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REVIEW

The Linkage between Reverse Gyrase and Hyperthermophiles: A Review of Their Invariable Association

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With the discovery of reverse gyrase in 1972, from Yellowstone National Park, isolated from *Sulfolobus acidocaldarius*, it has been speculated as to why reverse gyrase can be found in all hyperthermophiles and just what exactly its role is in hyperthermophilic organisms. Hyperthermophiles have been defined as organisms with an optimal growth temperature of above 85°C. Reverse gyrase is responsible for the introduction of positive supercoils into closed circular DNA. This review of reverse gyrase in hyperthermophilic microorganisms summarizes the last two decades of research performed on hyperthermophiles and reverse gyrase in an effort to provide an up to date synopsis of their invariable association. From the data gathered for this review it is reasonable to hypothesize that reverse gyrase is closely tied to hyperthermophilic life.

***Keywords*:** thermophiles, hyperthermophiles, reverse gyrase, positive supercoiling, negative supercoiling, type I topoisomerase, helicase

Since the discovery of hyperthermophilic microorganisms in the 1960's with *Thermus aquaticus*, and the continual discovery of more than 70 species of hyperthermophiles since then, there has been much speculation and research performed to deduce just how any organism could thrive at temperatures in which most organisms could not even sustain life (Lowe *et al*., 1993, Vieille and Zeikus, 2001; Charlier and Droogmans, 2005). It has become apparent that hyperthermophilic organisms do not just sustain life at these high temperatures but that they require the high temperatures in order to maintain life (Charlier and Droogmans, 2005). Bacterial communities of hyperthermophiles are composed of primary producers and decomposers of organic matter and they all function as chemolithotrophs as well. Thermophiles have been grouped into several categories over the years but a general consensus can be agreed upon under which hyperthermophiles are classified as organisms that have an optimal growth temperature of above 85°C (Lowe *et al*., 1993; Lebedinsky *et al*., 2007; Imanaka, 2008). From the research that has been performed, a common theme has been found regarding hyperthermophiles. That theme is the presence of an enzyme called reverse gyrase (De La Tour *et al*., 1990; De La Tour *et al*., 1991; Charbonnier and Forterre, 1994; Forterre, 1996; Zierenberg *et al*., 2000; Musgrave *et al*., 2002; Atomi *et al*., 2004; Charlier and Droogmans, 2005; Brochier-Armanet and Forterre, 2006; Imanaka, 2008).

Reverse gyrase is a catalytic enzyme composed of a putative helicase and a type I topoisomerase which work together to introduce positive supercoils into DNA (De La Tour *et al*., 1991; Krah *et al*., 1997; Lopez-Garcia *et al*., 2000; Klinger *et al*., 2003; Brochier-Armanet and Forterre, 2006; Boonyaratanakornkit *et al*., 2007). The introduction of positive supercoils has been shown to increase the linkage between strands of closed circular DNA which may be the cause of DNA stabilization (Charbonnier and Forterre, 1994; Forterre, 1996; Takami *et al*., 2004; Imanaka, 2008). In addition, reverse gyrase has been shown to recruit a protein that coats nicks made in the DNA strand which is known to contribute to the stabilization of DNA at high temperatures (Kampmann and Stock, 2004). The combined actions of linkage and protein coating may lead to the increased stability of DNA in hyperthermophilic environments making reverse gyrase essential for life under these conditions. Since the beginning of experimental research involving reverse gyrase and hyperthermophiles, it has been speculated as to why reverse gyrase can be found in all hyperthermophiles and just what exactly its role is in hyperthermophilic organisms. In this article, we attempt to summarize the last two decades of research performed on hyperthermophiles and reverse gyrase in an effort to provide an up to date synopsis of their invariable association.

Hyperthermophilic growth conditions

In order to begin examining hyperthermophiles, it must first be established what defines an organism as hyperther-

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Table 1. Classification of thermophiles based on optimal growth temperature

Classification	Optimal growth temperature
Thermophile	Above 55°C
Moderate thermophile	Above 65°C
Extreme thermophile	Above 75°C
Hyperthermophile	Above 85°C

Thermophiles can be classified into distinct categories based on their optimal growth temperature (Lowe *et al*., 1993; Charlier and Droogmans, 2005; Lebedinsky *et al*., 2007; Imanaka, 2008).

mophilic. This definition has changed since their discovery, but a general consensus has been determined. Thermophiles are separated into four distinct categories based on optimal growth temperatures (Table 1).

Although it seems unlikely that there could be a diverse array of habitats able to sustain temperatures of 85°C, hyperthermophiles have been isolated from several different sources, natural and man made. Examples of these sources include sun heated soils, litter, continental and submarine volcanoes, deep sea thermal vents, sulfatara fields, geothermal power plants, hot springs, oil reservoirs, and coal piles (Madigan, 2000; Vieille and Zeikus, 2001; Lebedinsky *et al*., 2007). The most famous sources of hyperthermophilic bacteria are the hot springs of Yellowstone National Park in which several species of hyperthermophiles have been discovered (Unsworth *et al*., 2007). In the 1980's, organisms were found to be thriving at temperatures of 100°C which lead to the speculation that the upper limit for hyperthermophilic lives is higher than 100°C but lower than 250°C (Lowe *et al*., 1993; Krah *et al*., 1997; Daniel and Cowan, 2000; Vieille and Zeikus, 2001; Takami *et al*., 2004). As of yet, the highest optimal growth temperature has been found to be 122°C for the Archaeal microorganism *Methanopyrus kandleri*, strain 116, which can be isolated from deep sea hydrothermal vents (Takai *et al*., 2008).

Challenges of thermophilic life

For hyperthermophilic organisms, and thermophiles in general, it is not a matter of withstanding high temperatures but it is the requirement of high temperatures in order to maintain their life. Due to this requirement, hyperthermophiles have to be equipped in order to deal with DNA damage, DNA packaging, DNA repair, elevated mutation rates, and replication in extreme heat temperatures (Guy *et al*., 2004). Therefore, hyperthermophiles have adapted to these environments with thermostable enzymes, efficient DNA repair mechanisms, DNA modifications that increase the melting temperature and stability of the tertiary structure and the formation of unique membrane lipids which regulate membrane fluidity (Lowe *et al*., 1993; Forterre, 1996; Charlier and Droogmans, 2005). Mesophiles, including eukaryotic cells, cannot survive at temperatures above 50°C due to the instability of their cellular molecules at elevated temperatures (Zierenberg *et al*., 2000). It has been found that tertiary enzymatic structures in mesophiles cannot withstand temperatures of above 70°C, however, the enzymes present in several hyperthermophiles exhibit optimal catalytic activity at 100°C, although they exhibit catalytic activity at temperatures lower than 100°C as well (Daniel and Cowan, 2000; Zierenberg *et al*., 2000). Enzymes present in hyperthermophiles, denoted hyperthermozymes, are inactive at moderate temperatures but are able to gain activity with rising temperatures to become fully active at the optimal temperature (Unsworth *et al*., 2007).

Several theories exist as to how DNA maintains stability in hyperthermophilic environments, such as elevated salt concentrations, the presence of polyamines and cationic proteins and the unpackaging of DNA, nevertheless, it is certain that DNA stabilization is not due to the G+C content of the cell (Daniel and Cowan, 2000; Takami *et al.*, 2004; Charlier and Droogmans, 2005; Unsworth *et al*., 2007). Of particular importance for DNA stabilization is the presence of the enzyme reverse gyrase in hyperthermophiles (Guy *et al.*, 2004). Although it has been highly disputed, reverse gyrase is the only enzyme that can be found in all hyperthermophilic organisms and it is believed to be responsible for the stabilization of DNA in extreme heat environments (De La Tour *et al*., 1991; Charbonnier and Forterre, 1994; Forterre, 1996; Zierenberg *et al*., 2000; Imanaka, 2008).

Reverse gyrase and its origin

In order to better understand reverse gyrase and its role in hyperthermophilic organisms, its evolution among hyperthermophiles must be examined. Reverse gyrase can be found in both Archaea and Eubacteria which suggests that it had a common ancestor before its point of divergence (De La Tour *et al*., 1991; Musgrave *et al*., 2002). The type I topoisomerase and helicase present in reverse gyrase are members of two distinct superfamilies, both of which have different ancestral proteins, implying that the domains must have evolved separately from one another before joining together (Atomi *et al.*, 2004). Based on the previous information, researchers were able to hypothesize that reverse gyrase evolved by the fusion of the two pre-existing enzymes, type I topoisomerase and helicase, in microorganisms (Musgrave *et al*., 2002). In 2005, research performed by Omelchenko *et al.* (2005) showed that the thermophilic microorganism *Thermus thermophilus* carries a pseudogene for reverse gyrase. This finding along with the presence of reverse gyrase in some thermophilic organisms suggests that reverse gyrase originated in thermophilic organisms and was then promoted from thermophiles to hyperthermophiles. The fact that, reverse gyrase can be found in all hyperthermophilic organisms indicates that the origin of reverse gyrase may have coincided with the appearance of the first hyperthermophilic organisms. Therefore, the acquisition of reverse gyrase may have been an evolutionary breakthrough in the adaptation of thermophiles to hyperthermophilic environments (Brochier-Armanet and Forterre, 2006).

It appears that reverse gyrase evolved separately between Bacterial hyperthermophiles and Archaeal hyperthermophiles. This is suspected due to the three clades present in Fig. 1; one clade represents Bacterial hyperthermophiles while the other two clades represent Archaeal hyperthermophiles. It is also apparent, from the two separate Archaeal clades, that reverse gyrase evolved independently within Archaeal hyperthermophiles. Regardless of how reverse gyrase evolved within and between Archaeal and Bacterial hyperthermo-

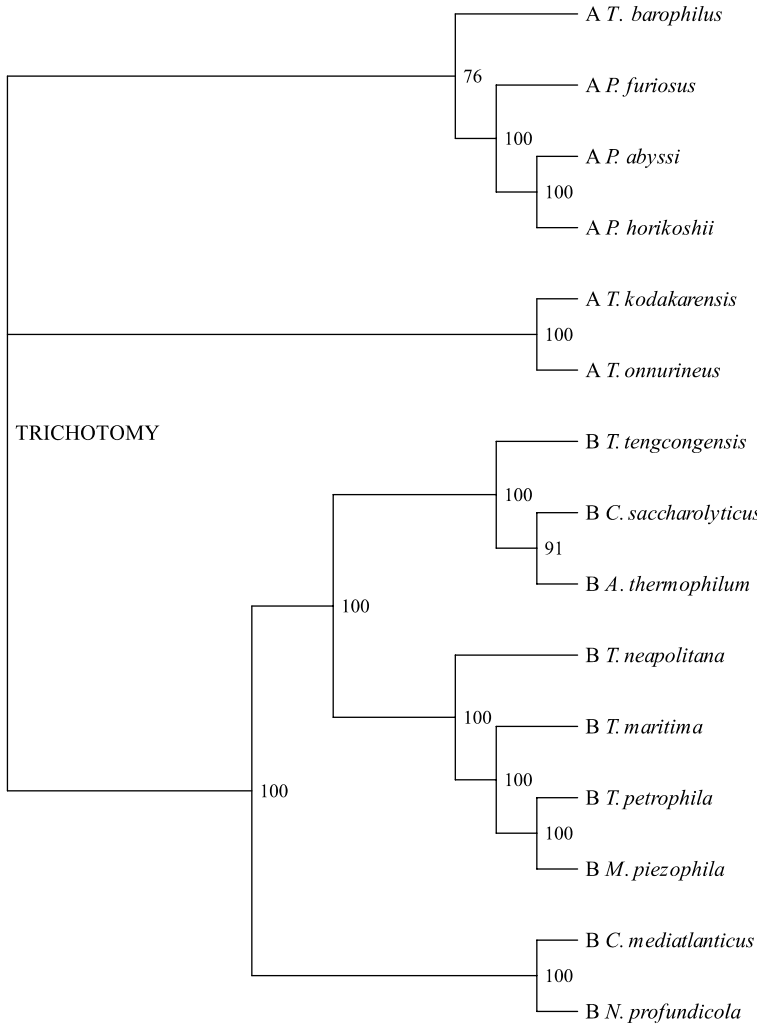

Fig. 1. A Phylogenetic tree constructed from of 15 reverse gyrase amino acid sequences consisting of 6 hyperthermophilic Archaeal and 9 hyperthermophilic Bacterial species. The phylogenetic analysis was carried out using the neighbor-joining algorithm in CLUSTAL X. The capital letter (appears before the genus name) A represents Archaea, B represents Bacteria. The following are the complete genus, species and accession numbers for the organisms included in the tree: *Thermococcus barophiluis* (YP_002579738.1), *Pyrococcus furiosus* (NP_578224.1), *Pyrococcus abyssi* (NP_126943.1), *Pyrococcus horikoshii* (NP_142736.1), *Thermococcus kodakarensis* (BAD26706.1), *Thermococcus onnurineus* (YP_002306713.1), *Thermoanaerobacter tengcongensis* (AAM24939.1), *Caldicellulosiruptor saccharolyticus* (ABP67172.1), *Anaerocellum thermophilum* (ACM60232.1), *Thermotoga neapolitana* (CAI44249.1), *Thermotoga maritima* (NP_227988.1), *Thermotoga petrophila* (ABQ46771.1), *Marinitoga piezophila* (EEB81126.1), *Caminibacter mediatlanticus* (ZP_01870961.1), *Nautilia profundicola* (YP_002607471.1).

philes, the large bootstrap values at the nodes of the clades indicate that the protein sequence of reverse gyrase is highly conserved among hyperthermophiles.

Reverse gyrase was first isolated in 1972 from the hyperthermophilic microorganism *Sulfolobus acidocaldarius*, which was found in the hot springs of Yellowstone National Park, and is the only known topoisomerase to exhibit reverse gyration (De La Tour *et al*., 1990, 1991, 1998; Brochier-Armanet and Forterre, 2006; Unsworth *et al*., 2007). Sequence analysis of reverse gyrase reveals that it exists as a single polypeptide that is linked to DNA by a 5' phosphotyrosol bond with putative helicase and type I toposiomerase domains located at the amino and carboxy terminals respectively (De La Tour *et al*., 1998; Musgrave *et al*., 2002; Charlier and Droogmans, 2005; Brochier-Armanet and Forterre, 2006). An ATP binding site along with nucleotide binding properties are found at the amino terminal corresponding to the helicase location (Confalonieri *et al*., 1993; Champoux, 2001; Del Toro Duany *et al*., 2008). The concerted action of the two domains with the addition of ATP and Mg^{2+} is the mechanism by which reverse gyrase introduces positive supercoils into closed circular DNA (De La Tour *et al*., 1991; Forterre, 1996; Zierenberg *et al*., 2000; Musgrave *et al*., 2002; Imanaka 2008). Reverse gyrase begins by making a single stranded break in the closed circular DNA and attaches itself to the 5' end of the broken DNA (Confalonieri *et al*., 1993; Kozyavkin *et al*., 1994; Champoux, 2001). It has been proposed that the helicase domain unwinds the DNA helix as it moves along the DNA strand creating positive supercoils in front of it and negative supercoils behind it. The topoisomerase domain

moves along the DNA behind the helicase domain and removes the negative supercoils that were just introduced, leaving the DNA with net positive supercoiling (Champoux, 2001). The introduction of positive supercoils leaves the circular DNA in what is called a relaxed state or a positively supercoiled state, which differs from that of mesophiles and thermophiles which are in a negatively supercoiled state (Charbonnier and Forterre, 1994; Lopez-Garcia *et al*., 2000; Charlier and Droogmans, 2005). In addition, it has been shown that the topoisomerase domain exhibits an inhibitory effect on the helicase domain of reverse gyrase. It is believed that this inhibitory effect permits inter-domain communication between the topoisomerase and helicase domains allowing for efficient coupling of ATP hydrolysis by the helicase and DNA supercoiling by the topoisomerase (Del Toro Duany *et al*., 2008; Valenti *et al*., 2008).

Positive DNA supercoiling increases the amount of links that can be found between the two strands of closed DNA, suggesting a more stabilized DNA conformation that can maintain the biological function of an organism in hyperthermophilic environments (Forterre, 1996; Lopez-Garcia and Forterre, 1999; Imanaka, 2008). As predicted, the percentage of linkage in plasmids isolated from hyperthermophiles is greater than that of plasmids isolated from mesophilic bacteria and archaea (Forterre, 1996; Lopez-Garcia and Forterre, 1999). Furthermore, an excess of DNA linkage has proven to be a novel feature of hyperthermophiles probably related to the presence of the type I topoisomerase of reverse gyrase (Lopez-Garcia and Forterre, 1997). In 2006 research performed by Hsieh and Plank demonstrated that reverse gyrase can act to renature DNA by annealing complementary single stranded DNA circles as well as positively supercoiled DNA that contains a single stranded bubble. The annealing of these two types on DNA creates an increase in DNA linkage (Hsieh and Plank, 2006). In addition, reverse gyrase activity has not been found to exist among mesophiles and is only very rarely found to exist among thermophiles (De La Tour *et al.*, 1990; Brochier-Armanet and Forterre, 2006; Imanaka, 2008).

Research performed by Kampmann and Stock in 2004 revealed an alternative mode of reverse gyrase DNA protection that was independent of DNA positive supercoiling. They were able to show that reverse gyrase recruits a protein that coats nicks in the DNA backbone at a rate that corresponds with DNA breakage. This protein is believed to hold the DNA backbone together until cellular mechanisms can make the necessary repairs. Through their experiments it was shown that in the absence of reverse gyrase, cellular DNA is completely degraded, whereas in the presence of reverse gyrase a fraction of intact DNA can be recovered. The rate of double stranded DNA breakage is reduced eight-fold in the presence of reverse gyrase. It was also shown that the thermoprotective activity of reverse gyrase is independent of supercoiling because linear as well as closed circular DNA was protected in the absence of ATP. Kinetic studies indicate that reverse gyrase is capable of inhibiting one or more steps in the thermodegradation pathway (Kampmann and Stock, 2004). Therefore, it can be said that reverse gyrase protects DNA from damage in extreme heat conditions due to heat-protective DNA chaperone activity as well as DNA renaturase activity (Del Toro Duany *et al*., 2008). With an optimal temperature of activity at 85°C and the previously described findings, there exists the strong suggestion that reverse gyrase activity must be a requirement for life under hyperthermophilic conditions but not necessarily the presence of positive supercoiling (De La Tour *et al*., 1990; Forterre, 1996; De La Tour *et al.*, 1998; Brochier-Armanet and Forterre, 2006).

The existence of negatively supercoiled DNA in hyperthermophiles

Although, reverse gyrase has been found to exist in all hyperthermophilic microorganisms, the same can not be said for the presence of positive DNA supercoiling (De La Tour *et al*., 1990; Zierenberg *et al*., 2000; Atomi *et al*., 2004; Charlier and Droogmans, 2005; Brochier-Armanet and Forterre, 2006; Imanaka, 2008). Initially, it was presumed that reverse gyrase was unique to Archaea and could not be found in Eubacteria, however, further research proved that reverse gyrase can be found in all hyperthermophilic organisms, sometimes independent of positive supercoiling (De La Tour *et al*., 1991; Lopez-Garcia *et al*., 2000). Gel electrophoresis research performed by Guipaud *et al*. in 1997 on the organism *Thermotoga maritima* revealed that this hyperthermophilic microorganism expresses DNA that is in the negatively supercoiled state even though reverse gyrase is present in the organism (De La Tour *et al*., 1998). In addition, this was the first experimental research performed wherein DNA gyrase, which is responsible for the introduction of negative supercoils into DNA, was found to exist in a hyperthermophilic microorganism (Guipaud *et al*., 1997). Further research performed in 1998 by De La Tour *et al*. demonstrated that *T. maritima* contains three different topoisomerases including reverse gyrase and gyrase (Lopez-Garcia *et al*., 2000).

These findings lead researchers toward the formulation of two postulates in order to explain this rare phenomenon. The first postulate hypothesized that the presence of gyrase dominates over the presence of reverse gyrase, which would produce negatively supercoiled DNA despite the existence of reverse gyrase in the cell. The second postulate hypothesized that the relaxed to positively supercoiled DNA state is a characteristic of specific hyperthermophilic Archaea. Research performed by Lopez-Garcia *et al.* in 2000 on the negatively supercoiled pGS5 plasmid isolated from the hyperthermophilic Archaeon, *Archaeoglobus profundus*, suggests that gyrase activity predominates over reverse gyrase activity whenever both topoisomerases are found to exist in cells. Therefore, although the DNA was not found to be in a relaxed or positively supercoiled state, the presence of reverse gyrase was still found to exist in hyperthermophilic microorganisms. This strongly supports the suggestion that reverse gyrase is a requirement for life under hyperthermophilic conditions.

Deletion of reverse gyrase from hyperthermophilic life

In an effort to determine whether reverse gyrase is essential for life under extreme heat conditions researchers sought a way to remove reverse gyrase from a hyperthermophilic ge-

nome and determine the microorganism's capability of existence in the absence of reverse gyrase. Research performed by Atomi *et al*. in 2004 tested the hypothesis that reverse gyrase is a requirement for life under hyperthermophilic conditions (Charlier and Droogmans, 2005; Brochier-Armanet and Forterre, 2006; Imanaka, 2008). Atomi accomplished this by disrupting reverse gyrase from the hyperthermophilic Archaeon, *Thermococcus kodakarensis*, resulting in the complete deletion of the reverse gyrase gene from the genome. The reverse gyrase gene was replaced with an auxotrophic tryptophan gene which was then used as a marker for Southern blot analyses. Experiments performed with the knock out mutant strain of *T. kodakarensis* were aimed at determining the ability of a hyperthermophilic organism to grow under extreme heat conditions without the presence of reverse gyrase.

To confirm the removal of the reverse gyrase gene, DNA supercoiling activity was monitored between the mutant strain and the host strain of *T. kodakarensis* through partial cell fractionation. In the presence of ATP, the wild type strain exhibited a significant amount of DNA supercoiling and relaxation, indicating the presence of reverse gyrase. In contrast, no observable amount of DNA supercoiling or relaxation was observed in the mutant strain. These results indicate that reverse gyrase was successfully removed from the mutant strain. The growth of the knock out mutant *T. kodakarensis* was then compared to the wild type strain under varying incubation conditions to determine the growth characteristics of the mutant strain. It was found that hyperthermophilic growth of *T. kodakarensis* was not inhibited at 90°C with the deletion of the reverse gyrase gene (Atomi *et al*., 2004). These results would indicate that life is possible under hyperthermophilic environmental conditions without the presence of reverse gyrase in the genome.

However, these results are somewhat misleading and need further interpretation. Growth was significantly reduced in the mutant strain, as incubation temperatures were increased from 60°C to 93°C, with a more marked growth reduction in the higher temperatures. Although the mutant strain was able to grow at 90°C, the growth observed was very poor when compared to that of the wild type strain. In addition, no growth of the mutant strain was observed at temperatures above 90°C in contrast to the wild type strain which continued to express growth up to 100°C (Atomi *et al*., 2004; Brochier-Armanet and Forterre, 2006; Imanaka, 2008). As suggested by Imanaka in 2008, these results imply that reverse gyrase is essential for the stabilization of chromosomal DNA at hyperthermophilic temperatures.

Conclusion

Is reverse gyrase a prerequisite for hyperthermophilic life

The question, is reverse gyrase a prerequisite for hyperthermophilic life, yet remains. With an optimal temperature of activity the same as the temperature that qualifies life as hyperthermophilic indicates that the enzyme reverse gyrase seems to be made specifically for hyperthermophilic conditions. In addition, its presence in all forms of hyperthermophiles strengthens the hypothesis that it is a prerequisite for hyperthermophilic life. The research performed by Atomi *et al*. in 2004, in which the reverse gyrase gene was deleted from the genome of *T. kodakarensis* proves that although hyperthermophiles can exist at high temperatures without the presence of reverse gyrase, they do not thrive but instead they barely sustain life. This signifies that in order for organisms to flourish, as they do in their natural habitats, reverse gyrase must be present in the cell. The research performed by Kampmann and Stock in 2004 provides a unique platform for the stabilization of DNA under hyperthermophilic environmental conditions. The ability of reverse gyrase to coat nicks in double stranded DNA along with an excess in DNA linkage provided by reverse gyrase provides strong evidence that reverse gyrase plays an essential role in hyperthermophilic life. From an evolutionary standpoint it appears that without reverse gyrase, thermophilic life would not have been granted the promotion to hyperthermophilic life.

It remains yet unclear as to why reverse gyrase is so important to hyperthermophiles but further research could lead to a definite explanation. It appears that the linkage between two strands of DNA created by reverse gyrase could be essential to its function in the cell but that is yet to be tested. Future research involving hyperthermophiles that posses negatively supercoiled DNA could be helpful in deducing the role of linkage in hyperthermophilic microorganisms. In addition, more research concerning the protein coat provided by reverse gyrase is necessary to determine under what conditions this coat is applied and if it is necessary for DNA stabilization. In any case, the more existence of organisms that succeed in temperatures where most could not even sustain life is evidence enough that hyperthermophiles are extraordinary topics of research.

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