Archaeal type IV prepilin-like signal peptidases
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CHAPTER 6

SUMMARY AND CONCLUDING REMARKS

Introduction to the Archaea and Sulfolobus

Researchers fascinated by the Archaea – no matter what particular aspect they work on – inevitably find themselves discussing the evolutionary position of this group of organisms. This is mostly because even 30 years after their recognition as a distinct major taxonomic group, the debate still continues whether Archaea deserve their own domain or whether they should simply be considered as an exotic group of bacteria.

The molecular tree of life (Figure 1), first presented by Woese and Fox in 1977 (237), clusters all living organisms into three major groups, the Bacteria, the Eukarya (or eukaryotes) and the Archaea. Eukarya derive their name from the presence of a nucleus, a membrane-bounded compartment which contains the hereditary information stored in DNA. Bacteria and archaea do not contain a nucleus and are therefore together referred to as “prokaryotes”. Despite the wide acceptance of the term – it has also been frequently used in this thesis – lumping together bacteria and archaea does not reflect the true evolutionary relationship between these organisms (157). Archaea resemble bacteria in morphology and metabolic complexity, but also have eukaryotic features as well as unique properties not found in the other two kingdoms of life. Importantly, information processing systems, including DNA replication, transcription and translation, are simplified versions of those found in eukaryotes. Because these systems rarely undergo horizontal gene transfer (87), it is assumed that the genetic lines of eukarya and archaea share a common evolutionary origin that is distinct from that giving rise to the bacteria. As a consequence, archaea are more closely related to eukaryotes than either of the two groups is to the bacteria.

Most archaea that can be grown in the laboratory are so-called extremophiles, as they inhabit ecosystems that humans might not consider suitable for the maintenance of life. Examples are solar salterns and salt lakes (halophiles), acidic volcanic springs (acidophilic hyperthermophiles), deep sea thermal vents (hyperthermophiles), and soda lakes (alkaliphiles). Current techniques that do not require laboratory cultivation but rather depend on direct analysis of environmental samples suggest that archaea are ubiquitous and constitute a significant part of our planet’s microbial biomass (188). An important feature which is exclusive to archaea is the presence of ether bonded rather than ester bonded isoprenoid lipids in the cell membranes. This difference has a major significance as it allows for a quantitative measurement of archaea in microbial biomass. In this way it became clear that archaea are significant “global players” of the microbial community. The domain archaea is in turn divided into distinct groups, the largest being Crenarchaea and Euryarchaea. The genus Sulfolobus belongs to the former. Sulfolobus species have been isolated from hot acidic sampling sites around the world. Examples are Beppu Hot springs in Japan (S. tokodaii), Yellowstone National...
Figure 1. Simplified view of a phylogenetic tree based on sequences of small subunit ribosomal RNA genes (adapted from refs. (15,204). The three main groups (domains) are Bacteria, Archaea and Eukarya. The two well-defined archaeal subdomains are the Crenarchaea and the Euryarchaea, while Korarchaeota are only characterized by environmental sequences. The existence of Nanoarchaeota as a taxonomic group is under debate.

Park (*S. solfataricus* and *S. acidocaldarius*) and Solfatara field in Naples, Italy (*S. solfataricus*). Common features of *Sulfolobus* species is an aerobic chemolithotrophic lifestyle and the requirement of high temperature (around 80°C) in combination with a low pH (2-3) to flourish. To deal with these two extremes, *Sulfolobus* membranes are particularly impermeable to protons even at high temperatures, thereby helping to maintain an internal pH which is close to neutral (221).

**Type IV pilins and archaeal flagellins**

As mentioned above, bacteria and archaea have several features in common, most of which belong to metabolic activities, also because these genes are more prone to horizontal gene transfer. Often, however, the archaean versions have their own “interpretation” of bacterial systems. One intriguing example is the functional divergence between type IV pilin-like proteins in bacteria and archaea. The bacterial type IV pilus is a retractile fiber-like extracytoplasmic structure that is built up of secreted pilin subunits (44). These proteins are synthesized with an N-terminal signal peptide which resembles secretory signal sequences owing to its tripartite structure: a short leader sequence characterized by the presence of positively charged amino acids, a hydrophobic domain of approximately 20-25 amino acids which can span the cytoplasmic membrane and a signal peptidase cleavage site. In secretory proteins, the entire signal sequence is removed upon or during secretion (158). In contrast, the distinct class of type IV pilin-like signal peptidases (TFPPs) only removes the short N-terminal leader. Furthermore, the two amino acids at which cleavage occurs are highly conserved. The hydrophobic stretch thus remains part of the mature proteins and constitutes the hydrophobic core of the assembled type IV pilus upon assembly by a membrane-bound machinery. Bacterial type IV pili can have a variety of functions, including DNA uptake, attachment to biotic and abiotic surfaces and twitching motility.
The best characterized type IV pilus-like structure of archaea is the flagellum, the function of which is to propel cells through liquid medium (23). In analogy with type IV pilins, the archaeal flagellins also contain an N-terminal type IV pilin-like signal peptide and are also assembled into a fiber-like helical structure ((41,213,217) and Chapter 4). Besides similarities of signal sequences, the components of the machineries that assemble bacterial pilins and archaeal flagellins are homologous and will be discussed in more detail below.

Cleavage of *S. solfataricus* sugar binding proteins and flagellin precursors

Sugar binding proteins from *S. solfataricus* were the first examples of non-flagellin proteins with a cleaved type IV pili-like signal sequence. These membrane localized proteins are genetically and functionally coupled to ABC transporters and are specific to certain sugars. The three characterized binding proteins with type IV pilin-like signals are GlcS (glucose), AraS (arabinose) and TreS (trehalose) (8,63). Screening the *S. solfataricus* genome sequence revealed additional binding proteins as well as proteins with unknown function ((7), Chapter 2 and 4). Among these proteins, some variations of cleavage site amino acid residues were observed. A major question is: why do certain sugar binding proteins make use of a signal sequence that is normally used to assemble extracellular fiber-like structures such as flagella? The binding proteins also appear to be assembled in a macromolecular structure, termed the bindosome which might serve to efficiently collect sugars from a nutrient-poor environment. However, the exact organization of this structure has remained elusive. Genetic evidence suggests that a type II/IV-like secretion system specific to *S. solfataricus* is essential for function and assembly of the bindosome (Albers et al., unpublished). The presence of a diverse set of type IV pilin-like substrates raised questions concerning the identity of the signal peptidase that cleaves these signal sequences. Furthermore, because of the considerable variation at the cleavage site amino acid residues was observed among the various (putative) substrates, the possibility arose that subclasses of substrate proteins are processed by different, dedicated signal peptidases (12,90).

Cloning and substrate specificity of PibD, the *S. solfataricus* homolog of type IV prepilin peptidases

To isolate the signal peptidase(s), an *in vitro* assay was established in which *E. coli* membranes containing uncleaved precursor proteins (the substrate) and native *S. solfataricus* membranes (containing the peptidase activity) were mixed in the presence of detergent and incubated at elevated temperature. Activity of the enzyme was monitored by SDS-PAGE electrophoresis in combination with Western blotting and detection of the substrate with an epitope tag specific antibody. Because the cleaved substrate has a lower molecular weight than the precursor, substrate cleavage can be identified as a shift of the band detected by the antibody ((5), *Chapter 2*). Initial studies were aimed at purifying the enzyme from *S. solfataricus* membranes. However, limited activity was observed in solubilized and fractionated membrane protein samples, suggesting that the peptidase is a low abundant enzyme. However, good
binding to a Concanavalin A column of the peptidase activity conferring protein was observed, suggesting that it might be glycosylated. Because at the time of these studies the *S. solfataricus* genome sequence became available, a sequence based homology search was performed. We used the COG database (208-210) to identify archaeal homologs of bacterial type IV prepilin peptidases. The COG database is generated using protein sequences from completely sequenced genomes by an all-against-all sequence comparison approach. In this way, similar proteins are grouped into clusters of orthologous groups (COGs). COG1989 (prepilin signal peptidase PulO and related peptidases) includes several archaeal members. These sequences were used to search the *S. solfataricus* database by Blast (16), and one candidate gene, SSO0131, was identified. Using the *in vitro* cleavage assay, recombinant SSO0131 (renamed PibD) expressed in *E. coli* membranes was shown to cleave both flagellin (FlaB) and glucose binding protein (GlcS) precursors. To determine the substrate specificity of PibD, important residues in the GlcS signal sequence were altered by site directed mutagenesis, in particular at the -1 and +1 positions (relative to the cleavage site). These experiments confirmed that at the -1 position glycine as well as alanine are equally tolerated. Furthermore, exchange of leucine at position +1 for several hydrophobic residues (isoleucine, valine, and phenylalanine) or alanine at the +1 position did not affect cleavage efficiency. Interestingly, the presence of a positive charge at the -2 position, which was suggested to be absolutely required for cleavage to occur, was not necessary for recognition of the substrate by PibD *(Chapter 2)*.

During the study of prepilin-like substrates and peptidases from the euryarchaeon *Methanococcus maripaludis* *(Chapter 4, and see below)*, we found that a substrate with glutamine at position +1 was also cleaved by PibD. This is interesting because glutamine is a polar amino acid while so far, only hydrophobic residues were anticipated at that position. Indeed, one putative PibD substrate encoded by the bindosome assembly operon (SSO2683) contains a glutamine at the +1 position. This confirms and strengthens our earlier conclusion of PibD being particularly efficient in cleaving a variety of substrates. An intriguing question that still remains to be answered is how this enzyme can be so flexible concerning its possible substrates while being exclusive to type IV pilin-like proteins. Clearly, there must be a strong selective pressure against amino acid combinations at the N-terminus of other secretory proteins that resemble to those recognized by PibD. In this respect, overexpression of wild-type and inactive *pibD* leads to growth arrest without extensive production of protein, both in *E. coli* as well as *S. solfataricus* (Chapters 2 and 3, and S.-V. Albers, unpublished). Possibly, an increased amount of this enzyme in the cytoplasmic membrane is toxic to the cell due to unspecific binding to membrane proteins. Alternatively, the conformation of the protein could prevent efficient translocation into the membrane. Interestingly, of the two non-redundant TFPP homologs from *M. maripaludis*, FlaK and EppA (see below), FlaK did not affect cell growth during expression while EppA did.

Data presented in Chapter 5 suggest that EppA is not selective in recognizing the hydrophobic domain of its substrate while FlaK is highly specific to the conserved H-domain of flagellins. This further supports the hypothesis that broader substrate specificity of a prepilin-like peptidase can lead to cytotoxic effects.
Catalytic residues and topology of PibD

In Chapter 3, the catalytic mechanism of PibD was studied by site directed mutagenesis. PibD contains two aspartic acid residues that are essential for cleavage activity. Moreover, an additional pair of aspartic acids in a large cytoplasmic loop is also important for function and is possibly involved in substrate recognition. These findings confirm the placement of PibD into the family of bacterial type IV pilin aspartic proteases (58). The requirement for two absolutely conserved aspartates was first shown for TcpJ from *Vibrio cholerae* (115). Because (similar to PibD) the two catalytic aspartates are in adjacent cytoplasmic loops, it was suggested that TFPPs are bilobed proteases, each lobe harboring one of the catalytic residues. In this model, the enzyme would embrace its substrate in a cleft between the two lobes. Unfortunately, to this date no structural information is available on this class of membrane-integrated proteases.

Sequence homology between bacterial and archaeal TFPPs is restricted to the active site motifs, a feature typical for many protease families. Interestingly, two classes of eukaryotic intramembrane aspartic proteases are related to TFPPs based on similar motifs around the catalytic residues. These are signal peptide peptidase and the catalytic subunit of γ-secretase, termed presenilin (128,238). Most striking is the conservation of a GxGD motif at the second catalytic aspartate residue (115,195). Glycine is the smallest and the only non-chiral amino acid. The presence of multiple glycines in this region suggests local structural flexibility. One could speculate that these residues might form a pocket in which a water molecule involved in peptide bond hydrolysis is coordinated. A common feature of aspartic acid proteases is that the catalytic residues do not directly cleave the substrate but coordinate and activate a water molecule which in turn hydrolyses the peptide bond (176). Although glycine itself does not have the capability to coordinate hydrophilic molecules, its peptide backbone oxygen and amino groups could play such a role. The glycine residue directly preceding the second catalytic aspartate is absolutely conserved in bacterial TFPPs, signal peptide peptidases and presenilin (195). This is also true for most euryarchaeal TFPP homologs, but with all crenarchaeal prepilin-like signal peptidases, the glycine is always replaced by another small amino acid: serine in APE0121 (*Aeropyrum pernix*) and alanine in PAE1599 (*Pyrobaculum aerophilum*), PibD, ST2258 and Saci_0139 (*Sulfolobus* spp.) and TpenDRAFT_0611 (draft genome of *Thermofilum pendens*).

Intriguingly, while the active sites of presenilin and SPPase are believed to be located within the membrane lipid bilayer, TFPPs cleave their substrates at the cytoplasmic face of the membrane. Their active sites are most likely exposed to the cytoplasm. This notion is supported by topology models of bacterial TFPPs (1,179) and of PibD (Chapter 3). Moreover, both TcpJ and PibD can be inactivated by treatment with the water-soluble 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (EDC or EDAC) and glycicinamide (115) and Chapter 3, which leads to covalent modification aspartate and glutamate residues. Because EDAC is membrane impermeable, an inhibitory effect suggests that the catalytic residues are solvent accessible and therefore not membrane embedded.

The membrane topology of PibD was determined by a prediction based strategy in combination with limited cysteine scanning mutagenesis. Based on these studies, a six
transmembrane model was suggested with the two catalytic aspartates exposed to the cytoplasmic side of the membrane. A common feature of all bacterial and archaeal TFPPs for which membrane topology information is available is that both the N- and C-terminal ends of the protein are located at the extracytoplasmic side of the membrane. A possible explanation for this observation could be the common evolutionary origin of all TFPPs and it suggests a functional relevance of the conserved membrane topology.

Finally, another sequence motif containing a pair of proline residues at the beginning or preceding the last transmembrane segment of archaeal TFPP homologs seems to be conserved (Figure 2). An alignment of these sequences suggests that the WxxPxxP consensus motif could have functional importance, although the tryptophan can be replaced by a positively charged residue in EppA-like enzymes. These amino acids could be necessary for recognition of, and interaction with the substrate, but their exact role should be established by future research.

Figure 2. Alignment of the C-terminal region in archaeal TFPPs. Conserved tryptophan and proline residues are in bold; transmembrane segments (predicted with Phobius) are underlined. Note that in some cases the conserved motif was assigned as part of the transmembrane segment by the topology prediction algorithm.
Flagellation in *S. solfataricus*

In contrast to most other archaea, the *S. solfataricus* flagellum only consists of one type of flagellin. Isolated flagella were studied by single particle electron microscopy and a structural similarity to bacterial type IV pili as well as the flagellum from *Halobacterium salinarum* was confirmed (Chapter 4). Transcriptional activation of the flagellin gene was observed at high cell densities and carbon source deprivation, as well as during growth on sugars as the sole carbon and energy source. An upregulation of flagellation under these conditions might be a strategy of *S. solfataricus* to escape nutrient poor environments.

The *S. solfataricus* fla operon is the smallest of those found in archaeal genomes. Possibly, additional components are encoded elsewhere in the genome, or *Sulfolobus* uses a minimal flagellum assembly apparatus. The highest degree of sequence conservation is found among the flaHIJ gene products. FlaH and FlaI contain Walker A and B motifs which identifies them as ATPases. FlaJ is the only polytopic membrane protein encoded by the fla locus and contains two GspII_F domains, which are also found in bacterial membrane proteins involved in type IV pilus assembly and type II secretion (163,173). The specific function of FlaH in flagellation remains elusive, but FlaI is similar to the typeII/IV secretion ATPases, which typically form hexamers and are thought to drive pilus assembly. Purified FlaI was indeed shown to hydrolyse ATP and the current hypothesis is that it energizes flagellum assembly in concert with FlaJ. The role of FlaJ in flagellum secretion and/or assembly was further demonstrated by targeted gene disruption of the corresponding gene (Chapter 4). Electron microscopy observations confirmed that the flaJ disruption mutant was not flagellated. Furthermore, loss of motility was demonstrated by a swarming assay on semi-solid gelrite plates.

Diversity of type IV pilin-like proteins in archaeal genomes

The astonishing diversity of type IV pilin-like proteins in *S. solfataricus* prompted us to investigate the situation in other archaea for which genome sequence data were available. To this end, a computational tool (FlaFind) was developed in collaboration with the groups of Profs. M. Pohlschröder and J.C. Kissinger (Chapter 5). A large number of previously unidentified substrates was found, including a novel group of type IV pilin-like proteins specific to certain methanogenic archaea. The two major characteristics of this group of proteins are a low predicted molecular weight (around 15 kDa) and the presence of a conserved Pfam domain at the N-terminus. Furthermore, the corresponding genes often co-localized with type IV pilin biogenesis homologs. The Pfam “Domain of unknown function (DUF) 361” comprises the conserved motif QXSXEXXXL. A more detailed analysis of the proteins containing this motif resulted in the hypothesis that this sequence could be part of the type IV pilin-like signal peptidase cleavage site with the consensus [RK][GA]Qh[SAT]h[DE] (cleavage site indicated by arrow; h is a hydrophobic amino acid). This motif is followed by a stretch of 16 mainly hydrophobic residues. Interestingly, a negatively charged glutamate at the +5 position is also found in almost all bacterial type IV pilins. This residue is thought to be engaged in an intermolecular salt bridge with the
positively charged N-terminal amino group of another subunit. In this way, the hydrophobic nature of the pilus core is maintained (46,159,161).

Two of the DUF361-like pilin encoding genes were cloned from *Methanococcus maripaludis* and shown to be processed by a dedicated signal peptidase which is encoded by the same operon as the pilins. Because this peptidase is only found in certain euryarchaea, it was called EppA (euryarchaeal prepilin peptidase) and the two pilins EpdA and EpdB (for EppA dependent). EppA belongs to a novel class of type IV prepilin-like peptidases, its distinctive feature is the larger molecular weight compared to PibD due to the presence of four additional transmembrane segments. A second previously described prepilin-like peptidase [flaK (20)] from *M. maripaludis* does not recognize the EpdA or EpdB but only cleaves flagellin precursors from the same organism. Results presented in Chapter 5 suggest that for EppA, presence of a glutamine residue at position +1 of the substrate protein is required for cleavage to occur. On the other hand, substrate recognition by FlaK is dependent on the conserved H-domain of flagellins rather than the identity of the amino acid at the +1 position. This notion is further supported by the level of sequence conservation of the N-terminal parts of flagellins and EpdA-like proteins in *M. maripaludis*. While this region is almost completely conserved in the flagellins, sequence homology in EpdA-like proteins is restricted to the DUF361-like motif.

An important conclusion from these data is that two different strategies which deal with multiple type IV pilin-like substrates seem to have evolved:

(i) Multiple (normally two) dedicated enzymes cleave distinct subclasses of substrates, as seen in *M. maripaludis*. A similar situation can be found in several bacteria, for example *Vibrio cholerae* and *Pseudomonas aeruginosa*. In *V. cholerae*, the toxin coregulated pilus subunit TcpA is cleaved by TcpJ (98,115) while another TFPP (VcpD) is required for processing of EpsI, a pilin-like component of the type II secretion machinery, which translocates enterotoxin through the outer membrane of *V. cholerae* (127). Although some cross-talk between the two peptidases occurs, they are not functionally redundant in vivo. In *Pseudomonas aeruginosa*, PilD cleaves the type IV pilin subunit PilA as well as pseudopilins involved in type II secretion (149,151). On the other hand, a second type of pilin (Flp-1) belonging to the type IVb subclass is cleaved by a smaller TFPP paralog, FppA (51).

(ii) Another strategy has been adopted by *S. solfataricus*. Depending on the stringency of the search criteria, some 19 to 23 substrate proteins can be identified in the genome. However, *S. solfataricus* only contains one type of prepilin-like signal peptidase, PibD. The considerable variation among the type IV pilin-like signal peptides from this organism is also reflected in the broad substrate specificity of PibD. Indeed, PibD also cleaved the two heterologous substrates, EpdA and flagellin from *M. maripaludis* (Chapter 5). Due to the functional variety of PibD substrates, one can expect that *pibD* is a constitutively expressed (housekeeping) gene. Indeed, no genes involved in secretion can be found in the genomic environment of *pibD* homologues from all *Sulfolobus* species.

**Outlook**

The primary aim of the studies presented here was the identification and characterization of the protein involved in processing of type IV pilin-like proteins in *S.
Summary

*solfataricus*. On the way towards this goal, a number of new questions arose which may be answered by future research. A selection of these is presented in the last paragraph of this summary.

The most desirable next step would be to study the PibD function *in vivo*. Isolation of a *pibD* disruption mutant was hampered by technical difficulties in the transformation and selection protocols (not shown). However, recent encouraging improvements of the gene disruption protocol ((113), S.-V. Albers, personal communication) show that genetic manipulation of *S. solfataricus* could become a more routine endeavour to anyone in the field, in the near future. This is important because the phenotype of a *pibD* disruption mutant could be severe and it is necessary to ensure that possible difficulties in obtaining a mutant are due to phenotypic effects rather than technical issues.

Although the catalytic aspartates can now be easily identified in almost all archaeal TFPP homologs, the mode of substrate recognition by this class of enzymes remains to be determined. One approach towards this aim could be the development of specific peptide inhibitors, based on the signal sequences cleaved by these enzymes. An inhibitory effect on *in vitro* substrate cleavage could then be studied. Additionally, direct interaction of the inhibitor with purified PibD (or a homologous protein, see below) can be measured by surface plasmon resonance or other techniques that monitor protein-ligand interactions. Alternatively, a short wavelength light activatable amino acid analog can be incorporated into the signal peptide to identify the docking site of the substrate. With this strategy, it was shown that the initial substrate binding site in γ-secretase is distinct from the actual catalytic center of the enzyme (109). In archaeal TFPP homologs a clear two-domain structure is observed: the N-terminal portion of four transmembrane segments includes the two catalytic aspartates while the C-terminal part consists of two to six transmembrane segments and a large cytoplasmic domain. The function of the latter is not yet clear, but it might be involved in initial substrate binding.

Despite considerable efforts to increase expression yields of PibD, a major challenge remains the production of sufficient amounts of protein for biochemical and structural studies. Alternatively, one could screen for increased production of PibD homologs from other archaea. For example, significantly higher expression levels in *E. coli* of *flaK* from *M. maripaludis* were obtained even prior to optimization of growth conditions. A PibD homolog that can be expressed and purified in considerable amounts is also a good candidate for structural studies of this important class of enzymes.

The finding that the two *M. maripaludis* TFPP homologs have distinct substrate requirements makes this system an interesting model for more detailed studies on these enzymes’ specificity. The major advantage is that cleavage activity can easily be monitored by co-expression of substrate and enzyme in *E. coli*. In this way, the hypothesis that the large cytoplasmic loop found in all archaeal TFPPs might be involved in substrate recognition can be tested. To this end, chimeras of EppA and FlaK could be constructed with junction points between the N-terminal and C-terminal domains. Then, the chimeric proteins can be tested for cleavage of EpdA or flagellin. Also, the conserved proline residues in the last transmembrane segment (see above) can be targeted by site directed mutagenesis. Here, exchange of proline for glycine might have a less severe effect than a more bulky or charged residue. Also, the *in vivo* function of EppA and the encoding operon could be studied, in particular to confirm
whether the corresponding operon indeed encodes an extracytoplasmic structure. In this context it is important to note that an *epdA* (MMP0233) disruption mutant has been isolated previously in a screen for acetate auxotrophic mutants of *M. maripaludis*. The mutant only grew well on complex media. Although the exact phenotype of the strain has not been investigated, our studies suggest that a membrane-associated process is affected.

The main focus of future research will most likely be on extracytoplasmic structures of *S. solfataricus*, in particular the bindosome. To this end, the *flaJ* disruption strain is a particularly valuable tool. However, flagellation itself might deserve further study, as the functions of accessory genes other than *flaI* and *flaJ* are still a mystery. One strategy to investigate the function of these accessory genes could be conditional overexpression in *S. solfataricus*. This might lead to distinct dominant negative effects, for example aberrant flagella or flagellated but non-motile cells.