Trends in Biochemical Sciences

CellPress

Review

A team of chaperones play to win in the bacterial periplasm

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The survival and virulence of Gram-negative bacteria require proper biogenesis and maintenance of the outer membrane (OM), which is densely packed with β-barrel OM proteins (OMPs). Before reaching the OM, precursor unfolded OMPs (uOMPs) must cross the whole cell envelope. A network of periplasmic chaperones and proteases maintains unfolded but folding-competent conformations of these membrane proteins in the aqueous periplasm while simultaneously preventing off-pathway aggregation. These periplasmic proteins utilize different strategies, including conformational heterogeneity, oligomerization, multivalency, and kinetic partitioning, to perform and regulate their functions. Redundant and unique characteristics of the individual periplasmic players synergize to create a protein quality control team capable responding to changing environmental stresses.

Highlights

Outer membrane protein (OMP) biogenesis is essential to bacterial cell survival and virulence in Gram-negative bacteria.

A robust and functionally redundant protein quality control network in the periplasm prevents the formation of toxic aggregates and facilitates proper OMP assembly at the OM.

Recent work demonstrates that, despite having overlapping functions, chaperones survival factor A (SurA), seventeen-kilodalton protein (Skp), and FK506 binding protein A (FkpA) and protease chaperone DegP also contribute uniquely to OMP biogenesis.

The complex 'teamwork' apparent in OMP biogenesis emerges from individual interactions between periplasmic players and unfolded OMP clients.

A team of periplasmic chaperones promotes OMP biogenesis

Pathogenic Gram-negative bacteria strive to survive, reproduce, and infect a host. Survival and virulence require a properly functioning cell envelope that can take up nutrients, provide mechanical stability and motility, adapt to changing environmental conditions, defend against external toxic molecules, and secrete virulence factors [1,2]. The cell envelope of a Gram-negative bacteria encompasses the **inner membrane (IM)** (see Glossary), the **OM**, the aqueous periplasm between the two membranes, and the **peptidoglycan** layer (Figure 1). The envelope is accessible through the semipermeable OM and is essential for bacteria viability and virulence. Therefore, current efforts to engineer new antibiotics often target cell envelope proteins and processes [3,4].

An important function localized to the cell envelope is the biogenesis of **OMPs**. OMPs densely pack the asymmetric OM with their β-barrel structures to act as porins, impart tensile strength to the membrane, and perform other essential cell envelope functions [1,2,5,6]. OMP biogenesis occurs in the following three general stages: (i) translocation across the IM through the **SecYEG translocon**, (ii) movement across the periplasm in an unfolded conformation, and (iii) insertion and folding into the OM with the aid of the **β-barrel assembly machine (BAM)** (Figure 1). Stage 2 is of particular interest as it involves the seemingly diffusive transit of unfolded membrane proteins through an oxidizing, energy-deficient (the cell envelope lacks ATP) [7], and aqueous compartment where they are prone to misfolding and aggregation [8,9].

Towards this point, a network of periplasmic chaperones and proteases has been shown to exist in the periplasm to bind **uOMPs**, degrade their toxic misfolded and aggregated states, facilitate proper folding into the OM, and prevent defective OMPs from compromising the integrity of the cell envelope before their insertion (Figure 1) [10,11]. The players involved in OMP biogenesis include the chaperones **survival factor A (SurA)**, **seventeen-kilodalton protein (Skp)**, and **FK506 binding protein A (FkpA)** as well as the protease-chaperone **DegP**, among others. Functional overlap between the periplasmic chaperones builds redundancy into the OMP biogenesis pathway. As a result, none are essential to cell survival on their own. However, each factor still contributes unique

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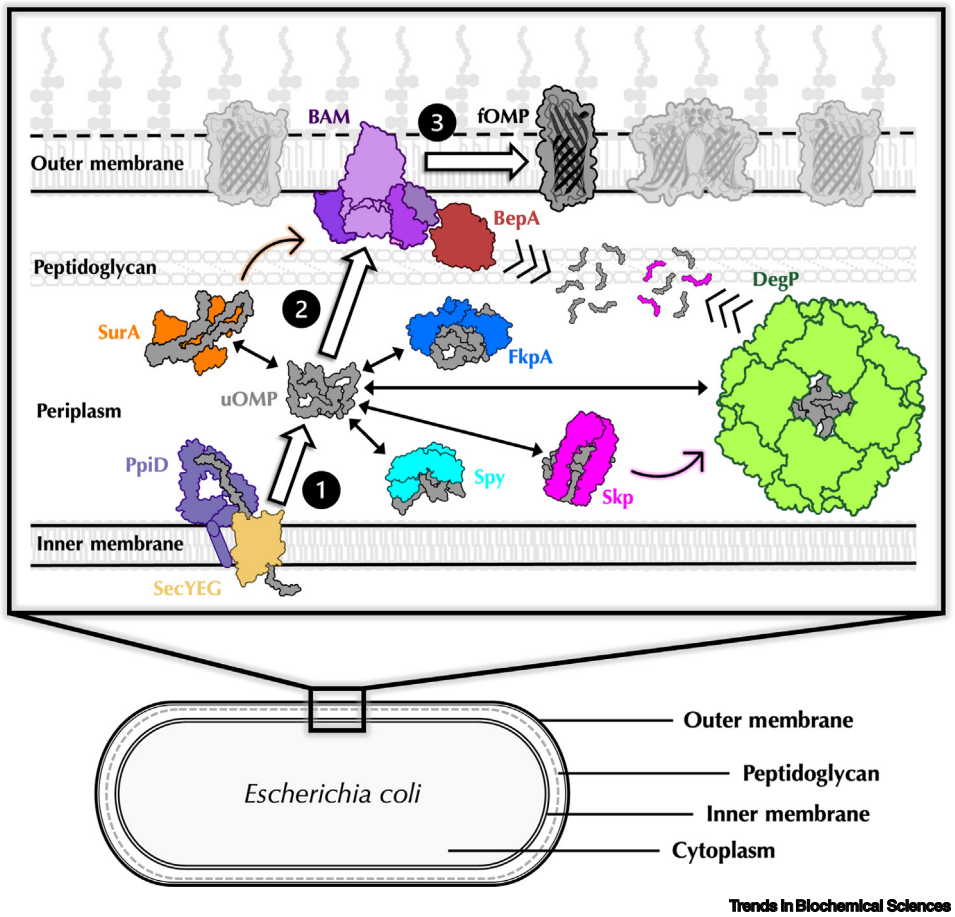

Figure 1. A periplasmic protein network promotes outer membrane protein (OMP) biogenesis in the cell envelope. A double membrane characterizes the cell envelope of Gram-negative bacteria. An inner membrane composed of phospholipids separates the cytoplasm and the cell envelope. A second asymmetric outer membrane (OM) is comprised of phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet. The OM is also densely packed with folded OMPs (fOMPs) that have a β-barrel structure. There exists an aqueous periplasm and a peptidoglycan cell wall between the two membranes. After first being translocated into the periplasm by the SecYEG translocon with assistance from peptidyl-prolyl isomerase D (PpiD) (1), unfolded OMPs (uOMPs) must traverse the periplasm without aggregating or misfolding (2) and then fold into the OM via β-barrel assembly machine (BAM) (3). Several periplasmic chaperones, including survival factor A (SurA), seventeen-kilodalton protein (Skp), FK506 binding protein A (FkpA), and spheroplast protein Y (Spy), and the protease-chaperone DegP assist in OMP biogenesis across the periplasm as part of the periplasmic protein network. Their reversible interactions with uOMPs are indicated by double-headed arrows. Specific interaction between SurA and BAM and between Skp and DegP are indicated by curved arrows. Proteases DegP and BepA degrade OMPs in the periplasm and stalled on BAM, respectively.

properties that, when combined, enable robust and adaptable OMP biogenesis in the face of environmental stress. Here, we review the current literature on the function of SurA, Skp, FkpA, DegP, and other periplasmic proteins involved in OMP biogenesis and their mechanisms of interaction with uOMPs. General strategies used by these periplasmic proteins to score folded OMPs and defend against toxic aggregation are discussed.

Introducing the players

SurA: most valuable player

If we consider the periplasmic factors needed for the OMP biogenesis network as members of a sports team, SurA is the most valuable player. As noted in the preceding text, no single deletion of

Glossary

β-Barrel assembly machine (BAM): a complex of the OMP BamA (formerly YaeT) and lipoproteins BamBCDE (formerly YfgL, NlpB, YfiO, and SmpA) that catalyzes the folding of other OMPs.
BepA: periplasmic protease chaperone implicated in OMP quality control at the BAM complex.
DegP: periplasmic serine endopeptidase and chaperone implicated in OMP biogenesis, also known as HtrA
DsbA: disulfide bond-forming enzyme A; periplasmic oxidoreductase involved in oxidizing disulfide bonds in periplasmic proteins and OMPs.
DsbC: disulfide bond-forming enzyme C; another periplasmic oxidoreductase involved in isomerizing disulfide bonds in periplasmic proteins and OMPs.
FK506 binding protein A (FkpA): dimeric periplasmic chaperone and *cis/trans* PPIase that binds unfolded OMPs.
Holdase: a type of chaperone that binds unfolded protein to prevent their misfolding or aggregation in an ATP-independent manner.
***Holo* translocon:** the set of components SecYEG-SecDF-YajC-YidC involved in translocating unfolded cell envelope proteins across the IM.
Inner membrane (IM): membrane separating the cytoplasm from the periplasm in Gram-negative bacteria.
Intrinsically disordered region (IDR): stretch of polypeptide without secondary structure.
Lipopolysaccharide (LPS) transport protein (LptD): essential OMP that inserts lipopolysaccharide into the outer leaflet of the OM, previously called Imp (increased membrane permeability).
LPS: major component of the outer leaflet of the outer membrane composed of a polysaccharide attached to lipid A.
OM: asymmetric and semipermeable outermost membrane in the cell envelope of Gram-negative bacteria.
OMP: β-barrel proteins found in the OM of Gram-negative bacteria.
Peptidoglycan: glycosaminoglycan chains linked together by short peptides that form the cell wall between the IM and OM in Gram-negative bacteria.
Peptidyl-prolyl isomerase (PPIase): an enzyme capable of isomerizing a *cis/trans* peptidyl-prolyl bond.
Peptidyl-prolyl isomerase D (PpiD): IM-associated chaperone that interacts

a periplasmic chaperone or protease kills *Escherichia coli* under typical laboratory conditions [12]; but unlike other single deletions, the Δ*surA* strain exhibits pronounced OM defects, increased susceptibility to antibiotics and environmental stressors, and reduced OMP levels [13–15]. Therefore, much work in recent years has been done to elucidate the mechanism of SurA and to structurally model a SurA–uOMP complex.

The structure of *apo*-SurA constitutes three main domains connected by flexible linkers: a 'core' domain folded from N- and C-terminal regions of the polypeptide and two **peptidyl-prolyl isomerase (PPIase)** domains called P1 and P2 (Figure 2A, domain architecture) [16]. Despite adopting a PPIase fold, neither domain efficiently catalyzes *cis-trans* peptidyl-prolyl isomerization reactions [14], and PPIase activity is not essential for SurA function [17]. In the crystal structure of SurA, P1 docks onto the core domain while P2 remains extended (Figure 2A, crystal structure). However, more recent data favor a heterogeneous ensemble of SurA conformations in solution

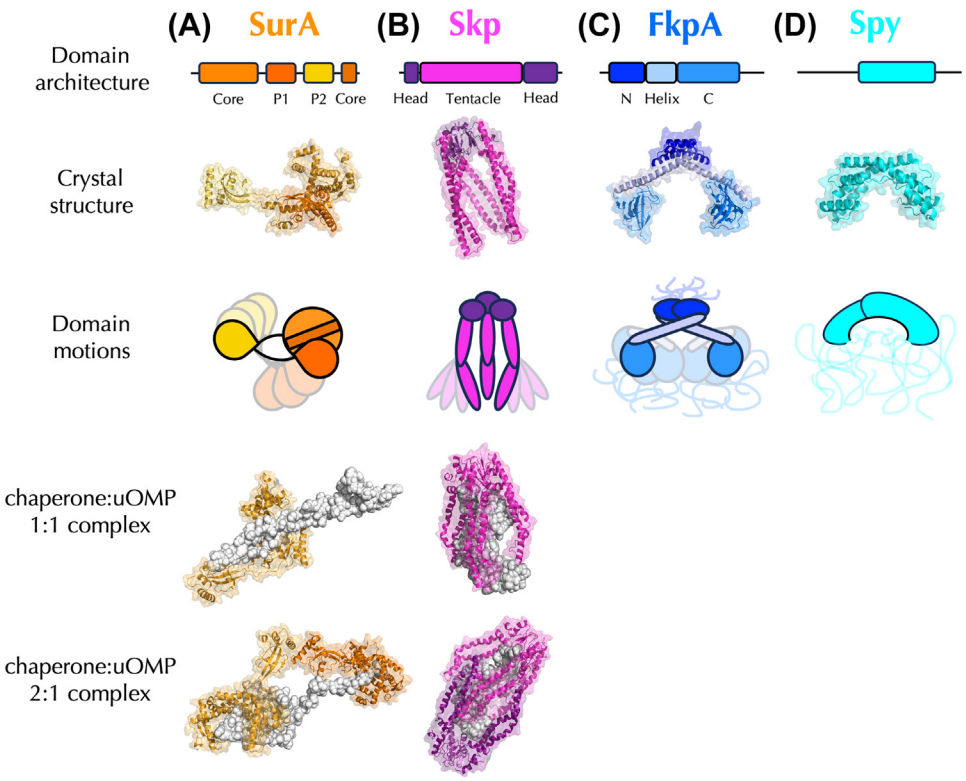

Figure 2. Periplasmic chaperones utilize oligomerization, conformational heterogeneity, and multivalency to bind unfolded outer membrane proteins (uOMPs). The periplasmic chaperones survival factor A (SurA), seventeen-kilodalton protein (Skp), FK506 binding protein A (FkpA), and spheroplast protein Y (Spy) function in different oligomeric states. (A) SurA [Protein Data Bank (PDB) 1M5Y] primarily functions as a monomer, (B) Skp (PDB 1U2M) chaperones uOMPs as a homotrimer, and (C) FkpA (PDB 1Q6U) and (D) Spy (PDB 3O39) both function as homodimers. All of the chaperones exist in conformationally heterogenous populations, and these domain motions and dynamics help regulate client binding and release (see Box 1 for specific examples). Structural models have recently been proposed for 1:1 and 2:1 complexes of SurA and Skp binding a uOMP, but no models of FkpA or Spy binding a uOMP have been published to date. Structural models of one and two SurA monomers binding the unfolded transmembrane barrel of OmpA ($OmpA_{171}$) are models o1s009 and o2s016 published in Marx *et al.* [22]. The model of a single Skp trimer binding $uOmpA_{171}$ is modified from the structure published in Zaccai *et al.* [54]. The interlocked conformation of a 2:1 Skp:uOMP complex has been proposed in the literature [50,55]; this model was made by simply docking a second Skp trimer onto the 1:1 complex in PyMOL.

with newly translocated polypeptides at the SecYEG translocon.
SecYEG translocon: main components of the machinery that translocate unfolded cell envelope proteins across the IM.
Seventeen-kilodalton protein (Skp): trimeric holdase chaperone that binds unfolded OMPs.
σ^E stress response: upregulates the expression of OMP biogenesis factors and downregulates the transcription of OMPs when unfolded OMPs accumulate in the periplasm.
Spheroplast protein Y (Spy): periplasmic chaperone and foldase that is heavily upregulated during stress responses and has recently been implicated in OMP biogenesis.
Survival factor A (SurA): monomeric periplasmic chaperone that binds unfolded OMPs and delivers them to BAM.
uOMP: unfolded but folding-competent ensemble that is solubilized by periplasmic chaperones before folding into the OM.
YcaL: OM-associated protease involved in OMP quality control at early folding stages on the BAM complex.

where the P2 domain, on average, resides closer to the core domain than in the crystal structure [18,19], and both the P1 and P2 domains are capable of docking to and releasing from the core domain in a competitive manner [20,21] (Figure 2A, domain motions). Importantly, this conformational flexibility in the *apo* state allows SurA to occasionally adopt an 'open' conformation that reveals a cryptic uOMP binding site [19,21,22].

uOMPs bind to the groove formed by the core and P1 domains in the 'open' conformation [22]. After binding, the P1 domain then clamps down to hold the uOMP [19]. However, most of the interaction interface is localized to the core domain [19], which explains why the core alone can complement the Δ*surA* phenotype [17] and bind uOMPs with similar affinity as full-length SurA [23]. Importantly, the conformation of a uOMP bound to SurA expands relative to the conformation of a uOMP free in solution [22,24–28] (Figure 2A, chaperone:uOMP 1:1 complex). The global expansion of the uOMP conformation when bound to SurA appears unique to the function of SurA and may help explain its importance as the primary chaperone for proper assembly of OMPs into the OM [13]. SurA binds its subset of known uOMP clients with low micromolar affinity (Table 1) [18,19,23,24,29–31], but how SurA recognizes its clients versus nonclients is an open question. It has been proposed that SurA generally recognizes an Ar-X-Ar motif (where Ar represents any aromatic residue, and X represents any amino acid) [32,33], but peptides lacking this motif also bind SurA [34]. More work is needed to identify the sequence or structural features of uOMPs that SurA (and other periplasmic chaperones) recognize and bind.

Why is SurA the primary periplasmic chaperone for uOMPs, the most valuable player of OMP biogenesis in *E. coli*? OM density significantly decreases, and the **σ^E stress response** turns on in full force to combat the loss of SurA [13,15,35]. The σ^E stress response detects the accumulation of uOMPs in the periplasm and upregulates the expression of many OMP biogenesis factors while simultaneously downregulating the transcription of OMPs to mediate the increased aggregation risk under these conditions. SurA binds uOMPs with low micromolar affinity and expands its clients, implying that SurA holds uOMPs in an extended conformation and easily relinquishes its clients for membrane insertion. Most saliently, SurA directly interacts with the BAM complex [13,36–38] and helps target uOMPs to the OM folding machinery [39,40]. Without SurA to mediate this handoff, the vitality of the bacteria suffers.

Skp: ball hog?

Skp functions as a quintessential **holdase**. It tightly sequesters uOMPs within its jellyfish-like structure to prevent and even disassemble the early formation of toxic aggregates in the periplasm [24,28,41–44], and it does so in an ATP-independent manner [7]. The crystal structure of trimeric Skp shows a series of short β-strands self-associating to form a barrel at the oligomerization interface, while long α-helical regions form the tentacles of the jellyfish-like structure (Figure 2B, domain architecture and crystal structure) [45,46]. However, Skp exists in a monomer–trimer equilibrium ($C_{1/2} \approx 1.5$ μM) at physiological concentrations [47,48]. Reports disagree as to whether the monomer is folded [47] or unfolded [48], but the literature unanimously concurs that Skp binds uOMPs as a trimer [49–51]. As a result, oligomerization and binding must be linked, although this linkage has yet to be thermodynamically characterized.

Skp binds uOMPs in the cavity formed by its three tentacles (Figure 2B, chaperone:uOMP 1:1 complex) [44,52–54]. Conformational flexibility at a hinge between the head and tentacles of the jellyfish-like structure allows for protein motions in the *apo* state [55] (Figure 2B, domain motions), but upon client binding, the Skp tentacles slightly clamp down on the bound uOMP, and the chaperone rigidifies [49,53,55]. Many short-lived (<1 ms) noncovalent interactions occur between Skp and the uOMP throughout the cavity and sum to a large binding energy by

Table 1. Reported dissociation constants for periplasmic chaperones binding uOMP clients

Chaperone	uOMP client	K_d	Temp (°C)[a]	Method[a]	Refs
SurA	$OmpA_{171}$[b]	1.8 (±0.1) μM	na	MST	[23,29]
	OmpC	0.11 (±0.08) μM	25	Fluorescence	[31]
	OmpC	0.17 (±0.02) μM	25	smFRET	[18]
	OmpF	5.2 (±1.7) μM	na	ELISA	[30]
	OmpG	0.44 (±0.09) μM	na	ELISA	[30]
	OmpT	9.3 (±0.5) μM	na	MST	[23]
	OmpX	0.80 (±0.04) μM	na	MST	[19]
	OmpX	8.2 μM	37	smFRET	[24]
	OmpX	1.0 μM	25	smFRET	[24]
Skp	BamA[c]	0.3 (±0.1) nM	25	Fluorescence	[51]
	OmpA	22 (±16) nM	25	Fluorescence	[51]
	OmpC	15.9 (±7.2) nM	25	FRET	[31]
	OmpC (1^{st} Skp binding)	0.55 (±0.04) nM	23	smFRET	[50]
	OmpC (2^{nd} Skp binding)	1.2 (±0.4) μM	23	smFRET	[50]
	OmpG	12 (±3) nM	25	Fluorescence	[51]
	OmpLA	10.8 (±0.2) nM	nr	Fluorescence	[58]
	OmpW	11.3 (±0.2) nM	nr	Fluorescence	[58]
	OmpX	359 nM	37	smFRET	[24]
	OmpX	3.6 nM	25	smFRET	[24]
	PagP	11.8 (±0.3) nM	nr	Fluorescence	[58]
FkpA	EspP	64.2 nM	nr	SPR	[72]
	$OmpA_{171}$[b]	8 (4–20) nM	20	SV-AUC	[59]
	OmpC	38.5 (±7.8) nM	25	Stopped-flow FRET	[62]
	OmpC	23.3 (±3.5) nM	37	Stopped-flow FRET	[62]
	OmpC	12.4 (±3.7) nM	44	Stopped-flow FRET	[62]
DegP S210A	OmpC	8.63 (±0.37) nM	25	Fluorescence	[31]

[a]Abbreviations: ELISA, enzyme-linked immunosorbent assay; FRET, Forster resonance energy transfer; MST, microscale thermophoresis; na, not applicable, method not performed at a single temperature; nr, not reported; smFRET, single-molecule FRET; SPR, surface plasmon resonance; SV-AUC, sedimentation velocity analytical ultracentrifugation.
[b]$OmpA_{171}$ is the transmembrane domain of OmpA only.
[c]Interaction with BamA is as an unfolded client, not as the folded protein in the OM.

avidity [53]. Binding between positively charged Skp (pI = ~10) and uOMPs is affected by salt concentration and lipid headgroup charges [51,56,57]. Therefore, electrostatic interactions in addition to the expected hydrophobic ones contribute to the overall binding between Skp and uOMPs.

Tight binding in the low nanomolar range characterizes the interaction between Skp and its clients (Table 1) [24,31,50,51,58]. As a result, Skp binding to mechanically and chemically denatured OMPs suppresses their aggregation and misfolding, but this also prevents their subsequent refolding [29,43,49,59]. Such a tight-binding chaperone hogging all the periplasmic uOMPs

and sequestering them away seems incongruous with a continued, directional flow of OMP biogenesis. Several explanations exist for the apparent ball-hogging behavior of Skp. The first considers kinetics rather than thermodynamics alone. Short-lived Skp–uOMP complexes [31] that dynamically bind and release clients on fast timescales easily allow for passing between members of the periplasmic chaperone network so that Skp is not really a ball hog at all [39,60]. Another compelling argument is that Skp plays a unique role in clearing away OMPs that assemble too slowly. Recent work shows that Skp facilitates the degradation of folding-impaired OMPs with mutated β-signals [40] or that are stalled on the BAM complex [61] as a sacrificial adaptor protein: the full Skp–uOMP complex is degraded by DegP [61]. In this case, long-lived binding triggers a turnover to DegP and degradation pathways. Additional explanations invoke interactions with lipid headgroups, BAM, or other cell envelope proteins to induce complex dissociation [29,53,56,57].

FkpA: in off the bench

Genetic knockouts of FkpA alone and in combination with other periplasmic factors seldom exhibit a noticeable phenotypic effect, obscuring the role of FkpA in the context of the full chaperone network [12]. However, FkpA rescues the synthetically lethal Δ*surA*/Δ*skp* double deletion at high temperatures (≥44 °C) [62], indicating that this chaperone plays an elevated role in controlling periplasmic homeostasis under heat shock conditions as a member of the σ^E operon [63–65].

FkpA forms homodimers as the functional unit of the chaperone, but unlike Skp, FkpA dimers predominate at the concentrations expected in the periplasm [59,66]. The FkpA structure comprises two domains: a C-terminal PPIase domain and a set of N-terminal α-helices that intercalate at the dimerization interface. The two are connected by a long and inherently flexible α-helix (Figure 2C, domain architecture and crystal structure) [67]. Breaking of this helix allows the C-terminal domains of the dimer to move independently of each other [68,69] (Figure 2C, domain motions). **Intrinsically disordered regions (IDRs)** exist at both the N and C termini, but no functional importance has been ascribed to these regions [59,67,68].

The PPIase domain of FkpA efficiently catalyzes the isomerization of *cis–trans* peptidyl-prolyl bonds in short peptides and full-length proteins, leading many to categorize FkpA as a folding catalyst in the periplasm [66,70,71]. However, FkpA also prevents the aggregation and improves the folding of several uOMPs [59,62,72], establishing the protein as a *bone fide* chaperone and not solely a PPIase enzyme. FkpA tightly binds uOMP clients with low nanomolar affinity (Table 1) [59,62,72], and the interaction energy is highly sensitive to low urea concentrations, indicating that an extensive binding interface is buried upon client binding [59]. This binding interface spans the concave surface of FkpA between the two 'arms' of the homodimer [59,68], although atomistic structural models like the ones of the SurA–uOMP and Skp–uOMP complexes do not yet exist for an FkpA–uOMP complex.

Much remains to be learned about the mechanisms of FkpA function. Current evidence supports that FkpA is a tight-binding holdase chaperone like Skp. However, the elevated status of FkpA heat shock conditions hints at additional unique properties [62]. FkpA may perform in a backup role without fanfare to maintain robust OMP biogenesis under optimal growth conditions; but when the stress ramps up, FkpA comes in off the bench to play an important part in cell survival.

Periplasmic proteases: on defense

Under ideal growth conditions, the vast majority of uOMPs translocated into the periplasm eventually fold into the OM [39], but stress conditions like high temperatures cause the accumulation of unfolded and misfolded OMPs. One of the best ways to prevent the inevitably harmful effects of

aggregation in this scenario is to reduce the uOMP concentration by degradation. DegP serves as the primary protease in the OMP biogenesis pathway to defend against toxic aggregation, especially during periods of high uOMP levels in the periplasm [13,39,73].

A monomer of DegP comprises a chymotrypsin-like core domain with serine protease activity and two peripheral PDZ domains that mediate the oligomeric structures of DegP (Figure 3, domain architecture) [74]. Even in the *apo* state, DegP adopts an ensemble of functional oligomeric states spanning trimers to 24-mers [75], but binding of a uOMP substrate shifts the ensemble towards higher proteolytic activity and larger cage-like or bowl-like structures that encapsulate substrate [76–80] (Figure 3, crystal structures and oligomerization). Coupling of oligomerization, client binding, and activity may regulate DegP functional cycles by maintaining a less active protein reservoir that becomes mobilized in response to accumulation of uOMPs in the periplasm, particularly under stress conditions. Both holdase chaperone and protease functions have been attributed to DegP [76,81–83]. In the context of OMP biogenesis, overexpression of the proteolytically inactive DegP S210A variant does complement in a Δ*degP* background, so an excess of holdase chaperone that sequesters uOMPs within its oligomeric structures can partially overcome the absence of a protease [82,83]. Nonetheless, proteolysis prevails as the dominant function in the periplasm to defend against the stress-induced accumulation of uOMPs [39,84].

In addition to DegP, periplasmic proteases **BepA** and **YcaL** act to degrade OMPs stalled on the BAM complex [73,85,86]. Experiments using **lipopolysaccharide (LPS) transport protein**

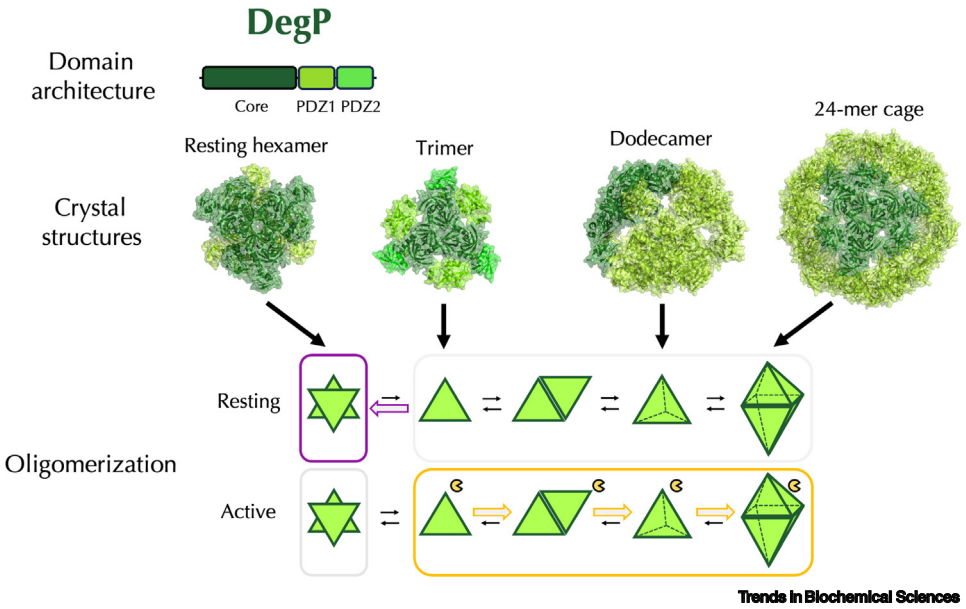

Figure 3. DegP oligomerization forms cages and increases proteolysis activity in response to substrate binding. Monomers of DegP comprise a core serine protease domain and two PDZ domains. These domains are colored on the structure of a DegP trimer [derived from Protein Data Bank (PDB) 1KY9]. DegP populates several different higher-order oligomers, including the resting hexamer (PDB 1KY9), trimer, dodecamer (PDB 3OTP), and 24-mer (PDB 3CS0) both under ideal and stress conditions. For simplicity, a single trimer is highlighted in a dark green color in the hexamer, dodecamer, and 24-mer structures. A cartoon representation of DegP oligomers where each trimer is a triangle illustrates how DegP populations shift in response to unfolded outer membrane protein (OMP) concentrations in the periplasm. At low concentrations of free uOMP, which is typical under ideal growth conditions, DegP favors its resting hexamer state and has low protease activity. Increased abundance of uOMPs in the periplasm under stress conditions increases substrate binding and shifts the equilibrium toward proteolytically active oligomers of DegP. Large cages form to both shield and degrade uOMPs.

(LptD) variants whose folding becomes comprised at various stages in the assembly process reveal that DegP degrades misfolded OMPs in the periplasm, YcaL proteolyzes BAM-associated but folding-impaired OMPs during early folding steps, and BepA rescues OMPs stalled in the late stages of folding on the BAM complex [73]. The presence of distinct proteases at various assembly steps highlights the need for carefully controlled protein quality control both in the periplasm and at the OM.

Rounding out the team: other periplasmic proteins implicated in OMP biogenesis

Many other folding factors, chaperones, and proteases in the periplasm are involved in the protein quality control of OMPs, lipoproteins, and soluble periplasmic proteins. To discuss them all would be beyond the scope of this review, but a few additional players in the OMP biogenesis pathway merit mention. The IM-associated chaperone **peptidyl-prolyl isomerase D (PpiD)** interacts with the SecYEG translocon and translocating polypeptides as they enter the periplasm [87–90]. Although chaperoning by PpiD is likely not specific to OMPs, it has been shown to interact with and assist with the release of OmpA during and after translocation [89,90]. Additional factors are disulfide bond oxidoreductases **DsbA** and **DsbC**, which are not chaperones but form and isomerize the disulfide bonds needed for proper assembly of essential OMPs LptD and BamA [91].

Finally, the small, dimeric chaperone **spheroplast protein Y (Spy)** becomes overexpressed as part of the Cpx and BaeSR stress response pathways and can account for almost 50% of the total periplasmic mass under stress conditions [92] (Figure 2D, domain architecture and crystal structure). The mechanism of Spy chaperone function has been worked out in detail using variants of the model protein Im7 (a binding partner for colicin E7) [92–97]. Initially, long-range electrostatic interactions facilitate quick substrate association. Then a mixture of short-range hydrophobic and hydrophilic interactions on the concave surface of Spy stabilizes the complex, and Im7 folds while bound. Lower affinity of Spy for the native Im7 structure than for unfolded or partially folded conformations drives client release, and the chaperone cycle starts again. In addition to serving as a general stress response chaperone, Spy has been implicated in the binding some uOMPs [98]. Overexpression of Spy functionally substitutes for decreased OMP levels in a Δ*skp*/Δ*fkpA* background [98,99]. This indicates that Spy can act as a similar holdase chaperone, although likely with weaker affinity for uOMPs than Skp or FkpA, hence the requirement for overexpression.

Strategies for winning

Staying in motion

Conformationally heterogenous ensembles are an important feature of periplasmic chaperones binding equally dynamic uOMP ensembles. Current research into both chaperone conformations and uOMP ensembles reflects this importance. Box 1 highlights three recent examples illustrating how protein motions influence function in periplasmic chaperones: (i) how SurA domain dynamics modulate the binding-competent chaperone pool, (ii) how structural flexibility in the tentacles of Skp accommodates a wider range of clients, and (iii) how binding of IDRs prompt client release in Spy. Understanding how uOMP binding alters the conformational landscape of a periplasmic chaperone to regulate the functional cycle of client binding and release in the otherwise energy-deficient periplasm is a continued avenue of research in the field.

Teaming up

Functional redundancy in the OMP biogenesis pathway ensures that no single factor is solely responsible for the transit of uOMPs across the periplasm; it is a team effort. Homo-oligomerization, multivalent binding, and heteroprotein supercomplexes all represent ways that molecules team up via contiguous structural interactions in the periplasm.

Box 1. Three examples of conformational flexibility in periplasmic chaperones

SurA

As discussed previously, both PPIase domains of SurA move relative to the core domain via flexible linkers [19,20] (see Figure 2A in main text, domain motions). Either PPIase domain can dock onto and competitively block the uOMP binding site on the core domain [21]. Consistent with this interpretation, 'locking' of the P1 domain onto the core using a disulfide bond precludes SurA chaperone function *in vivo* [104]. However, the conformational heterogeneity of SurA also implies that an open, binding-accessible conformation exists at low abundance in the *apo*-chaperone ensemble. As a result, autoinhibition of the uOMP binding site occurs in the absence of uOMPs, but elevated uOMP concentrations in the periplasm favor the open, binding-competent conformation of SurA through Le Chatelier's principle. Like coupled oligomerization and binding in Skp or DegP, these protein domain motions in SurA may tune the chaperone reservoir to respond to changing uOMP levels as a function of periplasmic stress.

Skp

Skp has been observed, either directly or indirectly, *in vivo* or *in vitro*, to interact with almost all OMPs of all sizes expressed at appreciable abundance in *E. coli* (see Table 1 in main text) [105]. However, the cavity in the crystal structure of Skp is only large enough to accommodate a small protein of ≤25 kDa [46]. Multivalency contributes to the binding of larger uOMPs, but the inherent conformational flexibility of Skp also plays an important role. In solution, the tentacles of the jellyfish-like structure move to expand the central binding cavity. A specific hinge region just below the trimerization interface allows individual tentacles to independently flip out (see Figure 2B in main text, domain motions). Possible concurrent rearrangement of all three tentacles significantly enlarges the cavity for binding of large uOMPs of >25 kDa (see Figure 2B in main text, domain motions) [55]. In this case, structural flexibility increases the size range of possible Skp substrates.

Spy

In the well-characterized interaction between Spy and its model substrate Im7, it is well established that folding of Im7 while bound to Spy decreases the affinity between the two proteins and facilitates Im7 release [94–96]. However, a second mechanism of client release involves the N-terminal IDR of Spy (see Figure 2D in main text, domain motions). Negative charges on the IDR interact with positive charges on the concave client-binding surface of Spy. As a result, the N-terminal IDR can competitively bind the overlapping interaction surface and displace a substrate, initiating client release [106]. As it is unlikely that uOMPs appreciably fold while bound to Spy, the IDR displacement mechanism provides an interesting explanation for how client release might occur in a possible Spy–uOMP complex.

Oligomerization in periplasmic chaperones generally increases the interaction surface to shield more of the client from the aqueous solvent while complexed, improving chaperone function by better preventing aggregation. For Skp and DegP, the formation of oligomers also regulates aspects of chaperone function [48,75,80]. Active and inactive oligomeric states sit poised at equilibrium, ready to respond to changing uOMP concentrations due to periplasmic stress (Figure 3, oligomerization).

Beyond oligomerization, multivalency in chaperone–uOMP complexes has increasingly been observed. While a simple bimolecular association does a good job of describing the binding of periplasmic chaperones to small uOMP clients (i.e., eight-stranded β-barrels), multiple copies of SurA, Skp, or FkpA can bind larger uOMPs [22,23,29,49,50,55,59]. Multivalent binding of SurA to a uOMP is currently evidenced by scattering data, cross-linking results, native mass spectrometry, and a Hill coefficient of ~1.5 [22,23,29]. This binding mode is modeled with a beads-on-a-string configuration, where each SurA monomer interacts with the uOMP but not with each other (Figure 2A, chaperone:uOMP 2:1 complex) [22]. Multiple Skp trimers binding uOMPs has also been observed [49,50,55]. In the favored model for 2:1 Skp:uOMP complexes, two Skp trimers interlock to encapsulate larger uOMPs (Figure 2B, chaperone:uOMP 2:1 complex). These preliminary models require continued experimental and structural validation but show how interactions between chaperone and uOMP may become more complex than bimolecular association and dissociation in the cell envelope.

Heteroprotein complexes like BAM and the ***holo* translocon** of SecYEG-SecDF-YajC-YidC perform essential insertase and translocase functions in the cell envelope. But do other macromolecular assemblies important to OMP biogenesis form in the periplasm? Evidence is strongest

for a direct interaction between SurA and BAM. First identified as a genetic interaction and later cross-linked *in vivo* [13,38,100], more recent work has used hydrogen–deuterium exchange mass spectrometry, cross-linking, and structural modeling with AlphaFold to visualize the SurA–BAM interaction mediated through contacts with BamA, BamB, and BamE [36,37,101]. Direct interaction between SurA and BAM helps explain the outsized importance of SurA if the majority of uOMP flux through the periplasm accesses BAM through SurA. Other heterocomplexes forming between PpiD and the *holo* translocon and between BAM and BepA have been captured by photo cross-linking [86,87,89] and modeled in AlphaFold [101], highlighting the importance of these multiprotein assemblies at the IM and OM, respectively

Set plays and free play

Direct interactions between proteins in supermolecular complexes have been proposed to set specific pathways of uOMP transfer, but free passing of uOMPs between members of the network also recapitulates biological observations. Kinetic modeling of all known oligomerization and binding interactions involved in OMP biogenesis reproduces *in silico* the OMP assembly defects associated with genetic deletions performed in the laboratory [39,60]. If the rate constants for uOMP binding to and release from periplasmic chaperones are fast (millisecond to second timescale or faster) and individual interactions are transient, chaperones freely pass uOMPs among themselves until the uOMP folds into the OM via BAM or becomes degraded by DegP. In this model, uOMPs remain bound to a chaperone for almost the entirety of their second to minute-long journey across the periplasm to prevent aggregation and misfolding [39,100]. Alternatively, proposed parallel pathways [13,15] or supercomplexes that span the periplasm [37,102] set up a distinct, ordered, and quick passage to the OM. Such organization is not required to effectively model real-life observations, as dynamic binding and unbinding in combination with kinetic partitioning [31] is sufficient. In the complexity of biology, both types of interactions likely occur. However, a diffusive passing model is the simplest and least energetically costly model that explains available phenotypic observations. So, do periplasmic chaperones freely bounce and pass uOMPs around like a basketball between players? Do supermolecular structures and defined pathways in the periplasm set the course of OMP biogenesis? Or do both mechanisms play out in the periplasm? Continued work determining the spatiotemporal distribution of cell envelope proteins will be needed to answer these questions.

Concluding remarks

How then do the individual properties of each periplasmic player contribute toward the goal of scoring a folded OMP? Biogenesis of the essential OMP LptD provides an excellent example. LptD inserts **LPS** into the outer leaflet of the OM, and deletion of LptD leads to extreme OM defects that result in cell death [103]. LptD has been identified as a 'true' client of SurA [35], but Δ*skp*/Δ*fkpA* strains are also deficient in LptD assembly [99]. Overexpression of SurA cannot rescue the Δ*skp*/Δ*fkpA* effect. Similarly, neither Skp nor FkpA overexpression rescues a Δ*surA* phenotype. Proper LptD assembly requires both SurA and a tight-binding holdase in Skp or FkpA [99]. As the case of LptD illustrates, there exists both functional overlap and distinct features of each chaperone.

More generally, robust OMP biogenesis in *E. coli* appears to need three features: (i) a folding primer (SurA), (ii) a tight-binding holdase (Skp or FkpA), and (iii) a protease (DegP) [60]. SurA is set apart by its intermediate to weak binding affinity for uOMPs, expansion of uOMPs in the bound complex, and direct interaction with BAM. Considering these features, we hypothesize that the primary role of SurA is to hand off of uOMPs to BAM in an optimal conformation for folding. Holdases like Skp and FkpA suppress aggregation, which is toxic to the organism. Other chaperones like Spy, DegP, and even SurA can provide this holdase function when

Outstanding questions

What is the spatiotemporal distribution of OMP biogenesis factors in the cell envelope? Is OMP biogenesis spatially or temporally coupled to other cellular processes like cell division?

How do conformational heterogeneity and structural dynamics regulate client binding and release in the absence of ATP?

What sequence or structural features determine specificity versus promiscuity in the chaperone–uOMP interaction?

What are the global and local structural properties of the unfolded OMP ensemble? How do these properties change upon binding a chaperone?

Do multivalent ternary complexes form between different chaperone types?

How does understanding of the OMP biogenesis network in *E. coli* translate to homologous systems (i.e., other Gram-negative bacteria and mitochondria)?

How do OMP biogenesis deficiencies affect not only survival but also virulence?

Key figure

The outer membrane protein (OMP) biogenesis team adapts to stressful conditions to score folded OMPs and defend against aggregation

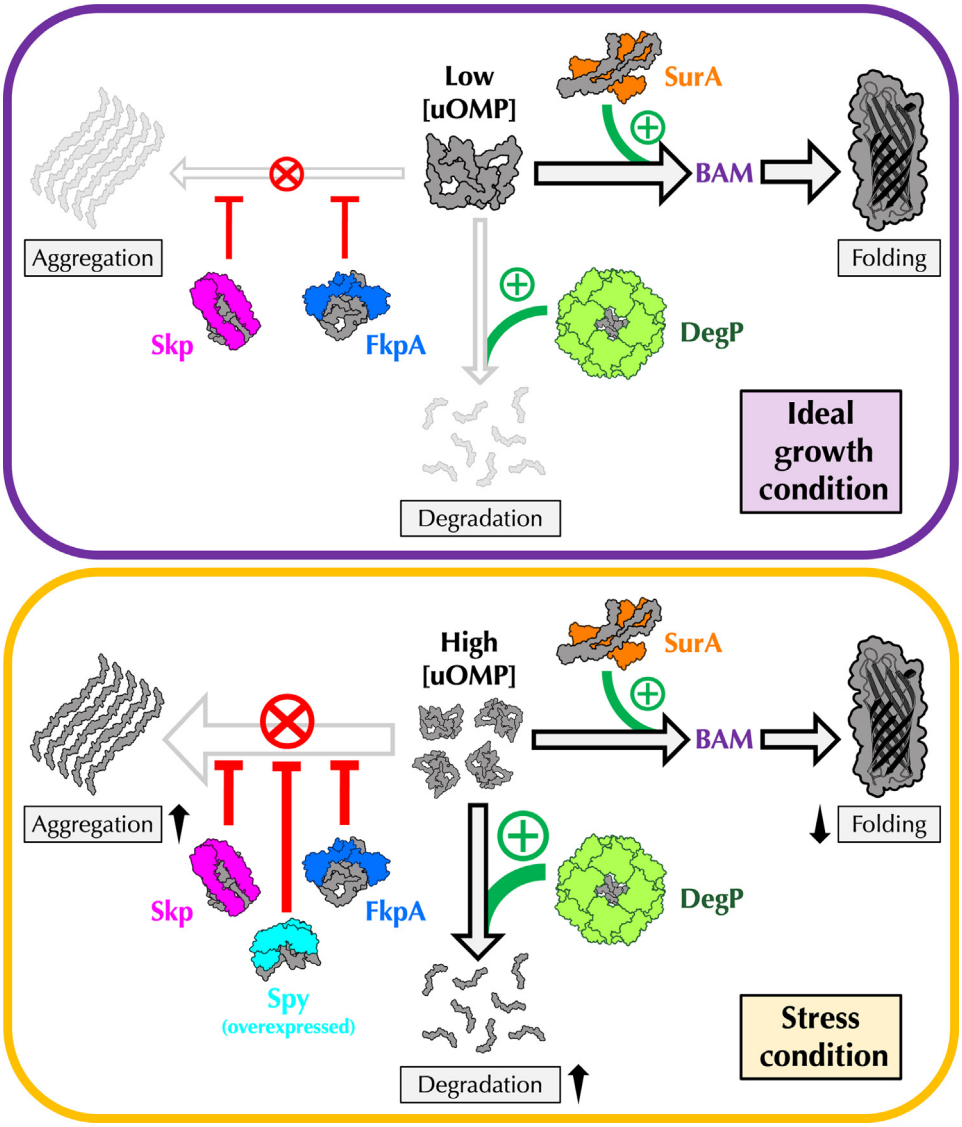

Figure 4. Under ideal growth conditions, holdases seventeen-kilodalton protein (Skp) and FK506 binding protein A (FkpA) prevent aggregation and misfolding, while survival factor A (SurA) promotes proper folding through the β-barrel assembly machine (BAM). Folding efficiency in the cell is maximized and almost all unfolded OMP (uOMP) in the periplasm is bound by a chaperone. Under stress conditions, such as heat shock stress, increased expression of holdase chaperones, including spheroplast protein Y (Spy), becomes essential to mitigate the increased risk of aggregation caused by higher concentrations of free uOMPs in the periplasm. Also, a significant fraction of uOMPs become degraded by DegP in this scenario, decreasing the levels of folded OMPs in the outer membrane. This network takes advantage of functional redundancy and unique chaperone properties to robustly respond to ideal and stressful growth conditions alike.

overexpressed, but some general holdase activity must exist in the periplasm for cell survival. Finally, a protease, primarily DegP, clears the accumulation of uOMPs in the periplasm and degrades misfolded conformations. Together the three categories of OMP biogenesis factors work as a team to score a folded OMP and defend against misfolding or aggregation (Figure 4, Key figure).

Much remains to be learned about OMP biogenesis in the context of cell survival and virulence (see Outstanding questions). Understanding the importance of spatiotemporal localization, binding specificity, regulation, and dynamics in OMP biogenesis will help illuminate how the functionally overlapping but complementary individual periplasmic players interact. The resulting network is complex and adaptable, ready to perform the essential duty of OMP biogenesis even under stresses such as high temperature, extreme pH, osmotic shock, or impaired folding at the BAM complex due to mutations or antibiotics (Figure 4). It is this resilience encoded into the periplasmic chaperone network that helps propel the bacterium to survive and win in the evolutionary game of life.

Acknowledgments

This work was supported by National Science Foundation grant MCB1931211 and National Institutes of Health (NIH) grant R35 GM148199 (K.G.F.). T.D. was supported by NIH training grant T32-GM008403. We thank Barbara Amann for providing feedback on the initial draft of this manuscript.

Declaration of interests

The authors declare no competing interests.

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