

Single Molecule Real Time (SMRT) DNA sequencing technology and an isothermal amplification method for next generation sequencing published in 2013 by Ma *et al.* (Ma, Z. *et al. Proc Natl Acad Sci U S A* 110(35): 14320-14323).

5 The current toolbox of polymerase enzymes which function well at ambient temperature is, however, very limited. Typically, different isothermal methods require reaction temperatures between 30-65°C which are mainly determined by the working range of the polymerases used in the reactions and are prone to inhibition by salt.

10 A cold-adapted polymerase from a *Psychrobacillus* sp. (PB) belonging to the A-family of DNA polymerases has been characterized. This enzyme possesses high polymerase activity at ambient temperatures but still has good stability at elevated temperatures up to 40 °C. Of particular interest, the marine derived enzyme also possesses good salt tolerance and strong strand-displacement activity (SDA) as well as proficient processivity at 25 °C, and is comparable with the state-
15 of-the art commercial enzymes (WO 2017/162765).

In many IA methods only a polymerase is required and the effectiveness of the method is heavily dependent on the SDA of that polymerase. Therefore anything which served to increase SDA of the PB or other polymerases used in IA would be highly desirable.

20 The present inventors have surprisingly found that a single point mutation in the finger domain of certain polymerases in the A family, in particular replacement of a single Asp residue, leads to significantly enhanced SDA.

Summary

25 Therefore, in a first example, the present disclosure provides a DNA polymerase, said DNA polymerase including the sequence of SEQ ID NO. 1 or a sequence which is at least 70%, preferably at least 75%, 78%, 80%, 82%, 85%, 88%, 90%, 92% or 95%, identical thereto, but wherein the aspartic acid residue at position 18 of SEQ ID NO. 1, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue.

30 ~~In one aspect, the present disclosure refers to a DNA polymerase including the sequence of SEQ ID NO. 1 or a variant sequence which is at least 70% identical to SEQ ID NO: 1, but wherein the aspartic acid residue at position 18 of SEQ ID NO. 1, or the equivalent aspartic acid residue in said variant sequence, has been replaced by a alanine.~~

35 SEQ ID NO. 1 is a fragment of the amino acid sequence of the PB polymerase I, it spans amino acids 405 to 436 in the truncated (lacking the 5'- 3'-exonuclease domain) wild type PB sequence. This region (405-436) within the finger domain is highly conserved amongst some of the DNA polymerase A family (also known as pol I family), see Figures 1 and 5. DNA polymerases of the
40 disclosure would typically be classed as of the DNA polymerase I or A type.

readily identified by using standard sequence alignment techniques such as Clustal X2 (Larkin, M.A. et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics, 23:2947-2948).

Of course, SEQ ID NO. 1 does not itself define a fully functional DNA polymerase. In preferred examples the DNA polymerase of the present disclosure is based on the amino acid sequence of PB DNA polymerase I, preferably which lacks the 5'-3'- exonuclease domain that is present in the wild-type *Psychrobacillus* species DNA polymerase I sequence. In preferred examples, the 5'-3'- exonuclease domain is absent from the DNA polymerase enzyme as 5'-3'- exonuclease activity is typically unwanted as it may degrade primers and/or products in an amplification mixture. This truncated wild-type PB sequence is referred to herein as SEQ ID NO. 2.

The disclosure provides a DNA polymerase comprising or consisting of the amino acid sequence of SEQ ID NO:2 or an amino acid sequence which is at least 60% identical to SEQ ID NO:2 but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue.

In another aspect, the present disclosure refers to a DNA polymerase comprising the amino acid sequence of SEQ ID NO. 2, ~~or a variant sequence which is at least 60% identical to SEQ ID NO. 2,~~ but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, ~~or the equivalent aspartic acid residue in said variant sequence,~~ has been replaced by alanine.

In preferred examples and examples, the DNA polymerase of the disclosure comprises (or consists of) an amino acid sequence that is at least 70%, or 75%, preferably at least 80%, 85%, 90% or 95%, e.g. at least 98% or 99% or 99.5%, identical to SEQ ID NO:2 but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue. It will be understood that position 18 in SEQ ID NO. 1 and 422 in SEQ ID NO. 2 are equivalent, SEQ ID NO. 1 is a fragment from position 405 to 436 of SEQ ID NO. 2.

Figure 5 shows an alignment of PB, Bst and Upts DNA polymerases. The key Asp residue is at position 422 in each case.

In further preferred examples, the DNA polymerase of the disclosure comprises (or consists of) an amino acid sequence that is at least 60%, 70% or 75%, preferably at least 80%, 85%, 90% or 95%, e.g. at least 98% or 99% or 99.5%, identical to SEQ ID NO. 11 or 12 but wherein the aspartic acid residue at position 422 of SEQ ID NO. 11 or 12, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue. Numbering is based on a sequence alignment according to Figure 5. SEQ

ID NOs. 11 and 12 are the (truncated) variants of the wild type Bst and Ubs polymerase sequences respectively.

In another aspect, the present disclosure refers to a DNA polymerase comprising the amino acid sequence of SEQ ID NO. 11 or 12, ~~or a variant sequence which is at least 60% identical to SEQ ID NO. 11 or 12,~~ but wherein the aspartic acid residue at position 422 of SEQ ID NO. 11, or 12, ~~or the equivalent aspartic acid residue in said variant sequence,~~ has been replaced by alanine.

Preferably, the DNA polymerase of the disclosure comprises or consists of the amino acid sequence of SEQ ID NO:2, 11 or 12 but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, 11 or 12, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue .

In one example, the DNA polymerase comprises (or consists of) the amino acid sequence of SEQ ID NO:4 (incorporating also the 5'→3' exonuclease domain) or a variant or fragment thereof but wherein the aspartic acid residue at position 719 of SEQ ID NO. 4, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue. The types of variants and fragments of SEQ ID NO:2 described herein apply, *mutatis mutandis*, to variants and fragments of SEQ ID NO:4, e.g. variants will have at least 70% preferably at least 80% or 90% sequence identity to SEQ ID.NO:4.

DNA polymerases of the disclosure include enzymatically active fragments of native polymerases. Enzymatically active fragments are fragments that have DNA polymerase activity. Enzymatically active fragments may be at least 400, at least 450, at least 475, at least 500, at least 525, at least 550, at least 560, at least 570 or at least 575 amino acids in length. Preferred fragments are at least 525, at least 550, at least 560, at least 570 or at least 575 amino acids in length. The fragments are at least 70%, preferably at least 80%, at least 85% or at least 90%, more preferably at least 95% (e.g. at least 98% or 99% or 99.5%), or 100% identical to the corresponding portion of SEQ ID NO:2, 11 or 12 but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, 11 or 12, or the equivalent aspartic acid residue in other sequences, has been replaced by a non-negatively charged amino acid residue.

DNA polymerase activity may be assessed using a molecular beacon that bears a loop structure and uses FAM as fluorescence donor and Dabcyl as an acceptor (non-fluorescent quencher) within an 8mer stem. This stem bears a 3'- extension that allows binding of a primer and acts as template for the DNA polymerase. The stem will be opened by the DNA polymerase when the extension proceeds. The following separation of the two labels is recorded by restoration of FAM emission. A suitable assay of this type is described in the Examples.

polymerase or product into a composition including at least one additional component, such as an acceptable buffer or carrier.

In another aspect, the present disclosure refers to a method of producing a DNA polymerase as disclosed herein, wherein the method comprises the steps of:

(i) culturing, in a growth medium, a host cell comprising one or more of the recombinant expression vectors as disclosed herein or one or more of the nucleic acid molecules as disclosed herein under conditions suitable for the expression of the encoded DNA polymerase; and optionally

(ii) isolating or obtaining the DNA polymerase from the host cell or from the growth medium or supernatant derived from said growth medium.

The DNA polymerase may be separated, or isolated, from the host cells/culture media using any of the purification techniques for protein known in the art and widely described in the literature or any combination thereof. Such techniques may include for example, precipitation, ultrafiltration, dialysis, various chromatographic techniques, e.g. size exclusion chromatography, ion-exchange chromatography, affinity chromatography, electrophoresis, centrifugation etc. As discussed above, the DNA polymerase of the disclosure may be modified to carry amino acid motifs or other protein or non-protein tags, e.g. polyhistidine tags (e.g. His₆-tag), to assist in isolation, solubilisation and/or purification or identification.

In another example, the present disclosure provides the use of a DNA polymerase of the disclosure for nucleotide (e.g. dNTP) polymerisation. Accordingly, DNA polymerases of the disclosure may be used to extend a nucleic acid (DNA) strand by one or more nucleotides.

In another aspect, the present disclosure refers to use of the DNA polymerase as disclosed herein for nucleotide polymerization.

In another example, the present disclosure provides the use of a DNA polymerase of the disclosure in a nucleic acid (DNA) amplification or sequencing reaction.

In another aspect, the present disclosure refers to use of the DNA polymerase as disclosed herein in a nucleic acid amplification or sequencing reaction.

In another example, the present disclosure provides the use of a DNA polymerase of the disclosure in a molecular beacon assay or in a strand displacement assay, e.g. as described herein.

Preferably, in uses and methods of the present disclosure, DNA polymerases of the present disclosure are used at a constant temperature, i.e. without thermal cycling. Accordingly, the use of DNA polymerases of the disclosure in isothermal reactions is particularly preferred.

The use of DNA polymerases of the disclosure in isothermal amplification reactions is particularly preferred. Isothermal reactions are performed at a constant temperature. Many isothermal amplification techniques are known in the art and include Loop mediated isothermal amplification (LAMP), rolling circle amplification (RCA), strand displacement amplification (SDA), multiple displacement amplification (MDA) and cross priming amplification (CPA).

In another example, the present disclosure provides a method of nucleotide polymerisation using a DNA polymerase of the present disclosure. Preferably, said method comprises providing a reaction mixture comprising a DNA polymerase of the present disclosure, a template nucleic acid molecule, an oligonucleotide primer

CLAIMS

1. ~~—A DNA polymerase including the sequence of SEQ ID NO. 1 or a variant sequence which is at least 70% identical to SEQ ID NO. 1, but wherein the aspartic acid residue at position 18 of SEQ ID NO. 1, or the equivalent aspartic acid residue in said variant sequence, has been replaced by alanine.~~

2. ~~—The DNA polymerase according to claim 1, wherein said DNA polymerase includes the sequence of SEQ ID NO. 1 or a variant sequence which is at least 88% identical to SEQ ID NO. 1, but wherein the aspartic acid residue at position 18 of SEQ ID NO. 1, or the equivalent aspartic acid residue in said variant sequence, has been replaced by alanine.~~

3. ~~—The DNA polymerase according to claim 1 or claim 2, wherein said DNA polymerase comprises the amino acid sequence of SEQ ID NO. 6, 7, 8, 9, or 10 but wherein the aspartic acid residue at position 18 of each sequence has been replaced by alanine.~~

14. A DNA polymerase comprising the amino acid sequence of SEQ ID NO. 2, ~~or a variant sequence which is at least 60% identical to SEQ ID NO. 2, but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, or the equivalent aspartic acid residue in said variant sequence,~~ has been replaced by alanine.

5. ~~—The DNA polymerase according to any one of the preceding claims, wherein said DNA polymerase comprises the amino acid sequence of SEQ ID NO. 2, or a variant sequence which is at least 90% identical to SEQ ID NO. 2, but wherein the aspartic acid residue at position 422 of SEQ ID NO. 2, or the equivalent aspartic acid residue in said variant sequence, has been replaced by alanine.~~

26. The DNA polymerase according to ~~any one of the preceding claims~~claim 1, wherein said DNA polymerase comprises the amino acid sequence of SEQ ID NO:4 ~~or a variant sequence which is at least 60% identical to SEQ ID NO. 4, but wherein the aspartic acid residue at position 719 of SEQ ID NO. 4, or the~~

~~equivalent aspartic acid residue in said variant sequence,~~ has been replaced by alanine.

37. A DNA polymerase comprising the amino acid sequence of SEQ ID NO. 11 or 12, ~~or a variant sequence which is at least 60% identical to SEQ ID NO. 11 or 12,~~ but wherein the aspartic acid residue at position 422 of SEQ ID NO. 11, or 12, ~~or the equivalent aspartic acid residue in said variant sequence,~~ has been replaced by alanine.

~~8. The DNA polymerase according to any one of the preceding claims, wherein said DNA polymerase comprises the amino acid sequence of SEQ ID NO: 11 or 12, or a variant sequence which is at least 90% identical to SEQ ID NO. 11 or 12, but wherein the aspartic acid residue at position 422 of SEQ ID NO. 11 or 12, or the equivalent aspartic acid residue in said variant sequence, has been replaced by alanine.~~

~~9. The DNA polymerase according to any one of the preceding claims, wherein said DNA polymerase has at least 30% greater strand displacement activity than a DNA polymerase with exactly the same sequence but with aspartic acid at position 18 or 422, relative to SEQ ID NO: 1 or 2 respectively, or at the equivalent position in said variant sequences.~~

~~10. The DNA polymerase according to any one of the preceding claims, wherein across a concentration range from 20mM to 200mM NaCl or KCl or a mixture thereof, said DNA polymerase exhibits at least 40% of its maximum polymerase activity.~~

411. A composition comprising the DNA polymerase according to any one of the preceding claims and a buffer.

512. A nucleic acid molecule comprising a nucleotide sequence that encodes the DNA polymerase according to any one of claims 1 to 310.

613. The nucleic acid molecule of claim 512, wherein the nucleotide sequence ~~has at least 70% sequence identity to~~ comprises SEQ ID NO: 13, 14 or 15.

714. An expression vector containing the nucleic acid molecule according to claim 512 or claim 613 and the necessary regulatory sequences for the transcription and translation of the protein sequence encoded by said nucleic acid molecule.

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815. A host cell or virus comprising one or more expression vectors according to claim 714 or one or more nucleic acid molecules according to claim 512 or claim 613.

10 916. A method of producing a DNA polymerase according to any one of claims 1 to 310, wherein the method comprises the steps of:

(i) culturing, in a growth medium, a host cell comprising one or more of the ~~recombinant~~ expression vectors according to claim 714 or one or more of the nucleic acid molecules according to claim 512 or claim 613 under conditions
15 suitable for the expression of the encoded DNA polymerase; and optionally

(ii) isolating or obtaining the DNA polymerase from the host cell or from the growth medium or supernatant derived from said growth medium.

20 1017. Use of the DNA polymerase according to any one of claims 1 to 310 for nucleotide polymerisation.

1118. Use of the DNA polymerase according to any one of claims 1 to 310 in a nucleic acid amplification or sequencing reaction.

25 1219. The use of claim 1118, wherein said reaction is an isothermal nucleic acid amplification reaction.

1320. The use of any one of claims 1017 to 1219, wherein said DNA polymerase is used at a constant temperature.

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1421. A method of nucleotide polymerisation using the DNA polymerase according to any one of claims 1 to 310, said method comprising:

(i) providing a reaction mixture comprising the DNA polymerase according to any one of claims 1 to 310, a template nucleic acid molecule, an oligonucleotide

primer which is capable of annealing to a portion of the template nucleic acid molecule and one or more species of nucleotide; and

(ii) incubating said reaction mixture under conditions whereby the oligonucleotide primer anneals to the template nucleic acid molecule and said DNA polymerase extends said oligonucleotide primer by polymerising one or more nucleotides.

1522. A method of amplifying a nucleic acid using the DNA polymerase according to any one of claims 1 to 340, said method comprising:

(i) providing a reaction mixture comprising the DNA polymerase according to any one of claims 1 to 340, a template nucleic acid molecule, an oligonucleotide primer(s) which is capable of annealing to a portion of the template nucleic acid molecule, and nucleotides; and

(ii) incubating said reaction mixture under conditions whereby the oligonucleotide primer(s) anneals to the template nucleic acid molecule and said DNA polymerase extends said oligonucleotide primer(s) by polymerising one or more nucleotides to generate a polynucleotide.

1623. The method of claim 1424 or claim 1522, wherein said method is performed at a constant temperature.

1724. The use of claim 1320 or the method of claim 1623, wherein said constant temperature is chosen from within the range 0°C to 42°C.

1825. The use or method of claim 1724, wherein said constant temperature is chosen from within the range 10°C to 25°C.