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DNA Polymerization in Icy Moon Abyssal Pressure Conditions

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Abstract :

Evidence of stable liquid water oceans beneath the ice crust of moons within the Solar System is of great interest for astrobiology. In particular, subglacial oceans may present hydrothermal processes in their abysses, similarly to terrestrial hydrothermal vents. Therefore, terrestrial extremophilic deep life can be considered a model for putative icy moon extraterrestrial life. However, the comparison between putative extraterrestrial abysses and their terrestrial counterparts suffers from a potentially determinant difference. Indeed, some icy moons oceans may be so deep that the hydrostatic pressure would exceed the maximal pressure at which hydrothermal vent organisms have been isolated. While terrestrial microorganisms that are able to survive in such conditions are known, the effect of high pressure on fundamental biochemical processes is still unclear. In this study, the effects of high hydrostatic pressure on DNA synthesis catalyzed by DNA polymerases are investigated for the first time. The effect on both strand displacement and primer extension activities is measured, and pressure tolerance is compared between enzymes of various thermophilic organisms isolated at different depths.

Introduction

Terrestrial life is possible under a large range of chemical and physical conditions. Extremophilic microorganisms push the boundaries of these ranges further than what can be expected from an anthropocentric point of view, inhabiting extreme environments which were previously thought to be too hostile for life. Yet, even extremophilic life is limited to environments above a certain level of water availability (Hallsworth *et al.*, 2007; Belilla *et al.*, 2019). Hence, looking for liquid water bodies remains essential for the search of extraterrestrial life and habitable environments. In this context, the subglacial oceans of icy moons of the Solar System are of particular interest (Jebbar *et al.*, 2020; Taubner *et al.*, 2020). Among them, Europa and Enceladus retain most of the attention. Several lines of evidence show that the water would be in interaction with rock, enabling enrichment of subglacial ocean with various ions, metals and organic molecules. Characterization of these interactions is indeed a major goal of future space missions (Pappalardo, 2012; Cable *et al.*, 2016). Moreover, these subglacial oceans may possess hydrothermal processes at their bottom. While stronger evidence support this hypothesis for Enceladus (Matson *et al.*, 2007; Hsu *et al.*, 2015; Sekine *et al.*, 2015; Waite *et al.*, 2017), geochemical models also argue in favor of abyssal hydrothermal processes for Europa (Lowell and DuBose, 2005; Zolotov, 2007).

Such extraterrestrial abyssal hydrothermal environments would be expected to show similarities with their terrestrial counterparts: hydrothermal vents and black smokers. On Earth, these oceanic environments are observed at various depths, ranging from shallow waters to 5km deep. While displaying extreme conditions such as temperature, pressure and sometimes alkaline pH or heavy metal abundancy, these environments are actually associated with complex and diverse ecosystems, offering energy sources and chemical and physical gradients for life. In the absence of light, the primary producers of hydrothermal vent environments are extremophilic chemolithotrophs. These microorganisms, either belonging to the *Bacteria* or *Archaea* domain, therefore make good models for testing habitability and have already been used as such (Taubner *et al.*, 2018).

However, the physical and chemical conditions may differ in extraterrestrial abysses. On Earth, deepest hydrothermal vent lies at approximatively 5km under the sea level with *in situ* pressure of 50MPa (Connelly *et al.*, 2012) and deepest point in the ocean is met in the Mariana Trench at 11km under the sea level at a pressure of approximatively 108MPa. In shear contrast, subglacial oceans of several moons could be hundreds kilometers deep (Schmidt and Manning, 2017). On Europa for example, the ocean is expected to be, depending on the model, up to 100-200km deep (Anderson *et al.*, 1997, 1998; Pappalardo *et al.*, 1999; Spohn and Schubert, 2003; Marion *et al.*, 2003; Thomas *et al.*, 2016) and beneath a 10km thick ice shell (Park *et al.*, 2015), reaching hydrostatic pressures as high as 130-260MPa (Naganuma and Uematsu, 1998).

Even though most of the terrestrial biosphere is permanently under high hydrostatic pressure (HHP), as 88% of the ocean volume is above 10MPa (Jebbar *et al.*, 2015), its effects on complex biological processes remains to be explored. Deep sea organisms have to cope with effects of HHP at the molecular level. Indeed, following Le Chatelier's principle, increases of HHP promotes reduction of volume through production of charges and condensation of biomolecules (Gross and Jaenicke, 1994; Abe *et al.*, 1999). In particular, dissociation of large

molecular assemblies such as ribosomes (Gross *et al.*, 1993) and DNA-protein complexes (Kawano *et al.*, 2005) is favored under HHP. Upon higher pressure increase, protein unfolding is facilitated (Jebbar *et al.*, 2020), most proteins losing their tertiary structure at 400MPa (Aertsen *et al.*, 2009). Before unfolding, HHP may have either an inhibitory or a stimulating effect on enzymatic activity, depending on the enzyme (Eisenmenger and Reyes-De-Corcuera, 2009). Notably, a peptidase complex from an abyssal hyperthermophilic *Archaea* have showed to be activated by HHP (Rosenbaum *et al.*, 2012). DNA is also reversibly compacted by HHP but remains exceptionally stable as it resists denaturation at even 1GPa (Hedén *et al.*, 1964; Girard *et al.*, 2007). Experimental data actually show that nucleic acids are stabilized by high hydrostatic pressure (Macgregor, 1996; Dubins *et al.*, 2001). As a consequence, all biological processes requiring an opening of the DNA double-helix, such as replication, transcription and translation, can be impaired by high hydrostatic pressure (Macgregor, 2002).

Yet, DNA synthesis has never been studied under HHP. DNA replication is one of the most conserved process, which must occur in every cell before cell division, thus, to ensure successful inheritance of genetic traits. *In cellulo* this process is catalyzed by a class of enzymes called DNA polymerases (DNAPs) which take part of a larger complex, the replisome. DNAPs require a DNA strand primed by a DNA or RNA strand with 3'-hydroxyl extremity, deoxyribonucleotide triphosphates (dNTPs) and divalent cations, Mg^{2+} or Ca^{2+} (Ralec *et al.*, 2017). The simplicity of this process has made possible all existing PCR technologies, allowing fast and unexpensive amplification of DNA. In this context, thermostable DNAPs from organisms isolated in hot environments are routinely used. In particular, many B-family DNA polymerases from hyperthermophilic *Archaea*, showing high thermostability, extension rate and fidelity have been characterized (Zhang *et al.*, 2015). Some were isolated from deep sea organisms which are permanently exposed to high hydrostatic pressure. While their activity is high at ambient pressure, there has been no characterization at physiological or even higher pressure conditions. To our knowledge, it has only been showed that a pressure of 89MPa strongly enhances thermostability of DNA polymerases from both deep sea and shallow waters (Summit *et al.*, 1998).

In this study, effects of HHP as high as 100MPa on activity of five thermostable DNAPs are investigated for the first time.

Material and methods

DNAPs production and purification

To facilitate interpretation of the effects of HHP on polymerase activity, we only used enzymes without 3 '→ 5' exonuclease activity, which allows proofreading, either naturally deprived of this activity or by using exonuclease-deficient mutants (exo$^-$).

Exonuclease-deficient *Pyrococcus abyssi* B-family DNAP (*Pab*PolB exo$^-$) (D215A) with a Nter his-tag sequence MGSSHHHHHHSSGLVPRGSH was produced and purified as previously described (Gueguen *et al.*, 2001; Gouge *et al.*, 2012). Briefly, enzyme was recombinantly expressed in *Escherichia coli* Rosetta 2 (DE3) cells transformed with a pET28 vector (Novagen) in which gene of PabPolB exo$^-$ was inserted between NdeI and BamHI restriction sites. Cells were grown to an optical density of 0.7 in LB medium at 37°C under shaking and in presence of kanamycine (50mg.mL^{-1}). Expression was then induced by addition of 1mM IPTG and cells were incubated overnight at 30°C. After centrifugation at 3000g for 20min at 4°C, cell pellets were suspended in 50mM Tris/HCl pH 7, 200mM NaCl, 9mM imidazole and 0.1% triton X-100 buffer to which were added protease inhibitors (Roche). Cells were lysed by sonication and lysate was incubated 1h at ambient temperature after addition of 50µg.mL^{-1} of DNase I. A heat treatment of 75°C during 30min was applied to the supernatant before purifying the protein by column chromatography. HisTrapHP™ with nickel-affinity resin, HiTrap™ Heparin HP with affinity for DNA-binding proteins and size exclusion Superdex™ 200 10/300 GL columns (GE Healthcare) were successively used. After dialysis, protein was stored at -20°C in a final buffer containing 50mM Tris–HCl, pH7.5, and 2mM $MgCl_2$. Quality and purity of sample was checked by electrospray ionization (ESI) at the mass spectrometry platform at IBS. Observed mass of dominant species was in agreement with a loss of MGSSHHH at Nter. A final concentration of 1.16µM (0.106mg/mL) of protein was measured with a NanoDrop™ device from ThermoFisher. Molar extinction coefficient was determined using an online calculator (https://web.expasy.org/cgi-bin/protparam/protparam).

Other proofreading-lacking DNAPs were all purchased. *Taq* from *Thermus aquaticus*, Vent® (*Tli*) exo$^-$ from *Thermococcus littoralis*, and Deep Vent® (*Psb)* exo$^-$ from *Pyrococcus* specie GB-D were purchased from New England Biolabs. *Pfu* exo$^-$ from *Pyrococcus furiosus* was obtained from Agilent. Concentrations of purchased polymerases were given by the manufacturers and were all equal to 2000units.mL^1.

Oligonucleotides and template DNA

For extension assays, template DNA was the M13mp18 singled-stranded circular DNA, 7249 nucleotides (nt) in length, purchased from New England Biolabs. For experiments at 55.5-65.0°C, the template was primed with a 30nt primer whose sequence was TGCCAAGCTTGCATGCCTGCAGGTCGACTC. For gel analysis experiments, this primer was labelled at the 5' end with a Cy5 fluorophore. Since primer-template interaction was no longer stable at higher temperatures, we used an 87nt primer instead whose sequence was TGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATC ATGGTCATAGCTGTTTCCTG for experiments at 70.0°C.

For strand displacement assays, template DNA was an oligonucleotide 87nt in length whose sequence was CAGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCA and which was labelled with a 6-FAM fluorophore at the 5'-end and primed with a 30t quencher whose sequence was TGCCAAGCTTGCATGCCTGCAGGTCGACTC. Fluorescence was initially quenched by a Deep Dark Quencher I 3'-modification harbored by a 30nt oligonucleotide, whose sequence was ATTCGTAATCATGGTCATAGCTGTTTCCTG, complementary to the sequence of the template from the 5'-end.

All oligonucleotides were purchased from Eurogentec (Seraing, Belgium).

High-pressure real-time fluorimetry

All fluorescence measurements were performed at various temperatures using a Jasco (Tokyo, Japan) FP-750 spectrofluorometer modified to accommodate a high-pressure cell manufactured by Top Industrie (Vaux-le-Pénil, France). 200µL of reaction mixtures were prepared to be put in custom rectangular quartz microcuvettes manufactured by Jasco (Tokyo, Japan) which were sealed with plastic film and joint as follows: approx 1cm^2 of plastic film was placed on top of the microcuvette and an O-ring joint from Sephat (Palaiseau, France) was placed over it to maintain the seal. Mixture volume slightly exceeded microcuvette volume to prevent the entrapment of an air bubble. No evidence of sample leak could be observed with experiments and controls. In particular, no sign of mechanical constraint on plastic film or O-ring joint was see. Excessive reaction mixture was removed prior to the experiment.

The measurement chamber was filled with distilled water at the desired temperature. The microcuvette was inserted in a custom metal adapter, which allows fluorescence measurement, then put inside the high-pressure cell while immediately starting signal acquisition. To prevent entrapment of an air bubble inside, excessive water was present in the chamber and removed during closure of the chamber. This allowed a better control and monitoring of temperature equilibrium of the sample.

After sealing, pressure was increased with a manual pump linked to the high-pressure cell through a water-filled metal capillary tube connected to the chamber. Pressure was monitored with a probe manufactured by Top Industrie (Vaux-le-Pénil, France), located between pump and high-pressure cell. HHP were typically reached in 1-2min.

Temperature inside the chamber was controlled through the circulation of a heat transfer fluid made with tap water supplemented with 30% ethylene glycol and anti-algae Aqua Stabil from Julabo (Seelbach, Germany). This fluid circulated within the high-pressure cell separately from the chamber and the capillary and was heated with a CORIO CD-200F refrigerated/heating circulator purchased from Julabo (Seelbach, Germany). The temperature to which the circulator had to be set to reach the desired temperature for the reaction was empirically determined by measuring it in the chamber under ambient pressure with a calibrated thermocouple.

Real-time strand displacement assays

For real-time strand displacement assays, we used a modified tripartite DNA hybrid (Dorjsuren *et al.*, 2009). Reaction mixtures consisted of 50mM Tris-Hcl pH8.8, 50mM KCl, 2mM

$MgCl_2$, 2mM DTT, 200µM dNTPs, 200nM tripartite DNA hybrid. *Pab*PolB exo$^-$ enzyme concentration was 46,4nM.

Excitation was made continuously at 480nm and emission at 525nm was measured every second. Total run length was 15min.

Real-time extension assays

For real-time extension assays, reaction mixtures consisted of 50mM Tris-Hcl pH8.8, 50mM KCl, 2mM $MgCl_2$, 2mM DTT, 200µM dNTPs, 418pM primed M13mp18. Picogreen™ (Thermofisher) was added at final 3200-fold dilution from the commercial solution. All commercial enzymes were incubated in the same buffer, except *Pfu* exo$^-$ which was incubated in the buffer provided by the manufacturer (Agilent). *Pab*PolB exo$^-$ enzyme concentration was 46.4 nM. Commercial enzymes were used at a concentration of 0.01 unit.mL^{-1}.

Excitation was made at 485nm every 20s and emission at 520nm was measured for 1s. Total run length was either 15 or 30min.

Gel analysis of products

Reactions were conducted similarly to the real-time extension assays in the same thermostated fluorescence cell with the fluorimeter turned off and without picogreen. The primer was labelled with a Cy5 fluorophore at the 5' end. After a 30min extension reaction under various pressures, decompression, chamber opening and sample retrieval were performed as fast as possible and reactions were stopped by adding an equal volume of 95% formamide, 10mM EDTA on ice. This procedure took approximatively 60s, from the start of decompression to the addition of the formamide. Primer extension products (20 µL) were loaded onto TAE-agarose 1% gels and separated by electrophoresis at 100V and 100mA for 5 h. Bands containing hybridized extended Cy5-labelled primer were revealed with a Gel Doc XR+ Gel Documentation System (Bio-Rad) used with the fluorescence mode.

Results

Gel analysis of products synthetized under HHP

We first determined effects of HHP on global DNA synthesis by *Pab*PolB exo$^-$ by analyzing, after decompression of the sample, the length of primer extension products synthetized under various pressure values and at 55.5 or 65.0°C. Fluorescent products labelled with Cy5 at the 5' end of the elongated 30nt primer were separated on agarose gel electrophoresis (Figure 1).

Products were longer at 65.0°C than at 55.5°C. In contrast, gradual decrease of product length as pressure increased was observed, from 50MPa at 55.5°C and from 25MPa at 65.0°C. Little or no increase in product length in comparison with the negative control were observed at 100MPa in both conditions.

Real-time monitoring of strand displacement activity under HHP

To analyze effects of HHP in real-time, we used a fluorescence-based tripartite oligonucleotide template system relying on strand-displacement activity by B-family DNAPs (Canceill *et al.*, 1999; Henneke, 2012). The increase of fluorescence caused by strand displacement of the oligonucleotide quenching the fluorescent signal of 87nt template was used as proxy for *Pab*PolB activity. Strand displacement assays were performed for 30min at 55.5°C under 0.1, 10, 50 and 100MPa three times independently (Figure 2).

Fluorescence signal of positive control (6-FAM labelled template annealed to extension primer without the quenching oligonucleotide) slightly decreased over time, presumably caused by photobleaching of the 6-FAM fluorophore. Pressure gradually delayed and slowed down the increase of fluorescence in the enzymatic assays, showing an inhibiting effect on strand displacement activity. The inhibition could be observed from 10MPa and little or no activity could be observed at 100MPa.

These experiments were also conducted at additional temperature (55.5, 60, 65, 70.0°C) and pressure values (0.1, 35, 50 and 100MPa) (Supplementary Figure 1). Fluorescence increase was faster at 60 and 65.0°C than at 55.5°C. In contrast, at every temperature, HHP gradually delayed fluorescence increase. Fluorescence curve of the unquenched template was slightly higher at 65.0°C than at 55.5 and 60.0°C, which may indicate that basal signal of the fluorophores is dependent on the temperature. At temperatures above theoretical melting temperatures (T_m) of primer in the presence of the quenching oligonucleotide, respectively 58.9°C and 67.1°C, spontaneous denaturation of the tripartite DNA may however have happened, as showed by the increase of fluorescence of the negative control at 70.0°C.

Real-time monitoring of primer extension activity under HHP

Primer extension activity was also directly monitored by measuring the fluorescence of picogreen which increases as it intercalates in increasing amounts of double-stranded DNA during primer extension. The DNA template was single-stranded M13mp18, allowing extension up to the order of the kbp. We monitored primer extension activity for 30min at 55.5°C under 0.1, 10, 50 and 100MPa three times independently (Figure 3).

Curves showed a clear biphasic behavior at 0.1, 10 and 50MPa. Duration of the second phase was reproducibly increased by HHP. In contrast, no clear biphasic behavior could be observed at 100MPa.

Variability was higher in this experiment compared to strand displacement assays, as indicated by larger standard deviation values, especially for the positive control (incubation at P_{amb} for 2h prior to the fluorescence measurement to achieve maximal primer extension). As with strand displacement experiments, fluorescence decreased over time. All other things being equal, pressure tended to increase all curves, showing a putative effect of HHP on intrinsic picogreen fluorescence or insertion in double-stranded DNA. Nonetheless, pressure-inhibition of extension activity was still observable as the plateau was reached later, or not reached at all, at 50 and 100MPa. The observed plateau unlikely resulted from dNTPs depletion as they were provided in large excess and could have resulted instead from unbounded picogreen shortage. In addition, maximum value of first derivative of the curves, corresponding to the maximum increase of fluorescence, decreased with pressure and the corresponding inflexion points, when applicable (0.1, 10 and 50MPa) were delayed (Supplementary Figure 2).

To obtain more precise and quantitative information about the fluorescence variation over time as a function of pressure, primer extension assays were replicated three times with additional pressure values and with shorter acquisitions (15min). The curves were transformed so that the value of fluorescence for positive control is equal to 100% and the value of fluorescence for the negative control is equal to 0%, both at every instant. This way, fluorescence was analyzed relative to controls and comparison between conditions was easier (Supplementary Figure 3). By calculating first derivative of these curves, we then determined relative increases of fluorescence as a function of time, roughly corresponding to elongation speeds. Fastest increase in fluorescence over the whole experiment and punctual fluorescence increase were calculated (Figure 4). Both maximum elongation efficiency achieved during the whole 15min run and fluorescence increase at various moments of the run were negatively affected by HHP above 10MPa. Milder pressure values (below 10MPa) had little effect on fluorescence increase.

Effect of prior pressure treatment on *Pab*PolB exo$^-$ primer extension activity at ambient pressure

To determine if the previously observed inhibiting effect of HHP on *Pab*PolB exo$^-$ was irreversible or not, samples were incubated at 55.5°C and 100MPa during 15, 30 or 60min. Upon decompression, measurement was immediately started and fluorescence was measured for 15min. The experiment was done three times independently and average relative fluorescence curves were determined (Figure 5).

Pre-incubation of the sample under HHP, even for longest durations, did not impair significantly the primer extension activity of the enzymes when pressure was decreased to ambient pressure. This shows that effect of pre-incubation on *Pab*PolB exo$^-$ activity was largely reversible.

Comparison of pressure sensitivity of various thermostable DNA polymerases at 55.5 and 70.0°C

We then compared sensitivity to HHP of DNAPs from various organisms: *Taq*Pol from the thermophilic bacteria *Thermus aquaticus* and B-family DNAPs from hyperthermophilic *Archaea*. Only exo$^-$ mutants of archaeal DNAPs were used: exo$^-$ *Pfu* Pol (Agilent) and Vent® exo$^-$ (NEB), respectively from *Pyrococcus furiosus* and *Thermococcus littoralis*, which have both been isolated in a shallow water hydrothermal vents (Lundberg *et al.*, 1991; Kong *et al.*, 1993) and Deep Ven Vent® exo$^-$ (NEB) from *Pyrococcus sp.* Strain GB-D , which has been isolated in a 2km deep hydrothermal vent (Jannasch *et al.*, 1992), like *Pyrococcus abyssi*. All archaeal enzymes had high sequence identity (Supplementary Figure 4).

Primer extension activity was measured during 30min three times independently for each enzyme at 0.1, 10, 50 and 100MPa, at 55.5 or 70.0°C. For reactions at 70.0°C, single-stranded m13mp18 was annealed to a longer primer (87nt rather than 30nt) to prevent melting of the hybrid. Curves were transformed as described previously and average maximum fluorescence increase observed during the whole run was determined. For each enzyme, the value at 0.1MPa was set to 100% in order to compare their elongation efficiency independently (Figure 6).

All commercial archaeal enzymes showed gradual inhibition by HHP at both 55.5 and 70.0°C. However, increasing the temperature compensated the decrease of activity induced by HHP. *Taq* enzyme turned to be more pressure-tolerant at 70.0°C than Vent® exo$^-$ and Deep Vent® exo$^-$. At 55.5°C, Deep Vent® exo$^-$ was the most pressure-sensitive DNAP as dramatical decrease of maximum achieved elongation speed already started at 10MPa. In contrast, *Pfu* exo$^-$ DNAP showed reduced inhibition by HHP in most conditions compared to the other commercial archaeal enzymes. However, *Pfu* exo$^-$ was the only enzyme used in the commercial buffer which contained triton X-100.

Unlike what was observed at 55.5°C, maximum elongation speed of *Pab*PolB exo$^-$ was significantly increased at both 10 and 50MPa when reaction occurred at 70.0°C. None of the other tested enzymes showed such behavior. Moreover, at such temperature the maximum observed speed was only halved by 100MPa exposure.

Discussion

We have showed here for the first time that, unlike many enzymes from both extremophiles and non-extremophiles whose activities can be enhanced by HHP (Eisenmenger and Reyes-De-Corcuera, 2009), DNA polymerase are inhibited by HHP above 10MPa. Effects of HHP on proteins are rather complex and depend on the protein. As a general rule, response to pressure is governed by Le Chatelier's principle which predicts that a system at the equilibrium will respond to an increase of pressure through a decrease of volume (Heremans, 2009; Jebbar *et al.*, 2015). For proteins, this decrease may result from two distinct effects: reversible compression, especially for proteins with large internal cavities, and unfolding which allows additional condensation of water molecules (Doster *et al.*, 2003; Eisenmenger and Reyes-De-Corcuera, 2009). At 400MPa, most proteins are, hence, fully unfolded (Aertsen *et al.*, 2009). Dissociations of molecular assemblies, such as the ribosome (Pande and Wishnia, 1986; Gross *et al.*, 1993), are also promoted under HHP as it releases sites for water condensation, most assemblies being fully dissociated at 200MPa (Aertsen *et al.*, 2009). Yet, it is not possible to impute the observed HHP inhibition of DNAPs on unfolding. In particular, the post-pressurization experiments show that, whatever its cause, the nearly full inhibition at 100MPa was reversible, just like what was observed with a RNA polymerase from a non-thermophilic abyssal bacteria (Kawano *et al.*, 2004).

While gel analysis of primer extension products provides a simple way to assess effects of HHP on DNA polymerization activity, the fact that this analysis can only be made after decompression may bias the results as fast pressure change could have specific effects such as sudden and quick stimulation of primer extension. Nonetheless, these experiments showed that the decrease of total elongated DNA synthetized by *Pab*PolB exo$^-$ was observable from 50MPa at 55.5°C and from 25MPa at 65.0°C, showing that pressure inhibition of DNAPs activity depends on temperature, as confirmed by the primer extension assays. It should also be pointed that *Pab*PolB could realize rolling circle replication of which requires displacement of elongated primer and, hence, relies on strand displacement activity which we showed to be inhibited by HHP. Such effect could contribute, to some extent, to the observed inhibition.

In contrast, it has been showed that HHP, up to 89MPa, actually increase thermostability of both DNAPs of *Pyroccocus* strains and *Taq* DNAP (Summit *et al.*, 1998). Hence, the HHP inhibition may result instead from subtle changes in protein structures and dynamics enhancing stability at the expense of activity. Psychrophilic enzymes, which present flexibility-inducing traits, may therefore be less sensitive to pressure inhibition. However, no experimental comparison of pressure-sensitivity between homologous thermophilic, mesophilic and psychrophilic deep-sea enzymes has been published, to our knowledge. Nonetheless, synergy between psychrophilic and piezophilic protein traits has been pointed (Capece *et al*., 2013; Carré *et al.*, 2022).

Strand displacement activity assays were limited by the fact that interaction between the DNA template and quencher-harbouring oligonucleotide is sensitive to pressure. While short simple double stranded DNA fragments can be destabilized by HHP (Hughes and Steiner, 1966; Gunter and Gunter, 1972), HHP actually tend to increase stability of DNA hybrids (Macgregor, 1996; Rayan and Macgregor, 2005). Therefore, if no interference caused by the Deep Dark Quencher I 3'-modification and 6-FAM fluorophore is assumed, interaction between the oligonucleotides was reinforced under HHP. Hence, in addition to any direct

effect on the enzyme, strand displacement activity was inhibited by the strengthened interaction between the DNA template and the strand that the enzyme had to displace. Indeed, total inhibition at 100MPa was observed during strand displacement assays whereas some activity could still be observed during primer extension assay. Therefore, assays that directly measure extension activity should be preferred to more indirect methods for HHP studies of DNAPs. One should notice that at higher temperatures the decreased stability of double-stranded DNA could compensate for HHP inhibition of strand displacement activity.

The biphasic behavior observed in primer extension activity curves is consistent with the biphasic model of DNAPs enzymatic activity. In such model, the first short phase corresponds to the addition of the first dNTP to the primer by all enzymes which is faster than the following dNTP polymerizations, which correspond to the second phase (Berdis, 2009). Our data suggest that this second phase is more affected by HHP as the quick initial increase of fluorescence was observed at 10 and 50MPa while the second more gradual increase appeared to be delayed at these pressure values and was mostly absent at 100MPa. This stresses the hypothesis that HHP impairs DNAPs activity through direct inhibition of primer extension rather than substrate binding.

Surprisingly high tolerance to HHP was observed with *Taq* DNAP, which originates from *Thermus aquaticus*, a surface thermophilic bacterium. Since *T. aquaticus* is less thermophilic than the archaea from which the other tested DNAPs were from, its proteins may be less thermostable, thus less resilient and less rigid (Aguilar *et al.*, 1997; Tehei and Zaccai, 2007; Zaccai, 2013), which could explain its low sensibility to HHP. However, *Taq* is A-family DNAP whereas all the other tested enzymes are from the B family. Depending on the fold and especially the internal cavities, intrinsic flexibility and, hence, sensitivity to HHP could vary between DNAP families.

When compared to the other exo$^-$ B-family archaeal DNAPs, *Pab*PolB showed increased tolerance to HHP. As little sequence variation is observed between these enzymes, subtle structural changes, at the level of internal cavities or interdomain hinges, may explain these differences. *Pab*PolB exo$^-$ was the only tested enzyme that was not purchased but produced and purified by us. In general, little information about piezophilic adaptation can be inferred from protein sequences as they are mostly dictated by temperature in abyssal environments (hot hydrothermal vents or cold waters and sediments) (Nath and Subbiah, 2016). Nonetheless, in comparison to their non-piezophilic homologs, enzymes from deep-sea organisms tend to have denser cores with smaller residues, less salt bridges (Carré *et al*., 2022).

Still, increasing temperature compensated HHP inhibition of all tested enzymes. This could result from two separate causes. First, the evaluated enzymes are hyperthermophilic, their optimal temperature being between 72 and 80°C for archaeal DNAPs (Zhang *et al.*, 2015), increasing the temperature from 55.5 to 70.0°C may hence be sufficient to account for the increase of the maximum elongation speed. Second, temperature may directly reduce the inhibiting effects of HHP through the increase of molecular motion and flexibility (Daniel and Cowan, 2000). Nonetheless, the present work stresses the importance of heat sources in deep environments where organisms are constantly under HHP. This is especially true for putative extraterrestrial abysses which could be deeper and exposed to pressure values above 100MPa (Naganuma and Uematsu, 1998).

Pressure adaptation of abyssal microorganisms still remains unclear. In particular, complex processes such as DNA replication can be affected by HHP in a complex manner as it

involves multiple protein, multimeric assemblies, proteins and DNA. Moreover, care should be taken when extrapolating data obtained *in vitro* on simple protein systems studied in buffers of given composition to the physiological intracellular context. Cytoplasmic crowding, interactions with piezolytes or with the other replisome proteins may indeed limit the effects of HHP on DNAPs. While HHP tend to disrupt multimeric assemblies and, hence, could impair replisome formation, depending on the volumetric parameters of each protein-protein and protein-DNA interaction, GINS or PCNA complexes could limit pressure-sensitivity of DNAPs. In particular, it has been shown that addition of PCNA improves strand displacement activity (Henneke *et al*., 2005) and fidelity (Castillo-Lizardo *et al*., 2014) of *Pab*PolB *in vitro*.

DNAPs could work better within the replisome by interacting with, for example, GINS or PCNA complexes. . In particular, interactions with GINS or PCNA complexes

Nonetheless, the deepest hydrothermal vents on Earth are found at a depth of 5km and most are found above 2km deep, limiting the pressure conditions to which such these organisms are actually exposed. Another possibility could be that high temperatures are sufficient to compensate inhibitory effects of HHP. Indeed, most of our experiments were made at 55.5°C, which is below the temperature range at which *Thermococcales* species such as *P. abyssi* grow (Erauso *et al.*, 1993).

This could stress even more the dependency on temperature of abyssal hyperthermophilic microorganisms and the need to search for heat sources in the extraterrestrial deep oceans.

Conclusion

In this work, effects of high hydrostatic pressure (HHP) on DNA polymerases (DNAPs) of abyssal and surface (hyper)thermophilic microorganisms was explored for the first time. By using various fluorescence-based approaches, including real-time fluorimetric assays, we showed that HHP inhibits total DNA synthesis, strand displacement activity and extension rate. These effects were showed to be reversible, mitigated by higher temperature and dependent on the enzymes.

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High-pressure experiments were conducted with the BioHP platform of the IBS.

Author contributions

All authors proposed and contributed in their respective expertise and participated in writing the manuscript. L.C. designed research, conducted experiments, analyzed data, wrote the paper and made the figures, G.H., E.H. and D.F. designed research and corrected the paper, E.G. and B.F. designed research, wrote and corrected the paper.

Disclosure statement

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

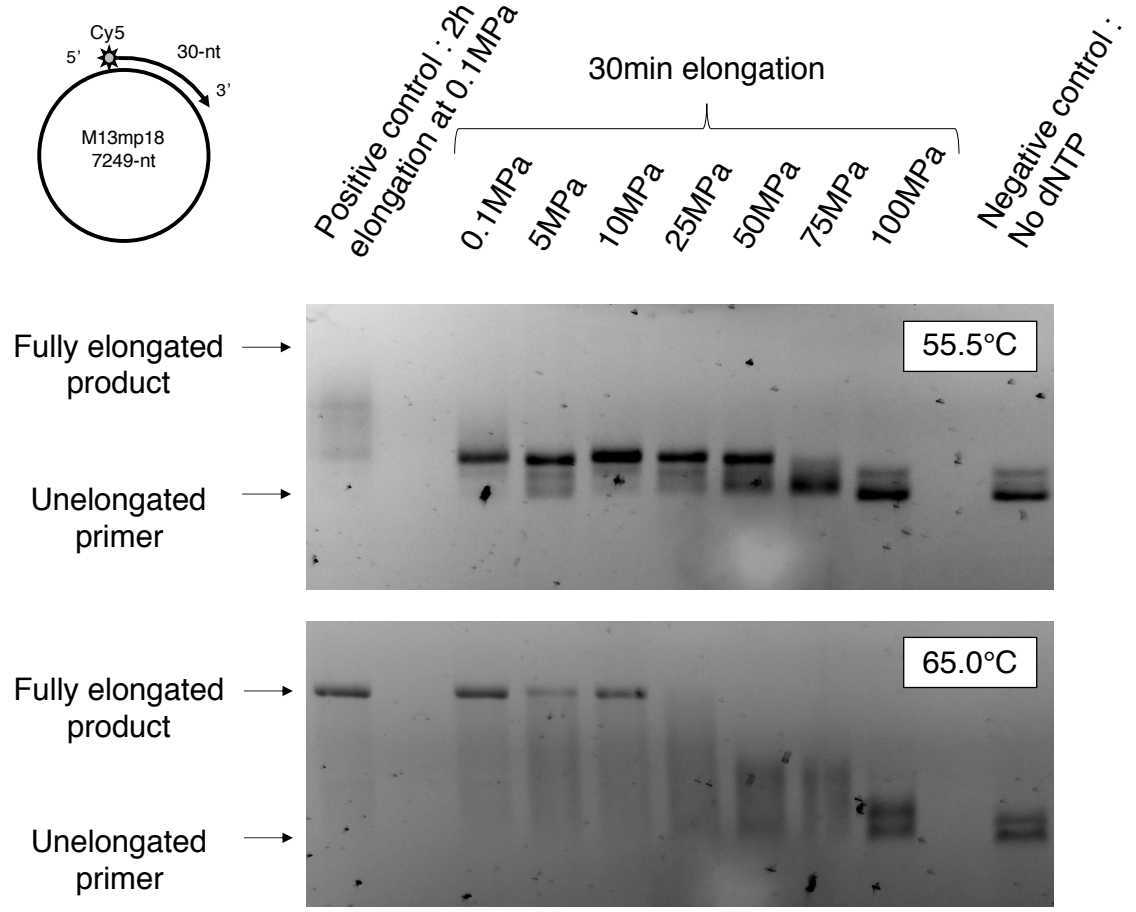


Figure 1: Gel analysis of products of *Pab*PolB exo⁻ under HHP

Extension of the Cy5-labeled 30nt long primer hybridized on a m13MP18 DNA template of 7249nt in length under pressure values indicated above and temperatures indicated on the left.

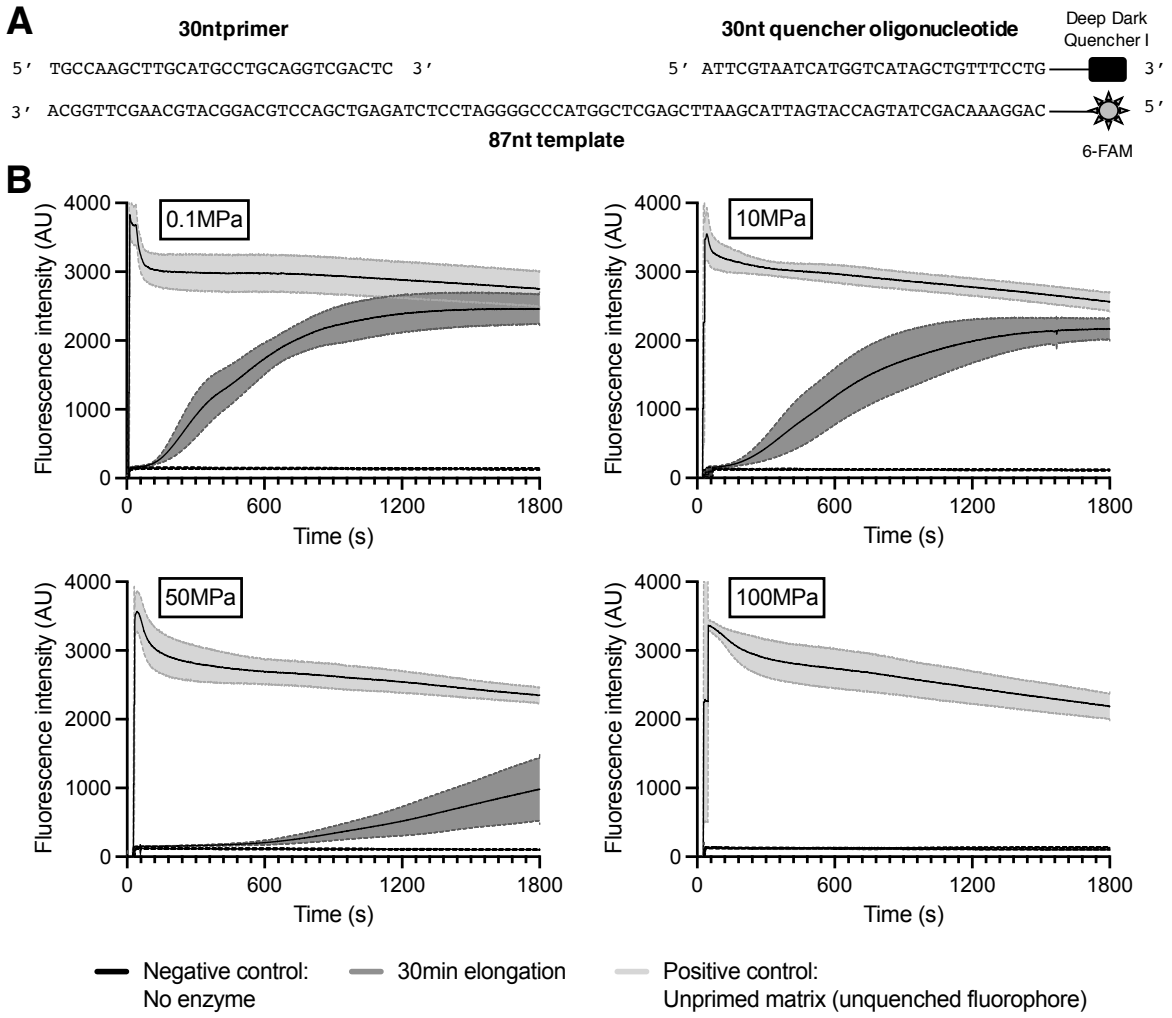


Figure 2: Realtime monitoring of strand displacement activity of *Pab*PolB exo⁻ under HHP

A: tripartite DNA hybrid used for these experiments consists of a 87nt template labelled at the 5' end with a 6-FAM modification hybridized to a 30nt primer and to a 30nt oligonucleotide labelled with a Deep Dark Quencher I modification at the 3' end. Unless this oligonucleotide is removed, the fluorescence of the 6-FAM is quenched. B: strand displacement activity of *Pab*PolB exo⁻ under various pressure conditions. The curves represent the average fluorescence over three independent experiments and the colored area corresponds to the standard deviation. Controls are indicated.

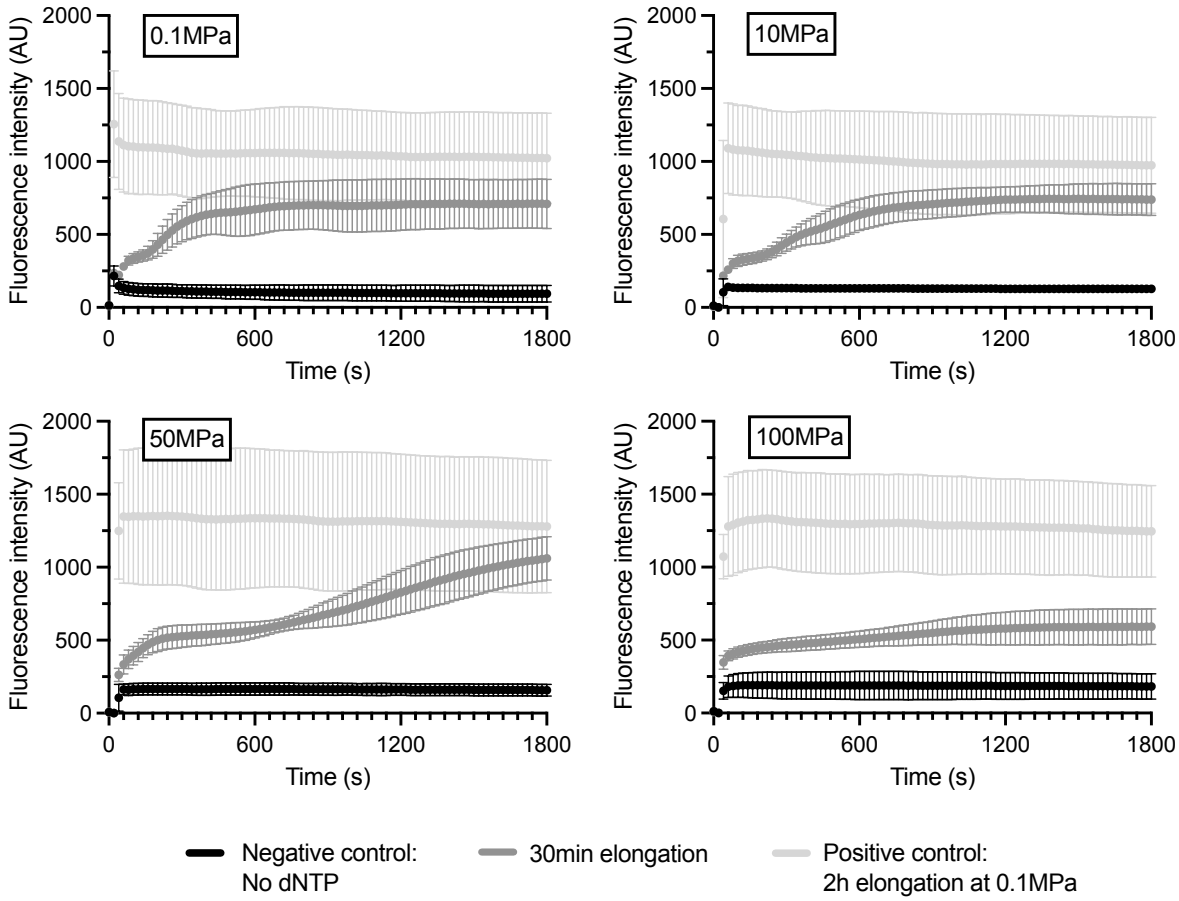
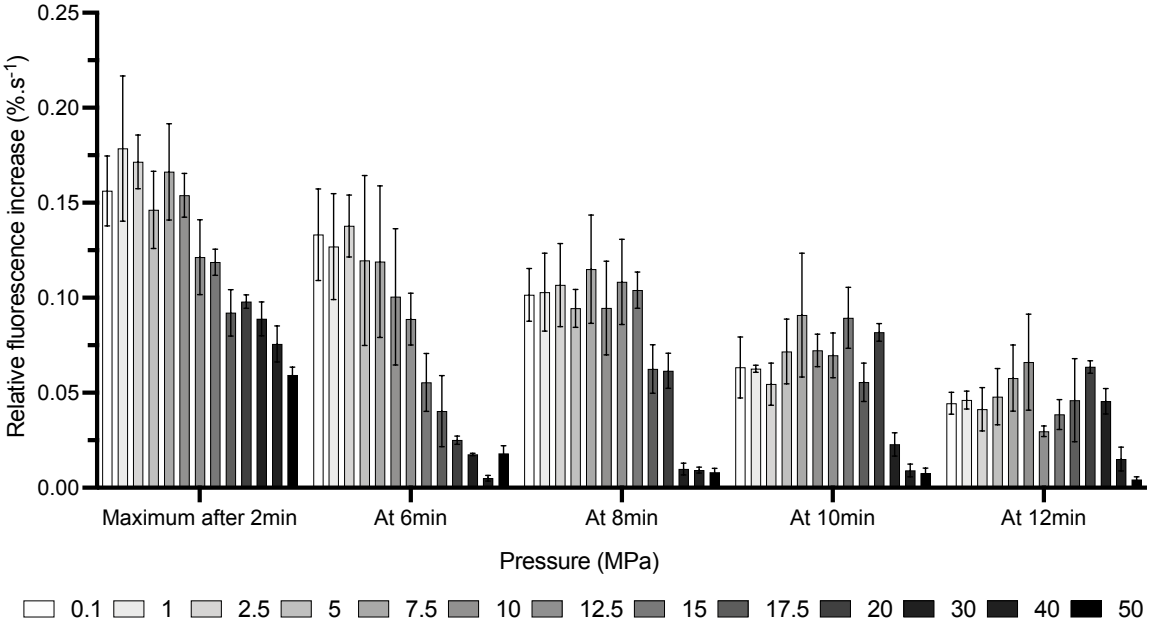


Figure 3: Realtime monitoring of primer extension activity of *Pab*PolB exo⁻ under HHP

Fluorescence signal of picogreen intercalant during the extension of the unlabelled 30nt long primer hybridized on a m13MP18 DNA template. The curves represent the average fluorescence over three independent experiments and the colored area corresponds to the standard deviation. Controls are indicated.



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504 Figure 4: Effect of HHP on elongation speed of *Pab*PolB exo⁻

505 Primer extension curves were normalized using positive and negative controls as, respectively,
506 superior and inferior limits. First derivatives were calculated for each of the three
507 independently obtained curves. Error bars correspond to standard deviation. Colors represent
508 pressure value (see legend).

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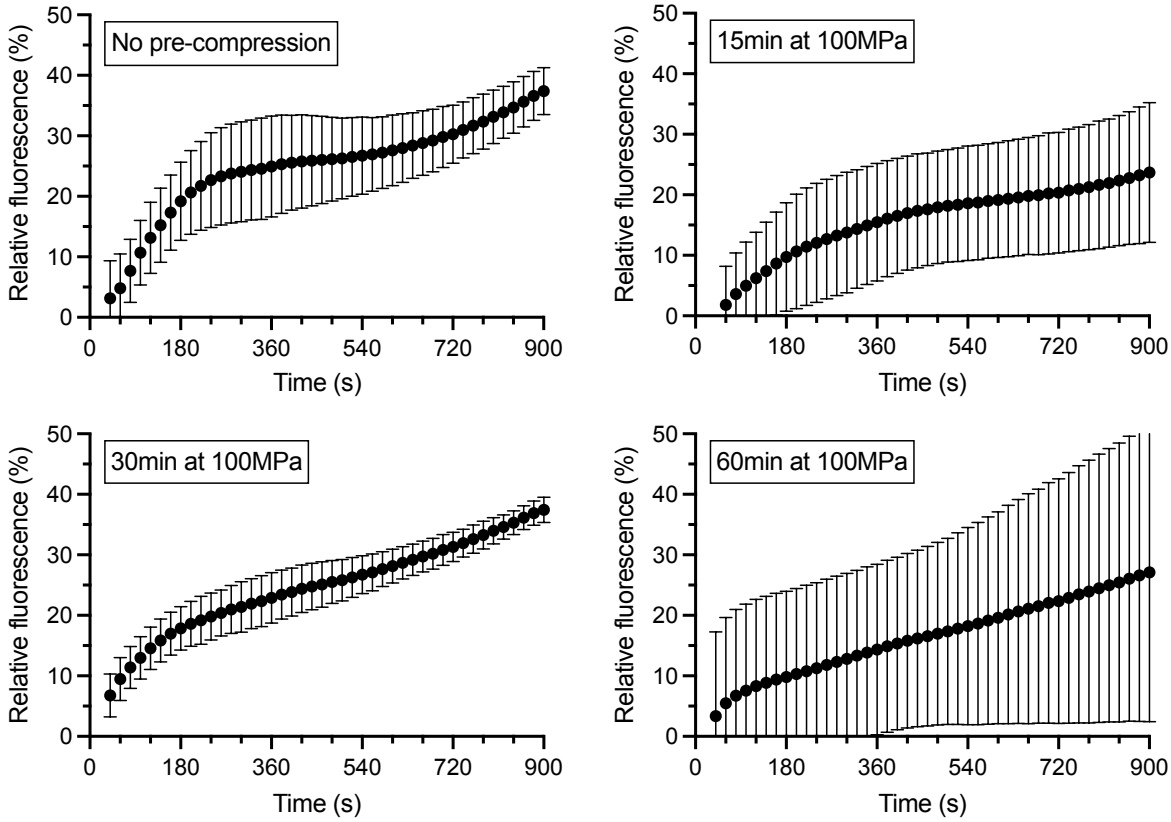


Figure 5: Effect of pre-compression on primer extension activity of *Pab*PolB exo$^-$ at P_{amb}

Primer extension curves were normalized using positive and negative controls as, respectively, superior (100%) and inferior (0%) limits. The curves represent the average fluorescence over three independent experiments. Incubation under HHP prior to fluorescence measurement is indicated. Error bars represent standard deviation.

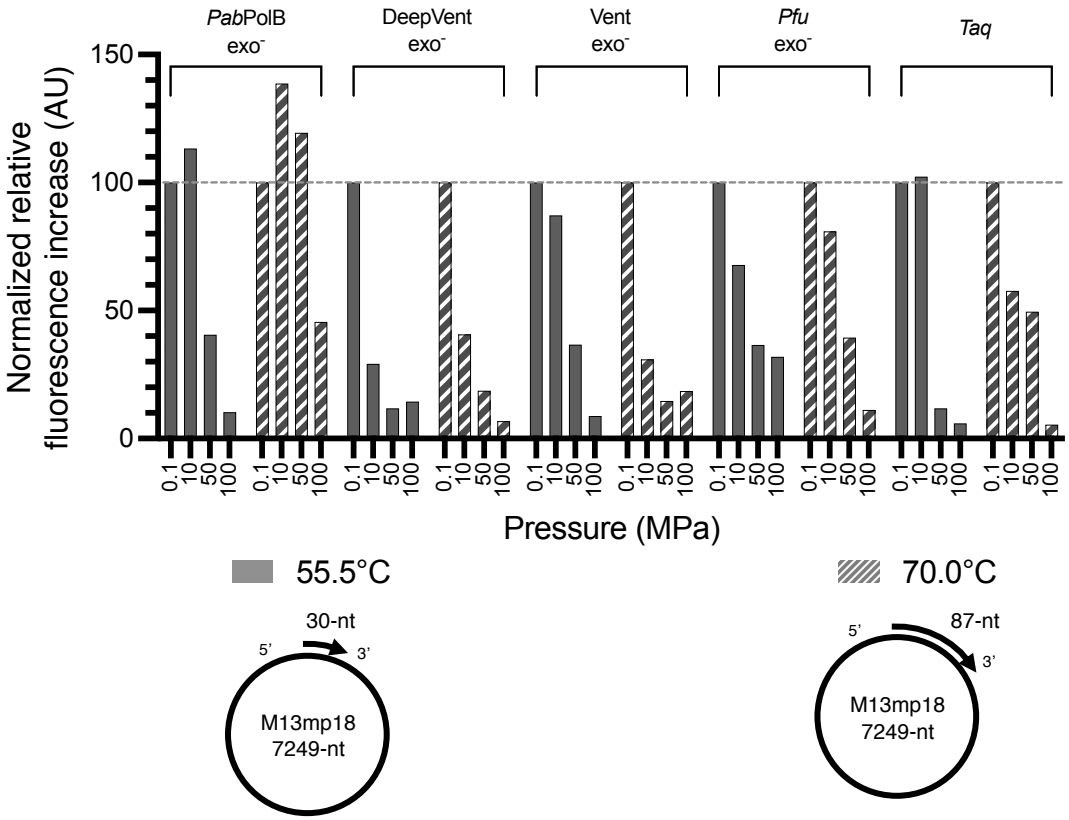
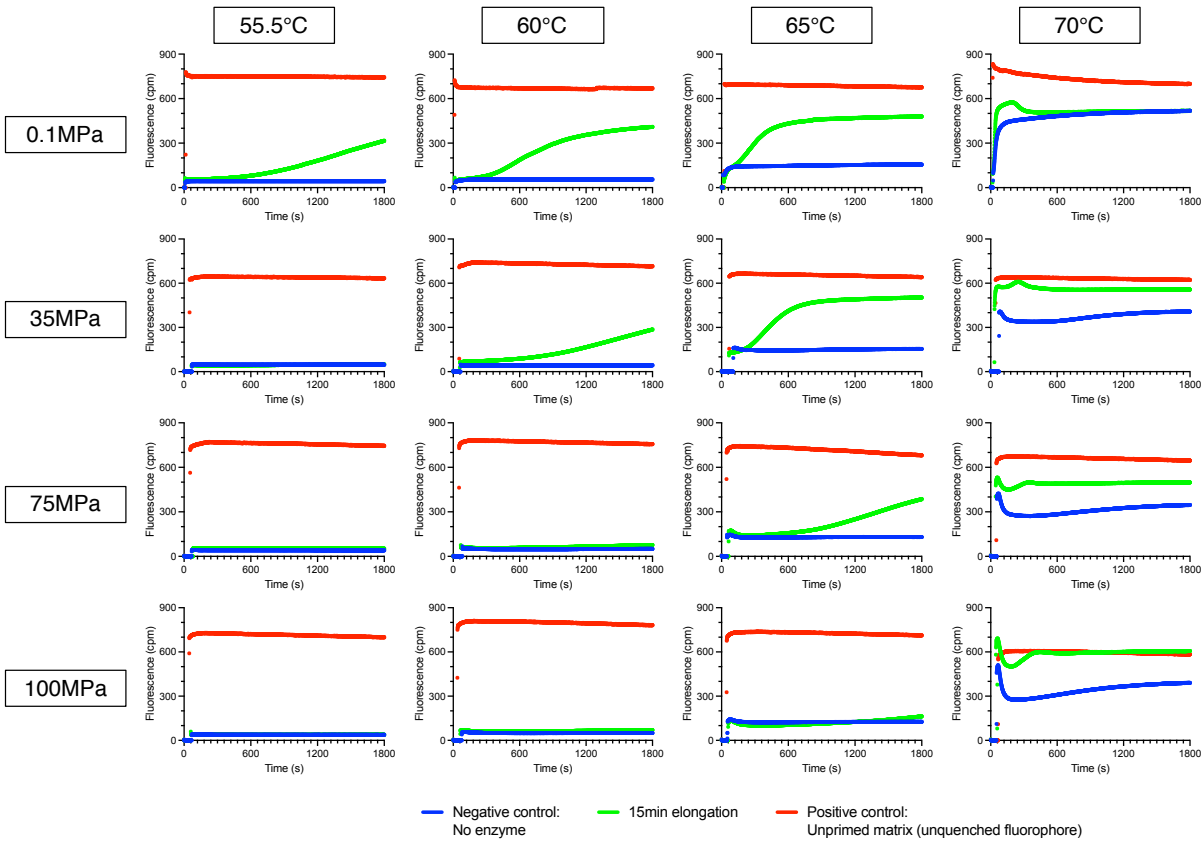


Figure 6: Comparison of pressure-sensitivity of various DNAPss

Primer extension curves were normalized using positive and negative controls as, respectively, superior and inferior limits. First derivatives were calculated for each of the three independently obtained curves. Average maximum observed speed is plotted as a function on the enzyme and pressure/temperature combination. To facilitate observation of the effect of HHP on elongation speed, for each temperature and enzyme, maximum speed at 0.1MPa was set to 100% and speeds at the other pressure values were compared to them. DNA template-primer hybrids used in each temperature condition are schematically represented below legend.



Supplementary Figure 1: Effect of HHP on strand displacement activity of *Pab*PolB exo⁻ at various temperatures

Fluorescence signal of the 6-FAM fluorophore harboured by the 87nt long DNA template increasing as the 30nt long oligonucleotide modified with a Deep Dark Quencher I is displaced by the enzyme. Curves were obtained only once. Controls are indicated. Notice that signal of the negative control at 70.0°C increases with heat as a consequence of spontaneous denaturation of DNA.

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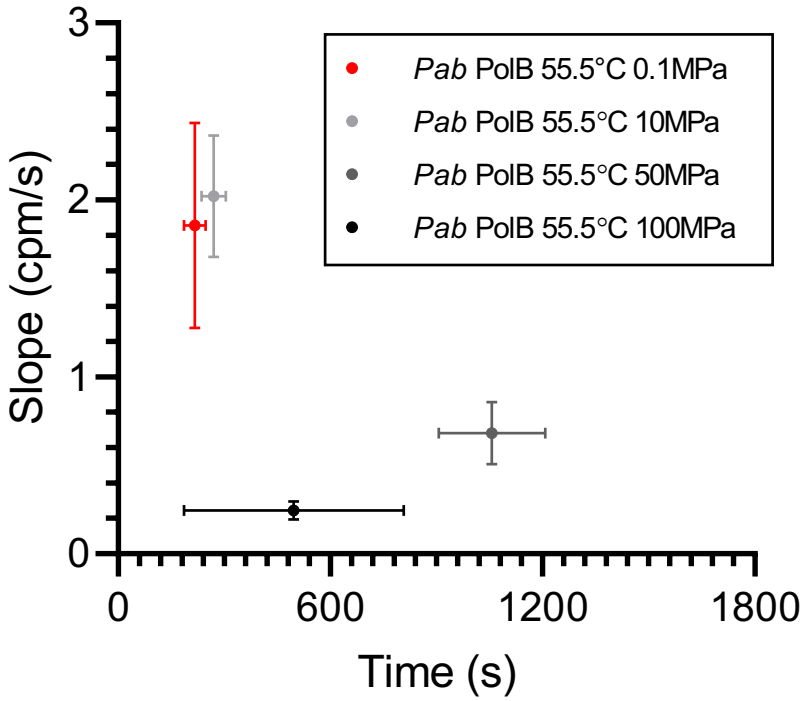

Supplementary Figure 2: Position and value of maximum fluorescence increase during primer-extension assays

Bars represent standard deviation. As no biphasic behaviour could be observed at 100MPa, only values obtained at 0.1, 10 and 50MPa correspond to an inflexion point.

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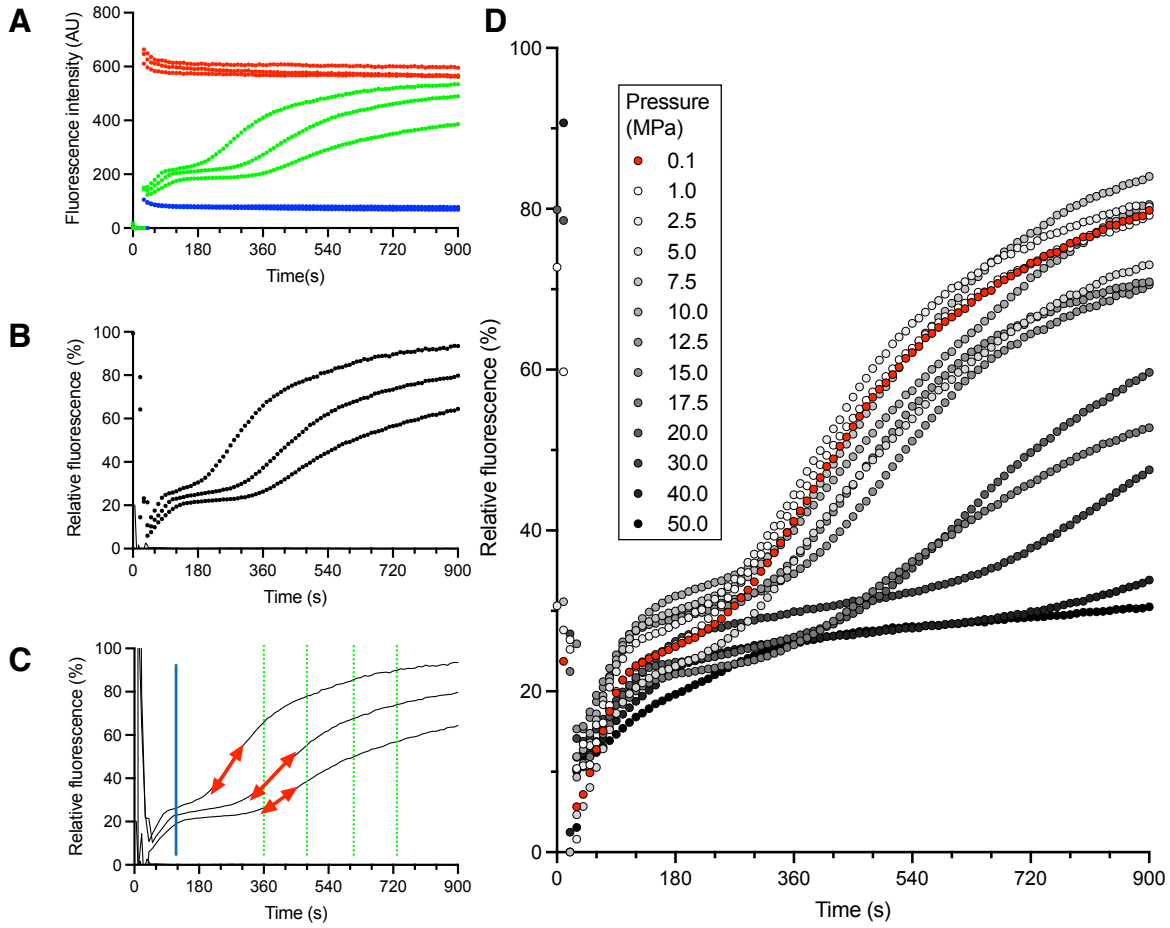
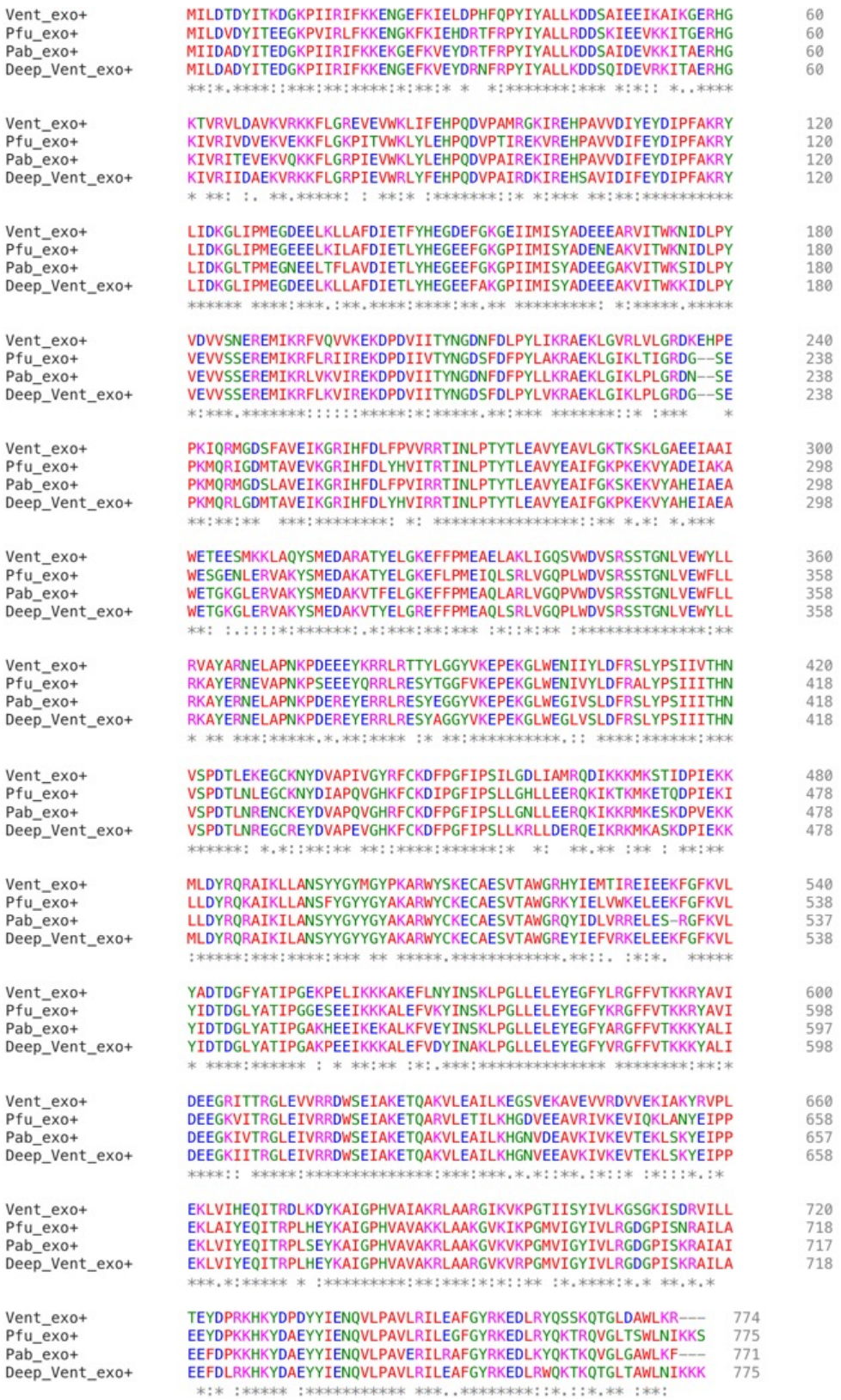


Supplementary Figure 3: HHP relative primer extension curves of *Pab*PolB exo⁻

A: Raw data for extension curves (green), positive (red) and negative (blue) controls, corresponding to the three independent measurements made at 1MPa and 55.5°C. B: relative fluorescence curves, based on raw data depicted in A, made using positive and negative controls as, respectively, superior (100%) and inferior (0%) limits. C: red arrows indicate localization of maximum value, after equilibration of pressure and temperature (blue line), of first derivative of relative fluorescence, corresponding to the maximum elongation speed; green dotted lines indicate additional timepoints at which values of first derivative were retrieved to be plotted in Figure 4. D: average relative fluorescence curves calculated over three independent experiments for every tested pressure values.



Supplementary Figure 4: Sequence alignments between exo$^+$ forms of tested archaeal enzymes

Since sequences of exo$^-$ mutants of commercial enzymes were unknown, intein-free sequences of wild-type exo$^+$ enzymes were compared instead. Sequences of

Pyrococcus specie GB-D, *Thermococcus littoralis* and *Pyrococcus furiosus* PolsB present sequence identity to *P. abyssi* PolB of 89.4%, 76.3% and 83.8%, respectively.

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