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# Translocation of proteins across archaeal cytoplasmic membranes

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**Abstract**

All cells need to transport proteins across hydrophobic membranes. Several mechanisms have evolved to facilitate this transport, including: (i) the universally-conserved Sec system, which transports proteins in an unfolded conformation and is thought to be the major translocation pathway in most organisms and (ii) the Tat system, which transports proteins that have already obtained some degree of tertiary structure. Here, we present the current understanding of these processes in the domain Archaea, and how they compare to the corresponding pathways in bacteria and eukaryotes.


*Keywords:* Sec system; Tat system; Protein translocation; Archaea

**Contents**



## 1. Introduction

### 1.1. The Archaea

Phylogenetic analyses of 16S ribosomal RNA sequences suggest that all living organisms can be classified into three domains [1]. Extensive biochemical and

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genetic data support this basic classification in which all eukaryotic organisms constitute one domain, while *Bacteria* and Archaea comprise two distinct prokaryotic domains. Despite the morphological resemblance between archaeal and bacterial cells, the archaea are as distantly related to the bacteria as they are to the eukaryotes. In fact, the structure of the archaeal genome (e.g., presence of histones and nucleosomes), as well as several aspects of the archaeal transcription machinery (e.g., archaeal homologs of transcription factor IIB (TFIIB) and RNA polymerase subunits; the presence of archaeal TATA boxes) more closely resemble their eukaryotic counterparts [2–4]. Thus, the domain Archaea represents a group of organisms distinct from the other two domains.

Based upon the habitats from which archaea have been isolated, it is evident that some of the organisms of this domain occupy unique environmental niches. The conditions of such environments may include a lack of oxygen, salt concentrations close to saturation, temperatures above the boiling point of water, pH levels as low as 0.5 or as high as 12, depths with dramatically increased hydrostatic pressures, or a combination of several of these conditions [5–7]. Recently, the availability of numerous complete archaeal genome sequences has provided a vast amount of data revealing unique adaptations used by these organisms for life in the ''extreme'' environments in which they are often found. However, while the archaea may be the dominant organisms in such ''extreme'' environments, they are ubiquitous and may be found in much more benign environments, such as ordinary garden soil [8–10]. Studying and understanding archaea and their adaptations to environmental parameters is undoubtedly crucial to identifying and understanding their ecological roles within a complex web of organisms from all domains of life. To date, in silico, in vivo, and in vitro analyses of archaeal cellular processes have not only led to a better understanding of life under extreme conditions, but have also provided novel insights into bacterial and eukaryotic cellular function. One such cellular process is that of protein translocation.

### 1.2. Protein translocation

The process of protein translocation into or across hydrophobic membranes is essential to all living organisms. The Sec pathway is most widely used for this process [11]. Transport of proteins via this pathway occurs either co- or post-translationally, and involves the recognition and targeting of the proteins to the Sec translocon, a proteinaceous membrane-spanning pore [12]. The core components of this pore, as well as the signal recognition particle (SRP), which plays an integral role in the targeting of co-translationally translocated proteins, are present in organisms of all domains of life, and have been well-characterized in bacteria and eukaryotes [12,13]. In addition to the universally conserved Sec components, archaea possess a combination of components found in both bacteria and/or eukaryotes (Tables 1 and 2). Interestingly, archaea lack a component with significant homology to the translocon-associated ATPases (SecA and Kar2p) required for the translocation of many bacterial and eukaryotic proteins, respectively [13]. Thus the mechanism and energetics of archaeal Sec-mediated protein translocation, which might have features distinct from those of the bacteria and eukaryotes, is poorly understood.

In addition to translocation via the Sec pathway, certain archaeal proteins are secreted via the twin arginine translocation (Tat) pathway which, unlike the Sec pathway, secretes folded proteins [14,15]. Translocation

Table 1
Conservation of SRP and SR components in the three domains of life

| Component | Eukaryotes | | Bacteria | | Archaea | |
|---|---|---|---|---|---|---|
| | *Homo sapiens* | *Saccharomyces cerevisiae* | *Escherichia coli* | *Bacillus subtilis* | *Halobacterium* sp. NRC-1 | *Sulfolobus solfataricus* |
| SRP complex | SRP9 | Srp21p[a] | – | – | – | – |
| | SRP14 | Srp14p[b] | – | – | – | – |
| | SRP19 | Sec65p | – | – | SRP19 | SRP19 |
| | SRP54 | Srp54p | Ffh | Ffh | SRP54 | SRP54 |
| | SRP68 | Srp68p | – | – | – | – |
| | SRP72 | Srp72p | – | – | – | – |
| | 7S RNA | 7S RNA | 4.5S RNA | ScRNA | 7S RNA | 7S RNA |
| | – | – | – | HBsu | – | – |
| SR | α | Srp101p | FtsY | Srb | Dpa | FtsY |
| | β | Srp102p | – | – | – | – |

–No homologs identified.
[a] Distant homolog of SRP9. See text for details.
[b] Two copies are present.

Table 2
Conservation of Sec components in the three domains of life

| Component | Eukaryotes[a] | | Bacteria | | Archaea | |
|---|---|---|---|---|---|---|
| | *Homo sapiens* | *Saccharomyces cerevisiae* | *Escherichia coli* | *Bacillus subtilis* | *Halobacterium* sp. NRC-1 | *Sulfolobus solfataricus* |
| Sec61α | • | Sec61p[b] | SecY | SecY | SecYh | SecYh |
| Sec61γ | • | Sss1p | SecE | SecE | SecEh | SecEh |
| Sec61β | • | Sbh1p[b] | – | – | Sec61βh | Sec61βh |
| TRAM | • | – | – | – | – | – |
| BiP | • | Kar2p | – | – | – | – |
| Sec62p | Sec62 | • | – | – | – | – |
| Sec63p | Sec63 | • | – | – | – | – |
| Sec71p | – | • | – | – | – | – |
| Sec72p | – | • | – | – | – | – |
| SecG | – | – | • | SecG | – | – |
| SecD | – | – | • | SecDF | SecDh | – |
| SecF | – | | • | SecDF | SecFh | – |
| YajC | – | – | • | YrbF | – | – |
| YidC | – | – | • | SpoIIIJ[b] | YidCh[b] | – |
| SecB | – | – | • | SecB | – | – |
| SecA | – | – | • | SecA | – | – |

– No homologs identified.
[a] Excluding mitochondria.
[b] Additional homologs present.
• Component present is the one listed.

by both mechanisms involves the recognition of an N-terminal signal sequence. These signal sequences show some structural similarities (Fig. 1), but differ enough such that each class can be distinguished by its corresponding secretion machinery.

The study of archaeal protein translocation is a relatively new endeavor, and the most well-characterized archaea are extremophiles, making the study of these processes challenging. However, recent advances in the field have led to in vivo data that complement extensive in silico data available for organisms of this domain. In this review, we will discuss the information revealed by genetic, genomic, and biochemical analyses concerning these pathways in the context of bacteria and eukaryotes, and how in some archaea one possible adaptation to extreme environments may involve the preferential use of the Tat pathway over the universally conserved Sec pathway.

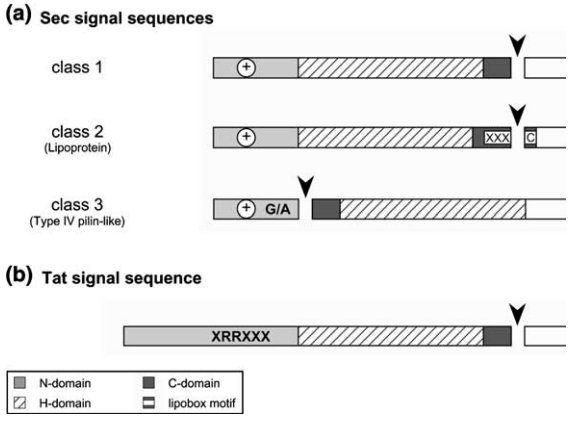


Fig. 1. Structures of (a) Sec and (b) Tat signal sequences. See text for details. Arrows designate signal peptidase cleavage sites.

## 2. Sec-mediated protein translocation

### 2.1. Targeting of proteins destined for Sec translocation

Prior to passage through the Sec translocon, proteins must be targeted to this pore. While post-translationally translocated proteins are directed to the translocon by chaperones, targeting of co-translationally translocated proteins requires the universally conserved SRP. The information required for targeting in both modes of translocation is encoded in the signal sequence, located in the extreme N-terminus of the preprotein.

#### 2.1.1. Sec signal sequences and their signal peptidases

Signal sequences of Sec substrates fall into three categories (Fig. 1(a)). While the majority of Sec substrates possess the universally conserved class 1 signal sequences, specific subsets of proteins are targeted to the Sec pore by two additional classes of signal sequences (classes 2 and 3; see below). All class 1 signal sequences, while showing almost no amino acid sequence homology to one another, share three structural characteristics: (i) a positively charged N-terminus (N-domain, 1–5 amino acids); (ii) a core of at least six hydrophobic amino acids

(H-domain, 7–15 amino acids); and (iii) an uncharged polar C-terminal region (C-domain, 3–7 amino acids) (Fig. 1(a)) [16]. Analyses of bacterial and eukaryotic class 1 signal sequences suggest that the charged amino acids of the N-domain interact with negatively charged phospholipids at the cytoplasmic face of the membrane, thus orienting the N-terminus of the signal sequence into the cytoplasm during translocation. The H-domain promotes insertion of the signal peptide into the membrane. The hydrophobicity of the H-domain is thought to be the primary determinant of whether it will be recognized by the SRP and secreted co-translationally, or by chaperones and secreted post-translationally [17,18]. The C-domain is recognized by the type I signal peptidase, which, upon translocation cleaves the signal sequence from the mature domain of the protein (see below).

One hallmark of these signal sequences is their interchangeability between eukaryotic and bacterial proteins [16]. Recent evidence suggests that this phenomenon is true for bacterial and archaeal class 1 signal sequences as well (Pohlschröder, unpublished data). A fusion of the Cell Surface Glycoprotein signal sequence (CSG-ss) from the archaeon *Haloferax volcanii* to the mature portion of the *Escherichia coli* periplasmic protein alkaline phosphatase (PhoA; normally active only in the periplasm), was expressed in *E. coli*. This fusion protein (CSG-ss/PhoA) exhibited enzymatic activity, indicating that it was properly targeted to the membrane and translocated into the periplasm. In addition, pulse-chase experiments revealed that the CSG-ss was cleaved from PhoA, albeit less efficiently than the native PhoA-ss, suggesting that it functions as a bona fide signal sequence (Fig. 2(b)). Computational analyses of signal sequences from the euryarchaeon *Methanocaldococcus jannaschii* [19] and the crenarchaeon *Sulfolobus solfataricus* [20] suggest that (i) these signal sequences possess a charge distribution similar to those of bacteria; (ii) the composition of their H-domain is unique, and (iii) their signal peptidase recognition site is similar to that of the eukaryotes. These observations, coupled with the observed low efficiency of translocation of the CSG-ss/PhoA fusion protein, suggest that some differences in class 1 signal sequences exist among the domains.

Class 1 signal sequences are cleaved from the preprotein by the membrane-associated type I signal peptidase (SPase), producing the mature protein. In bacteria, the so-called P-type signal peptidase consists of the catalytic core (domain I), and in some cases a domain II whose role is unknown [21]. The eukaryotic homolog of this SPase, SPC18, is part of the ER membrane-bound hetero-oligomeric signal peptidase complex (SPC) [22]. In contrast to the P-type SPase, SPC18 does not contain a domain II, and its catalytic site involves a Ser–His–Arg triad rather than the bacterial Ser–Lys dyad for cleavage [22–24]. It is intriguing that while all known archaeal type I SPases possess the Ser–His–Arg triad, some of them also possess a domain that exhibits homology to the bacterial domain II [20,25].

Two additional classes of bacterial Sec signal sequences that possess distinct signal peptidase recognition sites have been identified. Certain bacterial lipoproteins that are secreted via the Sec apparatus possess class 2 signal sequences (Fig. 1(a)) which contain a lipobox motif ([I/L/G/A]–[A/G/S]–C) that is recognized by the type II signal peptidase [26]. Cleavage of the class 2 signal sequence by this SPase results in a mature protein possessing an N-terminal cysteine residue, to which a diacylglycerol moiety is added, allowing membrane anchoring [27]. Archaea possess proteins with a motif that resembles the lipobox [28–30] and recent data suggest that lipid modification occurs at the conserved cysteine [31]. However, the fact that no type II SPase

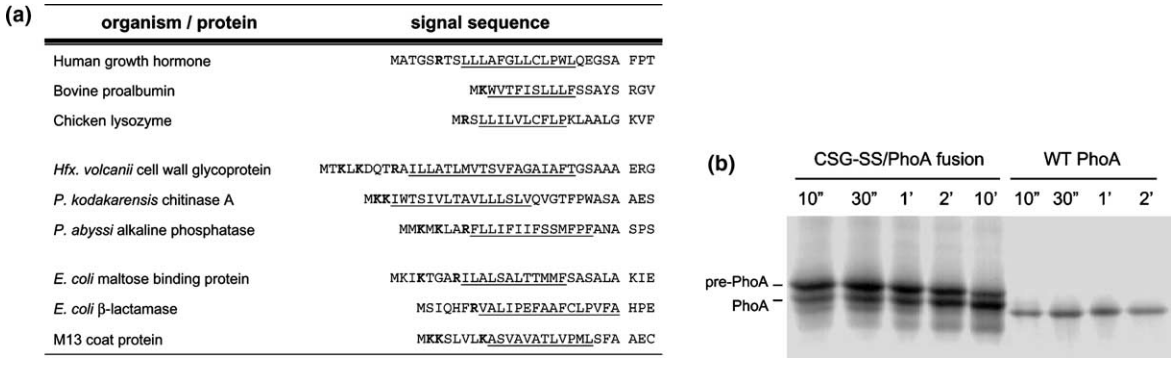


Fig. 2. (a) Class 1 Sec signal sequences demonstrate conservation of structure, but not amino acid sequence, throughout the three domains of life. The charged residue(s) preceding the H-domain (underlined) are in bold text; the signal peptidase cleavage site is represented by a space. (b) Recognition of an archaeal Sec signal sequence by *E. coli* and kinetics of signal sequence processing. Cells were pulse-labeled with [$^{35}$S]methionine for 20 s and chased for indicated times. Precursor and mature forms of the wild-type alkaline phosphatase (PhoA) and of a fusion of mature PhoA to the *Hfx. volcanii* cell surface glycoprotein signal sequence (CSG-ss/PhoA) were immunoprecipitated, separated by SDS–polyacrylamide gel electrophoresis, and autoradiographed.

homolog has been identified in organisms of this domain suggests that the archaea might employ a different enzyme for the cleavage of these signal peptides [20,30].

Finally, bacterial type IV pili are targeted to the Sec pore via class 3 signal sequences (Fig. 1(a)). Distinct from class 1 and class 2 signal sequences, class 3 signal sequences contain a highly conserved cleavage site between the N and H domains that is recognized by the type IV pilin peptidase (Fig. 1(a)) [32]. Cleavage of this signal sequence thus retains the hydrophobic region as part of the mature protein which plays an essential role in the biogenesis of the pilus. The archaea synthesize their flagella (which more closely resemble bacterial pili) as preproteins with class 3 signal sequences [33], which have been shown to be cleaved from the preprotein by a preflagellin peptidase [34]. Interestingly, recent analyses of signal sequences from the thermophilic archaeon *S. solfataricus* demonstrated that several sugar-binding proteins contain a typical class 3 signal peptide [20,35]. N-terminal sequencing of the mature protein has confirmed that cleavage occurs at the predicted motif [36]. Furthermore, the *S. solfataricus* homolog of the bacterial type IV prepilin peptidase has recently been shown to cleave both sugar-binding proteins and flagellin precursors [37]. While retention of the hydrophobic region of the flagellar subunit is likely to play a role in assembly similar to that in bacterial pili, it is not clear why the sugar-binding proteins retain their hydrophobic core.

### 2.1.2. Post-translational targeting

Post-translational translocation, or translocation of a protein after at least a portion of the mature protein has been synthesized, is the dominant mode of transport for secretory proteins in bacteria, and is employed for certain proteins destined for targeting to the yeast secretory pathway. Chaperones that are found in all domains of life, as well as those that are organism-specific, have been identified to be involved in targeting post-translationally translocated proteins to the Sec pore and maintaining their translocation competent states.

In *E. coli*, the cytoplasmic protein SecB binds preproteins as a homotetramer of 16 kDa subunits [38] and maintains the proteins in an unfolded conformation [39]. A hydrophobic region of SecB interacts with the mature portion of the preprotein preventing the formation of higher-order folded structures [40]. SecB can target precursor proteins to the translocon due to its ability to interact with SecA, a bacterial secretion-specific ATPase that is complexed to the bacterial translocation pore (SecYEG; see below) [41,42]. The probable sequence of events in targeting an envelope protein for export begins with the binding of SecB to the precursor protein. This results in targeting of the preprotein to SecYEG, through its affinity for SecA [43]. It is believed that this interaction triggers the release of the precursor protein from SecB, and its binding to the SecA–SecYEG translocase through the interaction of SecA and the signal sequence in a synchronous fashion.

While SecB is the best studied chaperone involved in post-translational protein translocation, it is dispensible for growth of *E. coli* and appears to be required for only a subset of exported proteins. Furthermore, SecB is not universally distributed within the domain bacteria, with homologs largely restricted to the Gram-negative bacteria. Other chaperones play roles in targeting and stabilization of unfolded preproteins, as over-expression of general cytoplasmic chaperones, such as DnaJ/K and GroEL, has been shown to complement some bacterial secretion defects and permit export of heterologous proteins [44]. Moreover, the general chaperone DnaK appears to play a role in post-translational export of native outer membrane proteins [45]. In contrast to SecB, DnaJ/K homologs, which have been identified in organisms of all domains of life [46,47], have also been shown to be crucial for eukaryotic protein translocation. In the yeast cytoplasm for example, Ydj1p and Ssa1p (DnaJ/Hsp40 and DnaK/Hsp70 type chaperones, respectively), maintain the translated polypeptide in a translocation competent state. Very little is known about post-translational targeting in archaea. To date, a highly divergent putative *secB* homolog has only been identified in *Methanococcus janaschii*, and this is likely due to a lateral gene transfer event from a bacterium to this archaeon. Furthermore, the putative role of general cytoplasmic chaperones in the targeting of archaeal proteins to the Sec pore has yet to be determined.

### 2.1.3. Co-translational targeting: The signal recognition particle-mediated pathway

It is generally believed that most (if not all) protein translocation into the mammalian ER, as well as insertion of proteins into the bacterial cytoplasmic membrane and yeast ER membrane, occurs co-translationally (reviewed in [12,48]). While post-translationally translocated proteins are targeted by chaperones, the cytoplasmic SRP and the membrane-associated SRP receptor (SR) are required for the targeting of co-translationally translocated proteins. Although universally conserved, the complexity and composition of the SRP varies substantially among the domains of life.

*2.1.3.1. The eukaryotic SRP-mediated targeting pathway.* The eukaryotic SRP is required for co-translational protein translocation and membrane protein insertion. Originally isolated from canine microsome preparations [49], the mammalian SRP and its mechanism of targeting are the most well-characterized.

*2.1.3.2. The mammalian SRP cycle.* Targeting by the mammalian SRP occurs in a cyclic manner, termed the SRP cycle. During the first step of the cycle, the SRP binds to the signal sequence of a nascent polypeptide emerging from a translating ribosome (Fig. 3(a)) [50,51].

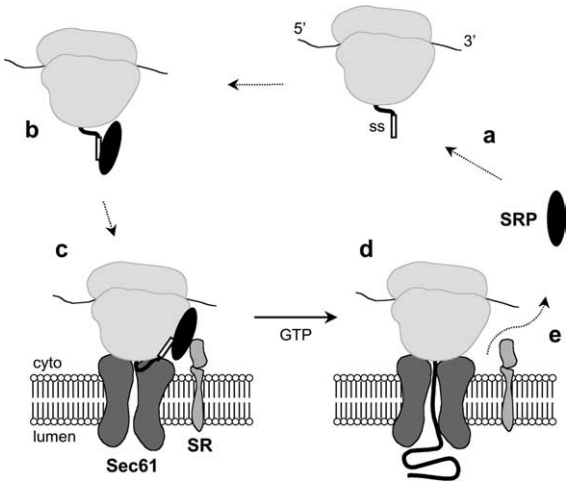


Fig. 3. The mammalian signal recognition particle (SRP) cycle. See text for details. SS, signal sequence; SR, signal recognition particle receptor.

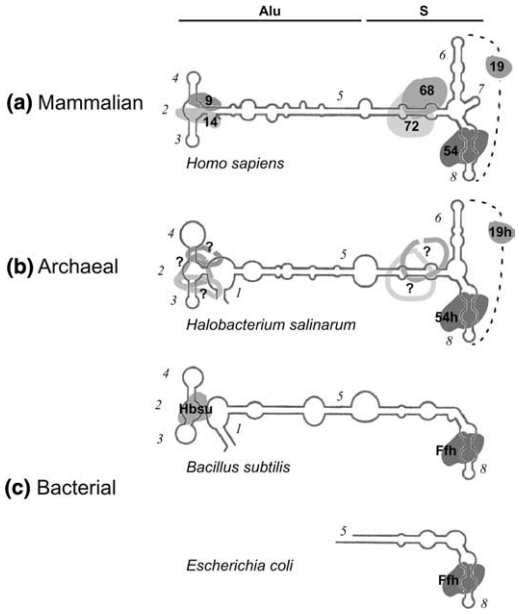


Fig. 4. The composition of SRP in the three domains. Numbers in italics represent RNA helix designations as defined by Larsen and Zwieb [59]. Non-italicized numbers label the corresponding SRP component, and question marks label components which have not been identified by sequence homology; h, homolog. For clarity, binding of SRP19 to the tips of helices 6/8 is represented by dashed lines. RNA size is exaggerated in comparison to protein components so that similarities and differences in helices can be more readily observed. Adapted from [51].

Once bound, translation of the protein is slowed by the SRP (termed ''translational arrest''; Fig. 3(b)), which prevents premature folding of the protein [52]. The SRP then binds the ER membrane-bound SR (Fig. 3(c)), and the ribosome binds the Sec pore [53]. A functional junction between the SRP/ribosome complex and the ER can only occur in the presence of GTP, which is required for three processes essential for co-translational translocation: (i) release of the signal sequence from the SRP; (ii) detachment of the SRP from the SR; and (iii) release of translational arrest and resumption of peptide elongation (Figs. 3(d) and (e)) [54,55]. Once released from the signal sequence and detached from the SR, the SRP then returns to the cytosol, thus completing the cycle.

*2.1.3.3. Components of the eukaryotic SRP*. The mammalian SRP is an 11S ribonucleoprotein complex consisting of a single 7S RNA molecule plus six proteins (SRP9, 14, 19, 54, 68, and 72, corresponding to their kilodalton masses; Fig. 4(a), Table 1) [49,56,57].

The 7S RNA subunit has long been characterized as a scaffold for SRP assembly (reviewed in [51]). However, recent in vitro crystallography data indicate that 7S RNA, in concert with the SRP54 component (see below), plays an integral role in the signal sequence recognition function of the SRP [58]. Its secondary structure can consist of 1–8 helices (depending on the organism), and can be divided into the Alu and S domains (Fig. 4) [59]. Helix 8 is found in all known 7S RNA homologs, and of the 8 helices it demonstrates the highest degree of sequence conservation throughout the domains of life [60].

The SRP54 subunit, required for the signal sequence-binding function of the SRP, binds helix 8 of 7S RNA (Fig. 4) [60]. Structurally, it consists of three domains: (i) an N-terminal N-domain; (ii) the ras-like G-domain, which binds GTP; and (iii) the C-terminal methionine-rich M-domain, which binds signal sequences of nascent polypeptides [51,61,62]. It is believed that binding of SRP54 to 7S RNA is facilitated by the binding of the SRP19 component to the tips of helices 6 and 8 of the 7S RNA S-region (Fig. 4) [60,63–67]; however, additional alternative functions cannot be excluded.

The translational arrest step of the SRP cycle (Fig. 3(b)) is facilitated by the SRP9/14 heterodimer, which binds the 7S RNA via helices 2–4, as well as part of helix 5 in the Alu region (Fig. 4) [68–70]. It has been observed that SRP complexes lacking this heterodimer do not arrest elongation, but still promote protein translocation [70,71].

Finally, the SRP68/72 heterodimer binds the S-region of 7S RNA [63], and is thought to function in the interaction of SRP with SR and in ribosome docking [51,72,73].

The composition of the *Saccharomyces cerevisiae* SRP differs slightly from that of the mammals, as (i) the mammalian 9/14 heterodimer is replaced by the Srp14p homodimer [74]; and (ii) the Srp21p component (identified to be a distant SRP9 homolog; E. Hartmann, personal communication) is present (Table 1) [73]. While loss of a functional SRP in *S. cerevisiae* (harboring a knockout in the gene encoding Srp54p) causes severe growth defects, it does not result in cell death [75].

*2.1.3.4. The eukaryotic SRP receptor*. Binding of the SRP to the ER membrane is mediated by the SR [76]. The heterodimeric SR [77] consists of an $\alpha$ subunit

(which binds the SRP54 subunit) and a β subunit (which anchors the SR heterodimer to the ER membrane). Both components exhibit GTPase activity [78,79], and in conjunction with SRP54 participate in the GTPase cycle involved in the recycling of the SRP [54,80].

*2.1.3.5. The bacterial and chloroplast SRP-mediated targeting pathways*. Previous work has demonstrated that the bacterial SRP-mediated targeting pathway functions primarily in the co-translational insertion of integral membrane proteins [81–83]. In contrast, the chloroplast SRP (cpSRP) is integral to the post-translational targeting of the light harvesting chlorophyll proteins (LHCP) to the thylakoid [84].

*2.1.3.6. The bacterial SRP*. While homologs of the 7S RNA and SRP54 components are conserved in all domains of life (Table 1) [13,65,83,85], the bacterial SRP appears to be much simpler than that of the eukaryotes (Fig. 4(c)). Extensive studies of the *E. coli* SRP indicate that it consists only of an SRP54 homolog (Ffh, or P48; binds the signal sequence of nascent proteins and exhibits GTPase activity) and a truncated homolog of the RNA component (4.5S RNA, or Ffs; lacks the Alu domain) (Fig. 4(c)) [86–89]. The scRNA (7S RNA homolog) of *Bacillus subtilis*, however, contains both Alu and S domains yet lacks helix 6 as well as a homolog of the SRP19 subunit (Fig. 4(c)) [90–92]. Copurification of the *B. subtilis* SRP complex revealed that the 10 kDa histone-like protein HBsu binds the Alu domain of the *B. subtilis* scRNA (Fig. 4(c)), and is a third component of the *B. subtilis* SRP (Table 1) [91]. The SRP in both organisms is essential, as depletion of SRP components results in cell death [93–95].

*2.1.3.7. The bacterial SRP receptor*. FtsY, the bacterial homolog of SRα, is thought to act as the bacterial SRP receptor, as it is essential, binds Ffh, and exhibits GTPase activity [61,87,88,96]. However, since (i) no bacterial homolog of SRβ has been identified and (ii) FtsY possesses no predicted membrane-spanning domains; the mechanism of membrane association of FtsY has been elusive. Recently, the N-terminal AN domain of FtsY was implicated in the binding of phosphatidylethanolamine (and an unidentified protein) in the *E. coli* cytoplasmic membrane, thus suggesting a mechanism by which FtsY associates with the membrane in the absence of an SRβ homolog [97]. The authors of this study suggested that this mechanism involves two steps, whereby FtsY first associates with the membrane via electrostatic interactions between the FtsY-AN domain and phosphatidylethanolamine. Once ''targeted'' to the membrane, FtsY can then bind the unidentified membrane protein component (hypothesized to be the translocon, but not examined), thus resulting in its proper localization on the membrane [97].

*2.1.3.8. The chloroplast SRP and SRP receptor*. The cpSRP consists of two subunits, cpSRP54 (SRP54 homolog) and cpSRP43 (no identified homolog), and lacks an RNA component [84,98]. Furthermore, a chloroplast homolog of FtsY has been shown to be involved in thylakoid targeting [99].

*2.1.3.9. The archaeal SRP-mediated targeting pathway*. Early studies examining the SRP of the haloarchaeon *Halobacterium salinarum* suggested that it is involved in the insertion of integral membrane proteins [100], similar to the *E. coli* SRP. However, studies employing (i) in silico analyses of completely sequenced archaeal genomes [13,67,85]; (ii) crystalization of archaeal SRP components [101,102]; (iii) in vitro reconstitution of heterologously expressed archaeal SRP components identified by sequence homology [67,103–105]; and (iv) in vivo copurification and characterization of the archaeal SRP [106], suggest that the archaeal SRP-mediated targeting pathway shares similarities with those of both the eukaryotic and bacterial pathways, while possessing unique archaeal characteristics.

*2.1.3.10. Components of the archaeal SRP*. Genome analyses employing homology searches have demonstrated that the archaeal SRP contains homologs of the universally conserved SRP54 and 7S RNA components, as well as a homolog of the SRP19 subunit (Table 1) [92]. Comparison of 7S RNA homologs among the three domains of life revealed that the high degree of sequence conservation observed in helix 8 does not seem to hold for the other helices, suggesting that the SRP may have different compositions among the domains of life (Fig. 4) [51,65]. The archaeal 7S RNA homolog was initially described in the halophile *H. salinarum* [107–109]. While its overall structure most closely resembles that of the eukaryotic 7S RNA (presence of helix 6 in S-domain; Fig. 4), it is interesting to note that the Alu domain of the archaeal 7S RNA more closely resembles that of the scRNA from the bacterium *B. subtilis* (presence of helix 1; compare Figs. 4(b) and (c)) [92].

Similar to the eukaryotic SRP54 and bacterial Ffh, the archaeal SRP54 homolog possesses GTPase activity and can specifically bind 7S RNA [103]. Furthermore, crystallization of the *Acidianus ambivalens* SRP54 homolog has confirmed the presence of a GTPase domain [101]. Amino acid sequence comparisons of SRP54 and its homologs from organisms of the three domains of life suggest that the archaeal SRP54 is more similar to the eukaryotic SRP54 than to the bacterial Ffh [106,110]. However, recent knockout studies demonstrating that a chromosomal deletion of the gene encoding the *Hfx. volcanii SRP54* homolog (*hv54h*) can only be obtained when a complementing *hv54h* is provided in trans, indicates that, as in *E. coli*, the archaeal SRP54 homolog is essential [106].

The identification of an SRP19 homolog in completely sequenced archaeal genomes is consistent with previous observations that the presence of helix 6 in an organism's 7S RNA was positively correlated with the presence of an SRP19 homolog [60]. Numerous recent biochemical and structural studies suggest that, as observed for the mammalian SRP, this component is required for the binding of the SRP54 homolog to 7S RNA. One in vitro reconstitution study using *Archaeoglobus fulgidus* SRP components demonstrated that significant binding of the SRP54 homolog to 7S RNA required the presence of the SRP19 homolog; however, a low level of binding occurred in its absence [67]. Furthermore, crystallization of the *M. jannaschii* SRP19 homolog bound to the S-domain of human 7S RNA suggests that the SRP19 homolog binds helices 6 and 8 of the RNA, resulting in a conformational change in the RNA which facilitates binding of the SRP54 subunit [102]. Two other independent studies, however, suggest that the SRP54 homologs of *A. fulgidus* [111] and *Pyrococcus furiosus* [104], unlike the eukaryotic SRP54, have a relatively high intrinsic affinity for their cognate 7S RNA homologs (~15 and ~18 nM, respectively). Thus, although the archaeal SRP19 homolog may in fact play an important role in SRP assembly, the requirement for this component in SRP assembly may not be absolute, and the SRP19 homolog may have additional, as yet undefined functions.

Although sequence homology searches have not identified archaeal homologs of SRP9, SRP14, SRP68, or SRP72, the conservation of 7S RNA nucleotide sequence and secondary structure within this domain suggests that additional, possibly archaea-specific, SRP components might be present. Recent in vivo studies have demonstrated that the archaeal homologs of the conserved SRP components (SRP54, SRP19, 7S RNA homologs) can be copurified from *Hfx. volcanii*, thus providing not only the first evidence that these components interact in vivo, but also the methodology to search for additional SRP components [106]. In fact, preliminary co-immunoprecipitation studies (using antibodies specific for the archaeal SRP54 homolog), as well as copurification studies (using affinity-tagged SRP components), suggest that the archaeal SRP contains additional subunits to those already identified (Rose and Pohlschröder, unpublished data). Further analyses will determine whether these components: (i) are homologs of known SRP components that have not yet been identified in the ongoing *Hfx. volcanii* genome sequencing efforts; (ii) have similar functions to known eukaryotic and/or bacterial SRP subunits, but show little to no sequence similarity; or (iii) possess unique, archaea-specific functions.

*2.1.3.11. The archaeal SRP receptor homolog*. Aspects of archaeal SRP recognition may also be universally conserved, as all sequenced organisms of this domain possess a homolog of the SRα/FtsY component [112,113]. However, similar homolog to bacteria, the archaea lack a homolog of the SRβ subunit, the membrane anchor of the eukaryotic SR. Interestingly, a study characterizing the *Sulfolobus acidocaldarius* FtsY homolog suggested that little (if any) FtsY homolog was associated with the cytoplasmic membrane [113]. Amino acid alignments of archaeal FtsY homologs demonstrate a short region of homology in the extreme N-terminus; however, little or no sequence conservation among FtsY homologs is observed in the remainder of the region corresponding to the *E. coli* FtsY-A domain (Fig. 5), which was recently shown to be important in the association of FtsY with the *E. coli* cytoplasmic membrane (see above) [97]. Furthermore, the FtsY homologs of two moderate halophilic archaea *Hfx. volcanii* (GenBank Accession No. AY187867) and *Haloarcula marismortui* (http://zdna2.umbi.umd.edu/cgi-bin/blast/blast.pl) possess extended N-termini, as compared to FtsY homologs of other archaea, including the extreme halophile *Halobacterium* sp. NRC-1 (Fig. 5). With the observed diversity in the N-termini of the FtsY homologs, in combination with the absence of an SRβ homolog, it will be interesting to characterize the way in which this protein interacts with the membrane, as well as its involvement in SRP-mediated targeting in the archaea.

Although many studies have focused on gaining a better understanding of the archaeal SRP-mediated targeting pathway, the role of this complex in archaea is still not understood. Further in vivo and in vitro analyses are needed to reveal the composition of the SRP, as well as the substrates targeted by this pathway.

### 2.2. The Sec translocator

Once targeted to the membrane, translocation of fully or partially synthesized Sec substrates occurs via the Sec translocon. Analyses of the bacterial and eukaryotic Sec machinery revealed that, while homologs of the core

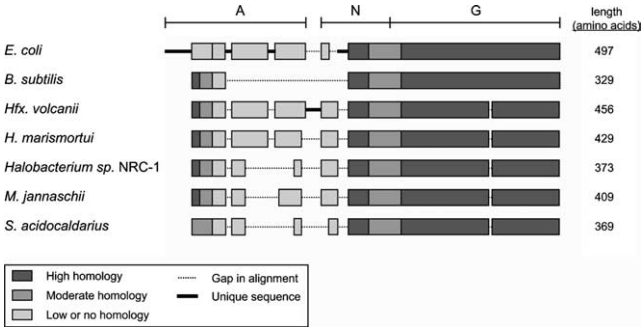


Fig. 5. Schematic representation of bacterial and archaeal FtsY homologs demonstrating the heterogeneity of the N-terminal A domain. See text for details. FtsY domain designations (A, N, and G) are based on the structure of the *E. coli* FtsY.

components (SecY/Sec61α) and (SecE/Sec61γ) are universally conserved, components associating with these subunits are distinct in the two domains [13,114]. Database searches reveal the presence of archaeal proteins with significant homology to both bacterial and eukaryotic Sec components (Table 2), and suggest that the archaeal Sec machinery is composed of a combination of homologs of components from the two other domains. Surprisingly, extensive genomic analyses have failed to identify putative translocation-associated ATPases in archaea [13]. In the absence of an obvious energy-coupling component, it is unclear how translocation of polypeptides across the cytoplasmic membrane is effected.

*2.2.1. The Sec translocation pore: SecYEG/Sec61αβγ*

The core components of the Sec membrane pore, SecY and SecE in the cytoplasmic membrane of bacteria, and the homologous proteins Sec61α and Sec61γ in the eukaryotic ER membrane, are universally conserved and have been shown to be essential for protein translocation both in vivo and in vitro [115–118].

Both the size and membrane topology of SecY/Sec61α are well-conserved. The 10 transmembrane domain topology of *E. coli* SecY is representative of its homologs in the other domains [119]. In contrast, the topologies of the SecE/Sec61γ homologs vary. While the majority of SecE homologs contain only one membrane-spanning segment, most proteobacteria, including *E. coli*, contain three membrane spanning segments. Interestingly, a truncated carboxy-terminal domain of this SecE, corresponding in sequence to the canonical single membrane spanning segment form, can functionally substitute for the intact protein [120]. Furthermore, a distant SecE homolog of *B. subtilis* that contains a single membrane spanning segment can substitute for *E. coli* SecE function in vivo [121]. In fact, subunits of the translocator from these divergent bacteria can complement one another [122].

In both bacteria and eukaryotes these components form a membrane complex together with a third membrane protein, designated SecG and Sec61β in bacteria and eukaryotes, respectively [114,115,123]. However, the evolutionary relationship between these third subunits is less clear. Although there is a short amino acid consensus motif suggestive of common ancestry, the overall amino acid sequence conservation of these small proteins is too weak to infer homology with certainty [124]. The *E. coli* protein SecG, is a small (11.5 kDa) highly hydrophobic protein with two transmembrane domains and a central hydrophilic cytoplasmic domain. SecG has been shown to stimulate ATP hydrolysis by SecA in vitro [125], and remarkably, the protein undergoes an apparent complete inversion in its membrane topology during each translocation cycle [126–128]. In contrast to the involvement of SecG in post-translational protein translocation, Sec61β, which generally possesses only a single membrane spanning segment in the C-terminal region, has been shown to be in close proximity to signal sequences of co-translationally translocated proteins [129,130].

Electron microscopic studies of the eukaryotic translocator reveal a proteinaceous ring surrounding an aqueous channel through the membrane [131–133]. The size of the ring structure suggests an oligomeric association of Sec61αβγ heterotrimers combining to form an active channel. Recent work by Bessoneau and colleagues suggest that SecYEG complexes assemble spontaneously and reversibly into dimers and higher-order multimers (of heterotrimers), and that the dimers represent the active translocation species [134]. The authors also demonstrated that the addition of crosslinker favors dissociation of the dimeric species into monomeric crosslinked SecYEG, which accounts for the fact that previous groups observed intra-trimer crosslinks, but no crosslinks between SecYEG trimers [134–136]. While a great deal of new data has accumulated in recent years, a consensus view of the structural aspects of the translocation process has yet to emerge. An encouraging advance in understanding the structure of the translocation complex has come from the work of Breyton and colleagues [137] who have used two-dimensional crystal lattices to produce a three-dimensional map of the *E. coli* SecYEG complex at an 8 Å resolution. In agreement with the work of Bessoneau et al., the structures observed are dimers of SecYEG complexes surrounding a $16 \times 25$ Å cavity. As models of the translocation complexes become more refined, increasing insight will be attained by comparative structural studies. In particular, it will be interesting to compare the overall congruence of the three dimensional structures among the domains of life.

Phylogenetic analyses of SecY/Sec61α and SecE/Sec61γ homologs in all domains of life indicate that the archaeal proteins are more closely related to their eukaryotic counterparts than to the corresponding bacterial subunits [13]. Consistent with this observation, archaeal proteins with significant homology to Sec61β have now been identified [124,138]. While the archaeal homolog contains the weak SecG/Sec61β consensus mentioned above [124,138], a second motif shared with the eukaryotic sequences identifies the archaeal proteins as Sec61β homologs (Fig. 6) [139]. Regardless of whether the archaeal Sec61β homolog and SecG share a common ancestor, it seems likely that the functions they perform have diverged, since neither the eukaryotes nor the archaea possess SecA homologs, whose stabilization is thought to be a major function of SecG (see above). Interestingly, while Sec61β is required during *Drosophila* development [140], the functions of SecG/Sec61β have been shown to be dispensible in both bacteria and eukaryotes [141–143]. A combination of in vivo and in vitro analyses will be necessary to decipher the roles of the individual translocation pore subunits in archaea.

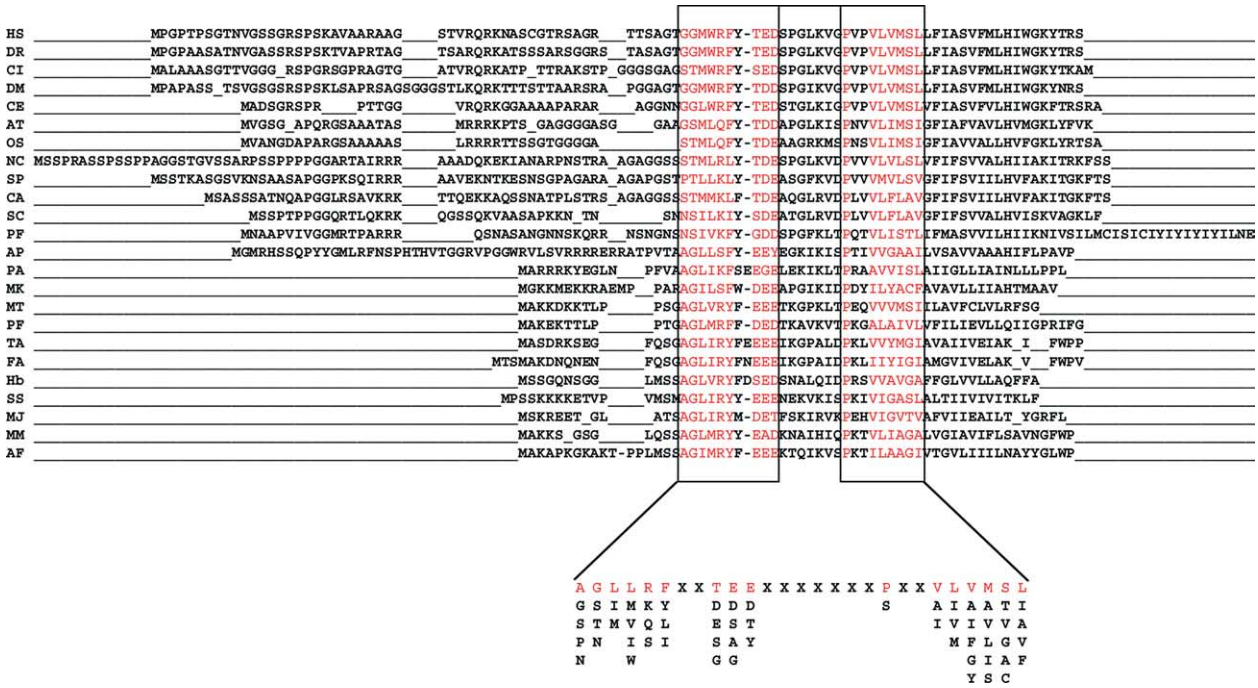

Fig. 6. Alignment of selected members of the Sec61β protein family. Sequence data were obtained from NCBI or from the Stanford Genome Technology Center website (at http://www-sequence.stanford.edu/group/candida). Sequence alignment was performed using ClustalX, and improved manually. The following motif derived from this sequence alignment identifies all known Sec61β homologs in the SwissProt/Trembl (http://us.expasy.org/sprot/) database, with no false positives [GSPAN]-[GSTN]-[LIM]-[MVILW]-[RKQS]-[FYLI]-x(1,2)-[DETSG]-[DESAG]-[DETY]- x(7,8)-[PS]-x(2)-[AVI]-[IVLM]-[AVIFGY]-[AVLIMS]-[TVSGAC]-[LIAVF] [139]. Residues that match the consensus for Sec61β listed above are printed in red. In the expanded box below the alignment, the most common residue at each position in the alignment is shown at the top of each column, with the other permitted residues listed below it. Sequences are abbreviated as follows: HS, *Homo sapiens* (gi|5803165|); DR, *Danio rerio* (gi|21317206|); CI, *Ciona intestinalis* (gi|24492721|); DM, *Drosophila melanogaster* (gi|21356199|); CE, *Caenorhabditis elegans* (gi|17543194|); AT, *Arabidopsis thaliana* (gi|15225401|); OS, *Oryza sativa* (gi|15528732|); NC, *Neurospora crassa* (gi|28927088|); SP, *Schizosaccharomyces pombe* (gi|19113224|); CA, *Candida albicans* (Contig6-2098); SC, *Saccharomyces cerevisiae* Sbh1 (gi|6320932|); PF, *Plasmodium falciparum* (gi|23612798|); AP, *Aeropyrum pernix* (gi|14600867|); PA, *Pyrobaculum aerophilum* (gi|18313309|); MK, *Methanopyrus kandleri* (gi|20093648|); MT, *Methanobacterium thermoautotrophicum* (ref|NC_000916.1|); PF, *Pyrococcus furiosus* (gi|18978388|); TA, *Thermoplasma acidophilum* (gi|16082665|); FA, *Ferroplasma acidarmanus* (gi|22406132|); Hb, *Halobacterium* sp. NRC-1 (gi|15791336|); SS, *Sulfolobus solfataricus* (ref|NC_002754.1|); MJ, *Methanocaldococcus jannaschii* (ref|NC_000909.1|); MM, *Methanosarcina mazei* (gi|21227474|); AF, *Archaeoglobus fulgidus* (gi|11499365|).

### 2.2.2. Accessory Sec components

Bacterial and eukaryotic translocons are associated with additional components, some of which are specific for co- or post-translational protein secretion, while others are involved in membrane protein insertion.

In *E. coli*, four integral membrane proteins co-purify with the SecYEG complex. Three of these proteins, SecD, SecF, and YajC are encoded by a single operon. Although the *secDFyajC* operon is not essential, mutations in the operon result in a strong general protein export defect [144–147]. While homologs of SecD and SecF are present in organisms of both prokaryotic domains, YajC is found only in some bacteria. The predicted topologies of SecD and SecF indicate that they have large periplasmic domains, suggestive of an important extracytoplasmic function [144]. One possibility is that these proteins play a role analogous to mammalian BiP in sealing the outside face of the idle translocator, thus preventing the non-specific passage of ions [133]. This, coupled with the observation that antibodies against SecD prevent the release of translocated proteins from the inner membrane of *E. coli* spheroplasts, suggested a role for SecDF at a late step in the translocation process [148]. However, in vitro analyses have also suggested that one role of these membrane proteins might be to regulate the membrane cycling of SecA [125,149,150]. In addition to the observations described above, recent biochemical studies showed that the heterotrimeric SecDFYajC complex mediates the interaction of a fourth accessory Sec subunit, YidC, with the core translocon [151–153]. At physiological protein concentrations, this membrane protein, which is intimately involved in the insertion of membrane-spanning segments into the cytoplasmic membrane [154], copurifies with the SecYEG complex only in the presence of the SecDFYajC complex [155]. Furthermore, overexpression of wild-type YidC complements the growth defect of a SecDFYajC-defective strain [155], and overexpressed YidC is stabilized by increasing the expression of SecDFYajC [154]. Taken together, these studies suggest that by virtue of its

interaction with YidC and the translocator SecDF may play an important role in membrane protein integration.

Although archaea lack SecA homologs, and the archaeal core translocon components are more similar to their eukaryotic counterparts, the majority of completely sequenced euryarchaeal genomes possess homologs of the bacterial SecD, SecF, and YidC components. While some bacteria and archaea contain a YidC homolog but no SecDF homologs, every organism that possesses SecDF homologs also has at least one YidC homolog. This observation supports the idea that in archaea, as is apparently the case in bacteria, the SecDF homologs play a role in membrane protein insertion. The fact that some bacteria and archaea appear to have a YidC homolog but no SecDF homologs does not contradict this hypothesis, as YidC homologs have been shown to mediate Sec-independent membrane protein insertion in both mitochondria and bacteria [156,157]. Alternatively, it is conceivable that the YidC homologs in microorganisms lacking SecDF can bind directly to the Sec pore.

Neither SecDF nor YidC are found in ER membranes. Instead, ER membrane protein insertion is thought to be mediated by TRAM, an ER membrane protein that binds to Sec61α. Upon lateral opening of the Sec61 translocator, TRAM has been suggested to facilitate release of nascent transmembrane segments into the lipid bilayer [158,159]. Although an analogous process is likely to occur in prokaryotes to allow the integration of membrane proteins, TRAM has no known prokaryotic homolog. The corresponding function in archaea and bacteria is presumed to be performed by YidC [160,161].

Additional accessory proteins in the yeast *S. cerevisiae*, designated Sec62p and Sec63p, form a heterotetrameric ER membrane complex (with Sec71p and Sec72p) that supports post-translational translocation when associated with a translocon containing one of two yeast homologs of Sec61α, Sec61p [143,162,163]. Sec63p has a DnaJ-type motif, and interacts with the lumenal DnaK-chaperone Kar2p. Interestingly, a second yeast translocator, which contains a distinct homolog of Sec61α, Ssh1p, does not interact with Sec62p/Sec63p, and does not support post-translational translocation [143]. However, translocation pores containing either Sec61α homolog are capable of supporting SRP-dependent co-translational translocation. Human homologs of Sec62p and Sec63p have been identified, and while these proteins co-purify with the ER Sec61 complex, it is unclear if they play a conserved role in post-translational secretion [164].

Homologs of neither the mammalian nor the yeast components associated with the Sec core have been found in any of the sequenced archaeal genomes to date, despite the fact that the archaeal core components show higher homology to the eukaryotic counterparts than to those in bacteria [13]. In vivo analyses, including copurification studies of the archaeal Sec pore components, as well as genetic analyses, should aid in determining whether additional components are involved in the archaeal Sec pathway.

### 2.3. Energetics

While bacteria export proteins mainly post-translationally, most eukaryotic protein export is thought to occur in a co-translational manner. The observation that archaea seemingly possess a mixture of components from these disparate systems prompts the question of whether co- or post-translational translocation predominates in archaea. At the heart of this issue is the fundamental question of the source of the energy driving archaeal protein export, particularly since no homologs of known bacterial and eukaryotic ATPases involved in protein translocation have been identified in archaea.

The bacterial SecA ATPase is required for post-translational protein secretion, and may also be involved in the translocation of large periplasmic domains of some cytoplasmic membrane proteins [165,166]. In yeast, Kar2p, an ER lumenal ATPase, is required for both co- and post-translational protein translocation, and acts as a "Brownian ratchet" providing directionality to protein translocation [167]. In in vitro assays, the directionality function provided by Kar2p can be substituted by antibodies [167] and in the case of biotinylated proteins, by avidin in the lumen of the proteoliposome vesicles [168]. Additional lumenal translocation functions are provided by Lhs1p (another DnaK-like chaperone) [169], and Sls1p/Sil1p (a GrpE-like nucleotide exchange factor) [170]. In contrast, no exogenous energy source is thought to be required for mammalian co-translational protein translocation, as in vitro reconstitution studies suggest that the elongation of nascent polypeptide chains is sufficient to drive proteins into the ER [171].

Since Kar2p functions extracytoplasmically, it is not surprising that homologs are absent from archaeal genomes, since ATP is not readily available in the extracellular environment of prokaryotes. It is remarkable, however, that no protein with significant homology to SecA has been identified in any of the completely-sequenced archaeal genomes. The failure to find an archaeal SecA or extracytoplasmic Kar2p homolog raises a number of fascinating issues.

Based on paradigms from known protein translocation systems, four models of the energetics of archaeal Sec-mediated protein export are depicted in Fig. 7. Post-translational export models, in which translation does not contribute to the energy of translocation, are depicted in Figs. 7(a–c).

In Fig. 7(a), a cytoplasmic motor protein or protein complex is pictured driving translocation through the Sec pore. If such a mode of export occurs in archaea, the

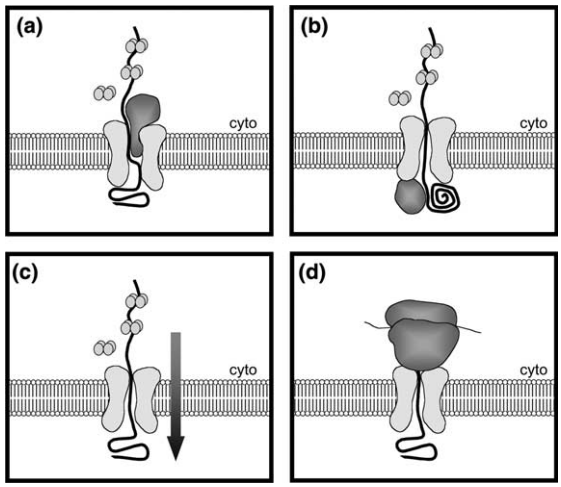


Fig. 7. Models of putative archaeal protein translocation energetics. See text for details (Section 2.3). (a) Post-translational translocation with a cytoplasmic energy-coupling protein. (b) Post-translational translocation with extracytoplasmic activity. (c) Post-translational translocation harnessing a gradient (e.g., ΔpH) across the cytoplasmic membrane. (d) Co-translational translocation.

failure to identify SecA homologs in these organisms may be due to the fact that either (i) the protein(s) involved, although performing a function analogous to SecA, are unrelated to SecA in structure and/or mechanism; or (ii) that an archaeal SecA-like protein with similar structure and function exists, but lacks any significant amino acid sequence conservation. Several such examples of structural and functional convergent evolution exist among the serine proteases [172–174], as well as in a number of viral coat proteins from eukaryotic viruses and bacteriophage [175–177].

In a second post-translational export model, as depicted in Fig. 7(b), one or more extracytoplasmic activities might drive protein translocation, that thermodynamically favor outward movement of the polypeptide. These activities may include alteration of the properties of the protein as it reaches the external side of the membrane, and might either be non-covalent (e.g., binding of the emerging polypeptide chain, or assisted folding), or might be due to a covalent addition, such as glycosylation. In either case this would provide directionality by preventing movement of the polypeptide chain back into the cytoplasm. Since the external environment of prokaryotes is not likely to contain useful concentrations of free nucleotides, either the extracytoplasmic proteins must function without an external energy source (as is the case for periplasmic chaperones in *E. coli*) or any energy requirements would have to be provided from within the cell and transduced across the membrane.

Fig. 7(c) shows a third possible energy-coupling mechanism. In this case, a gradient across the membrane (such as the proton motive force, pmf) provides the sole source of energy for protein translocation. In vitro data suggests that the pmf is sufficient to drive translocation of proteins via the Tat pore (see below). Alternatively, multiple energy coupling mechanisms may act together to effect transit through the archaeal translocon, as has been shown to be the case in *E. coli*, where the pmf facilitates secretion via the Sec pore in concert with the action of SecA [178,179].

In contrast, the co-translational mode of translocation may harness the energy of translation itself to extrude the polypeptide through the Sec pore to its extracytoplasmic destination, much like that in mammals (Fig. 7(d)). An exclusively co-translational mode of export would provide an appealing explanation for the absence of SecA- or Kar2p-like ATPases in archaea. However, it should be noted that recent pulse-chase analyses of two fully-translated, precursor hybrid proteins suggest that in the archaeon *Hfx. volcanii*, at least these proteins can be exported following a general translation arrest. In these experiments the signal sequence of a haloarchaeal S-layer glycoprotein was fused to either the *Hfx. volcanii* dihydrofolate reductase, or to the cellulose binding domain of the *Clostridium thermocellum* cellulosome [180]. In separate experiments, insertion of *Halobacterium* sp. NRC-1 bacterioopsin fusion constructs into the *Hfx. volcanii* cytoplasmic membrane was shown to occur post-translationally, however, membrane integration of bacterioopsin in its native host occurs co-translationally [181–183].

Further in vivo and in vitro analyses will be necessary to define the role of co- and post-translational protein translocation in the export of archaeal Sec substrates. An archaeal in vitro protein translocation assay will also be crucial to determine the energetics of co- and/or post-translational protein translocation. The ongoing development of such systems in a variety of archaeal species should reveal novel insights into archaeal Sec translocation mechanisms in the coming years.

## 3. The twin arginine translocation pathway

Investigation of protein translocation in chloroplasts and bacteria has recently led to the identification of a novel Sec-independent translocation mechanism (reviewed in [184,185]). Numerous in vitro studies have suggested that protein transport across cellular membranes via this route is driven solely by the proton motive force, although this feature has been challenged by recent in vivo results. [186–192]. While functional and sequence homologies exist between the chloroplast and bacterial pathways, separate designations for this pathway in the two domains have endured: ΔpH pathway in chloroplasts and twin arginine translocation (Tat) pathway in bacteria (for simplicity, the pathway will be referred to as the Tat pathway in this review). As in the Sec pathway, secreted

Tat substrates require an N-terminal signal sequence and translocation is thought to occur through a proteinaceous membrane pore [193,194]. However, unlike the Sec pathway, the translocation of Tat substrates can take place after the precursor has achieved some degree of tertiary structure in the bacterial cytoplasm or chloroplast stroma [14,195–197].

Recent in silico and in vivo analyses suggest that many archaea also employ this translocation pathway [198–200]. Here, the current understanding of the Tat pathway in bacteria and chloroplasts, and its proposed utilization by organisms of the archaeal domain are reviewed.

### 3.1. Tat signal sequences

Similar to the structure of class 1 Sec signal sequences, Tat signal sequences possess a conserved tripartite organization: an N-terminal region containing positively-charged amino acids (N-region) followed by a stretch of hydrophobic residues (H-region) and a C-terminal region that may contain a type I SPase cleavage site (C-region) (Fig. 1(b)) [201]. However, distinct from class 1 Sec signal sequences, Tat signal sequences exhibit amino acid sequence conservation, as they possess a highly conserved motif containing double arginine residues [193,194]. The significance of these double arginines in Tat targeting and transport was initially shown in chloroplasts, where substitution of the Arg–Arg with Gln–Gln or Arg–Lys blocked translocation, and substitution with Lys–Arg severely impaired translocation across the thylakoid membrane [193]. Similar analyses performed using additional chloroplast as well as bacterial and archaeal Tat signal sequences suggest that this motif is critical for proper precursor transport [196,199,202–205]. To date, only two bacterial Tat-dependent secretory proteins that lack the double arginines in their signal sequences have been identified [206,207].

In addition to the presence of double arginines, Tat signal sequences possess a number of defining characteristics, including sequence conservation flanking the double arginines (forming a consensus S/T-R-R-X-F-L-K motif), which was first noted in *E. coli* Tat substrates [194]. Although these surrounding amino acids are not as highly conserved as the double arginines, a subset of amino acids with similar biochemical properties are commonly found at each position in Tat signal sequences. The positions immediately before and after the double arginines are commonly occupied by polar amino acids, whereas nonpolar residues are frequently found in the two positions originally designated F and L. Furthermore, the Tat signal sequence H-region has a lower overall hydrophobicity compared to the H-region of Sec signal sequences. Studies in *E. coli* have shown that increasing the hydrophobicity of the signal sequence of a natural Tat substrate (TorA) directs a fusion protein to the Sec pathway [201]. In addition, the presence of a charged residue in the C-region of the Tat signal sequence has been suggested to act as a ''Sec-avoidance'' signal, as opposed to a Tat determinant [208,209]. However, the importance of this charge in Tat specific export remains ambiguous, as secretion of a Sec substrate (apocytochrome *c*) fused to a Tat signal sequence (HyaA) occurs in a Sec-dependent fashion in *E. coli* [210]. Finally, Tat signal sequences are typically longer than Sec signal sequences, which is commonly the result of an extended N-region. Despite this wealth of information on the structural and sequence characteristics of Tat signal sequences, the role of these features in substrate targeting and translocation is unclear.

The defining characteristics of Tat signal sequences have made it possible to analyze entire genomes for the presence of putative Tat substrates. Early genome-wide identification of Tat signal sequences using only the twin arginine motif followed by multiple hydrophobic amino acids as search criteria have been fruitful, but this method drastically overestimates the number of proteins secreted via the Tat pathway. Thus, a more stringent computational means of predicting Tat substrates is necessary, especially when dealing with organisms in which usage of the Tat pathway is not well characterized, as is the case for most archaea. A new approach to identify Tat substrates was inspired by the proposal that haloarchaea translocate the majority of their secretory proteins via the Tat pathway (http://www.sas.upenn.edu/~pohlschr/). The large pool of predicted secreted proteins containing typical Tat signal sequences (from a sequenced haloarchaeal genome, as well as numerous secreted proteins from a diverse group of prokaryotes), provided valuable information concerning the constraints of Tat signal sequences and led to the development of a Tat substrate-identifying Perl program, TATFIND [199]. This program defines a Tat substrate as any protein that possesses an $(X^{-1})R^{0}R^{+1}(X^{+2})(X^{+3})(X^{+4})$ motif within the first thirty-five amino acids of the protein (where each position X represents a defined set of permitted residues) and has an uncharged stretch (H-region) of at least thirteen amino acids. Although designed mainly using archaeal Tat signal sequences, TATFIND accurately identifies all previously characterized double arginine-containing Tat substrates, suggesting that the defining characteristics of Tat signal sequences are conserved in all prokaryotes.

### 3.2. Tat substrates

A number of characteristics of the Tat pathway distinguish it from the Sec system, most notably the ability to translocate cytoplasmically- or stromally-folded proteins. This observation originated from chloroplast translocation studies, in which analysis of Tat precursors revealed that the stromal intermediate of the chloroplast Tat substrate 23K is resistant to protease degradation

prior to translocation, suggesting that the protein is folded properly before its secretion [14]. Possibly the most convincing evidence for this phenomenon was generated by expressing GFP fused to the TorA signal sequence in *E. coli*, resulting in periplasmically localized and functional GFP. Since GFP is only capable of folding in the cytoplasm, this indicates the protein is folded prior to translocation via the Tat pathway [197].

The majority of originally-identified bacterial Tat substrates are cofactor-containing redox proteins, implying that the bacterial Tat pathway is a minor secretory pathway maintained primarily for the secretion of this subset of proteins [194]. Indeed, the recent development and use of TATFIND revealed that a number of organisms appear to use the Tat pathway mainly for the secretion of a small number of redox proteins [200]. The selective pressure driving translocation of redox proteins via the Tat pathway is most likely the necessity for cytoplasmic integration of co-factors. This process is expected to initiate or even require proper folding prior to transport, and therefore render these substrates incompatible with the Sec system.

However, in contrast to *E. coli*, some bacteria encode a large number of putative Tat substrates that are almost exclusively non-redox proteins, including binding proteins and metabolic enzymes (http://www.sas.upenn.edu/~pohlschr/tatprok.html). It is noteworthy that while some non-redox protein homologs seem to be secreted exclusively via the Tat pathway (e.g., alkaline phosphatase D and phospholipase C homologs), the secretory route of other proteins may be organism-specific. In addition, it appears that certain protein homologs within the same organism are secreted via different pathways. As opposed to redox Tat substrates, which fold cytoplasmically due to cofactor incorporation, the biochemical pressure responsible for targeting non-cofactor containing proteins to the Tat pathway is not understood. However, it is apparent that several bacteria use the Tat pathway as a general secretion pathway.

Similar to bacteria, it appears that archaea use the Tat pathway to highly varying extents (Table 3). Using TATFIND, identification of putative Tat substrates in completely-sequenced genomes revealed that some archaea use this pathway mainly for the secretion of a small number of redox proteins. For example, of the 7 annotated proteins predicted to be Tat substrates in the thermophilic *Pyrobaculum aerophilum*, all were redox proteins. In contrast, two archaea (*M. janaschii* and *Methanopyrus kandleri* AV19) were predicted to possess no Tat substrates, which is consistent with the absence of all of the necessary Tat machinery components in these organisms. Surprisingly, halophilic archaea seem to utilize this pathway extensively, as the majority of secreted proteins from two different haloarchaea possess putative Tat signal sequences. In analyzing the *Halobacterium* sp. NRC-1 genome for its utilization of the Sec and Tat pathways, it became apparent that the majority of its secreted proteins (including binding proteins, proteases, and metabolic enzymes) possessed putative Tat signal sequences, yet homologs of these proteins in non-halophilic organisms contained predicted Sec signal sequences [199]. Further analysis revealed that many of these putative Tat substrates in *Halobacterium* sp. NRC-1 were also predicted to be lipoproteins [30,199]. A similar trend was also recognized for the nearly-completed genome of the halophilic archaeon, *Hfx. volcanii* [30,199]. This routing of the secretome to the Tat pathway is a phenomenon which to date has been found only in halophilic archaea, strongly suggesting that it is an adaptation to the high intra- and extracellular salt

Table 3
Number of putative archaeal Tat substrates and components predicted using TATFIND and PSI-BLAST, respectively

| Organism[a] | Phylum | ORFs | Number of TATFIND 1.2 positives | Tat components | | |
|---|---|---|---|---|---|---|
| | | | | A/E | B | C |
| *Halobacterium* sp. NRC-1 | Euryarchaeota | 2446 | 68 | 1 | – | 2 |
| *Pyrobaculum aerophilum* | Euryarchaeota | 2605 | 14 | 1 | – | 1 |
| *Archaeoglobus fulgidus* | Euryarchaeota | 2420 | 9 | 2 | – | 2 |
| *Aeropyrum pernix* | Crenarchaeota | 1840 | 7 | 2 | – | 1 |
| *Methanosarcina mazei Goe1* | Euryarchaeota | 3371 | 6 | 2 | – | 2 |
| *Pyrococcus horikoshii* | Euryarchaeota | 1801 | 5 | – | – | – |
| *Methanosarcina acetivorans* str.C2A | Euryarchaeota | 4540 | 5 | 2 | – | 2 |
| *Sulfolobus solfataricus* | Crenarchaeota | 2977 | 5 | 3 | – | 2 |
| *Sulfolobus tokodaii* | Crenarchaeota | 2826 | 4 | 2 | – | 1 |
| *Pyrococcus furiosus* DSM3638 | Euryarchaeota | 2065 | 3 | – | – | – |
| *Pyrococcus abyssi* | Euryarchaeota | 1769 | 2 | – | – | – |
| *Thermoplasma acidophilum* | Euryarchaeota | 1482 | 2 | 1 | – | 1 |
| *Thermoplasma volcanium* | Euryarchaeota | 1500 | 2 | 1 | – | 1 |
| *Methanothermobacter thermautotrophicus* | Euryarchaeota | 1873 | 1 | – | – | – |
| *Methanocaldococcus janaschii* | Euryarchaeota | 1729 | – | – | – | – |
| *Methanopyrus kandleri* AV19 | Euryarchaeota | 1687 | – | 1 | – | – |

[a] Organisms with fully sequenced genomes.

conditions. When faced with salt concentrations approaching saturation, the process of protein folding is further complicated by a potential increase in protein aggregation and misfolding. It is possible that by routing secretory proteins to the Tat pathway, precursors are able to fold in the controlled cytoplasmic environment where chaperones are present. Once properly folded, the perils of aggregation and misfolding are greatly reduced. It is not clear why some proteins are still secreted via the Sec pathway. However, it is intriguing that in the haloarchaeal genomes analyzed, the minor subset of putative Sec substrates is nearly identical. This implies that there is a selective pressure stronger than protein misfolding in high salt conditions that directs these substrates for Tat-independent export.

### 3.3. Tat components

The proposed proteinaceous pore through which Tat substrates are translocated is composed of multiple membrane proteins. The Tat secretory apparatus, originally defined in chloroplasts and *E. coli*, can be composed of as many as three functionally distinct proteins: TatA, TatB and TatC homologs (Tha4, Hcf106, and cpTatC in chloroplasts, respectively) [188,190,211–214]. Alternative topology predictions suggest the presence of four [215] or six [190,211] membrane spanning segments for TatC (with N- and C-terminal loops located in the cytoplasm), while TatA and TatB homologs appear to contain a single membrane-spanning domain with a C-terminal cytoplasmic amphipathic helix [216,217]. Despite the structural similarities between TatA and TatB homologs, both proteins are required for the secretion of most Tat substrates in *E. coli* and therefore are functionally non-redundant [218]. In *E. coli*, these two proteins have been shown to co-purify in a complex containing a large excess of TatA relative to TatB. Negative stain electron microscopy of this complex revealed the formation of a pore-like structure [219]. In addition, TatA has been shown to purify as a 460 kDa homooligomeric complex. This and other data has led to the hypothesis that TatA is mainly responsible for forming a Tat pore through which substrates travel across the membrane [217]. Furthermore, elegant studies in chloroplasts and *E. coli* indicate that Tat substrate targeting to the membrane occurs via an interaction of the substrate with TatB and TatC, independent of TatA. However, translocation remained dependent on the presence and function of TatA [217,220,221]. These findings, along with additional biochemical studies in *E. coli*, indicate a role in substrate targeting for TatB and TatC [222].

Individual prokaryotes may possess up to three TatA homologs and two TatC homologs [198,223]. The multiple copies of TatA and/or TatC may result in the formation of distinct Tat translocases within the same organism. Indeed, Tat export of the alkaline phosphatase D in *B. subtilis*, which encodes three TatA and two TatC homologs, is dependent on only one of the two TatC homologs [224]. In contrast to *B. subtilis*, which possesses only 7 predicted Tat substrates, *Mesorhizobium loti* encodes 95 putative Tat substrates, but has only one TatA and one TatC homolog [200]. Hence, in prokaryotes there appears to be no correlation between the number of Tat substrates and the number of Tat component homologs present in a given genome. It appears that many organisms do not encode a TatB homolog, despite the finding that TatB is essential for the Tat-dependent transport of most substrates in chloroplasts and *E. coli* [188,218,223].

Knowledge of the composition of the archaeal Tat pathway is currently limited to the analyses of sequenced archaeal genomes, which were mined for Tat component homologs using PSI-BLAST. In agreement with the results from the TATFIND program, these analyses predicted that the archaea use this pathway to varying extents [200]. While a number of euryarchaeota lack the Tat machinery, all analyzed crenarchaeota and many euryarchaeota have at least one TatA and one TatC homolog (Table 3). However, no archaeal species analyzed encoded an identifiable TatB homolog. A variety of archaea possess multiple copies of TatA and/or TatC homologs, which suggests that distinct translocons may form, as noted for *B. subtilis*.

As observed in bacteria, there is no correlation between the number of archaeal Tat component homologs and the number of archaeal Tat substrates predicted by TATFIND. For example, *S. solfataricus* possesses three TatA homologs and two TatC homologs, yet only five putative Tat substrates. It is curious, however, that the only group of organisms that uses this pathway for the majority of their secreted proteins, the haloarchaea, possesses TatC homologs (TatC1 and TatC2) that have unique structural features [30,198]. The N-terminus of the haloarchaeal TatC1 contains an uncharacteristically long cytoplasmic loop, which is most pronounced in the TatC1 of *Hfx. volcanii*. In *Hfx. volcanii*, *Halobacterium* sp. NRC-1 and *Haloarcula marismortui*, the structure of TatC2 is even more distinctive, as it appears to be the result of a fusion between two copies of TatC homologs, and is approximately twice the length of any previously identified TatC homolog. These differences may reflect an adaptation to high salt, and/or be required for the secretion of a large number of proteins.

## 4. Concluding remarks

Two major routes of protein translocation have been identified in archaea, the universally-conserved Sec pathway and the twin arginine translocation pathway. Currently, most of the understanding of these processes

in archaea is based on our knowledge about bacterial and eukaryotic translocation machineries. However, the rapid expansion of the archaeal research community, the development of in vivo and in vitro tools, and an increasing wealth of genomic data are expected to soon allow for a better understanding of unique, archaea-specific aspects of protein translocation. Furthermore, there is no doubt that the analyses of a diverse group of organisms will allow us to better understand protein translocation in general. Finally, as many archaea thrive in environments that are hostile to most studied organisms, examining cellular processes (like protein translocation) in these organisms allows us to further our understanding of adaptations to such environments.

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