs increases gene targeting frequency in", JOURNAL OF INDUSTRIAL MICROBIOLOGY & BIOTECHNOLOGY ; OFFICIAL JOURNAL OF THE SOCIETY FOR INDUSTRIAL MICROBIOLOGY, SPRINGER, BERLIN, DE, vol. 39, no. 6, 21 February 2012 (2012-02-21), pages 917-925, XP035060031, ISSN: 1476-5535, DOI: 10.1007/S10295-012-1097-X page 919, column 2, paragraph 3 - page 923, column 2, paragraph 1	1,2,9,12
Y	US 2015/079680 A1 (BRADLEY ALLAN [GB] ET AL) 19 March 2015 (2015-03-19) paragraph [0010] - paragraph [0011]	1,2,9,12
A	THERESA SIEGL ET AL: "I-SceI endonuclease: a new tool for DNA repair studies and genetic manipulations in streptomycetes", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 87, no. 4, 15 May 2010 (2010-05-15), pages 1525-1532, XP019841641, ISSN: 1432-0614 page 1531, column 2, paragraph 1	1,2,9,12
A	WO 2014/065596 A1 (TOOLGEN INC [KR]) 1 May 2014 (2014-05-01) paragraph [0166]; claims; sequences 1,108 & DATABASE Geneseq [Online] 3 July 2014 (2014-07-03), "Streptococcus pyogens DNA encoding the Cas9 protein, SEQ 1.", retrieved from EBI accession no. GSN:BBF83954 Database accession no. BBF83954 sequence & DATABASE Geneseq [Online] 3 July 2014 (2014-07-03), "Streptococcus pyogens DNA encoding the Cas9 protein , SEQ 108.", retrieved from EBI accession no. GSN:BBF84061 Database accession no. BBF84061 sequence	1,2,9,12
A	WO 2015/013583 A2 (HARVARD COLLEGE [US]) 29 January 2015 (2015-01-29) sequence 22	1,2,9,12

-/--

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& DATABASE Geneseq [Online] 26 March 2015 (2015-03-26), "Streptococcus pyogenes cas9 protein, SEQ ID 22.", retrieved from EBI accession no. GSP:BBU25305 Database accession no. BBU25305 sequence ----- YAOJUN TONG ET AL: "CRISPR-Cas9 Based Engineering of Actinomycetal Genomes", ACS SYNTHETIC BIOLOGY, 25 March 2015 (2015-03-25), pages A-J, XP055204040, ISSN: 2161-5063, DOI: 10.1021/acssynbio.5b00038 & DATABASE EMBL [Online] 6 April 2015 (2015-04-06), "Actinomyces vector pCRISPR-Cas9, complete sequence.", retrieved from EBI accession no. EM_STD:KR011749 Database accession no. KR011749 sequence & DATABASE EMBL [Online] 6 April 2015 (2015-04-06), "Synthetic construct nuclease deficient Cas9 (dcas9) gene, complete cds.", retrieved from EBI accession no. EM_STD:KR011748 Database accession no. KR011748 sequence -----	
T		1,2,9,12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/055967

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2015079680 A1	19-03-2015	CN 105637087 A	01-06-2016
		DE 202014010413 U1	08-12-2015
		EP 2877571 A1	03-06-2015
		TW 201542816 A	16-11-2015
		US 2015079680 A1	19-03-2015
		US 2016177340 A1	23-06-2016
		US 2016207983 A1	21-07-2016
		WO 2015040402 A1	26-03-2015
WO 2014065596 A1	01-05-2014	AU 2013335451 A1	07-05-2015
		AU 2015218519 A1	17-09-2015
		CA 2888190 A1	01-05-2014
		CN 104968784 A	07-10-2015
		CN 105441440 A	30-03-2016
		EP 2912175 A1	02-09-2015
		HK 1212732 A1	17-06-2016
		JP 2016027807 A	25-02-2016
		JP 2016500003 A	07-01-2016
		KR 20150101446 A	03-09-2015
		KR 20150101476 A	03-09-2015
		KR 20150101477 A	03-09-2015
		KR 20150101478 A	03-09-2015
		SG 11201503059X A	29-06-2015
		US 2015284727 A1	08-10-2015
		US 2015322457 A1	12-11-2015
		US 2015344912 A1	03-12-2015
		WO 2014065596 A1	01-05-2014
WO 2015013583 A2	29-01-2015	AU 2014293015 A1	11-02-2016
		CA 2918540 A1	29-01-2015
		CN 105473773 A	06-04-2016
		EP 3024964 A2	01-06-2016
		KR 20160035017 A	30-03-2016
		SG 11201600217Q A	26-02-2016
		US 2015031132 A1	29-01-2015
		US 2015031133 A1	29-01-2015
		WO 2015013583 A2	29-01-2015

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 2, 9, 12(all partially)

   relating to a CRISPR-CAS9 based method for generating at least one deletion around at least one target nucleic acid sequence in a host cell having an at least partly deficient homologous end joining (NHEJ) pathway

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2. claims: 1, 2, 9, 12(all partially)

   relating to a CRISPR-CAS9 based method for generating at least one deletion around at least one target nucleic acid sequence in a host cell having an at least partly deficient NHEJ pathway and wherein the full functionality of the NHEJ pathway is restored

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3. claims: 3-8

   relating to the polynucleotide of SEQ ID NO:1 and products comprising said polynucleotide

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4. claims: 10, 11, 13

   relating to a method for selectively modulating transcription of at least one target nucleic acid sequence in a host cell by introducing at least one guiding means and a variant CAS9 that has reduced endodeoxyribonuclease activity

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