Marine DNA polymerases

The present invention relates to DNA polymerases. In particular, the present invention relates to DNA polymerases based on a DNA polymerase from a *Psychrobacillus* sp.

The gold standard of microbial identification still remains culturing and subsequent phenotypic differentiation of the causative agent, a process often taking several days to perform and analyze, and this delay may have major impact on morbidity and mortality of an infectious disease. In addition, many organisms cannot grow on culture media, hence, will be undetected by existing culturing methods. The Polymerase Chain Reaction (PCR) in many ways revolutionized the molecular genetics and diagnosis field. The workhorses in PCR technology, are thermostable high fidelity DNA polymerases which together with cyclic events of heating and cooling to obtain strand separation, primer annealing and elongation, lead to amplification of a target DNA sequence. PCR technology is now widely employed in biomedical and life science research as well as molecular diagnostics.

Point-of-care (POC) diagnostics are described as medical tools or devices enabling disease diagnosis in a patient´s community outside a hospital setting. The ideal diagnostic test should meet the "*ASSURED*" criteria: *Affordable*, *Sensitive*, *Specific*, *User*-friendly, *Rapid* and robust, *Equipment*-free and *Delivered* to those who need it. Although PCR technology has a high potential, it still has limitations and requires the use of high precision electrically powered thermal cycling equipment for repeated heating and cooling processes and skilled personnel to run the equipment. Non-specific amplification due to spurious priming in the annealing process is problematic, and PCR is also prone to inhibitory compounds in "crude" samples. In addition, the bulky design of PCR devices make PCR an imperfect solution for incorporation into POC technology platforms and make PCR-based methods difficult to employ as the major technology driver in POC diagnostics.

Lately, an increased focus on non-PCR based methods, or Isothermal Amplification methods, has emerged. In these methods, nucleic acid amplification takes place at constant (moderate) temperatures and has no need for high precision temperature cycling and control, or enzymes stable at high temperatures. Isothermal amplification methods are reported to have analytical sensitivities and

specificities comparable to PCR as well as a higher tolerance to inhibitory compounds, while allowing shorter time to results and easier use. These features make isothermal amplification methods highly desirable for those developing POC molecular diagnostics platforms and aiming to meet "ASSURED" criteria. A number of methods have in the last decade been published for isothermal amplification of nucleic acids (both RNA and DNA) (Reviewed by Gill, P. and A. Ghaemi (2008) *Nucleosides Nucleotides Nucleic Acids* 27(3): 224-243; Craw, P. and W. Balachandran (2012) *Lab Chip* 12(14): 2469-2486; de Paz, H. D. *et al*. (2014) *Expert Rev Mol Diagn* 14(7): 827-843; Yan, L. *et al*. (2014) *Mol Biosyst* 10(5): 970-1003). In several of the methods, success relies on the inherent strand displacement activity of the DNA polymerase used in the reaction setup. The term strand displacement describes the ability of the polymerase to displace downstream DNA encountered during synthesis.

In addition to diagnostics also other areas of interest benefit from isothermal amplification technology empowered by the DNA polymerase. In this regard, whole genome amplification (multiple displacement amplification) is important for genomic research especially when limited amount of DNA is present and/or in single cell approaches. Also in next-generation sequencing approaches, either commercially available or at the proof of concept stage, strand-displacing polymerases are important as exemplified by the Pacific Biosciences 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).

The current toolbox of polymerase enzymes used in the various isothermal technologies 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.

The *E.coli* Klenow fragment DNA polymerase, for example, exhibits useful levels of polymerase activity in only a relatively narrow temperatures range and has relatively low activity at low-to-moderate temperatures (e.g. at 25°C). What are needed in the art are DNA polymerases which exhibit useful levels of polymerase activity and good stability at a broad range of temperatures, in particular at low-to-moderate temperatures, but preferably also at higher temperatures. Such polymerases would be useful for isothermal amplification reactions performed at a broad range of temperatures, including temperatures at or close to room

temperature. The inventors have identified a DNA polymerase from a *Psychrobacillus* sp. which surprisingly fulfils these criteria. The *Psychrobacillus* sp. is cold adapted and its DNA polymerase I is active at low temperatures. However, unlike other marine DNA polymerases, the *Psychrobacillus* sp. DNA polymerase I is not rapidly inactivated above 20-25°C and thus represents a DNA polymerase that is very useful at a broad range of temperatures. Moreover, the *Psychrobacillus* sp. DNA polymerase I is much more active in general than other marine polymerases.

Therefore, in a first aspect the invention provides an isolated DNA polymerase or an enzymatically active fragment thereof, said DNA polymerase comprising the amino acid sequence of SEQ ID NO:1 or comprising an amino acid sequence which is at least 70% identical to SEQ ID NO:1.

The DNA polymerase of SEQ ID NO:1 is based on the amino acid sequence of DNA polymerase I from a *Psychrobacillus* species (also referred to herein as PB or Pb or PbI), a marine bacteria isolated in North of Norway. However, SEQ ID NO:1 lacks the 5'-3'- exonuclease domain that is present in the wild-type *Psychrobacillus* species DNA polymerase I sequence. In preferred embodiments, 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.

In some embodiments, the invention provides an isolated DNA polymerase or an enzymatically active fragment thereof, said DNA polymerase consisting of the amino acid sequence of SEQ ID NO:1 or consisting of an amino acid sequence which is at least 70% identical to SEQ ID NO:1.

In preferred embodiments, the DNA polymerase of the invention comprises (or consists of) an amino acid sequence that is at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, preferably at least 80%, 85%, 90% or 95%, e.g. at least 98% or 99% or 99.5%, identical to SEQ ID NO:1.

In some embodiments, the DNA polymerase of the invention comprises (or consists of) an amino acid sequence that has single or multiple amino acid alterations (additions, substitutions, insertions or deletions) compared to SEQ ID NO:1. Such sequences preferably may contain up to 5, e.g. only 1, 2, 3, 4 or 5, preferably 1, 2 or 3, more preferably 1 or 2, altered amino acids. Substitutions can be with conservative or non-conservative amino acids. Preferably said alterations are conservative amino acid substitutions.

Preferably, the DNA polymerase of the invention comprises the amino acid sequence of SEQ ID NO:1.

In a preferred embodiment, the DNA polymerase consists of the amino acid sequence of SEQ ID NO:1.

In one embodiment, the DNA polymerase comprises (or consists of) the amino acid sequence of SEQ ID NO:3. The present invention also provides variants and fragments of SEQ ID NO:3. The types of variants and fragments of SEQ ID NO:1 described herein apply, *mutatis mutandis*, to variants and fragments of SEQ ID NO:3.

Enzymatically active fragments of DNA polymerases of the invention are also provided. 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:1.

For any given DNA polymerase there is generally a temperature at which maximum polymerase activity is observed. For many DNA polymerases, when the temperature deviates (e.g. decreases or increases) from the temperature at which maximum activity is observed, there is generally a marked decrease in polymerase activity, even at only modest temperature deviations, meaning that many polymerases are only suitable for use in applications carried out in a fairly narrow temperature range.

The present inventors have found that, advantageously, at a broad range of temperatures, including at low to modest temperatures, DNA polymerase I of a *Psychrobacillus* species exhibits substantial DNA polymerase activity relative to the DNA polymerase activity at the temperature at which maximum polymerase activity is observed. Put another way, the inventors have found that unlike many DNA polymerases, the DNA polymerase I of this *Psychrobacillus* species exhibits a useful proportion of its maximum polymerase activity at temperatures which differ significantly from the temperature at which the maximum activity is observed.

Accordingly, in some embodiments DNA polymerases of the present invention exhibit across a broad range of temperatures a substantial proportion of their maximum polymerase activity.

In preferred embodiments, the temperature at which the DNA polymerase exhibits its maximum activity is about 37°C to 42°C, preferably 37°C-40°C (e.g. 37, 38, 39 or 40°C).

Some DNA polymerases of the present invention exhibit a substantial proportion of their maximum polymerase activity across the temperature range from 0°C to 35°C.

Some DNA polymerases of the present invention exhibit at least 30% of their maximum polymerase activity across the temperature range from 0°C to 35°C.

Some DNA polymerases of the present invention exhibit at least 40% of their maximum polymerase activity across the temperature range from 10°C to 35°C.

Some DNA polymerases of the present invention exhibit at least 50% of their maximum polymerase activity across the temperature range from 15°C to 35°C.

Some DNA polymerases of the present invention exhibit at least 60% of their maximum polymerase activity across the temperature range from 20°C to 35°C.

Some DNA polymerases of the present invention exhibit at least 70% (preferably at least 80% or at least 90%) of their maximum polymerase activity across the temperature range from 30°C to 35°C.

In some embodiments, at a temperature that deviates (increase or decrease) by up to about 10°C (e.g. 1, 2, 3, 4, 5, 6, 7, 9, or 10°C) from the temperature at which the polymerase exhibits maximum activity, DNA polymerases of the present invention exhibit at least about 30% of their maximum polymerase activity.

In some embodiments, at a temperature that deviates (increase or decrease) by up to about 5°C (e.g. 1, 2, 3, 4, 5 °C) from the temperature at which the polymerase exhibits maximum activity, DNA polymerases of the present invention exhibit at least about 60% of their maximum polymerase activity.

In some embodiments, at a temperature that is up to about 35°C to 40°C lower (e.g. 40°C, 30°C, 20°C, 10°C, or 5°C lower) than the temperature at which the

polymerase exhibits maximum activity, DNA polymerases of the present invention exhibit at least about 30% of their maximum polymerase activity.

In some embodiments, at a temperature that is up to about 12°C to about 15°C lower (e.g. 1, 2, 3, 4, 5, 10, 12 or 15 °C lower) than the temperature at which the polymerase exhibits maximum activity, DNA polymerases of the present invention exhibit at least about 60% of their maximum polymerase activity.

In some embodiments, at a temperature that is up to about 5°C to about 10°C lower (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 °C lower) than the temperature at which the polymerase exhibits maximum activity, DNA polymerases of the present invention exhibit at least about 70% of their maximum polymerase activity (preferably at least about 70%, 75%, 80%, 85%, 90% or 95% of their maximum polymerase activity).

Suitable assays for analysing DNA polymerase activity are known in the art. Such assays can thus be used to determine DNA polymerase activity at any given temperature relative to the DNA polymerase activity that is exhibited at the temperature at which the polymerase exhibits its maximum activity. DNA polymerase activity as discussed above may be assessed using a single-nucleotide incorporation assay. In an exemplary single-nucleotide incorporation assay, a primer consisting of 19 nucleotides, which is labelled with a fluorophore at its 5' end, is annealed to a template DNA strand consisting of 40 nucleotides; in the reaction set up the only dNTP present is dATP thus the polymerase can extend the primer in the 5'-3' direction only by one nucleotide at position 20 (as there is one T at the corresponding (complementary) position on the template strand); subsequent analysis on a denaturing polyacrylamide gel (e.g. 12 % polyacrylamide/7M urea) and scanning for the fluorophore labeled oligonucleotides shows the primer consisting of 19 oligonucleotides and the primer extended by the nucleotide adenine thus consisting of 20 oligonucleotides; enzyme activity (i.e. polymerase activity) is determined by densitometric measurement of bands representing the extended primer (intensity 1) and the unextended primer (intensity 0); the relative incorporation rate is calculated as follows: incorporation [%] = intensity 1/(intensity 0 + intensity 1)*100.

Thus, DNA polymerase activity may be assessed in an assay having the steps of (i) providing a primer consisting of 19 nucleotides, which is labelled with a fluorophore at its 5' end, (ii) annealing said primer to a template DNA strand consisting of 40 nucleotides to form a primer-template complex, (iii) incubating said

annealed primer-template complex with a DNA polymerase at the relevant temperature (i.e. temperature under investigation) in the presence of the only dNTP that is to be incorporated into the primer at position 20 at the 3' end of the primer (said dNTP is complementary to the relevant nucleotide on the annealed template DNA strand) and (iv) determining the amount of extended primer (20 nucleotides) compared with unextended primer (19 nucleotides) wherein the amount of extended primer relative to unextended primer corresponds to the level of polymerase activity (i.e. higher amounts of extended primer indicate higher polymerase activity).

Preferred primer and template strands are as described in the Example.

A particularly preferred single-nucleotide incorporation assay is described in the Example section herein. Thus, in preferred embodiments, relative DNA polymerase activities are as assessed in accordance with the single-nucleotide incorporation assay described in the Example section.

The present inventors have also surprisingly found that at a broad range of temperatures, including at temperatures significantly higher than its normal marine environment, the DNA polymerase I of this *Psychrobacillus* species shows good stability (temperature stability) in comparison with many other DNA polymerases, in particular in comparison with DNA polymerases from other marine organisms such as *Aliivibrio Salmonicida*. In this context, a "stable" DNA polymerase means that the DNA polymerase does not exhibit a substantial loss of polymerase activity after exposure to a broad range of temperatures. Alternatively viewed, good "stability" means that a DNA polymerase retains substantial polymerase activity (i.e. has substantial residual activity) after exposure to a broad range of temperatures.

Accordingly, some DNA polymerases of the present invention retain a substantial proportion of their maximum polymerase activity after incubation (pre-incubation) at a broad range of temperatures prior to assessing the DNA polymerase activity. In this context, the maximum polymerase activity may be the polymerase activity observed when the incubation (pre-incubation) of the DNA polymerase is done on ice (about 0^{o}C) prior to the initiation of the DNA polymerase activity assay.

Preferably, the incubation (pre-incubation) is for about 15 minutes (15 minutes is preferred). Preferably, irrespective of the temperature of the incubation (pre-incubation), the DNA polymerase is cooled on ice after said incubation (e.g. for about 5 minutes) prior to assessing DNA polymerase activity.

Some DNA polymerases of the present invention exhibit at least 40% of their maximum activity after incubation of the DNA polymerase at any temperature across the temperature range from 0°C to 40°C (e.g. from 0°C to 15°C, 0°C to 20°C, or 0°C to 25°C, or 0°C to 37°C) prior to assessing DNA polymerase activity. Preferably, the DNA polymerase activity is as assessed at about 25°C (25°C is preferred).

Some DNA polymerases of the present invention exhibit at least 60% (preferably at least 70% or at least 80%) of their maximum activity after incubation of the DNA polymerase at any temperature across the temperature range from 0°C to 37°C (e.g. from 0°C to 15°C, 0°C to 20°C, or 0°C to 25°C, 0°C to 30°C, or 0°C to 35°C) prior assessing DNA polymerase activity. Preferably, the DNA polymerase activity is as assessed at about 25°C (25°C is preferred).

Some DNA polymerases of the present invention exhibit at least 70% (preferably at least 80%, at least 90%, at least 95% or 100%) of their maximum activity after incubation of the DNA polymerase at any temperature across the temperature range from 0°C to 30°C (e.g. from 0°C to 15°C, 0°C to 20°C, or 0°C to 25°C) prior to assessing DNA polymerase activity assay. Preferably, the DNA polymerase activity is as assessed at about 25°C (25°C is preferred).

In some embodiments, after a 15 minute incubation at 37°C prior to the initiation of a DNA polymerase activity assay (e.g. a single-nucleotide extension reaction), DNA polymerases of the present invention exhibit at least 60% (preferably at least 70%, more preferably at least 80%) of the polymerase activity observed when the 15 minute incubation prior to the initiation of the DNA polymerase activity assay was done on ice (0°C), i.e. there is at least 60%, at least 70% or at least 80% “residual” activity.

In some embodiments, after a 15 minute incubation at 30°C prior to the initiation of a DNA polymerase activity assay (e.g. a single-nucleotide extension reaction), DNA polymerases of the present invention exhibit least 70% (preferably at least 80%, more preferably at least 80% or at least 90%, or 100%) of the polymerase activity observed when the 15 minute incubation prior to the initiation of the DNA polymerase activity assay was done on ice (0°C), i.e. there is at least 70%, at least 80%, at least 90% or 100% “residual” activity.

Suitable experiments for analysing the temperature stability of DNA polymerases are known in the art any of these may be used. DNA polymerase temperature stability may be assessed by incubating the DNA polymerase at a

temperature under test (for example 0°C (e.g. on ice), 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 37°C, 40°C or 45°C) for a period of time (e.g. 5 minutes to 25 minutes, preferably about 15 minutes) prior to performing an assay to determine polymerase activity. Preferably, DNA polymerase stability may be assessed by incubating the DNA polymerase at the temperature under test (for example 0°C (e.g. on ice), 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 37°C, 40°C or 45°C) for 15 minutes and subsequently cooling down the DNA polymerase on ice (e.g. for 5 minutes) prior to performing an assay to determine polymerase activity. Preferably, the DNA polymerase activity is as assessed at about 25°C (25°C is preferred). DNA polymerase activity may preferably be assessed using a single-nucleotide incorporation assay, preferably as described elsewhere herein. Particularly preferably, DNA polymerase activity is as assessed in accordance with the single-nucleotide incorporation assay described in the Example section.

Particularly preferred DNA polymerases of the present invention have good stability and also good activity over a broad range of temperatures.

Some DNA polymerases of the present invention have a broad pH stability profile (pH 6.0 to pH 8.5) as assessed by analysing DNA polymerase melting temperature (T_m). Some DNA polymerases of the present invention have a melting temperature of about 42°C-45°C, preferably about 43°C-44°C in the pH range 6.0 to 8.5. Melting temperature (T_m) may be determined by thermofluor experiments (Ericsson *et al.*, *Anal. Biochem.*, 2006, *357*, 289–298). A particularly preferred assay is described in the Example section.

Some DNA polymerases of the present invention have a higher DNA polymerase activity (specific activity) than some commercially available DNA polymerases.

Some DNA polymerases of the present invention have a higher DNA polymerase activity (specific activity) than some commercially available DNA polymerases at a temperature of about 25°C (25°C is preferred).

DNA polymerase activity (specific activity) can be analysed using any suitable assay and a skilled person is readily able to select a suitable assay. DNA polymerase activity as discussed herein may be assessed using an isothermal amplification assay. DNA polymerase activity as discussed herein may be assessed using an isothermal molecular beacon assay. In an exemplary molecular beacon assay, a molecular beacon template includes a loop region that is connected by a GC-rich stem region; the molecular beacon template has two

fluorphores that are in close proximity and thus quenched; upon extension by the DNA polymerase (DNA polymerase I) of a primer that is annealed to the molecular beacon template the stem is opened and the increase in distance between the two fluorophores is measured by the restoration of the fluorescence of a previously quenched fluorophore.

Thus, DNA polymerase activity may be assessed in an assay having the steps of (i) providing a template DNA molecule (molecular beacon template) which comprises a loop region that is connected by a GC-rich stem region and which comprises two fluorphores that are quenched by virtue of their close proximity to each other, (ii) annealing a primer to said template DNA molecule, (iii) incubating said template-primer complex with a DNA polymerase (e.g. at about 25°C, preferably 25 °C) and (iv) measuring the restoration in fluorescence of a previously quenched fluorophore, wherein said fluorescence is indicative of DNA polymerase activity.

In preferred embodiments, DNA polymerase activity is as assessed at about 25°C (25°C is preferred). DNA polymerase activity may be assessed using an isothermal molecular beacon assay that is carried out at about 25°C (25°C is preferred).

A preferred molecular beacon template and primer are as described in the Example.

A preferred DNA polymerase activity assay is the polymerase activity assay described in the Example section herein, which is an isothermal molecular beacon assay. Thus, in preferred embodiments DNA polymerase activity is as assessed in accordance with the polymerase activity assay described in the Example section.

Some DNA polymerases of the present invention have a higher polymerase activity (specific activity) than the *E.coli* Klenow fragment DNA polymerase (also referred to herein as KF), the *Geobacillus stearothermophilus* DNA polymerase (also referred to herein as Bst) and/or the commercially available variant of the *G. stearothermophilus* DNA polymerase Bst2.0. Preferably, the DNA polymerase activity is as assessed at about 25°C (25°C is preferred). DNA polymerase activity may be assessed using an isothermal molecular beacon assay (e.g. carried out at 25°C). More preferably, DNA polymerase activity is assessed in accordance with the polymerase activity assay described in the Example section.

Some DNA polymerases of the present invention have at least 50%, at least 75%, at least 100% or at least 150% higher polymerase activity (specific activity)

than the *E.coli* Klenow fragment DNA polymerase. Preferably, the DNA polymerase activity is as assessed at about 25°C (25°C is preferred). DNA polymerase activity may be assessed using an isothermal molecular beacon assay (e.g. carried out at 25°C). More preferably, DNA polymerase activity is assessed in accordance with the polymerase activity assay described in the Example section.

Some DNA polymerases of the present invention have at least 50%, at least 75%, at least 100%, at least 150%, at least 200%, at least 500% or at least 1000% higher polymerase activity (specific activity) than the *G. stearothermophilus* DNA polymerase. Preferably, the DNA polymerase activity is as assessed at about 25°C (25°C is preferred). DNA polymerase activity may be assessed using an isothermal molecular beacon assay (e.g. carried out at 25°C). More preferably, DNA polymerase activity is assessed in accordance with the polymerase activity assay described in the Example section.

Some DNA polymerases of the present invention have at least 50%, at least 75%, at least 100%, at least 150%, at least 200%, at least 300%, at least 400% or at least 500% higher polymerase activity (specific activity) than the commercially available variant of *G. stearothermophilus* DNA polymerase (Bst2.0). Preferably, the DNA polymerase activity is as assessed at about 25°C (25°C is preferred). DNA polymerase activity may be assessed using an isothermal molecular beacon assay (e.g. carried out at 25°C). More preferably, DNA polymerase activity is assessed in accordance with the polymerase activity assay described in the Example section.

Some DNA polymerases of the present invention have a higher DNA polymerase activity (specific activity) compared with other marine polymerases (e.g. DNA polymerase from *Aliivibrio Salmonicida*), in particular at a temperature of about 25°C (25°C is preferred). Some DNA polymerases of the present invention have at least 10%, at least 20%, at least 30%, at least 40% or at least 50% higher polymerase activity (specific activity) than the DNA polymerase from *Aliivibrio Salmonicida*. Preferably, the DNA polymerase activity is as assessed at about 25°C (25°C is preferred). DNA polymerase activity may be assessed using an isothermal molecular beacon assay (e.g. carried out at 25°C). More preferably, DNA polymerase activity is assessed in accordance with the polymerase activity assay described in the Example section.

Some DNA polymerases of the present invention have a DNA polymerase activity (specific activity) of at least 200,000 mRFU/min/µg, at least 250,000

mRFU/min/µg or at least 300,000 mRFU/min/µg when analysed at 25°C in accordance with a DNA polymerase activity assay described herein, preferably when analysed in accordance with the DNA polymerase activity assay described in the Example section herein.

Some DNA polymerases of the present invention have a high strand displacement activity. This is an important property as in many isothermal amplification methods success relies on the inherent strand displacement activity of the DNA polymerase used in the reaction setup. The term "strand displacement" describes the ability of the polymerase to displace downstream DNA encountered during synthesis.

Some DNA polymerases of the present invention have a higher strand displacement activity than other commercially available DNA polymerases at a temperature of about 25°C (preferably 25 °C).

Suitable assays to assess strand displacement activity of a DNA polymerase are known in the art and a skilled person is readily able to select a suitable assay. In an exemplary strand displacement activity assay, a "cold" primer and a reporter strand that is labelled with a fluorophore (e.g. TAMRA) at its 3' end are annealed to a template strand that has a quencher (e.g. BHQ2) at its 5' end (the fluorophore is thus quenched by the close proximity of the quencher) such that there is a one nucleotide gap between the 3' end of the annealed primer and the 5' end of the annealed reporter strand; upon strand displacement activity of the DNA polymerase the fluorophore labelled oligonucleotide (reporter strand) is displaced from the template strand and as a consequence the fluorophore and quencher are no longer in close proximity and an increase in fluorescence can be measured.

Strand displacement activity may be assessed in an assay having the steps of (i) providing a template DNA molecule that has a quencher (fluorescence quencher) at its 5' end, (ii) annealing to said template DNA molecule a cold primer (i.e. non-fluorescent oligonucleotide) and a reporter strand (reporter oligonucleotide) that is labelled with a fluorophore at its 3' end wherein there is a one nucleotide gap between the 3' end of the annealed primer and the 5' end of the annealed reporter strand, whereby the quencher quenches the fluorophore by virtue of their close proximity to each other, (iii) incubating said template-cold primer-reporter strand complex with a DNA polymerase (e.g. at about 25°C, preferably 25 °C) and (iv) measuring the fluorescence of the previously quenched fluorophore, wherein said fluorescence is indicative of strand displacement activity.

In preferred embodiments, strand displacement is as assessed in an assay that is carried out at 25°C.

Preferred primers, reporter strands and template strands are as described in the Example.

In a preferred embodiment strand displacement activity is as assessed in accordance with the strand displacement activity assay described in the Example section.

Some DNA polymerases of the present invention have at least 50%, at least 75%, at least 100%, at least 150%, at least 200%, at least 500%, at least 1000% or at least 2000% higher strand displacement activity than the *E.coli* Klenow fragment DNA polymerase. Preferably, the strand displacement activity is as assessed at about 25°C (25°C is preferred). Strand displacement activity may be assessed using a strand displacement assay as described herein (e.g. carried out at 25°C). More preferably, strand displacement activity is assessed in accordance with the strand displacement assay described in the Example section.

Some DNA polymerases of the present invention have at least 50%, at least 75%, at least 100%, at least 150%, at least 200%, at least 500, at least 600% or at least 700% higher strand displacement activity than the *G. stearothermophilus* DNA polymerase. Preferably, the strand displacement activity is as assessed at about 25°C (25°C is preferred). Strand displacement activity may be assessed using a strand displacement assay as described herein (e.g. carried out at 25°C). More preferably, strand displacement activity is assessed in accordance with the strand displacement assay described in the Example section.

Some DNA polymerases of the present invention have at least 50%, at least 75%, at least 100%, at least 150%, at least 200% or at least 400% higher strand displacement activity than the commercially available variant of *G. stearothermophilus* DNA polymerase (Bst2.0). Preferably, the strand displacement activity is as assessed at about 25°C (25°C is preferred). Strand displacement activity may be assessed using a strand displacement assay as described herein (e.g. carried out at 25°C). More preferably, strand displacement activity is assessed in accordance with the strand displacement assay described in the Example section.

Some DNA polymerases of the present invention have a higher strand displacement activity compared with other marine polymerases (e.g. DNA polymerase from *Aliivibrio Salmonicida*). Some DNA polymerases of the present

invention have a higher strand displacement activity than other marine DNA polymerases at a temperature of about 25°C (25°C is preferred). In some embodiments, DNA polymerases of the present invention have at least 50%, at least 75%, at least 100%, at least 150%, at least 200% or at least 500% higher strand displacement than the DNA polymerase from *Aliivibrio Salmonicida*. Preferably, the strand displacement activity is as assessed at about 25°C (25°C is preferred). Strand displacement activity may be assessed using a strand displacement assay as described herein (e.g. carried out at 25°C). More preferably, strand displacement activity is assessed in accordance with the strand displacement assay described in the Example section.

Some DNA polymerases of the present invention have a strand displacement activity of at least 20,000 mRFU/min/µg, at least 25,000 mRFU/min/µg or at least 30,000 mRFU/min/µg when analysed at 25°C in accordance with a strand displacement activity assay described herein, preferably in accordance with the strand displacement activity assay in the Example section herein.

Preferred DNA polymerases of the present invention have high processivity. "Processivity" in the context of DNA polymerases is the ability of a DNA polymerase to remain bound to the DNA template strand during synthesis (nucleotide incorporation) before dissociating, and the overall efficiency of DNA synthesis may therefore increase with increasing processivity of a given polymerase. Put another way, processivity represents a measure of the average number of nucleotides added by a DNA polymerase, per association event with the template strand. A DNA polymerase with high processivity may be of particular importance if longer stretches of DNA need to be synthesized.

Some DNA polymerases of the present invention have higher processivity than the commercial *G. stearothermophilus* DNA polymerase (BstpolI, Bst2.0) and *Bacillus subtilis* DNA polymerase (also referred to herein as BSU). In preferred embodiments, the higher processivity is significantly higher. In some preferred embodiments the DNA polymerases of the present invention have at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 75%, at least 100%, at least 200%, at least 500%, at least 1000%, at least 1500% or at least 2000% higher processivity than the commercial *G. stearothermophilus* DNA polymerase (BstpolI, Bst2.0) and/or the *B.subtilis* DNA polymerase (also referred to herein as BSU).

Some DNA polymerases of the present invention have at least 5000%, at least 10,000%, at least 15,000%, at least 20,000%, at least 25,000% or at least 30,000% higher processivity than the commercial *G. stearothermophilus* DNA polymerase (BstpolI, Bst2.0).

Some DNA polymerases of the present invention have at least 1000%, at least 1500% or at least 2000% higher processivity than the *B.subtilis* DNA polymerase (also referred to herein as BSU).

Suitable assays for analysing processivity are well known in the art. A suitable and particularly preferred processivity assay is described herein in the Example section.

Preferred DNA polymerases of the present invention have useful levels of polymerase activity across a range of salt (NaCl) concentrations. Put another way, DNA polymerases of the present invention exhibit across a broad range of salt concentrations a substantial proportion of the DNA polymerase activity observed at the salt concentration at which maximum polymerase activity is observed. Suitable assays for determining DNA polymerase activity are described elsewhere herein. A preferred assay for determining DNA polymerase activity is an isothermal molecular beacon assay, e.g. as described herein, preferably as described in the Example section.

In some embodiments, the NaCl concentration at which the DNA polymerase exhibits its maximum activity is about 65mM to 100mM (e.g. 70, 75, 80, 85 or 90mM).

In some embodiments, across a concentration range from about 0mM to 210mM NaCl, DNA polymerases of the present invention exhibit a substantial proportion (e.g. at least 30%) of their maximum polymerase activity.

In some embodiments, across a concentration range from about 20mM to 150mM NaCl, DNA polymerases of the present invention exhibit at least 60% of their maximum polymerase activity.

In some embodiments, across a concentration range from about 60mM to 120mM NaCl, DNA polymerases of the present invention exhibit at least 80% (preferably at least 90% or at least 95%) of their maximum polymerase activity.

Preferably, the above described abilities and properties are observed at a measurable or significant level and more preferably at a statistically significant level, when compared to appropriate control levels. Appropriate significance levels are discussed elsewhere herein. More preferably, one or more of the above described

abilities and properties are observed at a level which is measurably better, or more preferably significantly better, when compared to the abilities observed for prior art DNA polymerases.

In another aspect, the present invention provides an isolated DNA polymerase comprising (or consisting of) the amino acid sequence of SEQ ID NO:1 or comprising (or consisiting of) an amino acid sequence which is at least 70% identical to SEQ ID NO:1, wherein said DNA polymerase lacks a 5'-3' exonuclease domain (e.g. lacks some or all of the amino acid sequence of SEQ ID NO:5). Other features and properties of other aspects of the invention apply, *mutatis mutandis*, to this aspect of the invention.

In yet another aspect, the present invention provides an isolated DNA polymerase comprising (or consisting of) the amino acid sequence of SEQ ID NO:1 or comprising (or consisting of) an amino acid sequence which is at least 70% identical to SEQ ID NO:1, wherein said DNA polymerase is not a wildtype DNA polymerase from a *Psychrobacillus* sp. Other features and properties of other aspects of the invention apply, *mutatis mutandis*, to this aspect of the invention.

In a further aspect the present invention provides molecules (e.g. proteins) comprising DNA polymerases of the present invention.

As used throughout the entire application, the terms "a" and "an" are used in the sense that they mean "at least one", "at least a first", "one or more" or "a plurality" of the referenced components or steps, except in instances wherein an upper limit or exclusion is thereafter specifically stated. The operable limits and parameters of combinations, as with the amounts of any single agent, will be known to those of ordinary skill in the art in light of the present disclosure.

Nucleic acid molecules comprising nucleotide sequences that encode DNA polymerases of the present invention as defined herein or fragments thereof, or nucleic acid molecules substantially homologous thereto, form yet further aspects of the invention. A preferred nucleic acid molecule is a nucleic acid encoding the *Psychrobacillus* species DNA polymerase I sequence of SEQ ID NO:1, or a sequence substantially homologous thereto. A preferred nucleic acid molecule comprises (or consists of) the nucleotide sequence as set forth in SEQ ID NO:2, or is a sequence substantially homologous thereto. Optionally, the final three nucleotides of SEQ ID NO:2 may be omitted. Nucleic acid sequences of the invention include sequences having at least 70% or 75%, preferably at least 80%, and even more preferably at least 85%, 90%, 95%, 96%, 97%, 98%, 99% or 99.5%,

sequence identity to SEQ ID NO:2. Nucleic acid sequences of the invention thus include single or multiple base alterations (additions, substitutions, insertions or deletions) to the sequence of SEQ ID NO:2.

A particularly preferred nucleic acid molecule comprises the nucleotide sequence as set forth in SEQ ID NO:2.

Another preferred nucleic acid molecule consists of the nucleotide sequence as set forth in SEQ ID NO:2.

The present invention also extends to nucleic acid molecules comprising (or consisting of) nucleotide sequences which are degenerate versions of nucleic acid molecules described herein, e.g. degenerate versions of a nucleic acid molecule comprising (or consisting of) SEQ ID NO:2.

Nucleic acid molecules of the invention are preferably “isolated” or “purified”.

Homology (e.g. sequence identity) may be assessed by any convenient method. However, for determining the degree of homology (e.g. identity) between sequences, computer programs that make multiple alignments of sequences are useful, for instance Clustal W (Thompson, Higgins, Gibson, *Nucleic Acids Res.*, 22:4673-4680, 1994). If desired, the Clustal W algorithm can be used together with BLOSUM 62 scoring matrix (Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA*, 89:10915–10919, 1992) and a gap opening penalty of 10 and gap extension penalty of 0.1, so that the highest order match is obtained between two sequences wherein at least 50% of the total length of one of the sequences is involved in the alignment. Other methods that may be used to align sequences are the alignment method of Needleman and Wunsch (Needleman and Wunsch, *J. Mol. Biol.*, 48:443, 1970) as revised by Smith and Waterman (Smith and Waterman, *Adv. Appl. Math.*, 2:482, 1981) so that the highest order match is obtained between the two sequences and the number of identical amino acids is determined between the two sequences. Other methods to calculate the percentage identity between two amino acid sequences are generally art recognized and include, for example, those described by Carillo and Lipton (Carillo and Lipton, *SIAM J. Applied Math*., 48:1073, 1988) and those described in Computational Molecular Biology, Lesk, e.d. Oxford University Press, New York, 1988, Biocomputing: Informatics and Genomics Projects.

Generally, computer programs will be employed for such calculations. Programs that compare and align pairs of sequences, like ALIGN (Myers and Miller, *CABIOS*, 4:11-17, 1988), FASTA (Pearson and Lipman, *Proc. Natl. Acad. Sci. USA*,

85:2444-2448, 1988; Pearson, *Methods in Enzymology*, 183:63-98, 1990) and gapped BLAST (Altschul *et al.*, *Nucleic Acids Res.*, 25:3389-3402, 1997), BLASTP, BLASTN, or GCG (Devereux, Haeberli, Smithies, *Nucleic Acids Res.*, 12:387, 1984) are also useful for this purpose. Furthermore, the Dali server at the European Bioinformatics institute offers structure-based alignments of protein sequences (Holm, *Trends in Biochemical Sciences*, 20:478-480, 1995; Holm, *J. Mol. Biol.*, 233:123-38, 1993; Holm, *Nucleic Acid Res.*, 26:316-9, 1998).

By way of providing a reference point, sequences according to the present invention having 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% homology, sequence identity etc. may be determined using the ALIGN program with default parameters (for instance available on Internet at the GENESTREAM network server, IGH, Montpellier, France).

A "conservative amino acid substitution", as used herein, is one in which the amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art.

DNA polymerases of the present invention comprise genetically encoded amino acids, but may also contain one or more non-genetically encoded amino acids.

In addition to having DNA polymerase activity, variants or enzymatically active fragments of DNA polymerases of the invention described herein preferably have one or more of the other properties described herein, more preferably all of the other properties described herein.

The term "isolated" or "purified" as used herein in reference to nucleic acid molecules or sequences and proteins or polypeptides, e.g., DNA polymerases, refers to such molecules when isolated from, purified from, or substantially free of their natural environment, *e.g.,* isolated from or purified from an organism (if indeed they occur naturally), or refers to such molecules when produced by a technical process, i.e., includes recombinant and synthetically produced molecules.

Thus, when used in connection with a protein or polypeptide molecule such as a DNA polymerase, the term "isolated" or "purified" typically refers to a protein substantially free of cellular material or other proteins from the source from which it is derived. In some embodiments, such isolated or purified proteins are substantially free of culture medium when produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized.

Possible expression vectors include but are not limited to cosmids or plasmids, so long as the vector is compatible with the host cell used. The expression vectors are "suitable for transformation of a host cell", which means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences selected on the basis of the host cells to be used for expression, which are operatively linked to the nucleic acid molecule. Operatively linked is intended to mean that the nucleic acid is linked to regulatory sequences in a manner that allows expression of the nucleic acid.

In one aspect the present invention therefore provides an expression vector (preferably a recombinant expression vector) containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the protein sequence encoded by the nucleic acid molecule of the invention.

Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes and are well known in the art. Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector.

The recombinant expression vectors of the invention may also contain a selectable marker gene that facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention.

The recombinant expression vectors may also contain genes that encode a fusion moiety that provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a ligand in affinity purification (for example appropriate "tags" to enable purification and/or identification may be present, e.g., His tags or myc tags).

Recombinant expression vectors can be introduced into host cells to produce a transformed host cell. The terms "transformed with", "transfected with",

"transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g., a vector) into a cell by one of many possible techniques known in the art. Suitable methods for transforming and transfecting host cells can be found in Sambrook *et al*., 1989 (Sambrook, Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989) and other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic host cells and eukaryotic cells. Preferably, proteins of the invention may be expressed in bacterial host cells, such as *Escherichia coli*.

N-terminal or C-terminal fusion proteins comprising DNA polymerases and proteins of the invention conjugated to other molecules, such as proteins (e.g. epitope tags), may be prepared by fusing through recombinant techniques.

A yet further aspect provides a host cell or virus comprising one or more expression constructs or expression vectors of the invention. Also provided are host cells or viruses comprising one or more of the nucleic acid molecules of the invention. A host cell or virus capable of expressing a DNA polymerase of the invention forms a yet further aspect. Preferred host cells include Rosetta 2 (DE3) cells (Novagen).

Where appropriate, DNA polymerases of the invention may be isolated from a natural source, e.g. isolated from extracts of a *Psychrobacillus*, or produced recombinantly in a host cell and isolated and purified therefrom. The DNA polymerases of the invention may therefore be recombinant enzymes, in particular isolated recombinant enzymes. In certain embodiments the DNA polymerase is produced by recombinant techniques in a host cell that is not, or not from, an organism which is the same as that in which the DNA polymerase is found naturally, i.e. a heterologous host cell.

DNA polymerases of the present invention may be generated using recombinant DNA technology. Alternatively, a cell-free expression system can be used for production of the DNA polymerase. Alternatively, DNA polymerases of the present invention may be generated using chemical synthesis so that the DNA polymerase is generated by stepwise elongation, one amino acid at a time. Such chemical synthesis techniques (e.g. solid phase synthesis) are well known in the chemistry of proteins.

A further aspect of the invention provides a method of producing a DNA polymerase of the present invention comprising a step of culturing the host cells of

the invention. Preferred methods comprise the steps of (i) culturing a host cell comprising one or more of the recombinant expression vectors or one or more of the nucleic acid molecules of the invention under conditions suitable for the expression of the encoded DNA polymerase or protein; and optionally (ii) isolating or obtaining the DNA polymerase or protein from the host cell or from the growth medium/supernatant. Such methods of production may also comprise a step of purification of the DNA polymerase or protein product and/or formulating the DNA polymerase or product into a composition including at least one additional component, such as an acceptable buffer or carrier.

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 invention may be modified to carry amino acid motifs or other protein or non-protein tags, e.g. polyhistidine tags (e.g. His_6-tag), to assist in isolation, solubilisation and/or purification or identification.

A preferred method of producing a DNA polymerase of the invention is described in the Example section herein.

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

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

In another aspect, the present invention provides the use of a DNA polymerase of the invention in a molecular beacon assay, or in a strand displacement assay or in a single-nucleotide incorporation assay, e.g. as described herein.

Preferably, in uses and methods of the present invention, DNA polymerases of the present invention are used at a constant temperature, i.e. without thermal cycling. Accordingly, the use of DNA polymerases of the invention in isothermal reactions is particularly preferred. The use of DNA polymerases of the invention 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 aspect, the present invention provides a method of nucleotide polymerisation using a DNA polymerase of the present invention. Preferably, said method comprises providing a reaction mixture comprising a DNA polymerase of the present invention, 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 (e.g. deoxynucleoside triphosphates, dNTPS) and 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. Suitable conditions are well known in the art. Preferably a constant temperature is used and preferred temperatures are set out elsewhere herein. Optionally, the generation of the polynucleotide product is detected (e.g. via gel electrophoresis).

In another aspect, the present invention provides a method of amplifying a nucleic acid (DNA) using a DNA polymerase of the present invention. Typically, said method comprises providing a reaction mixture comprising a DNA polymerase of the present invention, a template nucleic acid molecule, an oligonucleotide primer(s) (e.g. 2 or more primers such as 2, 3, 4, 5 or 6 primers) which is capable of annealing to a portion of the template nucleic acid molecule acid molecule, and nucleotides (e.g. deoxynucleoside triphosphates, dNTPS) and 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. Suitable conditions are well known in the art. Preferred methods of nucleic acid amplification are isothermal amplification methods. Isothermal amplification methods of the invention are performed at a constant temperature and preferred temperatures are set out elsewhere herein. Optionally, the generation of the polynucleotide product is detected (e.g. via gel electrophoresis).

Exemplary isothermal amplification methods include Loop mediated isothermal amplification (LAMP), rolling circle amplification (RCA), strand

displacement amplification (SDA), multiple displacement amplification (MDA) and cross priming amplification (CPA).

Preferably, the constant temperature used in the methods and uses of the present invention is a low-to-moderate temperature, for example, 0°C to about 42°C, preferably about 10°C to about 40°C, or about 20°C to about 40°C, or about 25°C to about 40°C, or about 30°C to about 40°C or about 35°C to about 40°C, or about 37°C to about 40°C. In some embodiments, the constant temperature is about 10°C to about 15°C or is about 10°C to about 20°C. In some embodiments, the constant temperature is about 10°C to about 30°C. In some embodiments, the constant temperature is about 20°C to about 30°C. In some embodiments, the constant temperature is about 10°C to about 25°C. In some embodiments, the constant temperature is about 20°C to about 25°C. A constant temperature of about 25°C is preferred. In some embodiments, the constant temperature is 25°C.

A temperature may be considered constant when no active steps are taken to modify the temperature during the reaction, e.g. no thermal cycling. A 'constant' temperature may still allow temperature fluctuations during the method of up to about 5°C, typically no more than 3°C or 2°C.

DNA polymerases of the present invention may be used in point-of-care molecular diagnostics platforms.

DNA polymerases of the present invention may be used in whole genome amplification.

DNA polymerases of the present invention may be used in next-generation sequencing methods. So-called "next generation" or "second generation" sequencing approaches (in reference to the Sanger dideoxynucleotide method as the "first generation" approach) have become widespread. These newer techniques are characterised by high throughputs, e.g. as a consequence of the use of parallel, e.g. massively parallel sequencing reactions, or through less time-consuming steps. Various high throughput sequencing methods provide single molecule sequencing and employ techniques such as pyrosequencing, reversible terminator sequencing, cleavable probe sequencing by ligation, non-cleavable probe sequencing by ligation, DNA nanoballs, and real-time single molecule sequencing.

Uses and methods employing enzymatically active fragments of DNA polymerases of the invention are also provided and references herein to DNA polymerases of the invention encompass such active fragments unless otherwise clear from the context.

Uses and methods of the present invention are typically performed *in vitro*.

The present invention also provides compositions comprising a DNA polymerase of the invention. Such compositions preferably comprise a buffer. Optionally, compositions of the present invention further comprise one or more of the necessary reagents to carry out a nucleic acid amplification reaction (e.g. an isothermal amplification reaction), e.g. oligonucleotide primers capable of annealing to a region of the template DNA to be amplified and/or nucleotides (e.g. dNTPs). Typically compositions will be aqueous and buffered with a standard buffer such as Tris, HEPES, etc.

The invention further includes kits comprising one or more of the DNA polymerases of the invention, or one or more compositions of the invention, or one or more of the nucleic acid molecules of the invention, or one or more expression vectors of the invention, or one or more host cells or viruses of the invention. Preferably said kits are for use in the methods and uses as described herein, e.g., in nucleic acid amplification methods, such as isothermal amplification reactions. Preferably said kits comprise instructions for use of the kit components, for example for nucleic acid amplification.

NUCLEOTIDE AND AMINO ACID SEQUENCES DISCLOSED HEREIN AND THEIR SEQUENCE IDENTIFIERS (SEQ ID NOs)

All nucleotide sequences are recited herein 5' to 3' in line with convention in this technical field.

SEQ ID NO:1 – amino acid sequence of truncated DNA polymerase I isolated from a *Psychrobacillus* sp.

SEQ ID NO:1

TEVAFEIVEEIDSTILDKVMSVHLEMYDGQYHTSELLGIALSDGEKGYFAPADIAFQ
SKDFCSWLENATNKKYLADSKATQAVSRKHNVNVHGVEFDLLLAAYIVNPAISSED
VAAIAKEFGYFNLLTNDSVYGKGAKKTAPEIEKIAEHAVRKARAIWDLKEKLEVKLE
ENEQYALYKEIELPLASILGTMESDGVLVDKQILVEMGHELNIKLRAIEQDIYALAGE
TFNINSPKQLGVILFEKIGLTPIKKTKTGYSTAADVLEKLASEHEIIEQILLYRQLGKLN
STYIEGLLKEIHEDDGKIHTRYQQALTSTGRLSSINPNLQNIPVRLEEGRKIRKAFVP
SQPGWVMFAADYSQIELRVLAHMSEDENLVEAFNNDLDIHTKTAMDVFHVEQEAV
TSDMRRAAKAVNFGIVYGISDYGLSQNLDITRKEAATFIENYLNSFPGVKGYMDDIV
QDAKQTGYVTTILNRRRYLPEITSSNFNLRSFAERTAMNTPIQGSAADIIKKAMIDM
AERLISENMQTKMLLQVHDELIFEAPPEEIAMLEKIVPEVMENAIKLIVPLKVDYAFG
SSWYDTK

SEQ ID NO:2 – nucleic acid sequence encoding the *Psychrobacillus* species DNA polymerase I sequence of SEQ ID NO:1

SEQ ID NO:2

acagaagtagcattcgagattgttgaagaaattgactctacaatattagataaagtaatgtcagtccat
ttagaaatgtatgatgggcaatatcatacaagcgaattattaggtattgctttatcagat
ggagaaaagggttattttgctcctgctgatatagcttttcaatcgaaggatttttgttct
tggttagaaaatgctacgaataaaaagtatttagcagactccaaagcaacacaagcagtg
agtagaaaacataatgtgaatgtacatggagtggaattcgaccttcttttagcagcgtat
atagtaaatcctgctatctcttcagaggatgttgctgctattgctaaagaatttggatat
tttaacttgctgacaaacgatagtgtttatgggaaaggtgccaaaaaaaaccgcacctgaa
atcgagaaaattgcagaacatgccgtaagaaaagcaagggctatttgggacttgaaagaa
aagttagaagtaaaactggaagaaaatgaacaatatgcgttgtataaagaaatagagcta
ccgcttgcatctatccttggtacgatggaatcagatggggtgctggtggataaacaaatt
cttgtagaaatgggtcatgagcttaatattaagttacgagcgattgaacaagacatttat
gcgttagctggtgaaacgtttaatattaattcacctaaacaattaggtgtaatactattt
gaaaaaattggtcttacccctattaaaaagacaaaaacgggctattcaactgcagcagat
gttttggaaaaactagcaagtgaacatgaaataatagagcaaattttactatatcgtcaa
ttaggtaaactcaattccacatatatcgaaggattattaaaagagattcatgaagatgat
gggaagatccatacccgatatcaacaagccctaacttcaactgggcgtttgagttcgatc
aatccaaaccttcaaaatataccagttcgtttagaagaaggtagaaaaatacgtaaagcc
tttgttccttcacaaccgggatgggtaatgtttgcggcggattactctcaaattgaattg
cgtgttcttgcccatatgtctgaggatgaaaacctggtagaagcttttaataatgatctg
gatattcatactaaaacggctatggatgtattccatgtggagcaggaagcagtaacgtcc
gatatgcgccgtgctgctaaggcagttaactttgggattgtgtatggtattagtgattat
ggtttatcacaaaacctagatattactagaaaagaagcggcgacatttatcgagaattat
ttaaatagcttcccaggtgtaaaaggatatatggatgatatcgttcaagatgcgaaacaa
acaggctacgttacaacaattttgaatagacgaagatatttgcctgaaataacaagttct
aactttaatctccgcagttttgcagaacgtactgctatgaatacaccaattcaagggagt
gcagccgatattattaaaaaagcaatgatcgatatggcggaaagattaatatcagaaaat
atgcagaccaaaatgctactacaagtacatgatgaattaatttttgaggctccaccagag
gaaattgcaatgctagaaaaaatagtgccagaggtgatggaaaacgctattaaactgatt
gtacctttgaaagtggattatgcctttggttcatcttggtatgacacgaagtag

SEQ ID NO:3 – amino acid sequence of full-length DNA polymerase I isolated from a *Psychrobacillus* sp.

SEQ ID NO:3

MYLSTEKILLLDGNSLAYRAFFALPLLTNEHGIHTNAVYGFTMMLQKIMDEENPTHMLVA
FDAGKTTFRHSTFGDYKGGRQKTPPELSEQFPYIRKLIDAYGIKRYELEMYEADDIIGTL
SKRADEKGQQVVIVSGDKDLTQLATDKTTVYITRKGITDIEKYTPEHVQEKYGLTPLQII
DMKGLMGDASDNIPGVPGVGEKTAIKLLKEHGSVEDLYKALDTVSGVKLKEKLIANEEQA
IMSKALATIETAAPIQISIDDLSYTGPNMEEVIEVWKELAFKTLLEKSDYISEESETTEV
AFEIVEEIDSTILDKVMSVHLEMYDGQYHTSELLGIALSDGEKGYFAPADIAFQSKDFCS
WLENATNKKYLADSKATQAVSRKHNVNVHGVEFDLLLAAYIVNPAISSEDVAAIAKEFGY
FNLLTNDSVYGKGAKKTAPEIEKIAEHAVRKARAIWDLKEKLEVKLEENEQYALYKEIEL
PLASILGTMESDGVLVDKQILVEMGHELNIKLRAIEQDIYALAGETFNINSPKQLGVILF
EKIGLTPIKKTKTGYSTAADVLEKLASEHEIIEQILLYRQLGKLNSTYIEGLLKEIHEDD
GKIHTRYQQALTSTGRLSSINPNLQNIPVRLEEGRKIRKAFVPSQPGWVMFAADYSQIEL
RVLAHMSEDENLVEAFNNDLDIHTKTAMDVFHVEQEAVTSDMRRAAKAVNFGIVYGISDY
GLSQNLDITRKEAATFIENYLNSFPGVKGYMDDIVQDAKQTGYVTTILNRRRYLPEITSS
NFNLRSFAERTAMNTPIQGSAADIIKKAMIDMAERLISENMQTKMLLQVHDELIFEAPPE
EIAMLEKIVPEVMENAIKLIVPLKVDYAFGSSWYDTK

SEQ ID NO:4 – nucleic acid sequence encoding the *Psychrobacillus* sp. DNA polymerase I sequence of SEQ ID NO:3.

SEQ ID NO:4

atgtatttgtcaaccgagaaaatcctattattagacggcaatagtttggcataccgagct
ttttttgccctacctttattaacaaatgaacatggaatacatacaaacgcagtatatggc
tttacaatgatgctacaaaaaattatggatgaagaaaatcctactcatatgctcgtggca
tttgatgccgggaaaacgaccttccgtcactctacttttggggattataaaggtggaaga
caaaaaacaccaccagaactatcggaacaattcccttatatacgcaagttaatcgatgct
tatggtattaagcgatacgaactggaaatgtacgaagcagacgatattatcggtacttta
agcaagcgtgcagacgaaaaagggcagcaagttgtaattgtctcaggtgataaagattta
acacaactagctacagataaaacaactgtgtatatcacaagaaaaggcataaccgatatt
gaaaaatatacacctgaacatgtacaagaaaagtatggcttaactccattacagattata
gacatgaaaggtttaatgggagatgcttctgataatattccaggagttcctggtgtcgga
gaaaaaacagctattaagcttttaaaagaacatggttcggtagaggatttatataaagca
cttgatacagttagtggtgttaaactaaaggaaaaactcatcgccaacgaagagcaggca
attatgagtaaggcattagctacgattgaaacagctgcaccgatacagatttctatagac
gatctttcatatactggtcctaatatggaagaagtaattgaagtttggaaggaactagct
tttaaaactcttcttgagaaatctgactatatttctgaggaatccgaaactacagaagta
gcattcgagattgttgaagaaattgactctacaatattagataaagtaatgtcagtccat
ttagaaatgtatgatgggcaatatcatacaagcgaattattaggtattgctttatcagat
ggagaaaagggttattttgctcctgctgatatagcttttcaatcgaaggatttttgttct
tggttagaaaatgctacgaataaaaagtatttagcagactccaaagcaacacaagcagtg
agtagaaaacataatgtgaatgtacatggagtggaattcgaccttcttttagcagcgtat
atagtaaatcctgctatctcttcagaggatgttgctgctattgctaaagaatttggatat
tttaacttgctgacaaacgatagtgtttatgggaaaggtgccaaaaaaaccgcacctgaa
atcgagaaaattgcagaacatgccgtaagaaaagcaagggctatttgggacttgaaagaa
aagttagaagtaaaactggaagaaaatgaacaatatgcgttgtataaagaaatagagcta

ccgcttgcatctatccttggtacgatggaatcagatggggtgctggtggataaacaaatt
cttgtagaaatgggtcatgagcttaatattaagttacgagcgattgaacaagacatttat
gcgttagctggtgaaacgtttaatattaattcacctaaacaattaggtgtaatactattt
gaaaaaattggtcttacccctattaaaaagacaaaaacgggctattcaactgcagcagat
gttttggaaaaactagcaagtgaacatgaaataatagagcaaatttttactatatcgtcaa
ttaggtaaactcaattccacatatatcgaaggattattaaaagagattcatgaagatgat
gggaagatccatacccgatatcaacaagccctaacttcaactgggcgtttgagttcgatc
aatccaaaccttcaaaatataccagttcgtttagaagaaggtagaaaaatacgtaaagcc
tttgttccttcacaaccgggatgggtaatgtttgcggcggattactctcaaattgaattg
cgtgttcttgcccatatgtctgaggatgaaaacctggtagaagcttttaataatgatctg
gatattcatactaaaacggctatggatgtattccatgtggagcaggaagcagtaacgtcc
gatatgcgccgtgctgctaaggcagttaactttgggattgtgtatggtattagtgattat
ggtttatcacaaaacctagatattactagaaaagaagcggcgacatttatcgagaattat
ttaaatagcttcccaggtgtaaaaggatatatggatgatatcgttcaagatgcgaaacaa
acaggctacgttacaacaattttgaatagacgaagatatttgcctgaaataacaagttct
aactttaatctccgcagttttgcagaacgtactgctatgaatacaccaattcaagggagt
gcagccgatattattaaaaaagcaatgatcgatatggcggaaagattaatatcagaaaat
atgcagaccaaaatgctactacaagtacatgatgaattaatttttgaggctccaccagag
gaaattgcaatgctagaaaaaatagtgccagaggtgatggaaaacgctattaaactgatt
gtacctttgaaagtggattatgcctttggttcatcttggtatgacacgaagtag

SEQ ID NO:5 – sequence containing a 5'-3' exonuclease functional domain of DNA polymerase I isolated from a *Psychrobacillus* sp.

SEQ ID NO:5

MYLSTEKILLLDGNSLAYRAFFALPLLTNEHGIHTNAVYGFTMMLQKIMDEENPTHMLVAF
DAGKTTFRHSTFGDYKGGRQKTPPELSEQFPYIRKLIDAYGIKRYELEMYEADDIIGTLSK
RADEKGQQVVIVSGDKDLTQLATDKTTVYITRKGITDIEKYTPEHVQEKYGLTPLQIIDMK
GLMGDASDNIPGVPGVGEKTAIKLLKEHGSVEDLYKALDTVSGVKLKEKLIANEEQAIMSK
ALATIETAAPIQISIDDLSYTGPNMEEVIEVWKELAFKTLLEKSDYISEESET

SEQ ID NO:6 – amino acid sequence of the truncated DNA polymerase of *Aliivibrio salmonicida* used in experiments described herein.

SEQ ID NO:6

VDRSKYETIFTKEAFSAWLEKVNNAEVTAFDTETDSLDYMVANLIGLSFSVEEGEAAYVP
VAHDYLDAPEQLDRDWVLAQLKPYLEDETKAKVGQNLKYDASVLARYDIEMKGIKFDTML
ESYVYNSVAGKHNMDSLALRYLQHNTISFEEIAGKGKKQLTFNQIALEEAAPYAAEDADI
TLRLHNVLHAKLVTDEKLNAVFTDIELPLISVLSRMERKGVYIDDMLLSAQSLEIGQRLD
ELETASFEVAGQEFNMNSPKQLQTILFEKMELPVIKKTPSGAASTNEEVLQELALEYELP
KLILEYRGLAKLKSTYTDKLPKMINPSTGRVHTSYHQAVTATGRLSSTDPNLQNIPIRNK
EGRRIRQAFVAPHGWKILAVDYSQIELRIMAHLSQDRALLEAFSAGKDIHAATAAEVKGV

SIEEVTSEDRRRAKAINFGLIYGMSAFGLAKQIGISRGEAQDYMNVYFERYPGVMQYMEE
TRLLATEQGYVETLYGRRLYLPEINARNAIRRKAAERAAINAPMQGTAADIIKKAMILVD
NWIEAEGTGRVNLLMQVHDELVFEVKEDELEAITKQVTALMEAAVSLDVPLIAESGFGDN
WDEAH

Commercial enzymes used for comparison with the PB DNA polymerase, namely KF, Bst, Bst2.0, BSU, T4 and T7 DNA polymerase are all bought from New England Biolabs.

The invention will now be described by way of non-limiting Example with reference to the following figures in which:

Figure 1 shows a comparison of the polymerase activity at 25 °C (specific activity) of the recombinantly produced marine polymerase from a *Psychrobacillus* sp. (PB) with other polymerases. The commercially known polymerases are KF (Klenow Fragment *E. coli*), Bst (*G. stearothermophilus*), Bst 2.0 (*G. stearothermophilus*) and BSU (*B. subtilis*).

Figure 2 shows an overview of the strand-displacement activity assay setup. F= fluorophore. Q= Quencher.

Figure 3 shows a comparison of the strand-displacement activity at 25°C (specific activity) of the recombinantly produced marine polymerase from *Psychrobacillus* sp. (PB) with other polymerases. The commercially known polymerases are KF (Klenow Fragment *E. coli*), Bst (*G. stearothermophilus*), Bst 2.0 (*G. stearothermophilus*) and BSU (*B. subtilis*).

Figure 4 shows the polymerase activity of the PB polymerase and other polymerases at various NaCl concentrations. The commercially known polymerases are KF (Klenow Fragment *E. coli*), Bst (*G. stearothermophilus*), Bst 2.0 (*G. stearothermophilus*) and BSU (*B. subtilis*).

Figure 5 shows the effect of temperature on polymerase activity of PB polymerase.

Figure 6 shows the effect of temperature on the stability of the PB polymerase compared to KF. KF is Klenow Fragment (*E.coli*).

Figure 7 shows the melting temperature (T_m (^oC)) analysis of the PB polymerase I at different pH.

Figure 8 shows a comparison of processivity (lanes marked Pr) between the commercial thermophilic BstpolI (Bst2.0), mesophilic Bsu and PB polI (PbI). T7 is a known high processivity enzyme and is used as a positive control for high processivity. Lanes marked with P indicate the polymerase activity while Pr indicates processivity. Processivity is analysed based on the incubation of a non-labeled DNA trap, which will outcompete rebinding of the DNA polymerase to the labeled substrate if the enzyme dissociates from the template strand and less final product yield (+80 nucleotide incorporations) can be seen on the gel.

EXAMPLE

Cloning, gene expression and protein purification

Production of PB polymerase of SEQ ID NO:1

Based on structural knowledge of *Geobacillus stearothermophilus* DNA polymerase I and its truncated "large fragment" (PDB 1L3S), the unwanted 5´-3´-exonuclease domain has been removed in the expression constructs, i.e. the Forward primer is designed in such a way that the PCR product of the PB polymerase lacks the unwanted exonuclease domain. The gene encoding the DNA polymerase I from the *Psychrobacillus sp.* was cloned into the vector pET151/D-TOPO® using the Gateway® Technology (Thermo Fisher). The starting material for the polymerase chain reaction was the genomic DNA of *Psychrobacillus sp.*. By the use of the forward primer (5'- CACCACAGAAGTAGCATTCGAGATTGTT -3' (SEQ ID NO:7)) and reverse primer (5'- TTACTTCGTGTCATACCAAGATGAACC -3' (SEQ ID NO:8)) the gene has been truncated and is analogous to the so-called large fragment of the polymerase I.

Recombinant protein production was performed in Rosetta 2 (DE3) cells (Novagen®). The cells grew in Terrific Broth and gene expression was induced by addition of IPTG. Protein production was carried out at 15 °C for 6 h. In the first purification step the His_6-tagged protein was purified by immobilized Ni^{2+}-affinity chromatography. The second step was the cleavage of the tag by the tobacco etch virus (TEV) protease performed over night at 4 °C. To separate the protein from the His_6-tag and the His_6-tagged TEV protease a second Ni^{2+}-affinity chromatography has been performed in the third step. Fourth and final step of the protein purification was size-exclusion chromatography on a HiLoad 16/600 Superdex 200 pg (GE Healthcare). The final protein solution was concentrated and stored with 50 % glycerol at -20 °C.

A polymerase activity assay

This experiment compares the DNA polymerase activity of the PB polymerase with certain commercially available polymerases using a molecular beacon assay at 25°C.

The polymerase activity assay is based on a molecular beacon probe (modified from Summerer, *Methods Mol. Biol., 2008, 429, 225-235*). The molecular beacon template consists of a 23mer loop that is connected by a GC-rich 8mer stem region (sequence is indicated in italics) and a 43mer extension. Due to the loop formation the fluorophores Dabcyl and FAM are in close proximity and thus quenched. Upon extension by the DNA polymerase I of the primer that is annealed to the molecular beacon template the stem is opened and the increase in distance of the two fluorophores is measured by the restoration of FAM fluorescence (excitation 485 nm, emission 518 nm).

molecular beacon template

5´-

GGCCCGT^{Dabcyl}AGGAGGAAAGGACATCTTCTAGCA*T*^{FAM}*ACGGGCC*GTCAAGTT CATG GCCAGTCAAGTCGTCAGAAATTTCGCACCAC -3’ (SEQ ID NO:9)

primer

5´- GTGGTGCGAAATTTCTGAC -3’ (SEQ ID NO:10)

The molecular beacon substrate was produced by incubating 20 µl of 10 µM molecular beacon template and 15 µM primer in 10 mM Tris-HCl pH 8.0, 100 mM NaCl for 5 min at 95 °C. The reaction was then let to cool down at room temperature for 2 h. The substrate solution was stored at -20 °C with a final concentration of 10 µM.

Fifty microliter reactions consisted of 200 nM substrate and 200 µM dNTP (equimolar amounts of dATP, dGTP, dCTP and dTTP). For PB polymerase I the reaction further contained 5 mM $MgCl_2$ in 50 mM BIS-TRIS propane at pH 8.5, 100 mM NaCl, 1 mM DTT, 0.2 mg/ml BSA and 2 % glycerol. For the commercially known polymerase Is the respective reaction buffer supplied by New England

Biolabs has been used. Final salt concentration in the reaction buffer has been adjusted to 100 mM according to the optimal salt for the respective polymerases. The activity assay was carried out at 25 °C in black 96-well fluorescence assay plates (Corning®). The reaction was initiated by addition of protein solution (i.e. addition of polymerase). The increase in FAM fluorescence was measured as relative fluorescence units in appropriate time intervals by exciting at 485 nm and emission at 518 nm. The measurement was performed in a SpectraMax® Gemini Microplate Reader (Molecular Devices).

Results of this polymerase activity assay are shown in Figure 1. The PB polymerase shows higher polymerase activity than the commercially (New England Biolabs) available polymerases KF (Klenow fragment *E.coli*), Bst (polymerase from *G.stearothermophilus*), Bst 2.0 (polymerase from *G.stearothermophilus*). Only the BSU polymerase from *B. subtilis* shows a higher polymerase activity at this temperature.

The same type of polymerase activity assay was also performed in order to compare the DNA polymerase activity of the PB polymerase with other DNA polymerases from marine sources (data not shown). The PB polymerase had a markedly higher polymerase activity compared with the other marine polymerases tested (e.g. *Aliivibrio salmonicida* DNA polymerase).

Strand-displacement activity assay

This experiment compares the strand-displacement activity of the PB DNA polymerase with certain commercially available polymerases using a strand-displacement activity assay at 25°C. The ability of a polymerase to displace a strand, termed strand displacement, is important for many isothermal amplification methods.

Important in assessing the strand-displacement property is the design a robust assay. An overview of the assay setup is shown in Figure 2. The assay is based on an increase in fluorescence signal that is measured upon displacement of the quenched reporter strand which is only achievable through strand-displacement

activity of the DNA polymerase. As control the strand-displacement negative polymerase T4 is used and showed no activity in the assay (result not shown).

The substrate for the strand-displacement activity assay consists of a “cold” primer of 19 oligonucleotides (SEQ ID NO:11) and a reporter strand consisting of 20 oligonucleotides that is labeled with the TAMRA fluorophore (F) at its 3’ end (SEQ ID NO:12). The template strand consists of 40 oligonucleotides and is labeled with the Black Hole Quencher 2 (BHQ2) at its 5’ end (SEQ ID NO:13). The primers are annealed to the template strand leaving a one nucleotide gap at position 20 on the template strand. Furthermore are the labels in close proximity and thus the fluorophore TAMRA is quenched by BHQ2. Upon strand-displacement activity of the DNA polymerase I the TAMRA labeled oligonucleotide is displaced from the template strand. As a consequence the fluorophore and the quencher are no longer in close proximity and an increase in TAMRA fluorescence can be measured (excitation 525 nm, emission 598 nm).

5’- TATCCACCAATACTACCCT CGATACTTTGTCCACTCAAT[TAMRA] -3’
3’- ATAGGTGGTTATGATGGGATGCTATGAAACAGGTGAGTTA[BHQ2] -5’

The strand-displacement activity of PB polymerase expressed as mRFU/min/µg has been analysed using the above-described strand-displacement activity assay. The substrate for the strand-displacement activity assay was produced by incubating 20 µl of 10 µM “cold” primer, 10 µM reporter strand and 10 µM template strand in 10 mM Tris-HCl pH 8.0, 100 mM NaCl at 95 °C for 5 min. The reaction was then let to cool down at room temperature for 2 h. The substrate solution was stored at -20 °C with a final concentration of 10 µM.

Fifty microliter reactions consisted of 200 nM substrate and 200 µM dNTP (equimolar amounts of dATP, dGTP, dCTP and dTTP). For PB polymerase I the reaction further contained 5 mM $MgCl_2$ in 50 mM BIS-TRIS propane at pH 8.5, 100 mM NaCl, 1 mM DTT, 0.2 mg/ml BSA and 2 % glycerol. For the commercially known polymerase Is the respective reaction buffer supplied by New England Biolabs has been used. Final salt concentration in the reaction buffer has been adjusted to 100 mM according to the optimal salt for the respective polymerases. The activity assay was carried out at 25 °C in black 96-well fluorescence assay

plates (Corning®). The reaction was initiated by addition of protein solution (i.e. addition of polymerase). The increase in TAMRA fluorescence was measured as relative fluorescence units in appropriate time intervals by exciting at 525 nm and recording emission at 598 nm. The measurement was performed in a SpectraMax® M2e Microplate Reader (Molecular Devices).

Results of this strand-displacement activity assay are shown in Figure 3. The results show that the polymerase from *Psychrobacillus* sp. (PB) possesses superior strand displacement activity than the commercially available polymerases KF (Klenow fragment *E.coli*), Bst (polymerase from *G.stearothermophilus*), Bst 2.0 (polymerase from *G.stearothermophilus*). The *Psychrobacillus* sp. (PB) DNA polymerase shows comparable strand displacement-activity to the commercially available BSU (*B. subtilis*) polymerase.

The same type of strand-displacement activity assay was also performed in order to compare the DNA polymerase activity of the PB polymerase with other DNA polymerases from marine sources (data not shown). The PB polymerase had a markedly higher strand-displacement activity compared with the other marine polymerases tested (e.g. *Aliivibrio salmonicida* DNA polymerase).

Analysis of effect of salt concentration on polymerase activity

In crude samples, such as blood, there may be several compounds present that can inhibit the polymerase reaction. One such compound is salt (high salt content). An experiment to assess the effect of salt concentration on polymerase activity was performed as described below.

The optimum NaCl concentration for PB DNA polymerase has been analysed using the described polymerase activity assay varying the amount of salt present in the reaction buffer. One hundred percent activity refers to the salt concentration giving highest polymerase activity. Put another way, for each of the tested DNA polymerases the maximum polymerase activity observed is set as the 100% activity value. The other values (data points) in each curve are relative to the 100% activity for the respective polymerase.

Fifty microliter reactions consisted of 200 nM substrate and 200 µM dNTP (equimolar amounts of dATP, dGTP, dCTP and dTTP). For PB polymerase I the reaction further contained 5 mM $MgCl_2$ in 50 mM BIS-TRIS propane at pH 8.5, 1 mM DTT, 0.2 mg/ml BSA and 2 % glycerol. The buffers for the commercially known polymerase Is have been prepared according to the composition of the respective buffer supplied by New England Biolabs omitting the salt. The respective NaCl concentrations tested in this experiment have been added to each reaction individually. The activity assay was carried out at 25 °C in black 96-well fluorescence assay plates (Corning®). The reaction was initiated by addition of protein solution with 1 µg/ml for PB, 16 units/ml for Bst, 16 units/ml for Bst 2.0, 2.5 units/ml for BSU or 2 units/ml for KF. The increase in FAM fluorescence was measured as relative fluorescence units in appropriate time intervals by exciting at 485 nm and emission at 518 nm. The measurement was performed in a SpectraMax® Gemini Microplate Reader (Molecular Devices).

The results of this experiment are shown in Figure 4. As is evident from Figure 4, the PB DNA polymerase exhibits tolerance to range of different salt concentrations.

Effect of temperature on polymerase activity of PB polymerase

The effect of temperature on the polymerase activity of PB polymerase I has been investigated with the single-nucleotide incorporation assay.

Single-nucleotide incorporation assay

A primer consisting of 19 oligonucleotides (SEQ ID NO:15) is annealed to a template strand consisting of 40 oligonucleotides (SEQ ID NO:14). The primer is labeled with the FAM fluorophore at its 5' end. In the reaction set up only dATP will be provided thus the polymerase can extend the primer in 5'-3' direction only by one nucleotide at position 20. The subsequent analysis on a denaturing polyacrylamide gel (12 % polyacrylamide/7 M urea) and scanning for the FAM labeled oligonucleotides shows the primer consisting of 19 oligonucleotides and the primer extended by the nucleotide adenine thus consisting of 20 oligonucleotides. Enzyme activity is determined by densitometric measurement of bands representing the extended primer (intensity 1) and the unextended primer (intensity 0). The relative incorporation rate is calculated as follows:

incorporation [%] = intensity 1/(intensity 0 + intensity 1)*100

```
5'- ATTGAGTGGAGATAGTATCGTAGGGTAGTATTGGTGGATA - 3'      40mer
              3'- TCCCATCATAACCACCTAT[FAM] - 5'      19mer
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The substrate for the single-nucleotide incorporation assay was produced by incubating 20 µl of 0.5 µM fluorophore-labeled primer and 0.5 µM template DNA in 10 mM Tris-HCl pH 8.0, 100 mM NaCl at 75 °C for 5 min. The reaction was then let to cool down at room temperature for 2 h. The substrate solution was stored at -20 °C with a final concentration of 0.5 µM.

Ten microliter reactions contained 30 nM substrate and 10 µM dATP. For PB polymerase I the reaction further contained 5 mM $MgCl_2$ in 50 mM Tris at pH 8.5, 100 mM NaCl, 1 mM DTT, 0.2 mg/ml BSA and 2 % glycerol. The pH of the reaction buffer at room temperature was adjusted to be pH 8.5 at the respective incubation temperature. The reactions were initiated by addition of 0.018 ng/µl protein PB polymerase I and incubated for 15 min at the respective temperature (i.e. the temperatures corresponding to the data points on Figure 5). As a negative control protein dilution buffer has been used instead of protein solution.

Reactions were stopped by addition of 2.5 µl denaturing gel loading buffer (95 % formamide, 10 mM EDTA, 0.1 % xylene cyanol) and incubation at 95 °C for 5 minutes. For the denaturing polyacrylamide gel electrophoresis (12 % polyacrylamide/7 M urea) a sample volume of 6 µl was loaded onto the gel. The gel electrophoresis was performed in 0.5 x TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) at 50 W for 1 hour 15 minutes and the gel subsequently scanned with the PharosFX Plus Imager (Bio-Rad).

Results of this experiment are shown in Figure 5. One hundred percent activity refers to the temperature value with the highest activity. Put another way, for the tested PB DNA polymerase the maximum polymerase activity observed is set as the 100% activity value. The other values (data points) in each curve are *relative* to the 100% activity for the respective polymerase. By way of an example, at an incubation temperature of 20°C, the polymerase activity of the PB polymerase is

about 60% of the maximum PB polymerase activity observed, i.e. there is about 60% “relative” activity.

The results in Figure 5 show that the PB polymerase has significant polymerase activity across a broad range of temperatures.

Effect of temperature on the stability of PB polymerase compared to KF (Klenow Fragment).

The effect of temperature on the stability of PB polymerase I has been investigated with the single-nucleotide incorporation assay.

Single-nucleotide incorporation assay

A primer consisting of 19 oligonucleotides (SEQ ID NO:15) is annealed to a template strand consisting of 40 oligonucleotides (SEQ ID NO:14). The primer is labeled with the FAM fluorophore at its 5’ end. In the reaction set up only dATP will be provided thus the polymerase can extend the primer in 5’-3’ direction only by one nucleotide at position 20. The subsequent analysis on a denaturing polyacrylamide gel (12 % polyacrylamide/7 M urea) and scanning for the FAM labeled oligonucleotides shows the primer consisting of 19 oligonucleotides and the primer extended by the nucleotide adenine thus consisting of 20 oligonucleotides. Enzyme activity is determined by densitometric measurement of bands representing the extended primer (intensity 1) and the unextended primer (intensity 0). The relative incorporation rate is calculated as follows:

incorporation [%] = intensity 1/(intensity 0 + intensity 1)*100

```
5’- ATTGAGTGGAGATAGTATCGTAGGGTAGTATTGGTGGATA – 3'      40mer
                3’- TCCCATCATAACCACCTAT[FAM] – 5'      19mer
```

The substrate for the single-nucleotide incorporation assay was produced by incubating 20 µl of 0.5 µM fluorophore-labeled primer and 0.5 µM template DNA in 10 mM Tris-HCl pH 8.0, 100 mM NaCl at 75 °C for 5 min. The reaction was then let

to cool down at room temperature for 2 h. The substrate solution was stored at -20 °C with a final concentration of 0.5 µM.

For PB polymerase I 10 µl reactions were performed in 50 mM BIS-TRIS propane at pH 8.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.2 mg/ml BSA and 2 % glycerol. To test the inactivation temperature 0.018 ng/µl of PB polymerase I were added to the reaction buffer, incubated at the respective temperature (i.e. the temperatures corresponding to the data points on Figure 6) for 15 min and afterwards cooled down on ice for 5 min. For KF the reaction buffer has been prepared according to the composition of the respective buffer supplied by New England Biolabs with optimal pH at 25 °C. The protein has been added to the reaction buffer with 0.67 units/ml and the reactions have been incubated as described for the PB polymerase I. As a negative control protein dilution buffer has been used instead of protein solution.

The single-nucleotide extension reaction was initiated by addition of 30 nM substrate and 10 µM dATP and the mixture was incubated at 25 °C for 15 min. The reaction was stopped by addition of 2.5 µl denaturing gel loading buffer (95 % formamide, 10 mM EDTA, 0.1 % xylene cyanol) and incubation at 95 °C for 5 minutes. For the denaturing polyacrylamide gel electrophoresis (12 % polyacrylamide/7 M urea) a sample volume of 6 µl was loaded onto the gel. The gel electrophoresis was performed in 0.5 x TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) at 50 W for 1 hour 15 minutes and the gel subsequently scanned with the PharosFX Plus Imager (Bio-Rad).

Results of this experiment are shown in Figure 6. One hundred percent activity refers to enzyme sample incubated on ice (0°C). Put another way, for each of the tested DNA polymerases (PB and Klenow Fragment from *E.coli* (KF)) the polymerase activity observed when the polymerase was incubated on ice (0 °C) for 15 minutes prior to the initiation of the single-nucleotide extension reaction is set as the 100% activity value. The other values (data points) in each curve are *relative* to the 100% activity for the respective polymerase. By way of an example, after a 15 minute incubation at 37°C prior to the initiation of the single-nucleotide extension reaction, the polymerase activity of the PB polymerase is about 80% of the PB

polymerase activity observed when the 15 minute incubation prior to the initiation of the single-nucleotide extension reaction was done on ice (0°C), i.e. there is about 80% "residual" activity.

The results in Figure 6 show that in comparison with KF, the PB polymerase retains higher polymerase activity after prior incubations at a range of temperatures. Put another way, the PB polymerase is more temperature stable than the KF polymerase. For example, with an incubation at 30°C for 15 minutes prior to the initiation of the single-nucleotide extension reaction, the PB polymerase retains the same polymerase activity as when the incubation prior to the initiation of the single-nucleotide extension reaction was done on ice (0°C) (i.e. there is 100% residual activity with a 30°C incubation). In contrast, with an incubation at 30°C for 15 minutes prior to the initiation of the single-nucleotide extension reaction, the KF polymerase has only about 60% of the polymerase activity observed when the incubation prior to the initiation of the single-nucleotide extension reaction was done on ice (0°C) (i.e. there is about 60% residual activity with a 30°C incubation).

The same type of assay was also performed in order to compare the DNA polymerase activity of the PB polymerase with other DNA polymerases from marine sources (data not shown). The PB polymerase had a markedly higher stability compared with the other marine polymerases tested (e.g. *Aliivibrio salmonicida* DNA polymerase).

Biophysical data showing the melting temperature of PB polymerase in different pH buffers

The melting temperature (T_m) of the protein was determined by thermofluor experiments according to Ericsson *et al.* (*Anal. Biochem.*, 2006, *357*, 289–298). A number of buffers of pH 6.0 to pH 9.5 in the concentration of 50 mM have been tested. The respective buffer, SYPRO® Orange (Sigma Aldrich) in a final dilution of 6x and 12.5 µg of protein (i.e. PB polymerase) were mixed thoroughly in a well of a thin-wall PCR plate (Biorad). The wells were sealed with optical-quality sealing tape (Biorad). The volume of the final reaction was 25 µl. A temperature range of 10 – 90 °C with an increment of 0.3 °C at 3 seconds intervals has been scanned in the thermofluor experiment.

Results of this experiment are shown in Figure 7. PB polymerase I shows a broad pH stability profile at the range of pH 6 – 8.5 with a melting temperature averaging about 43-44 °C further proving its relative broad temperature application range.

Processivity

Processivity is the ability of the DNA polymerase to remain bound to the DNA template strand during synthesis (nucleotide incorporation) before dissociating, and the overall efficiency of DNA synthesis may therefore increase with increasing processivity of a given polymerase. This feature may be important especially if longer stretches of DNA needs to be synthesized. The processivity assay is therefore designed to measure the extent of polymerization after a single binding event. In order for the extension products to reflect the processivity of the polymerase, an excess of trap DNA was added in this case to ensure that each primer template is extended only once.

100-mer template
3'ATGTTGGTTCTCGTATGCTGCCGGTCACGGCTTAAGTGTGCCACAACACACAACCAACACCACCAC
AACACACCAACAACCACAACACACACAACCACAC-5' (SEQ ID NO:16)
Fam labeled 20-mer primer
5'FAM-TACAACCAAGAGCATACGAC (SEQ ID NO:17)

To prepare a primer-template DNA substrate, a 100 mer template (0.5 µM) was incubated with a 20 mer FAM labeled primer (0.5 µM) in the presence of 50 mM NaCl and 50 mM tris/HCl pH 7.5 for 5 minutes at 75 °C, and left to cool down to room temperature for at least 2 hours. The PB polymerase I and the commercial polymerases (New England Biolabs) tested herein were pre-incubated with the DNA substrate (25 nM) and dNTPs (10 µM) in 50 mM Tris-HCl (pH 8.5 for PB polymerase I and T7 DNA polymerase, pH 7.5 for Bst 2.0 and BSU), 50 mM KCl (PB polymerase I, Bst 2.0, T7 DNA polymerase) or 50 mM NaCl (BSU), 0.2 mg/ml BSA, 1 mM DTT and 2 % glycerol for 10 min at 25 °C.

The reaction (Pr) was initiated by adding 5 mM $MgCl_2$ and 1.1 mg/ml Herring sperm DNA as a trap and terminated after 2 minutes by adding stop solution (95 % formamide 10 mM EDTA, 0.05% bromophenol blue). To show the effectiveness of the trap, the same amount of PbI was pre incubated with 1.1 mg/ml trap and the

DNA substrate before initiation of the reaction with $MgCl_2$ (R0) in a parallel reaction. As a control for complete polymerization of the primed substrate, another reaction (P) was run without adding any trap, under the same reaction conditions. Amount of enzymes that provided equivalent polymerase activity in the P reaction was used as a starting point for processivity analysis. Thus, the following amounts of enzymes for processivity analysis were used: PB polymerase I 0.013 μg, Bst 2.0 0.14 μg and BSU 0.016 μg.

The reactions were heated for 5 minutes at 95 °C and the products were resolved on 10 % TBE-polyacrylamide gel containing 8 M Urea. The image was then visualized by Pharose FX Plus imager (BioRad).

Results of this experiment are shown in Figure 8. These results show that PB polymerase has a significantly higher processivity compared to Bsu and Bst2.0, where they perform in order PB>Bsu>Bst 2.0.

CLAIMS

1. An isolated DNA polymerase or an enzymatically active fragment thereof, said DNA polymerase comprising the amino acid sequence of SEQ ID NO:1 or comprising an amino acid sequence which is at least 70% identical to SEQ ID NO:1.

2. The isolated DNA polymerase or enzymatically active fragment thereof according to claim 1, wherein said DNA polymerase comprises an amino acid sequence that is at least 90% identical to SEQ ID NO:1.

3. The isolated DNA polymerase or enzymatically active fragment thereof according to claim 1 or claim 2, wherein said DNA polymerase comprises the amino acid sequence of SEQ ID NO:1.

4. The isolated DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 3, wherein said DNA polymerase exhibits at least 30% of its maximum polymerase activity across the temperature range from 0°C to 35°C.

5. The isolated DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 4, wherein said DNA polymerase exhibits at least 60% of its maximum polymerase activity across the temperature range from 20°C to 35°C.

6. The isolated DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 5, wherein at a temperature that deviates by up to about 10°C from the temperature at which said DNA polymerase exhibits maximum activity, said DNA polymerase exhibits at least 30% of its maximum polymerase activity.

7. The isolated DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 6, wherein said DNA polymerase exhibits at least 40% of its maximum activity after incubation for 15 minutes of said DNA

polymerase at any temperature across the temperature range from 0°C to 40°C prior to assessing DNA polymerase activity.

8. The isolated DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 7, wherein said DNA polymerase exhibits at least 60% of its maximum activity after incubation for 15 minutes of said DNA polymerase at any temperature across the temperature range from 0°C to 37°C prior to assessing DNA polymerase activity.

9. The isolated DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 8, wherein said DNA polymerase has a melting temperature of about 42°C-45°C in the pH range 6.0 to 8.5.

10. The isolated DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 9, wherein said DNA polymerase has a higher polymerase activity than the *E.coli* Klenow fragment DNA polymerase, the *Geobacillus stearothermophilus* DNA polymerase and/or DNA polymerase from *Aliivibrio Salmonicida*, wherein said DNA polymerase activity is as assessed at about 25°C.

11. The isolated DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 10, wherein said DNA polymerase has at least 50% higher strand displacement activity than the *E.coli* Klenow fragment DNA polymerase, the *Geobacillus stearothermophilus* DNA polymerase and/or the DNA polymerase from *Aliivibrio Salmonicida*.

12. The isolated DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 11, wherein said DNA polymerase has higher processivity than the *Bacillus subtilis* DNA polymerase.

13. The isolated DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 12, wherein across a concentration range from 0mM to 210mM NaCl, said DNA polymerase exhibits at least 30% of its maximum polymerase activity.

14. A composition comprising a DNA polymerase or an enzymatically active fragment thereof according to any one of claims 1 to 13 and a buffer.

15. A nucleic acid molecule comprising a nucleotide sequence that encodes a DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 13.

16. The nucleic acid molecule according to claim 15, wherein said nucleic acid molecule comprises the nucleotide sequence as set forth in SEQ ID NO:2, or comprises a nucleotide sequence that has at least 70% sequence identity to SEQ ID NO:2.

17. An expression vector containing a nucleic acid molecule according to claim 15 or claim 16 and the necessary regulatory sequences for the transcription and translation of the protein sequence encoded by said nucleic acid molecule.

18. A host cell comprising one or more expression vectors according to claim 17 or one or more nucleic acid molecules according to claim 15 or claim 16.

19. A method of producing a DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 13 comprising the steps of:

(i) culturing a host cell comprising one or more of the recombinant expression vectors according to claim 17 or one or more of the nucleic acid molecules according to claim 15 or claim 16 under conditions suitable for the expression of the encoded DNA polymerase; and

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

20. Use of a DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 13 for nucleotide polymerisation.

21. Use of a DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 13 in a nucleic acid amplification or sequencing reaction.

22. The use of claim 20 or claim 21, wherein said DNA polymerase or enzymatically active fragment thereof is used at a constant temperature.

23. The use of claim 21 or claim 22, wherein said reaction is an isothermal nucleic acid amplification reaction.

24. A method of nucleotide polymerisation using a DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 13, said method comprising the steps of:

(i) providing a reaction mixture comprising a DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 13, 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.

25. A method of amplifying a nucleic acid using a DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 13, said method comprising the steps of:

(i) providing a reaction mixture comprising a DNA polymerase or enzymatically active fragment thereof according to any one of claims 1 to 13, a template nucleic acid molecule, an oligonucleotide primer(s) which is capable of annealing to a portion of the template nucleic acid molecule acid molecule, and nucleotides;

(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.

26. The method of claim 24 or claim 25, wherein said method is performed at a constant temperature.

27. The use of claim 22 or the method of claim 26, wherein said constant temperature is 0°C to 42°C.

28. The use of claim 27 or the method of 27, wherein said constant temperature is 10°C to 25°C.