

**Small proteins as regulators of archaeal membrane
transporters in *Methanosarcina mazei***

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Summary

- Challenges in bioinformatical prediction and biochemical detection of small proteins with less than 100 amino acids (aa) have led to their under-annotation in most genomes. In the last decade, various methods have been adapted to the verification of small open reading frames (ORF) in order to identify those which are translated into functional proteins in the cell. Omics technologies such as transcriptomics, ribosome profiling and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) optimized for small proteins have been successfully applied for the identification and validation of small protein encoding ORFs.

Aiming to annotate the small proteome of the mesophilic methanoarchaeon *Methanosarcina mazei*, several studies used a variety of methods for the identification of small proteins particularly. In 2009, based on a genome wide differential RNA sequencing approach, a total of 42 small RNAs were predicted to encode potential small proteins ≤ 100 aa. This number has been expanded by different omics technologies like ribosome profiling and LC-MS/MS, which later resulted in the verification of over 400 small proteins. Most of them have no functional annotation and only one has been functionally characterized since then. Consequently, the aim of this work was the characterization and functional investigation of selected verified small proteins. Altogether four small proteins were characterized (sP36, MtrR, MttS and MttQ) and shown to be involved in the regulation of key membrane complexes: The ammonium transporter AmtB₁, the tetrahydrosarcinopterin-S-methyltransferase Mtr and the trimethylamine (TMA)-transporter MttP.

- The 61 aa protein **sP36**, identified by mass spectrometry, was shown to be highly upregulated under nitrogen limitation on the transcript and the protein level. While it is expressed under nitrogen (N) limited conditions, growth analysis with chromosomal deletion mutants demonstrate that sP36 has a function in response to an ammonium upshift. After increased ammonium concentration, sP36 specifically binds two proteins: the P_{II}-like N-sensing protein GlnK₁ and the ammonium transporter AmtB₁. Thereby, it facilitates the relocation of GlnK₁ to the membrane by enabling the interaction of GlnK₁ and AmtB₁ demonstrated by *in vivo* and *in vitro* experiments. This interaction results in the inactivation of AmtB₁ through GlnK₁, which decreases energy loss due to futile cycling of ammonium and prevents ammonium toxicity. In contrast, the bacterial AmtB regulation relies on the post-translational covalent modification (uridylylation) of GlnK depending on the internal glutamine concentration. Thus, the small protein mediated mechanism is proposed to represent the archaeal, more ancient way of regulating AmtB₁ activity.

- **MtrR** is a 49 aa protein, which was newly identified by ribosome profiling and additionally validated by LC-MS/MS in a previous study. Here, it was demonstrated that MtrR is localized exclusively at the membrane. Using heterologously expressed MtrR in a pulldown experiment

targeting the *M. mazei* membrane fraction, two subunits of the tetrahydrosarcinapterin-S-methyltransferase (Mtr) were copurified. The Mtr-complex is a key enzyme of methanogenesis, important for the transfer of a methyl group from tetrahydrosarcinapterin (H₄SPT) to coenzyme-M (CoM) coupled to a sodium ion translocation for energy conservation. Depending on the availability of reducing equivalents (molecular hydrogen, H₂), this reaction is either exergonic or endergonic, which impacts the direction of the methyl-transfer and the sodium transport. Through further *in vivo* and *in vitro* experiments MtrR was demonstrated to bind the MtrA subunit of the Mtr-complex with high affinity. Chromosomal deletion of *mtrR* (*M. mazei* ΔMtrR) resulted in impaired growth under H₂ presence compared to the wildtype (wt). This phenotype argues for the inability of *M. mazei* ΔMtrR to effectively use H₂ for methane production independent of the carbon source. Consequently, MtrR is proposed to modulate the Mtr-reaction: In the presence of H₂ as an electron donor, the Mtr-complex is regulated by MtrR to favor the reducing over the oxidizing pathway for most efficient energy conservation.

- Overall, the two studies have implication for the theoretical view on posttranslational protein regulation. The regulation of large membrane transport proteins by interaction with small accessory proteins is hypothesized to be a more general principle, which in addition to other modes of regulation serves as alternative fast and tunable mechanism. The results are in line with many other reports of small proteins being responsible for the regulation of transport activity, protein stability or specificity.

- Two further small proteins, **MttQ** (97 aa) and **MttS** (49 aa), are encoded in very close 3'-proximity of a gene encoding the putative transport protein MttP. Here, the three genes are demonstrated to be organized in one large operon including four other genes, which are all involved in the degradation of TMA and dimethylamine, which serve as energy and carbon source for *M. mazei*. Based on this genomic arrangement MttP was proposed to be a potential TMA transporter, which was experimentally validated for the first time in this study. MttP was shown to form a stable complex with the small proteins MttQ and MttS. Overexpression of MttP, MttQ and MttS enables *Escherichia coli*, which itself is not able to transport or degrade TMA, to import TMA, which was monitored via mass spectrometry. Further, a chromosomal deletion of *mttS* results in slower growth and lower maximum cell density in comparison to the wt with TMA as sole carbon and energy source. This demonstrates that the small protein is a crucial subunit for the TMA transport complex, potentially by increasing the transporters affinity to TMA resulting in increased import rates.

- In general, it is attractive to speculate on the evolution of small proteins. Similar to the examples of this study, small proteins are often conserved in very limited taxa, arguing for an evolutionary young adaption. They are reported to be able to evolve *de novo* from non-coding sequences as an adaptation to specific conditions. This way, small proteins serve as an accessory

proteome which evolves fast to increase the fitness of the host in an ecological niche by fine-tuning regulation. The findings of this work provide additional evidence for this hypothesis. sP36 is part of the archaeal mode of AmtB₁-regulation, setting it apart from bacteria. MttS and MttQ are two novel small subunits that associate with a structurally conserved membrane transporter MttP to form a unique protein complex. The core transporter, MttP, belongs to the Drug/Metabolite Exporter (DME) family, proteins that are broadly conserved across all domains of life. In complex with the small proteins it imports TMA, which stands in contrast to the usual role of DME transporters, which generally facilitate the export of metabolites. The adaptation of this transporter for import rather than export represents a significant functional divergence within the DME family. Last but not least, MtrR is only conserved in *Methanosarcina*, a genus with exceptionally broad substrate range, by which it is used to switch between different methanogenesis pathways.

- To put it in a nutshell, the biological relevance of this study lies within the improvement of our knowledge about the regulatory networks of methanogenesis and the nitrogen metabolism in *methanoarchaea*. On a broader scale, it contributes to our mechanistic understanding of the small proteome as a fast evolving regulatory treasure chest, with the special target of membrane complexes. Finally it represents a blueprint for the characterization of further small membrane-associated proteins in archaea and opens up new avenues to study small proteins together with a target complex by e.g. cryo-electron microscopy.