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Thermostabilities of DNA ligases and DNA polymerases from four genera of thermophilic eubacteria

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Thermophilic eubacteria were screened for thermostability of DNA ligases and DNA polymerases. A total of 103 and 248 strains were screened respectively. The strains belonged to four distantly related genera of *Thermus, Bacillus, Rhodothermus* and *Hydrogenobacter*. Thermostable DNA ligases were found in 22% of the strains and thermostable DNA polymerase were found in 15% of the strains. *Thermus* strains gave the highest frequency of both heat tolerant enzymes.

Introduction

All living organisms depend on DNA ligases and DNA polymerases for their DNA replication and for DNA repair. Polymerases replicate DNA by extending primers on the DNA strands and ligases form a covalent phosphate link between two strands of DNA. There are many different types of polymerases as replication involves many specialised functions. They are classified as families A, B, C and X corresponding to similarity with *E. coli* pol I, II, III and pol α. All ligases from bacteria studied so far use the high energy adenylate NAD prior to the formation of the phosphodiester bond. All other studied ligases from eukaryotes, archaea, viruses and bacteriophages use ATP for their adenylation.

DNA polymerases and DNA ligases are among the most important tools of modern gene technology. The use for thermophilic DNA polymerases in PCR has revolutionized the field of recombinant DNA technology and is still increasing. Use has been found for thermophilic DNA ligases in the ligase chain reaction (LCR) which is used to detect single base mutations (Barany, 1991). The use of thermostable ligase is also of considerable potential to construct sequencing primers from hexameric nucleotides (Szybalski, 1990). Sequences have been published for many thermophilic polymerases from both eubacteria and archaea (Perler *et al.*, 1996) and for three thermophilic ligases (Baraney and Gelfand, 1991; Lauer *et al.*, 1991; Jonsson *et al.*, 1994; Thorbjarnardottir *et al.*, 1995).

The properties of both thermostable polymerases and ligases are quite different even from the same genera of bacteria (Perler *et al.*, 1996). Values like optimum temperature, stability, K_m dNTP or K_m DNA, processivity, pH optimum and salt concentration are all factors that can be quite different for different enzymes. With this in mind it was decided to screen thermophilic eubacteria from four different genera (*Thermus*, *Bacillus*, *Rhodothermus* and *Hydrogenobacter*) for their heat stability to see if some correlation was found between optimum growth temperature and between the stability of the two different enzymes and how these properties were distributed among the different genera.

Materials and methods

The bacteria

The thermophilic bacteria used in the study were isolated from Icelandic hot springs. All of the strains had been identified at least to genus level.

Cultivation and disintegration

Strains were cultivated on agar plates with medium described by Degryse *et al.* (1978). *Rhodothermus* strains were grown on the same medium with 1% NaCl. Incubation was at 65°C for 12 h. Cell mass was removed from agar plates with a sterile loop. *Hydrogenobacter* was cultivated in liquid medium at 72°C (Kristjansson *et al.*, 1985). Cell mass was dissolved in 20% (w/v) of sterile buffer (10 mM Tris, pH 8.0, 10 mM MgCl, 50 mM NaCl and 1 mM dithiothreitol). Disintegration

was done in a French press cell (SLM Aminco), followed by centrifugation at 18.000 g for 30 min. Clear crude extract was kept at –70°C.

Selection criteria

For the polymerase screening a part of the crude extract was heated for 30 min at 95°C and centrifuged for 30 min at 10,000 g followed by measuring polymerase activity before and after heating. Polymerase was counted as heat stable if >10% of the activity remained after heating.

For the ligase screening the crude extract was heated for 30 min at 90°C to select strains with heat tolerant ligases followed by centrifugation as above. It was not possible to use the crude extract directly in the ligase assay, because of smearing in the gels from bacterial nucleic acids. Therefore proteins were precipitated using 80% ammonium sulphate. After centrifugation the pellet was dissolved in the same volume of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and desalted on Sephadex G-25 mini-columns. Ligase assay was done using the nick test indicating which strains had an active ligase after the heating procedure.

Preparation of nicked plasmid DNA

The plasmid pUC19 was isolated from the plasmid containing *E. coli*. The nicking of the supercoiled plasmid was done according to Barzilai (1973) and Takahashi *et al.* (1984). Reactions using 10 mM Tris-HCl pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 20 μg EtdBr/ml, 100 μg BSA/ml, 5 μg supercoiled DNA/ml and 0.5 μg DNase I/ml (Sigma Chemical Co., St. Louis, USA) were incubated for 30 min at 37°C followed by phenol extraction and precipitation of DNA. Quality control of the nicked plasmid product was done by ligating it with T4 ligase (Amersham Life Science) and running it on 1% agarose gel.

DNA ligase assay

Ligase activity was assayed for the ability to seal nicked plasmid DNA (pUC19) as monitored by electrophoresis on a 1% agarose gel. The ligated plasmid migrates more rapidly than the nicked DNA and can be visualised via UV light using ethidium bromide in the gel. The reactions were done in 10 μl volumes containing 200 ng of nicked pUC19 DNA, in ligase buffer (20 mM Tris-HCl, pH 8.0), 75 μM NAD, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 1 mM EDTA, 100 μg BSA/ml using 2 μl of pretreated crude extract. Negative and positive controls were run every time. The positive control was a cloned thermostable ligase from *Thermus scotoductus* (Jonsson *et al.*, 1994). Reactions were done at 65°C for 1 h followed by mixing with loading buffer (50 mM EDTA, 50% glycerol, 0.02% Bromophenol Blue).

Plasmid DNA was visualised on a UV transilluminator and photographing with Polaroid camera (FCR-10 DNA Photographic System, Fotodyne Incorporated New Berlin, USA). Strains were judged positive if ligating the nicked plasmid.

Thermostability of ligases

The strains having active DNA ligase after the above treatment were tested again for further heat stability. The pretreated crude extracts from positive strains were heated at 95°C for 5, 10, 20, 30 and 60 min followed by centrifugation for 15 min at 10,000 g. Ligase activity was then assayed.

Polymerase assay and unit definition

The DNA polymerase was determined in 70 μl reaction mixture of 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$ 50 mM KCl, 0.1% Triton-X-100, 200 μM of each dTTP, dGTP, dCTP, dATP, 1.75 μCi ^{3}HdATP, 0.2 mg/ml activated DNA and 5 μl of crude extract. Incubation was done for 0, 5, 10 and 15 min at 70°C and 50 μl pipetted onto 3 MM Whatman filterpapers

Table 1 Number of strains screened for DNA ligase and DNA polymerase activity.

Genera	*DNA ligase Screened*	*DNA ligase Active after 30 min at 90°C*	*DNA polymerase Screened*	*DNA polymerase with >10% activity remaining after 30 min at 95°C*
Thermus	68	23	85	31
Bacillus	14	0	88	1
Rhodothermus	12	0	43	4
Hydrogenobacter	9	0	32	0
TOTAL	103	23	248	36

which were directly washed twice in 600 ml of 10% trichloroacetic acid (TCA) for 20 min each wash. After washing twice in 50 ml propan-2-ol the filters were dried and placed in 5 ml aqueous scintillation fluid (Opti Fluor from Packard Instrument). The amount of ^{3}HdATP incorporated in acid-insoluble DNA was measured in a fluid scintillator (Packard). One unit was defined as the amount of DNA polymerase that incorporate 10 nanomoles of dNTPs into acid-insoluble form at 70°C in 30 min under stated assay condition.

Results and discussion

Selection of strains for screening

The bacteria used in this study were selected from the thermopile strain collection at the Technological Institute of Iceland which presently contains about 1100 strains, isolated from various geothermal areas in Iceland. They have been phenotypically and genotypically characterised to a varying degree. The *Bacillus* strains used in this work were quite diverse, but they have only been identified to the genus level. Some of the *Thermus* strains have been characterised to a species level (*T. ruber*, *T. scotoductus*, *T. brockianus*, *T. aquaticus*). At present only one species, *R. marinus*, has been described within the genus *Rhodothermus* (Alfredsson *et al.*, 1988). The *Hydrogenobacter* strains have only been identified to the genus level.

Occurrence of heat stable DNA ligases

Selection of heat stable ligases was done by heating the crude extract for 30 min at 90°C and the coagulated proteins separated by centrifugation. Out of a total of 103 strains, 23 (22%) showed thermostable ligase activity after the 90°C heating (Table 1). All of these stains were *Thermus* strains and most of them unidentified.

The *Thermus* strains were divided into three groups: the *Thermus scotoductus* group (Kristjansson *et al.*, 1994), *Thermus* strains growing >80°C and *Thermus* strains only growing <80°C. All 23 strains were tested for ligase heat tolerance by heating the crude extract further at 95°C for 5, 10, 20, 30 and 60 min. They gradually lost their activity as showed on Fig 1. The *Thermus scotoductus* group had the most thermostable ligases and the *Thermus* >80°C group showed more thermostability than the *Thermus* 80°C group. This indicates a correlation between growth temperature and stability of enzymes. However the *Hydrogenobacter* strains, which also grow at higher temperatures (72°C), showed no activity after the first heating. After heating the crude extract first for 30 min at 90°C and then for 60 min at 95°C, four strains still show DNA ligase activity. These were all purified but lost their thermostability after purification. The purification procedure needs to be developed further.

Occurrence of heat stable DNA polymerases

DNA polymerases with >10% activity remaining after heating for 30 min at 95°C were 36 (15%) out of 248 strains tested (Table 1). They were mostly *Thermus* (86%) but four from *Rhodothermus* (11%) and one from *Bacillus* (3%). By dividing the *Thermus* strains into three groups as was done in the ligase study, the *Thermus scotoductus* strains contained some heat stable DNA polymerases (Fig 2), but *Thermus* >80°C contained the most thermostable polymerases and they were much better than the *Thermus* <80°C. This is in good agreement with the results for the DNA ligases. The heat stability

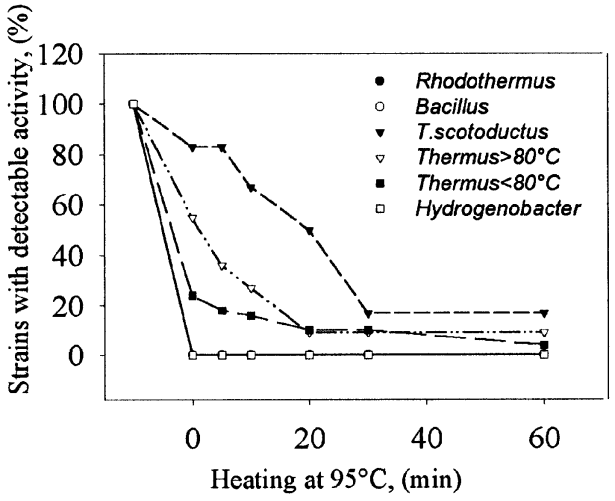

Figure 1 Thermostability of DNA ligases.

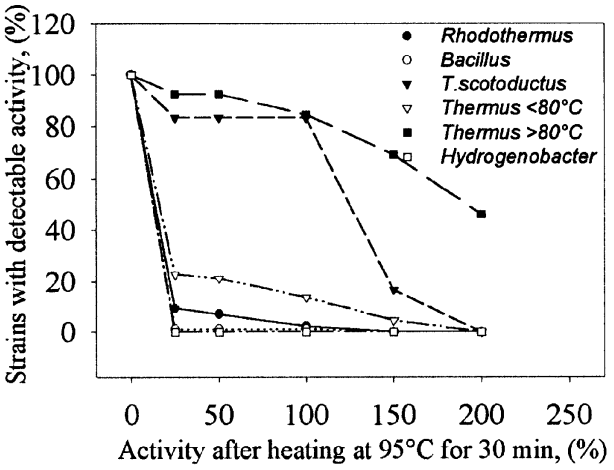

Figure 2 Thermostability of DNA polymerases.

of the enzymes from the 32 strains of *Hydrogenobacter*, was however expected to be better when compared to their high growth temperature.

Conclusion

Thermophilic microorganisms show a very different heat stability of their DNA ligases and DNA polymerases. It is not always in correlation to their growth temperatures but for *Thermus* which is clearly the genus that showed the most thermostable enzymes out of the four genera tested there is good correlation between growth temperature and thermostability of enzymes. There is also a good correlation between the two screenings since 88% of the strains that had heat stable ligases also had heat stable polymerases.

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