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Extremophiles and their application to veterinary medicine

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Extremophiles are organisms that can grow and thrive in harsh conditions, e.g., extremes of temperature, pH, salinity, radiation, pressure and oxygen tension. Thermophilic, halophilic and radiation-resistant organisms are all microbes, some of which are able to withstand multiple extremes. Psychrophiles, or cold-loving organisms, include not only microbes, but fish that live in polar waters and animals that can withstand freezing. Extremophiles are structurally adapted at a molecular level to withstand these conditions. Thermophiles have particularly stable proteins and cell membranes, psychrophiles have flexible cellular proteins and membranes and/or antifreeze proteins, salt-resistant halophiles contain compatible solutes or high concentrations of inorganic ions, and acidophiles and alkaliphiles are able to pump ions to keep their internal pH close to neutrality. Their interest to veterinary medicine resides in their capacity to be pathogenic, and as sources of enzymes and other molecules for diagnostic and pharmaceutical purposes. In particular, thermostable DNA polymerases are a mainstay of PCR-based diagnostics.

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Introduction

The term extremophile (lover of extremes) was coined over a quarter of a century ago (MacElroy, 1974). The word has been interpreted in a number of ways and, perhaps understandably, has become associated with those microorganisms that inhabit environments unsuitable to mammals. Conditions that are 'extreme' to one organism may be essential to another's survival, so the concept of extremophily is a relative one. Extremophiles span all three domains of life: bacteria, archaea and eukaryotes. They also include commensal microorganisms that are of fundamental significance to nutrition. A more complex form of extremophile, which will not be considered here, is the multi-stage parasite.There are many examples of these animals which, by rotating through several forms, suit themselves to multiple environments. Extremophiles are important not only because of what they can teach us about the fundamentals of biochemical and structural biodiversity but, also, because of their enormous potential as sources of enzymes and other biological materials with applications in biotechnology and medicine, both human and veterinary. Their unusual properties make them key targets for exploitation by biotech companies around the world.

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Temperature and life

The realm of extremophiles is remarkably diverse (see Table 1). Some extremophiles are adapted to temperature extremes. These include the thermophiles (or heat lovers), the hyperthermophiles (comprising archaea and bacteria that grow at temperatures above 80°C) and the cold-tolerant psychrophiles, which not only include microbes, but protoctists, algae, and some vertebrates like the Antarctic icefish, *Chaenocephalus aceratus.*

The greatest thermal extremes for growth occur among the archaea. *Pyrolobus fumarii* holds the record at present for the most thermophilic microorganism. It lives in the walls of 'black smoker' hydrothermal vent chimneys, with an upper temperature limit for growth of 113°C, and it cannot grow below 90°C. The organism

TABLE 1: Glossary

Environmental parameter	Description
Temperature	
Psychrophile	Optimal growth temperature ≤15°C, maximum ~ 20°C, minimum ≥ 0°C
Psychrotroph	Can grow at or below 5°C, maximum 25°C – 30°C
Mesophile	Optimal growth temperature approx. 37°C, often grows from 8°C or 10°C to 45°C or 50°C
Thermophile	Grows at > 50°C
Hyperthermophile	Grows at > 80°C
pH	
Acidophile	Grows at pH < 2
Alkaliphile	Grows at pH > 10
Salinity	
Halophile	Extreme halophiles (archaea) need 2.5 M – 5.0 M salt for growth. Moderate halophiles will grow in up to 15-20% NaCl
Oxygen tension	
Aerobes	Grow in ≥ 21% oxygen (level present in air)
Anaerobes	Grow only in the absence of oxygen
Microanaerobes	Grow at low levels of oxygen (not in air)
Facultative anaerobes	Can grow aerobically or anaerobically
Hydrostatic pressure	
Piezophiles	Obligate piezophiles do not grow at atmospheric pressure (1 atmosphere, 0.1 MPa). Piezotolerant bacteria grow at 1 atmosphere but will also grow at high pressures (up to 130 MPa)
Chemical extremes	
	Some bacteria are metal-tolerant, others can tolerate high levels of gases (e.g., carbon dioxide)

has novel metabolic properties and can survive autoclaving for one hour at 121°C (Blochl *et al.*, 1997). An upper temperature limit for survival is imposed by the denaturation of proteins and nucleic acids, as well as increased membrane fluidity with rising temperature. Archaea have unique membrane lipids with ether linkages and the hydrocarbon chains are polymers based on the monomer isoprene. They contain tetraethers and diethers, which are likely to be a major contributory factor in the ability of hyperthermophiles to withstand temperatures that would destroy membranes composed of a bilayer structure based on fatty acids and glycerol (Bullock, 2000). In addition, they are surrounded by a surface (S)-layer of glycoproteins, which help archaea to maintain structural integrity under extremes of temperature, pH, or salinity (Eichler, 2003). Thermophiles are more common than hyperthermophiles, and these include a wide range of microorganisms, including photosynthetic bacteria, various eubacteria (e.g., *Bacillus, Clostridium, Thiobacillus, Thermus* spp., lactic acid bacteria, actinomycetes, spirochetes, and many other genera) and numerous archaea. At the other end of the thermal spectrum, the lower temperature limit found so far for active microbial communities is -20°C in the liquid phase of sea ice (Deming, 2002), although bacteria survive under storage in liquid nitrogen at –196°C. Cold-adapted microbes, known as psychrophiles, have colonised the low-temperature habitats that cover most of the planet, including polar regions, mountains, and deep-sea waters. True psychrophiles have optimum growth temperatures of 15°C or less: psychrotrophs are more thermally adaptable and can grow at relatively low temperatures or at temperatures more suitable to mesophilic microorganisms (see Table 1).

Extremes of pH

Acidophiles and alkaliphiles thrive under conditions of low and high pH, respectively. *Helicobacter pylori*, which is associated with peptic ulcer disease in humans and animals, is not actually acidophilic but 'acid tolerant' due to its secretion of urease, which produces ammonium ions that buffer the hydrochloric acid. Truly acidophilic species such as *Picrophilus oshimae* grow optimally at pH values as low as 0.7 (Schleper et al., 1995). At the other extreme, alkaliphilic microbes that thrive at pH 10 to 11 are often found in soda lakes. These extremophiles maintain their internal pH at a value close to neutral by pumping hydrogen ions through their cell membranes. Some organisms are multiextremophiles, adapted to more than one type of extreme environment, such as the archaeon *Sulfolobus acidocaldarius*, which grows at pH 3 and 80°C. Acidic mine waters are a rich source of thermophilic acidophiles, some of which can oxidise iron (e.g., *Leptospirillum ferrooxidans*) and others can reduce iron (e.g., *Acidiphilum* spp.; Johnson and Hallberg, 2003). Thermophilic alkalitolerant bacteria have also been isolated, e.g., *Anaerobranca* spp. (Engle *et al.*, 1995), as well as haloalkaliphiles, which grow in high salt and high pH environments.

Salt and pressure

There are few organisms that can tolerate very high salt concentrations, such as those found in salt flats and marine hypersaline basins. For example, the Dead Sea, which contains about 30% salts, contains some halophilic archaea, the *Halobacteriaceae,* and one halophilic species of alga, *Dunaliella salina*. At lower salinities, bacteria, cyanobacteria, some green algae and unicellular eukaryotes can survive. Piezophiles, or pressure-lovers, can exist comfortably at depths of 10,500m under the ocean and tolerate pressures of up to 130MPa. Some of these are obligate piezophiles, which cannot grow at atmospheric pressure; other high-pressure habitats, such as the Marianas Trench, the deepest sea floor on the planet at 10,898m, contain microbes that are viable at atmospheric pressure. Bacteria have also been cultured from rock samples taken from depths of up to 4,000m (Gold, 1992).

Radiation and heavy metal-resistant organisms

Environmental extremes include high levels of ionising radiation. This affects cells by damaging DNA directly or by producing reactive oxygen radicals that can cause mutations in DNA or break the strands. However, some organisms can survive these conditions. The bacterium *Deinococcus radiodurans* has been isolated from the environs of nuclear reactors and can survive up to 20,000Gy of gamma radiation, enough to split its genome into small fragments. An extremely efficient DNA repair mechanism enables it to reassemble the fragmented DNA in a relatively short time (Battista, 1997).

Another remarkable illustration of radiation resistance was the survival of the bacterium *Streptococcus mitis* on a piece of foam in a television camera that had been left on the moon for almost three years and was brought back under sterile conditions by the crew of Apollo 12 in 1969. This microorganism was still culturable, despite exposure to vacuum, high radiation, and freezing temperatures in the absence of water or nutrients.

Other bacteria in the environment (e.g., *Ralstonia* sp. CH34) can tolerate concentrations of heavy metals lethal to other microbes and, consequently, may play a vital role in environmental remediation after heavy metal contamination (Nies, 2000). Even processed food does not escape from extremophile invasion. Osmophiles, which live in environments with a high concentration of sugar, are found in foods such as fruit cake, jams, cereals and dried fruit. These include some yeasts and other fungi.

Oxygen tension

The ability of organisms, particularly microbes, to adapt to aerobic environments is varied. Some microbes are obligate anaerobes, which do not have the ability to survive in aerobic environments: others are microaerophiles, which can survive at low oxygen tension but not in air, which contains 21% oxygen. Aerobic metabolism is more efficient than anaerobic metabolism, but metabolic reactions generate reactive oxygen species that can damage cells. Anaerobes have an impaired ability to detoxify and remove these damaging free radicals, confining them to anaerobic habitats.

Multicellular extremophiles

Eukaryotes cannot approach the thermal extremes tolerated by thermophilic microorganisms. Their upper limit for survival is about 60°C. However, some larger animals can tolerate temperatures below 0°C, due to various physiological adaptations. Some psychrophiles protect themselves against the formation of ice crystals, which destroy cell membranes, by secreting 'antifreeze' or cryoprotectant molecules, which lower the freezing point of water inside the cell. This strategy is employed by fish in Antarctic seas, which live at temperatures close to –1.8°C, the freezing point of seawater. It has also been reported in some insects, plants, fungi and microorganisms (Barrett, 2001). These proteins work by adsorbing to ice microcrystals, preventing the association of additional water molecules and preventing crystal growth (DeVries, 1988). Cells can also be protected by freezing of extracellular water during winter, and this has been observed in some species of frogs, turtles and even in one species of snake (Storey and Storey, 1996). However, the most resilient multicellular organism encountered so far is the tardigrade, or 'water bear'. This organism can go into a hibernation, or 'tun' state, in which it can survive temperatures from –253°C to 151°C, x-ray and vacuum exposure, and pressures in perfluorocarbon fluid of up to 600 MPa, 6,000 times greater than that at sea level (Seki and Toyoshima, 1998).

How do extremophiles adapt at a molecular level?

Temperature affects protein structure and function (Fields, 2001). A comparison of enzyme structures and amino acid sequences across the entire thermal range has shown certain conserved strategies for maintaining optimal stability and activity under different conditions. Methods employed by proteins to cope with high temperatures include increased ionic interactions and hydrogen bonds, increased hydrophobicity, decreased flexibility at room temperature, and smaller surface loops. The enzyme glutamate dehydrogenase provides a striking example of this. A comparison of the *Pyrococcus furiosus* glutamate dehydrogenase with the same enzyme from mesophiles revealed that the hyperthermophilic enzyme contained a sequence of ion-pairs that were formed by regions of the protein containing a high density of charged residues, but these were absent in the mesophilic enzymes. The ion-pair networks formed clusters at the inter-domain and inter-subunit surfaces (Rice *et al.*, 1996).Hyperthermophile proteins have very high temperature optima, higher than their growth temperatures. For example, a hyperthermophilic starch-degrading enzyme, amylopullanase, wasisolated and showed activity up to 142°C (Schuliger *et al.*, 1993). Chaperone proteins, which assist in protein folding, are particularly important in thermophiles. In *Pyrodictium occultum* grown at 108°C, 2°C below its upper growth limit, 80% of the soluble protein consists of a chaperone protein complex called the thermosome, which maintains the other cellular proteins in a functional conformation (Minuth et al., 1998).

At low temperatures, proteins tend to become less rigid, implying that increases in flexibility can increase function. This is indeed observed in psychrophilic proteins, which tend to show decreases in ionic interactions and hydrogen bonds, fewer hydrophobic groups and more charged groups on the protein surface, and longer surface loops (Feller and Gerday, 1997). Cold adaptation is accomplished by regulation of membrane fluidity by increasing the proportion of unsaturated fatty acids, the synthesis of cold-shock and antifreeze proteins, the regulation of ion channel permeability, alterations in enzyme kinetics that make the enzymes more efficient, and stabilising the polymerisation of microtubules (Georlette *et al.*, 2004).

Halophile proteins employ other adaptations, notably an excess of negatively-charged amino acids (glutamate and aspartate) on the protein surface. The surface binds hydrated ions and reduces the surface hydrophobicity, decreasing the tendency to aggregate at high salt concentrations (Hough and Danson, 1999). Halophiles respond to increases in osmotic pressure in different ways. The extremely halophilic archaea, the Halobacteriaceae, accumulate

TABLE 2: Applications of extremophiles in biotechnology, medicine and industry

Source	**Biomolecule**	**Process**
Thermophiles	DNA polymerase	Polymerase chain reaction (PCR) (diagnostic)
Thermophiles Psychrophiles	proteases	food processing (baking, brewing) cheese making and dairy production
Thermophiles	α-amylase	paper bleaching
Alkaliphiles Psychrophiles	Proteases, cellulases, amylases, lipases	detergents – polymer breakdown
Alkaliphiles	antibiotics	treatment of infections
Psychrophiles	unsaturated fatty acids	food supplement
Psychrophiles	dehydrogenases	biosensors
Halophiles	compatible solutes glycerol	pharmaceuticals
Halophiles	carotene	food additive
Psychrophiles		bioremediation of oil spills
Radiation-resistant		bioremediation of radioactive waste

K+. Other bacteria accumulate compatible solutes (e.g., glycine betaine, sugars, polyols, amino acids and ectoines), helping them to maintain an environment isotonic with the growth medium. These substances also help to protect cells against stresses like high temperature, desiccation and freezing (da Costa *et al*., 1998).

Acidophiles and alkaliphiles use proton pumps to keep their internal pH values close to neutral, but how their extracellular proteins operate at pH extremes is, as yet, poorly understood. Alkaliphiles have negatively charged cell-wall polymers in addition to peptidoglycan (Aono and Horikoshi, 1983) and these may reduce the pH value at the cell surface, which helps to stabilise the cell membrane. Acidophiles employ a range of mechanisms to enablethem to withstand low pH, including a positively charged surface (Kar and Dasgupta, 1996), high internal buffer capacity, overexpression of H+ export enzymes, and unique transpor ters (Rothschild and Mancinelli, 2001). Piezophile adaptations are as yet relatively unknown but, in some of these organisms, gene expression is known to be pressure-regulated (Abe and Horikoshi, 2001).

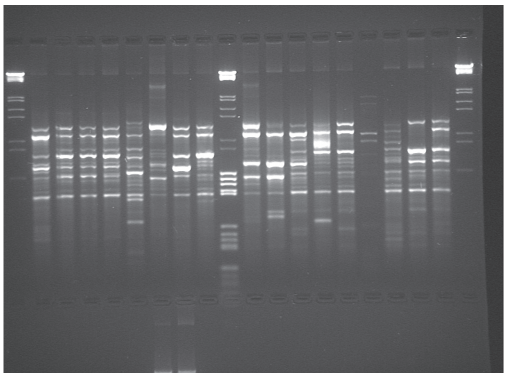
The interactions of extremophilic microorganisms with animals

Chemical extremes are common in nature. Anaerobes such as the archaeon *Methanococcus jannaschii* cannot tolerate oxygen, whereas microaerophiles like *Clostridium* sp. can tolerate low levels of oxygen. Anaerobic systems are of crucial importance to survival in many animals. In ruminants, the anaerobic environment within the rumen allows many fermentative reactions to proceed, includingmethanogenesis, which allows ruminants to survive on fibrous vegetation. The colon in monogastric species is also a largely anaerobic environment and plays host to hundreds of microbial species, many of which are oxygen intolerant. The ability to adapt to low oxygen tension is critical for some pathogens. Equine clostridial enterocolitis is being recognized with increasing frequency (Jones, 2000). *Mycobacterium tuberculosis* cells adapted to microaerophilic conditions survive for long periods without actively multiplying, and can tolerate higher doses of anti-tuberculosis antibiotics (Usha *et al*., 2002). In some cases, even thermophiles can be pathogens. *Mycobacterium xenopi*, a thermophilic bacterium, was found in a hospital's hot water system and three out of 87 patients exposed to this microorganism developed pulmonary mycobacteriosis (Lavy *et al*., 1992).

Exploitation of extremophile adaptation

Given this amazing structural and metabolic diversity, it is hardly surprising that these organisms are being exploited for a wide

Old Faithful in Yellowstone National Park, Wyoming, USA, where Taq DNA polymerase was first discovered.



Thermostable DNA polymerases are a mainstay of PCR-based diagnostics.

selection of applications (Table 2). So why are they of interest to veterinary medicine?

The term extremozyme refers to enzymes from extremophilic microorganisms (Hough and Danson, 1999). One of the prototypes of these biologically active proteins that has found its way into common everyday use in diagnostic and research laboratories worldwide is *Taq* DNA polymerase. This enzyme was originally isolated from the bacterium *Thermus aquaticus* (hence the name *Taq*). It was originally discovered in thermal springs in Yellowstone National Park,Wyoming (Brock and Freeze, 1969), but has since been isolated from many environments, including hot tap water. This enzyme is heat-stable at 95°C, ideal for use in the polymerase chain reaction (PCR), in which the DNA to be amplified is heated to denature and separate the strands prior to amplification by the polymerase. *Taq's* heat stability makes such reactions efficient enough to be possible on a routine basis, by reducing the need for adding extra polymerase during the reaction. More recently, other thermostable polymerases, each with different advantages for different PCR techniques, have become available. One of these, *Pfu* polymerase (isolated from the hyperthermophile *Pyrococcus furiosus*) has a higher replication fidelity than Taq.

PCR techniques are constantly being refined and are increasingly applied in veterinary research and diagnostics.The technology can be used to diagnose a wide range of viral (Belak and Thoren, 2001) and bacterial diseases in animals. Such assays allow quantification of viruses at levels where they would be undetectable by other techniques, as well as molecular genotyping of different strains. Diagnostic applications of PCR abound. These include quantification of feline herpesvirus 1 DNA in ocular fluid by real-time Taqman PCR (Vogtlin *et al.*, 2002), diagnosis of *Mycobacterium bovis* infection in calves sensitised by other mycobacteria (Amadori et al., 2002) and a multiplex PCR assay for detecting *Brucella* spp. and *Leptospira* spp. DNA in aborted bovine foetuses (Richtzenhain et al., 2002). In Ireland, sheep breeds are genotyped to determine whether they carry particular polymorphisms in the prion protein gene that confer susceptibility to scrapie (O'Doherty et al., 2001).

The untapped potential of extremophiles

The study of extremophiles has added greatly to our understanding of protein folding, structure and function. Efforts to discover more 'extreme' extremophiles have led to the discovery of many new microorganisms, increasing the phylogenetic data available, and have contributed enormously to our appreciation of the ways that living organisms evolve to meet an evolutionary challenge at the molecular level. However, in the commercial world, extremophile products have potential applications in many areas. The enzymes can be derived directly from the extremophiles themselves, or their structures can be compared to those of the homologous enzyme from a mesophilic organism and used as a basis to produce engineered enzymes with different thermostabilities or different catalytic properties appropriate for industrial use. One drawback of extremophile production is the difficulty of cultivating them in laboratory and industrial environments, and much work is ongoing into developing methods whereby thermophilic and hyperthermophilic organisms can be cultivated. Genes from thermophiles, hyperthermophiles and psychrophiles have been expressed successfully in a mesophilic host such as *E. coli* and, for thermophiles, heat precipitation of host cell protein has been used to purify the expressed protein. Continuous fermentation bioprocesses and different media have been explored to develop conditions that allow extremophilic microorganisms to thrive. A major challenge to extremophile exploitation is the development of bioreactors composed of materials that withstand the extremes of temperature, pH, and salinity that are required for extremophile cultivation (Schiraldi and DeRosa, 2002).

Both extremophile-derived enzymes and the whole microorganism are exploitable. Thermophiles have yielded stable a-amylases for starch hydrolysis, xylanases for paper bleaching, and proteases for food processing, baking, brewing, and for laundry detergents. Cellulases can be used for treatment of juices, colour brightening in detergents, and treating cellulose-containing biomass and forage crops to improve their digestibility and nutritional quality (Niehaus *et al.,* 1999). Alkaliphilic *Bacillus* strains have been isolated to produce enzymes appropriate for laundry and dishwashing detergents (Ito *et al.*, 1998), so that even cleaning can be enhanced by the products of extremophiles. Fabric processing can also benefit from extremophile enzymes: a thermo-stable and alkali-stable protease was used to improve wool properties and improve dye penetration (Schumacher *et al.,* 2001).

Psychrophiles are potential sources of enzymes suitable for food processing at low temperatures and for use in biosensors, as well as polyunsaturated fatty acids for the pharmaceutical industry, while halophiles are being explored as sources of carotene, compatible solutes and glycerol, and surfactants for pharmaceuticals (Rothschild and Mancinelli, 2001). Antarctic bacteria may be used in bioremediation of cold ocean waters after oil spills (Nichols *et al.*, 1999).

Extremophilic microorganisms may also comprise a largely untapped reservoir of novel therapeutic agents. For example, extremophile extracts were found to have activity against some *Candida* and *Aspergillus* spp., and an iron-binding antifungal compound called pyochelin was isolated from a novel species of *Pseudomonas* (Phoebe *et al.*, 2001). Dried *Dunaliella*, a halophile, is being investigated as a food supplement with antioxidant properties, and antifreeze proteins show potential as cryoprotectants for frozen organs (Rothschild and Mancinelli, 2001).

Unfortunately, not all potential applications of extremophiles are so benign. Engineered extremophiles may be targeted for development as biowarfare agents. *Deinococcus radiodurans*, for example, is being developed for biotechnology, using a combination of genetic engineering and genomic informatics (Daly, 2001). This bacterium is also being engineered for remediation of radioactive waste (Brim *et al.*, 2000).

Conclusions

From household detergents to molecular diagnostics, extremophiles or their products have revolutionised much of our domestic and working lives. It is not unlikely that further new and medically applicable discoveries will be made in the field of extremophile research; since the potential is so novel and enormous, applications of these entities may be limited only by human imagination.

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