Kinetics and Fidelity of Psychrophilic DNA

Polymerases

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Abstract: The discovery of extremophiles has enabled development of groundbreaking biotechnology. While most of the extremophile research is focused on thermophiles, organisms that adapt to living in cold temperature, known as psychrophiles, remain under-studied. We expressed and purified DNA polymerases PIPI and PIPB from *Psychromonas ingrahamii*, a psychrophile that can grow below water's freezing temperature. We demonstrate that they have *in vitro* DNA replication activity at temperatures as low as -19°C. To our knowledge, this is the coolest DNA polymerization reaction ever carried out in a laboratory. In exploring the behavior of a variety of polymerases as a function of temperature, we found that reaction temperature substantially increases substitution and deletion error rates of both psychrophilic and mesophilic DNA polymerases. Motif analysis further reveals that the substitution error profiles cluster according to phylogenetic similarity of polymerases. Our results provide a useful reference for

- 24 how reaction temperature, a crucial parameter of biochemistry, can affect the fidelity of DNA
- 25 polymerases adapted to a wide range of environment.

Introduction:

Life in cold environment is confronted with the thermodynamic obstacle of maintaining sufficient enzymatic activity; however, the abundance of various life-forms found in near or below freezing temperature suggests that biology has evolved ways to overcome such challenge. Contrary to what one would expect from naïve application of Arrhenius equation, life-forms that adapted to living in cold environment do not grow exponentially slower in the cold than their mesophilic cousins at room temperature^{1,2}. This suggests that efficiency of psychrophilic enzymes are evolutionarily adapted to sustain relevant metabolism in low temperature³⁻⁵. Low temperature adaptation of enzyme is thought to increase the catalytic rate by increasing local flexibility of active site, thus lowering activation energy barrier⁶⁻⁸. This can be contrasted with high temperature adaptation which often confers global structural robustness to an enzyme by increasing intra-molecular interactions that maintain functional tertiary shape. Studies based on directed evolution have shown that structural stability and enzymatic efficiency can be optimized simultaneously and are thought to be mutually compatible properties^{9,10}. Combining the observations helps to explain the general biochemical trend of cold-adapted enzymes (summarized in **Figure 1a**): an increase in enzyme's catalytic activity is often associated with a reduced binding affinity and a broader substrate specificity^{11,12}. However, a direct test of these ideas on orthologous enzymes from organisms adapted to low, medium, and high temperatures has not yet been performed.

As DNA polymerase is a central component of cellular replication and biotechnological platforms¹³, understanding how reaction temperature can affect fidelity is of vital interest. Polymerase error rate is tightly controlled to ensure the duplication of genetic material which can encode functional descendants. Replication error from DNA polymerase is a source of genetic variations and underlies the cause of many inheritable diseases¹⁴⁻¹⁷. Polymerase errors play a crucial role in biotechnology applications such as polymerase chain reaction (PCR) and multiple-displacement amplification (MDA)¹⁸⁻²⁰. Polymerase errors can introduce base mismatches and indels at an exponential rate, significantly impairing downstream interpretation. In high-

throughput sequencing, replication errors of polymerase can reduce accuracy and precision of base calling^{21–23}. To test the effect of temperature on polymerase fidelity, we characterized error rate of a variety of DNA polymerases as a function of temperature. Furthermore, we explored how temperature adaptation affects polymerase fidelity at different reaction temperatures by characterizing the error profiles of DNA polymerases of psychrophilic, mesophilic, and thermophilic origins. Combining unique molecular indices (UMIs) and high-throughput sequencing, we carried out a multiplexed measurement of substitution, insertion, and deletion error rate at the single-molecule level for seven different DNA polymerases, some of which are commonly used in molecular cloning and sequencing applications. Our data provide evidence that the error rate of cold-adapted DNA polymerases are more sensitized to fluctuations in reaction temperature than thermal-adapted ones. Our study reveals how temperature adaptation affects psychrophilic DNA polymerase kinetics and fidelity which may enable biotechnology development.

Materials and Methods:

Polymerase Cloning. *Psychromonas ingrahamii* 37 DNA Polymerases I and II sequences are found on Joint Genome Institute, Integrated Microbial Genomes & Microbiomes (IMG/G) portal (IMG id: 639633052). The sequences were codon optimized for *E. coli* expression. PIP1 gene fragment (1845 bp) was synthesized and cloned into pD454 T7 expression vector (Ampicillin resistance) by ATUM (formerly DNA 2.0) to generate a maltose-binding protein (MBP) fusion gene containing a single HRV 3C cleavage site. PIPB was synthesized by Integrated DNA Technologies (iDT) as a single DNA fragment (2430 bp). The fragment was amplified in PCR with primers containing 5'-NdeI and 3'-SalI sites, and the resulting product was digested and ligated into linearized pMAL-c5x vector (New England Biolabs). All cloning products were first subcloned in NEB-5α strains and then purified using ZymoPURE plasmid miniprep kits (Zymo Research). The sequences of the expression constructs were all verified with Sanger sequencing by Sequetech. Plasmid maps for the constructs used in this study are provided (**Supplemental text 1**).

Polymerase Purification. Several expression and purification conditions had been attempted to optimize yield and purity of active polymerase products. We first attempted to express a glutathione S-transferase (GST)-PIPB fusion construct in BL-21 DE3 grown in Luria Broth (LB) media. Isopropyl β -D-1-thiogalactopyranoside (IPTG) induction of protein expression was carried out at 16°C between 12 - 16 hours. Harvested cells were lysed with either 5 freeze-thaw cycles or a sonicator. We screened a variety of lysis buffer and found that most of the protein remained insoluble. We suspect that GST-tag does not promote protein solubility based on previous reports²⁴. In our second approach, we generated MBP-PIPI (112.4 kDa) and MBP-PIPB (134.8 kDa) fusion genes on a pMAL-c5x vector expressed in Arctic Express DE3 (Agilent Technologies), a bacterial expression cell line that overexpresses psychrophilic chaperones. Instead of LB, we grew the bacteria in a defined media M9ZB (**Supplemental text 2**) at 37°C until OD600 of 2.5 and induced expression with 1M IPTG at 10°C or 16°C for 20-24 hours. Cells were harvested in lysis buffer (20 mM Tris-HCl pH 7.50, 10% glycerol, 50 mM NaCl, 10 mM 2-Mercaptoethanol) with the additions of 400 U of DNase I and 1X protease inhibitor cocktail (No EDTA; ThermoFisher). Cells were lysed in French press homogenizer by passing through at 500 psi once, and at 10000 psi for three times. Clarified cell lysate was mixed with amylose resin at 4°C for three hours. Resin was washed with 20 column volumes of chaperone removal buffer (20 mM Tris-HCl pH 7.50, 10% glycerol, 50 mM NaCl, 10 mM 2-Mercaptoethanol, 50 mM KCl, 5 mM ATP) and washed with 10 column volumes of lysis buffer (with no addition of DNase I or protease inhibitor). Bound proteins were eluted by incubating at 4°C for 3 hours with 10 mM of maltose. MBP-PIPI was subjected to HRV-3C cleavage at 4°C overnight. We did not cleave MBP-PIPB as it was found to induce precipitation of the protein. Both proteins were further purified in HiLoad 16/60 Superdex 200 preparatory grade size exclusion column (GE Healthcare) to separate fractions of polymerase proteins from contaminating proteins. Purified proteins were eluted in lysis buffer, concentrated in Amicon Ultra protein filters, and then stored in -80°C until experiments.

Protein quantification. Protein concentration was quantified by loading proteins on denaturing SDS-PAGE gel and stained with SYPRO Red following the manufacturer's recommended protocol (ThermoFisher). Linear dynamic range of the quantification was

established by separately staining a titration series of known amount of MBP-paramyosin-Sal. A secondary quantification measurement with Bioanalyzer Protein Analysis Kits (Agilent) provided similar concentration values as SYPRO Red assay (data not shown).

Polymerase Activity Assay. Polymerase activity from 5°C to 50°C was measured with EvaEZ™ Fluorometric Polymerase Activity Assay Kit (Biotium) according to the provided protocol. Briefly, assay mix and polymerase of known concentrations were equilibrated to the reaction temperature separately for 5 minutes before mixing. Fluorescence signals of Eva dsDNA binding dye and ROX reference dye were measured in Bio-rad 96 well qPCR machine for 1 hour at constant reaction temperature with 30 seconds interval. Titrations of the polymerase were performed at 20°C to ensure linear response of measured activity at the given polymerase concentration. A positive control sample with saturating amount of Klenow Exo- polymerase was included to ensure that maximal fluorescence signals remained constant over the course of measurement at each reaction temperature. The initial slope of the fluorescence gain for each polymerase is used to estimate the steady-state speed of replication. The slopes are then normalized to the maximal slope measured across all temperature range for each corresponding polymerase.

Gel Extension Assay. For measurement of subzero polymerase activity, we prepared 9.5 µL of primed extension mix containing a final concentration in 10 µL volume of 10 nM primed template with 5'-ATTOTM 633 fluorescent label (iDT) (**Supplemental text 1**), 50 µM dNTP, 10 mM MOPS pH 8.50, 30% glycerol, 1.5 mM MgCl₂, 0.1 mg/mL BSA, 50 mM KCl. Reaction master mix and polymerase were separately equilibrated to -19°C on a TropiCooler™ (Boekel Scientific) for 30 minutes, then they were rapidly mixed with pre-chilled pipette tips. One microliter aliquot of the reaction was quenched in 9 µL of 90% formamide with 50 mM EDTA pH 8.0 and heat denatured at 95°C for 5 minutes before cooling on ice. For exonuclease assay, a similar reaction was prepared but without the addition of dNTP. After thermal equilibration, the polymerase was mixed with the reaction master mix and incubated on the thermal cooler. One microliter of the quenched mixture was resolved on 15% polyacrylamide TBE-Urea precast gels (Bio-rad) at 195 constant voltage for 50 minutes. Gels were imaged on Typhoon 9410 imager (GE).

Molecular Barcoding and Library Preparation. We purified homogeneous ($< 10^{-9}$ single nucleotide polymorphism per base) pUC19 plasmid template from a single clone of NEB-5 α (New England Biolabs) transformed with pUC19 grown in LB supplemented with ampicillin antibiotic. About 1 μ g of purified pUC19 was co-digested with 10 units of SapI and NdeI at 37°C for 24 minutes. Digested pUC19 template fragment was purified twice with 0.35X (V:V) of Ampure XP beads. Concentration and purity of the co-digested pUC19 template was determined on Bioanalyzer using high-sensitivity dsDNA quantification kit. Applying UMI barcoding strategy to measure polymerase errors on single-molecule level has been reported previously by the Xie group²⁵. We adapted their strategy to measure polymerase replication at different reaction temperatures. All reactions were conducted in a buffer made up of 10 mM MOPS pH 8.50, 30% glycerol, 1.5 mM MgCl₂, 0.1 mg/mL BSA, 50 mM KCl unless otherwise stated. We used MOPS buffer instead of Tris-based buffer as it has a much smaller $\Delta pK_a/\Delta T$ scaling which minimizes changes in buffer acidity at different temperatures. We increased the UMI length to 15 nucleotides on both ends, effectively increasing the combinatorial space to 4^{30} which minimizes the barcode collision rate between UMIs (**Supplemental text 1**). After annealing UMI index primer to pUC19 template (100:1 molar ratio), we pre-incubated the reaction mixture in 96 well plate at the desired reaction temperature for 2 minutes prior to the addition of polymerase (volume of reaction mixture to volume of polymerase is 19:1). Reaction was quenched with 50 mM EDTA after giving sufficient time to complete the reaction. Reaction time for each polymerase and condition was determined using fluorometric activity assays and qPCR measurement. We then purified the reaction mixture with Ampure beads and synthesized the complementary strand using the reverse UMI primer with Q5 DNA polymerase in its native reaction buffer (NEB) by incubating at 72°C for 10 minutes which is followed by EDTA quenching. We performed a second round of DNA purification and quantified molecular concentration of the resulting products using qPCR. A total input of 20000 barcoded molecules from each reaction was amplified in two rounds of 13 PCR cycles, with Ampure XP purification between each successive round of amplification. Agilent Bioanalyzer dsDNA kit was used to assess purity of the amplified product.

Replication Error Analysis. Library was sequenced on MiSeq using V3 2 x 300 bp chemistry kit. Each read is sorted into groups according to reaction indices. Then each read is trimmed to minima of 150 length and Q score of 20 (Phred+33 quality score), ensuring that the composite reads will cover the entirety of template sequence. Reads with the same reaction indices are then grouped according to their UMI barcodes and the barcodes are then stripped. Reads originating from the same replicated molecule would share the same UMI barcode, thus consensus for each position was determined by 90% majority within the UMI family that shares the same base. Bases with less than 90% majority consensus may contain PCR-induced errors and thus are not included for downstream analysis. Consensus sequence with non-matching bases in the first 5 positions are removed to avoid analysis of mis-primed products. We observed that the apparent error rate for a polymerase decreased and plateaued as consensus number increased, and we picked a minimum of 5 or 10 consensus threshold for each molecule to improve accuracy in replication error calling. Consensus sequence from each UMI family is then aligned to the reference sequence using BWA-MEM²⁶. Substitution, insertion, and deletion errors are determined by comparing the consensus sequence of each UMI molecule to the pUC19 reference sequence without counting ambiguous assignments.

Dataset and code availability

Dataset and code to reproduce the analysis will be available on public repository.

Results:

Psychrophilic DNA polymerases can replicate nucleic acid below zero degree Celsius

To better understand the effect of temperature-adaptation on polymerase fidelity, we characterized replication error rates of psychrophilic, mesophilic, and thermophilic DNA polymerases across a wide range of reaction temperatures. As no commercial source was available for psychrophilic DNA polymerases, we determined the coding sequences bioinformatically, and optimized a recombinant cloning and purification strategy for DNA

polymerases I and II from *Psychromonas ingrahamii*, a gram-negative bacteria previously shown to grow and replicate at -12°C in laboratory condition (**Supplementary Figure 1a**) (**Methods**)²⁷⁻²⁹. We named the DNA polymerases I and II from this psychrophilic bacteria as *Psychromonas ingrahamii* polymerase I (PIPI) and *Psychromonas ingrahamii* polymerase B (PIPB), respectively. PIPI was purified by maltose affinity column, cleaved off its tandem-fused maltose-binding protein (MBP) tag, and then re-purified with size exclusion chromatography (**Methods**). As we were unable to obtain a soluble version of PIPB after protease digestion of the fusion tag, we used MBP-PIPB for all of our experiments. A graphical overview of our study is summarized in **Figure 1b**.

To determine the temperature profile of polymerase replication, we adapted a dsDNA-binding fluorescent dye to detect accumulation of extended products in real-time in the presence of various DNA polymerases at different reaction temperature (**Methods**). The increase in fluorescence signal over time suggests that our purified psychrophilic polymerases are indeed biochemically active (left panel in **Figure 2a**). As positive controls for comparison, we show the temperature profiles of Klenow fragment lacking 3'→5' exonuclease domain (KleExo-), a mesophilic DNA polymerase (center panel in **Figure 2a**), and Taq polymerase, a thermophilic DNA polymerase commonly used in molecular assays (right panel in **Figure 2a**). Consistent with previous report, Klenow and Taq DNA polymerases are maximally active at or above 37°C^{30,31}. Intriguingly, PIPI and MBP-PIPB exhibit characteristically distinct temperature profiles for replication activity: PIPI reaches peak polymerase activity at 37°C, similar to KleExo- (left panel in **Figure 2a**). As observed previously, the optimal temperature of enzyme activity can differ from the temperature to which the host organism is adapted to^{31,32}. On the other hand, polymerase activity of MBP-PIPB is optimal at 20°C and becomes inactivated above 30°C. Combined, our results show that the temperature profiles are distinct for the two psychrophilic DNA polymerases, suggesting a different physiological requirement for their catalytic rates and structural robustness. This may not be surprising as family A and B polymerases are thought to carry out different roles *in vivo*.

As *Psychromonas ingrahamii* was characterized to grow and replicate at -12°C²⁸, we hypothesized that its polymerases would remain active in cold environment. Using 5'-

fluorescently labeled, primed M13 phage template (**Methods**), we measured extension activity of the two psychrophilic DNA polymerases at -19°C in aqueous solution supplemented with 30% glycerol. We discover that PIPI retains higher apparent extension activity as it completed a 5-mer extension within 7 hours, while MBP-PIPb did not (center panel in **Figure 2b**). MBP-PIPb failed to complete 5-mer extension at -19°C up to 23 hours; however, the product lengths noticeably increased (right panel in **Figure 2b**). As these two polymerases are predicted to contain 3' → 5' exonuclease domains, we also determined their exonuclease activity (**Figure 2c**). We find that MBP-PIPb can cleave single base on the timescale of minutes at 25°C, while PIPI fails to cleave at up to 2 hours, suggesting its exonuclease activity is slower than MBP-PIPb or inactive (**Supplementary Figure 1b**). The exonuclease activity of family B polymerases is thought to play a role in DNA repair. It may help explain the apparent discrepancy in polymerization rate at sub-zero temperature between the two polymerases.

Multiplexed measurement of polymerase error rate across reaction temperature

In order to profile polymerase errors across a range of reaction temperature and conditions, we adapted a high-throughput multiplexed sequencing approach as illustrated in **Figure 3a**²⁵. Briefly, a reaction mixture containing homogeneous single-stranded DNA (ssDNA) phage template (NEB) is equilibrated to the desired temperature prior to the addition of DNA polymerase. Unless otherwise noted, we have used 3-(*N*-morpholino)propanesulfonic acid (MOPS, pH 8.5) to buffer each reaction to pH changes, which can influence enzyme fidelity independently of temperature³³. After quenching and removal of the polymerase, the complementary strand is then synthesized with high-fidelity Q5 DNA polymerase to minimize errors introduced in the second strand. Both the first and second strand are synthesized using DNA primers that encode a predefined library index and unique molecular index (UMI) to barcode the identities of each reaction and individual molecules, respectively. The dsDNA products are then PCR amplified, purified, and pooled at equimolar concentrations for sequencing on a MiSeq 2 x 300 bp platform. We found no runoff amplification by-products in up to 26 rounds of PCR amplification, while 20 rounds of PCR amplification failed to yield sufficient DNA amount for downstream purification (**Supplementary Figure 2a**). The purified libraries are pooled and sequenced on Illumina MiSeq platform. We deconvolved the sequenced

reads by their reaction indices and UMIs, removed sequencing errors by trimming reads with low quality, and called consensus sequence of molecules as described in **Methods**. Quantification of base counts with high sequence consensus reveals an uniform coverage along the entire M13 template, indicating no substantial positional effect on measurement dropout.

Reaction temperature increases error rate of psychrophilic and mesophilic DNA polymerases

To quantify the error rate of each polymerase reaction, we determined base mismatch (substitution error) or indel (deletion or insertion error) in each unique molecule. As insertion rate is relatively low, we focused our analysis primarily on substitution and deletion rates of psychrophilic, mesophilic, and thermophilic DNA polymerases in **Figures 4a-b**. Quantification of Q5 DNA polymerase error rate in its native buffer (Q5NativeBuffer) at 72°C provides a baseline for our measurement, revealing an average substitution, deletion, and insertion rates of 4.44×10^{-6} , 9.50×10^{-7} , and 1.37×10^{-7} per base, respectively, which agrees well with previously reported values (**Table 1** and right panel in **Figure 4a**)^{25,34}. Phusion (ThermoFisher), another high-fidelity and commonly used thermophilic polymerase, has a similar substitution rate of 5.05×10^{-6} per base (**Table 1**) at 72°C in its native Phusion HF buffer (PhusionNativeBuffer; ThermoFisher). We discover that the substitution rate of Phusion DNA polymerase increases by more than a factor of two when switched from its native buffer to MOPS, a buffer with low $\Delta pK_a/\Delta T$ coefficient; furthermore, we notice an increased substitution rate of Phusion polymerase in its native buffer (PhusionNativeBuffer) in lower reaction temperature. As the composition of Phusion native buffer (ThermoFisher) is undisclosed, we postulate the $\Delta pK_a/\Delta T$ of its buffering reagent may be high and thus its acidity can change substantially upon temperature shift. which leads to changes in Phusion polymerase error rate. This would explain why, relative to the Phusion native buffer, the substitution rate of Phusion in MOPS buffer remains relatively invariant to temperature changes (**Table 1** and right panel in **Figure 4a**). Supporting this hypothesis, we do not observe substantial differences in the substitution rate of Q5 polymerase in MOPS buffer between 30°C and 72°C. Our finding thus suggests that biological assays that involve the use of thermophilic polymerases at low temperature consider the use of alternative buffering agent to ensure optimal fidelity.

Intriguingly, we observe a substantial temperature dependence for psychrophilic polymerase and mesophilic polymerase error rate. The temperature effect is most dramatic on PIPI and KleExo- whose substitution rate rose by more than 5 and 30 fold, respectively, between reaction temperatures of 10°C and 50°C (**Tables 2-3**) (left and center panels in **Figures 4a-b**). Similarly, the deletion rate also scales positively with reaction temperature for MBP-PIPB, PIPI, and KleExo- (**Tables 2-3**) (left and center panels in **Figures 4a-b**). Curiously, the temperature effect on substitution rate of KleLF and T4 polymerases is not monotonic, revealing a maxima of error rate at 37°C (center panel in **Figures 2b**). In general, we find a log linear regression describes well the correlation between substitution and deletion error rates across all polymerases and reaction conditions (**Supplementary Figure 2b**), while insertion rate occurs at much lower frequency compared to the other error types and remains largely temperature invariant (**Tables 1-3**). Next, we wondered if the substitution error spectrum is dependent on reaction temperature. We show that reaction temperature had the strongest effect on substitution spectrum of KleExo-. As expected, increase in reaction temperature is associated with increased frequency of transversion relative to transition errors (**Figure 4c**). Meanwhile, reaction temperature had a weak effect on the substitution spectrum of PIPI and MBP-PIPB and most other polymerases (**Supplementary Figure 2c**).

Distinct mutational profiles are driven by polymerase family type

Polymerase replication error rate is context dependent^{35,36}. We wondered whether phylogenetically related polymerases that experienced vastly different temperature adaptation histories would produce error profiles that are similar to each other across the range of reaction temperature we tested. To validate the phylogenetic relations, we aligned the primary peptide sequences of psychrophilic DNA polymerases to polymerases with known family assignment (**Supplementary Figure 2d**). To analyze the relations between polymerase family and sequence context of substitution errors, we convolved the errors by their corresponding +1 and -1 bases to generate 3-mer error spectrum. We only kept 3-mer errors that occurred at greater than 20% frequency in at least a single reaction, and removed the rest from further analysis. This filtering step allows us to narrow down 60 distinct 3-mer errors (~32% of the entire 3-mer spectrum) and focus on errors that are abundant in at least some of the reactions. We normalized the range of

error rate for each reaction: we divide the 3-mer error rate by error sum within the corresponding reaction and then apply standardization such that the mean and standard deviation of each 3-mer error rate are 0 and 1, respectively. Thus, a high Z-score indicates a particular error motif occurs more frequently in some polymerase reactions than others and *vice versa*. Hierarchical clustering of the standardized motif error rates reveals that error profiles cluster by the polymerase, indicating good concordance of error profiles for reactions at different temperatures (**Figure 5a**). Interestingly, we discover that error profiles of polymerase tended to cluster by their phylogenetic homology in which errors generated by families A and B largely separated into distinct clusters regardless of their respective temperature adaptation range. Our results thus provide evidence that conservation of polymerase sequence is coupled to the frequency of error profiles independent of temperature adaptation experience.

To identify mutational profiles that best explain the observed mutational profiles across all the reactions, we performed principal component analysis on the standardized the 3-mer error rate. This led us to identify principal components 1 and 3 that best explained the mutational profiles of the polymerase families. Projecting the data onto these two principal components reveals a natural boundary on this space that separates the mutational profiles between family A and B (**Figure 5b**). Overall, high principal component 1 score is associated with mutational profile of family B, while high principal component 3 score is associated with that of family A. To identify mutational motifs that best distinguish each polymerase family, we trained a random forest decision tree to classify origin of each 3-mer error motif (**Figure 5c**). We discover that polymerase family A more frequently produced A → G transition preceded by G/C at -1 position. On the other hand, polymerase family B more frequently produced pyrimidine (C/T) → A transversion. In addition, the error position is often preceded by a purine (A/G) in the -1 position. This suggests that conserved structural differences between the two polymerase families may underlie the propensity for polymerase to generate distinct mutational signatures.

Discussion:

DNA polymerases are the biological machinery underlying transmission of genetic information and hold the key to many biotechnological innovations. While the majority of studies have focused on polymerases of mesophilic or thermophilic origins, we are one of the first to provide a comprehensive biochemical characterization of activity and fidelity of bacterial DNA polymerases across a wide temperature adaptation range. To our knowledge, this is the first demonstration that DNA extension can be accomplished with psychrophilic DNA polymerases below water's freezing temperature (0°C). This opens the door towards enabling more efficient nucleic acid amplification techniques directly on cryopreserved samples³⁷. Reaction at low temperature is desirable as it can inhibit mammalian nuclease activity and reduce phototoxicity and DNA damages^{23,38}. Thus, the development of low temperature biotechnology should in theory enable more accurate and precise measurement of biological attributes at a state that is closer to the "ground-truth". Ice may also provide an unexpected environment for biological reactions. As early as 1964, groups have observed that biochemical reactions can be substantially catalyzed in partially frozen aqueous system due to increased concentration of reactant in the aqueous portions. These observations suggest that ice slurry may be an unexpected environment for the origin of living systems³⁹⁻⁴². Combined with cold-active enzymes, dynamic modulation of liquid-frozen phase may provide an under-exploited niche for biotechnology applications^{43,44}.

In this study, we have also explored one of the long standing questions in enzymology – is there a necessary trade-off between substrate specificity and activity? To address this question, we biochemically characterized bacterial DNA polymerase adapted to a wide range of environmental temperatures. Our results suggest that distinct temperature adaptation can differentially alter how the stability, kinetics, and fidelity of DNA polymerases change in response to fluctuations of environmental temperature. Our data shows that psychrophilic polymerases are more heat labile while thermophilic polymerases are largely heat resistant, as consistent with previous reports of other psychrophilic enzymes⁴⁵. Furthermore, increased reaction temperature substantially increased the error rate of psychrophilic DNA polymerases and *E. coli* DNA polymerase I (Klenow fragments) but not that of thermophilic polymerases such as Phusion and Q5. Nucleic acid analogues are of interest to a wide range of synthetic

biology and biological engineering research as a mean to expand orthogonal signals or building parts⁴⁶. Most often, this is achieved through a combination of rational engineering and directed evolution on mesophilic or thermophilic polymerase templates^{47–50}. Our results shown here suggest that using cold-adapted polymerases as a starting template may achieve higher substrate promiscuity and thus greater incorporation rate of unnatural nucleotides. This holds the possibility to improve most sequencing-by-synthesis sequencers available on the market as well as accelerating the application of nucleic acids analogues.

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Table 1. Error rates (per base) for thermophilic DNA polymerases.

Origin	Reaction	Temperature (°C)	Substitution rate (base ⁻¹)	Deletion rate (base ⁻¹)	Insertion rate (base ⁻¹)
Thermophile	Q5 ^{NB}	72	4.44 x 10 ⁻⁶	9.50 x 10 ⁻⁷	1.37 x 10 ⁻⁷
	Q5	30	6.04 x 10 ⁻⁶	7.45 x 10 ⁻⁷	5.31 x 10 ⁻⁷
	Q5	72	5.38 x 10 ⁻⁶	1.56 x 10 ⁻⁷	5.05 x 10 ⁻⁸
	Taq	30	6.32 x 10 ⁻⁵	2.27 x 10 ⁻⁶	2.01 x 10 ⁻⁶
	Taq	50	2.18 x 10 ⁻⁴	2.18 x 10 ⁻⁵	3.53 x 10 ⁻⁶
	Phusion ^{NB}	30	2.51 x 10 ⁻⁵	1.82 x 10 ⁻⁸	8.32 x 10 ⁻⁷
	Phusion ^{NB}	50	8.11 x 10 ⁻⁶	ND	1.95 x 10 ⁻⁶
	Phusion ^{NB}	72	5.05 x 10 ⁻⁶	ND	9.30 x 10 ⁻⁸
	Phusion	30	2.02 x 10 ⁻⁵	2.32 x 10 ⁻⁶	4.81 x 10 ⁻⁷
	Phusion	50	3.64 x 10 ⁻⁵	2.46 x 10 ⁻⁶	4.84 x 10 ⁻⁷
	Phusion	72	1.24 x 10 ⁻⁵	3.36 x 10 ⁻⁷	5.11 x 10 ⁻⁷

^{NB}=reaction carried out in native buffer supplied by the manufacturer.

ND= Error frequency below the detectable error baseline.

384 Table 2. Error rates (per base) for psychrophilic DNA polymerases.

Origin	Reaction	Temperature (°C)	Substitution rate (base ⁻¹)	Deletion rate (base ⁻¹)	Insertion rate (base ⁻¹)
Psychrophile	MBP-PIPB	10	9.52 x 10 ⁻⁶	1.84 x 10 ⁻⁶	1.26 x 10 ⁻⁶
	MBP-PIPB	20	2.55 x 10 ⁻⁵	2.20 x 10 ⁻⁵	1.09 x 10 ⁻⁶
	PIPI	10	3.87 x 10 ⁻⁵	1.48 x 10 ⁻⁶	1.62 x 10 ⁻⁷
	PIPI	20	6.34 x 10 ⁻⁵	4.15 x 10 ⁻⁶	2.20 x 10 ⁻⁷
	PIPI	37	1.83 x 10 ⁻⁴	1.59 x 10 ⁻⁵	ND
	PIPI	50	2.11 x 10 ⁻⁴	8.47 x 10 ⁻⁶	ND

385 ND= Error frequency below the detectable error baseline.

386 Table 3. Error rates (per base) for mesophilic DNA polymerases.

Origin	Reaction	Temperature (°C)	Substitution rate (base ⁻¹)	Deletion rate (base ⁻¹)	Insertion rate (base ⁻¹)
Mesophile	KleExo-	10	4.20 x 10 ⁻⁵	7.65 x 10 ⁻⁶	3.38 x 10 ⁻⁷
	KleExo-	20	6.91 x 10 ⁻⁵	8.55 x 10 ⁻⁶	ND
	KleExo-	37	1.07 x 10 ⁻³	1.75 x 10 ⁻⁴	4.39 x 10 ⁻⁷
	KleExo-	50	1.38 x 10 ⁻³	1.94 x 10 ⁻⁴	3.38 x 10 ⁻⁶
	KleLF	10	8.53 x 10 ⁻⁶	ND	2.29 x 10 ⁻⁸
	KleLF	20	1.56 x 10 ⁻⁵	1.12 x 10 ⁻⁷	2.14 x 10 ⁻⁷
	KleLF	37	2.99 x 10 ⁻⁵	1.20 x 10 ⁻⁷	1.43 x 10 ⁻⁷
	KleLF	50	2.00 x 10 ⁻⁵	ND	5.45 x 10 ⁻⁸
	T4	10	3.25 x 10 ⁻⁵	1.32 x 10 ⁻⁶	2.37 x 10 ⁻⁸
	T4	37	5.92 x 10 ⁻⁵	1.17 x 10 ⁻⁶	ND
	T4	50	1.45 x 10 ⁻⁵	ND	ND

387 ND= Error frequency below the detectable error baseline.

388 **Figure 1. (a)** A schematic illustrating the apparent trade-off between enzymatic speed versus
389 fidelity in the context of temperature adaptation. **(b)** Graphical summary of the study.

Figure 2. (a) Normalized extension speed of purified psychrophilic, mesophilic, and thermophilic DNA polymerases measured by a fluorometric assay. (b) Extension activity of purified psychrophilic DNA polymerases carried out at -19°C for 7 or 23 hours on a template with 5 base gap. (c) Exonuclease activity of psychrophilic DNA polymerases carried out at room temperature for 5, 10, or 20 minutes. Both measurements were repeated at least twice in two independent experiments.

397 **Figure 3. (a)** A schematic illustrating the experimental overview of multiplexed single-molecule
 398 measurement of polymerase error rate at various reaction temperature. Replication errors
 399 (highlighted by blue cross) are distinguished from sequencing errors (highlighted by orange
 400 cross) by comparing consensus sequence of individual molecules to the reference sequence of
 401 M13 phage template.

402 **Figure 4.** Substitution **(a)** and deletion **(b)** error rates of psychrophilic (left), mesophilic (center),
 403 and thermophilic (right) DNA polymerases as a function of reaction temperature. **(c)**
 404 Temperature-dependent variation in substitution spectrum across MBP-PIPB (left), PIPi (center),
 405 and KleExo- (right) polymeases.

Figure 5. (a) Heatmap of standardized 3-mer substitution rates ordered by hierarchical clustering. **(b)** Projection of polymerase reactions by principal components (PCs) 1 and 3 of standardized 3-mer substitution rates. Each dot is colored by the corresponding DNA polymerase and annotated with the reaction temperature. Dotted line is identity of PC1 and PC3 which separates substitution profiles between polymerase family A and B. **(c)** Motifs that distinguish error profiles between polymerase families A (blue) and B (red) are predicted by random forest classifier. Motifs predicted to have zero weights are removed and next we showcase here the top motifs with a difference in standard Z-score greater than 1. Logo plots reflect the frequency of non-zero weighted mutation motifs at -1, 0, and +1 positions that are enriched in polymerase families A (top) or B (bottom).

416 **Supplementary Figure 1. (a)** SDS-PAGE analysis of purified DNA polymerases. Lane L:
 417 PrecisionPlus Ladder (Bio-rad). Lane 1: MBP-paramyosin protein control (NEB). Lane 2:
 418 Klenow Exo- (NEB). Lane 3: Klenow LF (NEB). Lane 4: Q5 (NEB). Lane 5: Taq (NEB). Lane
 419 6: MBP-PIPB (134.8 kDa). Lane 7: PIPI (69.9 kDa). **(b)** Exonuclease activity assay of PIPI and
 420 MBP-PIPB at 25°C for 2 hours.

421 **Supplementary Figure 2. (a)** High-sensitivity Bioanalyzer DNA (Agilent) assay of replicated
 422 products after 20 or 26 rounds of PCR amplification in the flow-through (FT) or elution fractions
 423 of Ampure purification output. **(b)** Deletion and substitution rate (per base) is correlated as
 424 modeled by linear regression (r^2 : 0.98). **(c)** Substitution spectrum of mesophilic and thermophilic
 425 DNA polymerases across reaction temperature. **(d)** Multiple sequence alignment (Clustal
 426 Omega) of peptide sequences for polymerases used in this study. While Phusion DNA
 427 polymerase sequence is undisclosed, it is expected to be a derivative of Pyrococcus DNA
 428 polymerase II (Pfu). Family A cluster consists of Taq, PIP1, and Klenow polymerases. Family B
 429 polymerases consist of T4, Pfu, and PIPB.

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Figure 1

a

b

Figure 2

Figure 3

a

experimental overview

computational overview

Supplementary Figure 1

a

b

Supplementary Figure 2**a** **b** **c** **d**

Table 1. Error rates (per base) for thermophilic DNA polymerases.

Origin	Reaction	Temperature (°C)	Substitution rate (base ⁻¹)	Deletion rate (base ⁻¹)	Insertion rate (base ⁻¹)
<u>Thermophile</u>	Q5 ^{NB}	72	4.44 x 10 ⁻⁶	9.50 x 10 ⁻⁷	1.37 x 10 ⁻⁷
	Q5	30	6.04 x 10 ⁻⁶	7.45 x 10 ⁻⁷	5.31 x 10 ⁻⁷
	Q5	72	5.38 x 10 ⁻⁶	1.56 x 10 ⁻⁷	5.05 x 10 ⁻⁸
	<u>Taq</u>	30	6.32 x 10 ⁻⁵	2.27 x 10 ⁻⁶	2.01 x 10 ⁻⁶
	<u>Taq</u>	50	2.18 x 10 ⁻⁴	2.18 x 10 ⁻⁵	3.53 x 10 ⁻⁶
	<u>Phusion</u> ^{NB}	30	2.51 x 10 ⁻⁵	1.82 x 10 ⁻⁸	8.32 x 10 ⁻⁷
	<u>Phusion</u> ^{NB}	50	8.11 x 10 ⁻⁶	ND	1.95 x 10 ⁻⁶
	<u>Phusion</u> ^{NB}	72	5.05 x 10 ⁻⁶	ND	9.30 x 10 ⁻⁸
	<u>Phusion</u>	30	2.02 x 10 ⁻⁵	2.32 x 10 ⁻⁶	4.81 x 10 ⁻⁷
	<u>Phusion</u>	50	3.64 x 10 ⁻⁵	2.46 x 10 ⁻⁶	4.84 x 10 ⁻⁷
	<u>Phusion</u>	72	1.24 x 10 ⁻⁵	3.36 x 10 ⁻⁷	5.11 x 10 ⁻⁷

^{NB}=reaction carried out in native buffer supplied by the manufacturer.

ND= Error frequency below the detectable error baseline.

Table 2. Error rates (per base) for psychrophilic DNA polymerases.

Origin	Reaction	Temperature (°C)	Substitution rate (base ⁻¹)	Deletion rate (base ⁻¹)	Insertion rate (base ⁻¹)
<u>Psychrophile</u>	<u>MBP-PIPB</u>	10	9.52×10^{-6}	1.84×10^{-6}	1.26×10^{-6}
	<u>MBP-PIPB</u>	20	2.55×10^{-5}	2.20×10^{-5}	1.09×10^{-6}
	<u>PIPI</u>	10	3.87×10^{-5}	1.48×10^{-6}	1.62×10^{-7}
	<u>PIPI</u>	20	6.34×10^{-5}	4.15×10^{-6}	2.20×10^{-7}
	<u>PIPI</u>	37	1.83×10^{-4}	1.59×10^{-5}	ND
	<u>PIPI</u>	50	2.11×10^{-4}	8.47×10^{-6}	ND

ND= Error frequency below the detectable error baseline.

Table 3. Error rates (per base) for mesophilic DNA polymerases.

Origin	Reaction	Temperature (°C)	Substitution rate (base ⁻¹)	Deletion rate (base ⁻¹)	Insertion rate (base ⁻¹)
<u>Mesophile</u>	<u>KleExo-</u>	10	4.20×10^{-5}	7.65×10^{-6}	3.38×10^{-7}
	<u>KleExo-</u>	20	6.91×10^{-5}	8.55×10^{-6}	ND
	<u>KleExo-</u>	37	1.07×10^{-3}	1.75×10^{-4}	4.39×10^{-7}
	<u>KleExo-</u>	50	1.38×10^{-3}	1.94×10^{-4}	3.38×10^{-6}
	<u>KleLF</u>	10	8.53×10^{-6}	ND	2.29×10^{-8}
	<u>KleLF</u>	20	1.56×10^{-5}	1.12×10^{-7}	2.14×10^{-7}
	<u>KleLF</u>	37	2.99×10^{-5}	1.20×10^{-7}	1.43×10^{-7}
	<u>KleLF</u>	50	2.00×10^{-5}	ND	5.45×10^{-8}
	T4	10	3.25×10^{-5}	1.32×10^{-6}	2.37×10^{-8}
	T4	37	5.92×10^{-5}	1.17×10^{-6}	ND
	T4	50	1.45×10^{-5}	ND	ND

ND = Error frequency below the detectable error baseline.