



## Review

The Bam machine: A molecular cooper<sup>☆</sup>

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## ABSTRACT

The bacterial outer membrane (OM) is an exceptional biological structure with a unique composition that contributes significantly to the resiliency of Gram-negative bacteria. Since all OM components are synthesized in the cytosol, the cell must efficiently transport OM-specific lipids and proteins across the cell envelope and stably integrate them into a growing membrane. In this review, we discuss the challenges associated with these processes and detail the elegant solutions that cells have evolved to address the topological problem of OM biogenesis. Special attention will be paid to the Bam machine, a highly conserved multiprotein complex that facilitates OM  $\beta$ -barrel folding. This article is part of a Special Issue entitled: Protein Folding in Membranes.

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**Abbreviations:** LPS, lipopolysaccharide; IM, inner membrane; OM, outer membrane; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; OMP, OM  $\beta$ -barrel protein; GFP, green fluorescent protein; PMF, proton motive force; ATP, adenosine triphosphate; Psp, phage shock protein; NMR, nuclear magnetic resonance; PELDOR, pulsed-electro double resonance; SAXS, small-angle X-ray scattering; rmsd, root-mean-square deviation; AT, autotransporter; Bam,  $\beta$ -barrel assembly machine; POTRA, polypeptide translocation-associated; TPR, tetratricopeptide repeat; IL, interconnecting loop; EOM, ensemble optimization method; FHA, filamentous hemagglutinin; Mla, maintenance of lipid asymmetry

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## 1. Introduction

Perhaps the most distinguishing feature of the typical Gram-negative bacterium is the remarkable structure referred to as the outer membrane (OM). This robust bilayer accommodates a unique complement of OM-specific proteins and lipids that perform specialized cellular functions; as such, the OM can be regarded as an extra-cytoplasmic organelle. This membrane functions primarily as a highly selective permeability barrier that insulates the cell against a variety of potentially cytotoxic agents in the extracellular milieu. Incorporated in the OM are membrane proteins that participate in a diverse array of processes including transport (both passive and active), proteolysis, secretion, adhesion, signaling, homeostatic control, and biogenesis and reinforcement of the OM itself [1].

The targeting and assembly of membrane proteins in general is a complex process that requires multiple folding factors. OM protein biogenesis is further complicated by the fact that these proteins must traverse one lipid bilayer and then integrate specifically into another; the cell must be capable of discriminating between inner membrane (IM) and OM proteins in addition to coordinating the assembly of each.

A comprehensive review of membrane protein folding must include a discussion of integral  $\beta$ -barrel protein assembly in the Gram-negative OM, particularly because this system serves as a model for understanding mitochondrial and chloroplast biogenesis (as these organelles are the products of an ancient bacterial symbiosis; [2]). Because an appreciation of the topological problem of integral OM protein biogenesis requires some familiarity with OM anatomy and physiology, we preface our discussion of the topic with a primer on the individual components of the OM.

## 2. Description of OM and components

### 2.1. OM lipids

With respect to lipid content, the outer membrane in most Gram-negative bacteria is peculiar in that it is a composite of two major lipidic species (phospholipid and LPS) that are asymmetrically distributed in the membrane. These lipids, discussed below, have unique properties that substantially influence the fluidity and permeability of the OM.

#### 2.1.1. Phospholipids

The majority species of phospholipid in *E. coli* is the zwitterionic phosphatidylethanolamine (PE), which represents roughly 75–80% of the total phospholipid content in the cell. Negatively charged phosphatidylglycerol (PG) comprises much (15–20%) of the remainder, and a PG condensate known as cardiolipin (CL) is present in small amounts. This ratio is slightly altered in the OM, where PE represents an even larger proportion of total membrane phospholipid (~90%). The relatively high concentration of phospholipids with saturated acyl chains in the OM, along with the associated increase in membrane rigidity, further distinguishes the OM from the IM [3,4].

#### 2.1.2. LPS

The barrier property of the OM can largely be attributed to the presence and asymmetrical distribution of the well-known bacterial

glycolipid called lipopolysaccharide (LPS). This surface-molecule-turned-antigen is specifically targeted to the OM and is normally found exclusively on the outer surface of the cell [5]. LPS monomers in a bilayer exhibit strong lateral interactions with one another; this in combination with the aforementioned enrichment of fully saturated phospholipids in the inner leaflet greatly reduces the fluidity of the OM, which is rigid and gel-like in comparison to the IM [6].

LPS can be subdivided into three distinct fractions: lipid A (which secures LPS in the OM via a hexa-acylated sugar moiety), core oligosaccharide, and the distal O-antigen (which is not produced in derivatives of *E. coli* K-12). Synthesis of lipid A and addition of the core oligosaccharide occur at the cytoplasmic face of the IM, whereas O-antigen ligation only takes place after the nascent glycolipid is flipped to the periplasmic face of the IM [7]. LPS is shuttled to and integrated into the OM by a dedicated transport system that spans the *E. coli* cell envelope [7].

### 2.2. OM proteins

Despite the robustness of the OM, Gram-negative bacteria are capable of selective uptake of essential nutrients, toxin secretion (pathogenicity) or efflux (multidrug resistance), and assembly of complex surface-exposed macromolecules (as well as the cell envelope itself). This is accomplished through the regulated synthesis, assembly, and activity of OM proteins [6]. Although exceptions to the following generalization have been described, the vast majority of OM proteins present in Gram-negative bacteria belong to one of two major classes:

- 1) lipoproteins, which are tethered to the periplasmic face of the OM via N-terminal lipid modifications, and
- 2)  $\beta$ -barrels (OMPs), which span the OM and enable the cell to interact and mediate exchange with its environment.

#### 2.2.1. Lipoproteins

Many periplasmic proteins likely display diffuse localization patterns within the periplasm, as has been reported for periplasmic green fluorescent protein (GFP) [8]. However, a host of periplasmic proteins execute functions specifically at the inner or outer membrane. The N-terminal lipidation of membrane-associated periplasmic proteins serves to spatially restrict their activity, presumably enabling them to function more efficiently. Over 90 lipoproteins have been identified in *E. coli* [9], and these factors have been shown to participate in a host of processes including envelope biogenesis [10–14], cell division [15], secretion [16], and signaling [17]. Lipoproteins may interact with periplasmic proteins, integral membrane proteins, the murein layer, or other lipoproteins, and are sometimes found as vital components of transmembrane complexes.

Membrane anchoring of lipoproteins is not necessarily a prerequisite for function. Indeed, multiple essential lipoproteins in *E. coli* are known to function properly even when the N-terminal lipobox (required for lipidation) is deleted, so long as they are produced in excess [18,19]. This suggests that lipidation of some periplasmic proteins is simply a matter of economy. However, mislocalization of certain envelope lipoproteins abrogates function and in some cases causes toxicity. For example, ectopic IM localization of the peptidoglycan-associated lipoprotein Lpp leads to cell lysis [20], and rerouting of the OM-associated (penicillin-binding protein) PBP cofactor LpoA to the IM

renders it unable to participate in peptidoglycan biosynthesis [13]. Therefore, the proper localization of lipoproteins, generally speaking, is critical for growth and viability.

### 2.2.2. OMPs

In addition to the presence of LPS and the asymmetrical lipid distribution of the OM, another prominent characteristic that distinguishes it from the IM is the presence of integral membrane proteins that almost exclusively adopt a  $\beta$ -barrel conformation. The  $\beta$ -barrel structure can be imagined as an antiparallel  $\beta$ -sheet that wraps around a central pore to form a cylinder stabilized by an inter-strand main chain hydrogen bonding network; the barrel is closed by the noncovalent pairing of the first and last beta strands [21].

As with lipoproteins, membrane-integral  $\beta$ -barrel proteins carry out diverse functions in the outer membranes of Gram-negative bacteria (as well as in OMs of mitochondria and chloroplasts). The most abundant OMPs in *E. coli* serve as passive, relatively non-specific transporters that permit the diffusion of sugars, ions, and small hydrophilic molecules smaller than ~700 Da across the OM. Examples of these porins include OmpF, OmpC, and PhoE, all of which form highly stable trimeric complexes in the membrane. Other OMPs, such as the maltodextrin transporter LamB or the sucrose channel ScrY, facilitate diffusion of specific substrates. While the general and specific porins were among the earliest to be characterized, OMPs have since been shown to participate in an wide array of cellular processes including energy-dependent efflux (e.g. TolC), active transport (e.g. FhuA or BtuB), adhesion (e.g. Ag43), secretion (e.g. so-called autotransporters), pilus biogenesis (e.g. FimD), OM biogenesis (e.g. BamA or LptD), proteolysis (e.g. OmpT), peptidoglycan binding (e.g. OmpA), and the stress response (e.g. OMPLA) [22].

Although OM  $\beta$ -barrels can have widely different functions, available structural information reveals a number of physical characteristics that typify this family of proteins (Fig. 1). Every known bacterial OMP contains an even number of transmembrane (TM)  $\beta$ -strands such that the N- and C-termini of the barrel reside in the periplasm (it is worth noting that this is not necessarily the case for mitochondrial  $\beta$ -barrels). The  $\beta$ -strands are tilted relative to the transmembrane axis, and the length of each  $\beta$ -strand is correlated with the degree of the tilt. As the TM surface of an OM  $\beta$ -

barrel is lipid-exposed, it is not surprising that hydrophobic residues are present in a dyad repeat pattern in the TM  $\beta$ -strands (Fig. 2A); this pattern ensures a continuous hydrophobic surface around the barrel exterior, while permitting positioning of polar residues within the interior, often resulting in formation of a water-filled pore [22]. Short turns connect the  $\beta$ -strands at the periplasmic face of the OM, whereas long loops bridge the strands at the extracellular face. These loops may fold into and constrict (or occlude) the barrel pore, or they may interact with LPS; in certain cases, loops are critical for protein function (e.g. [23,24]).

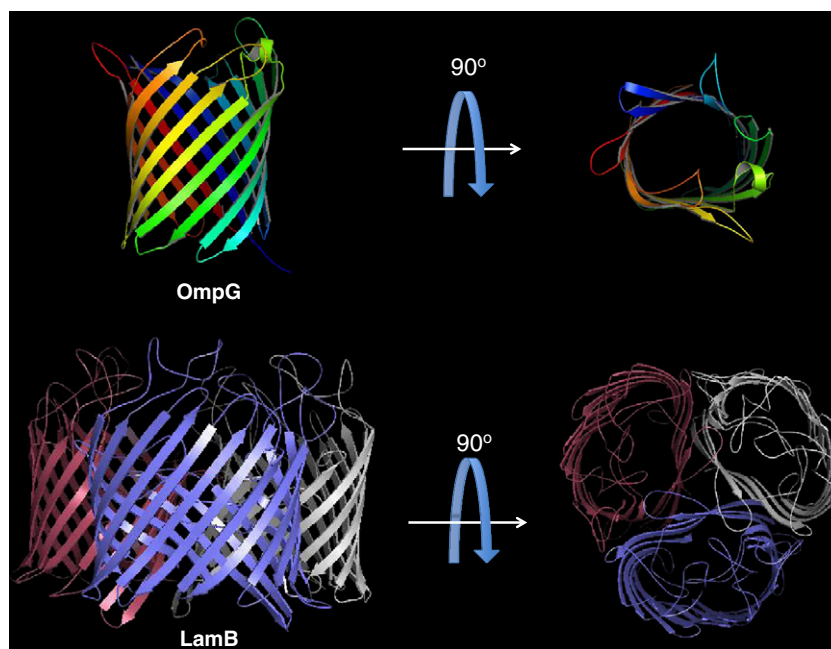
Some OMPs are hybrid proteins that contain sizeable periplasmic domains. Such domains may extend the function(s) of individual OMPs and serve as receptors, cell wall anchors, or platforms for multi-protein complex assembly [10,12].

### 2.2.3. Accessory proteins and surface structures

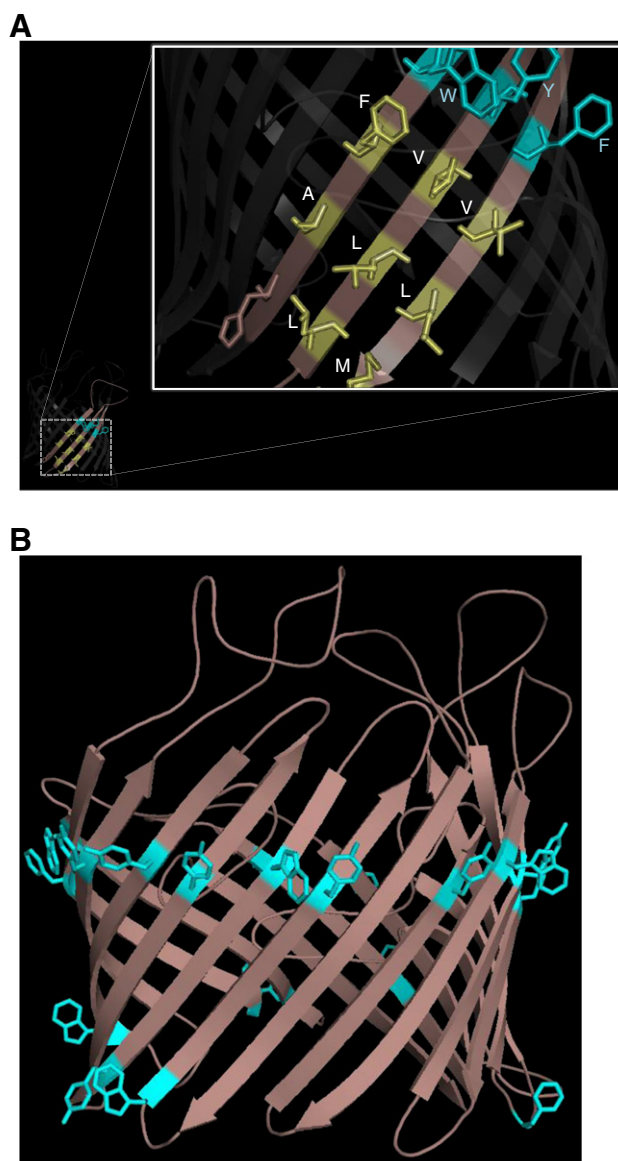
In addition to membrane lipids and integral proteins, the OM of *E. coli* accommodates a variety of complex surface-exposed accessory structures. The membrane serves as a substratum for assembly of appendages such as pili, flagella, and the injectisome, and it also anchors the capsular exopolysaccharide to the cell surface. It is not surprising, therefore, that defects in OM biogenesis can have deleterious effects on processes such as virulence, motility, and biofilm formation, which can be dependent on surface appendages or capsules. Recent comprehensive reviews of the biogenesis of these structures have been presented elsewhere (cf. [25–27]). Consequently, they will not be discussed in detail here, except to say that many of the factors required to build these appendages are themselves OM proteins (e.g. FimD, the type I pilus usher, is an OM  $\beta$ -barrel); therefore, proper assembly of surface structures can depend indirectly on proper assembly and folding of OM proteins.

### 2.3. Peptidoglycan (a disclaimer)

The peptidoglycan cell wall is an elastic mesh-like network of rigid glycan strands cross-linked by flexible peptide linkers. This essential organelle is required to resist turgor pressure, promote cell growth and septation, and maintain cell shape. The fact that this critical structural element is situated between the inner and outer membranes of Gram-



**Fig. 1.** The general  $\beta$ -barrel fold. These *E. coli* OMPs exhibit characteristics typical of bacterial OM  $\beta$ -barrels, including short periplasmic turns, long extracellular loops (some of which fold back into the barrel lumen in the case of LamB), and an even number of  $\beta$ -strands. Note that OmpG (top, PDB ID: 2X9K) is monomeric, whereas LamB (bottom, PDB ID: 1AF6) forms stable trimers in the OM.



**Fig. 2.** A. The alternating (dyad repeat) pattern for hydrophobic residues in TM strands 8–10 of the *E. coli* OM beta-barrel protein LamB (PDB ID: 1AF6). Non-polar, membrane-exposed residues are highlighted in yellow, and the aromatic side chains forming part of the aromatic girdle (see Fig. 2B) are highlighted in cyan. B. Residues defining the "aromatic girdle", shown in cyan on this structure of the LamB monomer from *E. coli* (PDB ID: 1AF6), demarcate the membrane boundaries.

negative bacteria might suggest that it represents a physical barrier for nascent OM components in transit through the periplasm; however, no available evidence supports this conclusion. Additionally, attempts to determine the porosity of the murein sacculus suggest that the peptidoglycan layer is permeable to globular proteins up to 100 kDa [28]. Although the role(s) of the peptidoglycan layer in OM biogenesis (if any) has generally been difficult to characterize, the available evidence suggests that involvement of the sacculus in transport or assembly of OM components is minimal at best. Accordingly, with all due reverence for this vital cellular factor and our colleagues who study it, we will largely ignore the peptidoglycan layer in our discussion.

### 3. The topological problem of OM biogenesis

None of the constituent parts of the OM are synthesized in situ. As a consequence, OM lipids and proteins must overcome a series of obstacles in order to traverse the cell envelope from their site of synthesis in the cytoplasm or the inner leaflet of the IM to their final location in the membrane. Following synthesis, OM-directed components

must first be translocated across the IM. This is an energetically unfavorable process for both lipids and proteins, so this step must be coupled to an exergonic reaction [29]. As membrane proteins and lipids necessarily contain highly hydrophobic domains, they must then be at least partially shielded from the aqueous periplasm in order to prevent aggregation and/or irreversible misfolding due to hydrophobic collapse. Additionally, these components must be specifically targeted to the OM, as the presence of OM components in the IM can cause disastrous effects [30,31]. As such, it is imperative that targeting of OM factors be specific and unidirectional. Lastly, OM lipids and proteins must be properly assembled into the OM, and in the case of OMPs, locally folded. To complicate matters further, OM integration must occur during growth in the absence of any obvious energy source (the periplasm is devoid of ATP and the OM is not energized by an ion gradient), all the while maintaining a robust permeability barrier [32].

The Gram-negative cell has evolved elegant solutions to the topological problem of OM biogenesis. Although each OM constituent (with the possible exception of phospholipid) has a dedicated periplasmic transport



and OM assembly system, the targeting process for all components can be generally described as follows:

- 1) OM components are synthesized in the cytosol (or at the cytosolic face of the IM),
- 2) energy derived from ATP hydrolysis promotes translocation across (and, in some cases, release from) the IM,
- 3) periplasmic chaperones shield hydrophobic patches and direct the components to the OM for assembly,
- 4) a dedicated OM protein (or protein complex) coordinates energy-independent substrate transfer and OM integration.

Each transport system accomplishes the steps above through the concerted efforts of envelope factors present in each cellular compartment. In this review, we will describe these systems in detail, with special attention paid to the transport and OM assembly of  $\beta$ -barrel proteins.

#### 4. Solutions to the topological problem of OM biogenesis

##### 4.1. Lipids

Every OM lipid is an IM emigrant. LPS and OM-destined phospholipids are synthesized at the cytoplasmic face of the IM, flipped to the periplasmic face by IM proteins, and directed to the OM [33]. The lipopolysaccharide transport (Lpt) has been extensively characterized, and recent studies (discussed below) have contributed significantly to our understanding of LPS biogenesis. In stark contrast, the mechanism of transmembrane phospholipid transport remains mysterious.

##### 4.1.1. LPS transport

The journey to the OM begins for LPS following synthesis at the cytoplasmic face of the IM (for review see [34]). “Rough” LPS (lipid A + core) is first flipped to the periplasmic face of the IM by the ATP-dependent translocase MsbA, which can also promote the translocation of phospholipids. Upon entering the periplasm, O-antigen is

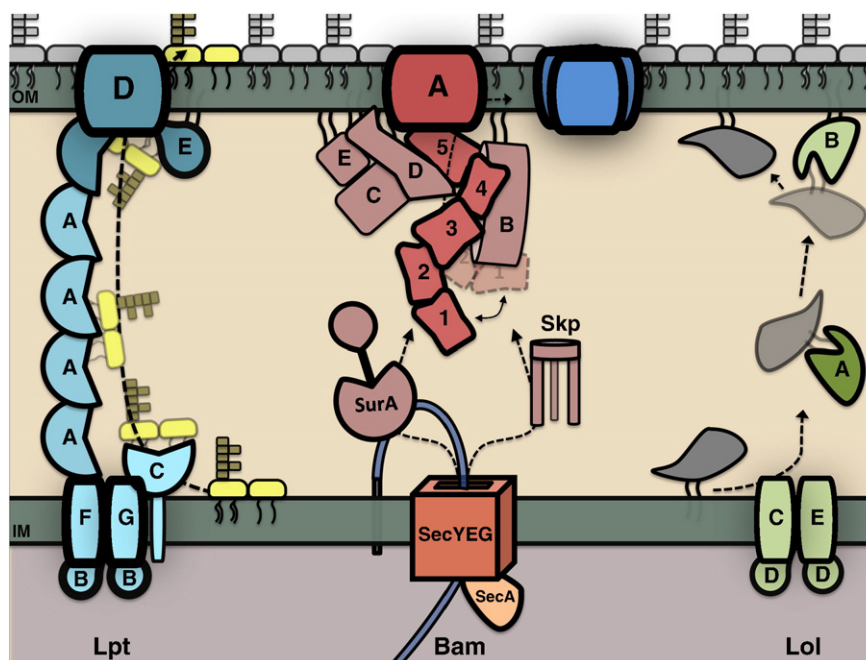
distally ligated onto rough LPS to form mature (“smooth”) LPS. The IM ABC transporter LptBFG likely energizes the release of LPS from the IM [35,36], and the bitopic membrane protein LptC binds LptA at the IM [37] and promotes transfer of the lipid A moiety of LPS to the soluble periplasmic factor LptA [38].

Until recently, it was not known whether LptA forms a soluble periplasmic intermediate with LPS or whether it serves as the periplasmic component of a transenvelope protein bridge, although results of previous studies were more consistent with the latter model [39]. Recent reports provide genetic and biochemical evidence that strongly supports the IM–OM bridge model for LPS transport [37,40].

Following IM extraction, the remaining steps in LPS transport and assembly proceed in an energy-independent fashion (Fig. 3). This likely involves the affinity-driven directional transfer of LPS monomers from the LPS binding factor at the IM (LptC), across the periplasmic transmembrane bridge (formed by LptA), to a two-protein OM complex composed of the  $\beta$ -barrel LptD and the OM lipoprotein LptE. The manner in which LptDE promotes the cell surface assembly of LPS remains to be determined, though an attractive model proposed by the Kahne group [18,41] posits that LPS is directly inserted into the OM outer leaflet through a lateral opening in the LptD barrel (perhaps gated by LptE). Such a model is especially appealing in light of the fact that OMPs permitting lateral diffusion of lipids into the OM have already been described [42].

##### 4.1.2. Phospholipid transport

Nothing is known about how phospholipids are shuttled from the IM to the OM. The translocation (“flip-flop”) of phospholipids to the periplasmic face of the IM can be mediated actively by MsbA or passively by  $\alpha$ -helical TM peptides [43–45], although the details of translocation by either route are not understood. Beyond this point, while it is clear that OM phospholipids originate in the IM [46], the mechanism of intermembrane transport remains elusive. Periplasmic lipid vesicles have not been observed visually, and it is unclear whether the peptidoglycan layer would permit the passage of vesicles across



**Fig. 3.** OM biogenesis in *E. coli*. All components of the bacterial OM are synthesized in the cytoplasm and translocated across the IM into the periplasmic space. Once in the periplasm, each OM constituent traverses the envelope and integrates into the OM via a dedicated transport system (shown above). The Lpt components form a transenvelope protein bridge that shields the lipid A moiety from the aqueous environment and funnels LPS through LptDE to the cell surface. OM  $\beta$ -barrels (OMPs) cross the IM through the Sec translocon and associate with periplasmic chaperones that target OMPs to the Bam machine for assembly. OM lipoproteins are extracted from the IM in an ATP-dependent fashion and transferred to an OM receptor (LolB) via a periplasmic carrier protein (LolA). Phospholipid transport is not shown, as the mechanism by which they are trafficked to the OM is not known.

the periplasm [4]. Zones of adhesion between the IM and OM have been postulated [47,48], though their existence remains controversial [49]. It is known that phospholipid shuttling does not require ATP or protein synthesis, but is heavily dependent on the proton motive force [50]. If the bulk of cellular phospholipid transport occurred through zones of membrane fusion or sites of membrane collision [51], it is not obvious why the proton motive force (PMF) would be required (unless IM–OM fusion itself requires the PMF).

Unlike protein and LPS transport, phospholipid transport is bidirectional [52]. A conserved system mediating retrograde phospholipid transport (OM to IM) has been identified in *E. coli* and was shown to contribute to the maintenance of lipid asymmetry (Mla) at the OM [53]. While the Mla system participates in phospholipid trafficking, it must not be the only means for phospholipid transport, as *mfa* mutant phenotypes are subtle and the system is dispensable for viability [53].

Numerous mechanisms of nonvesicular lipid transport have been observed or proposed in eukaryotes [51], and lipid trafficking in diderm prokaryotes could occur by one or more of these mechanisms in an analogous fashion. Elucidation of the lipid trafficking process(es) employed by bacteria will clearly require further inquiry.

#### 4.2. Proteins

All cellular proteins are synthesized in the cytoplasm, and proteins destined for the OM are no exception. Because export of lipoproteins and OMPs requires the Sec translocase, which only accommodates unfolded substrates [29], the earliest challenges facing nascent OM proteins involve stabilization of unfolded, aggregation-prone polypeptides in the cytoplasm and targeting of these preproteins to Sec for translocation.

Proteins bound for the cell envelope of *E. coli* can either be translocated during (SRP-dependent) or following (SecB-dependent) translation. In general, integral IM proteins are co-translationally targeted to Sec via the signal recognition particle (SRP)-dependent pathway, whereas exported proteins are maintained in an unfolded, translocation-competent state after translation by the cytoplasmic chaperone SecB, which binds regions of the mature, unfolded protein [54]. These secreted proteins are synthesized with an N-terminal signal sequence that serves as a recognition domain for SecA, the catalytic component of the translocon, which harnesses energy derived from ATP hydrolysis to drive polypeptide translocation through the channel formed by SecYEG [29].

Interestingly, OMPs are not strictly dependent on the post-translational, SecB-dependent targeting pathway for export. An increase in the hydrophobic character of OmpA or LamB signal sequences results in the co-translational, SecB-independent export of these OMPs, which are still correctly targeted to and assembled into the OM following translocation [55,56]. This suggests that post-translational secretion, while characteristic of all OM proteins, is not important for successive maturation steps following translocation.

Upon entry into the periplasmic compartment, the paths of lipoproteins and OMPs diverge. In this section, we discuss the OM targeting and assembly pathways for the two major types of OM protein after IM translocation.

##### 4.2.1. Lipoproteins

A lipoprotein is not born that way. The maturation process involves a series of post-translational modifications made to nascent lipoproteins following IM translocation. Specifically, prolipoproteins bearing a conserved lipobox sequence (present at the boundary between the signal sequence and cleavage product) are acylated at a conserved N-terminal cysteine residue both prior to and immediately following removal of the signal sequence. The resulting covalently affixed lipid moiety securely anchors the mature lipoprotein to the membrane [57]. Some lipoproteins are retained in the IM and

function there, but the majority (90%) are targeted to the OM by a dedicated periplasmic transport system called Lol [58].

Before a mature lipoprotein can be targeted to the OM it must first be liberated from the IM. Since lipoproteins are securely attached to membranes, it is not surprising that extraction of the lipid moiety from the IM is an energy-dependent process. This extraction is catalyzed by the concerted efforts of LolCDE (the constituent members of an IM-associated ATPase) and LolA (the periplasmic lipoprotein release factor). LolCDE energizes the transfer of the lipoprotein acyl chains from the IM to LolA, which forms a soluble periplasmic complex with the free lipoprotein. LolA then transfers the lipid moiety in a “mouth-to-mouth” fashion [59] to the structurally similar lipoprotein acceptor LolB, itself an OM lipoprotein, at which time the LolA–lipoprotein complex is dissociated and the lipid transferred to the inner leaflet of the OM via LolB. Thus, the localization of LolB at the OM and LolCDE at the IM ensures that lipoprotein transport is unidirectional and irreversible.

How is the Lol transport system able to distinguish OM lipoproteins from those that function at the IM? In *E. coli*, the identity of the residue(s) immediately following the acylated N-terminal cysteine specifies the fate of the lipoprotein. The presence of an aspartate at this +2 position causes IM retention of lipoproteins, and the presence of most other amino acids at +2 generally leads to OM localization. Asp(+2) serves as a “Lol avoidance signal” that prevents the release of lipoproteins from the IM, perhaps by promoting the formation of a tight lipoprotein–phospholipid complex that does not interact with LolCDE [60]. An elegant genetic selection conducted by the Pugsley group [61] led to the identification of alternative Lol avoidance +2 residues (e.g. Pro and Trp), however, these residues are not found in endogenous lipoboxes in *E. coli*. Additional studies have demonstrated a role for the residue at the +3 position in Lol avoidance [62], suggesting that the +2 residue is necessary but not sufficient for IM retention.

It is interesting to note that the Lol system has recently been implicated in the biogenesis of a non-lipoprotein [16]. The secretion of pullulanase is facilitated by the Pul secretin, a homododecamer of the OM protein PulD [63], and OM localization of PulD requires the OM lipoprotein PulS [64]. Indirect evidence for a LolA–PulS–PulD periplasmic complex suggests that PulD might “piggy-back” on PulS to co-opt Lol for OM localization [16]. Although the Lol–Pul heterotrimer could not be observed directly, its putative existence represents a novel targeting mechanism for membrane-spanning OM proteins.

##### 4.2.2. OMPs

**4.2.2.1. Late translocation steps.** Nascent  $\beta$ -barrel proteins enter the periplasm in an N- to C-terminal fashion during transit through the Sec translocon [29]. Since unfolded OMPs have a tendency to aggregate in solution, it is conceivable that interactions with periplasmic chaperones occur during or immediately following translocation. Indeed, a number of periplasmic factors have been implicated in facilitating late steps in translocation and/or release from the translocon. A recent report of the crystal structures of SecDF, two accessory components of the Sec machine, implies a role for these factors in mediating late steps in translocation in a manner dependent on the PMF [65]. Beyond the secretion machinery, there is evidence suggesting that the IM-associated periplasmic chaperone PpiD positively influences translocation of OMPs across the IM via early interactions with unfolded polypeptides exiting the Sec translocon [66]. This finding is intriguing insofar as it offers an explanation for the phenotype(s) associated with deletion of *ppiD* *in vivo*; however, all of the evidence supporting a role for PpiD in late steps of translocation was obtained in an *in vitro* system, potentially complicating interpretation of the results.

The periplasmic chaperone Skp (seventeen kilodalton protein) was also shown to interact with translocation intermediates of model OMPs at the periplasmic face of the IM [67]. This early interaction seems to

require release of the nascent polypeptide from ribosomes, which may suggest that some degree of secondary structure formation is required in order for Skp to bind an emerging OMP. It has been proposed that Skp promotes the release of newly translocated OmpA from the IM [67], but the fact that such evidence is derived from spheroplast experiments, combined with the demonstrated inability of Skp to enhance release of PhoE from Sec in a similar system [68] makes further inquiry necessary before firm conclusions can be drawn. Still, the processing delay observed for OMPs in the absence of certain periplasmic or OM-associated OMP biogenesis factors [69] may signify a role for extracytoplasmic proteins in accepting substrates before translocation is complete.

Following IM translocation, the mature OMP is liberated from its IM-associated signal sequence by periplasmic peptidases. Retarding or altogether preventing signal sequence cleavage by mutation of the processing site interferes with the targeting, assembly, and oligomerization of the trimeric OMP LamB, demonstrating the importance of signal sequence processing for OMP biogenesis. Additionally, the release of this processing-defective LamB variant from the IM is delayed, which may explain the observed activation of the phage shock (Psp) response [30], which is known to be sensitive to IM stress in general and disruption of the PMF in particular [70]. Assuming that Psp activation is an indicator of IM perturbation, it is possible that IM-tethered LamB may assemble aberrantly into the cytoplasmic membrane; such an event is likely to be toxic to the cell, and the reported toxicity associated with high-level production of processing-defective LamB further supports this notion.

**4.2.2.2. Interactions with periplasmic chaperones.** The importance of OMP-chaperone interactions in the periplasm is well established. Left to its own devices, a wayward beta barrel in the aqueous periplasmic space can wreak havoc in several respects: a) the surface hydrophobicity and insolubility of OMPs quickly leads to the toxic accumulation of misfolded aggregates, b) aberrant association with and assembly into the IM perturbs the membrane and causes stress response activation, and c) failure to correctly target chaperone-dependent OMPs (such as the essential LPS assembly factor LptD) compromises the OM permeability barrier and negatively affects cell viability. Periplasmic chaperones serve to maintain nascent OMPs in a folding-competent state while preventing off-pathway misfolding and aggregation, and they are critically important for specific targeting of OMPs to the OM [1].

In *E. coli*, the periplasmic protein SurA (so called because it was initially identified in a screen for genes required for stationary phase survival [71]) is regarded as the primary periplasmic OMP chaperone [72]. A role for SurA in extracytoplasmic folding (and OMP biogenesis in particular) was proposed simultaneously by several groups in the mid 1990's [73–75], and subsequent genetic, biochemical, and structural inquiry has led to a refined understanding of the function of this chaperone.

SurA was initially supposed to function in envelope protein folding in light of several *in vivo* observations:

- 1) overexpression of *surA* suppresses OM permeability defects and stress response induction associated with impaired OM biogenesis [75],
- 2) OMP maturation is retarded in a  $\Delta$ *surA* background [73,74],
- 3) deletion of *surA* results in activation of the  $\sigma^E$  extracytoplasmic stress response [74].

These findings implicated SurA in the targeting and/or folding of  $\beta$ -barrel proteins, raising the possibility that SurA is an OMP chaperone. Consistent with a role in OMP biogenesis, SurA was shown to interact specifically with unfolded (but not folded) OMPs [76,77], as well as with model peptides containing motifs that are enriched in OM  $\beta$ -barrels [77–82].

An unexpected structural motif found in all SurA homologs is the peptidyl-prolyl *cis-trans* isomerase (PPIase) domain of the parvulin type. The *surA* ORF encodes two PPIase domains referred to as P1

and P2 [73,75]. Both domains were shown to be dispensable for the chaperone activity of SurA, which instead resides in a molecular cradle formed by the association of the N- and C-terminal domains [76,83,84]. It remains unclear at this time what the importance of the PPIase domains might be, although the fact that deleting either parvulin domain from SurA causes novobiocin sensitivity in UPEC strains of *E. coli* [84] may imply a specific, parvulin-dependent function of SurA. It is conceivable that these domains are required to catalyze proline isomerization in some specific substrate (or substrates), thereby accelerating folding; indeed, P1 and P2 do endow SurA with the ability to isomerize peptidyl-prolyl bond [76,83]. However, mutation of catalytic site residues in P2 effectively abolishes the PPIase activity of SurA without affecting chaperone activity [76]. In addition, the P1 domain of SurA is conserved in the leptospiral homolog of SurA, but this domain is devoid of PPIase activity [85]. Taken together, these findings show that the contributions of the PPIase domains to the chaperone function of SurA are probably minimal. Still, the extraordinary conservation of at least one parvulin domain across SurA homologs implies a role for PPIase domains in somehow increasing the efficiency of OMP targeting/assembly, or in catalyzing an as-yet-unidentified secondary function of SurA. It is worth noting that the ability of SurA to interact with model peptides rich in aromatic residues is dependent on the P1 domain, which binds these peptides well even in isolation [82]. As such, the parvulin domains may influence substrate binding by promoting specific interactions.

SurA is known to enhance the rate of OMP monomer folding [69,73,74]. Additionally, the deletion or depletion of *surA* causes strong induction of the  $\sigma^E$  stress response (which is activated by unfolded OMPs), implying that  $\beta$ -barrel folding is compromised in the absence of SurA. This could reflect a general role for this chaperone in OMP assembly. To determine the substrate range of SurA, Collet and colleagues compared the OM proteome of a wild-type strain of *E. coli* to that of a  $\Delta$ *surA* mutant [86]. Surprisingly, they found that many OMPs (15 of the 23 observed) are *not* affected by the absence of SurA. Furthermore, many OMPs of reduced abundance in a  $\Delta$ *surA* strain are controlled at the level of synthesis by  $\sigma^E$ ; consequently, the relative contribution of SurA to folding of those OMPs could not be unambiguously determined, as depletion of *surA* effectively results in the downregulation of major OMP genes.

Despite this, proteomic analysis did lead to the identification of two OMPs whose decreased abundance in a  $\Delta$ *surA* mutant could not be explained by decreased synthesis: FhuA, a ferrichrome transporter, and LptD/Imp, the integral OM component of the Lpt transport system required for assembly of LPS into the OM. Levels of LptD in the membrane are reduced by an order of magnitude in the absence of SurA, despite the fact that *lptD* transcription is *increased* upon  $\sigma^E$  activation [86]. This suggests that LptD is a (perhaps *the*) primary substrate of SurA; it is certainly the most important substrate, given the significant OM permeability and growth defects associated with impaired LptD biogenesis [87]. In light of this, it is interesting to note that *lptD* and *surA* are organized into an operon [86,88].

It has been known for some time that defects in LPS biogenesis can have downstream effects on OMP assembly. For example, reduced OMP levels [89,90] and slowed kinetics of porin folding and trimerization [91] are observed in LPS truncation mutants, and the arrest of lipid synthesis by cerulenin treatment causes a block in OmpF assembly [92]. In addition, the increase in OM permeability associated with reduced expression [93] or mutation [94] of LptD has been described in detail (indeed, the original *lptD* gene name is *imp*, reflecting the increased membrane permeability of *lptD* mutants). Some have gone so far as to blame most of the pleiotropic effects associated with loss of SurA on reduced levels of LptD in a  $\Delta$ *surA* background [86]. This might suggest that the function of SurA in OMP biogenesis is secondary to its role as a *bona fide* LptD chaperone, at least in *E. coli*. However, the ability of SurA to greatly increase the efficiency of OmpT folding in an *in vitro*  $\beta$ -barrel assembly system [95] and the accumulation of unfolded porin observed



upon SurA depletion and change in OM density [72] clearly indicate a role for SurA as a general OMP chaperone. Furthermore, in addition to LptD, a requirement for SurA has been described for a number of specific OMPs, including the OM components of the chaperone/usher pilus assembly systems for both P (PapC) and type I (FimD) pili [84,96], the autotransporters EspP [97,98], Hbp [99], and IcsA [100], and the adhesin intimin [101].

Crystallographic studies on the tertiary structure of SurA across various species have highlighted the modular nature of SurA and homologous chaperones (including the cytoplasmic ribosome-associated chaperone Trigger Factor) [83,85,102]. The first solution structure of *E. coli* SurA revealed a core chaperone domain tightly associated with the P1 domain, whereas the “satellite” P2 domain is loosely tethered to the core module by a flexible linker [83][93]. However, a subsequent structure of SurA lacking the P2 domain (SurA $\Delta$ P2) and bound to a model peptide demonstrated a striking conformational rearrangement: in the presence of the aromatic-rich,  $\alpha$ -helical C-peptide, the P1 domain completely dissociates from the chaperone core to bind the peptide, and the chaperone domains of each SurA $\Delta$ P2 monomer directly interact such that an extensive dimer interface is formed [82]. It should be noted that a structural homolog of SurA present in *Bordetella* (Par27) also crystallizes as a dimer, although the dimer interface differs from that observed for SurA $\Delta$ P2 [102]. It is tempting to speculate that SurA can drastically alter its conformation or oligomeric state in order to interact with specific substrates, however, there is as yet no evidence that oligomers of SurA are physiologically relevant.

In addition to SurA, the other chaperone believed to play an important role in OMP biogenesis is Skp. Skp has a long and complicated history, having been originally identified as a DNA-binding factor [103], a dubious role for an extracytoplasmic protein. Skp was later implicated in late steps of IM translocation [104,105]; although Skp is not involved in translocation, this was not the last time that such a role for this chaperone was proposed (see Section 4.2.2.1).

It was not until 8 years after the discovery of Skp that its primary function was realized. Skp was isolated in an elegant biochemical screen for periplasmic proteins that selectively bind unfolded OMPs [106], and was independently identified in a genetic screen for transposition events that cause activation of the  $\sigma^E$  stress response [75]. Skp was later shown to promote formation of soluble periplasmic intermediates of OmpA [67]. Together, these findings strongly supported a role for Skp in OMP biogenesis.

The importance of Skp was fully appreciated when it was shown that it is essential for viability in a  $\Delta$ surA mutant background; that is, *surA* and *skp* constitute a synthetically lethal pair [72,107]. Deletion of *skp* causes slight stress response induction, but no significant effect on OMP assembly was observed [72]. Additionally, proteomic analysis suggests that no OMP in *E. coli* depends on Skp for assembly (J. Collet, personal communication). However, depletion of *skp* in a  $\Delta$ surA mutant leads to accumulation of unfolded OMPs, strong induction of the  $\sigma^E$  stress response, and cell death. Whereas the abundance of individual OMPs is not significantly decreased in a  $\Delta$ skp mutant, levels of all OMPs are reduced upon simultaneous disruption of *surA* and *skp* (J. Collet, personal communication). The synthetic lethality of the *skp surA* pair strongly suggests that these chaperones have OMP substrates in common (i.e. Skp and SurA are partially redundant), and that together they are responsible for transporting the bulk of *E. coli* OMPs to the OM. It should be noted that chaperone requirements in different organisms are quite diverse. For example, no synthetic phenotypes are observed when a  $\Delta$ surA  $\Delta$ skp double mutant is constructed in *N. meningitidis*; this may reflect the reduced importance of SurA in this organism relative to *E. coli* [108].

The structure of Skp has been aptly compared to the body plan of a jellyfish [109]. Skp is a homotrimer comprising a core “body” domain and three  $\alpha$ -helical “tentacles” that extend from it [110], bearing some resemblance to the structures of the archeal chaperone prefoldin [109] and the mitochondrial intermembrane space chaperone Tim9.10 [111][93]. The tentacle helices form the walls of a deep hydrophobic pit that is thought

to bind unfolded substrate proteins via a combination of hydrophobic and electrostatic interactions [112]. Indeed, NMR and site-directed fluorescence spectroscopy indicate that the OmpA  $\beta$ -barrel domain is buried deep within the cavity formed by the tentacles [113], whereas the hydrophilic periplasmic domain remains outside of the Skp cavity and assumes its native conformation [114]. The flexibility of the tentacles [109] theoretically enables Skp to accommodate OMPs of diverse sizes by tolerating large fluctuations in cavity volume. This would explain the ability of Skp to form 1:1 complexes with OMPs ranging in size from 19–89 kDa and containing 8–16  $\beta$ -strands [112]. *In vivo*, Skp exhibits a broad substrate spectrum that includes porins [68,106,112,115], OmpA [116], autotransporters [97,98,117], and intimin [101]. Skp has also been implicated in virulence in *Salmonella*, and indirect evidence suggests that it is important for cell–cell spread in *Shigella* [100,118].

Some evidence to support a role for minor periplasmic chaperones in OM biogenesis is available in the literature. For example, the IM-tethered, parvulin-like periplasmic protein PpiD was initially identified in a screen for genes that, when overexpressed, suppressed the OM permeability defect of a *surA* mutant. The results of ensuing experiments showed that *ppiD* could not be deleted in a strain lacking *surA*, leading the authors to conclude that these factors are at least partially redundant [119]. While these findings have been called into question [96,120], evidence is mounting that PpiD does indeed serve as a periplasmic chaperone, although its substrates may not necessarily be OMPs [120,121]. It has been proposed that PpiD functions as a “gatekeeper”, interacting with nascent envelope proteins as they are translocated into the periplasm [66] (see Section 4.2.2.1). The observed IM localization and *in vitro* chaperone activity of PpiD [120] are consistent with this hypothesis; however, because all evidence for involvement of PpiD in translocation (or translocon release) was obtained using spheroplasted cells [66][93], it remains to be seen whether or not PpiD facilitates late steps of translocation *in vivo*.

The chaperone function of FkpA, an FKBP-like [122], dimeric [123] periplasmic PPIase, has also been interrogated genetically and biochemically. Like SurA, FkpA exhibits chaperone activity *in vitro* [124,125] and *in vivo* [126,127] that is independent of its PPIase activity [125]. It is reportedly involved in cell invasion and virulence in various Gram-negatives [128–130], although the relative importance of FkpA in these processes has been disputed [131]. FkpA is also known to be essential for the toxicity of colicin M in a manner that *does* require PPIase activity [132], and while it has been implicated in maturation of the autotransporter EspP [133], colicin M remains the sole confirmed *in vivo* substrate of FkpA. Although periplasmic chaperone activity for this PPIase has been demonstrated, there is currently no evidence that FkpA is involved in OMP maturation in any direct way [134,135].

Considerably less is known about PpiA, the fourth and final PPIase present in the *E. coli* periplasm. Although the regulation of this factor by the CpxAR [136], which suggests a role for periplasmic PPIases in the envelope stress response, no *in vivo* substrates for PpiA have been identified, and no OMP biogenesis defects are observed in its absence [96]. Accordingly, PpiA is not thought to participate in OMP folding in any direct way [135].

The periplasmic ATP-independent serine protease DegP is a key housekeeping factor that can promote both refolding and proteolysis of unfolded, misfolded, or aggregated proteins in the envelope [137]. Particularly important during times of stress, the synthesis of DegP is activated in response to a wide variety of extracytoplasmic stresses [138]. This protease is also specifically induced by the presence of periplasmic OMPs and responds by degrading dead-end, off-pathway folding intermediates and/or forming massive molecular cages around folding-competent off-pathway substrates to promote proper assembly [139].

Although DegP is a potent protease, it also exhibits considerable chaperone activity even when proteolytic activity is abrogated by mutation of the active site serine [138]. The chaperone activity of



DegP is thought to predominate at lower temperatures, whereas a temperature-dependent conformational switch favors protease activity at higher temperatures [140]. DegP is often regarded as a periplasmic chaperone primarily because the simultaneous deletion of *surA* and *degP* is not tolerated [107]; this suggests that DegP is an essential component of a secondary OMP assembly pathway [72]. While the importance of DegP in maintaining homeostasis and responding to stress is clear, less is understood about how it might contribute to OM assembly of  $\beta$ -barrels. Overproduction of a protease-deficient variant of DegP (DegP<sup>S210A</sup>) has been shown to suppress the lethal effects associated with the expression of folding-defective OMPs [141,142], but the DegP<sup>S210A</sup> chaperone can only bind and sequester these OMPs in the periplasm; it cannot return them to the folding pathway. While DegP may be important for preventing toxic aggregation of misfolded or mistargeted OMPs through a combination of proteolysis and sequestration, it is not clear at this time whether it represents a dead-end for OMPs or whether they can re-enter the folding pathway after a productive association with DegP.

**4.2.2.3. OM targeting/substrate recognition.** The targeting of secretory proteins to the Sec translocon is known to depend on the presence of an N-terminal signal peptide that is recognized by components of the Sec apparatus. An important question in the field of OMP biogenesis involves the signal(s) and recognition factor(s) required for  $\beta$ -barrel assembly *in vivo*. The existence of a sorting signal for OMPs was initially proposed based on the observation that the C-terminal amino acid of  $\beta$ -barrels is absolutely required for OM assembly [143]. Phylogenetic analysis revealed a high degree of sequence conservation at this position; the C-terminal residue is almost always aromatic, and a phenylalanine side chain is found most often at this position [144]. While mutation or deletion of this C-terminal phenylalanine essentially blocks assembly and causes periplasmic accumulation *in vivo* [142], such OMP variants can still fold properly into bilayers *in vitro* [145], implying that these C-terminal mutants are folding-competent but are not recognized by an assembly factor.

Although the putative OMP recognition factor has yet to be identified in bacteria, a recent investigation into the targeting of mitochondrial  $\beta$ -barrels convincingly demonstrated the involvement of an OM peripheral membrane protein in selective recognition of  $\beta$ -barrels via the C-terminal  $\beta$ -strand, which contains a conserved sequence motif termed the “ $\beta$ -signal” [146]. This mitochondrial OMP recognition factor, Sam 35, is an essential component of a highly conserved multiprotein complex that is required for  $\beta$ -barrel assembly across phylogeny. These results strongly suggest that the cellular machinery that facilitates OMP assembly is able to identify and bind substrates by recognizing a conserved motif in the final  $\beta$ -strand [146]. The manner in which OMPs are assembled into a biological membrane by the bacterial homolog of this machine will be discussed in the following section.

**4.2.2.4. OM assembly.** Incorporation of peptides into the low-dielectric-constant lipid phase of a bilayer requires complete saturation of main-chain hydrogen bonding potential within membrane-spanning regions. In  $\alpha$ -helical TM proteins, the hydrogen bonding potential of the backbone is satisfied by the sequential formation of internal hydrogen bonds that stabilize the helix and permit partitioning into the bilayer (assuming the side chains to be exposed to the lipid phase are nonpolar). In  $\beta$ -barrel membrane proteins, however, the backbone polar groups are neutralized by hydrogen bonding between neighboring  $\beta$ -strands. As a consequence, whereas the TM  $\alpha$ -helices of IM proteins can be individually released into the membrane, the folding and membrane integration of OMPs are predicted to occur simultaneously, such that OMP assembly can be described as an “all-or-none” folding event [21]. This fundamental difference between IM and OM protein assembly suggests that the folding pathways of these membrane protein families are distinct.

In contrast to IM protein assembly, the mechanisms of OMP folding and membrane integration *in vivo* are poorly understood, although *in vitro* analysis of  $\beta$ -barrel folding into membranes affords some insight into the general characteristics of  $\beta$ -barrels that promote membrane folding. Since the exterior surface of the barrel is buried within a lipid bilayer, it is not surprising that side chains exposed to the hydrophobic core of the membrane are primarily short-chain aliphatic residues with high Kyte–Doolittle values [147]. Indeed, *in vitro* folding experiments with a model OMP showed that the majority of lipid-exposed  $\beta$ -barrel residues must be hydrophobic, and that prolines and charged residues are generally not tolerated among membrane-facing residues [148]. Aromatic side chains are overrepresented in OM  $\beta$ -barrels, particularly at the membrane–solvent interface [21], where they occur in rings around the barrel rims to form so-called “aromatic girdles” [6], presumably influencing the stability of the folded OMP within the membrane (Fig. 2B). It has also been shown that  $\beta$ -hairpins comprising a  $\beta$ -sheet need not necessarily be covalently linked, as fragments of a single  $\beta$ -barrel structure are still assembled properly into the membrane as a  $\beta$ -barrel monomer [149]. This implies that the minimal structural unit required for  $\beta$ -barrel assembly is shorter than the full-length OMP.

*In silico* modeling of OMP unfolding highlights the contribution of various structural features of  $\beta$ -barrel that can impact stability [150,151]. In addition to a barrel domain and extracellular loops, some OMPs exhibit unique features such as barrel plugs (which exhibit significant secondary structure with the barrel lumen), or helical “out-clamps” (which may reinforce weakly stable transmembrane  $\beta$ -strands at the membrane–solvent interface; [151]). Additionally, a number of OMPs oligomerize in the membrane, shielding high-energy interaction surfaces and stabilizing membrane strands [151]. The stability of some membrane-integral OMP trimers is striking; dissociation of porin trimers, for example, only occurs after heating at temperatures about 70 °C in the presence of detergent (ex. [69]).

While experimental systems devised to study assembly of  $\beta$ -barrels into biological membranes have shown the process to occur spontaneously (see [152]), the rate of OMP folding *in vitro* occurs far too slowly to be physiologically relevant. This implies the existence of folding factors at the OM that act to increase the rate of OMP assembly so as to support rapid growth. These folding factors have been recently identified [10,153,154], and a great deal of information about the so-called  $\beta$ -barrel assembly machine (Bam) has been generated since its discovery.

It is now clear that OMP assembly is dependent on Bam *in vivo* in every organism in which it has been tested [10,153–158]. Furthermore, Bam is extremely well conserved; functionally and structurally homologous cousins of BamA/Omp85, the most evolutionarily ancient Bam component, have been identified in mitochondria (Sam50/Tob55), chloroplasts (Toc75), chromalveolates [156] and all diderm bacteria. This suggests that Bam catalyzes a fundamental biological process, and that membrane  $\beta$ -barrels across phylogeny assemble by a nearly universal mechanism.

Bam is an oligomeric membrane-associated protein complex composed of the OM  $\beta$ -barrel BamA and a variable number of accessory OM lipoproteins that physically interact with it [10,159–163]. The complex was initially discovered in *E. coli* [10,87] and was found to comprise four lipoproteins (BamBCDE) that stably bind to the sizeable N-terminal periplasmic domain of BamA [10,161,162]. Attempts to determine the stoichiometry of Bam suggest that each complex contains one of each Bam component, although the low molecular weight of BamE makes it difficult to unambiguously determine whether one or two BamE molecules are present per complex [95].

The hub of the physical complex is the periplasmic domain of BamA, which is a helical beaded chain comprising five structurally homologous POTRA (polypeptide translocation associated) domains [161,164,165]. These domains, numbered P1–P5 from the N-terminus, each display a  $\beta$ - $\alpha$ - $\beta$ - $\beta$  architecture and assume a characteristic fold. Although

the BamA POTRA domains are strikingly similar with respect to structure (they can essentially be superimposed), they have surprisingly little primary sequence identity and domain deletion experiments imply distinct functional roles for individual POTRAs [161]. The BamA barrel and at least a subset of its POTRA domains are essential for viability [161,166]. The POTRAs, then, must participate in some critical step(s) in the OMP assembly process. Interestingly, diverse bacteria have different requirements for the periplasmic domain of BamA. In *E. coli*, for example, deletion of even a single POTRA domain leads to impaired  $\beta$ -barrel assembly and reduced viability [161]. The cell is able to tolerate deletion of P1 and/or P2; but removal of P3, P4, or P5 is lethal even in the presence of P1 and P2; the minimal BamA is P3–5 plus the C-terminal membrane domain [161]. However, *Neisseria meningitidis* is viable after deletion of P1–4 with only marginal effects on viability, suggesting that the majority of the periplasmic domain of BamA is dispensable in this species [166]. It is conceivable that this reflects a difference in the relative importance of particular Bam substrates in *E. coli* compared to *Neisseria*. It has not escaped our notice that the LPS assembly factor LptD, a Bam substrate and essential protein in *E. coli* [88], is dispensable for viability in *Neisseria* [167]. Although there are many possible explanations for the species-specific difference in the relative importance of the POTRAs, it is tempting to suggest that P1–P4 are, to different degrees, required for the assembly of LptD; this hypothesis correlates the dispensability of P1–P4 in *Neisseria* with the dispensability of LptD in that organism. In any case, the precise contribution of each individual POTRA domain to the assembly process remains to be determined.

Although it is unclear how the periplasmic domain of BamA interacts directly with substrates, quite a bit is known about how the POTRAs mediate Bam complex formation. The periplasmic domain of BamA is required for the physical associations between BamA and the remaining complex members. The stable association of BamA with BamB requires most of the BamA periplasmic domain; Removal of any POTRA except P1 disrupts the BamA–B connection [161]. In addition, point mutations or small insertions in P3 weaken or altogether disrupt the BamA–B interaction, perhaps indicating a particular requirement for P3 in nucleating or maintaining this association [161].

In contrast, only P5 is necessary for the physical association of BamA with BamC, D, and E, which form a lipoprotein subcomplex that interacts with BamA following BamCDE heterotrimerization [95,161]. From biochemical data presently available it is difficult to resolve the nature of the physical interactions between BamA and the individual members of the BamCDE subcomplex. It is tempting to conclude that the interactions between BamC/E and BamA occur indirectly through BamD, as C-terminal truncations of BamD prevent the association of BamA with both BamC and BamE [11,162]. This model is probably overly simplistic, however, as C-terminal BamD truncations (as well as *bamE* null mutations) also compromise the BamA–D interaction [162]. It is possible that BamC and/or BamE do associate directly with BamD, and in so doing stabilize a direct BamA–D interaction. While it is clear that our understanding of the Bam physical interaction network would benefit greatly from additional biochemical inquiry, it can at least be said (with some certainty) that the primary physical determinants for the BamA–CDE interaction(s) are contained within P5, the barrel-proximal POTRA domain. *bamD*, like *bamA*, encodes an essential member of the Bam complex. Because BamA and BamD, as the only essential members of an essential machine, likely interact in order to facilitate OMP assembly [11], it is not surprising that P5 is specifically required for viability in distantly related Gram-negatives. The exception that proves the rule is the mitochondrial BamA homolog Sam50, which contains just one POTRA domain in addition to its C-terminal  $\beta$ -barrel domain [165]. Unexpectedly, the lone POTRA domain of Sam50 is dispensable for viability, and  $\beta$ -barrel assembly appears to proceed unperturbed in its absence [146]. This may be a consequence of the fact that the essential accessory protein in this system (the peripheral membrane protein Sam35) associates with Sam50 not via the POTRA

domain but rather through the membrane-embedded  $\beta$ -barrel [146,168]. It will be interesting to determine whether the minimal requirement for P5 in bacteria reflects its importance as the complex assembly platform.

In addition to their discrete POTRA requirements, it is important to note that BamB and the BamCDE subcomplex interact with BamA independently; that is, mutations in or deletion of BamB does not affect the physical association between BamA and BamCDE, and vice versa [11,95,161,162]. This may suggest that BamB and BamCDE fulfill discrete functions and/or participate in separate steps of OMP assembly, although what these functions might be remains unclear.

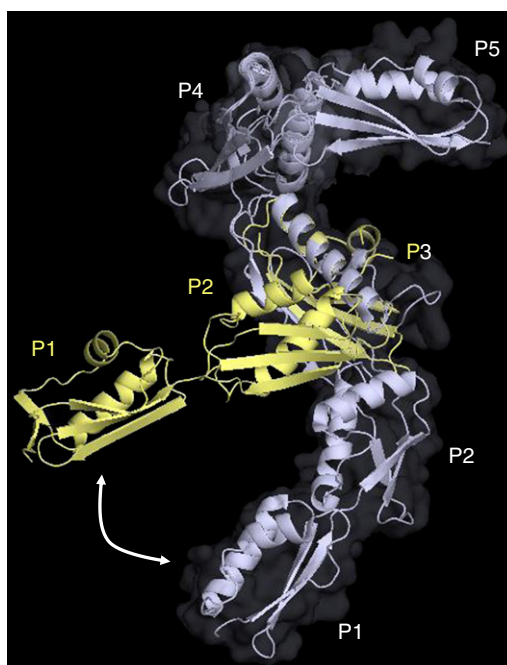
The precise roles of the accessory lipoproteins have not been conclusively determined, but genetic analysis of the complex has yielded some insight into the relative importance of these factors nonetheless. As mentioned above, BamD is an essential complex member (and the only essential lipoprotein); its depletion, much like the depletion of BamA [10], causes OMP assembly to stall altogether [11]. The nearly identical effects of depleting *bamA* and *bamD* implies that they either work together to catalyze an essential step in  $\beta$ -barrel assembly, or that they are each required to effect distinct but equally important sequential steps.

The remaining three lipoproteins are individually dispensable [10,11,162]. Deletion of either *bamC* or *bamE* has minimal effects on  $\beta$ -barrel assembly at best; these single mutants grow normally and exhibit subtle reductions in steady state OMP levels (if any). Null mutations in *bamB* cause more appreciable OM biogenesis defects, including decreased OMP levels, increased OM permeability, and activation of extracytoplasmic stress responses [10,87,162,169,170]. However, it is unlikely that BamB is important for the assembly of all Bam substrates, as deletion of this lipoprotein *enhances* the assembly of several OMPs, including the efflux pump TolC [169] and LptD4213, a folding-defective variant of the OMP that mediates surface assembly of LPS [87]. This may signify a more specialized role for BamB in assembly of some as-yet-undetermined subset of OM  $\beta$ -barrels.

Although single deletions of the non-essential Bam lipoproteins are tolerated, deleterious synthetic effects are observed when these deletions are combined with mutations in various OMP assembly factors. For example, null mutations in *bamC* or *bamE* (discussed above) have only mild effects on OM biogenesis, but simultaneous deletion of both factors causes significant induction of the  $\sigma^E$  stress response and a sharp reduction in OMP levels [162]. Even more striking is the effect of combining a *bamE* deletion with a null mutation in *bamB*—this double mutant is not viable [162]. These genetic interactions may imply partial functional redundancy between various Bam components, or they may reflect the inability of Bam to support growth when the efficiency of OMP assembly is reduced below some critical threshold. In any case, these synthetic effects demonstrate critically important roles for the non-essential lipoproteins, despite their individual dispensability.

While periplasmic chaperones should not be regarded as *bona fide* Bam complex members, the physical interaction between periplasmic factors and the Bam complex has been demonstrated. Specifically, binding of SurA to BamA can be detected in the presence of a chemical cross-linker, which traps this presumably transient association [171]. The SurA–BamA interaction is thought to facilitate the transfer of substrate from the chaperone to the assembly machine. The physical determinants of this association are not entirely clear, but there is biochemical evidence that support a role for P1 of BamA in SurA docking: point mutations within this POTRA domain diminish binding to the chaperone [171].

Interestingly, the SurA–BamA interaction does not require BamB [170]. This is somewhat unexpected given the apparent functional relationship between SurA and BamB: these assembly factors identically affect the kinetics of LamB assembly when they are deleted [69], and affect the folding of an overlapping subset of OMPs [86]. Furthermore, BamB was shown to be important for the assembly of Bam substrates delivered by SurA [95]. Since loss of BamB does not affect the ability of



**Fig. 4.** The periplasmic domain of BamA exhibits conformation flexibility about the hinge between POTRA domains 2 and 3. Two independently structures are superimposed at POTRA 3. The yellow structure (PDB ID: 2QDF) represents the “bent” conformation, and the purple conformation (PDB ID: 3EFC, 3OG5) represents the “extended” conformation.

SurA to interact with Bam, it is unlikely that SurA binds BamB directly (or cooperatively to BamA in the presence of BamB). BamB may instead exert some undetermined effect on BamA to promote assembly of SurA substrates [172].

Although attempts have been made to crosslink Skp and DegP to Bam, no such interactions have been observed [72]. It remains to be seen whether this reflects the transient nature of this interaction; it is formally possible that, in contrast with SurA, these chaperones do not directly interact with the Bam machine, but instead deliver substrate by some transfer mechanism that does not require docking to Bam.

## 5. A first glimpse at the physical Bam complex: structural insights

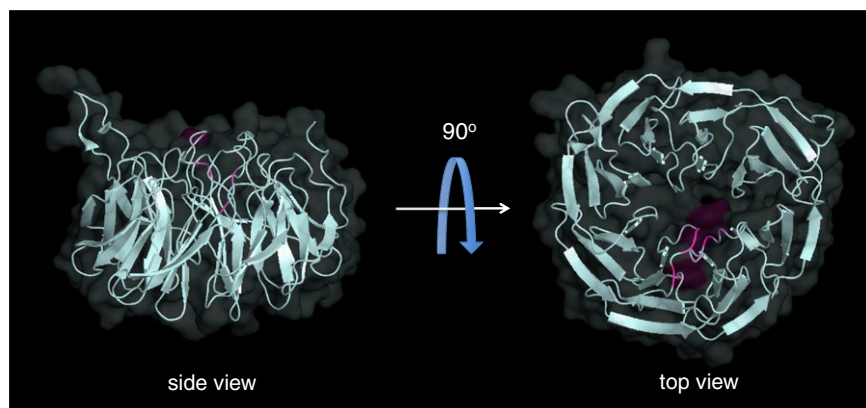
### 5.1. BamA

As an essential and nearly ubiquitous protein, BamA (along with its homologs) has received a tremendous amount of attention since its relatively recent discovery as a membrane biogenesis factor. While extensive genetic, biochemical, electrophysiological, structural,

and phylogenetic analyses have been conducted on this primeval protein, a detailed understanding of its function in  $\beta$ -barrel folding remains elusive. Still, recent investigations into the structure and function of BamA have yielded some insight into its activity.

The first glimpse of BamA came in the form of two atomic resolution structures of most of the periplasmic domain [161,173]. Bioinformatic analysis conducted prior to the solutions of the structure strongly suggested that the periplasmic domain of BamA is subdivided into five POTRA domains [164,165], but the low sequence homology between the POTRAs left open the possibility that these domains would be structurally distinct. In light of this, the first structures revealed something quite unexpected: the POTRAs present in the structures (P1–P4), despite lack of sequence homology, are so structurally homologous that the individual POTRA domains can be neatly superimposed with very little deviation ( $<1.80$  Å r.m.s.d.) from one POTRA to the next [173].

Despite the striking similarity between POTRA domains, there is one domain that displays some unique features. P3 differs from the others in two important respects: 1) the loop between the two  $\alpha$ -helices is significantly longer (10 additional residues) in P3 than in



**Fig. 5.** The solution structure of BamB (PDB ID: 3P1L). Residues shown previously to be involved in the BamA–BamB interaction are highlighted in magenta (Vuong et al. 2008).



the remaining POTRAs, and 2) the second  $\beta$ -strand contains a “ $\beta$ -bulge” which exposes a surface for binding of additional  $\beta$ -strands by  $\beta$ -augmentation [161,173]. The P1–P4 fragment crystallized as a dimer, and the dimer interface is created by the P3  $\beta$ -bulge binding to a short segment of P5 present in the construct which templates  $\beta$ -strand formation and extends the P3  $\beta$ -sheet. Interestingly, the observed  $\beta$ -augmentation occurred in a parallel fashion in one structure [161] and an antiparallel fashion in the other [173], suggesting that POTRAs may tolerate opposite binding orientations so as to accommodate a wide variety of OMP substrates. Whether or not this reflects the ability of BamA to associate with substrate in this way *in vivo* is far from certain, but these interactions provide an attractive mechanism for the binding of OMP  $\beta$ -strands (or  $\beta$ -hairpins) to POTRAs.

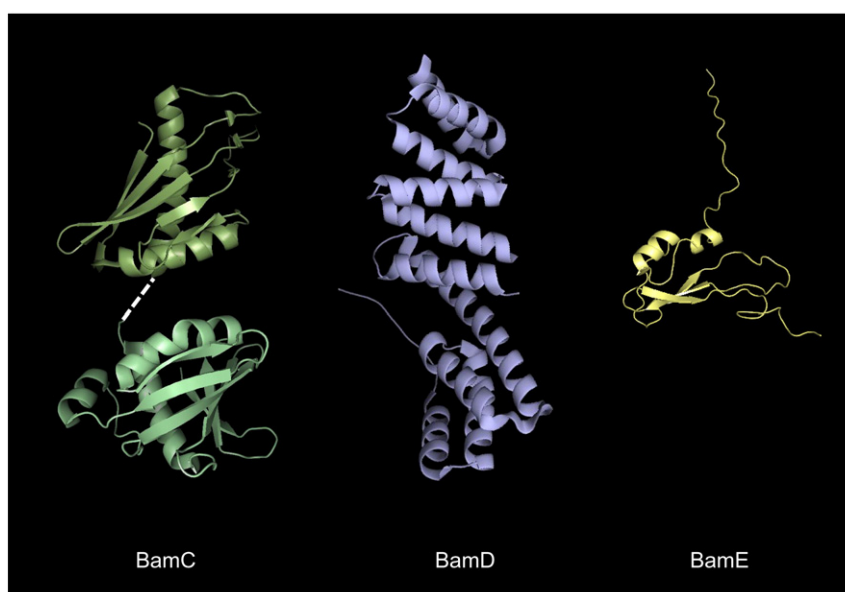
Comparing the two P1–P4 crystal structures revealed one of the most intriguing features of the periplasmic domain of BamA. While the relative orientations of the P1–P2 and P3–P4 domains are virtually identical in both structures, suggesting rigidity at these interfaces, a significant difference at the interdomain angle between P2 and P3 was readily apparent. Whereas the Sousa group observed a 130° angle between these POTRAs, placing the periplasmic domain in an extended conformation, the structure solved by Kahne and colleagues showed this angle to be smaller by 30°, resulting in a “bent” conformation relative to the Sousa structure (Fig. 4). There is extensive hydrogen bonding between P1–P2 and P3–P4, yet there are exceedingly few observed polar contacts between P2 and P3 [173]. This may imply a significant degree of conformational flexibility at the “hinge” between these two POTRA domains; indeed, this notion is supported by NMR, PELDOR, and SAXS data that confirm inflexibility at the P1–P2 joint [174,175] as well as the relative conformational freedom observed between P2 and P3 [176]. EOM analysis of the SAXS data further suggests that the two observed conformational states are not simply randomly sampled conformations that were trapped in the crystal lattice, but rather two preferred conformations assumed by BamA in solution [176]. The additional solution structures of the P4–P5 tandem pair, together with analysis of the behavior of that pair in solution, suggests that the P4–P5 joint is also rigid [176,177]. Taken together, the periplasmic domain of BamA can potentially be thought of as two rigid “arms” (comprising P1–P2 and P3–P5) that are connected by a flexible linker.

The functional relevance of this conformational flexibility, if any, is unclear. However, several related pieces of evidence may provide a clue. Aside from the difference in the P2–P3 angle observed between the Sousa and Kahne structures, there is an additional divergence that is apparent: the L2 loop between the two  $\alpha$ -helices in P3 is disordered in the Kahne structure (in which BamA assumes the “bent” conformation), but well-ordered and resolved in the Sousa structure. It is appealing to suggest that this may indicate a conformational switch between the bent and extended states that is regulated by a disorder-to-order transition at L2 of P3. While there is no available data to support such a notion, we note with interest the isolation of a partial loss-of-function mutation in BamA that maps precisely to the L2 loop mentioned above. This mutant, *bamA6*, exhibits a mild decrease in the steady state levels of OMPs, but enhances the folding of an OMP with a complex biogenesis pathway [178]. Whether or not the *bamA6* mutation biases the conformation of BamA towards one of the two observed states remains to be seen.

Although the structure of the complete periplasmic domain of BamA has been solved, there is no structural data available for the C-terminal  $\beta$ -barrel domain. However, the structure of the two-partner secretion transporter FhaC, a distantly related Omp85 homolog, was solved concurrently with BamA [179]. This structure reveals a 16-stranded  $\beta$ -barrel that contains an unusually long extracellular loop (L6). This loop, a common motif among Omp85 family members, is conserved in BamA, and is predicted to be even longer than in FhaC; assuming the loop extends into the lumen of the barrel, as the FhaC structure predicts, it is more than long enough to reach the periplasmic space [180]. This loop contains a highly conserved tetrad motif found in all Omp85 homologs, including BamA. Mutation or deletion of this motif in FhaC renders the transporter unable to secrete its passenger, the FHA adhesin [179,181]. No reports regarding the functional importance of the tetrad motif (or L6 in general) for BamA are available. It will indeed be interesting to see whether or not this conserved extracellular loop plays a role in OM biogenesis.

## 5.2. BamB

2011 proved to be a red-letter year for structural biology of the Bam complex. Four independent crystal structures of BamB were reported [182,183] in as many months! Unlike the previous structural



**Fig. 6.** The solution structures of *E. coli* BamCDE. The BamD (PDB ID: 2YHC) and BamE (PDB ID: 2KM7) structures are oriented with the N-termini pointing toward the top of the page. The structurally homologous helix grip domains of BamC (PDB ID: 2LAE, 2LAF) are shown on the left, with the extreme C-terminal domain at bottom. A dashed white line indicates the unresolved helix linking the two domains.



characterization of BamA, the independently-solved structures of BamB did not reveal significant conformational or architectural differences between the structures—structural comparisons between them show that they deviate very little from one another (r.m.s.d. = 0.5–0.7 Å; [182]). Even so, the individual groups contributing uniquely to our understanding of BamB and its contribution(s) to Bam-mediated OMP assembly.

*In silico* modeling of the BamB structure predicted that this lipoprotein assumes a  $\beta$ -propeller fold in the periplasm [160,170]; this was confirmed in each of the recently solved crystal structures. The BamB propeller has eight blades, each comprising four antiparallel  $\beta$ -strands. The blades are joined by long interconnecting loops (ILs) that form a surface at the narrower rim (“top”) of the propeller. A highly electronegative deep groove at the “bottom” of the propeller has been proposed to participate in protein–protein interactions [184].

In light of the BamB structure, previous identification of BamB residues critical for BamA binding (and vice versa) suggests a putative BamA–B interaction surface. Mutational analysis of BamB revealed five residues required for the physical association with BamA [170] that cluster to two adjacent  $\beta$ -blade interconnecting loops (IL4 and IL5) on the top of propeller (see Fig. 5). Mutation of these residues results in a *bamB* null phenotype and prevents the stable association of BamB and BamA (without compromising the stability of BamB itself; [170]). The BamB crystal shows that these residues are closely apposed and implies that they specify a BamA binding site [182,183]. Consistent with this, a deletion in a propeller blade connected to IL5 abrogates BamB function [87]. Truncation of this blade may cause IL5 to be buried within the central funnel, thus preventing this loop from accessing the solvent-exposed surface and interacting with BamA.

The reordering of residues in the unique  $\beta$ -bulge present in BamA–P3 was previously shown to disrupt the interaction between BamA and BamB, implying the presence of a binding site for BamB at the edge of the P3  $\beta$ -sheet [161]. Since an interaction between the  $\beta$ -bulge and a short fragment of P5 (comprising a single  $\beta$ -strand) was observed in both P1–P4 crystal structures, it was proposed that this mode of binding ( $\beta$ -augmentation) may be relevant for interactions between BamA and its substrates and/or interaction partners [161,173]. Intriguingly, a simulation of the BamA–B interaction using a protein docking algorithm suggests an attractive model for this association in which the  $\beta$ -bulge in P3 binds to IL4 of BamB by  $\beta$ -augmentation [172]. This model is satisfying because both BamB IL4 and the BamA  $\beta$ -bulge are known to be important for the BamA–B interaction, and because the  $\beta$ -bulge is known to bind  $\beta$ -strands by  $\beta$ -augmentation [161,170,173]. Additional evidence supporting this model includes the observed surface electronegativity of BamB in the region surrounding IL4–5, which is relevant in light of the high positive nature of P3 specifically. Electrostatic interactions at these domains may further stabilize the interaction between BamB and BamA P3 [172].

The hydrophobic pockets formed between neighboring BamB propeller blades have also been suggested to serve as OMP binding sites [183]. However, this proposal is based on intermolecular contacts observed between BamB monomers in the crystal lattice, and while BamB does increase the efficiency of OMP assembly [95], there is no evidence that it directly interacts with OMPs. The involvement of the P3  $\beta$ -bulge and IL4/5 in the BamA–B interaction was confirmed genetically and biochemically [161,170]; thorough dissection of the proposed role of BamB in OMP binding must also be performed before a direct OMP–BamB interaction can be confirmed.

### 5.3. BamC

Two separate groups solved the structure of BamC independently [182,185]. Both report a disordered N-terminus (comprising the first ~70–100 residues) that could not be resolved in either case. What

remains are two structurally homologous helix grip domains (Fig. 6) connected by a predicted  $\alpha$ -helical linker that was also not resolved in the crystal structure.

Unfortunately, the structures provide little insight into the function of BamC or the physical interaction(s) between BamC and other members of the complex. Firstly, unlike BamA and BamB, no BamC mutants have ever been reported (with the exception of a complete *bamC* deletion), making it difficult to identify residues or domains involved in function and/or protein–protein interactions. Furthermore, the intrinsic disorder of the extreme N-terminus leaves a significant gap in our structural understanding of this minor OMP assembly factor. Genetic and biochemical determination of functionally important BamC domains must be obtained before the relevance of its 3D structure can be appreciated.

### 5.4. BamD

The crystal structure of BamD was also reported independently by two groups. One group reported the structure of *E. coli* BamD [182], whereas the other recently solved structure is that of BamD from the distantly related *Rhodothermus marinus* [186]. Both show that BamD is composed of multiple tetratricopeptide (TPR) repeats packed together into a long superhelical structure (Fig. 6). Many TPR-repeat-containing proteins are known to scaffold multiprotein complexes [186], and structural homologs of BamD that serve as chaperone receptors have been shown to bind substrate via recognition of C-terminal targeting signals in a conserved binding pocket [187]. Since residues at the C-terminus of OMPs are known to be critically important for assembly [143], it has been suggested that one function of BamD may be to selectively bind OM  $\beta$ -barrels in the periplasm via recognition of their C-termini [182,186]. This is consistent with the finding that Sam35, an essential component of the OM complex that assembles  $\beta$ -barrels in mitochondria, directly binds nascent  $\beta$ -barrels in a manner that is entirely dependent on the presence of a C-terminal recognition signal [146]. It is tempting to speculate that BamD serves a similar function in the bacterial OM, selectively recognizing and binding OMPs prior to membrane integration.

A clear difference between the two available BamD structures is the shortened C-terminus of *E. coli* BamD relative to that of *Rhodothermus*. Generally speaking, successive TPR motifs pack against one another to stabilize each repeat, and the interaction surface of the terminal TPR is capped off by an  $\alpha$ -helix [186]. Whereas this capping helix is present in *Rhodothermus* BamD (where it terminates the TPR tandem array after the fifth TPR repeat), this helix is missing altogether in *E. coli* BamD [186]. Since C-terminal truncations of BamD in *E. coli* destabilize the interaction(s) of BamD with all other Bam complex members, it has been suggested that this domain is required for the association of BamD with the complex [11]. The variable conservation of the C-terminal capping helix may reflect variations in Bam complex constitution or binding interactions between complex members.

### 5.5. BamE

As with BamC, no partial loss-of-function mutations in BamE have been reported. Still, the recently solved structures of BamE [182,188] provide insight into function by highlighting potential protein–protein and protein–membrane interaction surfaces (Fig. 6). A thorough analysis of functionally important BamE residues was conducted by glycine, cysteine, and alanine-scanning mutagenesis [189], which revealed a cluster of surface-exposed residues that, when mutated, compromise the OM permeability barrier in a manner resembling a  $\Delta bamE$  mutant. A subset of these residues was further shown to influence binding to BamD (supposedly the only Bam component that interacts with BamE *in vitro*) and/or PG, an anionic minor OM phospholipid. The demonstrated interaction between BamE and PG *in vitro* may imply a fascinating and unexpected role for this lipoprotein: association of the Bam

complex with PG-rich regions of the OM [189]. In light of the fact that PG self-associates in the membrane [190] and that anionic phospholipids can promote protein translocation and drive interactions between membrane proteins and the bilayer [191], it is tempting to suggest that localizing OMP assembly to regions of the OM replete with negatively-charged PG may enhance the rate of  $\beta$ -barrel folding or membrane integration *in vivo*. It will be interesting to see whether the binding preference for PG exhibited by BamE is relevant in the periplasm.

Several groups reported the *in vitro* dimerization [188] or hexamerization [182] of BamE, and expression of BamE in the cytoplasm of *E. coli* also resulted in dimer formation [189]. While stoichiometric analysis of the purified Bam complex may be consistent with dimerization of BamE [95], analysis of the oligomeric state of BamE in the periplasm suggested that BamE is exclusively present as a monomer in its native environment [189], calling into question the physiological relevance of the BamE dimer.

## 6. Probing the function of the Bam complex

### 6.1. *In vivo* assembly kinetics

Prior to the discovery of the Bam complex, analysis of the  $\beta$ -barrel assembly mechanism was more or less limited to *in vitro* model OMP refolding studies (see [152], identification of intramolecular determinants of efficient  $\beta$ -barrel folding (ex.[134,148]), and elucidation of the folding pathway for individual OMPs by detection of folding intermediates (ex. [145,192–195]), although visualization of such intermediates was sometimes complicated by the fact that OMP assembly happens too quickly *in vivo* to reliably trap intermediates [69]. Identification and characterization of the Bam machine has contributed tremendously to our general understanding of  $\beta$ -barrel targeting and folding, and the results of several seminal studies have shed light on the manner in which OMP folding factors operate coordinately to facilitate OMP assembly.

The maturation of the abundant *E. coli* OMP LamB from cytoplasmic synthesis to OM assembly can be tracked *in vivo* by pulse-chase analysis [195]. Ureta et al. discovered that slowing cell growth by reducing the incubation temperature increased the half-life of LamB assembly intermediates, thereby permitting more accurate detection and quantification of the conformations LamB assumes during the normal assembly pathway. To probe the function of  $\beta$ -barrel folding factors and assess their contributions to OMP assembly, the kinetics of LamB folding were determined in strains deleted for various envelope proteins that had been previously implicated in OMP biogenesis [69].

In a wild-type cell grown at low temperature, LamB (along with the abundant trimeric porins) proceeds through a folding pathway in which the precursor is processed to a mature form in the periplasm, folded into a monomeric species, and finally converted to a stable LPS-associated trimer. In the absence of the periplasmic chaperone SurA or the Bam complex accessory lipoprotein BamB, the conversion of unfolded mature LamB to folded LamB monomer is dramatically delayed, whereas the trimerization of folded LamB occurs at a normal rate [69].

The importance of this kinetic analysis is underscored by the fact that assessing steady-state OMP levels in *surA* or *bamB* mutants only shows that OMP assembly is compromised without revealing *why*. Ureta et al. not only identified the LamB folding step that is accelerated by SurA and BamB (unfolded  $\rightarrow$  folded monomer), but also showed that *surA* and *bamB* null mutations have identical effects on LamB maturation. This finding suggests that these two factors promote the same step in OMP folding [69]; said differently, both SurA and BamB are needed for efficient folding assembly of a highly abundant OM protein.

### 6.2. Accommodating diverse substrates

OMPs come in a wide variety of shapes and sizes, and Bam must be sufficiently robust to accommodate the structural variation that

functional differentiation may require. OmpA, for example, is among the “lightweight” OMPs, its membrane domain comprising just eight  $\beta$ -strands [152]. This OMP functions primarily not as a diffusion channel but as a reinforcement factor, binding the murein sacculus and anchoring the cell wall to the OM [22]. In stark contrast, the wide-mouthed iron transporter FecA is required for energy-dependent import of an iron chelate through the gated pore formed by its large 22-strand  $\beta$ -barrel domain [22]. Clearly, the Bam complex must tolerate diversity among its substrates and adapt to facilitate the folding of unique and, in some cases, challenging substrates.

The OM LPS transporter LptD is one such substrate. Assembly of this essential OMP is complicated by several factors: 1) LptD contains a periplasmic N-terminal domain, which is involved in formation of an essential transenvelope Lpt protein bridge [37], 2) the OM lipoprotein LptE forms a plug in the LptD barrel [18,41] and is absolutely required for the biogenesis of LptD [196,197], 3) disulfide bonds linking the N-terminal periplasmic domain of LptD to the  $\beta$ -barrel must form properly in order for the protein to function [196], and 4) LptD assembly is exquisitely dependent on the chaperone SurA [86]. Clearly, folding and function of LptD depend heavily on multiple interactions with various envelope factors, including BamA.

The tight transmembrane association of LptDE and the requirement for LptE in LptD folding raise the question of whether BamA coordinates the assembly of the LptDE heterodimer. A recent report describes the isolation of *bamA* mutations that suppress the LptDE biogenesis defects associated with a partial loss-of-function mutation in *lptE* (*lptE6*, [197]). The LptE6 variant interferes with LptDE complex assembly; however, once formed, the LptDE6 complex is stable. Suppressor mutations that alter a specific proline residue in BamA P2 restore efficient LptDE6 complex assembly [197]. Although it is not yet clear exactly how BamA influences LptDE complex formation, the identification of *lptE* suppressors in *bamA* strongly implies a fascinating new role for Bam: coordinating the temporally and spatially regulated folding and heterooligomerization of an OMP together with its structural lipoprotein partner.

Autotransporters (ATs) provide another example of complex Bam substrates. ATs are OMPs that are thought to co-opt Bam for OM translocation of covalently-linked N-terminal passenger domains [97,198,199]. Compelling biochemical evidence from the recent elegant analysis of Bam-mediated AT assembly suggests that both the passenger and barrel domains of an AT interact with periplasmic chaperones and BamA, whereas the barrel domain additionally interacts with BamB and BamD [97,99,198]. Characterization of the temporal association between the *E. coli* AT EspP and Bam complex members shows that the association between Bam lipoproteins and EspP outlast the association between EspP and BamA [198]. It is tempting to speculate that the prolonged interactions between BamB/BamD and EspP reflect the late/terminal function(s) of these lipoproteins; since they are the last folding factors to contact EspP, they may well catalyze a final assembly step [198]. It will be very interesting to see if the Bam lipoproteins function in this fashion during the assembly of OM  $\beta$ -barrels in general.

The Bernstein group also proposed the existence of a checkpoint mechanism that prevents late steps in assembly of the AT barrel domain from occurring until the passenger domain has been completely secreted; this is supported by the finding that stalling of passenger domain translocation causes folding of the partially assembled  $\beta$ -barrel domain to stall as well [198]. This is an intriguing result in and of itself, as it suggests that the rate of Bam-mediated  $\beta$ -barrel assembly can be determined by a coupled event (in this case, OM translocation).

### 6.3. *In vitro* characterization of Bam function

For nearly 20 years [200], *in vitro* analysis of OMP folding into a lipid bilayer has been conducted in order to study the folding kinetics and physical determinants of  $\beta$ -barrel assembly, to determine the effects of variations in environmental conditions or phospholipid

content, or to dissect the interaction(s) between an OMP and a chaperone. Analysis of this kind yielded significant insight into the molecular events that occur during  $\beta$ -barrel folding and the conditions that favor it [152].

*In vivo*,  $\beta$ -barrel assembly requires the Bam complex. Does the *in vitro* folding pathway of a particular OMP resemble the folding pathway of the same OMP *in vivo*? This is a critically important question to consider if *in vitro* studies are to aid in our understanding of OMP folding in living cells. Ideally, to permit a mechanistic understanding of  $\beta$ -barrel folding in living cells, *in vitro* analysis of OMP assembly would include the purified Bam complex; such reconstitution systems have contributed greatly to the present understanding of other transport processes, including Sec-dependent IM translocation [201], maltose transport [202], and lipoprotein sorting [203]. To that end, the Kahne group at Harvard University recently reported a significant achievement: the *in vitro* reconstitution of the *E. coli* Bam machine [95].

The Kahne group found that the Bam holocomplex could be efficiently reconstituted by first purifying native BamA–B and BamCDE and then combining these subcomplexes to form the heteropentamer. Proteoliposomes containing the five-member Bam complex were able to increase the rate of *in vitro* OMP folding by several orders of magnitude [95]. Efficient OMP folding in this system requires the complete Bam complex and the SurA chaperone, but does not require LPS.

The beauty of the Bam reconstitution lies in the ingenious choice of substrate. Accurate kinetic analysis of OMP assembly *in vitro* requires a sensitive assay that permits detection of newly-folded substrate over time. To achieve this, the Kahne group took advantage of the protease activity of the OM  $\beta$ -barrel OmpT. A fluorogenic peptide added to proteoliposomes along with the unfolded OMP essentially serves as a reporter of OmpT folding: only folded OmpT cleaves the peptide, therefore the rate of fluorescence production is a function of the rate of OmpT folding [95]. This highly sensitive system affords real-time, quantitative analysis of Bam-assisted OMP folding kinetics.

The reconstitution of Bam *in vitro* is pivotal in part because it clearly demonstrates that the complete Bam complex is necessary for efficient  $\beta$ -barrel assembly. Moreover, it shows that the process occurs spontaneously and does not require an energy source. The rate of the folding reaction in this reconstituted system (on the order of seconds to minutes) is comparable to assembly rates observed for model OMPs *in vivo*, which suggests that Bam is sufficient for assembly of  $\beta$ -barrels on a physiologically relevant timescale [95].

Now that facilitated OMP assembly has been successfully reconstituted *in vitro*, it will be interesting to employ this system to ask additional mechanistic questions about the Bam apparatus. What experimental conditions favor increased folding efficiency? Does Bam turn over *in vitro* (i.e. can a single Bam complex process cycle through multiple rounds of substrate assembly)? Is the reconstituted machine competent to assemble a variety of OMPs? Can the folding of complex substrates (e.g. LptD or ATs) also be reconstituted *in vitro*, and are additional factors required? Omission of any one Bam component *in vitro* greatly reduces folding efficiency of OmpT [95]; is this generally true for OMPs in this system, or are individual Bam components dispensable for certain substrates? Deletion of the C-terminal phenylalanine of PhoE abrogates assembly *in vivo* without affecting *in vitro* refolding whatsoever [145]; does a mutation of this kind prevent OMP recognition by Bam *in vitro*? The reconstitution system makes the problem of  $\beta$ -barrel assembly far more tractable. The combined use of this system together with mutant OMP biogenesis factors that exhibit altered activity *in vivo* will likely provide mechanistic insight into this fascinating biological problem.

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