Trends Biotechnol. 34, 575–587 (2016)). These systems are among the simplest CRISPR-Cas systems known as they introduce targeted double stranded DNA breaks (DSBs) based on a ribonucleoprotein (RNP) complex formed by a single Cas endonuclease and an RNA guide.

To date, *Streptococcus pyogenes* Cas9 (SpCas9) is the best characterized and most widely employed Cas9 for genome engineering. Although a few other type-II systems have been characterized, none of them is derived from a thermophilic organism (Nakade, *et al*. Bioengineered 1–9 (2017). doi:10.1080/21655979.2017.1282018). Characterization of such CRISPR-Cas systems would be interesting to gain fundamental insights as well as to develop novel applications.

Although basic genetic tools are available for a number of thermophiles (Taylor *et al*. Microb. Biotechnol. 4, 438–448 (2011); Olson, et al. Curr. Opin. Biotechnol. 33, 130–141 (2015); Zeldes, *et al*. Front. Microbiol. 6, 1209 (2015)), the efficiency of these tools is still too low to enable full exploration and exploitation of this interesting group of organisms. Based on our finding that SpCas9 is not active *in vivo* ≥42°C, we have previously developed an SpCas9-based engineering tool for facultative thermophiles, combining homologous recombination at elevated temperatures and SpCas9-based counter-selection at moderate temperatures (Mougiakos *et al. ACS Synth. Biol.* 6, 849–861 (2017)). However, a Cas9-based editing and silencing tool for obligate thermophiles is not yet available as SpCas9 is not active at or above 42°C (Mougiakos *et al. ACS Synth. Biol.* 6, 849–861 (2017)) and to date no thermophilic Cas9 has been characterized.

SUMMARY

The inventors have discovered and characterised ThermoCas9: an RNA-guided DNA-endonuclease from the CRISPR-Cas type-IIC system of the thermophilic bacterium *Geobacillus thermodenitrificans* T12. The inventors have surprisingly shown its *in vitro* activity over a broad temperature range, demonstrated the importance of the sgRNA-structure for thermostability and applied ThermoCas9 for *in vivo* genome editing across a wide temperature range.

In one aspect, the present dicclosure refers to an *in vitro* method of modifying the genetic material of a eukaryotic cell, comprising (i) introducing a polynucleotide encoding a ThermoCas9 under the control of a first promoter into the genome of the cell, wherein the expressed ThermoCas9 comprises an amino acid sequence of SEQ ID NO: 1 or a sequence of at least 89% identity therewith; (ii) introducing into the cell an expression vector comprising a polynucleotide sequence encoding a guide RNA and under the control of a second promoter, wherein the guide RNA has a nucleic acid sequence which recognizes a nucleic acid sequence comprised at a desired target locus in the genome of the cell, and (iii) introducing into the cell an homologous recombination (HR) oligonucleotide.

In another aspect the present disclosure refers to an *in vitro* method of modifying the genetic material of a eukaryotic cell, comprising (i) introducing a polynucleotide encoding a ThermoCas9 under the control of a first promoter into the cell, wherein the expressed ThermoCas9 comprises an amino acid sequence of SEQ ID NO: 1 or a sequence of at least 89% identity therewith; and (ii) introducing into the cell an expression vector comprising a polynucleotide sequence encoding a guide RNA under the control of a separate first or a second promoter, wherein the guide RNA has a nucleic acid sequence which recognizes a nucleic acid sequence comprised at a desired target locus in the genome of the cell, and an HR oligonucleotide also under the control of the first or the second promoter, or a separate third promoter.

Accordingly, in one example, the present disclosure provides a method of modifying the genetic material of a eukaryotic cell, comprising (i) integrating a polynucleotide encoding a ThermoCas9 under the control of a first promoter into the genome of the cell, wherein the expressed ThermoCas9 comprises an amino acid sequence of SEQ ID NO: 1 or a sequence of at least 77% identity

a) a polynucleotide sequence encoding a ThermoCas9 nuclease, wherein the ThermoCas9 nuclease comprises an amino acid sequence of SEQ ID NO: 1 or a sequence of at least 89% identity therewith;

b) a polynucleotide sequence encoding a guide RNA, wherein the guide RNA has a nucleic acid sequence which recognizes a nucleic acid sequence comprised in the target locus;

c) a first promoter oriented with respect to polynucleotide sequences of (a) and (b) so as to drive expression thereof in the organism; and

d) a polynucleotide sequence which encodes an homologous recombination (HR) oligonucleotide under the control of the first promoter or a separate second promoter.

The present disclosure also provides a polynucleotide expression vector for modifying the genetic material at a target locus of a prokaryotic host organism which comprises the expression vector, the vector comprising:

a second expression vector comprising a polynucleotide sequence which encodes an homologous recombination (HR) fragment under the control of a promoter.

In another aspect, the present disclosure refers to an *in vitro* method of modifying the genetic material of an organism comprising introducing a first and second expression vector into the organism, the first expression vector being a vector comprising: a) a polynucleotide sequence encoding a ThermoCas9 nuclease, wherein the ThermoCas9 nuclease comprises an amino acid sequence of SEQ ID NO: 1 or a sequence of at least 89% identity therewith; b) a polynucleotide sequence encoding a guide RNA, wherein the guide RNA has a nucleic acid sequence which recognizes a nucleic acid sequence comprised in the target locus; and c) a first promoter oriented with respect to polynucleotide sequences of (a) and (b) so as to drive expression thereof in the organism; and a second expression vector comprising a polynucleotide sequence which encodes an homologous recombination (HR) oligonucleotide under the control of a promoter.

In yet another aspect, the present disclosure refers to an *in vitro* method of modifying the genetic material of a prokaryotic organism comprising introducing an expression vector as disclosed herein into the organism.

In yet another aspect, the present disclosure refers to a prokaryotic cell transfected, transformed or transduced with an expression vector as disclosed herein.

In another example, the disclosure provides a method of modifying the genetic material of a prokaryotic organism comprising transforming the organism with a single expression vector comprising (a), (b), and (c) above and also a polynucleotide sequence which encodes an homologous recombination (HR) fragment under the control of the said first promoter or a separate second promoter.

The organism may be transformed e.g. using electroporation.

In methods of the disclosure, the transformed organism is cultured for a period at a first temperature, and then cultured at a second temperature before or during induction of the promoter of the Cas9 sequence. Advantageously to improve the recombination efficiency, an incubation step may be carried out at the elevated temperature, at which the plasmid cannot replicate.

The first temperature may be 60 °C or less and the second temperature is a higher temperature which may be at least 55 °C. Alternativaely, the first temperature may be 55 °C or less and the second temperature may be greater than 55 °C. The different temperatures to be used may readily be selected by a person of average skill in the art, depending on the particular prokaryotic organism being modified, particularly a thermophilic bacterium.

CLAIMS

1. An *in vitro* method of modifying the genetic material of a eukaryotic cell, comprising (i) introducing a polynucleotide encoding a ThermoCas9 under the control of a first promoter into the genome of the cell, wherein the expressed ThermoCas9 comprises an amino acid sequence of SEQ ID NO: 1 or a sequence of at least 89% identity therewith; (ii) introducing into the cell an expression vector comprising a polynucleotide sequence encoding a guide RNA and under the control of a second promoter, wherein the guide RNA has a nucleic acid sequence which recognizes a nucleic acid sequence comprised at a desired target locus in the genome of the cell, and (iii) introducing into the cell an homologous recombination (HR) oligonucleotide.

2. An *in vitro* method of modifying the genetic material of a eukaryotic cell, comprising (i) introducing a polynucleotide encoding a ThermoCas9 under the control of a first promoter into the cell, wherein the expressed ThermoCas9 comprises an amino acid sequence of SEQ ID NO: 1 or a sequence of at least 89% identity therewith; and (ii) introducing into the cell an expression vector comprising a polynucleotide sequence encoding a guide RNA under the control of a separate first or a second promoter, wherein the guide RNA has a nucleic acid sequence which recognizes a nucleic acid sequence comprised at a desired target locus in the genome of the cell, and an HR oligonucleotide also under the control of the first or the second promoter, or a separate third promoter.

3. An *in vitro* method as claimed in claim 1 or claim 2, wherein the HR oligonucleotide is a double-stranded DNA HR oligonucleotide; optionally comprising a polynucleotide sequence for insertion into the genome of the cell by way of homologous recombination following guide RNA directed ThermoCas9 endonuclease cutting; preferably wherein:

   (a) the first promoter is a constitutive promoter; and/or

   (b) the first promoter is an inducible promoter; and/or

   (c) the second promoter is a constitutive or inducible promoter; and/or

   (d) wherein the third promoter is a constitutive or inducible promoter.

4. An *in vitro* method as claimed in claim 1 or claim 2, wherein the expression vector and/or the HR oligonucleotide are introduced into the cell by heat-shock; and/or wherein the cell is kept and/or grown at a temperature in the range 26 °C - 60 °C; preferably 31°C – 60 °C; more preferably 35 °C – 60 °C; even more preferably 34 °C – 41 °C, e.g. 37 °C.

5. A polynucleotide expression vector for modifying the genetic material at a target locus of a host organism which comprises the expression vector, the vector comprising:

    a) a polynucleotide sequence encoding a ThermoCas9 nuclease, wherein the ThermoCas9 nuclease comprises an amino acid sequence of SEQ ID NO: 1 or a sequence of at least 89% identity therewith;
    b) a polynucleotide sequence encoding a guide RNA, wherein the guide RNA has a nucleic acid sequence which recognizes a nucleic acid sequence comprised in the target locus;
    c) a first promoter oriented with respect to polynucleotide sequences of (a) and (b) so as to drive expression thereof in the organism; and
    d) a polynucleotide sequence which encodes an homologous recombination (HR) oligonucleotide under the control of the first promoter or a separate second promoter.

6. A vector as claimed in claim 5, wherein the sequence of (a) is 3' of the promoter and the sequence of (b) is 3' of the sequence of (a).

7. A vector as claimed in claim 5 or claim 6, wherein the first promoter is an inducible promoter; optionally wherein the inducible promoter is selected from a β-glucosidase promoter inducible with cellobiose or a Pm promoter inducible with 3-methylbenzoate.

8. A vector as claimed in any of claims 5 to 7, wherein the second promoter controlling the HR oligonucleotide is a constitutive promoter; optionally wherein the constitutive promoter is P3.

9. A vector as claimed in any of claims 5 to 8, wherein the arms of the HR oligonucleotide comprise a nucleic acid sequence which allowing recombination upstream and downstream, respectively, of a target locus in the host organism; preferably wherein the target locus comprises the target sequence; more preferably the target locus is a gene.

10. A vector as claimed in any of claims 5 to 9, wherein the HR oligonucleotide also comprises an insertion element between the upstream and downstream arms thereof; preferably wherein the insertion element is a gene of interest; optionally with a suitable promoter in operational orientation to provide for expression of the gene of interest in the host organism.

11. A vector as claimed in claim 9 or claim 10, wherein the target of interest comprises the PAM sequence 5'-NNNNCNN-3' located 3' of the target sequence; optionally at least 2, 3, 4, 5, 6 or more nucleotides from the target sequence.

12. A vector as claimed in any of claims 5 to 11, wherein the guide RNA is a single guide RNA (sgRNA).

13. An *in vitro* method of modifying the genetic material of an organism comprising introducing a first and second expression vector into the organism, the first expression vector being a vector comprising:

    a) a polynucleotide sequence encoding a ThermoCas9 nuclease, wherein the ThermoCas9 nuclease comprises an amino acid sequence of SEQ ID NO: 1 or a sequence of at least 89% identity therewith;
    b) a polynucleotide sequence encoding a guide RNA, wherein the guide RNA has a nucleic acid sequence which recognizes a nucleic acid sequence comprised in the target locus; and
    c) a first promoter oriented with respect to polynucleotide sequences of (a) and (b) so as to drive expression thereof in the organism;

    and a second expression vector comprising a polynucleotide sequence which encodes an homologous recombination (HR) oligonucleotide under the control of a promoter.

14. An *in vitro* method of modifying the genetic material of a prokaryotic organism comprising introducing an expression vector of any of claims 8 to 12 into the organism.

15. A method as claimed in claim 13 or claim 14, wherein the organism is cultured for a period at a first temperature, and then cultured at a second temperature before or during induction of the promoter of the ThermoCas9 sequence; preferably wherein the first temperature is 60 °C or less and the second temperature is a higher temperature which is at least 55 °C; more preferably wherein the first temperature is 55 °C or less and the second temperature is greater than 55 °C.

16. A method as claimed in any of claims 13 to 15, wherein the organism is a prokaryote; preferably wherein the prokaryote is:

    (a) a thermophilic bacterium, e.g. *Geobacillus thermoglucosidans*; or

(b) a bacterium with growth temperature optimum of at least 40 °C; preferably at least 45 °C., e.g. *Bacillus coagulans* or *Pseudomonas putida*; or

(c) a soil bacterium; preferably a saprotrophic soil bacterium, e.g. *Pseudomonas putida.*

17. A prokaryotic cell transfected, transformed or transduced with an expression vector of any of claims 5 to 12.