Biochimica et Biophysica Acta 1843 (2014) 1542–1550

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Assembly of β-barrel proteins into bacterial outer membranes ☆

Joel Selkrig [a,1], Denisse L. Leyton [a,b], Chaille T. Webb [a], Trevor Lithgow [a,*]

[a] Department of Biochemistry & Molecular Biology, Monash University, Melbourne 3800, Australia
[b] Department of Microbiology, Monash University, Melbourne 3800, Australia

ARTICLE INFO

Article history:
Received 26 June 2013
Received in revised form 5 October 2013
Accepted 8 October 2013
Available online 14 October 2013

Keywords:
β-Barrel protein
Outer membrane
Protein secretion
Membrane protein assembly
Membrane biogenesis

ABSTRACT

Membrane proteins with a β-barrel topology are found in the outer membranes of Gram-negative bacteria and in the plastids and mitochondria of eukaryotic cells. The assembly of these membrane proteins depends on a protein folding reaction (to create the barrel) and an insertion reaction (to integrate the barrel within the outer membrane). Experimental approaches using biophysics and biochemistry are detailing the steps in the assembly pathway, while genetics and bioinformatics have revealed a sophisticated production line of cellular components that catalyze the assembly pathway in vivo. This includes the modular BAM complex, several molecular chaperones and the translocation and assembly module (the TAM). Recent screens also suggest that further components of the pathway might remain to be discovered. We review what is known about the process of β-barrel protein assembly into membranes, and the components of the β-barrel assembly machinery. This article is part of a Special Issue entitled: Protein trafficking and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.

1. Introduction

The proteins assembled into bacterial outer membranes determine the organism's ability to compete and survive in a given environment. These proteins make up the pores and filters that enable nutrient import, the machines that drive locomotion, the extended structures that enable sensing of the environment, and attachment to environmental surfaces, along with efflux pumps that cleanse the cells of toxins and antibiotics. Furthermore, at least six types of protein secretion systems translocate protein effector molecules across the bacterial outer membrane, with the specific arsenal of proteins deployed by a given bacterial species contributing to its competitive advantage. All of these functions underpin bacterial survival and depend on the assembly of specific integral membrane proteins into bacterial outer membranes.

Unlike the bacterial inner membrane, that primarily houses integral membrane proteins composed of α-helical bundles, proteins that adopt a β-barrel topology dominate the outer membrane proteome. The process of integral membrane protein assembly into bacterial outer membranes is catalyzed by the β-barrel assembly machinery; a production line of factors that handle incoming polypeptides to ensure their efficient assembly into the outer membrane. Here we review research on the mechanism by which β-barrel proteins fold and insert into membranes, together with studies on the components of the β-barrel assembly machinery, and the way in which they drive the assembly of β-barrel proteins into bacterial outer membranes.

2. Outer membrane proteomes: complexity and topography

The vast majority of bacterial outer membrane proteins have a β-barrel topology, with the structures of 64 of these documented in a recent review [1]. A survey of literature reveals experimental studies on the assembly of at least 37 of these β-barrel proteins into the outer membrane (Table 1). These β-barrel proteins vary in the number of β-strands – and therefore vary in barrel circumference – and in the size and complexity of extra-membrane domains [1–3]. In all cases, the polypeptide chain folds into a series of anti-parallel β-strands and, as a result of hydrogen bonds between the first and last β-strands, comes to form a cylindrical β-barrel domain embedded in the lipid environment of the outer membrane. In many of the β-barrel proteins that have been crystallized, such as PagP, there is little or no N-terminal or C-terminal extension of the polypeptide into the periplasm (Fig. 1) and the extramembrane domain consists of a series of inter-strand loops that fold to produce a hydrophilic cap for the β-barrel protein, often allowing for access of small molecules into the lumen of the barrel.

It is becoming increasingly apparent that these relatively simple structures are not the only substrates that the β-barrel assembly machinery has to contend with. For example, the lipopolysaccharide transporter LptD has a complicated assembly process requiring participation of both the β-barrel assembly machinery and the LptE, a lipoprotein partner of LptD, to bring about a successful assembly reaction [4,5]. A second example is TolC [6,7]. In addition to having an

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* Corresponding author. Tel.: +61 3 9902 9217; fax: +61 3 9905 3726.
E-mail address: trevor.lithgow@monash.edu (T. Lithgow).
[1] Present address: School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore.

0167-4889/$ – see front matter.
http://dx.doi.org/10.1016/j.bbamcr.2013.10.009

Table 1
Experimental studies directed at the assembly of β-barrel proteins. Studies where biochemical or genetic analyses have documented outer membrane proteins in which the BAM complex participates in their assembly reaction.

Substrate	Method of detection	Reference
PorA, PorB, PilQ, FrpB, OMPLA, IgA1 protease	BamA depletion and direct BamA–PorA interaction by Far-Western blotting	[22]
LamB, OmpA	BamA depletion	[24]
LamB, OmpA	BamA depletion	[44]
OmpT	In vitro reconstitution of the BAM complex	[47]
PhoE	POTRA-PhoE peptide interaction in vitro (NMR)	[39]
Pet (autotransporter)	BamA and BamD depletion	[84]
Pet (autotransporter)	Direct protein–protein interaction by cross-linking to BamA and BamD	[97]
YadA (autotransporter)	BamA depletion	[98]
OmpF, LamB, TolC	BamA POTRA1 mis-sense and deletion mutations	[60]
PorA	In vitro PorA peptide changes recombinant BamA channel conductance	[99]
PorA, PorB, LbpA	POTRA deletion	[38]
BesC, p66, BB0405, surface exposed lipoproteins; CspA, OspA	BamA depletion	[100]
Hbp (autotransporter)	Direct protein–protein interaction by cross-linking to BamA and BamB	[82]
Int550	BamA depletion	[101]
EspP	Direct interaction with BamA and BamB by UV cross-linking	[68]
EspP	Direct interaction with BamA by UV cross-linking	[80]
EspP	Direct interaction with BamA, BamB and BamD by UV cross-linking	[52]
EspP	Direct EspP–BamA interaction by yeast two-hybrid	[86]
CpaC	BamE deletion strain	[102]
OmpC, OmpF, OmpA, BamA	BamA point mutations	[103]
LptD,OmpF, OmpC, OmpA, LamB	BamA point mutations	[104]
LptD	BamA depletion	[105]
IcsA, SepA, BrkA, AIDA-I (autotransporters)	BamA depletion	[83]
TolC	BamA and BamB depletion and BamA POTRA deletion	[25,106]
FimD	BamA and BamB depletion	[107]
SlpA	BamA depletion and direct BamA–SlpA interaction by Far Western blotting	[108]
OprF	BamA depletion	[109]
Invasin	BamA depletion	[110]
OmpA, OmpC, OmpF, OmpD	BamB isogenic mutant	[51]

extensive periplasmic domain that must be assembled together with two other partner proteins, including one which is embedded in the cytoplasmic membrane, TolC is itself a trimer: three copies of the TolC polypeptide must be assembled together to form a stable β-barrel structure in the outer membrane (Fig. 1). An extreme example of an even more complicated membrane protein topology is seen in the autotransporters, which have 12-stranded β-barrel domains but extracellular domains of extraordinary size — ranging in size from just a few kiloDaltons (e.g. EstA; [8]) to 1000 kDa or more (e.g. BigE; [9]). Assembly of autotransporters is not only a complicated protein-folding problem, but requires that a huge hydrophilic domain breach the outer membrane.

3. β-Barrel proteins: folding + insertion = assembly

Current research in several laboratories is aimed at dissecting how β-barrel proteins are assembled into the outer membrane. The assembly of a β-barrel consists of three parallel processes: (a) preventing the polypeptide from mis-folding, (b) folding of the various β-strands into the β-sheet, including interactions between the first and final strand forming a barrel shape, and (c) insertion of the barrel into the lipid phase of the outer membrane (Fig. 2). In a cellular context, the folding reaction for membrane-embedded β-barrel proteins has been viewed as a black box, but has been understood to involve a highly-synchronized process that encompasses both folding (of the barrel) and insertion (into the lipid environment).

Our understanding of the biophysical principles that must operate within this cellular black box has been advanced through direct measurements of purified β-barrel proteins, unfolded in urea, and then allowed to access the lipid environment of synthetic membranes. These pioneering studies have been reviewed elsewhere (reviewed in [10–15]). Taken together, these observations are consistent with the following: (i) the unfolded polypeptide substrate binds to the phospholipid surface, (ii) assumes some β-strand structure in a partially folded state, (iii) this favors a tilting of a proto-barrel structure and/or insertion of a β-sheet that is not yet a complete barrel, and (iv) the final steps of assembly of the β-barrel structure. These studies with purified proteins are moving towards a picture from where it can be distinguished when in this cascade of events a recognizable β-barrel would form. For example, fluorescence spectroscopy of OmpA applied to investigate the timing for formation of β-strands and their zippering into the β-barrel structure in the presence of pure phospholipid vesicles [16]. Studies such as this suggest that a hairpin of two neighboring β-strands occurs immediately and very soon after, the first β-strand can be measured to have annealed to the last β-strand. This partly-folded "proto-barrel" intermediate provides a continuous hydrophobic surface onto which lipids would adhere and ultimately accept the protein into the hydrophobic core of the membrane. In these purified systems, it is widely considered that the activation energy for the spontaneous assembly reaction is high, which may explain why the time-frame of these reactions is orders of magnitude higher than the assembly reaction as it occurs in bacteria.

In a recent detailed account for the β-barrel protein PagP, the folding reaction was found to be initiated from a membrane-adsorbed, yet highly unfolded conformation that lacks secondary structure [13]. Through a process involving multiple, reversible transition states, formation of the extracellular half of the β-barrel proceeds in which the C-terminal β-strands are highly structured, whereas the N-terminal β-strands and an N-terminal, periplasmic α-helix remain poorly organized [13]. This study on PagP is consistent with work on OmpA and FomA [10,12,17,18], and highlights that the assembly of membrane-embedded β-barrels can be considered in terms of the folding reactions for globular proteins first proposed by Anfinsen: the polypeptide has intrinsic information that would lead it to fold into a β-barrel structure, but has a complex folding pathway with many "off-pathway" detours that could be catastrophic if left uncontrolled [11]. By preventing the likelihood and extent of off-pathway detours, a native integral membrane protein embedded within a lipid bilayer results.

An additional layer of understanding can be super-imposed on this highly-synchronized process of folding and insertion if the point of view for each β-strand is considered. Using single-molecule force spectroscopy, Damaghi et al. [19] followed the re-folding of the β-barrel protein OmpG. OmpG can be reconstituted into a phospholipid bilayer adsorbed onto a layer of mica and analyzed by atomic force microscopy (AFM), whereby the AFM tip is used to extract the polypeptide from the membrane layer. The protein is then allowed to refold into the membrane and force measurements unambiguously showed individual β-hairpins of OmpG inserting into the membrane hairpin-by-hairpin [19]. This process demonstrates that there can be considerable reordering of lipid molecules during the membrane insertion of β-barrel proteins. By contrast, when the larger β-barrel protein FhuA was analyzed it was found that while β-hairpins could be removed from the membrane with the AFM tip, they did not subsequently reassemble into the membrane layer [20]. Whether because of difference in size or differences in sequence characteristics,

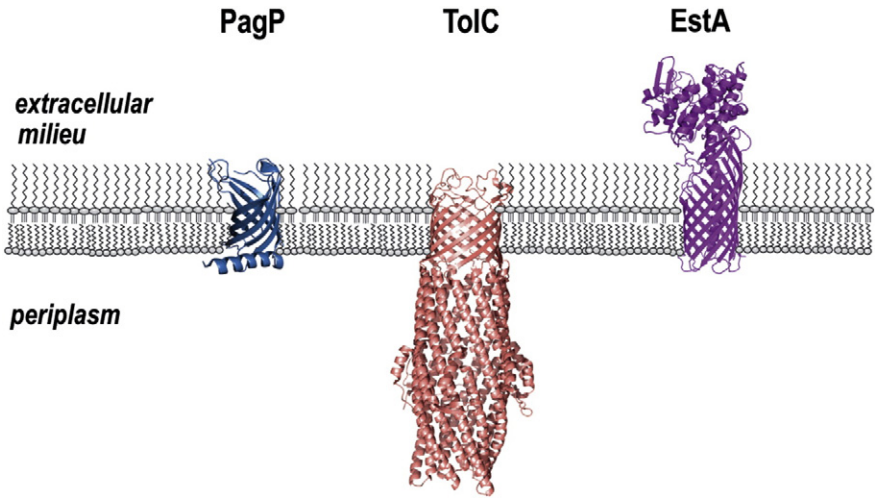

Fig. 1. Structure of β-barrel proteins in bacterial outer membranes. PagP (blue) is a β-barrel protein composed of 8 β-strands [13,96]. The N-terminus forms a short α-helix in the periplasm, and the eighth β-strand forms at the C-terminus of the polypeptide. TolC (red) is one of the largest β-barrel proteins structurally characterized [6]. It is composed of three copies of the TolC polypeptide, each of which contributes 4 β-strands to the 12-stranded β-barrel in the outer membrane. The N-terminal region of TolC is situated in the periplasm and interacts with partner proteins to form a drug-efflux system, capturing small molecules for their expulsion into the extracellular milieu. The esterase EstA (purple) is an autotransporter composed a C-terminal β-barrel domain with an N-terminal domain that folds on the surface of the cell [8].

or both, some β-barrel proteins are more readily inserted into membrane environments than other β-barrel proteins.

So what do biophysical studies with pure proteins tell us about the rate-limiting steps for β-barrel protein assembly in a cellular context? Firstly, in the proteinaceous and peptidoglycan-rich environment of the periplasm, unfolded polypeptides would be prone to off-pathway interactions, invoking a role for molecular chaperones in the periplasm. Secondly, folding of the various β-strands into the β-barrel is critical and may be serviced by factors that can provide domains for "β-augmentation" a process which affords a template for the formation of β-strand folding and thereby increase the overall assembly rate. Thirdly, while insertion of the barrel domain into a lipid phase can occur spontaneously in a purified system, factors that would accentuate disturbance in a stable bilayer would favor transition intermediates (such as those seen for the model protein PagP [13] and OmpA [16]), thereby catalyzing the membrane insertion steps in vivo. Finally, studies allowing direct comparison such as the AFM analysis of OmpG [19] and FhuA [20] suggest that individual characteristics of a given protein also need to be considered, with some β-barrels being perhaps more difficult to assemble than others. Indications that (i) the folding of the barrel and (ii) insertion into the lipid environment represent a synchronized process, thus suggest that the factors mediating folding and insertion are co-located in the outer membrane, and cooperate in lowering the activation energy for assembly of β-barrel proteins into bacterial outer membranes.

3.1. The BAM complex: a modular machine driving β-barrel assembly

An essential outer membrane protein, initially referred to as Omp85 and now called BamA, was identified as the key component of the β-barrel assembly machinery in *Neisseria meningitidis* [21,22]. BamA provides an essential function, in that the *bamA* gene is essential for bacterial viability. BamA was identified and purified from outer membranes of *Escherichia coli* and shown to exist in a hetero-oligomer, the BAM (*β*-barrel *a*ssembly *m*achinery) complex, associated tightly with four lipoprotein partners: BamB, BamC, BamD and BamE (Fig. 3) [23–29]. Both physically and conceptually, the BAM complex is a classic example of a modular molecular machine, as are so many protein transport systems [30]. It is widely believed that BamA forms the

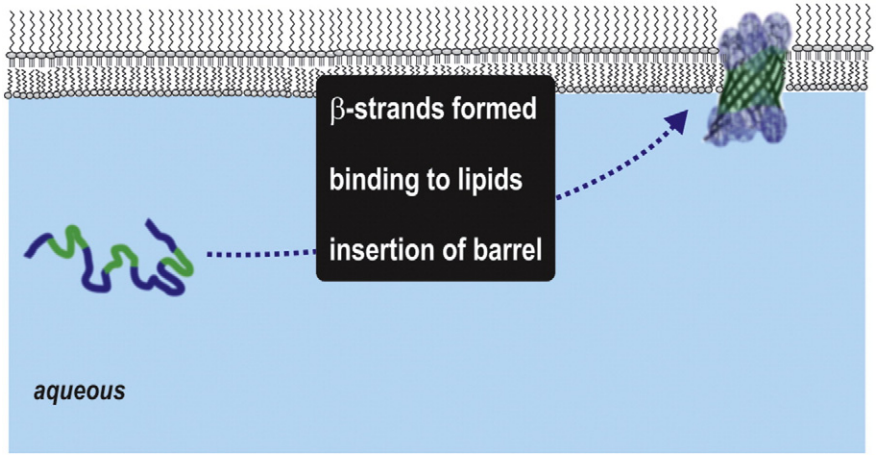

Fig. 2. The black box: folding and insertion of β-barrel proteins into the bacterial outer membrane. An unfolded polypeptide contains intrinsic information that will drive the formation of a β-barrel protein, where the antiparallel β-strands (colored green) coalesce into a cylinder that exposes hydrophobic side-chains on its outer face. The β-barrel will ultimately tilt into the membrane environment to bury all surface-exposed hydrophobicity, but this requires the hydrophilic loops (colored blue) to move through the lipid bilayer. The black box denotes that in vivo, the order of these events is only just becoming clear: distinct experimental approaches are providing pieces of the puzzle that together suggest lipid binding, formation of β-strands and insertion into the membrane may all occur as part of a single cooperative process.

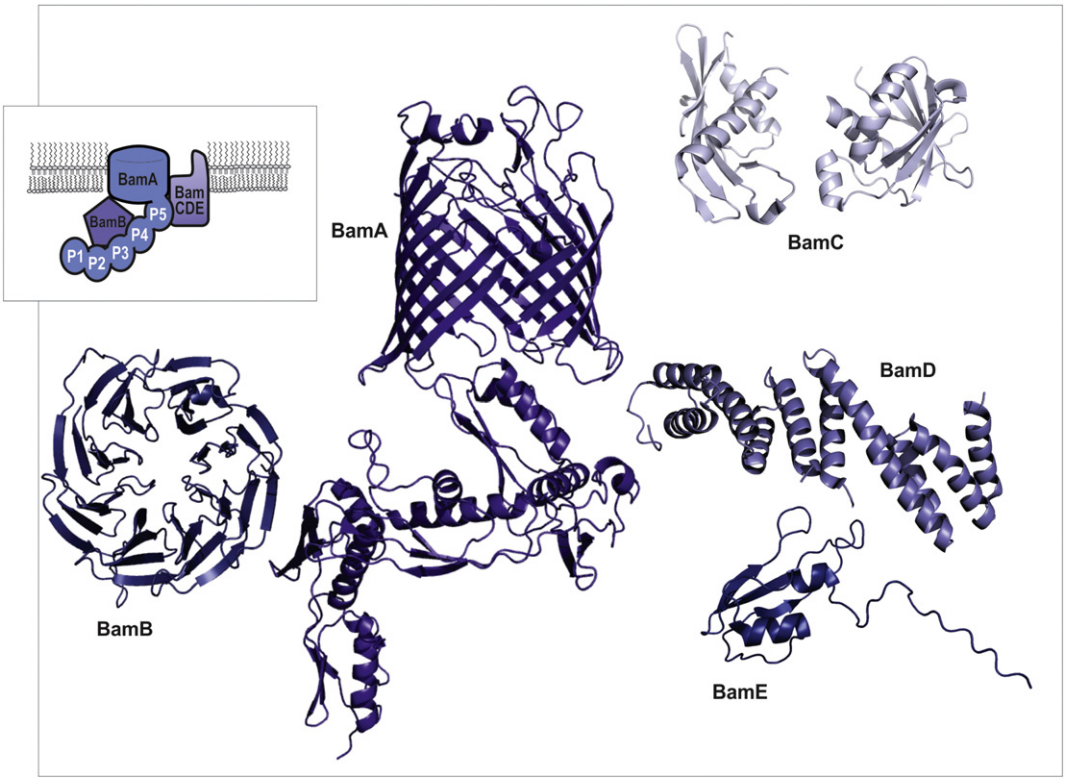

Fig. 3. Topology and structure of the BAM complex. The cartoon summarizes what is known about the architecture of the BAM complex, derived through the results of genetics and molecular interactions. Shown are the crystal structures available for each subunit of the BAM complex ordered according to the current knowledge of overall architecture (as depicted in the inset); BamA (*N. gonorrhoeae*; pdb 4K3B), BamB (*E. coli*, pdb 3Q7O), the N-terminal domain (residues 101–212) and C-terminal domain (residues 226–344) of BamC (*E. coli*; pdb 2YH6 and 2YH5 respectively), BamD (*E. coli*; pdb 3Q5M) and BamE (*E. coli*; pdb 2KM7).

protein:lipid interface at which outer membrane protein substrates enter into the lipid phase of the membrane [31], with BamB proposed to form a scaffold to assist β-barrel folding [32–35]. As detailed below, the other three subunits can be described as a BamC:D:E module and have been suggested to drive a conformational switch in the BAM complex that enables β-barrel insertion into the outer membrane.

3.2. The BAM complex: BamA

BamA has a highly-conserved, "D15-domain" which forms a 16-stranded β-barrel embedded in the outer membrane, and an N-terminal periplasmic domain comprised of five *p*olypeptide *t*ransport *a*ssociated (POTRA) domains [28,29,36–40]. POTRA domains were discovered first in the TpsB, BamA, Toc75 and FtsQ protein families [41], and have since been shown in many studies to function as protein–protein interaction domains. The isolated POTRA domains from BamA have been crystallized in two conformations: extended into a form that is ~100 Å long [36] or bent into a J-shaped arrangement [28]. It had been suggested that these conformations might represent two natural states that would be seen in a reaction cycle of BamA [36,42]. In various studies, the POTRA domains of BamA have been proposed to create strands that could assist β-augmentation for substrate folding [28], to provide a hydrophobic groove to take up a substrate polypeptide [36] and, as described below, to serve as the docking point for partner proteins of BamA (Fig. 3).

Work in the Buchanan laboratory recently revealed the structure of the complete BamA protein; crystallized in two conformations that demonstrate the nature of movements in the POTRA domains as well as unveiling conformational changes in the membrane-embedded D15 domain of BamA [43]. We now know that in bacteria BamA would sit as a cupola-like structure, with a domed "ceiling" rising above the plane of the outer membrane. The aqueous chamber thereby created within the bacterial outer membrane has a highly-charged internal surface: a chamber that would be repellent to a substrate β-barrel protein. Entry into the chamber from the periplasmic space is "open" when the POTRA domains sit in one conformation, but is closed when the POTRA domain switch to the alternative conformation [43].

The outer membrane environment does not provide access to energy sources such as ATP or electrochemical gradients, so the process of β-barrel protein assembly is presumably driven exclusively through the free-energy gain derived from protein folding. This in turn suggests that the role of the BAM complex would be to lower the activation energy of β-barrel protein assembly into the bacterial outer membrane. In this context, the placement of aromatic residues around the girth of the BamA molecule observed by Noinaj et al. was particularly striking: it suggests a local disruption of the lipid environment close to the position where the first and last β-strands of the D15 domain meet, and molecular dynamics studies illustrate that the movement of these first and last β-strands of BamA create a rift, opening up a continuity between the BamA chamber and the lipid core of the outer membrane [43]. It is reasonable to envisage that in the immediate vicinity of BamA, a localized patch of the outer membrane is made conducive to the penetration of nascent, partly- or largely-folded β-barrel proteins; the types of folding intermediates observed in biophysical analysis in synthetic membrane systems. This highly provocative picture of BamA provides insight into the pathway by which β-barrel proteins are assembled into the bacterial outer membrane, and a marvelous basis from which to address very specific experimental questions using biophysical, biochemical and cellular assay systems.

3.3. The BAM complex: the BamA:B module and the BamC:D:E module

A single mutation in POTRA5 of BamA separates the BAM complex into two modules: BamA:B and BamC:D:E [44]. Consistent with this, using controlled detergent conditions to solubilize the outer membrane of wild-type *E. coli*, the BAM complex disassociates into the BamA:B

module and the BamC:D:E module [45]; and a functional BAM complex can be reconstituted by docking a BamC:D:E module with a BamA:B module [46,47]. Recent work has been directed at determining the distinct biochemical functions provided by these modules.

In the BamA:B module, BamA is essential, while BamB is not: deletion strains of *E. coli* lacking BamB are viable, but display significant defects in β-barrel protein assembly [48–50]. The structure of BamB shows a very large protein surface that could provide a protective environment for a nascent β-barrel substrate in the process of folding [32–35]. Deletion of individual periplasmic domains of BamA: POTRA2, POTRA3 or POTRA4 causes disassociation of BamB [28], as do specific point mutations clustered in one specific "blade" of the β-propeller of BamB [29,35]. Based on crystal structures of the two individual proteins and the identity of point mutations that break the interaction between BamA and BamB, a structural model for the BamA:B module has been proposed [32–35]. According to this model, BamB binds directly to BamA through interaction with the POTRA2 and POTRA3 domain of BamA. Intriguingly, complementation analysis in *Salmonella* shows that BamB remains functional even when its interaction with BamA is 'broken' by the equivalent cluster of point mutations in BamB [51]. One explanation for this observation is that BamB might function in the vicinity of the BAM complex, without necessarily interacting tightly with BamA. With respect to function, the leading strands of the β-propeller structure of BamB (Fig. 3) have been suggested as attractive sites at which to facilitate β-augmentation for substrate folding [33], thereby assisting completion of β-strands into β-hairpins and partial β-sheets. Although the specific details around this idea remain controversial [35], recent cross-linking experiments with a β-barrel protein jammed in the BAM complex demonstrated a direct interaction of the substrate β-barrel protein with BamB [52].

The BamC:D:E module is thought to be involved in moderating the structural conformation of BamA, with single amino acid mutations in BamD, or deletion of BamE, inducing changes in the structure of BamA evident by various biochemical or genetic analyses [42,44,53]. BamC and BamE associate directly with BamD, to form the BamC:D:E module, which in turn binds to the POTRA5 domain of BamA via BamD [28]. This puts the BamC:D:E module in close proximity to the juncture of the first and last transmembrane β-strands of BamA, a place at which the outer membrane lipids may be in non-bilayer conformation [43]. Within the BamC:D:E module, BamD is the only essential lipoprotein conserved across the vast majority of Gram-negative bacteria. Conversely, BamC and BamE are not essential and deletion of either gene yields relatively minor effects on β-barrel protein assembly. Individual structures of BamC, BamD and BamE (Fig. 3) have shone light on this module, but the complete architecture remains to be determined. Most recently, the crystal structure of BamC:D was determined and depicts a large interface between the two proteins in which the unstructured N-terminus of BamC binds across the length of BamD [54]. By contrast, the C-terminal domains of BamC have been observed to sit on the extracellular face of the BAM complex, exposed to antibodies [45]. The reason for BamC to span the outer membrane, and the mechanism by which BamC and BamE act to support BamD in its essential role in β-barrel protein assembly, require further examination.

Analysis of species of bacteria other than *E. coli* raises important points with respect to the modularity of the BAM complex and the protein folding pathway for β-barrel substrate proteins. A most pertinent example concerns species of *Neisseria*, which are unique amongst the Beta-proteobacteria in that they do not encode a BamB protein [55]. Consistent with this, deletion of the POTRA domains 1–4 in *N. meningitidis* has only little effect on β-barrel protein assembly [38]. Conversely, deletion of the POTRA2, POTRA3 or POTRA4 domains in *E. coli* has a substantial effect on β-barrel protein assembly, as does deletion of the gene encoding BamB [28]. Both sets of experiments suggest that a major role for the POTRA2, POTRA3 or POTRA4 domains is to organize the BamA:B module, and this interaction makes for a module with a more effective means of driving the folding pathway for β-barrel proteins. What remains curious is the reason behind the loss of BamB in all sequenced species of *Neisseria*; perhaps a slower rate of β-barrel protein assembly can have a selective advantage in some environmental scenarios?

4. Periplasmic chaperones assist delivery and insertion into the outer membrane

Prevention of β-barrel mis-folding in the periplasm is assisted by the action of molecular chaperones [56–59]. The BAM complex is serviced by chaperones such as SurA, which bind substrate proteins in the periplasm and may coordinate the initial steps in substrate interaction with the BAM complex [60–63]. Deletion of the POTRA1 domain of BamA results in β-barrel protein assembly defects in *E. coli* [60], perhaps due to loss of a productive interaction with SurA [28]. In addition, two other periplasmic chaperones, Skp and DegP, participate in the biogenesis of outer membrane proteins [26,64]. The Skp chaperone binds unfolded proteins while they are still engaged in translocation through the Sec translocon [65,66]. They will remain associated as a soluble chaperone–substrate complex within the periplasm [67], but can also be detected in association with β-barrel proteins that are engaged at the BAM complex [68]. Biophysical experiments with purified proteins have shown that Skp interacts productively with unfolded β-barrel proteins, having a central cavity functioning as an Anfinsen cage for an unfolded OmpA polypeptide [57,69–74], and can pass the substrate directly on to BamA [75]. The relatively slow rate with which these processes operate in purified systems might reflect that in vivo Skp, SurA and DegP provide a network of chaperone interactions to minimize the time taken to productively interact with the outer membrane. In terms of the protein mass that has to be extruded through the membrane core, the assembly of model proteins such as OmpA and PagP can be considered relatively simple reactions, although they do have extracellular loops of considerable size that form a hydrophilic cap (Fig. 1). Much of the current work in the field is focused on autotransporters, as "extreme" substrates that represent promising models with which to dissect the β-barrel assembly machinery, including their folding state in the periplasm and at the surface of the outer membrane.

5. An extreme example of how timed protein folding really matters: autotransporters

Autotransporters are outer membrane proteins that contain both a β-barrel transmembrane domain and a passenger domain, which is the secreted functional moiety. Autotransporter assembly into the outer membrane is a major feat given the extensive extracellular domains in these β-barrel proteins. Current models for the assembly pathway suggest that the autotransporter β-domain is inserted into the outer membrane after which only a relatively small C-terminal portion of the extracellular domain, termed the autochaperone (AC) domain, folds, leaving substantial amounts of the polypeptide chain to move through the central channel of the β-barrel in order to finalize assembly (Fig. 4). Based on structural information and numerous important functional studies (recently reviewed in [76] and [77]), model autotransporter substrates have been designed to probe the component β-barrel assembly pathway using protein transport assays.

Translocation and folding of the AC domain are early steps in autotransporter biogenesis [78,79]. Mutations that affect the folding of the AC domain of the autotransporters Hbp and EspP greatly impact their rate of assembly and, as a result, translocation intermediates can be detected on the periplasmic side of the outer membrane in an unfolded state [80,81]. Cross-linking experiments have shown that these translocation intermediates interact with subunits of the BAM complex and periplasmic chaperones SurA and Skp [80,81]. Similar studies with translocation intermediates that are further along the biogenesis pathway (evidenced by passenger domains that are partly

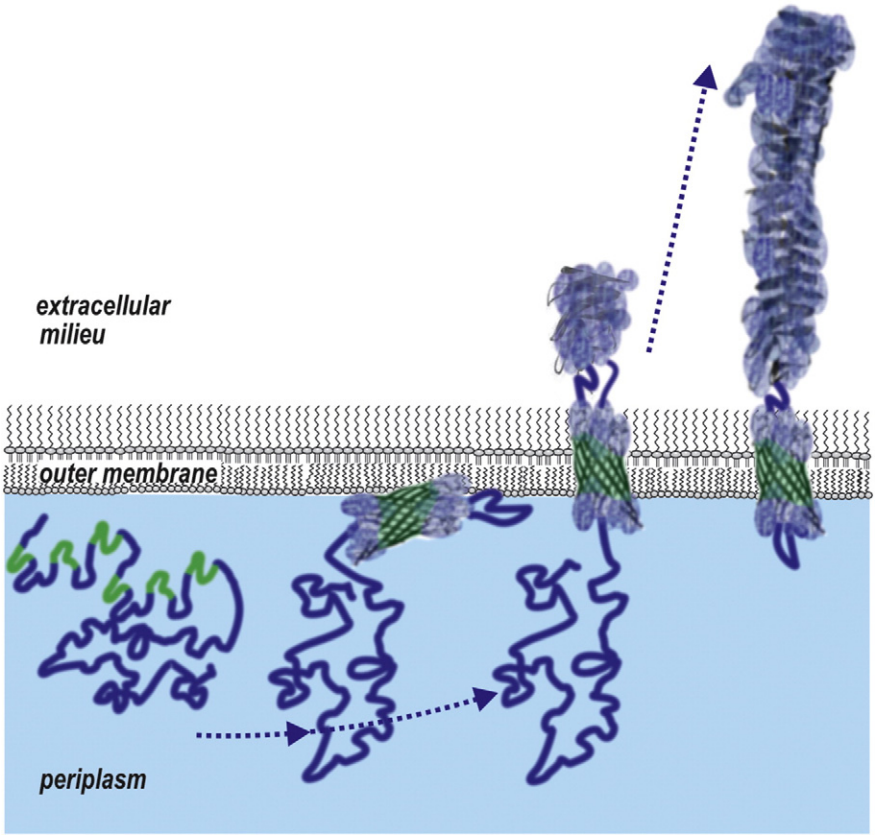

Fig. 4. β-Barrel protein topography and assembly pathways. Summary of the pathway for autotransporter folding and insertion into the outer membrane: (i) Initial folding to form the β-barrel is proposed to occur at the outer membrane, in order to engage a segment of polypeptide within the barrel lumen. (ii) After insertion of this translocation intermediate into the outer membrane, (iii) protein folding on the outer face of the outer membrane would drive the transfer of the nascent passenger domain through the β-barrel to assume its functional state in the extracellular milieu.

exposed as a hairpin at the cell surface) demonstrate that autotransporters continue to interact with components of the BAM complex and with periplasmic chaperones [68,82], in a manner that is sequentially and spatially regulated [52]. These results are consistent with those of other studies demonstrating that autotransporter biogenesis is impaired in *E. coli* and *Shigella flexneri* mutants depleted of BamA [83,84] or lacking periplasmic chaperones Skp, DegP or SurA [82,85–87] and in vitro studies showing direct interactions between unfolded autotransporters and BamA, Skp, DegP or SurA using yeast two-hybrid and surface plasmon resonance [61,86]. Collectively, these findings suggest that after mediating transit through the periplasm, molecular chaperones continue to serve the β-barrel assembly pathway until no unfolded regions of substrate polypeptide remain in the periplasm.

6. The translocation and assembly module (TAM)

The translocation and assembly module (TAM) functions in the β-barrel assembly pathway, and is composed of two integral membrane proteins: TamA and TamB. Like BamA, TamA has a "D15-domain" integrated into the outer membrane, and a series of POTRA domains that project into the periplasm (Fig. 5). TamB has an N-terminal signal-anchor sequence embedded in the inner membrane, and has a conserved C-terminal DUF490 domain exposed to the periplasm [88]. Neither the outer membrane protein TamA, nor the inner membrane protein TamB is essential for cell viability under laboratory conditions [88,89], but deletion of TAM causes an accumulation of autotransporter molecules in the periplasm, some of which are associated with the outer membrane but with at least part of the translocation intermediate stuck in the periplasm [88]. Similar defects in autotransporter assembly were observed in Δ*tamA* mutants, Δ*tamB* mutants, and in Δ*tamA*,Δ*tamB* double mutants suggesting that the loss of either TamA or TamB blocks TAM function [88]. These results indicate that the TAM is required for the efficient translocation of autotransporters across the outer membrane for presentation onto the cell surface.

It is as yet unclear whether the TAM participates in the assembly of other β-barrel proteins into the outer membrane. The benign phenotype of mutant bacteria lacking the TAM show that it is not the rate-limiting step for outer membrane protein assembly, and that the other elements of the β-barrel assembly machinery continue to function in its absence. Our current working model is that the TAM assists to provide another protein:lipid interface, analogous to that provided by BamA [43], to further assist insertion of at least some β-barrels into the outer membrane (Fig. 5). We suggest that some autotransporters, being challenging substrates to insert into the membrane, are particularly sensitive to the absence of the TAM. Future studies will be needed to address the range of substrates that might normally benefit from the activity of the TAM in the wild-type condition.

It also remains to be determined to what extent the TAM module might directly cooperate with the BAM complex to catalyze β-barrel protein assembly. In this regard, it is of great interest that sequence analysis of the spirochaetes *Borrelia burgdorferi* and *Treponema pallidum* reveals the presence of orthologs of BamA (BB0795 and TP0326 respectively) and TamB (BB_0794 and TP0325 respectively), whereas sequences corresponding to TamA are apparently absent. Curiously, the Spirochaete *tamB* orthologs are encoded directly upstream of their BamA orthologs. These tantalizing syntenic relationships between TamB and BamA may suggest that in spirochaetes, TamB and BamA functionally co-operate. BN-PAGE analysis of the *T. pallidum* BamA (TP0326) showed that it resides in a high molecular weight complex of ~300–400 kDa [90] and it will be of significant interest to determine whether TamB is a

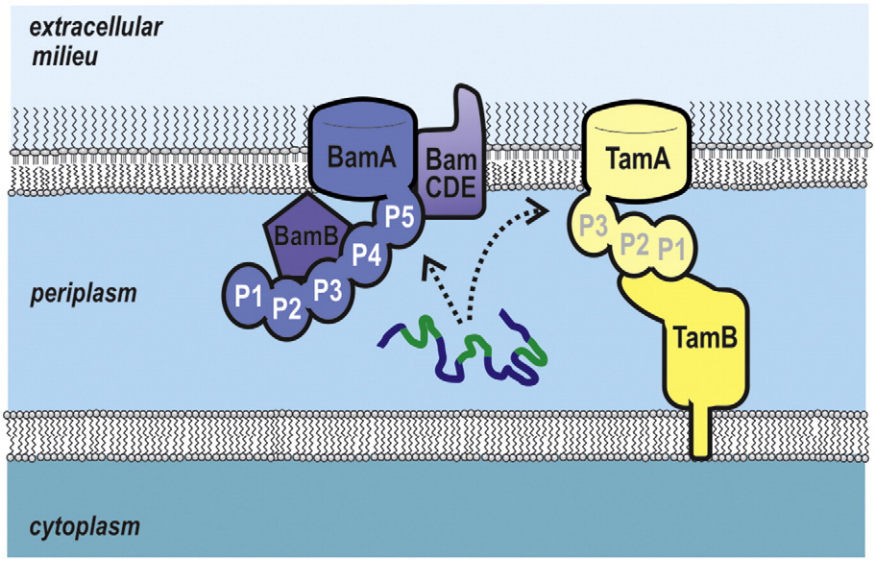

Fig. 5. The BAM complex and the TAM: insertion of β-barrel proteins into the outer membrane. The BamA:B module docks to the BamC:D:E module via an interaction of BamD and the POTRA5 domain of BamA. The BamC:D:E module has been proposed to drive at least some of the conformational changes in BamA that catalyze β-barrel protein assembly. The circumferential surface of the D15 domain of BamA (and perhaps regions of the BamC:D:E module) provide for a protein:lipid interface at which β-barrel protein insertion may be assisted. We speculate that the D15 domain of TamA might provide a similar type of assistance to the assembly of at least some β-barrel proteins.

subunit of that complex. Such a relationship would demonstrate an evolutionary rewiring in the modular structure of the β-barrel assembly machinery.

7. More new players: genetic screens and evolutionary insights

Even within the Proteobacteria, there is evidence that evolution has constructed distinct versions of the β-barrel assembly machinery. In the Alpha-proteobacteria, there is no BamC subunit in the BAM complex, but instead a small lipoprotein called BamF is present [55,91]. Whether this functions as part of a BamD:E:F module to drive a BamA conformational switch remains to be shown, the BamF subunit is released from the BAM complex together with BamD [55]. Comparative genomics revealed no evidence that any Delta-proteobacterium or Epsilon-proteobacterium encodes BamB, BamC or BamE subunits to interact with their BamA and BamD proteins [55]. It remains to be determined whether any of these species of bacteria have a minimalist BamA:D BAM complex, or have distinct protein partners functioning in the place of BamB, BamC and BamE.

There are also several reports that peptidoglycan-binding lipoproteins might serve as modules to the BAM complex. In *N. meningitidis* and in *Caulobacter crescentus*, the protein RmpM/Pal is present in stoichiometric amounts with the other lipoprotein subunits of the BAM complex [91,92]. In *E. coli*, a genetic screen uncovered the homologous peptidoglycan-binding lipoprotein YiaD as a suppressor of defects in a *bamD* mutant strain [93]. One possible explanation is that the peptidoglycan-binding domain of these proteins minimizes the distance of the BAM complex from the inner membrane, through latching it to the peptidoglycan layer. A more speculative possibility is that minimizing lateral mobility of the BAM complex might help coordinate its position with that of the sites for polypeptide entry (i.e. the Sec translocon) into the periplasm.

A recent synthetic lethality screen of *E. coli* mutants discovered two genes, *yfgH* and *yceK*, each of which when deleted in combination with a deletion of the *tamB* gene led to severe defects in growth and outer membrane integrity [94]. The two most direct explanations for synthetic phenotypes of this type are that (i) the novel protein and TamB are both subunits of the same complex (i.e. the TAM) and that deletion of two subunits leads to a total loss of TAM function and (ii) the novel protein and TamB act independently in the same pathway (i.e. the β-barrel assembly pathway) and that impacting two steps in this pathway generates a catastrophic defect not evident from impacting either step independently. It will be of great interest to learn the function of the two novel proteins, one of which has sequence features of a lipoprotein located in the inner membrane, the other of a lipoprotein located in the outer membrane.

8. Concluding remarks

Analyzing the structure of membrane proteins with a view to understanding how they are assembled has always been a hugely challenging problem, given technical challenges in purifying active membrane fractions and analyzing membrane protein structures [1,95]. Nonetheless, research from numerous laboratories working in distinct areas of membrane biology, biophysics, microbiology, biochemistry and structural biology is providing the means to appreciate the secretion and assembly of β-barrel proteins in bacterial outer membranes. As to the precise mechanism employed to insert and thereby assemble proteins into the hydrophobic core of the outer membrane, this remains perhaps the most hotly debated issue in the field. BamA might increase the kinetics of protein insertion by providing some local distortions in the lipid environment, assisting intermediate forms of a barrel to gradually assemble and enter the membrane. But the extent to which this occurs strand-by-strand, hairpin-by-hairpin or en bloc remains contentious. The TAM, and perhaps other factors, may serve as modular appendages of the BAM complex to promote efficient assembly of the diverse range of β-barrel proteins found in bacterial outer membranes.

Acknowledgements

We thank Eva Heinz and Victoria Hewitt for constructive comments on the manuscript. Work in the author's laboratory is supported by NHMRC Program Grant 606788. J.S. was supported by an NHMRC Postgraduate Scholarship, C.T.W. is supported by an NHMRC Training Fellowship, D.L.L. is supported by an Australian Research Council Super Science Fellowship and T.L. is an Australian Research Council Laureate Fellow.

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