Mini-Review

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Archaeal signal peptidases

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Signal peptidases are vital enzymes in the protein secretion pathway. In Archaea, type I signal peptidase, responsible for the cleavage of secretory signal peptides from the majority of secreted proteins, and prepilin peptidase-like signal peptidase, responsible for processing signal peptides from prepilin-like proteins like the preflagellins and various sugar-binding proteins, have been identified. In addition, the archaeal signal peptide peptidase, responsible for degradation of signal peptides after their removal from precursor proteins, has been characterized. These enzymes seem to have a mosaic of eukaryal and bacterial characteristics, and also possess unique archaeal traits. In this review, the most current knowledge with regard to these enzymes is summarized, including their cellular function, catalytic mechanism and distribution and conservation among archaeal species. Comparisons are drawn of these enzymes to their bacterial and eukaryal counterparts, and unique archaeal features highlighted.

Introduction

The Archaea constitutes a third domain of life, distinct from the Bacteria and the Eukarya (Woese *et al.*, 1990). Originally thought to inhabit only extreme environments, archaeal species have since been found in a wide variety of diverse habitats, where they play important roles in the ecosystem (Chaban *et al.*, 2006). Many characteristics of archaea often display an amalgam of bacterial, eukaryal and archaeal-specific features. This is apparently the case for the signal peptidases required for processing secreted proteins in archaea.

A functional conservation of the vital cellular processes of protein trafficking and secretion has been observed throughout all three domains of life (Pohlschroder *et al.*, 1997, 2005; Albers *et al.*, 2006). In particular, many proteins are synthesized as preproteins, with N-terminal signal peptides acting as sorting signals for recognition and targeting. These peptides are recognized and cleaved by their corresponding signal peptidases. Archaeal protein secretion is a relatively new research field. Current knowledge comes from identification, cloning and biochemical characterization of individual signal peptidases, as well as biochemical analysis of known secreted proteins and *in silico* examination of sequenced genomes. Within the last 5 years, examples of archaeal signal peptidase I (SPI), type IV prepilin peptidase (TFPP)-like enzyme and signal peptide peptidase (SPP) have been characterized, greatly extending our knowledge of protein processing and secretion in this domain. Archaeal SPI, TFPP-like and SPP candidates compiled from analyses of sequenced genomes or focused studies are presented in Table 1.

Signal peptides

Current knowledge on archaeal signal peptides comes mainly from examination of available genomes, since few

studies are available on individual secreted proteins. Various computer programs aimed at identification of signal-peptide-bearing proteins from sequenced archaeal genomes have predicted 8–32 % of the proteomes to possess signal peptides (Bardy *et al.*, 2003; Saleh *et al.*, 2001), falling within three categories (Pohlschroder *et al.*, 2005). Archaeal class I signal peptides are generally 20–30 amino acids in length (Bardy *et al.*, 2003) and contain the classical motif – a basic n-region followed by a hydrophobic core (h-region) and ending with a carboxyl-terminal region (c-region) containing the cleavage site (von Heijne, 1983). As in bacteria and eukarya, the archaeal cleavage site follows the (–3, –1) rule, where the –3 and –1 positions relative to the cleavage site are held by small, neutral amino acids (often alanine). *In silico* studies that focused on a single archaeon at a time have indicated certain eukaryal-like features of archaeal signal peptides (Albers & Driessen, 2002; Nielsen *et al.*, 1999). However, a subsequent genomic survey of 13 archaeal species indicated that their class I signal peptides were more like those of bacteria than eukaryotes in overall composition and amino acid preference at key positions around the cleavage site (Bardy *et al.*, 2003). Class I signal peptides are cleaved by SPI, which processes the majority of secreted proteins exported via the Sec system. Also processed by SPI may be substrates of the Tat translocon (used for folded proteins) (Paetzel *et al.*, 2002; Sargent *et al.*, 2006) that possess generally longer signal peptides distinguished by a less hydrophobic h-region and the presence of the twin arginine motif [(S/T)-R-R-X-Φ-Φ, where X can be any amino acid and Φ is any hydrophobic amino acid] (Berks 1996; Pohlschroder *et al.*, 2005). Apparent archaeal class II signal peptides containing a bacterial-like lipobox (–2[L/I/G/A]–1[A/G/S]+1C) have been detected in numerous archaeal proteins (Albers *et al.*, 2006; Bardy *et al.*, 2003); this is intriguing, given the unresolved issue of whether archaeal SPII homologues exist (see below). Archaeal class III

Table 1. Distribution of signal peptidases and signal peptide peptidases in Archaea

The GI numbers (protein) listed are from sequenced genomes found at NCBI Genomes (<http://www.ncbi.nlm.nih.gov/genomes/static/a.html>).

Organism	Signal peptidase I (SPI)	TFPP-like signal peptidase	Signal peptide peptidase (SPP)
Crenarchaeota			
<i>Aeropyrum pernix</i> (AP)	14601631	–	–
<i>Pyrobaculum aerophilum</i> (PAE)	18311818	–	18314240, 18313592
<i>Sulfolobus acidocaldarius</i> (SA)	70607524, 70607136	70605985	–
<i>Sulfolobus solfataricus</i> (SS)	15897803	15897089	–
<i>Sulfolobus tokodaii</i> (ST)	15921849, 15622258	15922589	–
<i>Thermophilum pendens</i> (TP)	110605194, 110605943	–	110606247
Euryarchaeota			
<i>Archaeoglobus fulgidus</i> (AF)	11499247, 11499660, 11499245, 11499380	11498541	–
<i>Ferroplasma acidarmanus</i> (FA)	68140881, 69268583	–	68140244
<i>Haloarcula marismortui</i> (HM)	55379569, 55379336, 55379337, 55232061, 55232062	55379184	55377494, 55378760
<i>Halobacterium salinarum</i> (HS)	15791199	15791095	15789821
<i>Haloferax volcanii</i> (HV)	61417364, 61417373	NA	NA
<i>Haloquadratum walsbyi</i> (HW)	110667554, 110666978	110666997	110667987, 110668686, 110668977
<i>Methanocaldococcus jannaschii</i> (MJ)	15668434	15669092, 15669469, 15669025	15668832
<i>Methanococcoides burtonii</i> (MB)	91773684	91772142	91773876
<i>Methanococcus maripaludis</i> (MMP)	45357950	45358118, 45357795	45358632
<i>Methanococcus voltae</i> (MV)	15029362	21070279	NA
<i>Methanoculleus marisnigri</i> (MMS)	110603338	110602842	–
<i>Methanopyrus kandleri</i> (MK)	20094467	20094140	–
<i>Methanosaeta thermophila</i> (MTP)	88952104	–	–
<i>Methanosarcina acetivorans</i> (MA)	20088935	20089408, 20091920, 20093290, 20091424	20092840
<i>Methanosarcina barkeri</i> (MBA)	73668537	73669011	73668088
<i>Methanosarcina mazei</i> (MMZ)	21227446	21227780	21226968
<i>Methanospiraera stadtmanae</i> (MS)	84490353	84489263	–
<i>Methanospirillum hungatei</i> (MH)	88603654	88604154	–
<i>Methanothermobacter</i> <i>thermautotrophicus</i> (MTH)	15679445	15678449	15678828
<i>Natronomonas pharaonis</i> (NP)	76800894	76801289	76800828, 76802382
<i>Picrophilus torridus</i> (PT)	48477996, 48478421	–	48477283
<i>Pyrococcus abyssi</i> (PA)	14521652, 14521681	14521786	14520806
<i>Pyrococcus furiosus</i> (PF)	33359468, 18976685	18976843	18977955
<i>Pyrococcus horikoshii</i> (PH)	14590486, 14590462	14590360	14591350
<i>Thermococcus kodakarensis</i> (TK)	57641972, 57641638	57639988	57641099
<i>Thermoplasma acidophilum</i> (TA)	16082489, 16081507	16081397	16081247
<i>Thermoplasma volcanium</i> (TV)	13541052	13542171	13540860
Nanoarchaeota			
<i>Nanoarchaeum equitans</i> (NE)	41615219	–	41615105

NA, Complete genome sequence is not publicly available for *Haloferax volcanii* or *Methanococcus voltae*; the GI numbers reported are from dedicated studies on the enzymes indicated for these organisms.

signal peptides are equivalent to bacterial type IV prepilin signal peptides. These signal peptides are distinct in that their cleavage site is directly after the n-region, leaving the hydrophobic h-region as part of the mature protein. This

property is essential to type IV pilin-like proteins for their proper anchoring and assembly into surface structures. In archaea, the preflagellin peptidase processes this class of signal peptides.

Signal peptidase I (SPI)

SPI is the essential housekeeping peptidase responsible for processing the majority of preproteins that are transported through either the Sec or the Tat secretion pathway (Sargent *et al.*, 2006; Pohlschroder *et al.*, 2005; Paetzel *et al.*, 2002). While limited sequence identity exists between SPI enzymes, all members of the SPI family possess highly conserved regions that are believed to have important roles in protein function. They are termed boxes A–E, with box A being the membrane-anchoring domain and boxes B–E participating in catalysis. While sequence alignments reveal a universally conserved serine residue within box B, two subgroups exist. The P-type enzyme (found in bacteria, mitochondria and chloroplasts) has a conserved essential lysine while the endoplasmic reticulum (ER)-type enzyme (found in eukarya, archaea and limited bacterial species) has a histidine residue in replacement (Dalbey *et al.*, 1997). In only rare cases, ER-type SPIs have been found in bacteria, with the best-studied example being *Bacillus subtilis*. This organism has five chromosomally encoded SPIs (Tjalsma *et al.*, 1999) with one, SipW, having a histidine replacement for the conserved lysine found in P-type enzymes. Presumably, SipW shares the catalytic mechanism of ER-type SPIs (Tjalsma *et al.*, 2000). The origin of the ER-type SPI found in bacteria is unclear. Several possibilities have been suggested, including convergent evolution of the P- and ER-type enzymes or a horizontal gene transfer event (Eichler, 2002).

It is generally accepted that P-type SPI enzymes utilize a Ser-Lys catalytic dyad and not the Ser-His-Asp triad commonly found in serine proteases (Dalbey *et al.*, 1997; Tuteja 2005). Site-directed mutagenesis studies in *Escherichia coli* revealed that Ser⁹⁰ and Lys¹⁴⁵ are critical amino residues for catalysis (Tschantz *et al.*, 1993), while subsequent mutagenesis work and analysis of the X-ray structure of the *E. coli* enzyme identified another residue, Ser²⁷⁸, as essential for optimal activity (Paetzel *et al.*, 2002). The proposed mechanism of catalysis is that Ser⁹⁰ O γ would perform a nucleophilic attack on the si-face of the scissile bond of the signal peptide, with Lys¹⁴⁵ N ζ acting as the general base.

The mechanism of action of the ER-type SPI is quite distinct. In the yeast Sec11p subunit of signal peptidase, mutagenesis targeting lysine residues revealed the non-essential nature of all lysines – suggesting that the enzyme might rely on a different catalytic mechanism (VanValkenburgh *et al.*, 1999). Although the exact mechanism of catalysis is unknown, it has been determined through mutagenesis that a conserved serine, histidine and two aspartic acids are important for activity (Dalbey *et al.*, 1997; VanValkenburgh *et al.*, 1999). Furthermore, studies on the ER-type SPI of *B. subtilis*, SipW, identified conserved Ser, His and Asp residues critical for activity, suggesting a Ser-His-Asp catalytic dyad or Ser-His catalytic dyad as potential mechanisms (Tjalsma *et al.*, 2000).

The archaeal SPI enzyme was first identified and biochemically characterized in *Methanococcus voltae* (Ng & Jarrell,

2003). The putative SPI gene was cloned and overexpressed in *E. coli*. Enzymic activity was demonstrated through an *in vitro* peptidase assay. Computer-based topological analyses of *M. voltae* and other archaeal SPI enzymes all indicate the active site to be on the external face of the cytoplasmic membrane. Thus, as in other SPIs, it seems that the archaeal enzyme cleaves the signal peptides from preproteins on the external face of the cytoplasmic membrane, thereby releasing the translocated protein from the membrane. In archaea, the potential processing of signal sequences of integral cytoplasmic membrane proteins has not been well studied. A recent proteomics survey of *Natronomonas pharaonis* and *Halobacterium salinarum* indicates that many, if not most, integral membrane proteins lack cleaved signal sequences (Falb *et al.*, 2006). In addition, there are no reports in archaea on the processing of Tat signal peptides by SPI.

In agreement with early insights gained from sequence analysis (Eichler, 2002), archaeal SPI apparently possesses traits from both its bacterial and eukaryal counterparts. Similar to bacterial SPI, archaeal SPI seems to operate independently and not as part of a eukarya-like complex. Genes corresponding to those encoding the subunits of the eukaryal signal peptidase complex have not been identified in genomic searches in archaea, and the overexpressed cloned version of the archaeal enzyme alone is biochemically active (Ng & Jarrell, 2003). The possibility remains, however, that the archaeal SPI enzyme functions optimally when complexed with additional subunits that are archaeal specific and simply not identified in genome searches (Fine *et al.*, 2006).

Regions with similarities to signature motifs of the SPI family were identified in all the known archaeal SPIs to date (Table 2). The apparent conservation of the catalytic residues in the archaeal SPI with its eukaryal counterpart raised questions as to whether it shared the ER-type SPI proposed mechanism of action. Site-directed mutagenesis was employed targeting the conserved Ser, His and Asp residues of SPI in *M. voltae* (Bardy *et al.*, 2005). A deviation from Sec11 was noted – while the yeast enzyme has a strict requirement for both conserved aspartic acids, Asp¹⁴⁸, but not Asp¹⁴², was proven to be essential in *M. voltae*. On the other hand, the essential nature of Ser⁵² and His¹²² was established; in particular, a histidine-to-lysine change led to inactivity of the enzyme, consistent with eukaryal Sec11 mutagenesis results. The mechanism of the peptidase likely involves the ER-type Ser-His-Asp triad. Indeed, almost all archaeal SPIs have the conserved Ser, His and two Asp residues separated by 5–8 amino acids corresponding to the ones targeted for mutagenesis in *M. voltae* (Table 2).

Certain archaeal species possess SPI enzymes with unique characteristics that set them apart from the majority of the archaeal SPIs. It was noted upon examination of archaeal SPI sequences that a few (e.g. in *Thermoplasma* species) contain a bacteria-like domain II region (Eichler, 2002). Domain II is absent in the ER-type SPI; it folds into a large β

Table 2. Partial alignment of archaeal signal peptidase I candidates indicating sequences around catalytically important amino acids

Abbreviations for organisms as in Table 1. ECO, *Escherichia coli*. BSU, *Bacillus subtilis*. SC, *Saccharomyces cerevisiae*. BSU_1731037 is the ER-type SipW enzyme. PF_18976685 is a partial sequence (missing some catalytic boxes) and was not included. Shading key: ■, 100 % conservation; ▒, >80 % conservation; ▒, >60 % conservation. Conserved substitutions are considered equal; S=T, V=I=L=M, D=N, K=R, F=Y=W. The proposed catalytic residue in third alignment row is in bold.

Bacteria and yeast							
ECO_987643	SGSMPTL	S90	DYIKRAVGLPGD	K145	DNRDNSADSR		D273
BSU_126186	GDSMYPTL	S43	HYVKRIIGLPD	K83	DNRRNSMDSR		D146
BSU_1731037	SGSMEPEF	S47	AVTHRIVDITKQ	H87	GDNNAAAD SAP		D106
SC_557828	SGSMEPAF	S44	PIVHRVLRQHNN	H83	GDNNAGNDISL		D103
Crenarchaeota							
AP_14601631	GRSMEPIL	S43	LITHRIIAVYQS	H79	GDNNPITDMGD		D105
PAE_18311818	SYSMEPTM	S38	WIIHRVYQKQNS	H81	GDNNPFPDQRV		D106
SA_70607136	GVSMPYVF	S33	YVIHQVIHIDGN	H70	GVDNITNYLPD		D95
SA_70607524	GYSMFYPL	S52	YIIHQVINVLPN	H99	GINDNFVDQIY		D121
SS_15897803	GVSMPYIF	S35	YVIHRVIATDNG	H73	GVDKITNPIPD		D98
ST_15622258	GVSMPYIF	S33	YVIHHVIKINYI	H71	GVDNITNPQSD		D99
ST_15921849	TNSMYPLL	S42	YVVHEVINVTKN	H81	GINNPTPDWPI		D102
TP_110605194	SWSMEPVL	S194	LIVHRIVLSVNG	H238	GDANPQADNIV		D251
TP_110605943	SWSMEPVL	S43	LIVHRIVLSVNG	H78	GDANPQADNIV		D99
Euryarchaeota							
AF_11499245	SSSMEPLM	S36	LITHRVVEIGDG	H76	GDAVEDVD PFD		D97
AF_11499247	GTSMLPEL	S32	LITHRVVEKTSE	H74	GDNLPREDEPV		D95
AF_11499380	SGSMEPHL	S45	PIIHRAIAYVHK	H101	GDNVRTNQLPD		D147
AF_11499660	SDSMTPTL	S40	WTVHRVVAETDG	H76	GDHNIATDQGG		D97
FA_68140881	GISMNPEF	S387	YITHRIHEISGD	H434	GDNNKVVDYMK		D455
FA_69268583	SYSMEHSA	S50	ILYHNSALNVVT	H103	GDSGFITVGDH		D174
HM_55232061	SPSMDPHI	S100	PIIHRAMLWVEA	H162	GDNNGRYDQVG		D213
HM_55232062	SGSMEPHI	S65	PIIHRMLWVDA	H129	GDNKVTNSKYD		D183
HM_55379336	SPSMDPHI	S100	PIIHRAMLWVEA	H162	GDNNGRYDQVG		D213
HM_55379337	SGSMEPHI	S65	PIIHRMLWVDA	H129	GDNKVTNSKYD		D183
HM_55379569	SGSMSPTI	S40	LTHRVVGETEQ	H83	GDANPFTDQDG		D104
HS_15791199	SGSMEPHI	S65	PIIHRARFWVDN	H131	GDNNRSNNYYD		D184
HV_61417364	SGSMEPHM	S88	PIIHRAMFWVDD	H153	GDNNPRYDQVS		D203
HV_61417373	SGSMQPTL	S81	PIIHRVFWVED	H143	GDDNDYYDQAR		D194
HW_110666978	SGSMEPEM	S122	PIIHRAHFYVED	H187	GDANARYDQAT		D237
HW_110667554	TGSMEPTL	S41	LTHRVVAETER	H84	GDANPFTDQDG		D105
MJ_15668434	SDSMYPIM	S18	PVIHRVIDKVEF	H120	GDNNPIHDPEL		D145
MB_91773684	SGSMEPHM	S55	PIIHRAMYVEE	H112	GDNIRTNIYFD		D149
MMP_45357950	SDSMVPVM	S34	PVIHRVIDTWTD	H120	GDNNPTYDPEL		D146
MV_15029362	SNSMYPIM	S52	PVIHRIIGNYTD	H122	GDNNQDRDPEL		D148
MMS_110603338	SESMVPM	S62	PIIHRAMTYVDT	H120	GDNNPYTDQPN		D158
MK_20094467	SESMYPY	S59	PVVHRVIAKTPE	H99	GDNNPLPD PWC		D120
MTP_88952104	SGSMVPM	S48	PIIHRAMRWVEM	H124	GDHNEVIDQMA		D159
MA_20088935	SGSMKPHM	S55	PIIHRAMYWVEA	H112	GDNPTTNKHYD		D150
MBA_73668537	SGSMEPHM	S55	PIIHRAMYRVEA	H112	GDNVVTNSHYD		D150
MMZ_21227446	SGSMEPHM	S55	PIIHRAMYRVEA	H112	GDNPVTNRHYD		D150
MS_84490353	SGSMEPSF	S56	PVIHRVIDINEI	H106	GDNNEVEDPYL		D132
MH_88603654	SESMVPHM	S62	PIIHRAMFWVEE	H120	GDNNPVIDQVG		D161
MTH_15679445	SGSMEPVF	S34	PVIHRVIGVETD	H82	GDNNQDPDPAP		D108
NP_76800894	SGSMEPNM	S99	QVIHRAMLWVED	H168	GDDNGDYDQVN		D219
PT_48477996	TGSMPYVI	S274	YYAHEIIRICYI	H315	GVANPSED PMP		D340
PT_48478421	SYSMEHSN	S43	IIHHRAMFYLEW	H103	GDSGFITAGDH		D165
PA_14521652	SGSMRPVF	S42	PIIHRVRGIKQV	H82	GDNNPVPDLYE		D111
PA_14521681	SDSMTPTI	S38	WTVHRVYAITES	H74	GDNNVATDQGD		D95
PF_33359468	SNSMYPITL	S43	WTVHRVYAITSE	H79	GDNNVATDQGG		D100

Table 2. cont.

PH_14590462	SDSMVPTI	S38	WTVHRVYAITEE	H74	GDNNVATDQQD	D95
PH_14590486	SGSMEPVF	S42	PIIHRVRGIKYI	H82	GDHNPVPDIYY	D111
TK_57641638	SESMTPTI	S39	WTVHRVYAIVDG	H76	GDHNVATDQQD	D97
TK_57641972	SGSMEPVF	S42	PIIHRVRYISTV	H83	GDNNPVPDPY	D112
TA_16081507	ESMTHSD	S31	TIHRAMFYLSW	H96	GDFNLAYRGIY	?
TA_16082489	ESMQHSS	S43	VIIHRAMFYLSW	H106	GDHNLATSPFF	S173
TV_13541052	ESMEHGS	S43	IIHRAMFYLTW	H106	GDHNLATSEKF	S173
Nanoarchaeota						
NE_41615219	TPSMQHNN	S59	PLVHRVIGLRG	H126	GDNNPTIDPFV	D163

sheet structure on top of the bacterial SPI catalytic core and its function is currently unknown. It has been hypothesized that domain II is a trait in primitive archaeal SPI that has been lost in some species upon diversification (Eichler, 2002). Interestingly, also noted in these SPIs is the absence of the second conserved Asp equivalent. A serine is found in these enzymes at that position, which may be equivalent to the Ser²⁷⁸ shown to be required for optimal activity in *E. coli* SPI. These unusual archaeal SPIs may have similarities in catalysis to the P-type SPI enzymes.

The identification of paralogous copies of SPI in some archaea (Table 1) is an interesting observation given the high overall similarity of the enzymes and that not all archaea express multiple versions of the enzyme. In many bacteria, such as *E. coli*, one P-type SPI seems to suffice for the processing of secretory preproteins, although *B. subtilis* possesses five SPI paralogues. The presence of two paralogous ER-type SPIs seems to be characteristic for most eukaryotic species (Dalbey *et al.*, 1997), though the yeast *Saccharomyces cerevisiae* contains only one. A recent study on *Haloferax volcanii* (Fine *et al.*, 2006) identified two signal peptidases, Sec11a and Sec11b, both containing conserved boxes A–E, with the conserved Ser and His. Both genes are expressed and both enzymes were active in an *in vitro* assay using a reporter substrate, although different cleavage efficiencies were observed. Gene deletion studies revealed that only *sec11b* was essential for viability. Possibilities raised to explain the presence of multiple SPI paralogues in archaea included differential expression in response to growth phase/environmental cues, substrate preferences and perhaps dedication of one of the enzymes to processing substrates that subsequently go through the Tat secretion system, and the possibility that the extra copy just serves as a backup when the amount of signal-peptide-bearing substrates increases (Fine *et al.*, 2006). Further experiments are required to evaluate these possibilities and to address the question of why only certain archaea seem to possess multiple paralogues.

Signal peptidase II (SPII)

Bacterial lipoprotein signal peptides possess conserved n-, h- and c-regions but also a lipobox in the c-region with the invariant +1 cysteine residue lipid-modified prior to processing by SPII. Signal peptides with such properties

have been reported in numerous archaeal proteins (Albers *et al.*, 2006; Bardy *et al.*, 2003). However, no SPII homologues have yet been reported in any annotated archaeal genome, nor were any found by extended searches through BLAST, COG or Pfam. Many potential Tat substrates in archaea also contain a lipobox in their signal peptides (Bolhuis, 2002; Rose *et al.*, 2002; Pohlschroder *et al.*, 2005). Due to the unusual nature of archaeal lipids that may be attached to archaeal lipoproteins, an archaeal SPII equivalent may possess unusual properties rendering them undetectable by current technology. In a recent large-scale proteomics survey of two haloarchaea, *Halobacterium salinarum* and *Natronomonas pharaonis*, it was revealed that as many as one-sixth of the archaeal proteins are acetylated. However, these occur via acetylation of the N-terminal residue by N-terminal acetyltransferase, a type of modification that is independent of a lipobox sequence or the cleavage of a signal sequence (Falb *et al.*, 2006).

Prepilin peptidase (TFPP)-like signal peptidases

Archaeal flagella are composed primarily of flagellins, which are unrelated to their namesakes in the bacterial domain (Ng *et al.*, 2006). Among several unique features of the archaeal motility apparatus is the presence of signal peptides at the N-terminus of preflagellins. These signal peptides and the N-termini of mature flagellins bear significant similarity to bacterial type IV pilins, the major structural proteins of type IV pili. Type IV pili are filamentous surface structures in bacteria which allow for surface motility called twitching (Mattick, 2002). These pilins typically have unusually short signal peptides often ending in lysine-glycine, followed by a hydrophobic stretch of ~20 amino acids. They are processed by a specific signal peptidase, termed the type IV prepilin peptidase (Lory & Strom, 1997). TFPP is responsible for processing other proteins, called pseudopilins, which have a pilin-like signal peptide and N-terminus. These pseudopilins are thought to represent minor pilins found in the type IV pilus structure as well as other proteins (e.g. PulG) needed to form the 'pseudopilus' in type II secretion systems (Mattick, 2002).

The archaeal TFPP-like enzyme (FlaK, preflagellin peptidase) is responsible for processing flagellins of the euryarchaeotes *Methanococcus maripaludis* and *M. voltae* (Bardy & Jarrell, 2002, 2003). The equivalent enzyme in the

crenarchaeote *Sulfolobus solfataricus* (PibD) processed not only flagellins but also certain sugar-binding proteins (Albers *et al.*, 2003; Szabo *et al.*, 2006). These sugar-binding proteins may assemble into a filament-like structure ('bindosome'), akin to flagella/pili, that extends from the cell surface (Albers *et al.*, 2006). Furthermore, several other potential substrates of PibD, including some seemingly unrelated to either flagellation or sugar-binding activity, were proposed based on genome analysis of proteins with pilin-like signal peptide cleavage sites and a hydrophobic mature N-terminus (Albers & Driessen, 2002). Since related *Sulfolobus* species seem to lack most of these other potential substrates (Albers *et al.*, 2003), and other archaea, such as methanogens, lack sugar-binding proteins, it has been suggested that PibD was unusual among the prepilin peptidase-like enzymes in archaea in having an abnormally large number of substrates (Ng *et al.*, 2006). However, subsequently it has been reported that substrates for TFPP-like enzymes may be more numerous than initially suspected throughout the entire archaeal domain (Albers *et al.*, 2006). Although experimental proof is lacking, there are genes in several archaea that could encode type IV pilins (see Albers *et al.*, 2006) that might form the thin filaments observed in electron micrographs of many archaea. It would not be unreasonable to assume that if these represent pilins, they would be processed by the same or closely related signal peptidases that process archaeal flagellins. Indeed, in at least *Methanocaldococcus jannaschii* and *Methanococcus maripaludis*, genes that encode prepilin peptidase-like enzymes are found within putative type IV pilin gene clusters (Table 3: *M. jannaschii* GI:15669025; *M. maripaludis* GI: 45357795).

Prepilin peptidase-like enzymes are found in most archaea, including both the Euryarchaeota and Crenarchaeota branches (Table 3). Since it is necessary for flagellin processing, this enzyme should be present in all flagellated archaea. Indeed, it is found in all such cases with rare exceptions such as *Pyrobaculum*. However, since the enzyme can process substrates unrelated to flagellins as in *S. solfataricus*, the enzyme could be found in nonflagellated archaea. This is also true, as attested by a homologue found in *Methanothermobacter thermautotrophicus*, for instance.

The archaeal TFPP-like enzymes are grouped with bacterial TFPPs in COG1989. Like the bacterial enzyme, the archaeal TFPP-like enzyme is believed to have its active site on the cytoplasmic side of the cytoplasmic membrane (Bardy & Jarrell, 2003; Szabo *et al.*, 2006). Bacterial TFPPs are usually bifunctional, performing signal peptide cleavage as well as methylation of the subsequent N-terminal amino acid (Lory & Strom, 1997). In archaea, there is no evidence for N-terminal amino acid modification in processed substrates, indicating that archaeal TFPPs lack methylase activity. Interestingly, a novel subclass of bacterial TFPPs that also lacks methylase activity (exemplified by TadV in *Actinobacillus actinomycetemcomitans*) (Tomich *et al.*, 2006) has recently been identified.

In both *Methanococcus voltae* FlaK and *S. solfataricus* PibD, analysis of key amino acid residues in the substrate necessary for signal peptide processing has been reported. The sequence prior to the cleavage site for the flagellins is highly conserved, with an almost universal presence of glycine at position -1 and with a basic amino acid (lysine or arginine) at position -2. In the case of FlaK (Thomas *et al.*, 2001), the -1 glycine and -2 basic amino acid were critical for *in vitro* preflagellin processing. Among several amino acids tested, only substitution of alanine at -1 allowed processing, albeit poorly, a case mirrored in TFPPs. Similar results were determined for PibD (Albers *et al.*, 2003) although a glycine or alanine at -1 led to similar levels of processing. PibD was less stringent than FlaK in the residues allowed at the -2 position, again supporting the broader substrate specificity of PibD. Requirements of the archaeal TFPP-like enzymes for amino acids at positions downstream of the processing site have not been as well explored. However, in all known archaeal flagellins, the +3 position is occupied by a glycine residue. In the case of *M. voltae* flagellins, a site-directed change of this glycine to valine resulted in nonprocessing, showing the importance of residues within the mature portion of the protein in the cleavage reaction (Thomas *et al.*, 2001). Inactivation of *flaK* in *M. voltae* led to nonprocessed flagellins and nonflagellated cells, indicating the necessity of proper flagellin processing in filament assembly (Bardy & Jarrell, 2003).

TFPPs represent a novel family of aspartic acid proteases (LaPointe & Taylor, 2000). Site-directed mutagenesis of FlaK and PibD indicated that both aspartic acids residues that aligned with the essential residues for bacterial TFPP activity were also essential in the archaeal enzymes (Bardy & Jarrell, 2003; Szabo *et al.*, 2006). These two essential aspartic acid residues are found in all archaeal prepilin peptidase-like enzymes (Table 3), clearly indicating the same catalytic mechanism for the enzymes from the two prokaryotic domains. Interestingly, the second conserved aspartic acid in archaeal and bacterial TFPPs is found within a motif resembling that found in presenilin aspartic acid proteases (γ -secretase), i.e. G(A)xGD (Steiner & Haass, 2000). The importance of the glycine residue immediately preceding the catalytic aspartic acid (Steiner & Haass, 2000), as well as the X amino acid (Yamasaki *et al.*, 2006), for catalytic function and substrate identification has been demonstrated in the case of the γ -secretases but no studies have been performed on the corresponding amino acids in the archaeal enzymes.

Signal peptide peptidase (SPP)

SPPs, found in all three domains of life, are enzymes that cleave signal peptides after their removal from the precursor proteins by signal peptidases. Eukaryotic SPPs are members of the polytopic GxGD aspartic acid proteases that include TFPPs and γ -secretases (Yamasaki *et al.*, 2006), whose catalytic mechanism is dependent on two aspartate residues. Eukaryotic SPPs are intramembrane enzymes that have been shown to cleave peptide bonds in the plane of the lipid bilayer, unlike conventional proteases with active sites

Table 3. Partial alignment of archaeal prepilin peptidase-like signal peptidase candidates indicating sequences around catalytically important amino acids

Abbreviations for organisms as in Table 1. ECO, *Escherichia coli*. PUA, *Pseudomonas aeruginosa*. Shading key as in Table 2. Numbers indicate the position of the catalytically important aspartic acid residues determined from the GenBank accession number. In some cases there are potential downstream in-frame start sites that yield positions for the catalytic aspartic acids that are more typical. These cases are shown in parentheses.

Bacteria					
ECO_7674168	SAWLTAAASVIDLDHQWLPDVFT	D143	LRKEALCMGDEVLLFAALGGWVG	D208	
PUA_15599724	TWGLLAMSLLIDADHQLLPDVLV	D149	TGKEGMCYGDFFKLLAMGAWGG	D213	
Crenarchaeota					
SA_70605985*	SIMLAHTSYLDIKKREVDLKIW	D21	YRFSMMCGADLILSLIISLSNA	D78	
SS_15897089	IIMLIHTSILDLKYREVDPKIW	D23	YKLSLLCGADLFLNVILSLANA	D80	
ST_15922589	SIMLIHTSFLDLKTRIDDKIWI	D27	YWLSLMCGADLFLSLIISFSNA	D84	
Euryarchaeota					
AF_11498541	LGFLLYACKLDLESRIVPNRVW	D21	YLIGAYCGADAKAIMAIAVIFP	D84	
HM_55379184†	VPVFGWAAYRDVKTTRVPNRTW	D24	WLIGGFGGADAKAFMLIAVLFP	D93	
HS_15791095*	VPALGWAAYRDIHTRRVSNTLW	D25	WYIGGFGGADAKAFMVLAVLFP	D92	
HW_110666997	MPILVWAAWRDVKTRRLPSYLW	D24	WAIGGFGGADAKVLMTIAILIP	D96	
MJ_15669025	FILLLLAAITDIKERIIPHKYT	D22	ILSIGMCGGDKVLFALAPIFA	D84	
MJ_15669092	AIGLLIASIYDLKSREIEDYVW	D22	MFLLGVGCGDGKILMGALIP	D83	
MJ_15669469	LICSTYGAVEDWRKREVTDFLW	D19	YYLNFMCGGDCCKFLMGLSYLKG	D116	
MB_91772142	LLFLLYSCYSDIKTRRVTKLW	D21	FYFGAFGGADAKILMVLISLIP	D82	
MMP_45357795	FLLLITATYTDIKERIIPHFVI	D26	ILGVGMCGGDKVMFTALSPILFA	D88	
MMP_45358118	VIGLLLASVQDFRSREIEDYIW	D18	MFLSGIGCGDGKILIGLALVP	D79	
MV_21070279	LLGLLIASIQDIKSREIENYIW	D18	MFVLGIGGADGKILMGALIP	D79	
MMS_110602842	GITLLIYASILDAKERRVPHKTW	D29 (18)	QAVNLFGGADAYALIIITACIP	D93 (82)	
MA_20094140	LAVATVAAITDLKWGIVPNRLT	D19	NVLGVLCGGDVKLLPSLTLFLH	D76	
MA_20089408	MPFLLYACYTDLKARRVSNKVV	D21	FQLGAFGGGDAKGLIVLSILFP	D82	
MA_20091424	SLFLVLACYTDLRYRRVSNRSC	D18	FKKGCFCGGDAKSLILISLFP	D72	
MA_20091920	MAFLLYSCYSDLKARRVSNGVW	D39 (21)	FHLGAFGGGDAKGLLVLSILFP	D100 (82)	
MA_20093290	LPFLLYSSYLDLETRRVPNRIW	D21	FRLRVFGGADAKALIVIGTLVP	D82	
MH_88604154	LITLLYGCREDIRERAVPVVMW	D33 (22)	TRFHLMCMAATKALILITVLVP	D98 (87)	
MBA_73669011	IPFLLYSCYSDLNSRRVSNKVV	D21	FQVGAFGGGDAKGLIVLSILFP	D82	
MMZ_21227780	MPFLLYGCYTDLKERRVSNKVV	D25 (21)	FQLGAFGGGDAKGLIVLSILFP	D86 (82)	
MS_84489263	TVCCIIATYTDVKYGIIPNKLT	D25	WYLGWLWAGGDKVLFALSTLLV	D85	
MTH_15678449	LLACFYASYSDIKRGIIPNRLT	D25 (22)	WRMVAVAGGDKVLFATVSLLP	D87 (84)	
NP_76801289	VPFAFAAAYRDIETRRIPNRTW	D53 (26)	WRIGGFGGADAKAIITLAVAFP	D127 (100)	
PA_14521786	VIVGILTSYTDIKTSYIIEEHF	D18	YYTGGWASGDVILGAYSALLP	D105	
PF_18976843	ILVGLTSYTDIKTSYIIEERF	D19	YYTGGWASGDVILAAFSLLP	D106	
PH_14590360	TIVGILTSYTDVKTSYIIEEHF	D18	YYTGGWASGDVILGAYSALLP	D105	
TK_57639988	LLTGILTSYTDIKTGFFVDVHA	D20	YYIGAWASGDVILAAFSALLP	D111	
TA_16081397	AATAVYSSVSDIKRRSVNSFTF	D27	FRETLCIGIDVKAETSyllAFQ	D118	
TV_13542171	LPLLIFGSHSDVKKRSINSFTF	D25	FGELLEFGIGDIKGIIVSVLLFS	D116	

*SA_70605985 and HS_15791095 as annotated are missing both conserved aspartic acid residues. However, using potential upstream in-frame start sites, both proteins align over their full length with other archaeal enzymes and the two conserved aspartic acid residues are found. These versions are used here.

†HM_55379184 as annotated starts after both conserved aspartic acid residues. We have used the TIGR annotation of this gene (NT01HMA2463), which uses an upstream start codon.

exposed to an aqueous environment (Lemberg & Martoglio, 2004). In bacteria, only limited data have been published on SPPs – while they have been identified in *E. coli* and *B. subtilis* as enzymes acting in concert with additional protease(s) to degrade signal peptides (Bolhuis *et al.*, 1999; Novak & Dev, 1998), their mechanism of action is currently unreported. First insight into the catalytic mechanism of prokaryotic SPPs comes from the enzyme SppA of the hyperthermophilic archaeon *Thermococcus*

kodakaraensis. SppA efficiently cleaved peptides with a relatively small side chain at the P1 position and a hydrophobic or aromatic residue at the P3 position. The positively charged Arg residue was preferred at the P4 position. Any substrates with an acidic residue at P2, P3 or P4 were not cleaved (Matsumi *et al.*, 2005).

Many of the amino acid residues that are highly conserved amongst the SPPs of bacteria and various archaeal species

Table 4. Partial alignment of archaeal signal peptide peptidase candidates indicating sequences around catalytically important amino acidsAbbreviations for organisms as in Table 1. BSU, *Bacillus subtilis*. Shading as in Table 2.

Bacteria				
BSU_2635437	GSMAASGGYYI	S147	KSGAHKDIMSPSREM	K199
Crenarchaeota				
PAE_18313592	NGLAASGAYYV	S113	TTGPLKYYGRELTDY	K162
PAE_18314240	AGLAASGAYYT	S158	TTGPFKYYGMDLTEF	K207
TP_110606247	KGLLASGAYMA	S125	KSGELKDVGSPYRPM	K177
Euryarchaeota				
FA_68140244	EGIGASGAYWL	S76	KVGKYKDINSFPRPM	K128
HM_55377494	TDVCASGGYDI	S151	TAGEYKDAGVPLKEM	K203
HM_55378760	EGTAASGGYYG	S120	RSGPDKAQISKDSLRL	K171
HS_15789821	RGPAASGGYYT	S119	KSAPDKGTTGPADKA	K166
HW_110667987	TDTCASGGYWI	S138	TAGEYKDAGVPLREI	K190
HW_110668686	EGTAASGGYFG	S122	RSGPDKAQITRDGLR	K173
HW_110668977	QGVASGAYYG	S120	RTGPDKAQPTTEQRR	K165
MJ_15668832	EGLDASGAYMV	S128	KAGKYKDIGSPFRPM	K180
MB_91773876	GDVAASAAYYI	S229	KLGSFKDVGGDWRL	K281
MMP_45358632	ENMGASAAYYQA	S121	TGGKYKDIGTPTRSM	K173
MA_20092840	GDLAASAAYYI	S210	KSGEFKDMGSSWRGL	K262
MBA_73668088	GDLAASAAYYI	S213	KSGEMKDMGSTWRGL	K265
MMZ_21226968	GDLAASAAYYI	S225	KSGEFKDMGGTWRGL	K277
MTH_15678828	SDSGTSGAYLA	S114	KAGEYKDMGADYRMI	K166
NP_76800828	TDACASGGYWI	S155	TAGEYKEAGVPFDDL	K207
NP_76802382	DTMGASGAYLS	S117	TTGPDKRGPSTEQQR	K161
PT_48477283	EGIGASGAYWL	S76	KIGKYKDINSFPRHM	K128
PA_14520806	SGYAYSGAYYI	S164	KTGPYKDMGADWRGL	K216
PF_18977955	SGYATSGGYI	S160	KTGPYKDMGADWRGL	K212
PH_14591350	SGYAYSGAYYI	S163	KTGPYKDMGADWRKL	K215
TK_57641099	GDIIASGGYYI	S162	KTGKHKDMGAEWRL	K214
TA_16081247	QGICASGAYWI	S76	KVGKYKDLSSYREP	K128
TV_13540860	LGICASGAYWI	S76	KSGKYKDMTSPFSEP	K128
Nanoarchaeota				
NE_41615105	AQYGTSAASYWI	S103	SKGKYKEFSNPLLPL	K155

were targeted for mutagenesis in SppA to determine those essential for enzymic activity. Ser¹⁶², believed to act as the nucleophilic residue, and Lys²¹⁴, believed to be the general base, are both invariant in archaeal SPPs (Table 4). Strikingly, histidine and aspartic acid residues that comprise the catalytic triad of many serine proteases are not conserved. Site-directed mutagenesis of various other amino acid residues in the *T. kodakaraensis* enzyme caused a reduction in activity and, though not essential or completely conserved, these residues (e.g. Arg²²¹) may play a role in catalysis. The data obtained suggest that the catalytic centre comprises a Ser-Lys dyad and not the usual Ser-His-Asp catalytic triad found in the majority of serine proteases. The alignment data clearly indicate that likely all archaea SPPs function in the same way. The proposed catalytic serine is present also in the SPPs of *B. subtilis* and *E. coli*. While the *B. subtilis* SPP also possesses the conserved lysine as the general base, the enzyme in *E. coli* does not; it has been suggested that this enzyme uses different residues for catalysis (Matsumi *et al.*, 2006).

Concluding remarks

While studies are still in their infancy, indications are that in regard to signal peptidases, archaea have their own unusual

twists. Archaeal SPI more closely resembles its eukaryal counterpart, requiring an essential His for catalysis but with the archaeal modification of needing only one Asp. Archaeal TFPPs have a catalytic mechanism that resembles that of the corresponding bacterial enzymes but again with archaeal specifics. The archaeal enzymes lack the methylase activity commonly associated with most bacterial TFPPs and the substrates include flagellins and sugar-binding proteins that are unique to archaea. Studies on the archaeal SPP pioneer the field of prokaryotic signal peptide peptidase research. Site-directed mutagenesis and alignment data clearly show archaeal SPPs to have a Ser-Lys catalytic dyad mechanism and this information may translate to bacterial systems as well. Still to be resolved is how archaeal proteins with apparent SPII signal peptides are processed, since SPII is as yet unidentified in any archaeon. One must also be cognizant of the fact that current programs used to detect archaeal signal peptides are trained on bacterial and eukaryal databases; it is highly possible that some archaeal-specific signal peptides have been missed. It has already been noted that some archaea express proteins with unusual signal peptides (Eichler, 2000). Proteomic analysis of secreted proteins from *Methanococcoides burtonii* is a recent, rare

example combining *in vivo* and *in silico* approaches to further refine our ability to recognize archaeal signal peptide sequences (Saunders *et al.*, 2006) and in turn contribute to our knowledge of archaeal signal peptidases.

Note added in proof

Recently, Szabo *et al.* (*J Bacteriol* 2006 Nov 17; [Epub ahead of print] PMID: 17114255) identified, throughout the archaea, a variety of diverse proteins, including possible pilins, that have class III signal peptides, which are cleaved by a prepilin peptidase-like enzyme distinct from FlaK/PibD.

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