NEW OPPORTUNITIES REVEALED BY BIOTECHNOLOGICAL EXPLORATIONS OF EXTREMOPHILES

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Summary

Over the past few decades the extremes at which life thrives has continued to challenge our understanding of biochemistry, biology and evolution. As more new extremophiles are brought into laboratory culture, they have provided a multitude of new potential applications for biotechnology. Furthermore, more recently, innovative culturing approaches, environmental genome sequencing and whole genome sequencing have provided new opportunities for biotechnological exploration of extremophiles.

1. Introduction

Organisms that live at the extremes of pH (>pH 8.5,< pH 5.0), temperature (>45°C, <15°C), pressure (>500 atm), salinity (>1.0M NaCl) and in high concentrations of recalcitrant substances or heavy metals (extremophiles) represent one of the last frontiers for biotechnological and industrial discovery. As we learn more about the extremes at which life can survive and thrive, more of these extremophiles are brought into culture and their genomes sequenced. In many cases, biotechnological applications of extremophiles and their biomolecules (e.g. enzymes) have been the driving force in both academic and industrial research of these organisms. Extremophiles and extremozymes occupy an important place in the multibillion dollars environmental biotechnology industry, with applications spanning agricultural, biomedical and industrial sectors. Due to the highly competitive nature of industrial R&D, in most cases the path from an extremophile to a successful commercial application is not documented in peer reviewed scientific publications and can be partially followed through patents, biotechnology meetings and company websites. In this review, we will consider a few new developments as several recent reviews focus on the biotechnological applications of extremophiles

2. Extremophiles and Biomolecules.

The most direct application of extremophiles in biotechnological processes involves the organisms themselves. Among the most established is biomining (bioleaching), in which microbial consortia are used to extract metals such as copper, cobalt, gold and uranium from ores. This has received considerable interest lately, propelled by developments in microbial isolation and the application of genomic approaches for studying the individual organisms and their community . These processes involve iron or sulphur oxidizing acidophilic microorganisms adapted to different temperature ranges, from mesophiles (bacteria such as *Acidithiobacillus*, *Leptospirillum* and the archaea *Ferroplasma*) to thermophiles (archaea from the genera *Sulfolobus*, *Metallospharea* and *Acidianus*) [see also – *Biotechnology of Archaea*]. When the activity of such extreme acidophiles is not controlled in biomining operations, they can lead to acid mine drainage (AMD), which causes considerable environmental damage. While the microbes responsible for bioleaching and AMD are inherently adapted to extremely low pH and high concentration of metals, they are part of an open and dynamic consortia and continuously adapt to the particular conditions they are facing. In commercial bioleaching operations this can result in the selection of more robust and efficient strains, with reduced sensitivity to metal toxicity. For example, arsenic resistance genes have been horizontally transferred via a transposon from an unidentified bacterium to *Acidithiobacillus caldus* and *Leptospirillum ferriphilum*, resulting in substantially increased resistance to arsenic in gold-bearing arsenopyrite bioreactors. This demonstrates that taxonomy alone is not necessarily predictive of the physiological fitness of an individual organism and that the community gene pool can impact the adaptability of the constituent members across taxonomic barriers. Such strain engineering and selection for improved biotechnological characteristics may be applied to a wide range of bioremediation projects [see also– *Bioremediation*] as has been done for contaminating petroleum hydrocarbons accidentally released in arctic environments.

Most of the applications involving extremophiles are based on their biomolecules, primarily enzymes but also other proteins (e.g. cryoprotectant antifreeze proteins), lipids, various small molecules. The most well known example of a successful application of an extremophile-derived product is *Taq* DNA polymerase which was isolated from *Thermus aquaticus*, first isolated from a geothermal spring from Yellowstone National Park. This enzyme approaches sales of about half a billion dollars per year. The molecular biology research tool enzymes not only include a wide range of other thermostable polymerases and ligases, but also enzymes isolated from psychrophilic organisms, such as the "Antarctic phosphatase" from New England Biolabs. Genencor commercialized one of the first industrial extremozymes for use in textiles detergents, a cellulase isolated from an alkaliphilic bacterium from an east African soda lake. Numerous other examples are reviewed by Antranikian [see – *Biotechnology of Archaea*].

Enzymes that have optimal activity at extreme temperatures and pH are widely used in household detergents and in the food, textile, pulp and paper, leather processing, chemical intermediates industries. For each application, the enzymes have to fulfill numerous requirements related to such features as activity and stability, substrate

specificity and enantioselectivity. As a result, natural enzymes often are not optimal for the desired biotechnological application. Consequently, a variety of approaches have been used to modify enzyme properties such as error-prone PCR, saturation mutagenesis, structure-based protein engineering and in vitro evolution approaches. Such approaches are best combined with genetic selection or high throughput screening, to identify the rare mutants that approach the target characteristics, followed by an iterative process of building fitness to the resulting variants. For example, thermostability was built into a suite of other optimal enzymatic characteristics of pectinases used in cotton fabric processing and increased alkaline stability was obtained for a previously engineered xylanase from the fungus *Trichoderma reessei* . Furthermore, based on genomics, structural data and computational modeling, certain protein architecture and amino acids usage trends provide clues to mechanisns of protein thermostablity and promise to lead to predictive protein thermostabilization.

3. Extremophile Genomics Exposing the Biotechnological Potential

Extremophiles, including eukarya (e.g. *Alvinella pompejana, Tetrahymena thermophila, Dunaliella salina*), have been prime subjects for genomic sequencing projects in an effort to understand the fundamental mechanisms of adaptation to specific environments and for practical applications. Recently, four psychrophilic bacteria have been published: two from arctic sediments, *Desulfotaleas psychrophila* and *Colwellia psycherythraea* , one from coastal Antarctic waters, *Pseudoalteromonas haloplankis* and *Photobacterium profundum*, a deep sea bacterium that is both adapted to low temperatures and high pressure. While there does not appear to be a distinct genomic trait unifying these cold adapted organisms, they share several characteristics, most notably a membrane with increased proportion of polyunsaturated and branched fatty acids to increase fluidity at low temperatures (and high pressure), and the presence of cold shock proteins which are believed to increase translation efficiency by destabilizing secondary structures in mRNA. Additionally, cryoprotectants increase the capacity for nutrient uptake. Due to higher solubility of oxygen at low temperatures and hence potential for oxidative damage within the cell, metabolic reactions that generate reactive oxygen are reduced and molybdopterin-dependent metabolism is eliminated (in *P. haplokantis*) or the number of catalase and superoxide dismutatase genes increased (in *C. psycherythraea* and *D. psychrophila*). While there are specific amino acid usage trends that appear to correlate with the psychrophilic proteomes, the most important adaptation for cold adapted enzymes appears to be a high specific activity at low temperatures. This is achieved by a highly flexible catalytic center at the expense of overall reduced protein stability and susceptibility for thermal denaturation. Such enzymatic characteristics are currently exploited in food biotechnological applications [see also– *Fermented Foods and their* Processing] that require low temperatures (e.g. milk and fruit juice processing). Beside cold adaptation, *P. profundum* is also able to withstand high pressure and has become a model organism for understanding piezophily. For example, transport processes and energy generation by cytochrome respiration are impacted by high pressure.

Similar to the situation in cold adaptation, intrinsic properties of nucleic acids, lipids and enzymes/proteins allow thermophiles to flourish in high temperature environments, and specific composition biases and structural adaptations have been identified. The

recent publications describing the genomes of two hyperthermophilic archaea, *Nanoarchaeum equitans* and *Thermococcus kodakaraensis*, one archaeal thermoacidophile (*Sulfolobus acidocaldarius*) and one thermophilic bacterium (*Carboxydothermus hydrogenoformans*) while not significantly changing the perception on high temperature adaptation, bring together a number of exciting novelties on various aspects of the biology, genome evolution and metabolic versatility in specific thermophilic environments.

The genome of *N. equitans* came shortly after the discovery of this unusual organism, which represents the first case of an archaeon that is an obligate symbiont or parasite on another archaeon, the marine crenarchaeote *Ignicoccus* sp.. The genome abounds in oddities, from being at the smallest spectrum of cellular genome sizes and encoding virtually no metabolic pathways, to containing a large number of split genes, including uniquely split tRNA genes. One of the split genes is represented by two separate ORFs encoding DNA polymerase B (also fused with a split intein) which has is functional *in vitro* and *trans* spliced at high temperature thus restoring a functional polymerase.

The *Sulfolobus acidocaldarius* genome sequence represents an important landmark in archaeal genomics as this organism is one of the few laboratory genetic systems in Archaea. *S. acidocadarius* genome has a number of similarities and differences with two other *Sulfolobus* species, and will ease future experimental studies and facilitate overall biotechnological applications of these Crenarchaeaota. The bacterial thermophile, *Carboxydothermus hydrogenoformans* (35), has a remarkable efficiency to carry out carbon monooxidase metabolism due to the presence of five anaerobic CO dehydrogenase complexes.

This genomic blueprint should allow detailed studies of hydrogenogenesis, which could become an important industrial process for generating hydrogen. The most thermoacidophilic organism known, *Picrophilus torridus* inhabits solfataric environments with pH below 1 and about 60^{o}C , and its low pH adapted enzymes will most likely be found useful for biotechnological applications requiring acidic conditions. To cope with such conditions, *P. torridus* has evolved a membrane with low proton permeability and special lipid composition as well as efficient transport mechanisms to maintain the internal pH at values compatible with biochemical functions. Unlike other organisms, which use sodium ion and ATP-driven primary transporters, *P. torridus* predominantly uses the internal high proton concentration (pH 4) to power a large number of solute secondary transporters.

At the other extreme, the genome of a haloalkaliphilic archaeon from highly alkaline soda lakes, *Natronomonas pharaonis*, has revealed several adaptations to this environment. These include an overall modification of the proteome to increase the fraction of acidic amino acids and reduce protein hydrophobicity, a coating of the cell membrane with glycoproteins and secreted enzymes attached by lipid anchors as well as an efficient transport system for heavy metals and nitrogen compounds which are scarce in hypersaline environments. The halophilic bacterium *Salinibacter ruber* displays similar adaptation mechanisms to hypersaline environments and some of these may have been acquired via lateral gene transfer. No doubt, many more surprises are hidden in these genomes that can be exploited for biotechnological purposes.

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Biographical Sketches

Anna-Louise Reysenbach obtained her PhD in Microbiology from the University of Cape Town in 1987, where she studied the physiology of the obligate anaerobe *Clostridium acetobutylicum*. She then joined Dr. Jody Demings laboratory at the University of Washington where her research career with extremophiles from deep-sea vents started. In 1990, Anna-Louise joined Dr. Norman Pace's laboratory, where she worked on the natural phylogenetic diversity of hot spring environments in Yellowstone National Park. She then took a faculty position at Rutgers University and later at Portland State University where her research interests cover the ecology, physiology and genomics of novel and hard to grow thermophilic Archaea and Bacteria. She has served on numerous NASA, US- National Research Council, US-National Science Foundation committees and panels. Her research takes her to remote hot spring environments and she has studied thermophiles from deep-sea vents in the Indian, Pacific and Atlantic oceans.

Mircea Podar obtained a diploma in Biology at the University of Cluj, Romania. He then continued doctoral studies at the University of Texas Southwestern Medical Center in Dallas, Txeas, where he studied the biochemistry and genetics of self-splicing group II introns in yeast mtochodria and obtained a PhD in 1997. He then pursued evolutionary studies of ctenophores (comb jellies) as well as microbial diversity and mobile genetic elements in deap sea hydrothermal vents, as a postdoctoral scholar at the Woods Hole Oceanographic Institution and the Marine Biological Laboratories in Woods Hole, Massachusetts. After a short second postdoctoral appointment at the Salk Institute in La Jolla, California, in 2000 he took a scientist position at Diversa Corporation in San Diego. There, he studied the evolution of enzyme gene families, comparative microbial genomics and metagenomics, from fundamental and biotechnological perspectives. He left Diversa in 2006 and is presently an adjunct professor at Portland State University and a scientist at Oak Ridge National Laboratory. His interests range from ctenophores biology to microbial genomics and metagenomics and their impact on understanding ecology and evolution.