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The *Escherichia coli* outer membrane protein OmpA acquires secondary structure prior to its integration into the membrane

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Almost all proteins that reside in the outer membrane (OM) of Gram-negative bacteria contain a membrane-spanning segment that folds into a unique β barrel structure and inserts into the membrane by an unknown mechanism. To obtain further insight into outer membrane protein (OMP) biogenesis, we revisited the surprising observation reported over 20 years ago that the *Escherichia coli* OmpA β barrel can be assembled into a native structure *in vivo* when it is expressed as two noncovalently linked fragments. Here, we show that disulfide bonds between β strand 4 in the N-terminal fragment and β strand 5 in the C-terminal fragment can form in the periplasmic space and greatly increase the efficiency of assembly of "split" OmpA, but only if the cysteine residues are engineered in perfect register (*i.e.*, they are aligned in the fully folded β barrel). In contrast, we observed only weak disulfide bonding between β strand 1 in the N-terminal fragment and β strand 8 in the C-terminal fragment that would form a closed or circularly permutated β barrel. Our results not only demonstrate that β barrels begin to fold into a β-sheet-like structure before they are integrated into the OM but also help to discriminate among the different models of OMP biogenesis that have been proposed.

Gram-negative bacteria have a cell envelope composed of two membranes, the inner membrane and the outer membrane (OM), and an enclosed space known as the periplasm. The proteins embedded in the OM mediate a variety of physiological functions including protecting bacteria against external environmental stresses and transporting nutrients to maintain cellular metabolism (1). Almost all outer membrane proteins (OMPs) are anchored in the OM by a "β barrel," a closed cylindrical structure composed of antiparallel β strands (2). β barrels vary in size from 8 to 36 β strands, and some OMPs form homodimers or homooligomers (2–4). Besides containing a common β barrel domain, some OMPs also have segments that reside inside the β barrel lumen and/or extracellular or periplasmic domains.

After newly synthesized OMPs are transported across the inner membrane through the Sec machinery, they interact with a variety of periplasmic chaperones including SurA, Skp, DegP, and OsmY that maintain them in an insertion-competent conformation and facilitate their integration into the OM (5–10). These periplasmic chaperones play not only partially redundant roles but also distinct roles in OMP biogenesis. Skp is a jellyfish-like homotrimer that can accommodate unfolded or partially folded OMPs in its central cavity to protect them from misfolding in the aqueous environment (11, 12). Skp either transfers OMPs to other chaperones such as SurA or identifies defective OMPs and directs them to proteases for degradation (13, 14). SurA targets OMPs to a heteroligomer known as the *b*arrel *a*ssembly *m*achinery (Bam) complex that catalyzes their insertion into the OM. The Bam complex consists of a highly conserved subunit (BamA) that contains a 16-stranded β barrel integrated into the OM and five globular *po*lypeptide *tr*ansport-*a*ssociated (POTRA) domains located in the periplasmic space as well as a variable number of lipoproteins (BamB–E) that bind to the POTRA domains (15, 16). Only one of the lipoproteins (BamD) is conserved and essential for viability (17, 18).

Although the structure of the Bam complex has been solved (19–22), the mechanism by which it catalyzes the assembly of OMPs is still not well understood. The crystal structure of BamA and molecular dynamic simulations suggested that the seam between β strands β1 and β16 open laterally (23–25). Experiments in which the BamA β barrel is locked in a closed state provided evidence that this opening is important for function and led to several different models for OMP assembly. In the "threading" (or "budding") model, it was proposed that OMPs enter into the BamA pore as fully unfolded proteins and then fold into β hairpins that pass sequentially into the plane of the membrane through a lateral gate formed during the transient opening of BamA β barrel. In an alternative "assisted" model, it was proposed that the lateral opening of the BamA barrel gate perturbs the local lipid bilayer to promote the insertion of folded or partially folded OMPs into the OM (24, 26, 27). Recently, intermolecular disulfide-bond crosslinking was used to map interactions between an arrested OMP assembly intermediate and BamA (28). This study provided evidence that BamA forms a hybrid barrel with an incoming OMP through a stable interaction between β1 and the C-terminal β strand of the client (a segment that contains the conserved "β signal" motif GXXΦXΦ, where Φ is an aromatic amino acid) (13) and a more dynamic interaction

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between β15/β16 and N-terminal β-strands of the client (28). Consistent with the biochemical data, a high-resolution structure of the same assembly intermediate bound to the Bam complex determined by cryo-EM confirmed both the interaction of BamA β1 with the β signal and the extreme dynamics of the interaction on the other side of the hybrid barrel (29). Although these studies strongly support an entirely new model in which the β signal first binds to BamA and then catalyzes the insertion of the remainder of the barrel by a "swing" mechanism, the data do not fully address the structure of the client protein at early stages before it is integrated into the OM.

To help elucidate the folded state of a generic monomeric β barrel prior to its insertion into the OM and thereby gain insight into the mechanism of OMP assembly, we exploited an intriguing but unexplained observation that was reported over 20 years ago. In an analysis of *Escherichia coli* OmpA, a well-characterized and highly abundant OMP that contains an empty N-terminal eight-stranded β barrel domain and a C-terminal periplasmic domain, Koebnik (30) found that when the OmpA β barrel was split at the third periplasmic turn into two fragments, the protein was able to fold and function properly *in vivo*. It was not determined, however, if the two halves of the protein interacted in the periplasmic space and acquired any secondary or tertiary structure prior to integration, or if they were each inserted into the OM independently and then formed a folded β barrel at a postinsertion stage. To address these questions, we examined the folding of both split OmpA and split OmpA mutants in which cysteine residues were engineered into adjacent strands in the final structure that, in principle, would permit the two halves to form disulfide bonds. We found that although split OmpA was assembled very inefficiently, a subset of cysteine mutants formed disulfide bonds and greatly enhanced assembly. Interestingly, only cysteine residues that are perfectly aligned in internal β strands (but not those that would connect the interface or "seam" between the first and last β strands) formed disulfide bonds efficiently. Furthermore, disulfide-bond formation was only observed in the presence of the periplasmic thiol-disulfide oxidoreductase DsbA. As expected, the disulfide-bonded forms of split OmpA required an active Bam complex for assembly. These constraints indicate that the OmpA sequence has sufficient information to facilitate the interaction of the two halves of the protein and the formation of at least some secondary structure but does not facilitate the formation of a closed barrel structure prior to membrane integration. The data not only provide information about the status of OmpA in the periplasm but also help us to discriminate between various models of OMP assembly.

Results

The formation of artificial disulfide bonds enhances split OmpA assembly in vivo

To obtain further insight into OMP biogenesis, we wished to confirm the observation that when the OmpA β barrel is split into two coexpressed fragments that each contain four of the eight β strands, the two fragments can insert into the *E. coli* OM and fold into a native structure (30). To this end, we generated a plasmid (pXW22) that coexpresses *E. coli* K-12 OmpA(β1–β4) and OmpA(β5–β8) with independent OmpA signal peptides under the control of the *trc* promoter (Fig. 1*A*). We first transformed XW100 (MC4100 *ΔompA*) with pXW22 or an empty vector. To estimate the assembly of split OmpA, we exploited the observation that OmpA is the receptor for several T-even phages including K3 (31). Cells were assayed for K3 sensitivity by monitoring plaque formation after serial dilution of a phage stock. While cells that were transformed with the empty vector were completely resistant to phage infection, cells that expressed split OmpA were sensitive to phage, albeit ∼100-fold less sensitive than the wildtype MC4100 strain (Fig. 1*C*). To further examine the assembly of split OmpA, we grew cells overnight in LB medium and determined the level of split OmpA assembly by Western blotting using an antiserum against an OmpA loop 1 peptide. Based on previous studies, we predicted that if the OmpA fragments formed a properly folded β barrel, we would observe a phenomenon known as "heat modifiability" (30). In that case, the unheated protein would be resistant to SDS denaturation and migrate more rapidly than its predicted molecular weight on SDS-PAGE (32). As expected, a relatively rapidly migrating OmpA polypeptide that corresponds to the full-length β barrel was observed in the absence of heat but only when gels were overloaded (Fig. 1*C*, lanes 3–4 and Fig. 1*D*). Because we detected only a low level of free OmpA(β1–β4), most of the fragments were presumably degraded in the periplasm. Taken together, the results show that split OmpA folds into a functional β barrel under our experimental conditions but only very inefficiently.

Based on the possibility that OmpA(β1–β4) and OmpA(β5–β8) interact to form a native folding intermediate *in vivo* but then assemble into unstable β barrels, we hypothesized that if intermolecular disulfide bonds that lock the fragments together can be formed, then the efficiency of assembly might increase. To test this idea, we mutated four pairs of residues aligned in adjacent β strands in the fully folded OmpA β barrel structure to cysteine (Fig. 1*A*). In the structure of fully folded *E. coli* OmpA (from the Castellani and Chalmers strain; Protein Data Bank: 2GE4, see Ref. (33)) and the predicted structure of the nearly identical *E. coli* K-12 OmpA (34), all the mutated residues are facing inward in the barrel (Fig. 1*B*). The distance between the two Cα atoms in each pair is ∼4.5 to 7.0 Å and is within the range required for disulfide-bond formation (35). Two pairs of mutations, G97C/W123C and Q99C/M121C, would facilitate the formation of a disulfide bond between two internal β strands, β4 and β5, that, in principle, should not interfere with assembly. The other two pairs, G35C/S184C and S37C/M182C, would facilitate the formation of a disulfide bond between β1 and β8 that might either create a circularly permutated variant (an assembly competent variant in which β5–β8 would be N terminal to β1–β4; see Ref. (36)) or lock together the two β strands that form the seam of the β barrel.

Consistent with our hypothesis, the phage sensitivity assays showed that cells that expressed OmpA(β1–β4)^{G97C}/

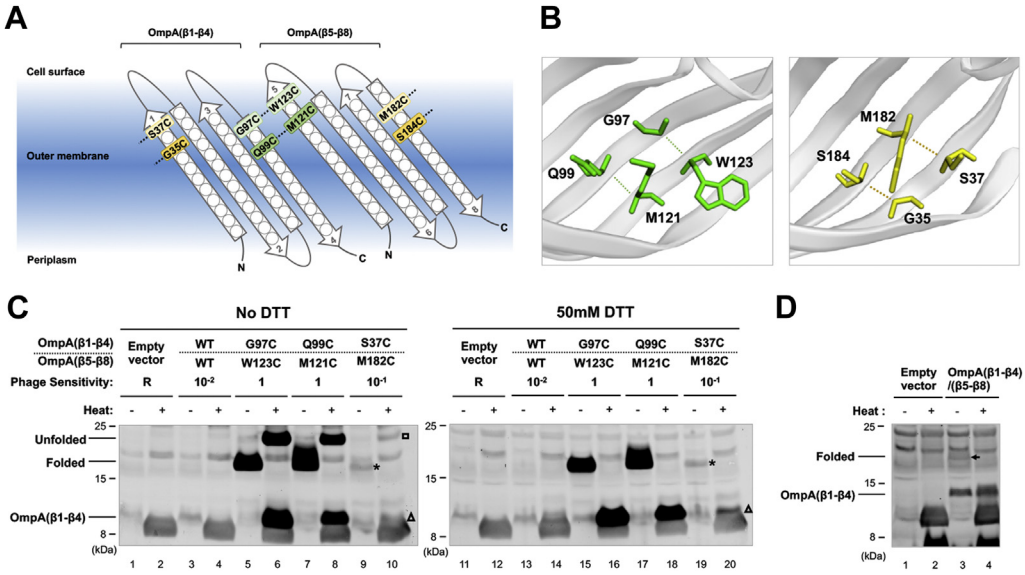

Figure 1. The formation of in-register artificial disulfide bonds enhances split OmpA assembly *in vivo*. *A*, diagram of split OmpA shows the residues mutated to cysteine to form artificial disulfide bonds between β strands 4 and 5 (*green*) or β strands 1 and 8 (*yellow*). *B*, topology diagram based on the structure of *Escherichia coli* K-12 OmpA predicted by AlphaFold and plotted using the EzMol server (34, 52, 53). In this model, the mutated residues in split OmpA β4 and β5 (*green*) or β1 and β8 (*yellow*) all face inward. *C* and *D*, XW100 (MC4100 Δ*ompA*) was transformed with pXW22 (P_{trc}-OmpA(β1–β4)/(β5–β8)) or a derivative encoding a split OmpA cysteine mutant pair. Cells were grown in LB overnight. Lysates from either 0.4 at an absorbance of 600 nm (*C*) or 0.8 at an absorbance of 600 nm (*D*) cell equivalents were mixed with SDS-PAGE loading buffer in the absence or the presence of 50 mM DTT and analyzed by Western blot using an antiserum against an OmpA loop 1 peptide. The K3 phage sensitivity of strains that contained each plasmid was determined by plaque assay and normalized to the phage sensitivity of *E. coli* MC4100. R, resistant to K3 phage. *B*, the folded and unfolded forms of assembled OmpA(β1–β4)^{S37C}/(β5–β8)^{M182C} are denoted with an *asterisk* and a *square*, respectively, and the OmpA(β1–β4)^{S37C} fragment is denoted with a *triangle*. *C*, the folded form of OmpA(β1–β4)/(β5–β8) is denoted with an *arrow*.

OmpA(β5–β8)^{W123C} or OmpA(β1–β4)^{Q99C}/OmpA(β5–β8)^{M121C} were as sensitive to K3 phage as wildtype MC4100 (Fig. 1*C*). Furthermore, Western blot analysis showed a strikingly high level of a rapidly migrating form of the full-length OmpA β barrel in unheated samples when the two cysteine mutant pairs that can lock β4–β5 were coexpressed (Fig. 1*C*, lanes 5 and 7). In the heated samples, the protein migrated at the molecular weight expected for the unfolded form (∼20 kDa), although some of the linked fragments dissociated during processing (Fig. 1*C*, lanes 6 and 8). To confirm that the ∼17 to 20 kDa bands result from the assembly of the two halves of the OmpA β barrel, we showed that they are recognized by antisera generated against the OmpA loop 4 peptide as well as the loop 1 peptide (Fig. S1). The finding that the unfolded form of the protein completely dissociated into fragments when heated samples were treated with DTT (Fig. 1*C*, lanes 16 and 18) confirmed that the ∼20 kDa band was formed by intermolecular disulfide-bond formation. It should be noted that the more rapidly migrating form of the OmpA β barrel observed in unheated samples did not dissociate in the presence of DTT and was therefore presumably folded into the OM with a highly stable structure that did not require the disulfide bond to remain intact (Fig. 1*C*, lanes 15 and 17). The results support the hypothesis that the disulfide bond is required for an early stage of assembly when the two halves come together, rather than for the maintenance of stability once they assemble.

Interestingly, very different results were obtained when cells expressed derivatives of split OmpA that contained cysteine mutations in β1 and β8. Cells that expressed OmpA(β1–β4)^{S37C}/OmpA(β5–β8)^{M182C} were ∼10-fold less sensitive to phage than MC4100, and cells that expressed OmpA(β1–β4)^{G35C}/OmpA(β5–β8)^{S184C} were completely resistant (Fig. 1*C* and data not shown). Moreover, only a low level of the assembled OmpA β barrel was observed in cells that produced OmpA(β1–β4)^{S37C}/OmpA(β5–β8)^{M182C} (Fig. 1*C*, lanes 9–10). Because no folded OmpA was detected in cells that produced OmpA(β1–β4)^{G35C}/OmpA(β5–β8)^{S184C} (data not shown), this pair was not studied further. Taken together, the results imply that the ability of disulfide bonds to lock the two halves of OmpA together and stabilize the protein is highly dependent on the location of the strands that are covalently linked in the final folded structure of the protein.

We next wished to obtain further evidence that the remarkable increase in the assembly of coexpressed derivatives of OmpA(β1–β4) and OmpA(β5–β8) that form disulfide bonds between β4 and β5 is due to the stabilization of a *bona fide* folding intermediate and not simply a fortuitous locking of the two halves of the protein together. To distinguish between these two possibilities, we tested whether split OmpA could fold when OmpA(β1–β4) and OmpA(β5–β8) derivatives that have slightly out-of-register cysteine mutations were coexpressed. We transformed XW100 with a plasmid encoding a matched pair or the out-of-register pair OmpA(β1–β4)^{G97C}/OmpA(β5–β8)^{M121C}, OmpA(β1–β4)^{Q99C}/OmpA(β5–β8)^{W123C}, OmpA(β1–β4)^{S37C}/OmpA(β5–β8)^{S184C}, or OmpA(β1–β4)^{G35C}/OmpA(β5–β8)^{M182C} and monitored OmpA assembly by Western blotting. Although the Cα atoms of one of the out-of-register pairs are only 6.8 Å apart in the folded structure of the OmpA β barrel (at the far end of the range at which disulfide bonds can form (35)), we observed a strong reduction in the level of assembly (Fig. 2*A*, compare lanes 3–6 and 7–8). In all the other cases (in which the Cα atoms were separated

by 7.2–10.3 Å), no folding was observed (Fig. 2*A*, lanes 9–10 and Fig. 2*B*, lanes 5–8). The out-of-register cysteine residues formed disulfide bonds efficiently when urea-denatured forms of the mutant fragments were mixed together *in vitro* (Fig. S2), so the mismatch does not cause a fundamental block in disulfide-bond formation. Taken together, these results suggest that even though OmpA(β1–β4) and OmpA(β5–β8) are not covalently linked when they enter the periplasm, at some stage the two halves of the protein interact closely in a way that aligns β4 and β5 (presumably through the formation of at least secondary structure) in the same conformation that is seen in the final structure.

The assembly of split OmpA is initiated in the periplasmic space

Two observations strongly suggested that the two halves of OmpA interact in the periplasmic space. First, the inefficient assembly and instability of wildtype split OmpA seems inconsistent with a model in which the two halves of the protein insert into the OM independently and then form a β barrel structure. Second, the finding that the formation of disulfide bonds between OmpA β1 and β8 is only weakly compatible with β barrel assembly suggests that these disulfide bonds close the barrel and block the interaction between BamA and the β signal in β8. Nevertheless, we next wished to analyze the timing of disulfide-bond formation further. To this end, we examined whether disulfide bonds form in the absence of DsbA, the major enzyme that catalyzes disulfide oxidation of closely aligned cysteine residues in the periplasm (37), or if the disulfide bonds result from a spontaneous oxidation reaction. Because the residues that we mutated to cysteine are all situated close to the cell surface, it is very unlikely that DsbA would catalyze disulfide-bond formation after split OmpA inserts completely into the OM. To distinguish between these two mechanisms of oxidation, we compared the assembly of the coexpressed cysteine mutants in XW100 and a *dsbA*-negative strain (XW106) using Western blot assays. As expected, all the matched split OmpA cysteine mutants folded into the OM *via* disulfide bridges that could be broken with DTT in the wildtype strain (Fig. 3, *top gels*, lanes 7–8; *bottom gels*, lanes 3–4 and 7–8). In contrast, no folded split OmpA was detected in the *dsbA*-negative strain (Fig. 3, *top gels*, lanes 9–10; *bottom gels*, lanes 5–6 and 9–10), whereas the folding of full-length OmpA and the OmpA β barrel (TM-OmpA, residues 21–195) was not significantly affected by the absence of DsbA (Fig. S3). These results strongly suggest that the disulfide bonds form either in the periplasm or near the periplasmic surface of the OM.

Split OmpA is integrated into the OM by the Bam complex

We next wished to determine if split OmpA, like the native OmpA β barrel, is assembled by the Bam complex. Consistent with this possibility, we found that in a *bamA101* strain (XW107) that produces ∼10-fold less BamA than wildtype MC4100 (Fig. S4*A*), the folding of the paired cysteine mutants decreased by at least 50-fold (Fig. S4*B*), whereas the assembly of wildtype OmpA and TM-OmpA was not significantly

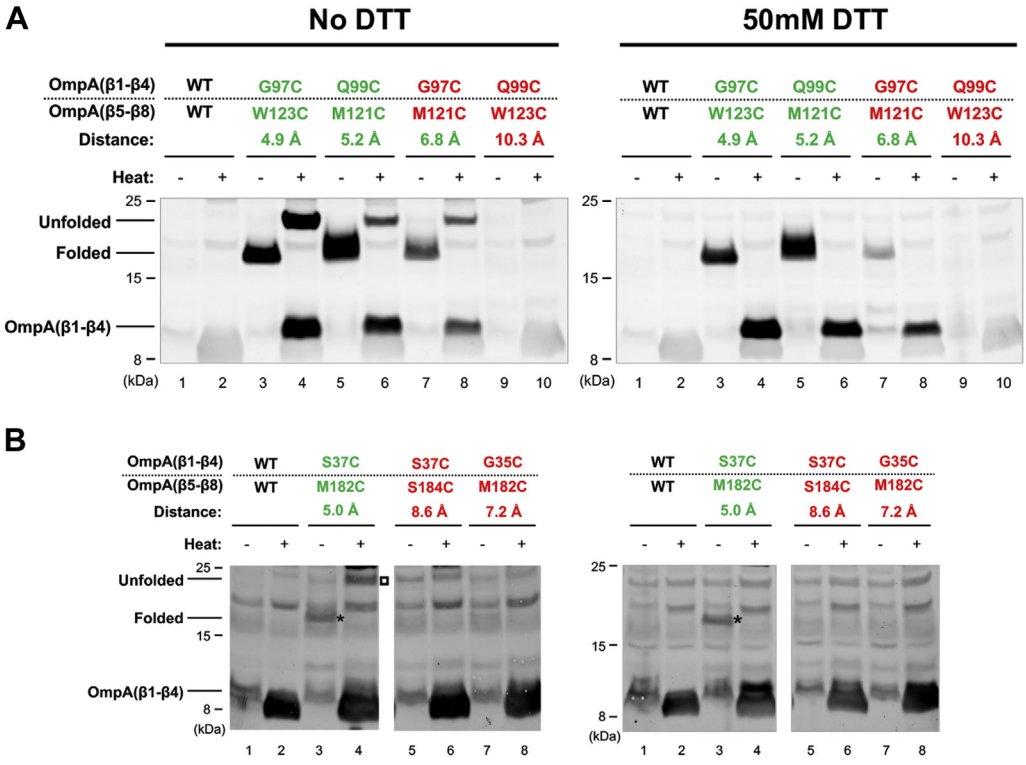

Figure 2. Out-of-register cysteine residue pairs do not improve split OmpA folding. Matched (*green*) or out-of-register (*red*) cysteine mutations were introduced between β strands 4 and 5 (*A*) or β strands 1 and 8 (*B*) of split OmpA. Cells expressing one of the split OmpA variants were grown in LB overnight, and lysates were analyzed by Western blot as described for Figure 1C. The distances between Cα atoms of the two mutated residues were measured based on Protein Data Bank (code: 2GE4) (33) (*green*: <7.0 Å; *red*: >7.0 Å). For OmpA(β1–β4)^{S37C}/(β5–β8)^{M182C}, the bands corresponding to folded and unfolded forms of assembled OmpA(β1–β4)/(β5–β8) are denoted with an *asterisk* and a *square*, respectively.

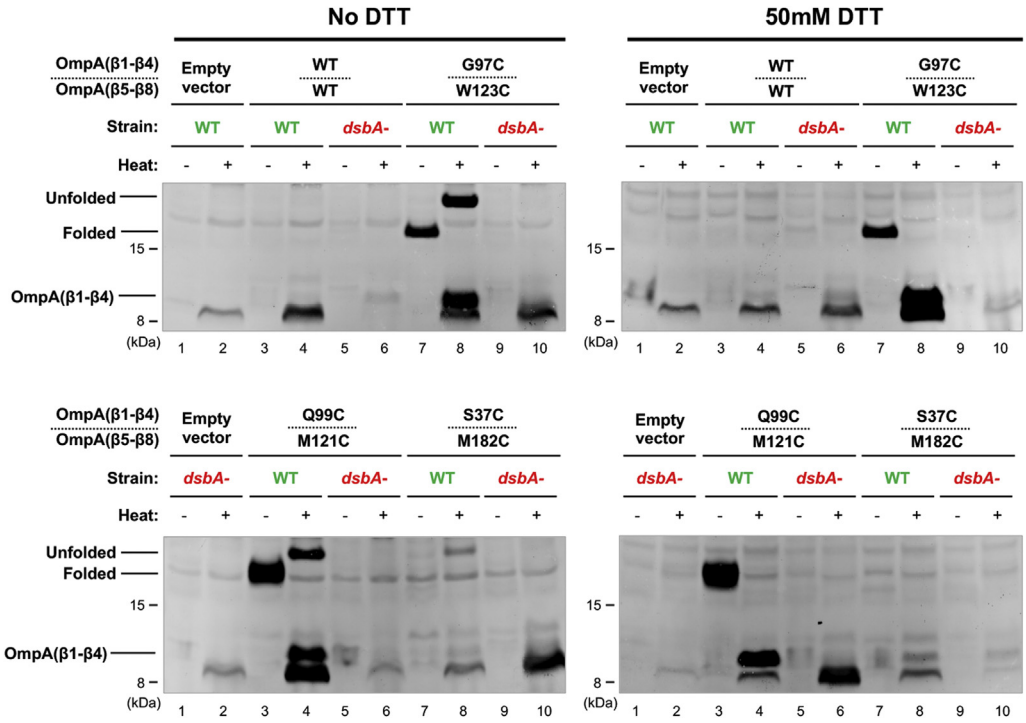

Figure 3. The absence of DsbA suppresses the folding of split OmpA cysteine pairs. XW100 (MC4100 Δ*ompA*; WT) and an isogenic *dsbA*-negative strain (XW106) were transformed with pXW22 (P_{trc}-OmpA(β1–β4)/(β5–β8)) or a derivative encoding a split OmpA cysteine mutant pair. Cell lysates of overnight cultures were analyzed by Western blot as described for Figure 1*C*. Loading controls are shown in Fig. S5*A*.

affected (Fig. S4*B*). To examine the role of the Bam complex further, we examined the effect of the novel antibiotic darobactin, which inhibits OMP insertion into the OM by binding to the β signal–binding site on BamA (38, 39), on the assembly of split OmpA. In these experiments, we transformed XW100 with a plasmid that encodes wildtype OmpA(β1–β4) and OmpA(β5–β8) or a pair of matching cysteine mutants under the control of a tightly regulated rhamnose-inducible promoter. When the cell cultures reached midlog phase, half of each culture was treated with 2 μg/ml darobactin, whereas the other half was left untreated. The folding of split OmpA was then assessed by Western blot. While the levels and properties of the folded forms of OmpA were similar to those observed in previous experiments in control samples, no folded protein could be detected when darobactin was added (Fig. 4). In addition, only very low levels of the OmpA (β1–β4) fragment were observed in darobactin-treated samples, but we were able to use a high molecular weight cross-reactive protein as a control to show that equal amounts of cell extract were added in each lane (Fig. S5*B*). These results strongly suggest that BamA is required both to promote the insertion of split OmpA into the OM and to maintain its stability in the periplasm.

Finally, to obtain additional evidence that the Bam complex plays an important role in the assembly of split OmpA, we introduced mutations into the β signal motif (**G**VS**Y**R**F**) located in OmpA(β5–β8) to disrupt its interaction with BamA. We mutated the conserved aromatic residues in the β signal of OmpA(β5–β8) at positions -1 and -3 (Y189 and F191) to alanine. The same mutations were recently shown to slow the targeting (and presumably the binding) of the full-length OmpA β barrel to the Bam complex and thereby increase its sensitivity to proteases (13). We examined the effect of β signal mutations on the folding of paired split OmpA cysteine mutants in XW100 by Western blotting. While single mutations in the β signal of both OmpA(β1–β4)^{G97C}/OmpA(β5–β8)^{W123C} and OmpA(β1–β4)^{Q99C}/OmpA(β5–β8)^{M121C} greatly reduced the folding efficiency of split OmpA and enhanced protein degradation (Fig. 5, *A* and *B*, lanes 5–8), a double β signal mutation (Y189A and F191A) completely blocked assembly (Fig. 5, *A* and *B*, lanes 9–10). By using the detection of a crossreactive protein as a control, we confirmed that equal amounts of cell extract were loaded in each lane (Fig. S5, *C* and *D*). Based on the observation that the elimination of the periplasmic chaperone Skp reduces the degradation of OMPs that contain β signal mutations and enhances their assembly (13, 14), we next wished to see if the level of the split OmpA mutants would increase in strain XW102 (MC4100 Δ*ompA* Δ*skp*). Interestingly, the assembly of the single mutants was almost completely restored, while the folded form of the double mutant became detectable (Fig. 5, *C* and *D*). These observations provide further evidence that after split OmpA is linked by specific disulfide bonds in the periplasm, the protein is assembled by the same pathway (and subject to the same constraints) as native OMPs.

Discussion

In this report, we reproduced the striking observation published in 1996 that split OmpA is assembled into a functional protein *in vivo* and exploited this classical finding to

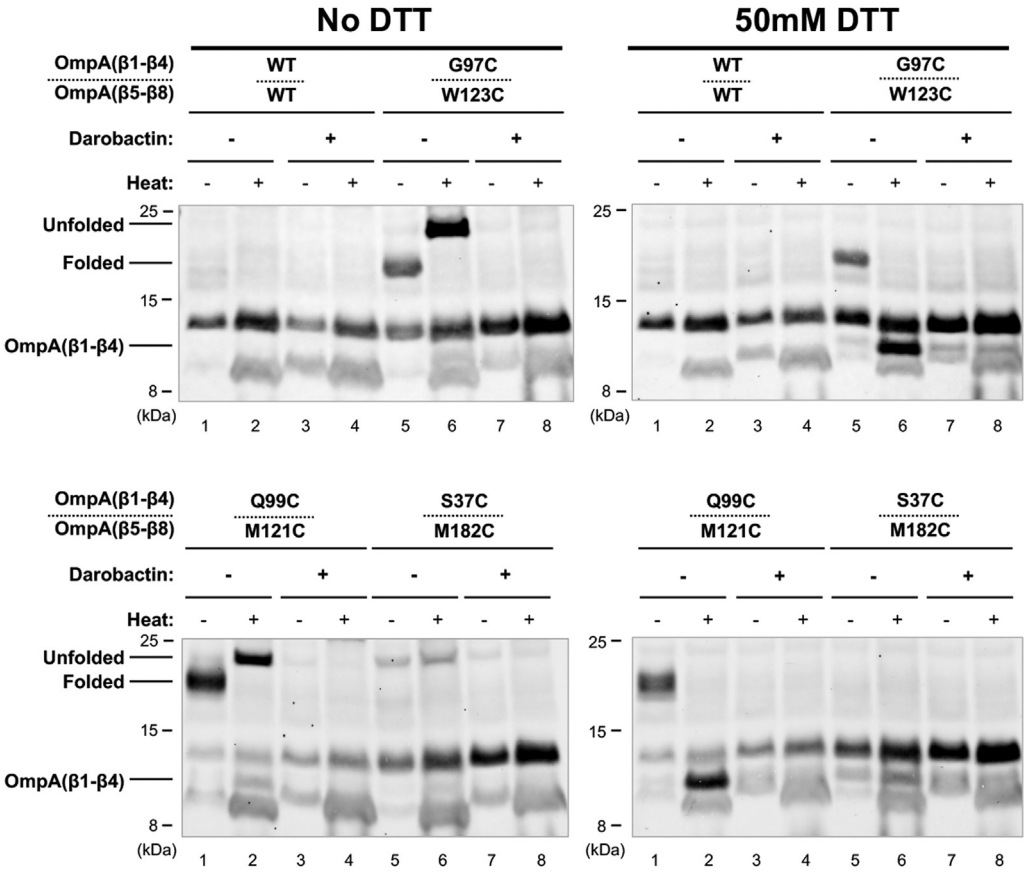

Figure 4. Darobactin inhibits the assembly of split OmpA cysteine mutants. XW100 (MC4100 *ΔompA*) was transformed with pXW37 (P_{rha}-OmpA(β1–β4)/(β5–β8)) or a derivative encoding a split OmpA cysteine mutant pair. Cells were grown in LB to midlog phase and treated with 0 or 2 μg/ml darobactin for 5 min. The expression of split OmpA was then induced by the addition of 0.2% rhamnose. Cells were harvested 3 h later, and cell lysates were analyzed by Western blot as described for Figure 1*C*. Loading controls are shown in Fig. S5*B*.

obtain insight into the mechanism of OMP assembly. At least under the experimental conditions that we used, we found that the assembly is very inefficient. Assembly was strongly enhanced by the formation of an artificial disulfide bond between β4 and β5, but only when the cysteine residues were located in positions that are in close proximity in the final folded structure. In contrast, the formation of disulfide bonds between β1 and β8 only slightly improved the folding of split OmpA or, in one instance, completely abolished assembly. One plausible explanation for this observation is that disulfide bonds that close the OmpA β barrel (between β1 and β8) inhibit recognition of the β signal located in β8 by BamA or by other factors such as the accessory lipoprotein BamD (40). Alternatively, the formation of a disulfide bond between β1 and β8 might stabilize split OmpA in a circularly permuted form in which the relocation of the β signal to an internal site or the loss of critical conformational information might impair recognition by chaperones and/or the Bam complex. This scenario appears less likely, however, because the circularly permuted form of OmpA in which the order of β1–β4 and β5–β8 is switched but the two halves remain covalently linked has previously been shown to be assembled efficiently *in vivo* (36). We also obtained several lines of evidence that the assembly of the linked forms of split OmpA required the Bam complex, so the presence of the disulfide bonds does not cause the assembly to go off pathway.

Our results clearly support the hypothesis that the assembly of split OmpA is initiated in the periplasmic space. Of particular note, we found that the periplasmic oxidase DsbA is required for the formation of disulfide bonds that greatly improve split OmpA folding, and no spontaneous disulfide oxidation was observed in a *dsbA*-negative strain. Because the cysteine mutations that we tested are all located close to the cell surface in the final structure of OmpA, it is very unlikely that DsbA catalyzes disulfide oxidation after the split OmpA completely inserts into the lipid bilayer (41). We were unable to determine, however, if the split OmpA fragments interact in the periplasm and are subsequently targeted together to the OM (perhaps by chaperones such as SurA), or if the OmpA(β5–β8) fragment interacts with OmpA(β1–β4) only after it binds to BamA *via* its β signal. In either case, the results imply that a covalent link between the two fragments is required to stabilize their interaction. As a corollary, the inefficient assembly of wildtype split OmpA suggests that the two halves do not first insert into the OM and subsequently interact to form a β barrel structure. Indeed, given the amphipathic nature of OMP β barrels, it seems unlikely that the two halves could insert stably into a hydrophobic lipid bilayer.

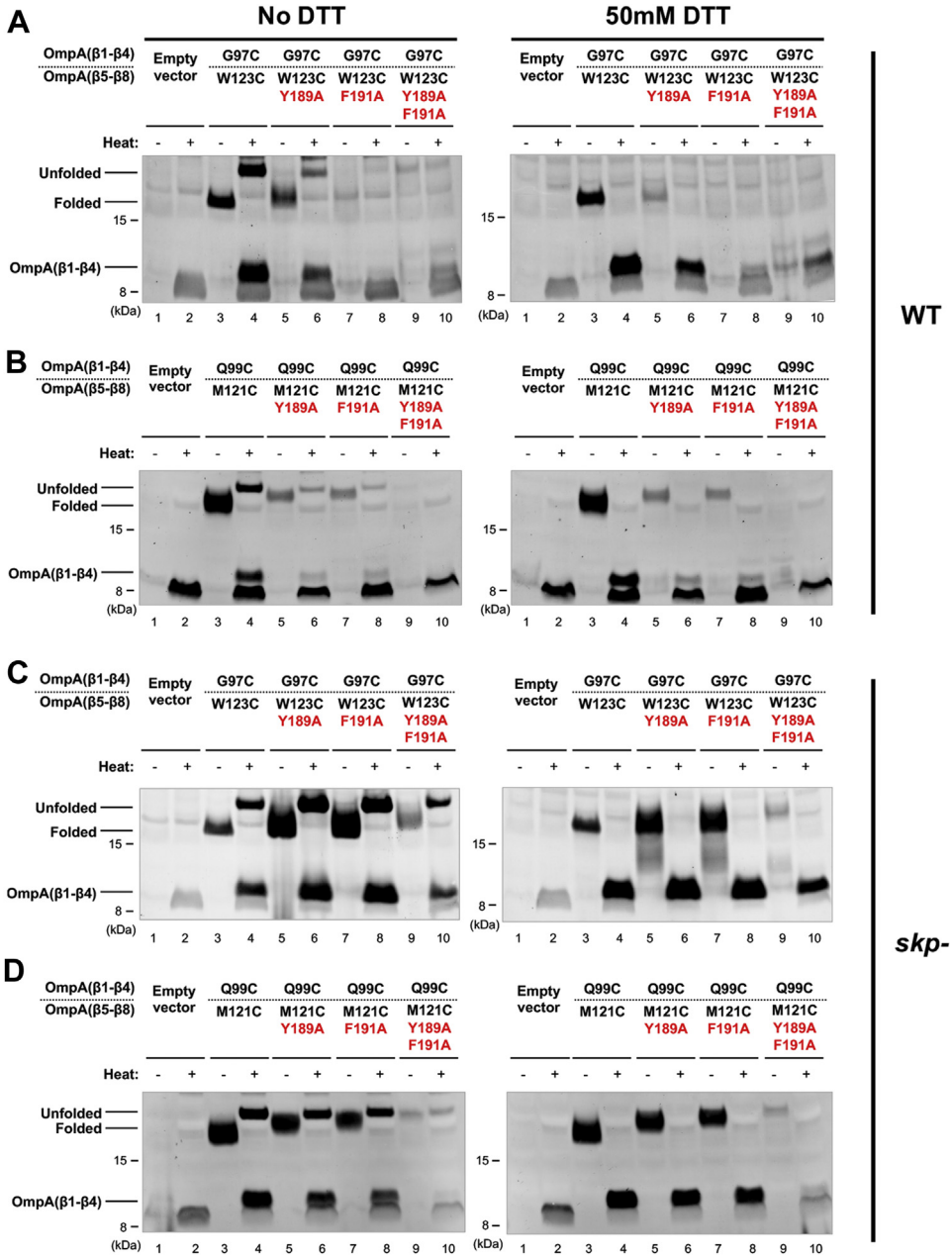

Figure 5. β-signal mutations disrupt the assembly of split OmpA. XW100 (MC4100 *ΔompA*; WT) (*A* and *B*) and an isogenic *skp*-negative strain (XW102) (*C* and *D*) were transformed with a plasmid encoding the indicated split OmpA mutant. Cells were grown in LB overnight, and cell lysates were analyzed by Western blot as described for Figure 1*C*. Loading controls are shown in Fig. S5, *C* and *D*.

The finding that only a subset of paired cysteine residues formed disulfide bonds that increase assembly efficiency provides interesting insights into the status of split OmpA prior to its insertion into the OM. Because only in-register disulfide bonds could form, the two halves must interact in the periplasm and begin initial stages of folding into a β sheet. The precision of alignment that was required is striking given that the folding of OmpA appears to be remarkably insensitive to mutations (42, 43). At a minimum, it is likely that a β sheet structure begins to form that aligns β4 and β5 and perhaps allows β1 and β8 to form less stable interactions. Although we cannot determine if any tertiary structure is formed, previous research on a class of OMPs known as autotransporters has suggested that at least partially curved β sheets can form prior to membrane insertion. In one study, it was shown that an α-helical linker segment that spans the β barrel of a "classical" autotransporter and connects it to an extracellular domain becomes resistant to proteinase K digestion before integration (44). In an analysis of the trimeric autotransporter UpaG, an OMP that contains a 12-stranded β barrel assembled from three unlinked four-stranded subunits (that each also contribute an embedded linker segment), stable dimeric and unstable trimeric assembly intermediates were detected in the periplasm (45). Of course, the embedded segments of these specialized OMPs may nucleate assembly and impose constraints that are not found in typical empty β barrel proteins

like OmpA. It is also important to note that we cannot determine if the interaction between the two split OmpA fragments that initiates folding is an assisted process or an unassisted process. It is conceivable, for example, that chaperones such as SurA or Skp interact with the two fragments and promote the formation of bonds that keep the two halves together at least transiently. Alternatively, the sequence itself might promote the formation of a low energy folding intermediate in the periplasmic space. In any case, because OMP engineering has proven useful in the generation of nanopore channels and bacterial display systems for high-throughput screening of peptide libraries (43, 46), the finding that the formation of specific disulfide bonds between adjacent β strands of split OmpA strongly enhances assembly might be useful for the future design of stable novel OMPs.

With respect to the mechanism of OMP integration, our data are consistent with models in which substrate proteins are targeted to the Bam complex either as partially folded proteins or as unfolded polypeptides that acquire secondary structure prior to insertion (*e.g.*, the barrel elongation model) and then integrate into the membrane by forming a hybrid barrel assembly intermediate with an open form of BamA. In contrast, our results do not support models such as the threading model in which newly synthesized OMPs have been proposed to pass through the BamA lumen in a completely unfolded state and then form β hairpins that integrate into the OM through a lateral gate in a step-wise fashion (47). At the other extreme, our data do not support models in which the Bam complex promotes the membrane integration of fully folded β barrels simply by perturbing the structure of the lipid bilayer. Although it has been reported that BamA lowers the kinetic barrier to allow the integration of OMPs into membrane vesicles even in thick lipid bilayers (26, 27), it seems likely that the function of the Bam complex is more elaborate and that it facilitates OMP assembly by a multistep process.

Experimental procedures

Strains, antibiotics, phage, and antisera

The *E. coli* K-12 strains used in this study were MC4100 [*araD139 Δ(argF-lac)169 λ-e14-flhD5301 Δ(fruK-yeiR) 725(fruA25) relA1 rpsL150 rbsR22 Δ(fimB-fimE)632(::IS1) deoC1*], XW100 (MC4100 Δ*ompA*) (13), XW102 (MC4100 Δ*ompA* Δ*skp*) (13), XW106 (MC4100 Δ*ompA dsbA::cm*), and XW107 (MC4100 Δ*ompA bamA101*). To create strains XW106 and XW107, the *dsbA::cm* allele from strain RI2 (44) and the *bamA101* allele from strain DPR959 (48) were introduced into XW100 by P1 transduction. Ampicillin (100 μg/ml), kanamycin (30 μg/ml), and trimethoprim (50 μg/ml) were added to media as needed. Rabbit polyclonal antisera generated against OmpA extracellular loop 1 and 4 peptides and an BamA C-terminal peptide have been described (13, 49).

Plasmid construction

The plasmids used in this study are listed in Table S1. Plasmids pXW01 and pXW04, which encode *TM-ompA*, and plasmid pXW02 encoding a circularly permutated OmpA β barrel, have been described previously (13). To construct pXW22 (P_{trc}-OmpA(β1–β4)/(β5–β8)), DNA fragments encoding the OmpA signal peptide with OmpA(β1–β4) and OmpA(β5–β8) were amplified by PCR using pXW01 and pXW02 as templates and primer pairs XW01/XW39 and XW02/XW38 (oligonucleotide primers used in this study are listed in Table S2). The PCR fragments were then assembled into pTrc99A by Gibson assembly (50). To construct pXW37 (P_{rha}-OmpA(β1–β4)/(β5–β8)), an NdeI site was introduced into pXW22 in front of the *ompA* ribosome-binding site using primers XW11 and XW12. The DNA fragment encoding split OmpA was then subcloned into pSCrhaB2 (51) using the NdeI and BamHI restriction sites. To construct pXW41 (P_{T7}-OmpA(β1–β4)) and pXW42 (P_{T7}-OmpA(β5–β8)), the mature region of *ompA(β1–β4)* and *ompA(β5–β8)* were amplified by PCR using *E. coli* MC4100 genomic DNA and primer pairs XW42/XW43 and XW44/XW45, and the resulting PCR product was ligated to pET28b using Gibson assembly. Mutations were introduced into each of the aforementioned plasmids using the QuikChange Mutagenesis kit (Agilent) with appropriate primers. To construct pXW47 (P_{trc}-OmpA), the DNA fragment encoding the full-length OmpA was amplified by PCR using primers XW01 and XW62 and was ligated to pTrc99A by Gibson assembly.

Analysis of split OmpA folding in overnight cultures

Strains XW100, XW102, XW106, and XW107 transformed with pXW22 or a pXW22 derivative were grown in LB overnight without inducer. Cells (three equivalents at an absorbance at 600 nm) were collected by centrifugation (3000*g*, 6 min, 4 °C), washed with 1 ml cold PBS, resuspended in 131 μl BugBuster Master Mix (Novagen) containing EDTA-free protease inhibitors (Roche), and incubated at room temperature for 20 min. The lysate was then mixed with 4× LDS sample buffer (Thermo Fisher Scientific) and 50 mM DTT if needed. Half of the lysate was heated at 95 °C for 15 min, whereas the other half was kept on ice. Proteins were resolved on 12% Bis–Tris minigels (Thermo Fisher Scientific) using MES buffer. The folded and unfolded forms of split OmpA were detected by Western blot using the anti-OmpA loop 1 antiserum.

Phage assay

Phage sensitivity was determined using a previously described protocol (13). Cells were grown in LB overnight. A 100 μl aliquot of each overnight culture was then mixed with 4 ml 0.7% (w/v) LB agar containing 100 μg/ml ampicillin and poured onto an LB–ampicillin agar plate. A K3 phage stock was serially diluted by 10-fold up to a 10^7-fold dilution, and 5 μl of each phage dilution was spotted onto the plates. The plates were incubated at 30 °C overnight. The phage sensitivity was determined by the highest dilution at which plaques were observed and was normalized to the phage sensitivity of *E. coli* MC4100 (10^6-fold dilution).

Darobactin inhibition assay

Strain XW100 transformed with pXW37 or a pXW37 derivative was grown in LB at 37 °C overnight. The overnight

cultures were diluted into LB medium at an absorbance of 0.02 at 600 nm and grown to an absorbance of ∼0.4 to 0.5 at 600 nm. Darobactin was then added to a final concentration of 2 μM. After 5 min, 0.2% rhamnose was added to induce the expression of split OmpA variants for 3 h. The assembly of split OmpA was analyzed by Western blot as described previously.

In vitro *disulfide-bond formation assay*

Urea-denatured OmpA(β1–β4) and OmpA(β5–β8) derivatives were expressed and purified as described (13). The two split OmpA fragments were then added to 20 mM Tris (pH 8.0) at a concentration of 0.2 μM and incubated at 30 °C for 45 min. Disulfide-bond formation between the two fragments was examined by Western blots as described previously.

Data availability

All the relevant data are contained within the article and the supporting information.

Supporting information—This article contains supporting information (13).

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