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Halophiles and their enzymes: negativity put to good use

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Halophilic microorganisms possess stable enzymes that function in very high salinity, an extreme condition that leads to denaturation, aggregation, and precipitation of most other proteins. Genomic and structural analyses have established that the enzymes of halophilic Archaea and many halophilic Bacteria are negatively charged due to an excess of acidic over basic residues, and altered hydrophobicity, which enhance solubility and promote function in low water activity conditions. Here, we provide an update on recent bioinformatic analysis of predicted halophilic proteomes as well as experimental molecular studies on individual halophilic enzymes. Recent efforts on discovery and utilization of halophiles and their enzymes for biotechnology, including biofuel applications are also considered.

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Introduction

Halophiles thrive from sea salinity (~0.6 M) up to saturation salinity (>5 M NaCl), and include Archaea, Bacteria, and Eukarya [1]. Many halophilic microorganisms have been isolated from diverse environments, ranging from artificial solar salterns, to natural brines in coastal and submarine pools, and deep salt mines. Some of the most commonly observed halophiles are those flourishing in salterns used for salt production, e.g. *Halobacterium* spp. (a misnomer, being members of the domain Archaea), *Salinibacter ruber* (a member of the Bacteroidetes phylum), and *Dunaliella salina* (green alga of the Chlorophyceae class) (Table 1). Halophilic microorganisms also have long been recognized as agents of spoilage of fish and meat preserved with solar salt and some varieties have been used for fermentation of protein-rich foods.

Over the past few decades, adaptation of halophilic microorganisms to their environment has been the subject of increasing interest, with methodology for culturing, manipulation, and genetic engineering steadily advancing. Our understanding of the adaptation of halophiles to high salinity includes several different mechanisms for balancing the osmotic stress of the external medium. Halophilic Archaea (Haloarchaea) primarily use a 'salt-in' strategy, accumulating concentrations of KCl equal to NaCl in their environment, and where examined, their enzymes tolerate or require 4–5 M salt [2]. In contrast, most halophilic Bacteria and Eukarya, largely use a 'salt-out' strategy, excluding salts and accumulating or synthesizing de novo compatible solutes (e.g. glycine betaine and other zwitterionic compounds for Bacteria, and glycerol and other polyols for Eukarya) [3]. Among some halophiles, a combination of adaptive mechanisms may operate.

Early microbiologists addressing the adaptation of halophilic enzymes to high salinity discovered two primary features: a substantial number of protein charges and increased hydrophobicity [4]. Dissolved ions shielded electrostatic repulsions at low (<1 M) concentrations of salts and increased hydrophobic effects occurred at higher concentrations, from 4 M to saturating conditions. Roles for specific ion pairs were also sometimes suggested, e.g. in stabilizing active site regions or promoting subunit interactions. The combined effects of these forces were hypothesized to result in improved function in hypersaline conditions, where most non-halophilic proteins are inactivated by low water activity and limiting solvation, resulting in their denaturation, aggregation, and precipitation.

In the 1990s, the availability of the first solved structure of a halophilic enzyme and a halophile genome sequence provided a much more detailed molecular perspective on halophilic adaptations than previously available [5–7]. Subsequently, over the next two decades, there has been a veritable explosion in studies of halophiles and their enzymes [8]. In this article, we review the key features of halophilic proteins and enzymes revealed from bioinformatic, structural, genetic, and biochemical studies over the past few years and address some potential applications to biotechnology.

Insights from bioinformatic analysis

The striking negativity of the halophilic proteome was first revealed by genome sequencing of *Halobacterium* sp. NRC-1 (Table 1) [6–9]. A unimodal distribution of protein isoelectric points (pI) with a mean of 5.0 and mode of 4.2 was observed, in stark contrast to all non-halophilic proteomes which possess bimodal distribution with acidic and basic proteins and an average pI very close to neutrality (Figure 1). *Halobacterium* exhibited an excess of

Table 1

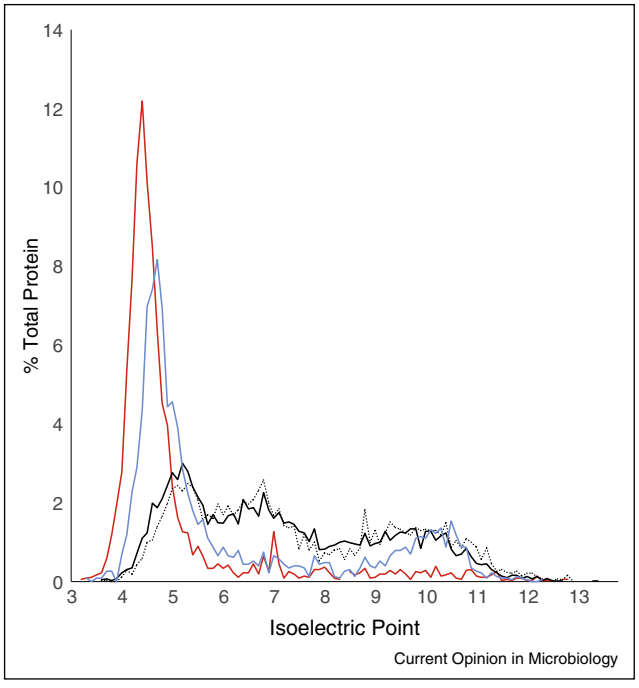
Representative halophilic microorganisms

Domain and organism	Physiology and isolation	Mean pI[a]
Archaea		
Halobacterium sp. NRC-1	Laboratory model, phototrophic facultative anaerobe	5.0
Haloferax volcanii	Laboratory model, prototroph, from Dead Sea mud	5.0
Haloferax mediterranei	Versatile metabolism, PHA producer from Spanish saltern	4.8
Halorubrum lacusprofundi	Cold-adapted halophile from Antarctic lake	4.8
Bacteria		
Acetohalobium arabaticum	Methylotrophic homoacetogenic Fermicute from Arabat lagoon	5.9
Chromohalobacter salexigens	Anaerobic chemoorganotrophic Proteobacterium from Bonair	6.6
Halomonas elongata	Ectoine producing Proteobacterium from Bonaire solar saltern	6.3
Halanaerobium hydrogeniformans	Hydrogen producing haloalkalophilic Firmicute from Soap Lake	6.6
Halorhodospira halochloris	Anaerobic purple sulfur Proteobacterium, Wadi Nantrun lake	6.7
Halorhodospira halophila	Anaerobic purple sulfur Proteobacterium, Summer Lake mud	6.3
Salinibacter ruber	Heterotrophic Bacteriodetes from Spanish solar saltern	5.9
Eukarya		
Debaryomyces hansenii	Hemiascomycetous yeast from New Zealand soil	6.9
Dunaliella salina	Unicellular microalga/Chlorophyceae common in salterns	NA
Wallemia ichthyophaga	Xerophilic Basidomycete from Slovenian saltern	7.2

NA, not available.
[a] Calculated from NCBI protein database using EMBOSS 6.3.1 IEP program at Pasteur Institute.

acidic (glutamic and aspartic acid) and a deficit of basic (lysine and arginine) amino acids. Excess negative charges were localized to the surface of modeled proteins, consistent with available structural work [5,9,10]. With subsequent sequencing of many additional genomes, bioinformatic studies confirmed the great dominance of acidic residues and a deficit of basic residues, especially lysine, for halophilic prokaryotes, but not necessarily halophilic eukaryotes (Table 1) [11].

Figure 1



Distribution of protein isoelectric points predicted from genome sequences. Percent total protein is plotted versus pI in 0.1 increments for *Halorubrum lacusprofundi* (red), *Acetohalobium arabaticum* (blue), *Debaryomyces hansenii* (black line), and *Escherichia coli* (black dots).

For Haloarchaea, orthologous proteins from more than a dozen genomes have recently been identified via reciprocal BLAST analysis to generate haloarchaeal protein families (called Haloarchaeal Orthologous Groups or HOGs) [12•]. This investigation resulted in the finding of nearly 800 acidic protein clusters (conserved HOGs or cHOGs) present in all Haloarchaea examined, including a subclass of unique proteins found only within this group. This work clearly established the acidic nature of all conserved haloarchaeal proteins in contrast to their non-halophilic orthologs. Databases of haloarchaeal genomes and/or protein families have been made available on dedicated websites, even while the number of halophile genomes has continued to grow [13–15].

In addition to their negative nature, halophilic and especially haloarchaeal proteins have been found to contain a slightly lower composition of bulky hydrophobic side chains (phenylalanine, isoleucine, and leucine) on their surface compared to small and borderline hydrophobic (glycine, alanine, serine, and threonine) amino acid residues [16]. These findings are consistent with increased flexibility and surface hydration of halophilic proteins, which may account for the observed similarities of halophilic proteins to psychrophilic proteins [8,17••]. In a detailed study comparing 15 homologous pairs of halophilic and non-halophilic proteins, general trends of increased acidic amino acids and reduced large nonpolar amino acids were recently confirmed [18•]. Dipeptide