

PHAGE ϕ 29 DNA POLYMERASE CHIMERA

The present invention is encompassed within the biotechnology field. Specifically, it relates to a DNA polymerase chimera comprising an amino-terminal (N-terminal) region encoding a ϕ 29 type DNA polymerase and a carboxyl-terminal (C-terminal) region comprising at least one HhH domain which are bound by means of a connecting amino acid sequence and to the use thereof for replicating, amplifying or sequencing a template DNA. Likewise, the present invention provides a method for replicating, amplifying or sequencing a deoxyribonucleic acid with said DNA polymerase chimera and a kit for carrying out said method.

Prior State of the Art

The only enzyme required by the bacteriophage ϕ 29 to replicate its genome is its DNA polymerase, a 66 KDa monomeric protein capable of catalyzing both the initiation of the replication and the elongation of the synthesized strand. For the initiation, this polymerase is bound to a protein known as "terminal" (TP), recognizes the end of the ϕ 29 DNA and catalyzes the formation of a TP-dAMP covalent complex. After the polymerization of 10 nucleotides, the DNA polymerase/TP heterodimer disassociates and the elongation of the strand coming from DNA is carried out.

Replicative DNA polymerases require the interaction with accessory proteins which stabilize the binding between the enzyme and the DNA (Kuriyan and O'Donnell. J Mol Biol. 1993; 234: 915-925). On the other hand, said DNA polymerases need to couple the polymerization upon the detachment of the DNA strand which is not being copied for which they require the functional association thereof to helicase type proteins. In this sense, the DNA polymerase of the bacteriophage ϕ 29 has various intrinsic functional characteristics making it unique:

a) High processivity (defined as the number of nucleotides

incorporated by binding event).

b) High strand detachment capacity which allows replicating the genome of said bacteriophage in the absence of helicase type accessory proteins. These two characteristics, processivity and strand detachment allow the $\phi 29$ DNA polymerase to be capable of synthesizing DNA strands of more than 70 kb in length (Blanco *et al.* J Biol Chem. 1989; 264: 8935-8940).

c) High accuracy in the insertion of nucleotides in the new strand (Esteban *et al.* J Biol Chem. 1993; 268: 2719-2726).

All these characteristics have led to the development of a great variety of isothermal process (at constant temperature) protocols for amplifying double stranded DNA (dsDNA) based on the use of this polymerase. In a simple configuration, the capacity of the $\phi 29$ DNA polymerase to use circular single stranded DNA (ssDNA) allows amplifying DNA by the rolling circle method (or RCA - rolling-circle amplification), producing ssDNA molecules of great length and containing more than 10 copies of the circular template (Blanco *et al.* J Biol Chem. 1989; 264: 8935-8940; US5001050, US5198543 and US5576204). In the process for amplifying dsDNA developed by Amersham Biosciences / Molecular Staging (Dean *et al.* Genome Res. 2001; 11: 1095-1099; Dean *et al.* Proc Natl Acad Sci USA. 2002; 99: 5261-5266), the combination of the use of the $\phi 29$ DNA polymerase with the use of hexamers (hexanucleotides) random sequence primers allows obtaining amplification factors of 10^4 - 10^6 starting from picograms of circular plasmid DNA [Templiphi™ of GE Healthcare] or from 10 nanograms of Genomic DNA [Genomiphi™ of GE Healthcare and Repli-G® of Qiagen]. The products produced are of high quality and can be digested or sequenced directly without the need of prior purification, it has been demonstrated that the $\phi 29$ DNA polymerase is the most robust enzyme for this purpose. The common buffer for carrying out the amplification reactions

with the $\phi 29$ DNA polymerase contains tris-HCl (pH 7.5) plus different concentrations (in the millimolar order) of NaCl or KCl and $MgCl_2$ (US20030207267). However, in spite of the satisfactoriness of these protocols in very diverse situations, the development of other protocols which allow starting from lesser DNA amounts is a growing need.

The HhH ("helix-hairpin-helix") motifs bind the DNA regardless of its sequence and are found in various DNA polymerases, ligases and glycosylases (Shao and Grishin. Nucleic Acids Res. 2000; 28: 2643-2650; Doherty *et al.* Nucleic Acids Res. 1996; 24:2488-2497). These motifs contain a pair of anti-parallel α -helixes connected by a "hairpin" type loop. The second α -helix does not protrude from the structure and therefore, unlike other DNA binding motifs, it cannot be intercalated in the major groove of the DNA. Crystallographic studies suggest that the protein-DNA interactions are established through the "loop" between the two α -helixes. This loop is involved in establishing nonspecific interactions with the DNA and normally contains the consensus sequence GhG, wherein *h* is a hydrophobic residue normally I, V, or L. The resolution of crystallographic structures suggests that the interactions are established between the nitrogen of the polypeptide strand and the oxygen of the phosphates of the DNA. Furthermore, polar amino acids which would establish additional interactions with the phosphate groups tend to exist in the positions 2 and 3 with respect to the second G. The last G of the consensus sequence forms the N-terminal part of the second α -helix and the hydrophobic residue *h* contributes to the interactions between the two α -helixes of the motif. The two α -helixes are packaged forming an angle of 25-50° between one another dictating the characteristic hydrophobic pattern in the sequences. The HhH motifs generally form part of major structures known as (HhH)₂ made up by two HhH motifs bound by an α -helix, forming a mirror symmetry with

respect to that of the DNA facilitating the stable binding thereof to the same (Shao and Grishin. *Nucleic Acids Res.* 2000; 28: 2643-2650; Doherty *et al.* *Nucleic Acids Res.* 1996; 24:2488-2497; Thayer *et al.* *EMBO J.* 1995; 14: 4108-4120).

5 The formation of chimeras between heat stable DNA polymerases such as Taq and Pfu and nonspecific DNA binding motifs has been previously used to increase the DNA binding capacity by said polymerases. (Pavlov *et al.* *Proc Natl Acad Sci USA.* 2002; 99: 13510-13515; W02004013279; Wang *et al.* 10 *Nucleic Acids Res.* 2004; 32: 1197-1207).

 The crystallographic resolution of the structure of the ϕ 29 DNA polymerase has provided the molecular bases responsible for the processive polymerization coupled to the strand detachment, a specific characteristic of this enzyme 15 (Kamtekar *et al.* 2006; *EMBO J* 25: 1335-1343).

 The comparative analysis with other DNA polymerases of the eukaryotic type (family B) shows a similar general folding: a C-terminal polymerization domain formed by the universal subdomains *fingers*, *palm* and *thumb* and forming a 20 channel through which the DNA is bound; and a 3'-5' N-terminal exonuclease domain responsible for removing the nucleotides mistakenly incorporated during the polymerization. The main structural difference between the DNA polymerases of known structure and that of ϕ 29 is the presence, in the latter, of 25 two additional subdomains in its polymerization domain, both corresponding to insertions of conserved sequence in the subgroup of the DNA polymerases using a protein called TPR1 and TPR2 as primer. The subdomain TPR1 is located close to *palm* and contacts with the DNA duplex. The subdomain TPR2, 30 with a β -*hairpin* structure forms, close to the subdomains *thumb*, *palm* and *fingers*, an annular structure which would completely surround the newly synthesized DNA, binding the DNA polymerase to the DNA, required for replicating in a processive manner. Likewise, the subdomain TPR2, together with 35 the subdomains *fingers*, *palm* and the exonuclease domain,

participates in the formation of a narrow channel through which the template strand passes to access the active center during replication, forcing the separation of the double stranded DNA as the polymerase is displaced, acting in a manner similar to how a helicase would act and providing the polymerase with its capacity of coupling the polymerization to the strand detachment (Kamtekar *et al.* 2006; *EMBO J* 25: 1335-1343; Rodríguez *et al.* 2005; *Proc Natl Acad Sci USA* 102: 6407-6412). Such significant differences in the polymerization domain of the ϕ 29 DNA polymerase with respect to the rest has the effect that the fusion of a peptide in the C-terminal end thereof would have unpredictable binding properties thereof in the polymerization and DNA.

Summary of the Invention

The present invention relates to a DNA polymerase chimera comprising a N-terminal region encoding a ϕ 29 type DNA polymerase and a C-terminal region comprising, at least one HhH domain which are bound by means of a connecting amino acid sequence and to the use thereof for replicating, amplifying or sequencing a template DNA. Likewise, the present invention provides a method for replicating, amplifying or sequencing a deoxyribonucleic acid with said DNA polymerase chimera and a kit for carrying out said method.

The phage ϕ 29 DNA polymerase has several characteristics of great interest for amplifying DNA such as: a high processivity without the need of the participation of any accessory protein and a high strand detachment capacity allowing it to replicate the genome of said bacteriophage in a single binding event to the DNA, as well as a high accuracy in the insertion of nucleotides in the new strand. These characteristics have lead to the development of a great variety of protocols for the isothermal amplification of DNA based on the use of this polymerase which allow obtaining products of high quality that can be digested or sequenced directly without the need of prior purification. However,

there is a need for protocols which allow the amplification of DNA from lesser amounts thereof. The present invention responds to this need by means of two approaches: 1) the development of a composition which significantly improves the specificity and the yield of the reaction, and 2) the formation of chimeras between the ϕ 29 DNA polymerase and nonspecific DNA binding motifs increasing the DNA binding capacity of the enzyme.

In the examples of this patent, it is shown that the simultaneous addition of polyoxyethylenated sorbitan monolaurate (Tween[®] 20) and an ammonium salt to the buffer commonly used for the amplification with the ϕ 29 DNA polymerase, on the one hand, prevents the non-specific DNA amplification and, on the other hand allows the detectable and specific amplification from limiting amounts of 0.1 femtograms (fg) of plasmid DNA and 10 fg of genomic DNA as template.

With respect to the increase of the DNA binding capacity, in the present invention HhH motifs of the topoisomerase V of *Methanopyrus kandleri* have been fused to the carboxyl end of the ϕ 29 DNA polymerase through a connecting sequence or *linker* originating from 4 different chimeras. These chimeras are capable of amplifying the template DNA from an amount less than that required by the natural ϕ 29 DNA polymerase.

A first aspect of the present invention relates to a DNA polymerase chimera (hereinafter, DNA polymerase chimera of the invention) comprising:

- a) an amino acid sequence encoding a ϕ 29 type DNA polymerase bound by its C-terminal end to
- b) a connecting amino acid sequence bound by its C-terminal end to
- c) an amino acid sequence comprising at least one helix-hairpin-helix domain (HhH).

As used in the present description, the term "DNA

polymerase" relates to an enzyme capable of catalyzing the polymerization of deoxynucleoside triphosphates. Generally, the enzyme initiates the synthesis in the 3' end of a primer hybridized with a template DNA sequence and proceeds towards the 5' end of the template DNA strand.

As used in the present description, the term "chimera" relates to a protein, the amino acid sequence of which is a product of fusion of amino acid sequences of at least two different proteins. A chimeric protein is generally produced by means of its expression from a chimeric DNA encoding the chimeric amino acid sequence.

As used in the present description, the terms "DNA polymerase chimera" or "chimeric DNA polymerase" relate to a protein, the amino acid sequence of which is a product of fusion of amino acid sequences of at least two different proteins, of which one is a DNA polymerase, and which is capable of catalyzing the polymerization of deoxynucleoside triphosphates. The DNA polymerase chimera of the invention comprises an N-terminal region encoding a ϕ 29 type DNA polymerase (a), and a C-terminal region comprising at least one HhH domain (c) which are bound by means of a connecting amino acid sequence (b).

As used in the present invention, the term " ϕ 29 type DNA polymerase" relates to any DNA polymerase containing TPR1 and TPR2 subdomains in its polymerization domain providing the polymerase with the capacity of coupling the processive polymerization to the strand detachment. Examples of ϕ 29 type DNA polymerases that can be used in the present invention are selected from the list comprising the DNA polymerases isolated from the following phages: ϕ 29, Cp-1, PRD-1, ϕ 15, ϕ 21, PZE, PZA, Nf, M2Y, B103, GA-1, SF5, Cp-5, Cp-7, PR4, PR5, PR722, L17 or Acidianus Bottle-shaped virus (ABV).

In a preferred embodiment of this first aspect of the invention, the amino acid sequence of (a) of the DNA

polymerase chimera of the invention encodes a ϕ 29 type DNA polymerase which is selected from the DNA polymerases isolated from the following phages: ϕ 29, Cp-1, PRD-1, ϕ 15, ϕ 21, PZE, PZA, Nf, M2Y, B103, GA-1, SF5, Cp-5, Cp-7, PR4, PR5, PR722, L17 or Acidianus Bottle-shaped virus (ABV).

Preferably, the amino acid sequence of (a) of the DNA polymerase chimera of the invention has a identity of at least 80% with SEQ ID NO: 1. More preferably, the amino acid sequence of (a) of the chimera of the invention has an identity of at least 90% with SEQ ID NO: 1. Still more preferably, the amino acid sequence of (a) of the chimera of the invention is SEQ ID NO: 1.

The exonuclease domain of the ϕ 29 type DNA polymerases is known and can be modified to reduce the exonuclease activity retaining a high processivity and strand detachment capacity. These modified DNA polymerases are especially useful for sequencing large molecules.

In a preferred embodiment of this first aspect of the invention, the amino acid sequence of (a) of the DNA polymerase chimera of the invention encodes a ϕ 29 type DNA polymerase having modification in the exonuclease domain, wherein said modified DNA polymerase has preferably, less than 10% of exonuclease activity and, more preferably, less than 1% of exonuclease activity than the corresponding naturally occurring DNA polymerase or "wild type". In a still more preferred embodiment, said modified ϕ 29 type DNA polymerase lacks detectable exonuclease activity with respect to the corresponding naturally occurring DNA polymerase.

The HhH ("helix-hairpin-helix") motifs are non-sequence dependent DNA binding motifs. Structurally, the HhH motifs are formed by a pair of anti-parallel α -helixes connected by a hook-type loop. This loop is involved in the interaction with the DNA and has in general a consensus sequence consisting of glycine-amino acid hydrophobic-glycine (GhG) wherein h is a

hydrophobic amino acid residue, commonly, leucine, isoleucine or valine. The last glycine of the consensus sequence serves as N-terminal residue of the second α -helix and the hydrophobic residue h contributes to the interactions between the two α -helices of the motif.

Examples of proteins having a HhH motif in their sequence are, but not limited to, the DNA topoisomerase V of *Methanopyrus kandleri*, the proteins MutY, Nth, MutM/Fpg, Nei, UvrC, DinP, RecR, UmuC, DnaE or DnIJ of *Escherichia coli* or the proteins RAD1, RAD2, RAD10, RAD27, RAD 55, RAD 57, REV1, OGG1, NTG1, NTG2, DIN-7 or EXO-1 of yeasts, as well as the homologs of these proteins in other organisms such as for example, but not limited to, *Bacillus subtilis*, *Caenorhabditis elegans*, *Haemophilus influenzae*, *Methanococcus jannaschii*, *Micrococcus luteus*, *Methanobacterium thermoformicum* or *Salmonella typhimurium*.

Therefore, in a preferred embodiment of this first aspect of the invention, the amino acid sequence of (c) of the DNA polymerase chimera of the invention comprises at least one HhH domain of a protein which is selected from the list comprising:

- topoisomerase V of *M.kandleri*,
- MutY, Nth, MutM/Fpg, Nei, UvrC, DinP, RecR, UmuC, DnaE or DnIJ of *E. coli*,
- RAD1, RAD2, RAD10, RAD27, RAD 55, RAD 57, REV1, OGG1, NTG1, NTG2, DIN-7 or EXO-1 of yeasts, or
- a homologous protein of the above in *B. subtilis*,
- *C. elegans*, *H. influenzae*, *M. jannaschii*, *M. luteus*, *M. thermoformicum* or *S. typhimurium*.

The C-terminal end of the topoisomerase V of *M. kandler* is organized into 12 repeats of about 50 amino acids each known as A-L domains and each having two HhH motifs.

In a preferred embodiment of this first aspect of the invention, the amino acid sequence of (c) of the DNA polymerase chimera of the invention, comprises at least one

HhH domain derived from the topoisomerase V of *M. kandleri*, the amino acid sequence of which is SEQ ID NO: 2.

5 In a more preferred embodiment of this first aspect of the invention, the amino acid sequence of (c) of the DNA polymerase chimera of the invention is SEQ ID NO: 3 corresponding to the sequence of the H domain of the topoisomerase V of *M. kandleri*.

10 In another more preferred embodiment of this first aspect of the invention, the amino acid sequence of (c) of the DNA polymerase chimera of the invention is SEQ ID NO: 3 bound by its C-terminal end to SEQ ID NO: 4 corresponding to the sequence of the H domain of the topoisomerase V of *M. kandleri* bound by its C-terminal end to the I domain of the topoisomerase V of *M. kandleri*.

15 As used in the present invention, the term "connecting amino acid sequence" relates to a short amino acid sequence of at least 2 amino acids in length which allows maintaining the functionality of the amino acid sequences of (a) and (c) of the DNA polymerase chimera of the invention. Preferably, the
20 connecting amino acid sequence of (b) is of 6 amino acids. More preferably, the connecting amino acid sequence of (b) is SEQ ID NO: 5 or SEQ ID NO: 6.

25 Another aspect of the present invention relates to the use of the DNA polymerase chimera of the invention for replicating, amplifying or sequencing a template DNA.

Another aspect of the present invention relates to a method for replicating, amplifying or sequencing a template DNA which comprises contacting said DNA with a reaction
30 mixture comprising at least:

- a) the DNA polymerase chimera of the invention,
- b) a buffer,
- c) magnesium chloride,
- d) a primer, and
- 35 e) nucleoside triphosphates.

A preferred embodiment of this aspect of the invention relates to a method for replicating, amplifying or sequencing a template DNA which comprises contacting said DNA with a reaction mixture comprising the aforementioned elements (a)-(e) and further comprising polyoxyethylenated sorbitan monolaurate, an ammonium salt, a potassium salt or a combination of any of the above.

In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the DNA polymerase chimera of the invention is at a concentration between 5 nM and 75 nM. In a more preferred embodiment, the DNA polymerase chimera of the invention is at a concentration between 25 nM and 60 nM. In a still more preferred embodiment, the DNA polymerase chimera of the invention is at a concentration of approximately 50 nM.

In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the polyoxyethylenated sorbitan monolaurate (Tween® 20) is at a concentration between 0.003% and 0.1% of the total volume of the reaction. In a more preferred embodiment, the polyoxyethylenated sorbitan monolaurate is in a proportion between 0.006% and 0.05% of the total volume of the reaction. In a still more preferred embodiment, the polyoxyethylenated sorbitan monolaurate is in a proportion between 0.01% and 0.03% of the total volume of the reaction. In a still more preferred embodiment, the polyoxyethylenated sorbitan monolaurate is in a proportion of approximately 0.025% of the total volume of the reaction. "Total volume of the reaction", is understood as the resulting volume after the addition of the template DNA to the reaction mixture.

In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the ammonium salt is selected from the list comprising: ammonium sulfate, ammonium chloride or ammonium acetate.

In a preferred embodiment of the method for replicating,

amplifying or sequencing of the invention, the ammonium salt is ammonium sulfate. In a more preferred embodiment, the ammonium sulfate is at a concentration between 30 mM and 60 mM. In a still more preferred embodiment, the ammonium sulfate is at a concentration between 40 mM and 50 mM. In a still more preferred embodiment, the ammonium sulfate is at a concentration of approximately 45 mM.

In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the ammonium salt is ammonium chloride. In a more preferred embodiment, the ammonium chloride is at a concentration between 60 mM and 120 mM. In a still more preferred embodiment, the ammonium chloride is at a concentration between 80 mM and 100 mM. In a still more preferred embodiment, the ammonium chloride is at a concentration of approximately 90 mM.

In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the ammonium salt is ammonium acetate. In a more preferred embodiment, the ammonium acetate is at a concentration between 60 mM and 120 mM. In a still more preferred embodiment, the ammonium acetate is at a concentration between 80 mM and 100 mM. In a still more preferred embodiment, the ammonium acetate is at a concentration of approximately 90 mM.

In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the buffer is at a pH between 7.0 and 8.5. In a more preferred embodiment, the buffer is at a pH between 7.2 and 8. In a still more preferred embodiment, the buffer is at a pH of approximately 7.5.

In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the buffer is tris-hydrochloric, tris-acetic or HEPES. In a more preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the buffer tris-hydrochloric, tris-acetic or HEPES is at a pH between 7.0 and 8.5. In a still more preferred embodiment, the buffer tris-hydrochloric,

tris-acetic or HEPES is at a pH between 7.2 and 8. In a still more preferred embodiment, the buffer tris-hydrochloric, tris-acetic or HEPES is at a pH of approximately 7.5.

5 In a preferred embodiment of this aspect of the method for replicating, amplifying or sequencing of the invention, the buffer tris-hydrochloric, tris-acetic or HEPES is at a concentration between 25 mM and 50 mM. In a more preferred embodiment, the buffer tris-hydrochloric, tris-acetic or HEPES is at a concentration between 30 mM and 45 mM. In a still more preferred embodiment, the buffer tris-hydrochloric, tris-acetic or HEPES is at a concentration of approximately 40 mM.

15 In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the potassium salt is potassium chloride or potassium acetate. In a more preferred embodiment, the potassium chloride or the potassium acetate is at a concentration between 30 mM and 70 mM. In a still more preferred embodiment, the potassium chloride or the potassium acetate is at a concentration between 40 mM and 60 mM. In a still more preferred embodiment, the potassium chloride or the potassium acetate is at a concentration of approximately 50 mM.

25 In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the magnesium chloride is at a concentration between 2 mM and 20 mM. In a more preferred embodiment, the magnesium chloride is at a concentration between 5 mM and 15 mM. In a still more preferred embodiment, the magnesium chloride is at approximately 10 mM.

30 In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the polyoxyethylenated sorbitan monolaurate is in a proportion between 0.01% and 0.03% of the total volume, the ammonium sulfate is at a concentration between 40 mM and 50 mM, the buffer tris-hydrochloric, tris-acetic or HEPES is at a concentration between 30 mM and 45 mM and at a pH between 7.2

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and 8.0, the magnesium chloride is at a concentration between 5 mM and 15 mM and the potassium chloride or the potassium acetate is at a concentration between 40 mM and 60 mM.

5 In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the polyoxyethylenated sorbitan monolaurate is in a concentration of 0.025% of the total volume, the ammonium sulfate is at a concentration of 45 mM, the buffer tris-hydrochloric, tris-acetic or HEPES is at a concentration of 40 mM and at a pH of
10 7.5, the magnesium chloride is at a concentration of 10 mM and the potassium chloride or the potassium acetate is at a concentration of 50 mM.

In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the
15 polyoxyethylenated sorbitan monolaurate is in a proportion between 0.01% and 0.03% of the total volume, the ammonium chloride is at a concentration between 80 mM and 100 mM, the buffer tris-hydrochloric, tris-acetic or HEPES is at a concentration between 30 mM and 45 mM and at a pH between 7.2
20 and 8.0, the magnesium chloride is at a concentration between 5 mM and 15 mM and the potassium chloride or the potassium acetate is at a concentration between 40 mM and 60 mM.

In a more preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the
25 polyoxyethylenated sorbitan monolaurate is in a concentration of 0.025% of the total volume, the ammonium chloride is at a concentration of 90 mM, the buffer tris-hydrochloric, tris-acetic or HEPES is at a concentration of 40 mM and at a pH of 7.5, the magnesium chloride is at a concentration of 10 mM and
30 the potassium chloride or the potassium acetate is at a concentration of 50 mM.

In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the
polyoxyethylenated sorbitan monolaurate is in a proportion
35 between 0.01% and 0.03% of the total volume, the ammonium

acetate is at a concentration between 80 mM and 100 mM, the buffer tris-hydrochloric, tris-acetic or HEPES is at a concentration between 30 mM and 45 mM and at a pH between 7.2 and 8.0, the magnesium chloride is at a concentration between 5 mM and 15 mM and the potassium chloride or the potassium acetate is at a concentration between 40 mM and 60 mM.

In a more preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the polyoxyethylenated sorbitan monolaurate is in a concentration of 0.025% of the total volume, the ammonium acetate is at a concentration of 90 mM, the buffer tris-hydrochloric, tris-acetic or HEPES is at a concentration of 40 mM and at a pH of 7.5, the magnesium chloride is at a concentration of 10 mM and the potassium chloride or the potassium acetate is at a concentration of 50 mM.

As used in the present description, the term "replication" relates to the synthesis of a complementary DNA from a template DNA.

As used in the present description, the term "amplification" relates to the increase of the number of copies of a template DNA.

As used in the present description, the term "sequencing" relates to the determination of the order of the nucleotides of a template DNA.

"Contacting" is understood as the fact that the template DNA and the reaction mixture are incubated in primer extension conditions.

As used herein, the term "primer" relates to an oligonucleotide capable of acting as the starting point of the DNA synthesis when it is in primer extension conditions. Preferably, the primer is a deoxyribose oligonucleotide.

The primers can be prepared by means of any suitable method, including for example, but not limited to, the direct chemical synthesis. The primers can be designed to hybridize with specific deoxynucleotide sequences in the template DNA

(specific primers) or can be randomly synthesized (arbitrary primers).

As used in the present description, the term "specific primer" relates to a primer the sequence of which is complementary to a specific deoxynucleotide sequence in the template DNA to be amplified.

"Complementary" is understood as the fact that the primer can be hybridized with a region of the template DNA such that it can act as the starting point of the DNA synthesis when it is in primer extension conditions. Preferably, that region has a 100% complementarity with a region of the template DNA. In other words, each nucleotide in the region of complementarity with the primer can form hydrogen bonds with a nucleotide present in the single stranded template. However, those with a normal experience in the field will acknowledge that primers having a region with less than 100% complementarity with respect to the template DNA will function to carry out the method for replicating, amplifying or sequencing of the present invention.

The term "arbitrary primer" relates to a primer the sequence of which is randomly synthesized and which is used to initiate the DNA synthesis in random positions of the template DNA. Generally, in the method for replicating, amplifying or sequencing of the present invention a population of arbitrary primers is used. The term "arbitrary primers" relates to a set of primers with a random sequence and which are used to initiate the DNA synthesis in random positions of the template DNA.

In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the primer is specific.

In another preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the primer is arbitrary. Preferably, the arbitrary primer is protected against the action of 3'-5' exonucleases. And more

preferably, the arbitrary primer is an oligonucleotide of 6 nucleotides, "hexanucleotide" or "hexamer" protected against the action of 3'-5' exonucleases.

5 As used in the present description, the expression "protected against the action of exonucleases" relates to a modified primer such that it is resistant to the nucleolytic degradation by any 3'-5' exonuclease activity present in the DNA polymerase chimera of the invention.

10 In the method for replicating, amplifying or sequencing of the invention, more than one primer can be used, being able to use specific and/or arbitrary primers.

In a preferred embodiment of the method for replicating, amplifying or sequencing of the invention, the primer is at a concentration between 2 μ M and 100 μ M. In a more preferred
15 embodiment, the primer is at a concentration between 20 μ M and 80 μ M. In a still more preferred embodiment, the primer is at a concentration between 40 μ M and 60 μ M. In a still more preferred embodiment, the primer is at a concentration of approximately 50 μ M.

20 As used in the present description the term "nucleoside triphosphates" relates to organic molecules formed by the covalent bond of a pentose, a nitrogen base and three phosphate groups.

The term nucleoside triphosphates include
25 deoxynucleoside triphosphates (dNTPs) such as for example, but not limited to dATP, dCTP, dITP, dUTP, dGTP, dTTP, or derivatives thereof. Preferably, the deoxynucleoside triphosphates are dATP, dTTP, dGTP and dCTP. Still more preferably, these four dNTPs are in equimolar conditions. In a
30 preferred embodiment of this aspect of the invention, the deoxynucleoside triphosphates are at a concentration between 100 μ M and 800 μ M. In a more preferred embodiment, the deoxynucleoside triphosphates are at a concentration between 200 μ M and 600 μ M. In a still more preferred embodiment, the
35 deoxynucleoside triphosphates are at a concentration of

approximately 500µM.

The term nucleoside triphosphates also includes dideoxynucleoside triphosphates (ddNTPs) such as for example, but not limited to, ddATP, ddCTP, ddITP, ddUTP, ddGTP, ddTTP, or derivatives thereof.

In some preferred embodiments of the method for replicating, amplifying or sequencing of the invention, at least one nucleoside triphosphate or one primer is labelled by means of techniques well known in the state of the art. The nucleotide labelled can be, for example, a deoxynucleoside triphosphate or a dideoxynucleoside triphosphate. Detectable labels include, for example, radioactive isotopes, fluorescent labels, chemiluminescent labels, bioluminescent labels or enzymatic labels.

As used in the present description, the term "template DNA" relates to a DNA molecule that can serve as a substrate for synthesizing a complementary DNA strand; i.e., it relates to a DNA molecule to be replicated, amplified or sequenced. In a preferred embodiment the template DNA is plasmid DNA. In another preferred embodiment, the template DNA is genomic DNA.

Replicating, amplifying or sequencing of the template DNA is carried out in primer extension conditions. The expression "primer extension conditions" refers to the conditions in which the template DNA-dependent synthesis initiated in a primer can take place.

The template DNA synthesis according to the method for replicating, amplifying or sequencing of the present invention can take place by means of a thermal cycling process or at an essentially constant temperature.

"Isothermal conditions" is understood as essentially constant temperature. Preferably, the template DNA synthesis according to the method for replicating, amplifying or sequencing of the present invention takes place at an essentially constant temperature. More preferably, at an essentially constant temperature between 25 and 40°C, and

still more preferably at approximately 30°C.

5 A large number of methods allowing DNA amplification are known in the state of the art. Some methods require a thermal cycling process such as, for example, but not limited to the polymerase chain reaction (PCR). Other methods do not require a thermal cycling process, rather they are performed at a essentially constant temperature such as, for example, but not limited to the rolling circle amplification (RCA), the multiple detachment amplification (MDA), the strand displacement amplification (SDA) or the loop mediated amplification (LAMP). The amplification of a template DNA according to the method of the present invention can take place by means of a thermal cycling process or at an essentially constant temperature.

15 Preferably, the amplification of the template DNA according to the method for amplifying of the present invention takes place by means of rolling circle amplification (RCA), by means of multiple detachment amplification (MDA), strand displacement amplification (SDA) or loop mediated amplification (LAMP).

20 Another aspect of the present invention relates to a kit or device comprising elements suitable for carrying out the method for replicating, amplifying or sequencing of the present invention.

25 Another aspect of the present invention relates to a kit for carrying out the method for replicating, amplifying or sequencing of the present invention comprising:

- 30 a) the DNA polymerase chimera of the invention,
- b) a buffer, and
- c) magnesium chloride.

In a preferred embodiment of this aspect of the invention, the kit further comprises polyoxyethylenated sorbitan monolaurate, an ammonium salt, a potassium salt or a combination of any the above.

35 Preferably, said ammonium salt is selected from the list

comprising: ammonium sulfate, ammonium chloride or ammonium acetate.

Preferably, said potassium salt is potassium chloride or potassium acetate.

5 In a preferred embodiment of this aspect of the invention, the kit further comprises a primer. In a more preferred embodiment, the primer is an arbitrary primer which is protected against the action of 3'-5' exonucleases.

10 In a preferred embodiment of this aspect of the invention, the kit further comprises nucleoside triphosphates. For example, in a more preferred embodiment of this aspect of the invention, the kit further comprises deoxynucleoside triphosphates and/or a dideoxynucleoside triphosphate.

15 In a preferred embodiment of this aspect of the invention, the kit comprises at least one triphosphate nucleoside or one labelled primer. The labelled nucleoside can be, for example, a deoxynucleoside triphosphate or a dideoxynucleoside triphosphate.

20 The kit can further include, without any form of limitation, buffers, agents to prevent contamination, etc. On the other hand, the kit can include all the supports and recipients necessary for putting it into practice and for its optimization. Preferably, the kit further comprises the instructions for carrying out the method of the invention.

25 Throughout the description and claims, the word "comprises" and its variants do not exclude other technical features, additives, components or steps. For the persons skilled in the art, other objects, advantages and features of the invention will be inferred in part from the description and in part from the practice of the invention. The following drawings and examples are provided by way of illustration and do not limit the present invention.

30 Description of the Drawings

35 Figure 1 shows the effect of Tween® 20 and (NH₄)₂SO₄ in the amplification capacity of the ϕ 29 DNA polymerase. The assay

was carried out as described in the main text in the presence of the indicated amounts of plasmid DNA (4.2 kpb). After incubating at 30°C for 5 h, the reactions were analyzed as described in the main text. On the left, the linear DNA fragments obtained after digesting the ϕ 29 DNA with *HindIII* used as DNA length markers.

Figure 2 shows the amplification of different amounts of plasmid DNA (in the order of femtograms) by the ϕ 29 DNA polymerase in the presence of Tween® 20 and $(\text{NH}_4)_2\text{SO}_4$. The assay was carried out as described in the main text in the presence of 0.025% Tween® 20 and of 45 mM $(\text{NH}_4)_2\text{SO}_4$. The DNA length markers are the same as those used in Figure 1.

Figure 3 shows the effect of the NH_4^+ ion in the amplification capacity of the ϕ 29 DNA polymerase. The assay was carried out as described in the main text in the presence of 0.025% Tween® 20 and the indicated ammonium salt as well as the indicated amounts of plasmid DNA (4.2 kpb). After incubating at 30°C for 6 h, the reactions were analyzed as described in the main text. The DNA length markers are the same as those used in Figure 1.

Figure 4 shows the amplification of different amounts of *Bacillus subtilis* genomic DNA by the ϕ 29 DNA polymerase in the presence of Tween® 20 and $(\text{NH}_4)_2\text{SO}_4$. The assay was carried out as described in the main text in the presence of 0.025% Tween® 20 and 45 mM $(\text{NH}_4)_2\text{SO}_4$. The DNA length markers are the same as those used in Figure 1.

Figure 5 shows the significant improvement depicted by the addition of 0.025% Tween® 20 and 45 mM $(\text{NH}_4)_2\text{SO}_4$ to the current reaction buffer of a commercial kit for the amplification of DNA based on the ϕ 29 DNA polymerase (Illustra kit of General Electrics HealthCare). The assay was carried out as described in the main text. The DNA length markers are the same as those used in Figure 1.

Figure 6 shows a diagram of the different steps followed to construct the chimeras HAY, HGT, HIAIY and HIGT.

Figure 7 shows the retardation in gel of the primer/template DNA molecules by the natural and chimeric $\phi 29$ DNA polymerases. The hybridized molecule of 15 bases/21 bases labelled in 5' (dsDNA) was incubated with natural $\phi 29$ DNA polymerase or with the chimeric DNA polymerase indicated in the conditions described in the text. The mobilities of the free dsDNA and of the polymerase-DNA complex were detected by autoradiography after the separation thereof by electrophoresis in 4% (w/v) native polyacrylamide gels (80:1 monomer: bisacrylamide).

Figure 8 shows the processive replication of DNA by the natural and chimeric $\phi 29$ DNA polymerases. (A) Replication of M13 DNA coupled to strand detachment by the natural and chimeric $\phi 29$ DNA polymerases. The replication of 250 ng of M13 DNA with a single primer was carried out as has been described in the text using natural or chimeric $\phi 29$ DNA polymerases (30 nM). The position of the M13 DNA of unit in length is shown on the right. (B) Processive synthesis by the natural and chimeric $\phi 29$ DNA polymerases. The assay was carried out in the same conditions as in (A), using decreasing concentrations of the DNA polymerase indicated. After incubating at 30°C for 20 min, the samples were processed as has been described in (A).

Figure 9 shows the rolling circle amplification with multiple priming of 10 fg plasmid DNA by the natural and chimeric $\phi 29$ DNA polymerases. The assay was carried out as described in the main text in the presence of buffer B and 50 nM of the DNA polymerase indicated. The DNA length markers are the same as those used in Figure 1.

Figure 10 shows the amplification of the entire genome with multiple priming of 100 fg *B. subtilis* genomic DNA with natural and chimeric $\phi 29$ DNA polymerases. The assay was carried out as described in the text in the presence of buffer

B and 50 nM of the DNA polymerase indicated. The DNA length markers are the same as those used in Figure 1.

EXAMPLES

The following specific examples provided in this patent document serve to illustrate the nature of the present invention. These examples are only included for illustrative purposes and must not be interpreted as limitations to the invention claimed herein. Therefore, the examples described below illustrate the invention without limiting the field of application thereof.

EXAMPLE 1 Optimization of the experimental conditions for amplifying DNA with multiple priming by the ϕ 29 DNA polymerase.

It has been shown that the ϕ 29 DNA polymerase amplifies 10^4 - 10^6 times starting from several picograms of circular DNA. For this purpose a reaction buffer containing 40 mM tris-HCl, pH 7.5, 50 mM KCl and 10 mM $MgCl_2$ (hereinafter Buffer A) was used. After testing the influence of different detergent and salt conditions on the DNA amplification capacity of the DNA polymerase of ϕ 29, it is found that the simultaneous addition of 0.025% Tween® 20 and 45 mM $(NH_4)_2SO_4$ to the Buffer A highly improve the amplification of the limited amounts of provided DNA.

Reaction conditions for amplifying plasmid DNA.- The incubation mixture contained 12.5 μ l of buffer A, 50 μ M of hexamers protected against the action of the 3'-5' exonuclease, 500 μ M of each of the deoxynucleoside triphosphates (dCTP, dGTP, dTTP and dATP), the indicated amounts of a plasmid DNA (with a size of 4.2 kbp) and, where indicated, $(NH_4)_2SO_4$ 45 mM or 0.025% Tween® 20 or a combination of both was added. The DNA was denatured by incubation at 95°C for 3 minutes and subsequent cooling in ice for 5 min. The reaction was initiated upon adding 50 nM ϕ 29 DNA polymerase and it was stopped after the incubation at 30°C by means of

heating to 65°C for 10 min. To analyze the results, 1 µl samples were taken from the reactions, the amplified DNA was digested with the EcoRI restriction endonuclease and was subjected to electrophoresis in 0.7% agarose gel. The DNA was detected by means of staining the gels with ethidium bromide.

Reaction conditions for amplifying genomic DNA.- The incubation mixture contained 12.5 µl of buffer A, 45 mM (NH₄)₂SO₄, 0.025% Tween® 20, 50 µM of hexamers protected against the action of the 3'-5' exonuclease, 500 µM of each of the deoxynucleoside triphosphates (dCTP, dGTP, dTTP and dATP) and the indicated amounts of *Bacillus subtilis* genomic DNA (with a size of 4 Mpb). The DNA denatured by incubation at 95°C for 3 minutes and subsequent cooling in ice for 5 min. The reaction was initiated upon adding 50 nM φ29 DNA polymerase and it was stopped after the incubation at 30°C by means of heating to 65°C for 10 min. To analyze the results, 1 µl samples were taken from the reactions and were subjected to electrophoresis in 0.7% agarose gel. The DNA was detected by means of staining the gels with ethidium bromide.

Figure 1 shows the effect of adding 45 mM (NH₄)₂SO₄ and 0.025% Tween® 20 in the amplification of small amounts of provided plasmid DNA. As shown, the φ29 DNA polymerase did not give any amplification product detectable with the standard Buffer A when 100 fg of provided DNA were used. In these reaction conditions, the addition of 0.025% Tween® 20 in the absence of DNA caused the appearance of trace DNA products, most probably as a consequence of the nonspecific DNA amplification caused by the hybridization and elongation of the random hexamer primers. The same trace was observed with 10 fg of provided DNA. However, in the presence of 100 fg of provided DNA, the addition of 0.025% Tween® 20 allowed the φ29 DNA polymerase to produce a detectable amount of amplified plasmid. The total production of specific or nonspecific amplified DNA indicates that the addition of 0.025% Tween® 20

to the Buffer A powers the amplification capacity of the ϕ 29 DNA polymerase. A similar effect was observed with the NP40 detergent. Contrarily, other analyzed detergents such as Triton X100 and Triton X114 did not power the amplification capacity of the ϕ 29 DNA polymerase (not shown). The simultaneous addition of 0.025% Tween® 20 and 45 mM $(\text{NH}_4)_2\text{SO}_4$ to the buffer A has two consequences in the yield and the specificity of the amplified products: 1) DNA amplification in the absence of provided DNA was not detected; 2) several μg of plasmid DNA of unit length were obtained by amplification even when the provided amount of DNA was as low as 10 fg. As control, the addition of 45 mM $(\text{NH}_4)_2\text{SO}_4$ to the Buffer A did not produce any improvement in the amplification capacity of the ϕ 29 DNA polymerase.

Therefore, it can be concluded that the simultaneous addition of 0.025% Tween® 20 and 45 mM $(\text{NH}_4)_2\text{SO}_4$ to the Buffer A (hereinafter Buffer B) produces a clear optimization of the experimental conditions for carrying out the amplification with multiple priming of circular DNA by the ϕ 29 DNA polymerase, both being absolutely necessary reactants to amplify limited amounts (10 fg) of provided DNA. In fact, as can be seen in Figure 2, the use of Buffer B allowed the ϕ 29 DNA polymerase to synthesize micrograms of DNA by using a provided amount of plasmid as low as 0.1 fg (~24 molecules) after 6 hours of reaction. As quality control, the digestion of the amplification products with *EcoRI* generated linear dsDNA fragments of 4.2 kb which indicated that the amplification products were really tandem repeats of the original plasmid. Again, the Buffer B also prevented the nonspecific DNA amplification (see Figure 2 the lanes corresponding to the reactions carried out without provided DNA).

Figure 3 shows the effect of the ammonium ions and 0.025% Tween® 20 in improving the amplification of small

amounts of plasmid DNA. The assay was carried out in the previously mentioned conditions in the presence of 0.025% Tween® 20 and the indicated ammonium salt. As can be observed in Figure 3 both the NH_4Cl and the $\text{NH}_4\text{CH}_3\text{COO}$ had a similar effect to the $(\text{NH}_4)_2\text{SO}_4$ both in the yield and in the specificity of the amplified products. This result indicates that the aforementioned effect of the $(\text{NH}_4)_2\text{SO}_4$ in the amplification of limiting amounts of plasmid DNA is due to the NH_4^+ ions.

To determine if the optimized conditions described above was also applied to the amplification of genomic DNA, the same type of assays performed in the presence of limited concentrations of *B. subtilis* genomic DNA (4 Mpb in length) was carried out. As shown in Figure 4, the presence of 0.025% Tween® 20 and 45 mM $(\text{NH}_4)_2\text{SO}_4$ in the buffer B, on the one hand prevented the nonspecific DNA amplification (lanes without provided DNA), and on the other hand, allowed the $\phi 29$ DNA polymerase to give detectable and specific genomic DNA amplification even when 10 fg of provided DNA were used, i.e., an amount 10^6 times lower than that recommended in the current commercial genomic amplification kits.

To determine if the simultaneous addition of 0.025% Tween® 20 and 45 mM $(\text{NH}_4)_2\text{SO}_4$ increases the amplification efficiency of the current commercial kits for amplifying DNA based on the $\phi 29$ DNA polymerase, the same type of plasmid DNA amplification assays described in Figures 1, 2 and 3 was carried out. Figure 5 shows the significant improvements depicted by the addition of 0.025% Tween® 20 and 45 mM $(\text{NH}_4)_2\text{SO}_4$ to the current reaction buffer of the Illustra kit (GE HealthCare). As can be observed, by following the recommendation of the supplier, with the Illustra kit only amounts of plasmid provided equal to or greater than 10 pg can be amplified in a detectable manner in agarose gel. Contrarily, the simultaneous addition of 0.025% Tween® 20 and 45 mM $(\text{NH}_4)_2\text{SO}_4$ to the reaction buffer of the Illustra kit

significantly reduces the needed amount of DNA which can be amplified, amplification products from provided 1 fg of plasmid DNA being observed, involving an improvement of four orders of magnitude in the amplification.

5 EXAMPLE 2. Improvement of the amplification capacity of the ϕ 29 DNA polymerase by the addition of HhH domains.

ϕ 29 DNA polymerase was fused with one or two HhH domains to construct new DNA polymerases with an improved DNA binding capacity which allows using less amounts of provided DNA. The
10 inspection of the structure of the ϕ 29 DNA polymerase lead to selecting the fusion of the HhH domains with the C-terminal end of the enzyme since this end (the end of the subdomain *thumb*) is close to the tunnel entrance of the top dsDNA sequence. The fusion of the HhH domains with the N-terminal
15 end of the ϕ 29 DNA polymerase would compromise the intrinsic capacity of strand detachment since the biochemical and structural data demonstrated that the unwinding of the parent DNA takes place near the amino end. Furthermore, positioning the HhH domains in the N-terminal end would not be suitable
20 one for improving the binding of the dsDNA part formed by hybridization of the hexamer primer with the template DNA. In this sense, the fusion of a sequence (His)₆ at the N-terminal end of the ϕ 29 DNA polymerase has a damaging effect in the amplification.

25 2.1 Chimeric DNA polymerase construct.

To make the chimeras HIAY and HIGT, GenScript Corporation was entrusted to synthesise a DNA fragment which contained the nucleotides encoding the HhH domains, H (56 amino acids) and I (51 amino acids) of the topoisomerase V of
30 *M. kandleri* (code in GenBank AF311944 and (Pavlov *et al.* Proc Natl Acad Sci USA. 2002; 99: 13510-13515), and the latter was cloned between the *EcoRV* sites of the commercial vector pUC57. The resulting plasmid pUC57-HhH was used as template for amplifying a DNA fragment encoding the domains H and I by PCR.

Therefore, the primer 3 (SEQ ID NO: 7) together with the primers 1 (SEQ ID NO: 8) or 2 (SEQ ID NO: 9) gave the DNA I and II fragments of 369 bp, respectively. In addition to a *KasI* site introduced by both primers, primer 1 also introduced the sequence encoding the SEQ ID NO: 5 connector (Pavlov *et al.* Proc Natl Acad Sci USA. 2002; 99: 13510-13515), whereas the primer 2 introduced the nucleotide sequence encoding the SEQ ID NO: 6 connector (a SEQ ID NO: 10 connector derivative previously described in Sun *et al.* Proteins. 2006; 65: 231-238). The primer 3 contained the sequence encoding the 6 histidine residues followed by a stop codon and a *BamHI* site (see in Figure 6 a simplified diagram of the chimeric DNA polymerase construct).

At the same time, a pJLw2 plasmid derivative (Lázaro *et al.* Methods Enzymol. 1995; 262: 42-49) containing the gene encoding the ϕ 29 DNA polymerase (572 amino acids) was used as a template for a PCR reaction carried out with the primer 4 (SEQ ID NO: 11) including a 5' *HindIII* site and the primers 5 (SEQ ID NO: 12) or 6 (SEQ ID NO: 13), to obtain the fragments III and IV of 1757 bp, respectively. The fragments III and IV will therefore contain the DNA encoding the ϕ 29 DNA polymerase followed by the SEQ ID NO: 5 (Fragment III) and SEQ ID NO: 6 (fragment IV) sequences which also include a *KasI* site. The fragments I-IV were purified in 0.7% agarose gels and were then digested with *KasI*. The digested DNA fragments I and III, and II and IV were ligated with the T4 DNA ligase to obtain a linear DNA of 2108 bp encoding the chimera HIAY (Fragment V) and HIGT (Fragment VI), respectively. The ligated products were purified in 0.7% agarose gels and were then digested with the *BamHI* and *HindIII* endonucleases. The digested products were purified by electrophoresis in agarose gels. The fragments V and VI were finally cloned in the vector pT7-4 (Tabor *et al.* Proc Natl Acad Sci USA. 1985; 82: 1074-1078). The chimeras HIAY (ϕ 29 DNA polymerase + SEQ ID NO: 5 connector

+ domains H and I of topoV) and HIGT (ϕ 29 DNA polymerase + SEQ ID NO: 6 connector + domains H and I of topoV) were used as the template to construct the chimeras HGT and HAY, respectively, by inserting a stop codon after the H fragment of TopoV by means of the QuikChange® (Stratagene) directed mutagenesis kit. The confirmation of the DNA sequence and the absence of additional mutations was carried out by means of sequencing the entire gene. The chimeric DNA polymerases were expressed in BL21(DE3) cells of *E. coli* which housed the cloned chimeric gene in a pJLw2 plasmid derivative, and were then essentially purified as described in (Lázaro *et al.* Methods Enzymol. 1995; 262: 42-49).

In summary, the chimeric DNA polymerases obtained were the following:

HAY: ϕ 29 DNA polymerase -SEQ ID NO: 5- HhH H (635 aa; ~73 kDa)

HGT: ϕ 29 DNA polymerase -SEQ ID NO: 6- HhH H (635 aa; ~73 kDa))

HIAY: ϕ 29 DNA polymerase -SEQ ID NO: 5- HhH H-I (692 aa; ~80 kDa)

HIGT: ϕ 29 DNA polymerase -SEQ ID NO: 6- HhH H-I (692 aa; ~80 kDa)

2.2. DNA binding capacity of the chimeric DNA polymerases.

To determine if the fusion of the HhH motifs at the end of the ϕ 29 DNA polymerase conferred an improved binding capacity of the chimeras to the DNA, the retardation analysis of the electrophoretic mobility of the DNA in gel was carried out.

Conditions of the assay.- The oligonucleotides of 15 bases (SEQ ID NO: 14) and 21 bases (SEQ ID NO: 15) having a 5' extension of six nucleotides in addition to the sequence complementary to the oligonucleotide of 15 bases were supplied by Isogen. The oligonucleotide of 15 bases was labelled in 5' with [γ -³²P] ATP and the T4 kinase polynucleotide. The oligonucleotide of 15 bases labelled in 5' was hybridized with

that of 21 bases in the presence of 0.2 M NaCl and 60 mM (pH 7.5) tris-HCl. The hybridized oligonucleotide molecule of 15 bases labelled in 5'/oligonucleotide of 21 bases was used to analyze the interaction with the natural or chimeric ϕ 29 DNA polymerases. The incubation mixture, in a final volume of 20 μ l contained 50 mM (pH 7.5) tris-HCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, 20 mM ammonium sulfate, 0.1 mg/ml bovine serum albumin (BSA), 4% glycerol, 1 nM oligonucleotide molecule of 15 bases/21 bases, and the indicated concentration of the natural or chimeric ϕ 29 DNA polymerase. After incubation for 5 min at 4 °C, the samples were subjected to electrophoresis in 4% polyacrylamide gels (w/v) (monomer:bis 80:1) which contained 12 mM (pH 7.5) tris-acetate and 1 mM EDTA and were developed at 4 °C in the same buffer at 8 V/cm, essentially as described by (Carthew *et al.* Cell. 1985; 43: 439-448). After autoradiography, the polymerase-dsDNA complex was detected as a detachment of the mobility (retardation) in the migration position of the labelled DNA.

In these conditions, the natural ϕ 29 DNA polymerase produces a single retardation band using the labelled hybridized oligonucleotide molecule of 15 bases/21 bases (see Figure 7) which has been interpreted as a stable enzyme-DNA complex competent for polymerization (Méndez *et al.* J Biol Chem. 1994; 269: 30030-30038). The chimeras HAY, HGT and HIGT showed a DNA binding capacity greater than the natural enzyme since the larger part of the substrate was detached with a 9.5 nM concentration unlike the natural DNA polymerase which needed an approximately 2 times higher concentration. The chimera HIAY had a DNA binding capacity similar to, or even less than, that of the natural polymerase. From these results, it can be concluded that, in general, the addition of the HhH H and H+I domains of Topo V to the C-terminal end of the ϕ 29 DNA polymerase confers an improved DNA binding capacity, although there are exceptions such as in the case of the

chimera HIAY.

2.3. Rolling circle replication by the chimeric DNA polymerases.

To determine if the improvement in the DNA binding obtained by the addition of the HhH domains of the TopoV to the C-terminal end of the ϕ 29 DNA polymerase affected, on the one hand, the polymerization activity, and on the other, the processive synthesis of DNA coupled to the strand detachment, replication assays with M13 primer in which the DNA polymerase starts the polymerization from the 3'-OH group of a DNA oligonucleotide were carried out. In this assay, the first replication cycle does not require strand detachment, but once it is completed, the polymerase finds the 5' end of the primer, thus requiring an active detachment to continue the following replication cycles (rolling circle type).

Conditions of the assay.- The M13mp18 ssDNA was hybridized with the universal primer in the presence of 0.2 M NaCl and 60 mM (pH 7.5) tris-HCl, and the resulting molecule was used as a primer/template to analyze the polymerization of processive DNA coupled to the strand detachment by the chimeric DNA polymerases. The incubation mixture contained in 25 μ l, 50 mM (pH 7.5) tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml BSA, 40 μ M dCTP, dGTP, dTTP, and [α - ^{32}P]dATP (1 μ Ci), 250 ng M13mp18 ssDNA primed with the oligonucleotide of sequence SEQ ID NO: 16, and 30 nM natural or chimeric ϕ 29 DNA polymerase. After incubating for the indicated times at 30°C, the reactions were stopped by the addition of 0.1% EDTA 10 mM -SDS, and the samples were filtered through Sephadex G-50 columns. The relative activity was calculated from the Cerenkov radiation corresponding to the excluded volume. For size analysis, the labelled DNA was denatured by treatment with 0.7 M NaOH and was subjected to electrophoresis in 0.7% alkaline agarose gels as described in (McDonnell *et al.* J Mol Biol. 1977; 110: 119-146). After the

electrophoresis, the M13mp8 ssDNA of unit in length was detected by staining with ethidium bromide, and the gels were then dried and autoradiographed.

As shown in Figure 8A, a significant discovery is that the presence of the HhH domains does not interfere with the strand detachment capacity of the chimeric DNA polymerases. The amount of DNA synthesized by the chimeras HAY, HGT, HIAY and HIGT was greater than that synthesized by the natural ϕ 29 DNA polymerase (4, 5, 5 and 7 times, respectively). The replication speed was similar (in the case of the chimeras HAY and HGT) or even faster (chimeras HIGT and HIAY) than that obtained with the natural ϕ 29 DNA polymerase. This result indicates that the presence of HhH domains in the C-terminal end of the ϕ 29 DNA polymerase in the chimeras improves the use of the template DNA during the rolling circle replication.

2.4. Processive polymerization by the chimeric DNA polymerases.

ϕ 29 DNA polymerase is a paradigm for the processive DNA replication since it is capable of incorporating more than 70 kb without dissociating from the DNA in the absence of auxiliary proteins. Therefore, whether the fusion of the HhH domains in the C-terminal end of the ϕ 29 DNA polymerase in the chimeric DNA polymerases affected the processivity of the polymerization was analyzed.

Conditions of the assay.- The processivity of the chimeric DNA polymerases was analyzed with different proportions of enzyme/DNA. The incubation mixture contained, in 25 μ l, 50 mM (pH 7.5) tris-HCL, 10 mM $MgCl_2$, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml BSA, 40 μ M dCTP, dGTP, dTTP, and [α - ^{32}P]dATP (1 μ Ci), 250 ng primed M13mp18 ssDNA, and the indicated decreasing amounts of the natural ϕ 29 DNA polymerase or chimeric DNA polymerases. After incubating for 20 min at 30°C, the reactions were stopped by the addition of 10 mM EDTA -0.1% SDS, and the samples were filtered through

Sephadex G-50 columns. For size analysis, the labelled DNA was denatured by treatment with 0.7 M NaOH and was subjected to electrophoresis in 0.7% alkaline agarose gel. The processivity of the polymerization was evaluated by analyzing the length of the replication products with decreasing proportions of DNA polymerase/DNA.

As shown in Figure 8B, decreasing proportions of enzyme/DNA did not alter the length of the elongation products synthesized by the natural or chimeric $\phi 29$ DNA polymerases, according to a processive DNA polymerization model.

2.5. Rolling circle amplification (RCA) with multiple priming of plasmid DNA by the natural and chimeric $\phi 29$ DNA polymerases.

As has been described above, both the high processivity and the strand detachment capacity which the $\phi 29$ DNA polymerase has were the base for the development of one of the more efficient processes for amplifying isothermal ssDNA by Amersham Biosciences/Molecular Staging, in which the $\phi 29$ DNA polymerase combined with random hexamer primers achieves the isothermal and accurate amplification of 10^4 to 10^6 times by strand detachment of picograms of circular plasmids [Templiphi™ (www.gehealthcare.com)]. The results with a $\phi 29$ DNA polymerase which contained a (His)₆ sequence fused in the C-terminal end thereof showed that, despite the efficiency thereof, during the rolling circle replication with a single primer, it was incapable of giving amplification products detectable during the RCA with multiple priming. The same was certain for other mutant derivatives of the $\phi 29$ DNA polymerase which showed greater affinity for the dNTP, replicating the M13 DNA at the level of the natural DNA polymerase, but could not give amplification products. Therefore, although the fusion of HhH domains to the C-terminal end of the $\phi 29$ DNA polymerase improved the intrinsic capacity of the natural enzyme to perform the rolling circle replication with a single

primer, a similar efficiency increase during the RCA with multiple priming could not be anticipated.

5 Conditions of the assay.- The incubation mixture contained, in 12.5 μ l of buffer B, 10 fg plasmid DNA (4.2 kpb) as supply. To denature the DNA provided, the samples were incubated for 3 min at 95°C and were then cooled in ice for 5 min. The reactions were initiated by the addition of 50 nM natural or chimeric ϕ 29 DNA polymerase. After incubating at 30°C for the indicated times, the reactions were stopped by
10 incubating the samples for 10 min at 65°C. 1 μ l of each reaction was digested with EcoRI and was then analyzed by electrophoresis in 0.7% agarose gel. After electrophoresis, the amplified DNA was detected by staining with ethidium bromide.

15 As shown in Figure 9, the natural ϕ 29 DNA polymerase gave a detectable amplification product from 4 h of reaction. The chimera HAY gave a detectable amplification product from 4 h, the total production amount of amplification after 5 h being twice of that obtained with the natural ϕ 29 DNA
20 polymerase. The chimera HGT produced an amount of amplified DNA comparable to that obtained with the chimera HAY; it is interesting that the yield of maximum amplification was obtained in the shorter reaction time (3 h). The maximum amount of amplified DNA with the chimeras HIAY and HIGT was
25 similar to that obtained with the natural ϕ 29 DNA polymerase, although as in the case of the chimera HGT, said maximum yield was achieved from 3 h of reaction. After digesting the synthesized DNA with the four chimeras with EcoRI, more than
30 80% of the amplified DNA gave a dsDNA fragment of 4.2 kb which indicated that the large part of the amplification product really consisted of tandem repeats of the original plasmid.

 This result indicates a greater capacity of the chimeric DNA polymerases to amplify limited amounts (10 fg) of the plasmid DNA than the natural ϕ 29 DNA polymerase.

2.6 Amplification of the DNA with multiple priming of genomic DNA by the natural and chimeric ϕ 29 DNA polymerases.

In addition to the RCA technology with multiple priming described above, a process for amplifying entire genome known as multiple displacement amplification (MDA) was developed based on the properties of the ϕ 29 DNA polymerase combined with the use of random hexamer primers (Dean *et al.* Proc Natl Acad Sci USA. 2002; 99: 5261-5266). The Genomiphi™ (GE Healthcare) and Repli-G® (Qiagen) kits, based on this process, require a minimum amount of 10 ng of genomic DNA. To investigate if the chimeric DNA polymerases have improved capacity for amplifying limited amounts of genomic DNA with respect to the natural ϕ 29 DNA polymerase, MDA of the *B. subtilis* genomic DNA was performed.

Conditions of the assay.- The incubation mixture contained, in 12.5 μ l of buffer B, 100 fg *B. subtilis* genomic DNA. To denature the DNA, the samples were incubated for 3 min at 95°C and were then cooled with ice for 5 min. The reactions were initiated by the addition of 50 nM natural or chimeric ϕ 29 DNA polymerase. After incubating at 30°C for the indicated times, the reactions were stopped by incubating the samples for 10 min at 65°C. 1 μ l of each reaction was analyzed by electrophoresis in 0.7% agarose gel. After electrophoresis, the amplified DNA was detected by staining with ethidium bromide.

As shown in Figure 10, in the experimental conditions described above, the natural ϕ 29 DNA polymerase gave detectable amplification products after 5 h of incubation. The chimera HAY also produced amplification products in this time, but the total amount of amplified DNA after 6 h was much greater than that obtained with the natural ϕ 29 DNA polymerase. As can also be observed, the rest of the chimeras, HGT, HIAY and HIGT gave clear amplification products in the shorter assay time (3 h), the total amplified amount in this

time being similar to (HIAV and HIGT) or much greater (HGT) than that observed after 6 h with the natural ϕ 29 DNA polymerase. These results indicate that the presence of the HhH domains provides the chimeric DNA polymerases an improved capacity for amplifying the genomic DNA with respect to the natural ϕ 29 DNA polymerase.

2.7 Measurement of the accuracy of the chimeric DNA polymerases.

3 μ l of each of the samples from the experiment showed in Figure 9, corresponding to the multiple rolling circle amplification of 10 fg plasmid DNA were incubated in the presence of 17 μ l of the restriction mixture (2 μ l New England Biolabs (NEB) 10X EcoRI Buffer, 0.5 μ l [10 units] of the NEB EcoRI endonuclease and 14.5 μ l H₂O) to obtain linear monomers of the amplified plasmid. After incubating for 1 hour at 37°C, the DNA was purified through Qiagen Gel-Extraction Kit Columns and was eluted in 30 μ l TE buffer (10 mM (pH 7.5) tris-HCl, 1 mM EDTA). 10 μ l of each elution were religated by incubating them with 2 μ l NEB 10X Ligase Buffer, 8 μ l H₂O and 0.5 μ l (200 units) NEB T4 DNA ligase. The reactions were incubated for one night at 16 °C, and *E. coli* XL-1 Blue competent cells were transformed with 2 μ l of each. Approximately 1000 transformants were obtained with each of the amplification samples, whereas none were obtained with the control samples which contained 10 fg of a plasmid of 4.2 kpb treated in the same manner as each of the samples described above. Two clones were selected from each transformation and the corresponding plasmids were purified and sequenced according to standard processes. The oligonucleotides used for the sequencing were: pT7-N (SEQ ID NO: 17), sp4+10 (SEQ ID NO: 18) and sp10+7 (SEQ ID NO: 19). In total, 4918 non-overlapping nucleotides of each of the plasmids amplified by the natural and chimeric DNA polymerases were sequenced. The results are shown in Table 1 and they indicate a synthesis accuracy of the chimeric DNA polymerases similar to the natural enzyme.

Table 1. Polymerization accuracy of the natural and chimeric ϕ 29 DNA polymerases

Polymerase	Mutations
Natural	0
HAY	0
HGT	0
HIAY	1 (transversion G to T)
HIGT	0

4918 non-overlapping nucleotides of each of the plasmids amplified by the natural and chimeric DNA polymerases were sequenced as described in the main text.

CLAIMS

1.- A DNA polymerase chimera comprising:

- a) an amino acid sequence encoding a ϕ 29 type DNA polymerase, bound by its C-terminal end to
- b) a connecting amino acid sequence bound by its C-terminal end to
- c) an amino acid sequence comprising at least one helix-hairpin-helix (HhH) domain.

2.- The DNA polymerase chimera according to claim 1, wherein the amino acid sequence of (c) comprises at least one HhH domain of a protein which is selected from the list comprising:

- topoisomerase V of *Methanopyrus kandleri*,
- MutY, Nth, MutM/Fpg, Nei, UvrC, DinP, RecR, UmuC, DnaE or DnIJ of *Escherichia coli*,
- RAD1, RAD2, RAD10, RAD27, RAD 55, RAD 57, REV1, OGG1, NTG1, NTG2, DIN-7 or EXO-1 of yeasts, or
- a homologous protein of the above in *Bacillus subtilis*, *Caenorhabditis elegans*, *Haemophilus influenzae*, *Methanococcus jannaschii*, *Micrococcus luteus*, *Methanobacterium thermoformicum* or *Salmonella typhimurium*.

3.- The DNA polymerase chimera according to claim 2, wherein the amino acid sequence of (c) comprises at least one HhH domain derived from the topoisomerase V of *Methanopyrus kandleri*.

4.- The DNA polymerase chimera according to claim 3, wherein the amino acid sequence of (c) is SEQ ID NO: 3.

5.- The DNA polymerase chimera according to claim 3, wherein the amino acid sequence of (c) is SEQ ID NO: 3 bound by its C-terminal end to SEQ ID NO: 4.

6.- The DNA polymerase chimera according to any of claims 1 to 5, wherein the connecting amino acid sequence of

(b) is SEQ ID NO: 5 or SEQ ID NO: 6.

7.- The DNA polymerase chimera according to any of claims 1 to 6, wherein the ϕ 29 type DNA polymerase of (a) is selected from the DNA polymerases isolated from the following phages: ϕ 29, Cp-1, PRD-1, ϕ 15, ϕ 21, PZE, PZA, Nf, M2Y, B103, GA-1, SF5, Cp-5, Cp-7, PR4, PR5, PR722, L17 or ABV.

8.- The DNA polymerase chimera according to any of claims 1 to 7, wherein the ϕ 29 type DNA polymerase of (a) has an amino acid sequence having an identity of at least 80% with SEQ ID NO: 1.

9.- The DNA polymerase chimera according to claim 8, wherein the ϕ 29 type DNA polymerase of (a) has an amino acid sequence having an identity of at least 90% with SEQ ID NO: 1.

10.- The DNA polymerase chimera according to claim 9, wherein the ϕ 29 type DNA polymerase of (a) has the amino acid sequence SEQ ID NO: 1.

11.- The DNA polymerase chimera according to any of claims 1 to 10, wherein the ϕ 29 type DNA polymerase of (a) has a modification in the exonuclease domain and wherein said modified DNA polymerase has less than 10% of exonuclease activity than the corresponding naturally occurring DNA polymerase.

12.- The DNA polymerase chimera according to claim 11, wherein the modified ϕ 29 type DNA polymerase of (a) has less than 1% of exonuclease activity than the corresponding naturally occurring DNA polymerase.

13.- The DNA polymerase chimera according to claim 12, wherein the modified ϕ 29 type DNA polymerase of (a) lacks detectable exonuclease activity with respect to the corresponding naturally occurring DNA polymerase.

14.- Use of the DNA polymerase chimera according to any of claims 1 to 13 for replicating, amplifying or sequencing a template DNA.

15.- Method for replicating, amplifying or sequencing a

template DNA which comprises contacting said DNA with a reaction mixture comprising at least:

- a) the DNA polymerase chimera according to any of claims 1 to 12,
- 5 b) a buffer,
- c) magnesium chloride,
- d) a primer, and
- e) nucleoside triphosphates.

10 16.- The method according to claim 15, wherein the reaction mixture further comprises polyoxyethylenated sorbitan monolaurate.

17.- The method according to either of claims 15 or 16, wherein the reaction mixture further comprises an ammonium salt.

15 18.- The method according to any of claims 15 to 17, wherein the reaction mixture further comprises a potassium salt.

19.- The method according to claim 18, wherein the potassium salt is potassium chloride or potassium acetate.

20 20.- The method according to any of claims 16 to 19, wherein the polyoxyethylenated sorbitan monolaurate is in a proportion between 0.003% and 0.1% of the total volume of the reaction.

21.- The method according to claim 20, wherein the polyoxyethylenated sorbitan monolaurate is in a proportion between 0.006% and 0.05% of the total volume of the reaction.

22.- The method according to claim 21, wherein the polyoxyethylenated sorbitan monolaurate is in a proportion between 0.01% and 0.03% of the total volume of the reaction.

30 23.- The method according to any of claims 17 to 22, wherein the ammonium salt is selected from the list comprising: ammonium sulfate, ammonium chloride or ammonium acetate.

35 24.- The method according to claim 23, wherein the ammonium salt is ammonium sulfate.

25.- The method according to claim 24, wherein the ammonium sulfate is at a concentration between 30 mM and 60 mM.

5 26.- The method according to claim 25, wherein the ammonium sulfate is at a concentration between 40 mM and 50 mM.

27.- The method according to claim 23, wherein the ammonium salt is ammonium chloride or ammonium acetate.

10 28.- The method according to claim 27, wherein the ammonium chloride or the ammonium acetate is at a concentration between 60 mM and 120 mM.

29.- The method according to claim 28, wherein the ammonium chloride or the ammonium acetate is at a concentration between 80 mM and 100 mM.

15 30.- The method according to any of claims 15 to 29, wherein the buffer is tris-hydrochloric, tris-acetic or HEPES.

31.- The method according to any of claims 15 to 30, wherein the buffer is at a pH between 7.0 and 8.5.

20 32.- The method according to any of claims 15 to 31, wherein the magnesium chloride is at a concentration between 2 mM and 20 mM.

33.- The method according to claim 32, wherein the magnesium chloride is at a concentration between 5 mM and 15 mM.

25 34.- The method according to any of claims 18 to 33, wherein the potassium chloride or the potassium acetate is at a concentration between 30 mM and 70 mM.

30 35.- The method according to claim 34, wherein the potassium chloride or the potassium acetate is at a concentration between 40 mM and 60 mM.

36.- The method according to any of claims 15 to 35, wherein the nucleoside triphosphates are dCTP, dGTP, dTTP and dATP.

35 37.- The method according to claim 36, wherein the dCTP, dGTP, dTTP and dATP nucleoside triphosphates are in equimolar

amounts.

38.- The method according to any of claims 15 to 37, wherein the primer is arbitrary and is protected against the action of exonucleases.

5 39.- The method according to any of claims 15 to 38, wherein the template DNA is plasmid DNA.

40.- The method according to any of claims 15 to 38, wherein the template DNA is genomic DNA.

10 41.- The method according to any of claims 15 to 40, wherein the amplification is performed at an essentially constant temperature between 25 and 40 °C.

15 42.- The method for amplifying a template DNA according to any of claims 15 to 41, wherein the amplification takes place by means of rolling circle amplification (RCA), multiple displacement amplification (MDA), strand displacement amplification (SDA) or loop mediated amplification (LAMP).

43.- The method according to any of claims 15 to 42, wherein at least one nucleoside triphosphate or one primer is labelled.

20 44.- Kit for carrying out a method according to claims 15 to 43 comprising:

- a) the DNA polymerase chimera according to any of claims 1 to 13,
- b) a buffer, and
- 25 c) magnesium chloride.

45.- Kit according to claim 44, further comprising polyoxyethylenated sorbitan monolaurate.

46.- Kit according to either of claims 44 or 45, further comprising an ammonium salt.

30 47.- Kit according to any of claims 44 to 46, further comprising a potassium salt.

48.- Kit according to either of claims 44 or 47, further comprising a primer.

35 49.- Kit according to any of claims 44 to 48, further comprising the primer according to claim 38.

50.- Kit according to any of claims 44 to 49, further comprising nucleoside triphosphates.

51.- Kit according to claims 48 to 50, wherein at least one nucleoside triphosphate or one primer is labelled.