

# The power of the small: the underestimated role of small proteins in bacterial and archaeal physiology

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Small proteins encoded by small open-reading frames (sORFs) ( $\leq 70$  aa) were overlooked for decades due to methodological reasons and are thus often missing in genome annotations. Novel detection methods such as ribosome profiling (Ribo-Seq) and mass spectrometry optimized for small proteins (peptidomics) have opened up a new field of interest and several catalogs of small proteins in bacteria and archaea have been recently reported. Many translated sORFs have been discovered in genomic locations previously thought to be noncoding, such as 5' or 3' untranslated regions or well-studied regulatory small RNAs (sRNAs). Even within longer ORFs, additional functional sORFs have been detected. Today, only a small proportion is characterized, but those small proteins indicate important and diverse functions in cellular physiology. Here, we summarize recently characterized small proteins involved in microbial metabolism.

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## Introduction

In all three domains of life, small proteins play important roles in diverse cellular processes such as metabolism, stress response, or regulation of transcription and translation (reviewed in, e.g. [1–3]). Since different definitions of their size are used, here, we focus exclusively on small proteins that are less than 70 amino acids (aa) in length. They are usually translated from small open-reading frames (sORFs) and are not products of processing [4,5]. Whereas in earlier times small proteins were found rather by serendipity discovering, for example, the today-well-studied small canonical proteins from *E. coli* (MgrB, AcrZ, and CytX), today, large-scale

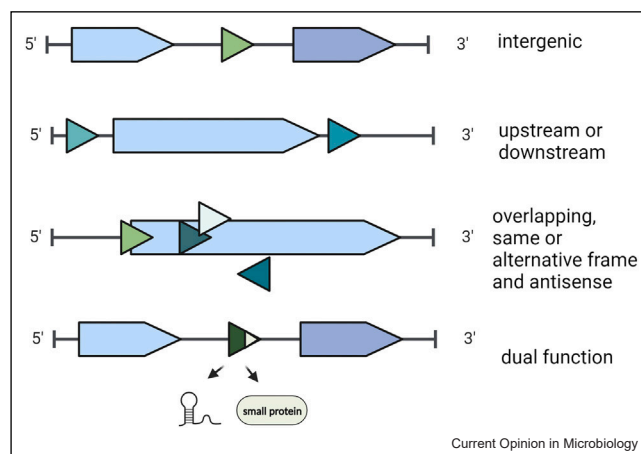
genome-wide studies by methods such as ribosome profiling (Ribo-Seq) combined with RNA sequencing (RNA-Seq) or mass spectrometry (MS) optimized for small proteins (peptidomics) are the gold standard [6–10]. Thereby, sORFs encoding small proteins were identified at almost all possible locations in the genomes. In addition to intergenic locations, sORF can be encoded in longer ORFs in the same or in an alternative frame, in antisense orientation or overlapping with the transcriptional start of longer ORFs. Small proteins can also be encoded in the 5' or 3' untranslated regions (UTR) of mRNAs or within regulatory RNAs (consequently called dual-function RNAs) (see [Figure 1](#)) (e.g. reviewed in [2]). Technical limitations often complicate the functional characterization of small proteins, for example, due to their low molecular mass, their sometimes nonexistent charge, or hydrophobic properties. Consequently, standard biochemical methods have to be adapted to study such small proteins. Moreover, small proteins < 50 aa are exponentially harder to detect and to characterize than small proteins between 50 and 100 aa. The scope of this review is to cover highlights of the past five years.

Since 2017, a German-wide priority program dedicated to study small proteins in prokaryotes has been funded by the German Research Foundation, DFG (SPP2002 “Small Proteins in Prokaryotes, an unexplored world”, 24 laboratories, <https://www.spp2002.uni-kiel.de/wordpress/>). The SPP2002 mainly focused on (i) establishment and optimization of methods to identify small proteins genome-wide (Ribo-Seq and peptidomics), (ii) global profiling of small proteins in several (pathogenic) bacteria and archaea, as well as (iii) functional characterization of selected identified small proteins, for example, in infection processes. Overall, the program aimed to unveil the new layer of mechanisms in prokaryotes hidden in the plethora of small proteins.

## Ribosome profiling

One of the crucial methods that has advanced small protein studies in recent years is Ribo-Seq. Polysomes actively translating mRNA are isolated followed by sequencing the mRNA that was protected by ribosomes during RNase treatment, generating footprints. Comparing those footprints to the total RNA-Seq of the same sample can distinguish between translated and UTR. Trimming away UTRs reveals ORF boundaries, allowing more detailed mapping of ORFs, sORFs, and alternative ORFs within

Figure 1



Genomic localization of small proteins (created with Biorender.com).

longer genes (reviewed in [8]). Ribo-Seq can detect sORFs internal to longer ORFs by ribosome trapping at the translation initiation site (TIS) and translation termination site (TTS) to determine start and stop codons [11,12]. Currently, comprehensive Ribo-Seq datasets emerged for several microorganisms. Just recently, Ribo-Seq was performed for five bacterial species: the pathogenic *Streptococcus pneumoniae* D39 [13], *Campylobacter jejuni* [14], *Escherichia coli* M1655 [15], *Salmonella typhimurium* [16] and the plant symbiont *Sinorhizobium meliloti* [17]. Often, Ribo-Seq analysis is combined with other methods such as MS (peptidomics) to verify small proteins (see Table 1), and the results are validated in vivo by epitope tagging followed by immunological detection. In archaea, small proteins as well move into the focus of interest. Recently, two independent Ribo-Seq datasets were generated for *Haloferax volcanii*. Gelsinger and colleagues established the Ribo-Seq method for *H. volcanii* and identified TIS by trapping the ribosomes [18]. Shortly after, Hadjeras and colleagues performed a combination of Ribo-Seq and peptidomics to analyze the *H. volcanii* small proteome [19]. Here, translation was shown for 212 of the 317 annotated sORFs under one growth condition (67%). In addition, 47 novel small proteins were identified, some of which were verified by expression analysis. In direct comparison, Ribo-Seq appears to be the more sensitive detection method as MS for translation of sORFs [19]. To gain further information about the existence and quantity of the small proteins, MS can support those studies. New strategies in MS such as top-down or bottom-up proteomics, ion mobility, or data-independent acquisition methods expand the identification and quantification of small proteins [6,20,21].

## Small proteins in bacteria

### Newly described small proteins involved in the metabolism of cyanobacteria

The small cyanobacterial protein NblD (66 aa) was recently shown to be essential for pycobilisome degradation under N-limitation, to maintain the cellular pool of aa, N-containing metabolites, and organic acids. Thus, it plays an important role in adapting to N-limiting growth conditions and metabolic homeostasis. CpcB, the chromophorylated (holo) form of the phycocyanin beta subunit, was identified as the target of NblD. Furthermore, the authors speculate that NblD has also additional functions under N-sufficiency such as remodeling of pycobilisome composition. Homologs of NblD were detected in many other cyanobacteria, underlining its crucial biological role [22,23].

Photosystem (PS) I and II are known to contain small proteins [18]. The small protein PsbO (33 kDa) in the thermophilic cyanobacterium *Thermosynechococcus vestitus* BP-1 has been recently described. With a Strep-tagged version of the subunit PsbO, two highly active PSII assembly intermediates could be purified and characterized [24]. The small cyanobacterial protein Atp $\Theta$  (48 aa) from *Synechocystis* 6803 has been shown to interact with the F<sub>0</sub>F<sub>1</sub>-type ATP (adenosine triphosphate) synthase and inhibits the ATP synthase from hydrolyzing ATP in the reverse reaction. As the photosynthetic ATP synthesis takes place in light, a mechanism to reduce ATP hydrolysis in the dark is crucially important for the energy conservation in cyanobacteria. Atp $\Theta$  plays an important role in this mechanism as it can decrease the ATPase activity of the ATP synthase up to 40% [25].

Table 1

## Recent Ribo-Seq studies in combination with experimental validations.

Organism	Standard Ribo-Seq	TIS	TTS	MS	WB	Number of identified novel sORFs	Reference
<i>Salmonella typhimurium</i>	x				x	139 (< 100 aa)	[22]
<i>Streptococcus pneumoniae</i> D39		x			x	114 (5–43 aa)	[13]
<i>Campylobacter jejuni</i>		x	x		x	42 (≤70 aa)	[14]
<i>Sinorhizobium meliloti</i>	x			x	x	37 (≤70 aa)	[17]
<i>Escherichia coli</i> BL21		x	x		x	365 (≤50 aa)	[11]
<i>E. coli</i> M1655	x					41	[15]
<i>Haloflex volcanii</i>		x				68 (<50 aa)	[18]
<i>H. volcanii</i>	x			x	x	47 (≤70 aa)	[19]
<i>Methanosarcina mazei</i>	x			x	x	314 (≤70 aa)	(Jordan et al., unpublished data)

WB, western blot analysis. MS, mass spectrometry. TIS, translation initiation site. TTS, translation termination site

The cyanobacterial small protein AcnSP (44 aa) in *Synechocystis* sp. PCC6803 regulates the activity of aconitase by increasing its substrate affinity. This affects the carbon flow in the oxidative branch of the open TCA (tricarboxylic acid) cycle toward 2-oxoglutarate. AcnSP is plasmid-encoded and appears to have arisen by duplication of the N-terminal part of chromosomal *acnB* (encoding aconitase) [26,27].

### Domain of unknown function 1127 containing small proteins

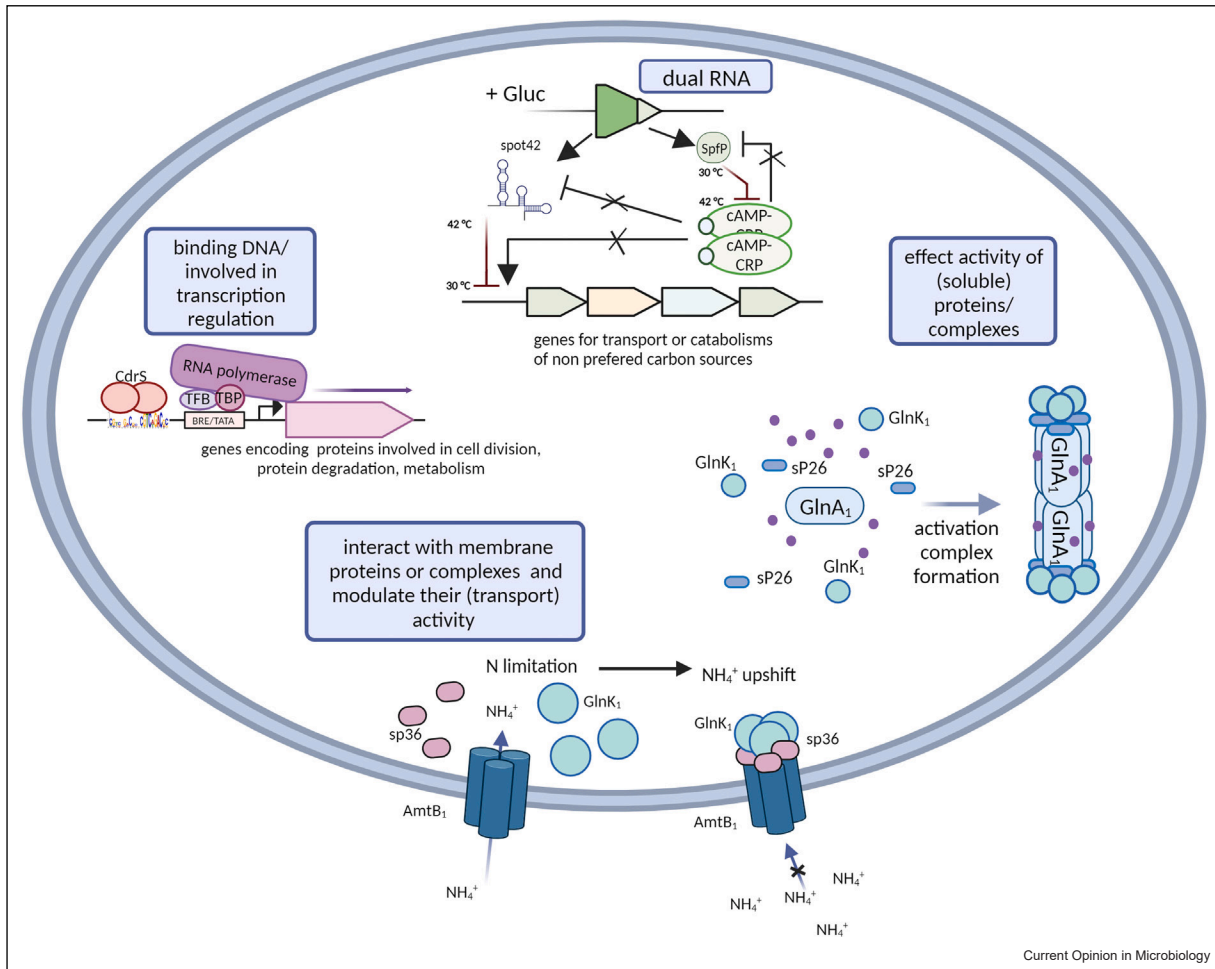
In some cases, bacterial small proteins have been identified with conserved but uncharacterized domains. A widespread, enigmatic family of bacterial small proteins has been identified that is characterized by a highly conserved domain of unknown function (DUF). This DUF1127 is found in 17 000 proteins in 4000 bacterial species. Most of these proteins have a length of < 100 aa. In the plant pathogen *Agrobacterium tumefaciens*, seven DUF1127 proteins have been identified. Three are shorter than 50 aa and therefore termed short DUF1127 proteins (SDPs). Deletion of the corresponding sORFs leads to phenotypical and transcriptome changes. Most of differentially expressed genes in deletion mutants were involved in phosphate uptake, glycine/serine homeostasis, and nitrate respiration suggesting a regulatory function of the SDP proteins in these processes [28]. A recently identified DUF1127 protein in *Rhodobacter sphaeroides*, CcaF1 (71 aa), can bind different types of RNA and therefore modulate the bacterial transcriptome [29].

### Dual-function small RNA

New detection methods for small proteins have brought a new class of regulatory small RNA (sRNA) into focus, the so-called dual-function sRNAs [5]. These sRNAs are mainly well-studied regulatory sRNA in which translated sORFs have subsequently been identified. For several examples, a function for the respective small protein has been discovered. The most prominent example is sRNA Spot42 from *E. coli*, originally detected in 1973 [12,30,31], encoding the 15-aa small protein SpfP recently identified by Ribo-Seq [12,30,31]. The sRNA Spot42 is known to repress genes involved in the metabolism of nonpreferred carbon sources and is Hfq-dependent. The small protein SpfP itself blocks the CRP (cAMP receptor protein)-mediated activation of its target genes, and consequently is also an important player in catabolite repression. Furthermore, the group of Gisela Storz showed an increased activity of the small protein at higher temperature, indicating the importance of the regulation by a small protein when efficiency of base pairing is decreased [32].

Another recently detected dual-function sRNA in *E. coli* is AzuCR (164 nt) encoding for the 28-aa small protein AzuC. AzuC interacts with GlpD involved in glycolysis and

Figure 2



Examples for small protein functions in prokaryotic cells (created with Biorender.com).

phospholipid biosynthesis and increases its dehydrogenase activity. In parallel, the sRNA represses the translation of *cadA*-, *galETKM*-, and possibly other target mRNAs [33]. The translation of *AzuC* itself is regulated by other Hfq-dependent sRNAs, indicating a tight and multilevel control of the regulatory functions of this dual-function sRNA under different environmental conditions [33].

VcdRP (~306 nt) was recently described for *Vibrio cholerae*, acting as a repressor of cholera toxin (CTX) production. In addition to the base-pairing RNA sequence (VcdR), VcdRP also encodes a 29-aa small protein (VrdP) [34]. The dual RNA appears to have a function in optimal utilization of carbohydrate sources by balancing the metabolite flux between glycolysis and the TCA cycle. VcdRP expression is dependent on available carbohydrate sources and is under control of the cAMP-dependent transcriptional regulator CRP. The base-pairing regulatory function is Hfq-dependent and uses a conserved stretch at its 3' end to repress the translation

of proteins of the PTS (phosphotransferase system) sugar transport system, such as *PtsHI*. The exact mechanism of how VrdR disrupts the expression of CTX is not known, but it is hypothesized that by inhibiting the translation of *ptsH* or *ptsI*, it results in higher cAMP levels. This results in activation of CRP, which then blocks expression of an operon of the virulence cascade (*tcpPH*), and thus affects CTX expression. The small protein VcdP has been shown to bind to citrate synthase (GltA) and to increase the activity of GltA, which is probably due to the reduction of the inhibitory effect of NADH on GltA [34].

#### Small proteins in archaea

Besides the global approach to study the transcriptome and identify new small ORFs (see above), there are very few sORFs of *H. volcanii* functionally characterized. These include sORF HVO\_2753, which encodes a small protein that affects both swarming activity and biofilm formation [35] and the recently discovered small

transcriptional regulator protein CdrS (61 aa). *cdrS* is cotranscribed with the tubulin-like cell division protein (FtsZ2) and contains a ribbon–helix–helix motif, exclusively known for bacterial transcription factors. ChipSeq identified 18 specific binding sites, the majority of which were located between 110 and 41 nt upstream from the transcriptional start site, suggesting an activating function in transcription. Liao et al. further show that CdrS regulates the transcription of genes, the products of which are involved in cell division (e.g. FtsZ1 or DacZ (adenylate cyclase)), metabolism, and metabolite transport/regulation [36].

The influence of a small archaeal protein on the activity of a transporter in *Methanosarcina mazei* was recently shown. Here, the small protein sP36 (61 aa) is mediating the inhibition of the ammonium transporter AmtB<sub>1</sub> by the PII-like protein GlnK<sub>1</sub>. Upon an increase in ammonium concentration after a period of N-limitation, sP36 binds both proteins and relocates at the membrane resulting in inhibition of AmtB<sub>1</sub> [37]. Rapid adaptation to nitrogen availability by inhibition of AmtB<sub>1</sub> is crucial since active transport of ammonium into the cell is energy-consuming [38,39].

Another small protein from *M. mazei* sP26 (23 aa) positively affects the glutamine synthetase (GlnA<sub>1</sub>) activity by stabilizing its dodecamer structure independently of the presence of 2-oxoglutarate or GlnK<sub>1</sub> [40].

## Conclusion

While in recent years increasing numbers of small proteins have been identified genome-wide in prokaryotes using methods such as Ribo-Seq, RNA-Seq, or peptidomics, only a few of those small proteins have been functionally characterized so far. The diminutive size of small proteins and the frequent lack of charged or stainable aa makes characterizing those more challenging. Methods still have to be optimized, especially for the small proteins below the limitations of standard methods to characterize their physiological functions. The here-discussed small protein functions changed our understanding of cellular physiology as they often appear to have an enhanced influence on well-described pathways and regulate cellular processes in a way researchers could not imagine in the last decades. A big open field is looking at small proteins of phages and genome defense islands (including phage defense by toxin–antitoxin systems). Just recently, a small protein (smORF<sub>72</sub>) in the prophage72 of the polylysogenic isolate *Vibrio cyclitrophicus* 1F-97 was identified to control the induction of the phage independently of DNA damage and therefore plays a fundamental role in phage–host biology and interprophage competition [41]. Examples of cellular locations and physiological pathways where small proteins have been shown to function

in prokaryotes are depicted in Figure 2. Prominent examples of new ways of regulation are dual-function sRNA, which were formerly dedicated as nontranslated regulatory RNAs, until Ribo-Seq studies discovered translated regions of those sRNAs, promoting a completely new understanding of regulatory processes in the cell. Future knowledge on the full repertoires and functions of small proteins in prokaryotes will also provide insights into sORF phylogeny and evolution of sORFs encoded small proteins. This may shed light on the origins of sORFs in microbial genomes. The resulting deeper understanding of how sORFs evolved (e.g. origin and evolutionary rates) as well as the determination of the selection pressure on codon usage will help to establish computational tools for genome-wide detection using machine learning tools. Furthermore, the discovery and characterization of phage-encoded sORF, for example, hold enormous potential for novel active agents such as alternative antibiotics.

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## Data Availability

No data were used for the research described in the article.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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