Published in final edited form as:

Curr Opin Microbiol. 2012 June ; 15(3): 351–356. doi:10.1016/j.mib.2012.02.002.

Extreme Challenges and Advances in Archaeal Proteomics

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Abstract

Archaea display amazing physiological properties that are of interest to understand at the molecular level including the ability to thrive at extreme environmental conditions, the presence of novel metabolic pathways (e.g., methanogenesis, methylaspartate cycle) and the use of eukaryotic-like protein machineries for basic cellular functions. Coupling traditional genetic and biochemical approaches with advanced technologies, such as genomics and proteomics, provides an avenue for scientists to discover new aspects related to the molecular physiology of archaea. This review emphasizes the unusual properties of archaeal proteomes and how high-throughput and specialized mass spectrometry-based proteomic studies have provided insight into the molecular properties of archaeal cells.

Introduction

While proteomic studies of archaea are simplified by their typically unicellular structure and relatively small genome size (0.49 to 5.75 Mbp), archaea are also distinguished by a number of unusual properties that can complicate MS-based analysis of their proteome. In particular, most model archaea are extremophilic, able to withstand amazingly harsh conditions such as temperatures over 100 °C, saturating salt and/or extreme pH [1]. Thus, many of the proteins of archaea are relatively resistant to the standard chemical and thermal treatments used to denature proteins for separation by 2D-PAGE or trypsin digest [2]. MS-based identification of archaeal proteins is also complicated by an inability to correctly identify translation start sites due to a limited knowledge of ribosomal binding site and promoter consensus sequences. This lack of knowledge results in under- and/or over-prediction of the coding sequence for genes during genome annotation and can compromise the database used for MS-based searches [3]. An additional drawback is that many archaea require rich media for laboratory growth or can only be detected by metagenomic sequencing of environmental samples, thus, increasing complexity for MS. Haloarchaeal proteomes are further complicated by their radical adaptation to molar concentrations of intracellular salt in which charged amino acids on the protein surface allow the retention of water molecules for

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catalytic function [4]. Thus, haloarchaeal proteomes are very acidic, specifically depleted in lysine residues and must be extracted from molar concentrations of salt which can compromise the depth of sample coverage by isoelectric focusing, isotope-coded protein labeling (ICPL) of free amino groups and other proteomic approaches [5;6]. Nevertheless, as detailed below, significant progress has been made in using MS-based proteomics as a tool to further understand the molecular physiology of archaea.

High-throughput proteomic surveys

A number of high-throughput proteomic surveys have been conducted in the Archaea, some of which are summarized in Table 1 [2;7–19]. Many of these surveys relied upon non-traditional approaches to overcome the specific limitations of standard proteomic methods. For example, harsh chemical and physical manipulations have been used to improve the proteolytic digestibility and coverage of the denaturation-resistant proteomes of hyperthermophilic archaea such as *Pyrococcus furiosus* [2]. The inherently acidic proteomes of the haloarchaea have necessitated variation of pH gradient range, carrier ampholytes and physical size of 2D gels for improved resolution [5]. In higher throughput processes, tandem HPLC separation strategies and combinatorial serine protease digestions have been employed to marginalize the lack of separation achievable through isoelectric focusing alone [20;21]. Likewise, algorithms that identify interrupted gene sequences among archaeal genomes have been developed by examining –1 and +1 frame shifts and have been successfully applied to proteomic surveys of archaea such as *Sulfolobus solfataricus* [16]. Archaeal genome annotations are also plagued by instances of false start sites, unexpected small open reading frames or the occurrence of rare start codons. Sophisticated peptide search algorithms and labor-intensive database management have been combined to alleviate some of these issues [14]. However, portions of the collective archaeal proteome have proven difficult to access due to the physical and spatial properties of certain protein subpopulations.

Localization of proteins within the cell

Proteomics is a common tool used to localize proteins to subcellular fractions such as the extracellular matrix, membrane and cytosol of archaea. One of the more challenging sectors of the archaeal proteome to investigate has been that which is membrane-associated. Due, in large part, to an inherent insolubility and more importantly, poor peptide fragmentation, many of these proteins are unavailable for separation with conventional gel-based techniques and are difficult to desorb via MALDI ionization methods [22]. Resolution has been achieved with advanced, high-throughput processes which rely on electrospray ionization (ESI), liquid chromatography-based separation of peptides, tandem mass analysis and also through more uniform peptide fragment generation via alternative digestion strategies [3;6;20;22;23]. Successful analysis of the secreted sub-proteomes of various *Sulfolobus* species have been achieved through the use of modified separation and identification techniques as well as enrichment methods for hydrophobic or glycosylated proteins that are secreted directly or associated with the membrane or secreted membrane vesicle [24–26]. Many of these sub-proteomes, particularly in *Methanococcoides*, have also been profiled under multiple growth conditions to better assign adaptive functions to the organism and its encoded proteins [27–30].

Identification of protein complexes and networks

Proteomic approaches have been successfully applied to elucidating archaeal protein complexes and their associated networks. Investigation of ribosomal proteins in both *Pyrobaculum aerophilum* and *Sulfolobus acidocaldarius* resulted in the identification of 100% and 97% of all predicted ribosomal proteins, respectively, as well as three new

putative ribosomal proteins not previously characterized [31]. In *Halobacterium salinarum*, 10 of the 14 predicted gas vesicle proteins were identified by LC- MS/MS [32]. Furthermore, molecular sieve chromatography and glycerol gradient ultracentrifugation techniques were paired with MS technology to identify subunits of larger macromolecular complexes in *Thermoplasma acidophilum* [33;34]. DNA replication machinery of the archaea has also been studied as a dynamic complex in order to identify key elements that exist in common with other domains and those that remain uniquely archaeal. Nineteen strains of *Thermococcus kodakarensis* were developed, each expressing an individual His₆-tagged subunit of the archaeal replisome [35]. A network of interacting proteins was identified through the use of traditional protein purification and MS analysis. In proteomic experiments designed to identify proteins of a functional relatedness in uncharacterized biochemical pathways, immobilized metabolites have been used as bait for interacting proteins. Such is the case in *Pyrococcus furiosus* where (*N*²-(N-acetylaminopropyl) spermidine) was used to identify 11 proteins involved in its synthesis and modification [36].

Co- and post-translational modification of proteins

While high-throughput MS-based surveys have provided global perspectives on archaeal proteomes, often times it is desirable to know additional information that may not be easily obtained through shotgun proteomic approaches. When identifying and/or mapping sites of co- or post-translational modification, it is often necessary to use additional separation or enrichment techniques to select for specific peptides or modifications in order to obtain higher resolution data. Separation strategies have been developed for the enrichment and purification of peptides that may be modified such as immobilized metal affinity chromatography (IMAC) or metal oxide affinity chromatography (TiO₂/MOAC) for phosphoproteomes or combined fractional diagonal chromatography (COFRDIC) for N-terminal peptides. A variety of these strategies have been utilized to demonstrate a number of different protein modifications on archaeal proteins including glycosylation (reviewed in [37]), phosphorylation [38–41], acetylation ([8;15;40;42–44], reviewed in [45]), deamination/methylation [41;46;47], and ubiquitin-like SAMPylation [48] (Table 2).

Protein acetylation

Proteomic technologies have provided evidence that archaeal proteins can be acetylated at either the α-amine of N-terminal residues or the ε-amine of lysine residues, as is the case with Alba (reviewed in reference [45]). Prior to high-throughput proteomic studies, the prevalence of protein Nα-acetylation was assumed to be infrequent among prokaryotes (bacteria and archaea) [45]. In contrast, protein Nα-acetylation was known to be one of the most common modifications in eukaryotes, occurring on an estimated 50 – 90% of soluble proteins [45]. Recent MS-based studies of several haloarchaea and the crenarchaeon *Sulfolobus solfataricus* demonstrate numerous proteins of various functions are Nα-acetylated [8;15;42–44]. In the absence of intentional enrichment of acetylated peptides or N-termini, a shotgun proteomic study of *Haloferax volcanii* demonstrated at least 5% of the proteome was Nα-acetylated [8]. Enrichment of the *Halobacterium salinarum* and *Natronomonas pharaonis* proteomes by COFRDIC revealed 18% and 13% of the proteins were Nα-acetylated, respectively [15;42;43]. In addition, 17 Nα-acetylated proteins were identified by MS analysis of large proteins and protein complexes enriched by gel filtration from *S. solfataricus* [44]. Thus, Nα-acetylation appears to be a relatively common form of protein modification among archaea. In contrast, although the acetylation of lysine residues has been detected for select proteins, it remains to be determined how widespread this modification is among archaea [45].

Phosphoproteomes

Several studies focusing on individual proteins or complexes demonstrated that serine and threonine residues are phosphorylated in archaea [39–41]. Since protein phosphorylation is a reversible modification, there may be a mixture of modified and unmodified proteins in the cell simultaneously. A common strategy employed in two different phosphoproteomes of haloarchaea is to use IMAC or MOAC to enrich for phosphopeptides upstream of mass spectrometry analysis [38;39]. By enriching for phosphopeptides, the complexity of the solution is decreased and the effective concentration of phosphosites is increased. This enhances the likelihood of identifying a phosphorylation site. By using titanium oxide (TiO₂) beads (MOAC), 90 phosphopeptides were identified in 69 different proteins in *Halobacterium salinarum* [39]. A comparable study in *Haloferax volcanii* looked at IMAC and MOAC enriched phosphopeptides and identified 195 putative phosphoproteins and 9 phosphosites on 8 proteins [38]. In addition to demonstrating that there were potentially hundreds of phosphorylation targets in these organisms, these two studies also showed that archaeal organisms phosphorylate tyrosine residues.

Ubiquitin-like protein modification

Small ubiquitin-like archaeal modifier proteins were identified to form protein conjugates in *Haloferax volcanii* using a strategy that combined genetics with MS-based proteomics [48]. Similarly to ubiquitylation of eukaryotes, SAMPylation involves the formation of an isopeptide bond between the C-terminus of SAMP and the ϵ -amine of a lysine residue of a substrate protein. Protein conjugation partners were simultaneously purified with affinity tagged SAMP1 and SAMP2. Analysis of the SAMP containing fractions revealed both the identities and the sites of modification on dozens of protein targets including some of the SAMPylation machinery [48].

Metalloproteomes

Metalloenzymes and metal-associated proteins are an important component of the molecular physiology of archaea. Many archaea are strict anaerobes and/or thrive in environments of high metal content such as acid mine drainage and thermal marine sediments. To probe the full spectrum of metals which may associate with archaeal proteins, a MS-based proteomic survey was performed using the hyperthermophile *P. furiosus* as a model, one of the few organisms known to possess enzymes containing tungsten. In this survey, 343 different metal ion containing proteins were detected by proteomic methods [49]. Out of these proteins, 158 had not been previously annotated as metalloproteins and many were associated with novel metals (Pb, Mn, Mo, Ur, and V) [49]. Comparative analysis of iron-metalloproteomes has also been performed by 2D-non-denaturing PAGE and peroxidation staining [50]. With this approach, the *Ferroplasma acidiphilum* proteome was found to be dominated by iron-metalloproteins (unlike the proteomes of its archaeal relatives or bacterial neighbor *Acidithiobacillus ferrooxidans*). Many of the ‘iron-positive’ spots were identified by 2ESI-Q-TOF to be proteins not previously described as iron-metalloproteins and, thus, *F. acidiphilum* proteins are thought to be commonly organized and stabilized by iron atoms [50].

Differential proteomics

A number of genome-wide proteomic studies have been performed on archaea grown under different culture conditions or with isogenic mutations to further understand their molecular physiology. One highlight is the use of proteomics to discover an unconventional energetic pathway for the reduction of carbon dioxide to methane in carbon monoxide-grown *Methanosarvina acetivorans*, a marine cytochrome-producing methanogen [51]. In this study, cells were grown on carbon monoxide versus methanol or acetate as the carbon and

energy source and differential proteins were quantified by labeling with $^{14}\text{N}/^{15}\text{N}$ and detection by LTQ/FTICR-MS [51].

Proteomics of model microbial communities and co-cultures

Proteomics tools have been used to investigate the molecular physiology of archaea not only in pure culture but also in co-cultures and microbial communities. For example, the hyperthermophilic *Nanoarchaeum equitans* is dependent on physical and specific cell-to-cell contact with *Ignioccus hospitalis*, and this relationship has been analyzed by proteomics [9;52]. MS-analysis of proteins solubilized from membrane fractions of *I. hospitalis*-*N. equitans* in co-culture reveals proteins which may form the contact sites [52]. In addition, shotgun proteomics suggests *I. hospitalis* may increase its energetic, protein processing and cellular membrane functions in lieu of replication and transcription while in co-culture with *N. equitans* [9]. The proteomes of syntrophically-growing dechlorinating bacteria (*Dehalococcoides* and *Desulfovibrio* spp.) have also been analyzed in culture with *Methanobacterium congolense* but found to change little in the presence of this hydrogenotrophic methanogen [53]. Stable isotope probing (SIP) has also been developed and used to track ^{15}N -flux into the proteomes of multispecies biofilm communities of acid mine drainage (AMD) from Richmond Mine at Iron Mountain, CA, USA [54]. While these iron-oxidizing AMD biofilms are dominated by *Leptospirillum* spp. (bacteria), the proportion of archaea is estimated to rise to 29 % as the biofilm thickens and diversifies during later stages of growth [55]. Community-based quantitative proteomics has revealed that, as conditions in these biofilms are shifted to low pH (0.85), there is an increased detection of proteins from low abundance archaea including *Ferroplasma* type II and *G-plasma* (an uncharacterized *Thermoplasmatales* lineage) [56]. The types of proteins (*e.g.*, energy conversion and ribosomal proteins) augmented suggests a growth advantage or strong response in certain archaea as the pH drops in these AMD communities [56].

Perspectives

While archaeal proteomic studies remain at an early stage, important discoveries have already been achieved using this new technology including the identification of a new pathway of energy conservation in methanogens, a novel system of ubiquitin-like protein conjugation and usual metalloproteins. Since many archaea have been difficult to culture in the laboratory, it is exciting to see that proteomics is moving out into the field suggesting the future really is limitless.

Acknowledgments

We apologize for not citing many important references that have contributed to this review due to space limitations. This work was funded in part by grants to JAMF from the National Institutes of Health (GM57498) and the Department of Energy Office of Basic Energy Sciences (DE-FG02-05ER15650). MAH's research funding is provided by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, Bethesda, MD.

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Highlights

- Archaea display amazing physiological properties that are of interest to understand at the molecular level.
- Proteomics provides an avenue for scientists to discover new aspects related to the molecular physiology of archaea.
- Unusual properties of archaeal proteomes are highlighted in this review.
- High-throughput and specialized mass spectrometry-based proteomic studies, which provide insight into the molecular properties of archaea, are also described.

Table 1

Comparison of proteome coverage among commonly studied archaeal species.

Species	No. Proteins Identified*	Est. Coverage of Proteome	Ref (s)
<i>Halobacterium salinarum</i>	1,461	62 %	[7]
<i>Haloferax volcanii</i>	1,296	32 %	[8]
<i>Ignicoccus hospitalis</i>	1,058	73 %	[9]
<i>Methanocaldococcus jannaschii</i>	963	54 %	[10]
<i>Methanococcus maripaludis</i>	939	55 %	[11]
<i>Methanosarcina acetivorans</i>	593	14 %	[12–14]
<i>Nanoarchaeum equitans</i>	476	85 %	[9]
<i>Natromonas pharaonis</i>	929	41 %	[15]
<i>Pyrococcus furiosus</i>	932	44 %	[2]
<i>Sulfolobus solfataricus P2</i>	1,590	53 %	[16]
<i>Thermococcus gammatolerans</i>	951	44 %	[17]
<i>Thermococcus onnurineus NA1</i>	1,395	71 %	[18]
<i>Thermoplasma acidophilum</i>	1,025	52 %	[19]

* Includes cumulative, non-overlapping data from all references indicated.

Table 2

Post-translational modifications identified in Archaea including those detected by MS-based proteomics.

Residue	Modification	Organism	Occurrence ^a	MS-based Detection	Ref (s)
Asparagine	N-glycosylation	many	many ^b	NA ^c	Reviewed in [37]
Aspartic acid	Methyl esterification	<i>Hfx. volcanii</i>	1	ESI-Q-ToF	[14;41]
		<i>M. acetivorans</i>	1 ^d	Q-FT MS	
Glutamic acid	Methyl esterification	<i>Hfx. volcanii</i>	4	ESI-Q-ToF	[41;46]
		<i>Hbt. salinarum</i>	19	ESI-Q-ToF	
Glutamine	Deamidation/methyl esterification	<i>Hbt. salinarum</i>	3	MALDI-ToF ESI-Q-ToF	[46]
Lysine	Nε-acetylation	<i>Hfx. volcanii</i>	1	NA ^c	Reviewed in [45]
		<i>Hbt. salinarum</i>	1	NA ^c	
		<i>S. solfataricus</i>	1	NA ^c	
Lysine	SAMPylation	<i>Hfx. volcanii</i>	45	LTQ-ETD/CID	[48]
Serine	Phosphorylation	<i>Hbt. salinarum</i>	70	LTQ-Orbitrap	[38–41]
		<i>Hfx. volcanii</i>	7	ESI-Q-ToF	
Threonine	Phosphorylation	<i>Hbt. salinarum</i>	10	LTQ-Orbitrap	[38–41]
		<i>Hfx. volcanii</i>	5	ESI-Q-ToF	
Tyrosine	Phosphorylation	<i>Hbt. salinarum</i>	1	LTQ-Orbitrap	[38;39]
		<i>Hfx. volcanii</i>	1	ESI-Q-ToF	
N-terminus	Nα-acetylation	<i>S. solfataricus</i>	17	ESI-Q-ToF	[8;15;42–44]
		<i>Hfx. volcanii</i>	68	LCQ MS/MS	
		<i>Hbt. salinarum</i>	112	ESI-Q-ToF LTQ-FT	
		<i>Nmn. pharaonis</i>	39	ESI-Q-ToF LTQ-FT	

^aNumber of unique modifications identified in the literature to date.

^b Several proteins in multiple species of archaea are glycosylated but beyond the scope of this review.

^c Non-mass spectrometry based methods, such as genetic screening and NMR, were used to detect modification.

^d Site of modification not clear.