How the assembly and protection of the bacterial cell envelope depend on cysteine residues

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The cell envelope of Gram-negative bacteria is a multilayered structure essential for bacterial viability; the peptidoglycan cell wall provides shape and osmotic protection to the cell, and the outer membrane serves as a permeability barrier against noxious compounds in the external environment. Assembling the envelope properly and maintaining its integrity are matters of life and death for bacteria. Our understanding of the mechanisms of envelope assembly and maintenance has increased tremendously over the past two decades. Here, we review the major achievements made during this time, giving central stage to the amino acid cysteine, one of the least abundant amino acid residues in proteins, whose unique chemical and physical properties often critically support biological processes. First, we review how cysteines contribute to envelope homeostasis by forming stabilizing disulfides in crucial bacterial assembly factors (LptD, BamA, and FtsN) and stress sensors (RcsF and NlpE). Second, we highlight the emerging role of enzymes that use cysteine residues to catalyze reactions that are necessary for proper envelope assembly, and we also explain how these enzymes are protected from oxidative inactivation. Finally, we suggest future areas of investigation, including a discussion of how cysteine residues could contribute to envelope homeostasis by functioning as redox switches. By highlighting the redox pathways that are active in the envelope of *Escherichia coli*, we provide a timely overview of the assembly of a cellular compartment that is the hallmark of Gram-negative bacteria.

The cell envelope of Gram-negative bacteria is a complex macromolecular structure that consists of an inner membrane surrounding the cytoplasm and an outer membrane that separates the cell from the environment. While the inner membrane is a classic phospholipid bilayer, the outer membrane is asymmetric, with phospholipids in the inner leaflet and lipopolysaccharides in the outer leaflet (1). The two membranes are separated by the periplasm, a viscous compartment that represents 10–20% of the total cell volume (2) and contains a thin layer of peptidoglycan. The peptidoglycan, also referred to as the cell wall, is a polymer made of repeating units of a disaccharide (GlcNAc-*N*-acetylmuramic acid) cross-linked by short peptides (3, 4). In enterobacteria, the outer membrane and the peptidoglycan are covalently attached by protein tethers (5, 6). In the model bacterium *Escherichia coli*, approximately one-third of cellular proteins are destined for the cell envelope (7). Soluble proteins are present in the periplasm, where they engage in a variety of functions, including peptidoglycan assembly, protein folding, and nutrient import. Integral membrane proteins are present in both membranes. While inner membrane proteins cross the lipid bilayer via hydrophobic α-helices, proteins inserted in the outer membrane contain amphipathic β-strands that are arranged in a linear antiparallel β-sheet; this β-sheet folds into a barrel by establishing hydrogen bonds between the first and last β-strands (1, 8). Some of these so-called β-barrels function as passive diffusion channels, allowing small hydrophilic molecules to enter the cell, when others, connected to energy sources in the inner membrane, actively import specific compounds (9). Other important envelope proteins are the lipoproteins, globular proteins anchored to a membrane by a lipid moiety. Although some lipoproteins remain in the inner membrane, most of them are targeted to the outer membrane (10, 11).

Each envelope layer is essential for viability; the outer membrane serves as a permeability barrier against toxic compounds present in the surroundings (8), the peptidoglycan provides shape and osmotic protection to the cell (3), and the inner membrane delimits the cytoplasm and hosts many vital cellular processes, including respiratory systems. The crucial importance of the envelope is nicely illustrated by the fact that several antibiotics (3) and antibacterial toxins (12) target the mechanisms of peptidoglycan assembly while others, such as colistin (a last resort antibiotic), destabilize the outer membrane. Assembling the envelope properly is challenging, in part because the complex machineries involved in the biogenesis of its different layers need to coordinate and to adapt their activities to the growth rate. In addition, most of the envelope building blocks necessary for synthesis, being produced in the cytoplasm or in the inner membrane, need to be transported to their final destination in an assembly-competent state and correctly integrated in the construction despite the lack of an obvious energy source (there is no ATP in the periplasm (13)). In the case of envelope proteins, the large majority of which enter the periplasm in an unfolded state that is prone to aggregation (14), correct folding often involves the formation of one or more disulfide bonds.

The understanding of the mechanisms of envelope assembly and maintenance has increased tremendously during the past two decades. For instance, the machineries involved in the biogenesis of the outer membrane have been identified and their characterization has been initiated. Further, elegant mechanisms

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used by cells to monitor the integrity of their envelopes have started to be elucidated. These major achievements are reviewed here; however, they are discussed from an unusual perspective. Indeed, we have chosen to give central stage to the amino acid cysteine, one of the least abundant amino acid residues in proteins, whose unique chemical and physical properties make it often critical in biological processes. Putting cysteine residues under the spotlight brings to the surface often overlooked connections between essential cellular processes; it also highlights the important role played by redox-dependent mechanisms in cell envelope homeostasis. In the envelope, cysteine residues have two major functions that are reviewed here. First, they are involved in the formation of disulfide bonds that give envelope proteins further stability. Second, through their ability to function as nucleophiles in enzymatic reactions, cysteine residues are central to the activity of enzymes that are required for proper envelope assembly. Using nucleophilic cysteines comes with a price, however; because cysteines are highly vulnerable to oxidizing molecules that target bacteria during infection, cysteine-based enzymes are susceptible to irreversible inactivation and therefore need specific protection mechanisms. This is true both in the cytoplasm and in the periplasm. However, the reducing equivalents used by rescuing systems often originate from the cytoplasmic pool of NADPH (15). As a result, the protection of cysteine-based enzymes functioning in the envelope offers an additional challenge, as it involves transporting electrons across the inner membrane. Here, after briefly introducing the pathways of disulfide formation, we discuss the importance of cysteine residues in the folding of three essential assembly factors (FtsN, LptD, and BamA) and two proteins that cells use to monitor envelope integrity (RcsF and NlpE). Next, we focus on enzymes that utilize cysteine side chains as part of their catalytic machinery, and we address how these proteins are maintained as active in the oxidizing environment of the periplasm.

How disulfides are formed in the periplasm: a brief overview

The formation of a disulfide bond between two cysteine residues stabilizes a protein structure, mainly by decreasing the conformational entropy of the denatured state. This stabilizing effect can be up to ~4 kcal/mol per disulfide formed (16, 17). Disulfide bond formation is vital for the stability of many secreted proteins, both in bacteria and in eukaryotes. Proteins that are secreted to extracytoplasmic compartments such as the cell envelope or to the extracellular milieu benefit from stabilizing disulfides to remain folded in environments that lack ATP-dependent chaperones and often are rich in proteases and destabilizing compounds. Although disulfide bonds can form spontaneously in the presence of molecular oxygen, the process is rather slow and needs to be catalyzed *in vivo*. The first catalyst of disulfide bond formation identified in bacteria was *E. coli* DsbA (for Disulfide bond), a small (23-kDa) soluble periplasmic protein with a thioredoxin (Trx) fold. The biochemical characterization of DsbA established this protein as a highly oxidizing oxidoreductase (redox potential of −119 mV) (18) with a CXXC catalytic motif, mostly found oxidized *in vivo*. The oxidizing power of DsbA comes from the fact that reduction of the CXXC motif increases the stability of DsbA, thereby favoring the transfer of its catalytic disulfide to newly synthesized proteins entering the periplasm (19). The search for a protein capable of reoxidizing inactive DsbA led to the discovery of a small (20-kDa) inner membrane protein, DsbB (20) (Fig. 1 and Table 1). DsbB has two pairs of essential cysteine residues that mediate the transfer of electrons from DsbA to the electron transport chain and ultimately to molecular oxygen (21). Under anaerobic conditions, electrons flow from DsbB to anaerobic electron acceptors, such as nitrate and fumarate (21).

DsbA catalyzes disulfide bond formation as cysteines in its substrates enter the periplasm. Therefore, when disulfides need to be formed between cysteine residues that are nonconsecutive in the substrate sequence, DsbA often catalyzes the formation of nonnative disulfides, causing protein misfolding, aggregation, and/or degradation. DsbC, a V-shaped dimeric (47-kDa) oxidoreductase of the Trx family, was identified as a disulfide isomerase that corrects the errors of DsbA (22) (Fig. 1). DsbC harbors a CXXC catalytic motif, which, in contrast to DsbA, is found reduced in the periplasm (23), and displays an extended cleft whose inner surface is patched with uncharged and hydrophobic residues (24). These two features allow DsbC to recognize misfolded substrates; first, the N-terminal cysteine of the DsbC active site attacks a nonnative disulfide in the substrate, which results in the formation of an unstable mixed-disulfide complex. Next, the mixed disulfide is resolved either by the attack of another cysteine from the misfolded protein or by the C-terminal cysteine of DsbC itself. In the first case, DsbC acts as an isomerase that catalyzes the reshuffling of the disulfide in the substrate. In the second case, DsbC functions as a reductase, giving DsbA another chance to oxidize the substrate protein. Either way, the active site of DsbC needs to be kept reduced and active, which is the function of DsbD (Fig. 1 and Table 1) (23, 25), a 59-kDa protein with three domains; two domains, DsbDα and DsbDγ, are located in the periplasm, and the third domain, DsbDβ, is embedded in the inner membrane. DsbD uniquely transfers electrons across the membrane, from the cytoplasmic Trx system and NADPH (26) to DsbC (23, 25), via a cascade of thiol-disulfide exchange reactions (27, 28). The actual mechanism by which DsbD transfers electrons between cytoplasmic and periplasmic oxidoreductases is not fully understood but likely involves major conformational changes within DsbDβ, as suggested by structural studies with CcdA, a DsbDβ homolog (29, 30).

Three essential assembly factors have disulfide-bonded cysteines in their native conformations

There is strong bias against cysteine residues in envelope proteins; in a bacterium like *E. coli*, only ~40% of envelope proteins have cysteines, compared with ~85% of cytoplasmic proteins (31). Remarkably, about 70% of cysteine-containing envelope proteins have even numbers of cysteines, which has been shown to be a marker of disulfide bond formation (31). In cells lacking DsbA, these proteins do not fold properly and are subjected to proteolysis (32), which impairs cellular processes. For instance, cells lacking DsbA are nonmotile because FlgI, a flagellum component and a DsbA substrate, is not properly

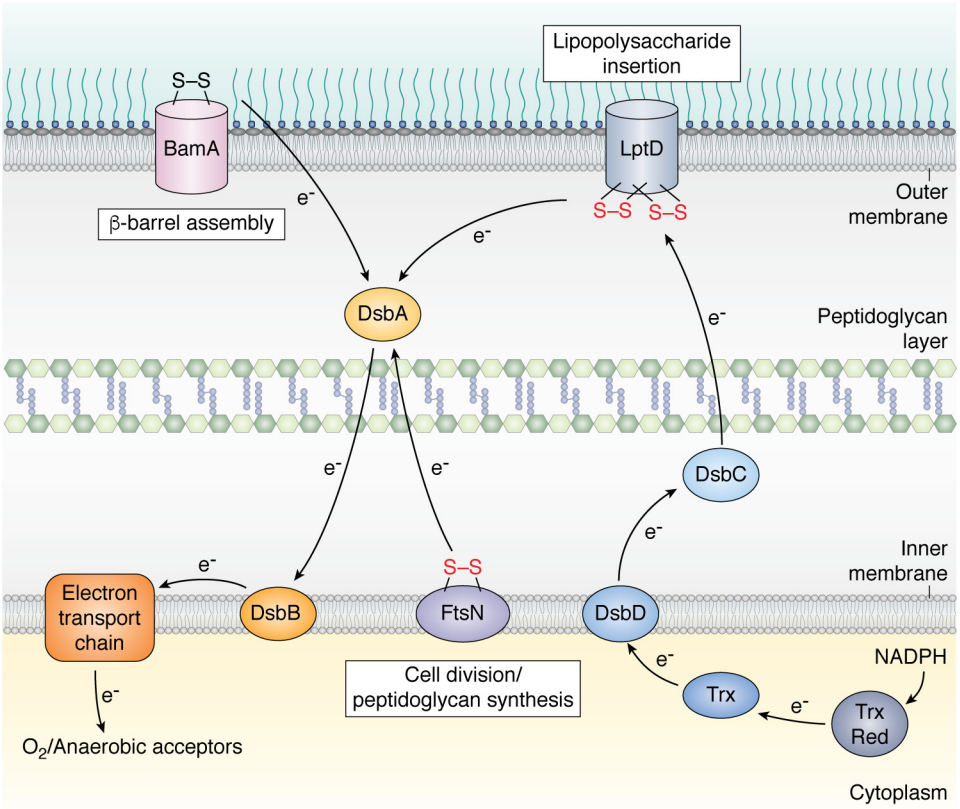

Figure 1. Proteins that are essential for envelope assembly contain disulfide bonds in their native conformation. Formation of these disulfides is required for two of them, FtsN, an inner membrane protein involved in peptidoglycan synthesis during cell division, and LptD, an outer membrane β-barrel that inserts lipopolysaccharide molecules in the outer leaflet of the outer membrane. Essential disulfides are in *red*. In contrast, disulfide formation is not required, at least under the tested conditions, for the folding of BamA, the core component of the machinery that introduces β-barrel proteins into the outer membrane. Disulfide bond formation in envelope proteins is catalyzed by DsbA, which is recycled by transferring the electrons received from the substrate to the inner membrane protein DsbB. DsbB then shuttles electrons to the electron transport chain and ultimately to molecular oxygen or anaerobic acceptors. Several envelope proteins, like LptD, contain disulfides between cysteines that are not consecutive in the sequence. In this case, their folding also involves DsbC, a protein disulfide isomerase that corrects the errors of DsbA. DsbC is kept reduced by the inner membrane protein DsbD, which receives reducing equivalents from the cytoplasmic Trx system at the expense of NADPH. The Trx system consists of Trx and Trx reductase (*Trx Red*). The *black arrows* show the electron flow.

oxidized (33). In the same line, deleting *dsbA* decreases virulence in pathogenic strains of *E. coli* and other Gram-negative bacteria due to the misfolding of virulence factors involved in adhesion, secretion, and toxicity (34). For instance, in *Pseudomonas aeruginosa*, cells lacking DsbA fail to fold the protease LasB (35), the pilus component PilA (36), and other proteins important for pathogenicity (37). Thus, the proteins involved in disulfide formation are attractive targets for the design of innovative antivirulence strategies (38). Note, however, that the degree of protein misfolding observed in the absence of DsbA varies from protein to protein; while some can barely be detected in cells lacking *dsbA* (39), others, like the β-barrel OmpA, appear to be stable (40).

Strikingly, of the ~40 essential proteins belonging to the machineries that assemble the peptidoglycan and the outer membrane, only 3 contain cysteine residues in their sequences (or in their periplasmic segments, in the case of inner membrane proteins). In all 3 cases, these cysteines are involved in disulfide formation. This remarkably small number of cysteine groups in the components of the assembly complexes suggests a negative selection for cysteine residues (31), potentially to protect these machineries from oxidative inactivation.

One of the assembly proteins with two cysteines is the inner membrane protein FtsN (Fig. 1 and Table 1), an essential constituent of the large protein complex that mediates cell division (41). Although the exact function of FtsN remains elusive, this protein likely regulates peptidoglycan synthesis during cytokinesis (septation) (42). The two cysteines of FtsN have been shown to form a disulfide in the large (~30-kDa) periplasmic SPOR (sporulation-related repeat) domain of the protein (41), a region that is important for binding to denuded peptidoglycan (glycan strands lacking the stem peptides that normally cross-link the glycan polymers) (43). Mutation of the two cysteines of the SPOR domain decreases intracellular FtsN levels and causes cells to grow as filaments, a phenotype indicative of an impaired cell division process. Thus, the disulfide bond of the SPOR domain of FtsN stabilizes the structure of this protein and is important for function (41).

A second essential assembly protein with cysteine residues is LptD (Fig. 1 and Table 1), one of the components of the Lpt (Lipopolysaccharide transport) system that transports lipopolysaccharide molecules across the cell envelope (44). The biosynthesis of lipopolysaccharide, a glycolipid made of three distinct moieties (the lipid A anchor, the core oligosaccharides, and the

Table 1
Key actors in cysteine-mediated envelope homeostasis in *E. coli*

Enzyme	Function
Envelope oxidoreductases	
Disulfide bond formation	
DsbA	Catalyzes disulfide bond formation in the periplasm
DsbB	Inner membrane protein that recycles DsbA
Disulfide bond isomerization	
DsbC	Catalyzes disulfide bond isomerization in the periplasm
DsbD	Inner membrane protein that recycles DsbC
Cysteine protection	
DsbG	Rescues periplasmic single cysteine residues from oxidative damage
DsbD	Inner membrane protein that recycles DsbG
Essential envelope assembly factors with structural disulfide bonds	
LptD	Outer membrane protein that inserts lipopolysaccharide molecules into the membrane; two nonconsecutive disulfides; formation of at least one disulfide is essential
BamA	Outer membrane protein that inserts β-barrel proteins into the outer membrane; one nonessential disulfide bond
FtsN	Inner membrane protein with a large periplasmic domain regulating peptidoglycan synthesis; one essential disulfide bond in the periplasmic domain.
Disulfide-containing stress sensors monitoring envelope integrity	
RcsF	Outer membrane lipoprotein monitoring the integrity of the peptidoglycan and of the outer membrane; two nonconsecutive disulfides required for folding; induces the Rcs phosphorelay pathway under stress
NlpE	Outer membrane lipoprotein monitoring lipoprotein trafficking to the outer membrane; two consecutive disulfides required for folding; induces the Cpx system when lipoprotein transport is perturbed
Envelope assembly enzymes with a catalytic cysteine residue	
LdtA, LdtB, and LdtC	L,D-Transpeptidases catalyzing the attachment of the Braun lipoprotein Lpp to the peptidoglycan
LdtD and LdtE	L,D-Transpeptidases catalyzing the formation of 3-3 cross-links between two *meso*-diaminopimelic acid residues of adjacent stem peptides

O-antigen, a sugar polymer of variable composition (45)), takes place in the cytoplasm and in the inner membrane; lipopolysaccharide molecules are then extracted from the inner membrane by the $LptB_2FGC$ ABC transporter, transferred to the periplasmic protein LptA, and finally delivered to the outer membrane translocon, made of the large β-barrel LptD and the lipoprotein LptE. Together, the Lpt proteins form a membrane-to-membrane bridge for the unidirectional transport of lipopolysaccharides (46); ATP hydrolysis by $LptB_2FGC$ powers the entire process (47).

LptD contains four cysteine residues that form two nonconsecutive disulfides (Cys1-Cys3 and Cys2-Cys4) connecting the C-terminal β-barrel, in which LptE forms a plug (48, 49), to an N-terminal domain present in the periplasm (50, 51) (Fig. 2). Formation of at least one of these disulfides is required for function (50). The folding pathway of LptD is particularly complex; following translocation of the nascent protein into the periplasm, a first disulfide is introduced by DsbA between the first and second cysteines. Subsequent rearrangement of this disulfide into a Cys2-Cys4 bridge involves LptE (50, 51) and the protein disulfide isomerase DsbC (52). Formation of the second disulfide by DsbA can then occur. Given the essential function of LptD, one would expect Δ*dsbA* cells not to be viable. It is indeed the case, but only under anaerobic conditions in which LptD accumulates in a reduced, inactive form (53). In the presence of oxygen, however, Δ*dsbA* cells grow like WT cells, presumably because oxygen-dependent background oxidation, catalyzed by low-molecular-weight thiol-oxidizing compounds present in the periplasm, is sufficient for survival.

The third assembly protein with two cysteines forming a disulfide is the outer membrane protein BamA, the core component of the β-barrel assembly machinery (BAM) that assembles β-barrel proteins in the outer membrane (Fig. 1 and Table 1). β-Barrel precursors are synthesized in the cytoplasm with an N-terminal signal peptide that targets them to the Sec translocon for transport across the inner membrane. Note that it was recently shown that the mature domains of the proteins destined for secretion contain multiple, degenerate, interchangeable hydrophobic stretches that also play a role in targeting to the translocase machinery (54). Upon emerging from the translocon on the periplasmic side of the inner membrane, the signal peptide is cleaved off and unfolded β-barrels interact with periplasmic chaperones for transport across the periplasm and delivery to BAM (14, 55).

BamA is made of a 16-stranded C-terminal β-barrel embedded in the membrane and a large N-terminal periplasmic extension consisting of five POTRA (polypeptide transport-associated) domains (56) (Fig. 3). Structures of BAM have shown that BamA can adopt two conformations, namely, an outward-open conformation (57–59) in which the β-barrel domain opens between the first and last β-strands, opening a lateral gate to the membrane, and an inward-open conformation (59, 60) in which the lateral gate is closed while a periplasmic entry pore to the barrel lumen is open. In addition to BamA, BAM includes four accessory lipoproteins (BamB, BamC, BamD, and BamE) (61). All components are required for efficient β-barrel assembly, but only BamA and BamD are essential (56, 62). The disulfide bond of BamA is present in an extracellular loop (loop 6 in Fig. 3) of the β-barrel domain (63). Although this loop contains the most highly conserved segment of BamA and has been shown to be important for β-barrel assembly (64, 65), the two cysteine residues are not well conserved among BamA homologs (Iorga B. I., unpublished results), calling into question their functional importance. Accordingly, their mutation has no impact on BAM function (66). Despite important structural and functional insights, crucial questions remain unresolved regarding the mechanism of BAM (56, 67–69).

Finally, DsbA was shown to catalyze the formation of disulfides in several BAM substrates (39). For instance, CirA, the outer membrane colicin 1 receptor protein, and FhuA, a β-barrel that functions as a ferrichrome iron receptor, contain one and two disulfide bonds, respectively. When and where these disulfides are formed (before or after BAM folding) remain to be established.

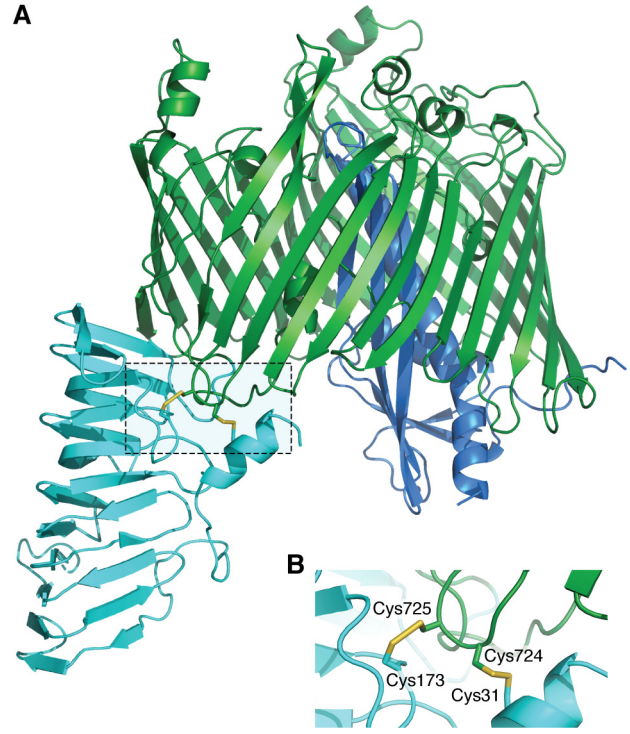

Figure 2. Structure of *E. coli* LptD. *A*, cartoon representation of *E. coli* LptD (the N-terminal periplasmic domain is in *cyan* and the C-terminal β-barrel is in *green*) in complex with the lipoprotein LptE (dark *blue*). *B*, the two nonconsecutive disulfide bonds (represented as *sticks*) of LptD link the N-terminal domain to the C-terminal domain. Formation of at least one of them is required for folding and activity. This is a homology model generated using MODELLER v9.22 (108) and the PDB structures 4RHB (partial *E. coli* LptD (residues 230–784) in complex with LptE) and 4Q35 (full-length *Shigella flexneri* LptD in complex with LptE) (49) as templates.

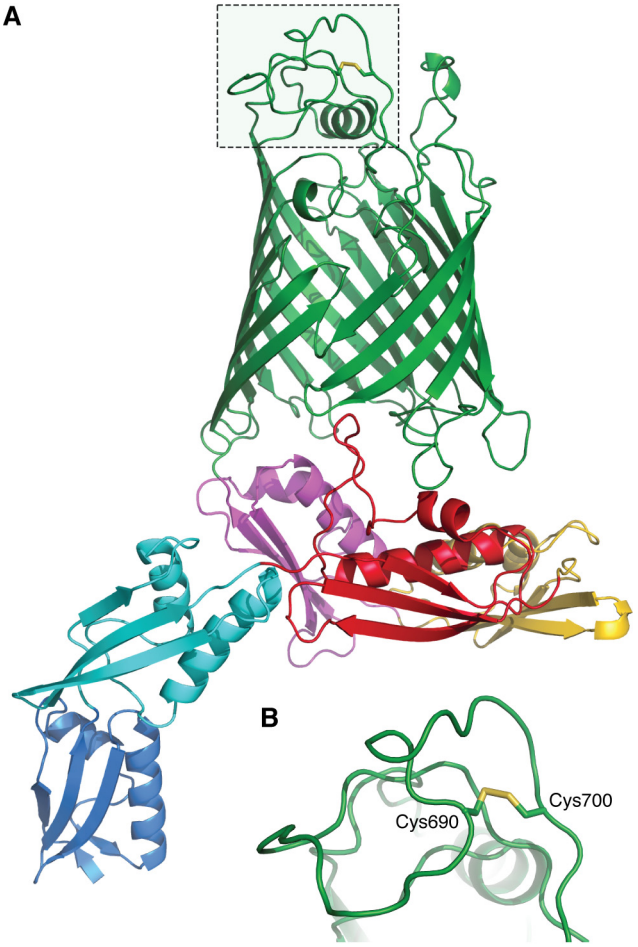

Figure 3. Structure of *E. coli* BamA. *A*, cartoon representation of *E. coli* BamA from PDB structure 5D0O (59). The transmembrane β-barrel domain of BamA is colored *green* and the five periplasmic domains POTRA1, POTRA2, POTRA3, POTRA4, and POTRA5 in *blue*, *cyan*, *red*, *yellow*, and *magenta*, respectively. *B*, the disulfide bond present in extracellular loop 6 of BamA (represented as *sticks*) is dispensable for folding and activity.

Disulfide bond formation is required for the folding of two important envelope stress sensors

Bacteria evolve in always-changing environments in which they can be exposed to molecules or conditions that alter envelope integrity. Given the vital importance of this compartment, bacteria rely on stress sensor proteins to detect perturbations in their envelope and to respond in a fast and adequate manner to inflicted damage. In *E. coli*, two major envelope stress sensors, the outer membrane lipoproteins RcsF and NlpE (Fig. 4 and Table 1), both contain disulfide-linked cysteine residues in their native conformations.

RcsF is a small (11-kDa) surface-exposed lipoprotein that monitors the integrity of the outer part of the envelope, *i.e.* the outer membrane and the peptidoglycan, in enterobacteria (70). In particular, RcsF detects alterations caused by exposure to polymyxin B (71), a cationic antimicrobial peptide that disrupts the lipopolysaccharide leaflet, or to mecillinam (72), a β-lactam that interferes with peptidoglycan synthesis by inhibiting the essential transpeptidase penicillin-binding protein 2 (PBP2). As a result, RcsF triggers a complex signaling cascade known as the Rcs phosphorelay pathway, which tries to contain the inflicted damage by modulating the expression of dozens of genes, including those producing capsular oligosaccharides (70). It is remarkable that the folding of RcsF, a protein required to sense most Rcs-inducing cues, critically depends on two nonconsecutive disulfide bonds (Fig. 4); in cells impaired in disulfide formation (Δ*dsbA* or Δ*dsbB*) or disulfide isomerization (Δ*dsbC* or Δ*dsbD*), RcsF does not fold (40, 73) and is degraded by periplasmic proteases. Interestingly, one of the two RcsF disulfides connects two adjacent antiparallel β-strands (73), which is rarely seen in proteins (74). This unusual feature led to the proposal that this disulfide might function as a redox switch controlling the ability of the protein to detect stress (73). However, no evidence supporting this hypothesis has been reported so far. Instead, although the exact mechanism by which RcsF monitors envelope integrity is still a matter of debate (75–77), it is clear that the ability of RcsF to sense stress is linked to the unusual presence of this protein on the cell surface (the general view is that *E. coli* outer membrane lipoproteins face the periplasm). Interestingly, the export of RcsF to the surface is mediated by BAM via the assembly of complexes between RcsF and abundant β-barrel proteins, such as OmpC and OmpF (Fig. 4). The structure of a BamA-RcsF complex, which forms as an intermediate in the assembly of the complexes between RcsF and its β-barrel partners, was solved recently. In this complex, RcsF is lodged deep inside the lumen of the BamA barrel,

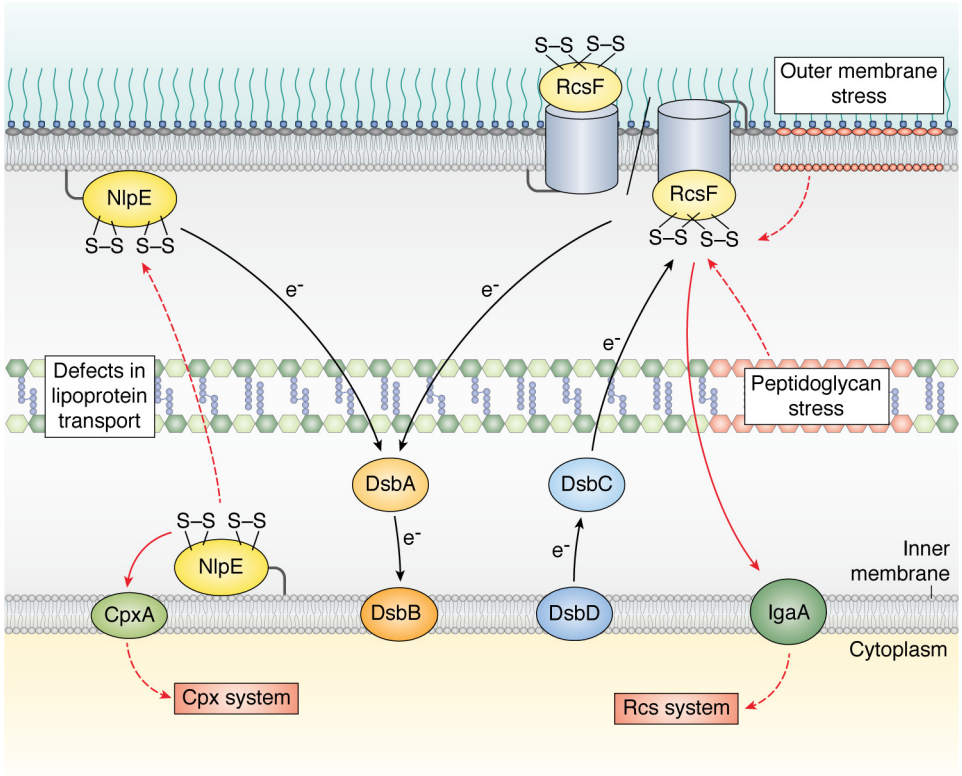

Figure 4. RcsF and NlpE, two outer membrane lipoproteins that monitor the integrity of the *E. coli* cell envelope, contain disulfide bonds in their native conformations. RcsF detects damage in the peptidoglycan and in the outer membrane. Upon stress, it activates the Rcs system by interacting with the inner membrane protein IgaA (109). NlpE detects perturbations in the transport of lipoproteins to the outer membrane; it accumulates in the inner membrane where it interacts with CpxA, activating the Cpx system. Disulfide bond formation in NlpE and RcsF is catalyzed by the DsbA-DsbB system (see the legend to Fig. 1). RcsF contains disulfides between cysteines that are not consecutive in the sequence; its folding involves the DsbC-DsbD isomerization system (see the legend to Fig. 1). The *black arrows* show the electron flow. The plain *red arrows* indicate proteins that directly interact under stress. The *dotted arrows* indicate processes that occur under stress.

which is observed in the inward-open conformation (78). Introduction of artificial disulfides again proved useful in revealing that RcsF does not bind to BamA when it is locked in the outward-open conformation (78).

A second envelope stress sensor with disulfide-bonded cysteines in its native conformation is the outer membrane lipoprotein NlpE (Fig. 4), which activates the Cpx stress response when lipoprotein trafficking to the outer membrane is perturbed (79, 80). In this case, NlpE accumulates in the inner membrane, where it can physically interact with the inner membrane sensor histidine kinase CpxA, triggering Cpx (79, 80). As a result, CpxA autophosphorylates and transfers its phosphoryl group to the cytoplasmic response regulator CpxR, which then binds DNA to regulate the expression of a large set of genes (81, 82).

NlpE consists of two distinct structural domains, namely, an N-terminal domain, which interacts with CpxA (80) and is homologous to the lipocalin Blc, a bacterial lipoprotein that binds hydrophobic ligands, and a C-terminal domain, which adopts an oligonucleotide/oligosaccharide-binding fold (83). Each domain of NlpE contains a disulfide bond that is introduced by DsbA (80) (Fig. 4). Interestingly, failure to form the C-terminal disulfide causes NlpE to induce Cpx (80), thus suggesting that the C-terminal disulfide functions as a molecular sensor for redox perturbations. Because *dsbA* is a Cpx regulon member, this sensing would establish a neat feedback loop. The molecular mechanism of this redox-regulated Cpx induction remains to be determined, however. It is noteworthy that, whereas RcsF occupies a critical position in Rcs, NlpE is not central for Cpx function. Indeed, most Cpx-inducing cues, such as accumulation of misfolded proteins in the periplasm, inner membrane stress, and cell wall perturbations (81, 82), are NlpE independent.

The activity of a family of enzymes important for envelope integrity depends on a single reduced cysteine residue

In *E. coli*, most envelope proteins either do not have any cysteine residues or have cysteine residues that are involved in disulfides. In the previous paragraphs, we discussed the importance of forming correct disulfide bonds in proteins that are required for envelope biogenesis and protection. In the following, we focus instead on the important role played by cysteine residues that are part of catalytic machineries and therefore need to remain reduced in the envelope. In fact, only a small group of enzymes use cysteine-based chemistry in the *E. coli* envelope, and these enzymes all belong to the L,D-transpeptidase family.

E. coli expresses six L,D-transpeptidases, but other bacteria, such as *Bdellovibrio bacteriovorus*, express more than 20 (84). Three of the *E. coli* L,D-transpeptidases, *i.e.* LdtA, LdtB, and LdtC, attach the C-terminal lysine residue of the outer membrane lipoprotein Lpp, the numerically most abundant protein in *E. coli* (also known as the Braun lipoprotein), to a diaminopimelic acid

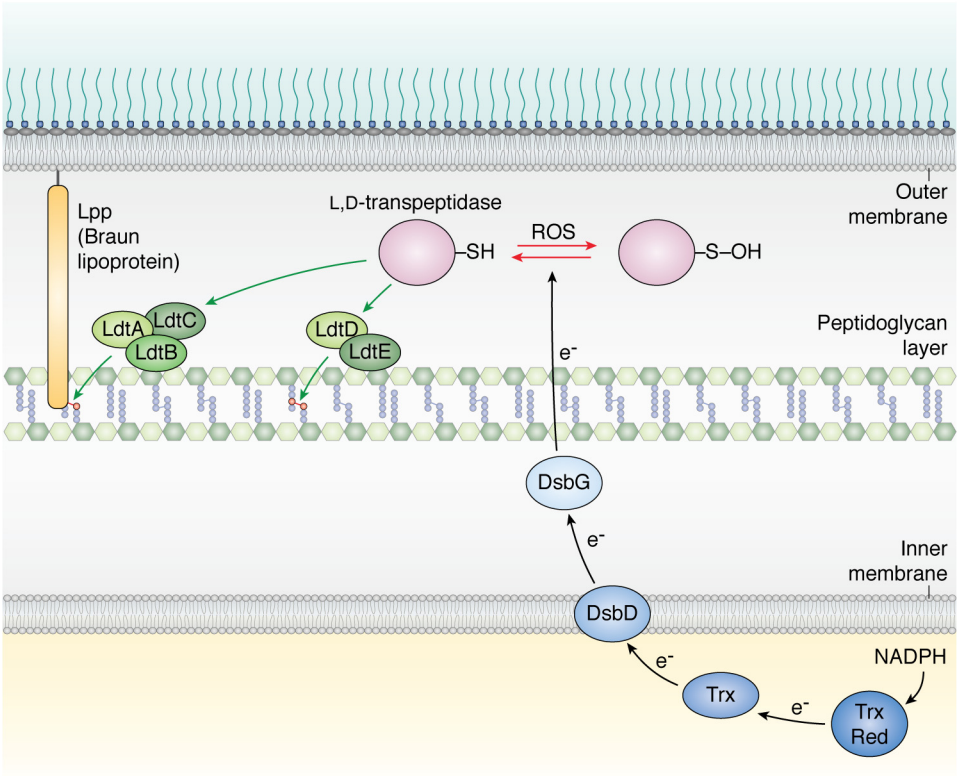

Figure 5. A family of L,D-transpeptidases catalyze reactions that are crucial for envelope assembly using a catalytic cysteine residue. Three L,D-transpeptidases (LdtA, LdtB, and LdtC) catalyze the covalent attachment of the Braun lipoprotein Lpp, the numerically most abundant protein in *E. coli*, to the peptidoglycan. Two other L,D-transpeptidases (LdtD and LdtE) catalyze the formation of 3-3 cross-links between two *meso*-diaminopimelic acid residues of adjacent stem peptides of the peptidoglycan. The catalytic cysteine residue of L,D-transpeptidases is prone to oxidation to a sulfenic acid (-SOH) when exposed to reactive oxygen species (*ROS*). Sulfenic acids can be further oxidized to sulfinic and sulfonic acids (not shown), two irreversible modifications. L,D-Transpeptidases are maintained as reduced and active in the periplasm by DsbG. Electrons are delivered to DsbG by the inner membrane protein DsbD. Electrons are delivered to DsbD as explained in the legend to Fig. 1. The *black arrows* show the electron flow.

residue in the peptide stems of the peptidoglycan (6, 85) (Fig. 5 and Table 1). This reaction, which provides the only covalent connection between the outer membrane and the peptidoglycan, is required for envelope stiffness (86) and stress sensing (87). Furthermore, in cells lacking LdtA, LdtB, and LdtC, the architecture of the envelope is compromised; the intermembrane distance is modified (88), which impairs Rcs functioning and the ability to detect and to respond to envelope defects (87). The other L,D-transpeptidases expressed by *E. coli* have a different function. LdtD and LdtE catalyze the formation of 3-3 cross-links between two *meso*-diaminopimelic acid residues of adjacent stem peptides during peptidoglycan synthesis (89, 90) (Fig. 5 and Table 1), while the enzymatic activity of LdtF (YafK) remains unknown. In *E. coli*, there are only 2–10% 3-3 cross-links, with the majority of cross-links being between D-Ala and *meso*-diaminopimelic acid residues (4-3 cross-links) (91). Formation of 3-3 cross-links increases, however, when cells enter stationary phase and when the transport of lipopolysaccharides to the outer membrane is impaired (90, 91).

The functional importance of L,D-transpeptidases in the assembly of the envelope is beginning to be fully appreciated, not only in *E. coli* but also in a large number of bacteria, including mycobacteria, where they play a major role in peptidoglycan assembly by catalyzing abundant 3-3 cross-links (92). These enzymes have an Achilles' heel, however; their activity involves a catalytic cysteine residue that needs to be kept reduced (93) (Fig. 5), which is challenging, given that cysteine residues are particularly sensitive to oxidation because of the electron-rich sulfur atom in their side chain (15). When exposed to oxidants produced by phagocytic cells (94), the thiol side chains of cysteine residues are indeed oxidized to sulfenic acids (-SOH), which are highly reactive and can be irreversibly oxidized to sulfinic acids ($-SO_2H$) and sulfonic acids ($-SO_3H$) (15). These latter two modifications are often detrimental for protein function and inactivate L,D-transpeptidases. Accordingly, it was recently shown that exposure of *E. coli* to copper, a redox-active metal that is able to catalyze cysteine oxidation in the presence of oxygen (95), inhibits L,D-transpeptidases, compromising Lpp attachment and 3-3 cross-link formation (96). Protecting the thiol functional group of L,D-transpeptidases from oxidation is the function of DsbG (93) (Fig. 5 and Table 1), a periplasmic dimeric oxidoreductase with a Trx-like domain and a CXXC catalytic motif. As in DsbC, this CXXC motif is maintained as reduced by electrons provided by DsbD (97) (Fig. 5). Thus, intracellular metabolism (DsbD is recycled at the expense of NADPH) provides the reducing equivalents to keep cysteine-based envelope enzymes functional, thus maintaining envelope integrity.

Conclusions and perspectives

Since the discovery of DsbA in 1991, an impressive body of research has revealed the critical role played by cysteine

residues in the biogenesis and maintenance of the bacterial cell envelope. In the previous sections, we discussed the importance of disulfide bond formation for the folding and stability of envelope proteins, including crucial assembly factors and stress sensors, and we highlighted the functional relevance of enzymes that use cysteine-based chemistry to build the cell envelope properly. It is likely that additional examples of envelope assembly factors with cysteine residues important for folding and/or activity will be identified in the future, in *E. coli* or in other Gram-negative bacteria. In addition, future research will probably identify novel antioxidant factors protecting envelope proteins from oxidation, an area that has been less well explored than that of oxidative protein folding.

As we reach the end of this review, we would like to suggest the hypothesis that cysteine residues may play an additional role in the envelope by serving as regulatory switches controlling processes necessary for envelope homeostasis. Given their ability to undergo reversible redox modifications, cysteine residues indeed often act as powerful molecular switches allowing organisms to adapt to changes in the environment, as has been extensively described for the bacterial cytoplasm and for higher organisms (98, 99). To our knowledge, no such example has been described so far for the mechanisms that participate in envelope biogenesis. However, several envelope proteins display features that hint at potential redox regulation. For instance, as discussed above, the uncommon presence of a disulfide bond between two adjacent β-strands in RcsF (73) is intriguing and suggests that a layer of redox regulation remains to be discovered for this protein. In addition, both the stress sensor NlpE and PBP1a (an enzyme required for peptidoglycan synthesis) have a disulfide bond between two cysteine residues that are found in a CXXC motif. The fact that CXXC motifs can function as redox switches (74) suggests that these two proteins also may undergo redox regulation. Note that, in the case of NlpE, the redox state of the CXXC motif does not affect Cpx activation (79). Another intriguing case is the abundant outer membrane protein OmpA, which is important for envelope integrity. OmpA is composed of an N-terminal 8-stranded β-barrel and a C-terminal periplasmic domain binding to the peptidoglycan (100, 101). Although this two-domain conformation is well established, some studies have proposed that OmpA can also fold into a 16-stranded β-barrel with a large central pore (102, 103). Interestingly, a disulfide bond present in the C-terminal domain might function as a redox switch controlling the OmpA conformation, as suggested by work in *Salmonella enterica* serovar Typhymurium (104). Finally, a third envelope protein that could potentially be redox regulated is PBP5, a D,D-carboxypeptidase that cleaves the terminal D-alanine from the pentapeptide side chains in peptidoglycan (3). This enzyme has a single cysteine residing close to the active site but without being involved in catalysis (105). In an old study, however, it was shown that cysteine-modifying reagents inhibit PBP5 (106), strongly suggesting that PBP5 (as well as PBP6a and PBP6b, two *E. coli* paralogs in which the cysteine residue is conserved (3)) might be subject to redox regulation. The recent development of specific probes designed to monitor the redox state of cysteine residues (107) will facilitate further exploration of the versatile function and crucial roles of the amino acid cysteine in the bacterial cell envelope. In the same line, the fact that the cell envelope is an environment in which disulfides can be formed will prove very useful in studying the mechanism of crucial assembly and surveillance processes. Indeed, in addition to their native roles, disulfides can be artificially introduced into proteins to affect their structures, allowing inference of their function. In the case of BamA, for instance, using artificial disulfides has led to major mechanistic insights by demonstrating the importance of BamA cycling between an outward-open conformation and an inward-open conformation (57–59).

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Abbreviations—The abbreviations used are: Trx, thioredoxin; PBP, penicillin-binding protein; BAM, β-barrel assembly machinery.

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