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Molecular breeding of polymerases for resistance to environmental inhibitors

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ABSTRACT

Potent inhibitors limit the use of PCR assays in a wide spectrum of specimens. Here, we describe the engineering of polymerases with a broad resistance to complex environmental inhibitors using molecular breeding of eight different polymerase orthologues from the genus *Thermus* and directed evolution by CSR in the presence of inhibitors. Selecting for resistance to the inhibitory effects of *Neomylodon* bone powder, we isolated 2D9, a chimeric polymerase comprising sequence elements derived from DNA polymerases from *Thermus aquaticus, Thermus oshimai, Thermus thermophilus* and *Thermus brockianus*. 2D9 displayed a striking resistance to a broad spectrum of complex inhibitors of highly divergent composition including humic acid, bone dust, coprolite, peat extract, clay-rich soil, cave sediment and tar. The selected polymerase promises to have utility in PCR-based applications in a wide range of fields including palaeobiology, archaeology, conservation biology, forensic and historic medicine.

INTRODUCTION

The polymerase chain reaction (PCR) enables the detection, amplification and interrogation of DNA sequences from minute starting quantities down to single DNA molecules (1). This has enabled a wealth of applications in medicine and biology ranging from clinical diagnostics, prognostics, forensics to molecular genetics including molecular archaeology and palaeobiology (2,3). However, the utility of PCR assays and the recovery of amplicons from such specimens can be greatly hindered or even abrogated by the presence of potent inhibitors. A wide range of substances are known to strongly inhibit polymerase activity and limit the use of the PCR in samples where they are present (4,5). These may either be part of the sample composition, such as haem (and its degradation products such as bilirubin) in blood and faeces (5,6), or impregnate the sample during its burial time. Examples of the latter include Humic and Fulvic acids, complex mixtures of polyphenolic compounds (7) produced by the decomposition of terrestrial vegetation. Humic and Fulvic acids are ubiquitous in soil and water, and thus are present in any sample exposed to these environments (8,9). Inhibition of PCR by Humic acids is thus especially relevant for samples of palaeontological, archaeological or forensic interest, which are buried in soil (or cave sediment) for extended periods of time. While various DNA extraction methods have been described (10–14) and optimized (15–17), residual amounts of inhibitor are difficult to remove and can degrade PCR efficiency (18).

Attempts have also been made to mitigate the inhibitory effect on polymerase activity by increasing the concentration of polymerase (19) or by the inclusion of various additives. Some of these such as betaine, BSA, T4 gp32 or salmon sperm DNA have been reported to relieve inhibition of polymerase activity, but need to be added at substantial concentrations (typically >0.2 mg/ml) (20–22). Furthermore, although addition of e.g. BSA might help to reduce the inhibitory effects of some contaminants in ancient DNA extracts (16), the effect can be sample specific and is not always sufficient to ensure optimal PCR efficiency.

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While all polymerases are inhibited to some extent, there are both inhibitor- and polymerase-specific differences. For example, some 'non-Taq' polymerases such as Tth, Tfl, HotTub™ and Pwo have been reported to tolerate higher concentrations of blood in PCR than Taq (23). Likewise protein engineering can be employed to isolate polymerases with improved performance in the presence of relevant inhibitors. For example, screening of repertoires of KlenTaq polymerase yielded mutants that displayed increased resistance to inhibition by blood and crude soil samples (24).

Here, we have explored polymerase evolution by compartmentalized self-replication (CSR) (25) as a strategy to isolate polymerases with a broad resistance to environmental inhibitors. Starting from a diverse repertoire of chimeric polymerase genes prepared by the molecular breeding of eight orthologues from the genus *Thermus* and *Deinococcus,* we performed CSR selections directly in the presence of complex multicomponent inhibitor cocktails to yield polymerases with a generic resistance profile. Using this approach, we have isolated several polymerases with resistance to environmental inhibitors found frequently in specimens of paleontological, archaeological and forensic interest (such as humic acid or soil). CSR selection in the presence of *Neomylodon* bone powder yielded a novel chimeric polymerase with a substantial broad-spectrum resistance to a wide range of unrelated environmental inhibitors including tar, clay-rich soil or coprolites.

MATERIALS AND METHODS

Cloning of polymerases from *Thermus filiformis*, *Thermus brockianus*, *Thermus oshimai* and *Thermus scotoductus*

Thermus filiformis, T. brockianus, T. oshimai and *T. scotoductus* were obtained from the Japanese collection of microorganisms. They were grown in the following media (4 g/l yeast extract, 8 g/l bacto-peptone, 2 g/l NaCl, 0.1% Nitch's Trace elements, 0.0012% $FeCl_3$, 0.048 g/l $CaCl_2$, 0.1 g/l $MgS0_4 \cdot 7H_2O$, 0.7 g/l $NaNO_3$, 0.11 g/l Na_2HPO_4, where Nitch's trace elements are 0.5 ml concentrated H_2SO_4, 2.46 g $MnSO_4$, 0.89 g $ZnSO_4 \cdot 7H_20$, 0.5 H_3BO_3, 0.025 $CuSO_4 \cdot 5H_2O$, 0.029 g $Na_2MO_4 \cdot 2H_2O$, 0.046 g $CoCl_2 \cdot 6H_2O$ in 1l). *Thermus filiformis* was grown at 60°C and the others at 65°C until their optical density at 600 nm was >1. Genomic DNA was purified using the QiaAmp DNA mini kit (Qiagen) as per manufacturer's instructions, following bacterial protocol C. The *T. filiformis* DNA polymerase I gene was amplified with primers (5′-ATG ACC CCA CTT TTT GAC CTG GAG G/5′-TCA ATC CTG CTT CGC CTC CAG CCA G), whereas *T. brockianus, T. oshimai* and *T. scotoductus* were amplified using primers (5′-CCC ACC TCC ACC TCC AGG GGC AC/5′-CGG GTC CTC CTG GTG GAC GGC CAC C). PCR conditions were 1× *Pfu* buffer (Stratagene), 200 µM dNTPs, 1 µM primers, 2% formamide, 2.5U *Pfu* polymerase (Stratagene), 5 µl genomic DNA, thermocycled at: 94°C 5 min, 30× (94°C 30 s, 50°C 30 s, 72°C 15 min), 65°C 10 min. Amplification products (AP) were resolved by agarose gel electrophoresis, 2.5 kb bands excised and purified using QiaQuick gel extraction kit, re-amplified with *Pfu* polymerase using primers 5′-CAG GAA ACA GCT ATG ACA AAA ATC TAG ATA ACG AGG GCA A/5′-GTA AAA CGA CGG CCA GTA CCA CCG AAC TGC GGG TGA CGC CAA GCG, cut with XbaI and SalI, cloned into pASK75 with T4 DNA ligase and transformed into *Escherichia coli* Ace6 (28). Polymerases were expressed and assayed for PCR activity.

Molecular breeding library preparation

The pASK75 plasmid DNA containing the DNA polymerase I genes of *T. filiformis* (Tfi), *T. brockianus* (Tbr), *T. oshimai* (Tos), *T. scotoductus* (Tsc), *T. thermophilus* (Tth), *T. flavus* (Tfl) and *T. aquaticus* (Taq) as well as *Deinococcus radiodurans* (Dei) was purified using a HiSpeed plasmid midi kit (Qiagen) as per manufacturer's instructions, with an additional isopropanol precipitation step to reduce the final volume 10-fold. The plasmids were mixed in an equimolar fashion (final concentration 770 µg/ml) and StEP shuffled (26) in 1× *Taq* buffer, 200 µM dNTPs, 1 µM primers 5, 6, 7.5 µl plasmid mix and 1 µl SuperTaq. Cycling conditions were 94°C 10 min, 60× (94°C 30 s, 55°C 1 s), 65°C 10 min. The resulting DNA was resolved on and cut out of an agarose gel as before. It was then reamplified with biotinylated primers (5′ Biotin-GTA AAA CGA CGG CCA GTA CC/5′-Biotin-CAG GAA ACA GCT ATG ACA AA), cut with XbaI and SalI as before, purified as before on an agarose gel, then incubated with M-280 Streptavidin Dynabeads (Invitrogen) as per manufacturer's instructions to remove cut-off end fragments as well as uncut (or partially cut) APs, then cloned into pASK75 and transformed into *E. coli* Ace6, yielding a molecular breeding library size of 1×10^8 cfu (colony forming units). A diagnostic restriction digest of 20 clones produced 20 unique restriction patterns, indicating that the library was diverse. Subsequent sequencing of selected chimeras showed an average of 4–6 crossovers per gene.

Production of peat extract (Humic acid)

A sample of peat soil was broken into small pieces and water was added. The sample was then heated to 50°C for 1 h to aid solubilization. The resulting samples were spun down at 13 000 rpm for 30 min and the water phase was recovered. The volume was then reduced 10-fold by using a concentrator. The final peat extract had a dark brown colour and a pH of 6.5. The inhibitory activity of the resulting peat extract (rich in Humic acid) was tested by doing a 30 cycle PCR: (94°C 10 min, 30 × (94°C 30 s, 50°C 30 s, 72°C 1 min), 65°C 10 min in the presence of a 2-fold dilution series of Humic acid from 60% to 0.03%. The PCR conditions were 1× SuperTaq buffer (HT Biotechnology Ltd.) [10 mM Tris–HCl, pH 9.0 (25 C),1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100]. 0.2 mM dNTP, 1 µM primers (5′-AAA AAT CTA GAT AAC GAG GGC AA/5′-ACC ACC GAA CTG CGG GTG ACG CCA AGC G), 1 µl SuperTaq (HT Biotechnology) and 0.01 µl of pASK75 as template (100 µm stock), water and Humic acid as required. PCR

assays were also performed in the presence of *E. coli* cell debris as it is known that DNA and protein counteract to an extent the inhibitory effects of Humic acid.

Other inhibitors: provenance and preparation

SP61 (powdered *Neomylodon* bone, Patagonia, Chile), Tar [tar-impregnated bone material (La Brea tar pit, USA)], SP448 and SP475 (Giant ground sloth coprolites, Argentina), 1736 (cave sediment, Vindija Cave, Croatia) were kindly provided by Max-Planck Institute for Molecular Anthropology, Leipzig, Germany. SP61, Tar and 1736 were used as 25% (w/v) suspensions in 1× SuperTaq buffer [10 mM Tris–HCl, pH 9.0 (25°C), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100]. SP448 and SP475 were prepared as a 10% (w/v) suspension in 1× SuperTaq buffer. Humic acid (SIGMA) was prepared as saturated solution in 1× SuperTaq buffer. The samples were neither heated nor concentrated. For clay-rich soil (Cambridge, UK), a slurry in 1× SuperTaq buffer was incubated for 2 h at 50°C, followed by 20 min at 90°C. The extract was then centrifuged at 8000 rpm for 10 min and the supernatant was kept as inhibitory solution. These suspensions (solution for soil) were then used to determine inhibitory concentrations for wild-type Taq lysate.

Selection of Humic acid resistant clones

All selections were performed in 1× SuperTaq buffer. CSR emulsification and selection were performed essentially as described (27) using library 3T (28), primers (5: 5′-CAG GAA ACA GCT ATG ACA AAA ATC TAG ATA ACG AGG GCA A/6: 5′-GTA AAA CGA CGG CCA GTA CCA CCG AAC TGC GGG TGA CGC CAA GCG) and 94°C 5 min, 20× (94°C 30 s, 50°C 30 s, 72°C 5 min), 65°C 5 min but with the addition of Humic acid to the water phase of the emulsion as the source of selective pressure. The highest amount of Humic acid which produced a positive selection was 20% (v/v). The aqueous phase was retrieved essentially as described (27) from the emulsion by ether extraction, content CSR products were purified using a Qiagen PCR prep kit (Qiagen, Chatsworth, CA, USA) with an additional 35% GnHCl wash, digested with DpnI to remove plasmid DNA, digested with ExoSAP (USB) to remove residual primers, reamplified with out-nested primers (5′ Biotin-GTA AAA CGA CGG CCA GTA CC/ 5′-Biotin-CAG GAA ACA GCT ATG ACA AA), recloned and transformed into *E. coli* Ace6 as above. The resultant clones were screened and ranked in order using a PCR assay. Briefly, 2.5 µl of induced cells were added to 20 µl of PCR mix [1× SuperTaq buffer, 0.2 mM dNTPs, 1 µM primers, 0.01 µl of pASK75 (100 µM stock), water and Humic acid as required] with the relevant primers (5′-AAA AAT CTA GAT AAC GAG GGC AA/5′-ACC ACC GAA CTG CGG GTG ACG CCA AGC G).

Selection for resistance to soil and *Neomylodon* bone powder

For selection of resistant polymerase clones, the CSR (25,27) was performed in the presence of inhibitory amounts of soil or ground bone powder (SP61, 250 µg/µl suspension in 1× SuperTaq buffer) at a concentration at which the wild-type enzyme is completely inhibited. To determine inhibitory concentrations, Taq polymerase was expressed as described previously. Briefly, overnight cultures of cells expressing the wild-type *Taq* polymerase in pASK75 were diluted 1:100 in 200 µl 2× TY supplemented with Ampicilline (100 µg/ml) and grown for 2 h at 37°C, 220 rpm, induced with anhydrotetracycline (0.1 µg/ml final), continued shaking for 4 h (37°C, 220 rpm) and harvested by centrifugation (3800 rpm, 6 min). The pellet was resuspended in 50 µl 1× SuperTaq buffer (HT Biotechnology Ltd), heated for 5 min at 94°C and cleared by high-speed centrifugation to give a 4× lysate supernatant. These lysates were used directly or stored at −20°C. Lysates were diluted 4× and incubated with a range of concentrations of soil or bone powder suspension to determine minimal inhibitory concentration, where the wild-type Taq enzyme is completely inhibited to optimize selective pressure in CSR. The concentration used in the CSR reaction was 12.5 µg/µl (final concentration) of *Neomylodon* bone powder suspension or 3% of soil extract.

As inhibitor suspensions destabilized 'classic' Span80 emulsions (25,29), we used a modified CSR protocol, whereby 2×10^8 T8 library cells (induced and washed as above) were suspended in 200 µl of CSR mix as described and emulsified in 600 µl of [7% (v/v) ABIL WE09, 20% (v/v) mineral oil and 73% (vol/vol) Tegosoft] using a TissueLyser bead mill (Qiagen) and 5 mm stainless steel beads (Qiagen) with the following settings: 15 Hz for 10 s, 17 Hz for 7 s (30). The emulsion was then equally split into four thin-walled 0.5 ml reaction tubes and thermocycled at 94°C for 5 min, 10× (94°C 30 s, 50°C 30 s, 72°C 5 min), 65°C 10 min. After the CSR reaction, the emulsion was broken by extraction with Hexanol (400 µl), aqueous phases were pooled and centrifuged (5 min, 13 000 rpm). The cleared aqueous phase was collected and subjected to PCR purification (QIAquick PCR Purification Kit) and finally eluted in 50 µl EB buffer (65°C) (Qiagen). To remove plasmid DNA and primers, the reaction was then DpnI/ExoSAP treated and reamplified for cloning using primers (5′-CAG GAA ACA GCT ATG ACA AAA ATC TAG ATA ACG AGG GCA A/5′-GTA AAA CGA CGG CCA GTA CCA CCG AAC TGC GGG TGA CGC CAA GCG/ 5′-Biotin-GTA AAA CGA CGG CCA GTA CC/ 5′-Biotin-CAG GAA ACA GCT ATG ACA AA) and the following cycling conditions: 94°C 2 min, 94°C 30 s, 50°C 30 s, 72°C 5 min, 65°C 10 min, 25 cycles. Reamplified library amplicons were cut with XbaI and SalI, purified on an agarose gel, then incubated with M-280 Streptavidin Dynabeads (Invitrogen), cloned into pASK75 and transformed into *E. coli* Ace6 (library size: 4×10^8 cfu) and selected for a second round of CSR.

Screening, protein expression and characterization

Individual colonies representing polymerase clones were picked and grown in 200 µl 2 × TY medium with ampicillin (100 µg/ml). The polymerase gene was then induced

with anhydrotetracycline (0.1 μg/ml), the cells were harvested after 4 h at 37°C (3800 rpm, 6 min) and resuspended in 50 μl 1× SuperTaq buffer (HT Biotechnology Ltd.) to result in a 4× lysate. PCR screening was performed using primers (5′-AAA AAT CTA GAT AAC GAG GGC AA/5′-ACC ACC GAA CTG CGG GTG ACG CCA AGC G) and 0.01 μl of pASK75 as template (100 μM stock), and the following cycling conditions: 94°C 1 min, 25× (94°C 10 s, 50°C 30 s, 72°C 1 min), 65°C 2 min. Lysates which resulted in an AP (representing active polymerase clones) were then screened for their resistance towards the different inhibitors in inhibition assays. Promising polymerase clones were expressed and purified as described (28) using a 16/10 Hi-Prep Heparin FF Column (Amersham Pharmacia Biotech). Polymerase fractions eluted around 0.3 M NaCl and were concentrated and dia-filtrated into 50 Mm Tris pH 7.4, 1 mM DTT, 50% glycerol and stored at −20°C. Purified polymerases were activity normalized to Taq (5 U/μl) using primers 5′-AAA AAT CTA GAT AAC GAG GGC AA/5′-ACC ACC GAA CTG CGG GTG ACG CCA AGC G, 0.01 μl of pASK75 as template (100 μM stock), and the following cycling conditions: 94°C, 1 min, 25× (94°C 10 s, 50°C 30 s, 72°C 1 min), 65°C 2 min.

Determination of inhibition profiles

The resistance to inhibitory concentrations of different substances [bone powder (SP61), tar, clay-rich soil, cave sediment (1736), coprolites (SP475, SP448), Humic acid and peat extract] was then assayed using the very same PCR conditions. Briefly, inhibitor suspensions were prepared as in 'Other inhibitors: provenance and preparation' section and added at 100% inhibitory concentration to PCR mix A comprising 1× SuperTaq buffer (10 mM Tris–HCl, pH 9.0 (25°C), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), dNTPs (20 μM final), primers 5′-AAA AAT CTA GAT AAC GAG GGC AA/5′-ACC ACC GAA CTG CGG GTG ACG CCA AGC G (at 0.1 μM each), 1 pM of pASK75 template. This mix was then serially diluted into inhibitor-free PCR mix B (PCR mix A supplemented with 2.5 U of either SuperTaq or 2D9 DNA polymerase) and aliquoted into a 96-well Thermowell PCR plate (Costar). Reactions were overlaid with a drop of light mineral oil (SIGMA) and thermocycled in a MJ Tetrad Thermocycler using the following cycling conditions: 94°C 1 min, 25× (94°C 10 s, 50°C 30 s, 72°C, 1 min), 65°C 2 min. After PCR was completed, APs were resolved by gel electrophoresis on 2% agarose gels. Inhibition profiles were determined by densiometric scanning of AP bands (with background correction) using Image J and plotted as a function of inhibitor concentrations. Resistances were defined as IC_{50} (50% inhibitory concentrations) as determined by the midpoint of the sigmoidal inhibition curves. Relative average resistances (Figure 4b) were calculated from the average IC_{50} values of multiple experiments ($n = 3$ or more) and normalized to (i.e. divided by the average resistance of) Taq DNA polymerase. We chose to express resistance in relative terms (as normalized to Taq) as the heterogeneity and particulate nature of inhibitor samples made it challenging to prepare inhibitor slurries of precise concentration for independent experiments. The remaining variability of resistance is expressed by standard error bars (Figure 4).

To determine the dependence of inhibition on extension times in PCR (Supplementary Data and Figure S5), PCR were set up as in 'Determination of inhibition profiles' section, but with modified cycling conditions: 94°C 1 min, 25× (94°C 10 s, 50°C, 15 s, 72°C 15 s), 65°C 2 min. As above, APs were resolved by gel electrophoresis on 2% agarose gels.

Inhibition profiles for AmpliTaq Gold 360 DNA polymerase (Applied Biosystems) were determined using an identical PCR set-up but using AmpliTaq Gold buffer and thermocycling conditions according to manufacturer's recommendations. Briefly, inhibitor suspensions were prepared as in 'Other inhibitors: provenance and preparation' section and added at 100% inhibitory concentration to PCR mix AT comprising 1× AmpliTaqGold buffer supplemented with 2 mM $MgCl_2$, dNTPs (20 μM final), primers 5′-AAA AAT CTA GAT AAC GAG GGC AA/5′-ACC ACC GAA CTG CGG GTG ACG CCA AGC G (at 0.1 μM each), 1 pM of pASK75 template. This mix was then serially diluted into inhibitor-free PCR mix BT (PCR mix AT supplemented with 2.5 U AmpliTaq Gold DNA polymerase) and aliquoted into a 96-well Thermowell PCR plate (Costar). Reactions were overlaid with a drop of light mineral oil (Sigma) and thermocycled in a MJ Tetrad Thermocycler using the following cycling conditions: 95°C 10 min, 25× (95°C 20 s, 50°C 30 s, 72°C 1 min), 65°C 2 min. Manual hot-start PCR for 2D9 was carried out as above using PCR mix A except 2D9 enzyme was not added until the PCR block had reached 94°C. As above, APs were resolved by gel electrophoresis on 2% agarose gels, inhibition profiles were determined by densiometric scanning and relative average resistances were calculated from the average IC_{50} values of multiple experiments ($n = 3$) and normalized to (i.e. divided by the average resistance of) AmpliTaq Gold DNA polymerase (Figure 5).

Fidelity determination

Mutation rates of purified polymerases were determined using a well-established *in vitro* gap-filling assay (31). The assay scores errors generated in the *LacZ* α-complementation gene in M13mp2 during synthesis to fill a 407-nt gap. Reaction mixtures (25 μl) contained 0.2 nM M13mp2 gapped DNA substrate, 40 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM dithiothreitol, 5 μg of bovine serum albumin, 2.5% glycerol and 200 μM each of dATP, dGTP, dCTP and dTTP. Polymerization reactions were initiated by adding Taq (1.2 nM) or 2D9 polymerase (17 nM), and were incubated at 65°C for 10 min (Taq) or 5 min (2D9), and terminated by adding EDTA to 20 mM. When reaction products were analysed by agarose gel electrophoresis, the results indicated that the gap had been completely filled. In this assay, correct synthesis produces M13mp2 DNA that yields dark blue phage plaques upon introduction into an *E. coli* α-complementation strain and plating on indicator plates.

Errors are scored as light blue or colourless mutant M13 plaques. DNA from independent mutant clones was sequenced to define the *lacZ* mutation, and this information was used to calculate error rates, as described (31).

PCR amplification of ancient DNA from purified extracts

Ancient DNA (aDNA) was extracted essentially as described (32) under clean-room conditions. Essentially, bone was ground with mortar and pistil. Ten milliliter extraction buffer containing 0.45 M EDTA (pH 8), 0.5% *N*-Lauroylsarcosine, 1% Polyvinylpyrolidone, 50 mM DTT, 2.5 mM PTB and 0.25 mg/ml Proteinase-K were added to 200 mg of bone powder and incubated for 16 h at 37°C under rotation. The remaining bone powder was collected by centrifugation and only the supernatant was used for further processing. aDNA was purified by binding to silica. Forty milliliter of L2 buffer (5.5 M guanidinium–isothiocyanate, 25 mM NaCl, 100 mM Tris pH 8) and 50 µl of silica suspension were added to 10 ml supernatant and incubated for ~30 min. The pellet was collected by brief centrifugation, the supernatant discarded, and aDNA was further purified as in ref. (32), except that the silica pellet was washed in buffer L2 and once with NewWash (Bio 101, La Jolla, CA, USA). After drying the pellet, the DNA was eluted at 56°C in aliquots of 100 µl TE (10 mM Tris pH 7.4, 1 mM EDTA). Mock extractions were performed alongside all extractions. The final volume of the extract was 100 µl. After normalization of the mutant purified enzyme compared to the wild-type Taq on a modern DNA template, amounts of the enzymes representing the same activity were used for subsequent functional tests on ancient samples. Due to the high risk of contamination, these experiments were performed in the purpose-built ancient DNA laboratory at the Max-Planck-Institute in Leipzig, Germany. The cave bear sample used in the following PCR reactions was extracted following the protocol described (15). The primers used (1F: 5′-GTGGTGTCATGCATTTGG-3′, 129R: 5′-ACTGCGACGAGACCTTTAC-3′) amplify a part of the mitochondrial control region of the cave bear (*Ursus spelaeus*). Cave bear mtDNA was amplified by 1-step PCR. Briefly, 2 µl of ancient sample were added to a 20 µl PCR in SuperTaq buffer (HT Biotech) with 2 µl 10× SuperTaq buffer, 0.2 µl dNTPs [25mM each], 0.2 µl enzyme, 14.1 µl double distilled water (ddw), 0.5 µl equimolar primer mix (1F/129R, 10 µM each), 3 µl template (1:150 dilution). This PCR was set-up in a clean room following precautions appropriate for aDNA. The PCR was performed on cave bear mtDNA at limiting dilutions (MPI Leipzig) and the cycling conditions were: 95°C, 2 min, 60× (95°C 30 s, 56°C 30 s, 72°C 30 s) 72°C 4 min.

RESULTS

Preparation and characterization of the Step 8 library (8T)

We had previously shown that molecular breeding (33) of polymerase genes is a productive approach for the evolution of polymerase function. We previously constructed a molecular breeding library (3T) by recombining three polA genes from the thermophilic genus *Thermus*: Taq (*T. aquaticus*), Tth (*T. thermophilus*) and Tfl (*T. flavus*) using staggered extension process (StEP) DNA shuffling (26). We demonstrated that this library was a rich source of polymerases with different phenotypes notably the ability to amplify damaged DNA and enhance the recovery of ancient DNA from Pleistocene specimens (28) as well as the ability to efficiently utilize hydrophobic base analogues (34). Sequence diversity is a crucial factor in any gene library intended for directed evolution as it increases the likelihood of producing a novel phenotype. Molecular breeding is no exception in this regard. However, selected polymerases from this initial molecular breeding library displayed a surprisingly uniform chimeric gene structure. Most of the polymerases selected from 3T were simple *Tth*/*Taq* chimeras and shared an arrangement of gene segments, whereby the N-terminal region (comprising part of the 5′–3′ exonuclease domain) derived entirely from *Tth,* whereas the main polymerase domain derived mainly from *Taq* (28,34).

In order to access more diverse chimeric polymerase genotypes, we decided to construct a molecular breeding library utilizing a greater number of orthologues. To this end, we cloned several members of the polA family from the genus *Thermus* such as *T. brockianus* (Tbr), *T. filiformis* (Tfi), *T. scotoductus* (Tsc) and *T. oshimai* (Tos) as well as the DNA polymerase I from *D. radiodurans* (Dra) as functional polymerases (Supplementary Data and Figure S1) and expressed them in *E. coli* (data not shown). DNA shuffling requires significant regions of sequence homology both for fragment annealing and to prevent misalignment of shuffled sequence elements. Sequencing revealed the *Thermus* genes (Tbr, Tfi, Tsc, Tos) to display 58% identity with Taq on the DNA level. The *Deinococcus* gene is more divergent (37% overall identity between the seven *Thermus* genes and *D. radiodurans*), but was included as a potential additional source of diversity. We then proceeded by constructing a molecular breeding library by recombining the cloned genes of Tbr, Tfi, Tsc, Tos and Dra with the previously cloned Taq, Tth and Tfl genes using StEP shuffling (26) to obtain 8T, a 2×10^8 cfu library of chimeric polymerases. Sequencing of unselected clones suggested significant diversity, revealing chimeras of Taq × Tbr, Tbr × Tsc and Tbr × Tos × Tth (data not shown). Initial screening of activity revealed ca. 30% active clones (in PCR), which is significantly higher than typical activity levels in a random-mutant library (1–5%) (25) but lower than the levels seen with the less diverse molecular breeding library 3T (>70%) (28).

Determination of levels of inhibition of wild-type Taq polymerase in PCR

We first detemined levels of inhibition of wild-type (wt) Taq polymerase in PCR by a range of inhibitors by performing PCR amplification experiments with wt Taq polymerase in the presence of increasing amounts of inhibitor added to the PCRs. We used cellular lysate of *E. coli* cells expressing Taq polymerase to simulate the

conditions within the emulsion compartments during CSR selection. During CSR, cells expressing the polymerase are lysed by an initial heating step releasing the polymerase and encoding plasmid into the lumen of the aqueous emulsion compartment. The resulting discharge of protein, membrane fragments and nucleic acids (genomic DNA, rRNA, tRNA) into the reaction volume can significantly affect inhibition profiles. Using a crude cellular lysate for inhibitor titration mimics this environment.

We first tested the inhibitory potential of a number of specimens of palaeontological interest known to contain high concentrations of PCR inhibitors including a variety of powdered bone and coprolite samples from Pleistocene fauna. For example, we tested bone powder (SP61) and coprolite (SP448) from the extinct giant ground sloth (*Neomyolodon*) of Patagonia as well as tar-impregnated bone samples from the *la Brea* tar pits. To determine the inhibitory concentrations of SP61, we performed standard PCR reactions in the presence of increasing amounts of bone powder slurry (resuspended in 1× Taq buffer) (see subsection 'Other inhibitors: provenance and preparation' in 'Materials and Methods'). SP61 proved strongly inhibitory up to concentrations of 4–5 μg/μl bone slurry. We also examined SP448 (sloth coprolite): this substance showed the strongest inhibitory effect of all materials tested with as little as 12 ng/μl sufficient for complete inhibition of PCR by wtTaq polymerase. Finally, we tested samples from the *la Brea* tar pits, which contain one of the richest and best studied assemblages of Pleistocene vertebrate remains. Unsurprisingly, tar-impregnated bone also proved to be a very potent inhibitor with 30 ng/μl sufficient to completely inhibit wtTaq polymerase. We chose *Neomylodon* bone powder and coprolite for selection. We chose bone powder because of its generic importance in ancient samples and coprolite, because of its powerful inhibitory effect, which we hoped would provide strong selection pressure for the evolution of adaptive resistance. We also tested a number of environmental samples of which both peat and clay-rich garden soil provided strong inhibition at 5% (w/v) (peat) and 3% (w/v) (soil) inhibitory concentrations and these too were chosen as selection substrates.

Polymerase selections

To isolate polymerase mutants with an increased resistance to desired inhibitors we performed selection experiments using CSR (25) starting initially from the previously described molecular breeding library 3T (Taq, Tth, Tfl) (28) to establish favourable selection conditions and parameters. In later selection experiments, we used the herein described more diverse molecular breeding library 8T (Taq, Tth, Tfl, Tbr, Tsc, Tos, Tfi, Dra).

CSR is based on a simple feedback loop, whereby a polymerase replicates only its own encoding gene. CSR reactions occur within the aqueous compartments of a water-in-oil (w/o) emulsion (29), which is critical to ensure the linkage of genotype and phenotype during CSR. Thus, only polymerases capable of replicating their own encoding gene under the selection conditions (here the presence of potent inhibitors) are able to produce 'offspring' and survive to the next round, while genes encoding polymerases that are inhibited are not replicated and are lost from the gene pool (Figure 1). CSR has proven a powerful method for the directed evolution of polymerase function including thermostability, resistance to inhibitors, expanded substrate spectrum and the ability to replicate damaged DNA or hydrophobic base analogues (25,28,34,35).

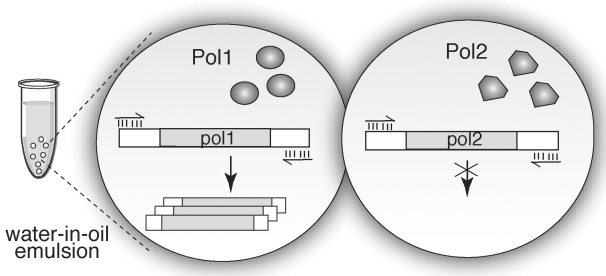

Figure 1. Principle of CSR selection (25). CSR is based on a simple feedback loop, whereby a polymerase replicates only its own encoding gene. Compartmentalization in the aqueous compartments of a w/o emulsion (29) isolates individual self-replication reactions from each other ensuring clonality. Two independent emulsion compartments are shown. Polymerases [such as Pol1 (left compartment)] that are capable of replicating their one encoding gene (*pol*1) in the presence of inhibitor within the compartment increase their copy number in the post-selection population, while polymerases like Pol2 (right compartment) that are unable to function with inhibitor present disappear from the gene pool.

Environmental inhibitors present special challenges for the evolution of resistance, as they often contain not just one but a variety of PCR inhibitors of distinct chemical makeup, potentially requiring the simultaneous acquisition of multiple disparate adaptations. It was therefore not obvious that it would be possible to select polymerases with substantial resistance to such inhibitor 'cocktails'.

Selection for resistance to Humic acid (peat soil extract). To explore selection parameters, we first performed selections using the 3T library, which previously had proven a reliable source of polymerases with novel phenotypes (28,34). CSR selections for resistance to Humic acid-rich peat soil extract were initially carried out using 'classical' Span80-based w/o emulsions (25,29) but these proved unstable in the presence of inhibitor slurries such as bone powder. We therefore changed to a different emulsion formulation comprising inert silicone-based surfactants (30,36) for later CSR selections. This emulsion mixture was adapted to standard CSR protocols and provided excellent stability, uniformity and performance in CSR providing up to three fold increased recovery of CSR products (data not shown). Using these emulsion mixes, we performed single or multiple rounds of CSR selections in the presence of increasing amounts of inhibitors and screened selected clones by PCR.

We first performed CSR selections in the presence of a Humic acid-rich peat extract with a slightly acidic pH (pH 6.5). The highest amount of peat extract, which produced a positive selection signal from the 3T library

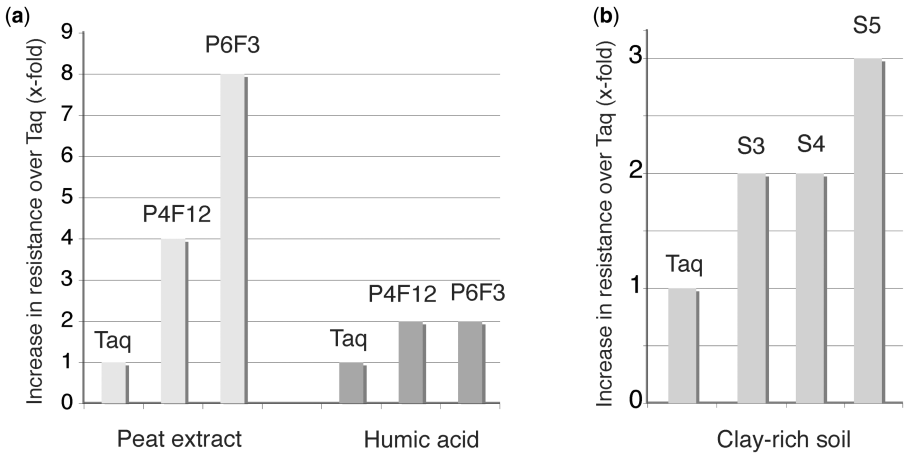

Figure 2. Inhibition profiles of selected polymerases. (**a**) Average increase in resistance ($n = 3$) of selected polymerase chimeras P4F12 and P6F3 (Supplementary Data and Figure S2) relative to wild-type Taq polymerase in PCR in the presence of either Humic acid-rich peat extract (left panel) or pure Humic acid (right panel). (**b**) Increase in resistance of selected polymerase chimeras S3–S5 (Supplementary Data and Figure S3) relative to wild-type Taq polymerase in PCR in the presence of clay-rich garden soil.

was 20% (v/v), significantly higher than the maximum inhibitory concentrations of wtTaq polymerase. After two rounds of selection, we screened polymerases for the ability to perform PCR in the presence of increasing concentrations of peat extract and identified a number of clones with increased resistance. Two polymerases in particular, P4F12 (Hu1) and P6F3 displayed an up to 8-fold increased resistance to peat extract (Figure 2a).

Both selected polymerases share a similar arrangement of gene segments. While diverging in detail, in both Hu1 and P6F3, the N-terminal region (comprising part or all of the 5′–3′ exonuclease domain) as well as the C-terminal region derive mainly from Tth, whereas the protein core derives mainly from Taq (Supplementary Data and Figure S2), as observed previously for selections from the 3T library. Both Hu1 and P6F3 also contain a very short segment of Tfl in the main polymerase domain as well as numerous point mutations not present in either of the parent genes [Hu1: F92L, P114Q; K508R / P6F3: V14A, T27A, E90Q, A184T, K220M, A271V, S739G (Taq numbering)].

We also compared the resistance of the selected polymerases to purified Humic acid from a commercial source. While most of the selected polymerases, including P4F12 and P6F3 also showed increased resistance to pure Humic acid, the level of resistance was much lower (2-fold) than for the peat extract. Humic acid-rich peat extract and pure Humic acid are clearly distinct inhibitors and their different interaction with the selected polymerases may reflect the heterogeneous nature of Humic substances. Although Hu1 and P6F3 already display some level of general resistance to Humic acid, it may be desirable for future selections to alternate between different Humic and/or Fulvic acid preparations to ensure a more generic resistance to Humic substances.

Selection for resistance to clay-rich garden soil. Having established that it was possible, in principle, to obtain polymerases with resistance to complex inhibitors, we next performed selections from the new 8T library using soil slurry as the selection agent. The clay-rich soil sample used in the experiments was collected in Cambridge (UK) and showed a slightly alkaline pH (pH 8.5). After two rounds of CSR selection, we isolated several polymerases (S3–S5) with an increased [up to 3-fold (S5)] resistance to inhibition by soil (Figure 2b). Again the selected chimeric polymerases shared a similar arrangement of gene segments, with the N-terminal region deriving mainly from Tth, and the polymerase core mainly from Taq (S4, S5) (Supplementary Data and Figure S3), while S3 is derived mainly from Taq polymerase. All three polymerases also contain additional point mutations not present in either of the parent genes [S3: A54V/S4: H20Y, M809L/S5: P114Q, I138V, G371A, V443A, M648V, F694L, F726L (Taq numbering)].

Selection for resistance to bone powder. Although the resistance improvements obtained from these experiments were modest, success in obtaining improved polymerases from the 8T library encouraged us to perform selections using a different selection agent. We performed CSR selections in the presence of inhibitory concentrations of *Neomylodon* bone powder (SP61) (12.5 μg/μl final concentration). After just one round of selection, we isolated a number of polymerases with significantly increased resistance compared to wtTaq polymerase. Among those, 2D9 outperformed all other identified polymerases and was chosen for further characterization. 2D9 displays an arrangement of sequence segments unlike any other chimeric polymerase previously isolated from the molecular breeding repertoires, comprising segments from at least four different polymerases comprising *T. thermophilus*, *T. oshimai*, *T. brockianus* and Taq polymerase (Figure 3, Supplementary Data and Figure S4).

Characterization of 2D9

We purified 2D9 and investigated its resistance to a range of inhibitors, in particular those present in samples

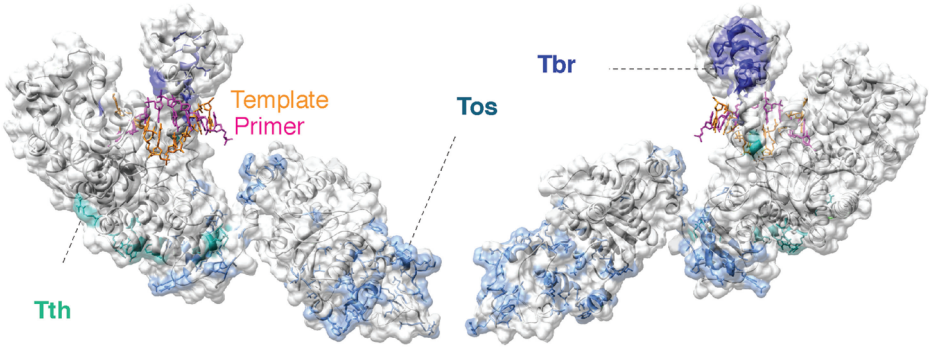

Figure 3. 2D9 polymerase structure. Changes from the Taq consensus in the selected polymerase 2D9 (Supplementary Data and Figure S4) are mapped on a ribbon and surface representation of Taq DNA polymerase [1TAU.pdb (42)]. The Taq consensus is coloured in white, gene segments deriving from Tos DNA polymerase are coloured light blue, those deriving from Tth DNA polymerase are green–blue, those deriving from Tbr DNA polymerase are dark blue. Segments deriving from Tos are found mainly in the N-terminal 5′–3′ exonuclease domain, while the main polymerase domain contains segments from Tos, Tbr and Tth. A primer-template strand duplex bound in the polymerase active site is also shown.

relevant to ancient DNA research (2,3). These included various samples of powdered bone (including the *Neomylodon* sample used for selections) and coprolites as well as extracts from bone material derived from the *la Brea* tar pits. Specimens such as these often contain not just one but a whole cocktail of organic and inorganic PCR inhibitors (37). For example, bone samples contain hydroxyapatite, collagen and haem, while coprolites contain bile salts, complex polysaccharides, bilirubin and hemin, all of which inhibit PCR amplifications to various degrees. Bone material from tar pits, in addition to the inhibitors already present in bone, is impregnated by polycyclic aromatic hydrocarbons. While the latter represents a special case, they are of potential relevance as tar pits are a rich source of Pleistocene specimens, some of which may contain amplifiable DNA. Finally, we also tested 2D9 with other samples relevant to biomedical PCR and known to contain complex mixtures of inhibitors. These included blood [containing at least three strongly inhibitory compounds (haem, lactoferrin, IgG)], soil (containing various amounts of Humic substances, plant polysaccharides and iron) as well as two different sources of Humic acid, either extracted from peat or in pure form from a commercial source.

Remarkably, 2D9 displayed a substantial resistance to a broad range of inhibitory substances, chemically distinct from those present in the bone powder used for selection (Figure 4), including coprolites soil and cave sediment. While tar pit bone samples should be superficially similar (comprising bone-specific inhibitors such as collagen, hydroxyapatite and haem), these also contain a complex mixture of polycyclic hydrocarbons.

Surprisingly, we found that the resistance of 2D9 to inhibition by tar impregnated bone, soil and coprolite was actually higher than that for *Neomylodon* bone. In addition 2D9 also showed improved resistance to other inhibitors such as cave sediment, Humic acid-rich peat extract and pure Humic acid (sodium salt). Thus 2D9 appears to display a generically improved resistance to a range of environmental inhibitors. However, its resistance is not universal. For example, 2D9 did not display any improved resistance to the inhibitory effects of whole blood (Y. Xu and T. Evans, New England Biolabs, personal communication), indicating that whole blood contains (a) substance(s) to which 2D9 is not resistant.

Resistance (where present) was found to be independent of primer annealing temperatures during PCR (data not shown) but showing some dependence on extension times with 2D9 showing greater resistance to tar (compared to Taq) at shorter extension times (15 s versus 1 min) (Supplementary Data and Figure S5).

2D9 fidelity and PCR performance

Evolution of novel properties in DNA polymerases can occur at the expense of core properties such as fidelity or PCR sensitivity. We determined 2D9 misincorporation rates using a well-established gap-filling assay (31) and found it to be very similar to the error rate of Taq in the same assay (Supplementary Data and Table 1). This may reflect a general property of CSR selected polymerases. CSR inherently selects for polymerase fidelity as self-replication must operate within the 'error threshold' (38). Polymerases with high error rates will introduce random mutations into their own gene during CSR selection thereby reducing the fitness of their 'offspring'.

In previous work on polymerase evolution, we had isolated chimeric polymerases (from the 3T library) with an ability to amplify damaged DNA (28). In that case, although the selected polymerases outperformed the parent Taq polymerase in the amplification from DNA templates containing lesions as well as on ancient DNA, the acquisition of the translesion synthesis phenotype appeared to have come at the expense of PCR sensitivity. We therefore tested the 2D9 polymerase on challenging PCR amplifications of low-copy number targets. We performed four independent PCR amplification experiments of 24 separate PCRs (total: 96) of mitochondrial DNA (mtDNA) at limiting dilutions of extract derived from Pleistocene cave bear (*Ursus spelaeus*) bone as well as an equal number (96) of no template controls (PCR reactions to which no cave bear extract was added). We then scored successful amplifications for Taq respectively 2D9. We found that 2D9 yielded comparable numbers of APs compared to Taq (2D9: 70 positive PCRs of 96;

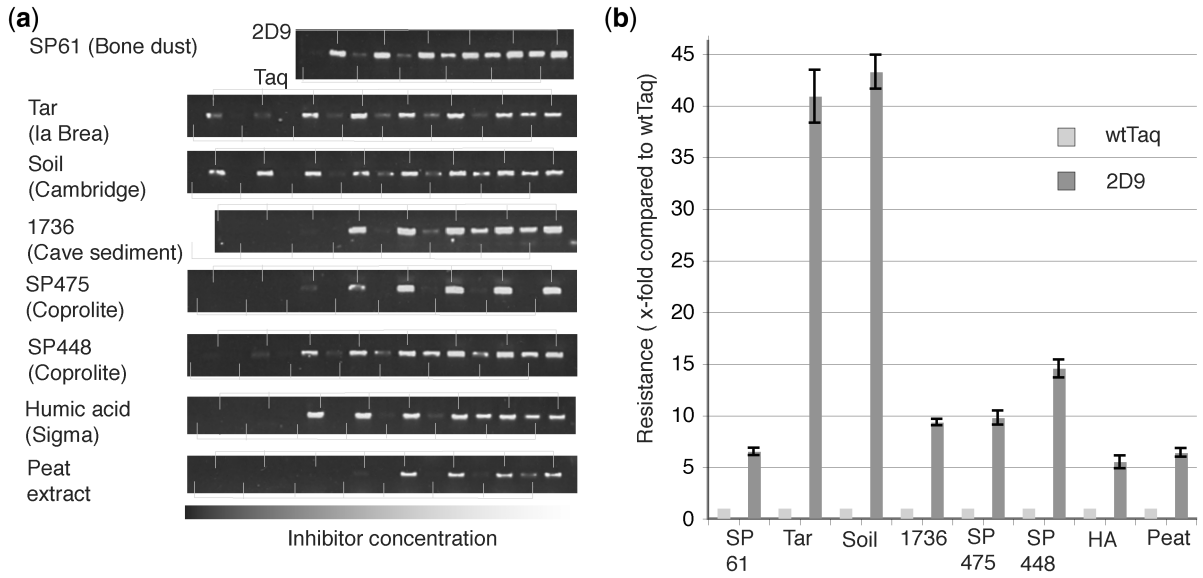

Figure 4. Inhibition profiles of Taq versus 2D9 in PCR. (**a**) The effect of various inhibitors on polymerase activity of Taq and 2D9 in PCR was determined using a PCR assay (see subsection 'Other inhibitors: provenance and preparation' in 'Materials and Methods' section) in the presence of decreasing amounts of inhibitor. (**b**) The average relative resistance to various inhibitors was calculated from the ratio of the average IC_{50} (50% inhibitory concentration) of several independent experiments ($n = 3$) of 2D9 in relation to the IC_{50} of Taq for the same inhibitor. Error bars represent the standard error of these experiments. 2D9 is significantly more resistant to inhibition for all inhibitors tested.

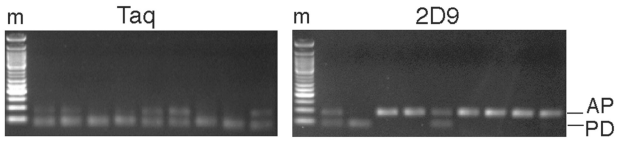

Figure 5. PCR from low-copy number targets. Comparison of 2D9 with standard Taq DNA polymerase in the amplification of mitochondrial DNA from cave bear bone extracts at limiting dilution. Panels show sections of typical post-amplification gels. 2D9 provides comparable numbers of amplicons but in general yields cleaner and higher yield APs with a reduced formation of primer–dimers.

Taq: 56/96), showing that its PCR sensitivity was not compromised. No template controls were always clean (i.e. contained no AP). Notably, 2D9 consistently yielded clearer and stronger APs (Figure 5) (compared to Taq), presumably due to an apparent reduced tendency to produce primer–dimers.

DISCUSSION

Here, we describe the isolation of novel chimeric polymerases with enhanced resistance to several environmental inhibitors with relevance for PCR applications. Starting from a diverse library of polymerase chimeras prepared by molecular breeding (33) of the DNA pol I genes from members of the genus *Thermus* and *Deinococcus*, we performed CSR selections in the presence of complex inhibitor cocktails. We isolated a number of 'specialist' polymerases with specifically increased resistance to a given inhibitor such as Humic acid and clay-rich soil. From CSR selections using *Neomylodon* bone powder as the inhibitor, a polymerase with broad spectrum of resistance emerged (Figure 4). This polymerase (2D9) displayed a chimeric gene structure unlike any we had previously observed comprising segments from four different polymerases: Taq (1–109), Tos (110–388), Tth (389–456), Tbr (457–471) and Taq again (472–834), resulting in 81 mutations from the Taq consensus (Figure 3 and Supplementary Data and Figure S4). As with previously selected polymerases, the bulk of the main polymerase domain derives from Taq, presumably because a reaction buffer optimized for Taq was used for both selections and screening. Even subtle variations in reaction buffer composition can significantly affect PCR performance (39). Although most of the other *Thermus* enzymes (e.g. Tos, Tbr, Tfl and Tsc) showed good activity in Taq buffer, Tth and Tfi did not (data not shown). Thus a different set of polymerases may emerge using different buffer conditions (optimized e.g. for Tth) in the CSR selections.

All of the inhibitors examined in this study comprise complex, largely indescribable mixtures. Humic acid is a complex blend of poly-phenols (7) while coprolites comprise a whole cocktail of substances that are potent inhibitors including bile salts, complex polysaccharides, bilirubin and hemin (4,37,40). The tar samples comprise, in addition to inhibitors found in bone such as collagen and haem, diverse mixtures of polycyclic aromatic hydrocarbons (asphaltenes). Resistance to these diverse inhibitor cocktails may reflect resistance to a common inhibitory component present in all of these, but it is far from clear what this substance would be. Furthermore, 2D9 does display some selectivity in its resistance both in the degree of resistance (e.g. coprolites and Humic acids of different provenance) as well as in the absence of resistance (in comparison to Taq polymerase) to inhibition by complex samples containing related inhibitors such as human blood.

An alternative explanation would be that all of these substances share a common mechanism of inhibition. Indeed, one attractive explanation would be that the inhibitory activity of these different substance mixtures is due to their capacity for non-specific interaction with proteins and/or nucleic acids and the resulting sequestration of polymerase and template DNA from solution into an inaccessible form. Consistent with this hypothesis is the fact that the inhibition of both Humic acid and coprolite extracts can (at least partially) be overcome by the use of increased amounts of polymerase/primer oligonucleotides as well as the addition of non-specific protein (BSA) or DNA (salmon sperm DNA). Furthermore, this attenuating effect is additive (data not shown). Indeed, one of the problems faced during selections was a significant reduction of inhibitor potency in bacterial lysates. Aqueous CSR compartments during selection contain significant amounts of denatured protein, bacterial nucleic acids (mostly genomic DNA and rRNA) and membrane lipids from bacterial lysis and these were found to attenuate the inhibitory effect.

While it is unclear what the mechanism of non-specific adsorption would be, it is notable that Humic and Fulvic substances (as well as Asphaltenes) are known to form colloids. Indeed, most of the inhibitors appear as suspensions of fine particulate matter. Adsorption to the large surface area of colloid particles in suspension would provide an effective route to sequester significant quantities of protein and nucleic acids and offers a plausible mechanism of inhibition. Adsorbed to the surface of the colloids, the reagents would be inaccessible and unable to participate in PCR. Thus, 2D9 may simply display reduced interaction with colloids. However, inspection of surface charge or hydrophobicity distribution of Taq polymerase and a Phyre-threaded homology model (41) of 2D9 do not reveal obvious localized differences, which could mediate such differential interactions, despite significant divergence at the sequence level.

Other potential explanations such as enhanced PCR performance of 2D9 (e.g. due to increased bypass of template abasic sites generated by thermocycling or exposure to chemicals in the inhibitor samples) or increased processivity do not appear to hold. Taq and 2D9 polymerase display a similar ability to bypass DNA lesions (Supplementay Data and Figure S6), generate similar profiles of DNA products in primer extension reactions (Supplementary Data and Figure S7a), and have similar processivity (both in the presence and absence of inhibitors), when measured under conditions that allow only a single cycle of DNA synthesis (Supplementary Data and Figure S7b).

The discrepancy in inhibition profiles between primer extension reactions and PCR may (at least partially) be reconciled by consideration of the iterative nature of PCR, which may amplify fairly subtle differences, which would be challenging to detect in primer extension reactions. For example, an efficiency differential of as little as 10% in the presence of inhibitors could yield differences of up to 20-fold over a 30 cycle PCR. Inhibitor action during PCR thermocycling may also differ from extension reactions because of e.g. inhibitor effects on polymerase stability, primer/template rebinding or because of inhibitor action is temperature dependent.

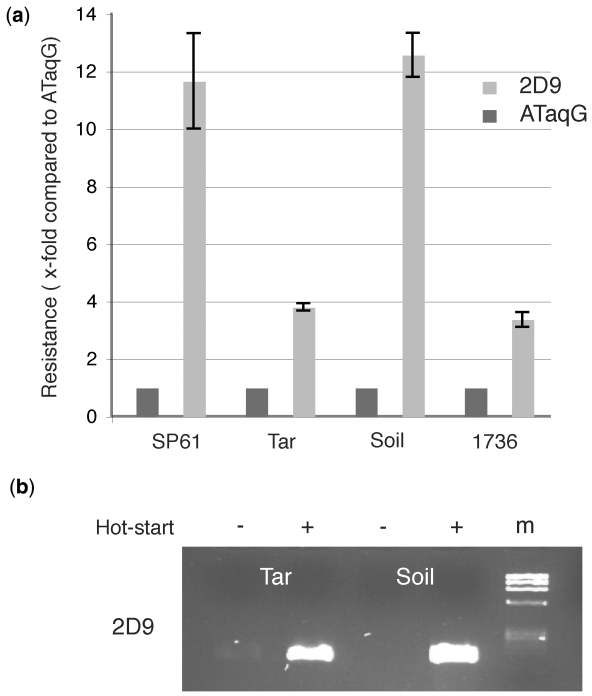

Figure 6. Inhibition and the effect of hot-start. (**a**) Inhibition profiles of 2D9 versus AmpliTaq Gold 360 (ATaqG) in PCR. The effect of various inhibitors (SP61: bone dust, Tar, Soil, 1736; cave sediment) on polymerase activity of 2D9 and ATaqG as determined by a PCR assay in the presence of decreasing amounts of inhibitor and plotted as the average relative resistance ($n = 3$, standard error) (as in Figure 4) of 2D9 and ATaqG with respect to ATaqG as a reference (resistance = 1). (**b**) The effect of manual hot-start on resistance of 2D9 polymerase to inhibitors tar and soil at minimal limiting amounts of inhibitor. Hot-start increases inhibitor resistance by at least a factor of two.

The 2D9 polymerase showed greatly enhanced resistance (up to 47×) in the presence of some environmental inhibitors, notably clay-rich soil and tar-impregnated bone material, while showing enhanced resistance (5–15×) to the inhibitory effects of a broad range of other complex inhibitors such as coprolites, cave sediment, bone powder, peat and pure Humic acid (Figure 4). At the same time, it retained an ability to function under more challenging PCR conditions, like amplifying ancient DNA extracted from cave–bear bone at limiting dilutions (Figure 5). However, its sensitivity to amplify low-copy number targets (e.g. in ancient DNA PCR), although essentially identical to conventional Taq, was lower than that of an engineered hot-start variant of Taq (AmpliTaq Gold) (data not shown). Furthermore, when we examined AmpliTaq Gold (ATaqG) resistance to inhibitors we found that, although 2D9 outperforms ATaqG on most inhibitors (Figure 6a), ATaqG is generally much less sensitive to inhibition than standard Taq, although based on the same enzyme. ATaqG is a chemically modified version of Taq DNA polymerase. The chemical modification renders the enzyme inactive at room temperature but high temperature and low pH (encountered in Tris-buffered PCR at 92–95°C) reverse the chemical modification and restore enzyme activity resulting both

in hot-start PCR as well as a continued release of active enzyme during PCR thermocycling, which increases yields of specific APs. Amplification of low-copy number targets is especially vulnerable to side-reactions caused by extension of primers mis-annealed to non-target sequences or themselves (primer–dimers), which preferentially form at low temperatures. Hot-start PCR prevents extension of such illegitimate priming events by delaying polymerase activity until proper primer annealing temperatures are reached. There must therefore be also a distinct advantage in hot-start PCR in relation to inhibition sensitivity. Indeed, when we re-examined the resistance profile of 2D9 using a manual hot-start procedure, we found that hot-starting increased the already high resistance of 2D9 to inhibition by at least 2-fold (Figure 6b).

The fact that a simple 'manual' hot-start further improved the already substantial resistance of 2D9 suggests that hot-starting contributes to ATaqG's improved resistance compared to 'standard' Taq. This in turn indicates that the mechanism of inhibitor action may, in addition to the proposed sequestration of reagents, involve the promotion of mispriming events during early PCR cycles. Presumably, the proposed sequestration of template DNA and primers renders even abundant templates effectively low-copy number targets, which may then benefit from the increased specificity of hot-start amplification. Provided that there are no inhibitor absorbing components in the proprietary ATaqG buffer or that residual chemical modification of ATaqG does not alter its propensity to interact with the inhibitor cocktails, a further contribution to inhibitor resistance would likely derive from continued release of active enzyme during the course of PCR. Indeed, the resistance gain by (manual) hot-start (average: 2×) is significantly smaller than the differential between standard Taq and ATaqG (average: ~5×). The development of a chemical hot-start capability for 2D9 should allow disentanglement of these disparate effects and, by analogy with ATaqG, would be expected to lead to significant further increases in resistance.

In conclusion, CSR selection for resistance to complex environmental inhibitors yielded 2D9, a chimeric polymerase with significant resistance to inhibition by divergent organic and inorganic inhibitor cocktails such as coprolite, bone dust, soil, Humic acid and tar. The broad spectrum resistance of 2D9 to a variety of environmental inhibitors is promising benefits in a number of PCR applications where such inhibitors are present. However, but the full realization of the potential of 2D9 may require the development of a chemically- or antibody-based hot-start capability for this polymerase. The fact that just a single round of CSR selection yielded a polymerase with a substantially and broadly increased resistance underlines the power of the CSR method for the isolation of novel polymerase phenotypes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B. and Erlich,H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487–491.
2. Hofreiter,M., Serre,D., Poinar,H.N., Kuch,M. and Paabo,S. (2001) Ancient DNA. *Nat. Rev. Genet.*, **2**, 353–359.
3. Paabo,S., Poinar,H., Serre,D., Jaenicke-Despres,V., Hebler,J., Rohland,N., Kuch,M., Krause,J., Vigilant,L. and Hofreiter,M. (2004) Genetic analyses from ancient DNA. *Annu. Rev. Genet.*, **38**, 645–679.
4. Wilson,I.G. (1997) Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.*, **63**, 3741–3751.
5. Lantz,P.G., Abu al-Soud,W., Knutsson,R., Hahn-Hagerdal,B. and Radstrom,P. (2000) Biotechnical use of polymerase chain reaction for microbiological analysis of biological samples. *Biotechnol. Annu. Rev.*, **5**, 87–130.
6. Al-Soud,W.A., Jonsson,L.J. and Radstrom,P. (2000) Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. *J. Clin. Microbiol.*, **38**, 345–350.
7. Baigorri,R., Fuentes,M., Gonzalez-Gaitano,G., Garcia-Mina,J.M., Almendros,G. and Gonzalez-Vila,F.J. (2009) Complementary multianalytical approach to study the distinctive structural features of the main humic fractions in solution: gray humic acid, brown humic acid, and fulvic acid. *J. Agric. Food Chem.*, **57**, 3266–3272.
8. LaMontagne,M.G., Michel,F.C. Jr, Holden,P.A. and Reddy,C.A. (2002) Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. *J. Microbiol. Methods*, **49**, 255–264.
9. Howeler,M., Ghiorse,W.C. and Walker,L.P. (2003) A quantitative analysis of DNA extraction and purification from compost. *J. Microbiol. Methods*, **54**, 37–45.
10. Hoss,M. and Paabo,S. (1993) DNA extraction from Pleistocene bones by a silica-based purification method. *Nucleic Acids Res.*, **21**, 3913–3914.
11. Hanni,C., Brousseau,T., Laudet,V. and Stehelin,D. (1995) Isopropanol precipitation removes PCR inhibitors from ancient bone extracts. *Nucleic Acids Res.*, **23**, 881–882.
12. Kalmar,T., Bachrati,C.Z., Marcsik,A. and Rasko,I. (2000) A simple and efficient method for PCR amplifiable DNA extraction from ancient bones. *Nucleic Acids Res.*, **28**, E67.

13. Keyser-Tracqui,C. and Ludes,B. (2005) Methods for the study of ancient DNA. *Methods Mol. Biol.*, **297**, 253–264.
14. Moreira,D. (1998) Efficient removal of PCR inhibitors using agarose-embedded DNA preparations. *Nucleic Acids Res.*, **26**, 3309–3310.
15. Rohland,N. and Hofreiter,M. (2007) Ancient DNA extraction from bones and teeth. *Nat. Protoc.*, **2**, 1756–1762.
16. Rohland,N. and Hofreiter,M. (2007) Comparison and optimization of ancient DNA extraction. *Biotechniques*, **42**, 343–352.
17. Rohland,N., Siedel,H. and Hofreiter,M. (2004) Nondestructive DNA extraction method for mitochondrial DNA analyses of museum specimens. *Biotechniques*, **36**, 814–816, 818–821.
18. Al-Soud,W.A. and Radstrom,P. (2001) Purification and characterization of PCR-inhibitory components in blood cells. *J. Clin. Microbiol.*, **39**, 485–493.
19. Sutlovic,D., Definis Gojanovic,M., Andelinovic,S., Gugic,D. and Primorac,D. (2005) Taq polymerase reverses inhibition of quantitative real time polymerase chain reaction by humic acid. *Croat Med. J.*, **46**, 556–562.
20. Abu Al-Soud,W. and Radstrom,P. (2000) Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. *J. Clin. Microbiol.*, **38**, 4463–4470.
21. Tebbe,C.C. and Vahjen,W. (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Appl. Environ. Microbiol.*, **59**, 2657–2665.
22. Kreader,C.A. (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl. Environ. Microbiol.*, **62**, 1102–1106.
23. Abu Al-Soud,W. and Radstrom,P. (1998) Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. *Appl. Environ. Microbiol.*, **64**, 3748–3753.
24. Kermekchiev,M.B., Kirilova,L.I., Vail,E.E. and Barnes,W.M. (2009) Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. *Nucleic Acids Res.*, **37**, e40.
25. Ghadessy,F.J., Ong,J.L. and Holliger,P. (2001) Directed evolution of polymerase function by compartmentalized self-replication. *Proc. Natl Acad. Sci. USA*, **98**, 4552–4557.
26. Zhao,H., Giver,L., Shao,Z., Affholter,J.A. and Arnold,F.H. (1998) Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nat. Biotechnol.*, **16**, 258–261.
27. Ghadessy,F.J. and Holliger,P. (2007) Compartmentalized self-replication: a novel method for the directed evolution of polymerases and other enzymes. *Methods Mol. Biol.*, **352**, 237–248.
28. d'Abbadie,M., Hofreiter,M., Vaisman,A., Loakes,D., Gasparutto,D., Cadet,J., Woodgate,R., Paabo,S. and Holliger,P. (2007) Molecular breeding of polymerases for amplification of ancient DNA. *Nat. Biotechnol.*, **25**, 939–943.
29. Tawfik,D.S. and Griffiths,A.D. (1998) Man-made cell-like compartments for molecular evolution. *Nat. Biotechnol.*, **16**, 652–656.
30. Diehl,F., Li,M., He,Y., Kinzler,K.W., Vogelstein,B. and Dressman,D. (2006) BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nature Methods*, **3**, 551–559.
31. Bebenek,K. and Kunkel,T.A. (1995) Analyzing fidelity of DNA polymerases. *Methods Enzymol.*, **262**, 217–232.
32. Hofreiter,M., Rabeder,G., Jaenicke-Despres,V., Withalm,G., Nagel,D., Paunovic,M., Jambresic,G. and Paabo,S. (2004) Evidence for reproductive isolation between cave bear populations. *Curr. Biol.*, **14**, 40–43.
33. Crameri,A., Raillard,S.A., Bermudez,E. and Stemmer,W.P. (1998) DNA shuffling of a family of genes from diverse species accelerates directed evolution. *Nature*, **391**, 288–291.
34. Loakes,D., Gallego,J., Pinheiro,V.B., Kool,E.T. and Holliger,P. (2009) Evolving a polymerase for hydrophobic base analogues. *J. Am. Chem. Soc.*, **131**, 14827–14837.
35. Ghadessy,F.J., Ramsay,N., Boudsocq,F., Loakes,D., Brown,A., Iwai,S., Vaisman,A., Woodgate,R. and Holliger,P. (2004) Generic expansion of the substrate spectrum of a DNA polymerase by directed evolution. *Nat. Biotechnol.*, **22**, 755–759.
36. Ghadessy,F.J. and Holliger,P. (2004) A novel emulsion mixture for in vitro compartmentalization of transcription and translation in the rabbit reticulocyte system. *Protein Eng. Des. Sel.*, **17**, 201–204.
37. Poinar,H.N., Hofreiter,M., Spaulding,W.G., Martin,P.S., Stankiewicz,B.A., Bland,H., Evershed,R.P., Possnert,G. and Paabo,S. (1998) Molecular coproscopy: dung and diet of the extinct ground sloth Nothrotheriops shastensis. *Science*, **281**, 402–406.
38. Eigen,M. (1971) Selforganization of matter and the evolution of biological macromolecules. *Naturwissenschaften*, **58**, 465–523.
39. Wolffs,P., Grage,H., Hagberg,O. and Radstrom,P. (2004) Impact of DNA polymerases and their buffer systems on quantitative real-time PCR. *J. Clin. Microbiol.*, **42**, 408–411.
40. Loreille,O., Roumat,E., Verneau,O., Bouchet,F. and Hanni,C. (2001) Ancient DNA from Ascaris: extraction amplification and sequences from eggs collected in coprolites. *Int. J. Parasitol.*, **31**, 1101–1106.
41. Bennett-Lovsey,R.M., Herbert,A.D., Sternberg,M.J. and Kelley,L.A. (2008) Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. *Proteins*, **70**, 611–625.
42. Eom,S.H., Wang,J. and Steitz,T.A. (1996) Structure of Taq polymerase with DNA at the polymerase active site. *Nature*, **382**, 278–281.