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An expanding universe of small proteins

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Historically, small proteins (sproteins) of less than 50 amino acids, in their final processed forms or genetically encoded as such, have been understudied. However, both serendipity and more recent focused efforts have led to the identification of a number of new sproteins in both Gram-negative and Gram-positive bacteria. Increasing evidence demonstrates that sproteins participate in a wide array of cellular processes and exhibit great diversity in their mechanisms of action, yet general principles of sprotein function are emerging. This review highlights examples of sproteins that participate in cell signaling, act as antibiotics and toxins, and serve as structural proteins. We also describe roles for sproteins in detecting and altering membrane features, acting as chaperones, and regulating the functions of larger proteins.

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Introduction

Small proteins have been understudied, even though the few characterized ones exhibit robust deletion phenotypes and display great diversity in their forms and functions [1[•],2,3[•],4[•],5–7]. A number of small proteins discovered to date were uncovered in biochemical studies as components of larger complexes or as processed peptides arising from larger precursors. Others were found in genetic screens as suppressor mutations or interacting partners in yeast two-hybrid analyses. These discoveries have prompted more directed bioinformatic and tiling microarray analyses which have revealed a growing number of genes encoding proteins 50 amino acids or less in their original forms, defined here as small proteins (sproteins) [2,8[•],9,10]. We use the term peptide for sproteins processed from larger proteins. Genes encoding sproteins have been found as single loci on the chromosome and as

parts of operons, and over half of the 60 sproteins detected in *Escherichia coli* are predicted to contain α -helical trans-membrane domains. We summarize what is known about sproteins and propose additional functions for these proteins inside and outside of the cell (Table 1).

Secreted peptides as signals

Bacterial communities survive in their natural habitats as a result of their ability to perceive environmental changes and respond accordingly. Secreted peptides derived from neighboring microorganisms of the same or different species form integral parts of these responses. These peptides allow the cell to adapt to new conditions and confer competitive advantages against other organisms.

At an intraspecies level, secreted small peptides play roles in communication, differentiation, and establishing clonal behaviors. In *Bacillus subtilis*, ComX (translated as a 55 aa protein, but 10 aa in its active form) acts as a pheromone, stimulating natural competence in response to crowding through activation of the ComP histidine kinase [11]. ComX presumably activates ComP through a direct interaction, but this has yet to be demonstrated. Also in *B. subtilis*, the sporulation killing factor (Skf) (translated as a 55 aa protein, but a cyclic 26 aa peptide in its active form) is secreted by endospore-forming cells to kill their siblings by an unknown mechanism in a cannibalistic process [12,13^{••}]. In addition, Skf promotes robust biofilm formation [5].

Other small secreted peptides affect interspecies relationships as antimicrobial peptides (Figure 1). Lantibiotics and circular bacteriocins are representative of the many post-translationally modified antimicrobial peptides secreted by Gram-positive bacteria to limit competition within ecological niches [14]. These compounds can inhibit peptidoglycan synthesis or form pores that depolarize cell membranes [15]. A few secreted antimicrobial peptides like the processed form of nisin in *Lactococcus lactis* (57 aa cleaved to 34 aa) have a dual role and also serve as intercellular signals between sibling cells [16,17].

Some sproteins play integral roles in host–pathogen interactions. The phenol soluble modulins (PSM) peptides secreted by various *Staphylococcus* species are involved in both promoting and inhibiting pathogenesis. *S. aureus* PSM peptides induce the lysis of human neutrophils and are major virulence determinants; in fact, the increased production of PSM peptides probably contributes to the enhanced virulence of community associated-MRSA as compared to healthcare associated-MRSA [7]. In contrast,

Table 1

Functions of small proteins in bacteria^a

Name	Length ^b	Organism ^c	Predicted transmembrane domain and other biochemical properties ^d	Proposed functions	Reference
Secreted signal peptides					
ComX	55 (5–10)	<i>B. subtilis</i>	No; isoprenyl-modified at tryptophan	Quorum sensing signal that stimulates competence	[11]
SkfA	55 (26)	<i>B. subtilis</i>	No; modified with disulfide bond and cyclized	Cannibalism factor	[5,12,13**]
Nisin	57 (34)	<i>L. lactis</i>	No; dehydrated at serines and threonines and cyclized	Antimicrobial peptide	[15]
PSM	44	<i>S. aureus</i> , <i>S. epidermis</i>	No	Induces lysis of leukocytes, modulates host immune response	[7,18*]
Membrane components					
SpoVM	26	<i>B. subtilis</i>	Amphipathic α -helix	Recognizes positive membrane curvature	[25**]
Metal and nucleic acid chaperones					
FbpC	29	<i>B. subtilis</i>	No	Assists function of small regulatory RNA FsrA in iron-sparing response	[26]
MntS	42	<i>E. coli</i>	No	Binds manganese and maintains manganese homeostasis	(LS Waters, M Sandoval and G Storz, unpublished data)
Stabilizing factors					
PetG	38	<i>Synechocystis</i> sp.	Yes	Promotes assembly and/or stability of cytochrome <i>b₆f</i> complex	[6]
PetN	29	<i>Synechocystis</i> sp.	Yes	Promotes assembly and/or stability of cytochrome <i>b₆f</i> complex	[6]
KdpF	29	<i>E. coli</i>	Yes	Stabilizes K ⁺ -translocating P-type ATPase KdpFABC	[29]
AcrZ	49	<i>E. coli</i>	Yes	Associates with AcrAB–TolC multidrug efflux pump	(EC Hobbs, BJ Paul, JL Astarita and G Storz, unpublished data)
Regulators					
SgrT	43	<i>E. coli</i>	No	Inhibits activity of glucose transporter PtsG	[30]
MciZ	40	<i>B. subtilis</i>	No	Inhibits GTPase activity of tubulin-like protein FtsZ	[3*]
Sda	46	<i>B. subtilis</i>	No	Inhibits autophosphorylation of histidine kinase KinA	[33]
MgrB	47	<i>E. coli</i>	Yes	Inhibits histidine kinase PhoQ in feedback loop	[34**]
MgtR	30	<i>S. typhimurium</i>	Yes	Modulates membrane-bound AAA+ protease FtsH	[1*]

^a Small toxins are not included in this table. For a comprehensive review about small toxins see [19].

^b Number of amino acids in full length form (number of amino acids in active form, if applicable).

^c The organisms listed in this table are limited to those in which the function of the small protein has been studied experimentally.

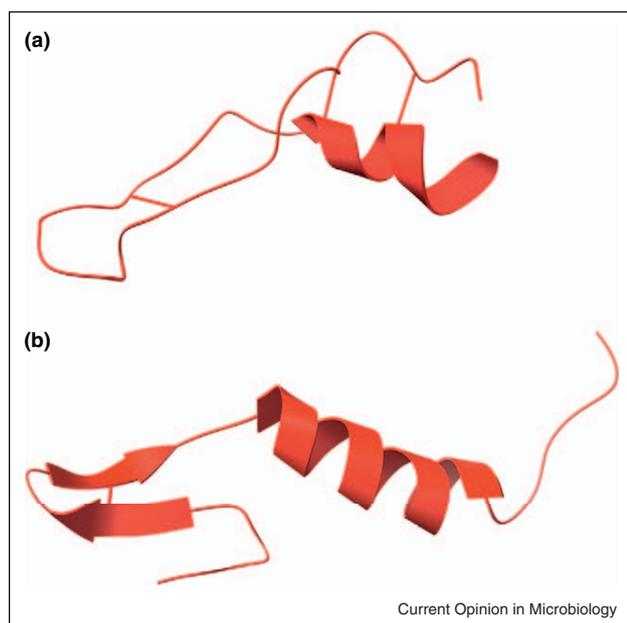
^d TM domain is predicted by TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>).

PSM gamma toxins secreted by *Staphylococci epidermis* cooperate with host-derived antimicrobial peptides of the innate immune system to reduce survival of a *Streptococcus* group A strain, an important human pathogen [18*].

Secreted peptides are probably the most extensively studied class of s proteins; however, it is likely that many

more are undiscovered. Imaging mass spectrometry (IMS) shows great promise in illuminating the full complement of secreted proteins [13**]. In IMS, bacteria are incubated directly on a MALDI mass spectrometry plate containing growth media. The metabolites secreted by the bacteria are subsequently identified. This technique was recently applied to identify the mature forms of the

Figure 1



Small secreted proteins exhibit diversity in their three-dimensional structures and can contain unique intramolecular linkages or modified amino acids. For example, the mature form of (a) subtilisin (PDB: 1PXQ) is cyclized in a head-to-tail fashion (omitted here for clarity) and contains three unique linkages between cysteine sulfur atoms and α -carbons of phenylalanine and threonine. In contrast, (b) leucocin A (PDB: 1CW6) has a single disulfide bond [41,42].

B. subtilis SkfA and SdpC cannibalism toxins [13^{**}]. Since distinct populations of cells can be grown next to one another, IMS can also be employed to discover bioactive molecules secreted by one species in response to the presence of another.

Small proteins as toxins

The toxins of type I toxin–antitoxin modules are a particularly mysterious class of sroteins. They tend to have a highly hydrophobic α -helical transmembrane domain followed by a small C-terminal region rich in aromatic or polar residues, reminiscent of the phage holins [19]. These toxin characteristics are shared by antimicrobial peptides, but there is no evidence that type I toxins are secreted. Instead, one of the toxins has been shown to cofractionate with the inner membrane [20]. While type I toxins kill the cell when overproduced, synthesis from their corresponding mRNAs is tightly regulated by *cis*-encoded small regulatory RNA (sRNA) antitoxins. Most toxin deletion mutants have no phenotype, and yet these genes are widespread through Firmicutes and Enterobacteriaceae, suggesting they serve conserved but undiscovered functions [21^{*}].

In *E. coli*, the overexpression of the type I toxin genes *hok*, *srnB*, *pndA*, *fst*, *ibsC*, *tisB*, *ldrD*, and *shoB* leads to common

global effects such as nucleoid compaction, membrane depolarization, and membrane disruption (F Fontaine and G Storz, unpublished data) [20,22]. However, microarray data as well as differences in the kinetics of cell death suggest that type I toxins exhibit individual specificities in their mechanisms of action [22]. Little is known about the physiological roles that small toxins serve, but the SOS-induced TisB has been implicated in forming ‘persistent’ subpopulations that are resistant to DNA-damaging antibiotics like ciprofloxacin [23]. More generally, type I toxins might allow bacterial populations to slow the metabolism of a fraction of cells under conditions of stress, thereby increasing their survivability, until conditions are more favorable for growth. Cells may also induce toxins as a form of programmed cell death to limit the propagation of phage infections. In addition or alternatively, type I toxin–antitoxin systems could play a role in chromosome maintenance, ensuring that regions immediately adjacent to toxin loci are not lost under nonselective growth conditions [24].

Small proteins as membrane components

A longstanding problem in biology is understanding how proteins are targeted to subcellular compartments. Most proteins in bacteria are localized by the presence of other proteins. However, at some point one of these proteins has to recognize fundamental physical features that make a cellular address unique. The amphipathic α -helical sroprotein SpoVM (26 aa) from *B. subtilis* serves such a function and recognizes positive (convex) membrane curvature, acting as a cue for the deposition of the endospore coat [25^{**}]. Moreover, SpoVM also tethers the endospore coat to the developing forespore. SpoVM most likely is too small to sense membrane curvature as a monomer, but rather forms higher order complexes that bind to convex surfaces.

We postulate additional roles for sroteins in detecting membrane features or even in modulating cell membrane thickness or fluidity to respond to changing environmental conditions. For instance, a membrane-spanning sroprotein with an especially high affinity for phospholipids might be induced in response to cold shock to affect membrane fluidity. Remodeling the cell envelope through alterations in lipid composition is a time-consuming process, requiring transcriptional reprogramming and protein synthesis followed by the manufacture, trafficking, and insertion of new lipid moieties. One can imagine that the synthesis and membrane insertion of an sroprotein would be less time-intensive and energy-intensive. In addition to altering membrane fluidity, small single transmembrane domain proteins could aggregate and form structures reminiscent of lipid rafts, recruiting or tethering larger proteins to specific subcellular addresses.

Small proteins as chaperones of metals and nucleic acids

Although sroteins are probably too small to act as enzymes *per se*, they may facilitate cellular processes by

presenting substrates and cofactors in a state that is accessible to other entities. For example, there is evidence that *B. subtilis* employs the small basic proteins FbpA (59 aa), FbpB (53 aa), and FbpC (29 aa) to promote interactions between the sRNA FsrA and its mRNA targets and thereby maintain iron homeostasis [26]. In *E. coli*, MntS (42 aa) binds to and is hypothesized to deliver manganese to other proteins (LS Waters, M Sandoval and G Storz, unpublished data). In addition to acting as chaperones, sroteins could be involved in coordinating metal-containing compounds in larger complexes. For example, the paralogous YbgT and YccbB proteins in *E. coli* are encoded in cytochrome oxidase operons and contain conserved cysteine residues within their transmembrane domains that could coordinate heme groups in cytochrome oxidases [8^{*}].

Other sroteins could play an opposite role by repackaging substrates to make them inaccessible to the cell. For instance, it is well established that slightly larger basic proteins (~60–80 aa) repackage and render DNA UV-resistant during endospore formation in *Bacillus* species, and redox active metals like iron are sequestered by Dps (167 aa) and ferritins (~160 aa) [27]. It seems reasonable that sroteins could play similar roles in organisms faced with long periods of dormancy and extreme environmental conditions.

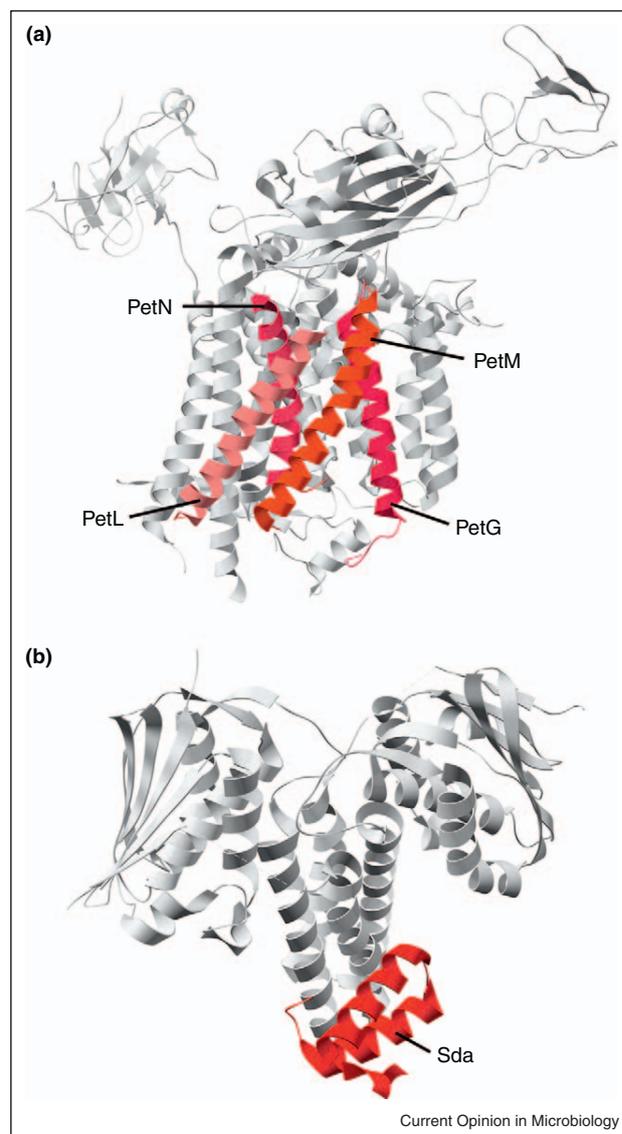
Small proteins as stabilizing factors for larger protein complexes

Macromolecular machines play central roles in the cell, conducting fundamental processes such as protein synthesis, cell division, photosynthesis, solute flux, and cell signaling. A number of sroteins participate in the assembly or maintenance of cellular complexes integral to these pathways. For example, highly conserved sroteins found in both plants and cyanobacteria are integral components of photosystems I and II. The cytochrome *b₆f* complex in *Synechocystis* PCC 6803 contains four sroteins: PetG (37 aa), PetL (32 aa), PetM (35 aa), and PetN (29 aa) [6,28]. PetG and PetN are essential for the stability of the complex, PetM is thought to have a regulatory role, and PetL has no known function (Figure 2a) [6,28]. In *E. coli*, KdpF (29 aa) stabilizes the KdpABC potassium transporter *in vitro* [29]. It is unknown how AcrZ (49 aa) impacts antibiotic efflux mediated by AcrAB–TolC, but an attractive hypothesis is that it stabilizes the formation of AcrB trimers in the membrane (EC Hobbs, BJ Paul, JL Astarita and G Storz, unpublished data). We expect that as biochemical methodologies for the detection of sroteins improve, others will be found to serve as stabilizing factors in larger protein complexes.

Small proteins as regulators

Sroteins are also induced to modulate a number of different pathways by interacting with proteins of various functions. For example, in *E. coli*, SgrT (43 aa) inhibits

Figure 2



Small proteins (shown in red) interact with integral membrane proteins in a number of different ways. (a) PetG and PetN are transmembrane proteins that associate with the periphery of the cytochrome *b₆f* complex (PDB: 2E74) and are required for its stabilization in *Synechocystis* PCC 6803 [6,43]. PetM is thought to have a regulatory role [28]. (b) Sda forms a complex with the soluble portion of KinB (PDB: 3D36) to block the initiation of endospore formation in *Bacillus* species [44].

glucose import through the PtsG transporter [30], while MciZ (40 aa) binds to FtsZ and inhibits its GTPase activity to prevent the formation of aberrant Z-rings during endospore formation in *B. subtilis* [3^{*}]. The sroteins could also alter the ribosome during adaptation to stress conditions. An example of this may be found in *E. coli* where a srotein paralogous to the L36 ribosomal protein, YkgO (46 aa), is synthesized upon zinc limitation,

perhaps to alleviate the requirement for zinc when L36 is part of the ribosome [31].

Several sroteins have been described that promote or inhibit the regulated proteolysis of other proteins. For example, degradation of the MgtC virulence factor by FtsH protease is promoted by MgtR (30 aa) in *Salmonella enterica* [1*]. Many proteins subject to regulated proteolysis are recognized by their cognate protease only when they are in complex with a specialized adaptor protein. Small antiadaptor proteins are induced by cells to competitively inhibit the activities of adaptor proteins. For example, *B. subtilis* induces synthesis of the ComS anti-adaptor protein (46 aa) in response to accumulation of ComX peptide, which allows transcription factors to accumulate and direct changes in gene activation [32].

One final group of sroteins regulates the activities of histidine kinases. Sda (46 aa) is synthesized by *Bacillus* species in response to DNA damage and inhibits endospore formation by preventing the autophosphorylation of an initiator kinase (Figure 2b) [33]. In *E. coli*, MgrB (47 aa) participates in a negative feedback loop to inactivate PhoQP through direct interaction with PhoQ histidine kinase [34**]. Other srotein regulators that act in similar as well as in novel ways, perhaps by altering the DNA-binding capacity of a transcription factor or by altering the specificity of a membrane transporter, are likely to be discovered.

Applications

Sroteins have immediate and future applications. The lantibiotic nisin, a small secreted antimicrobial peptide, has been used extensively as a food preservative for more than 20 years. Antimicrobial peptides have also been assayed for diverse medical applications in humans such as therapies against cancers as well as bacteria resistant to multiple antibiotics [35–37]. Surprisingly, subtilosin, which has been proposed as a treatment for bacterial vaginosis, inhibits the motility of human spermatozoa and may have additional use as a spermicide (Figure 1) [38].

In addition to being developed as antimicrobial agents themselves, small hydrophobic peptides may also have potential as highly specific vehicles for drug delivery. Very often, drugs are effective only if they target specific cell types. They usually must also penetrate the cell membrane. Sroteins show promise in addressing these problems. For example, the synthetic pHLIP (pH Low Insertion Peptide) has been employed to target the cell impermeable toxin phalloidin to tumors [35]. Under the slightly acidic conditions associated with certain cancers, pHLIP inserts into membranes as a transmembrane α -helix, carrying the conjugated phalloidin moiety into the cytoplasm where it is released to kill the cell. Finally, applications could be derived from characterizing the

abilities of sroteins to modulate regulatory networks. This knowledge could be employed to rewire regulation, selectively boosting metabolic pathways responsible for the production of economically valuable metabolites or the consumption of hazardous waste.

Perspectives

As is the case with sRNAs, sroteins largely have been missed or ignored in genome annotations. However, improved bioinformatic and biochemical approaches should facilitate the discovery of many more sroteins on bacterial as well as bacteriophage chromosomes. Global genomic analysis will also indicate how widely distributed sroteins are in all genomes and address other questions. Does the distribution of sroteins vary with the lifestyle or habitat of a bacterium? How do genes encoding sroteins arise, and do they evolve more quickly or more slowly than larger proteins? Many of the techniques currently used to characterize and identify sRNAs should be broadly applicable to studies that address these questions.

Our definition of a srotein as being less than 50-amino-acid-long is arbitrary, and other slightly larger proteins serve similar interesting functions. For instance, while MgrB (described above) can inactivate a histidine kinase [34**], SafA (65 aa) is induced in *E. coli* by one two-component system to activate another and thereby connect the activation of disparate signaling pathways [39]. *B. subtilis* employs anti- σ factors like Fin (76 aa) and Gin (64 aa) to switch between different σ -factors as endospore formation progresses [40]. It is becoming clear that sroteins can serve in any one of a number of functions, and the discovery and elucidation of others will further illuminate the unique roles they play in the cell.

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