Periplasmic folding factors in Gram-negative bacteria

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Summary for laymen

All organisms need a system to regulate the uptake of substances from their external milieu, allowing nutrients to enter and preventing toxic substances from doing so. In Gram-negative bacteria the cell envelope is responsible for determining which substances can enter the cell. It consists of two membranes, each with different selective permeability properties, and an aqueous space between the membranes known as the periplasm. Both the membranes and the periplasm contain many proteins, biological macromolecules that perform a wide variety of crucial cellular functions. Proteins are long polymeric chains of building blocks known as amino acids. In order to perform their cellular functions these chains must adopt a particular three-dimensional structure, a process that is known as protein folding. All organisms contain proteins that assist in the protein folding process, known collectively as folding factors. The periplasm of Gram-negative bacteria contains various folding factors that facilitate the folding of cell envelope proteins, enabling them to fulfil their cellular function. These folding factors function by catalysing certain rate-limiting steps of the protein folding process (e.g. disulphide forming enzymes) or binding to unfolded or misfolded proteins and preventing them from aggregating (e.g. molecular chaperones). Studying these periplasmic folding factors is not only of fundamental interest to microbiologists and biochemists, but is also of importance to the fields of biotechnology, as protein folding is often a limiting factor in the production of biotechnological products, and medicine, as periplasmic folding factors could be potential drug targets. This thesis provides an overview of the current state of our knowledge on the functions and modes of action of periplasmic folding factors in Gram-negative bacteria. The main focus is on the periplasmic folding factors that have been identified in *Escherichia coli*, as this is a commonly studied model organism. The situation in other Gram-negative bacteria is also briefly discussed, as well as the perspectives for future research into periplasmic folding factors.

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The Gram-negative outer membrane

Bacteria can be divided into two groups depending on the architecture of their cell envelope. Gram-positive bacteria have a single membrane surrounded by the peptidoglycan cell wall (1). Gram-negative bacteria have a more complex cell envelope structure consisting of two membranes with the peptidoglycan layer located within the aqueous compartment between the two membranes, the periplasm (Fig. 1) (1). The outer membrane of Gram-negative bacteria functions as a selective barrier. Small, hydrophilic substances can cross the membrane through proteins known as porins, but the membrane is impermeable to hydrophobic molecules and hydrophilic molecules larger than approximately 600 Da (2). This barrier function protects bacteria from harmful substances in their environment, such as antibiotics and detergents. Defects in the biogenesis of the outer membrane are known to increase the sensitivity of bacteria for such harmful substances (3).

The outer membrane is an asymmetric bilayer consisting of an inner leaflet composed of phospholipids and an outer leaflet predominantly composed of the glycolipid lipopolysaccharide (LPS). LPS consists of a lipid moiety known as lipid A, an oligo-acylated β-1-6 disaccharide (usually hexa-acylated), bound to an oligosaccharide core moiety, which in some bacteria is attached to a long oligosaccharide repeat known as the O-antigen (4). The acyl chains in LPS are predominantly fully saturated and form a gel-like layer of low fluidity (4). In combination with the strong lateral interactions between LPS molecules in the presence of divalent cations, this makes the LPS layer impermeable to hydrophobic molecules (4).

The outer membrane contains many integral outer membrane proteins (OMPs). A characteristic feature of these proteins is that they adopt a β-barrel conformation, consisting of a cylinder of transmembrane β-strands (5, 6). The β-strands are positioned at an angle with respect to the lipid bilayer and the structure is stabilised by hydrogen bonds between backbone atoms of the β-strands (5). The β-strands are connected by short turns at the periplasmic side of the membrane and by longer loops at the extracellular side (6). The membrane-exposed surface of the β-barrel is characterised by the presence of hydrophobic residues, whereas the interior of the barrel usually contains small or polar residues, allowing the formation of aqueous pores (5). Aromatic residues are often highly enriched close to the membrane interface (5). Bacterial outer membrane proteins have their N-and C-termini located on the periplasmic side of the membrane (6).

Many OMPs contribute to the selective barrier properties of the outer membrane by functioning as non-specific pores, examples include OmpC and OmpF which are both trimeric outer membrane porins that allow the diffusion of small, hydrophilic molecules into the periplasm of *E. coli* (1). Other OMPs function as specific transporters, e.g. in *E. coli* the trimeric LamB functions as a maltose transporter (1). The outer membrane also contains lipoproteins that are anchored to the inner leaflet of the membrane by a lipid tail, which is attached to an N-terminal cysteine residue (1). Outer membrane lipoproteins have been implicated in the assembly of OMPs at the outer membrane, the insertion of LPS into the outer membrane and the anchoring of the outer membrane to the peptidoglycan layer (1, 4, 6).

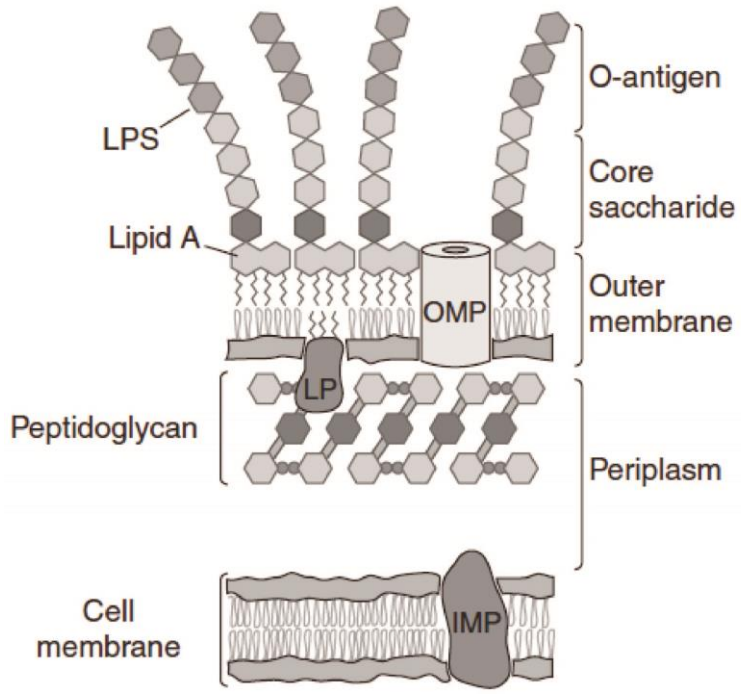

Figure 1: The structure of the Gram-negative cell envelope. The cell envelope consists of two membranes with the aqueous periplasm and the peptidoglycan cell wall in between them. The outer membrane is an asymmetric bilayer with an inner leaflet composed of phospholipids and an outer leaflet composed of LPS, a glycolipid with a lipid moiety (Lipid A) attached to a core saccharide moiety and, in some bacteria, to an oligosaccharide repeat known as the O-antigen. The outer membrane contains integral membrane proteins with a β-barrel structure and lipoproteins anchored to the membrane by a lipid moiety. The rigid peptidoglycan layer is anchored to the outer membrane by a lipoprotein known as Braun's lipoprotein. The inner membrane is a traditional phospholipid bilayer and contains integral membrane proteins with α-helical transmembrane segments. Figure taken from (1).

The periplasm and peptidoglycan layer

The aqueous space between the outer and inner membranes is known as the periplasm. The periplasm is packed with proteins, including soluble periplasmic proteins, periplasmic domains of inner membrane proteins, OMPs and lipoproteins and parts of envelope-spanning structures, such as the flagella (1). Based on the structures of periplasm-spanning protein complexes it has been proposed that the periplasm is approximately 170-180 Å thick (1). Unlike the cytoplasm, the periplasm is an oxidising environment and thus periplasmic proteins can contain disulphide bonds (7). The periplasm does not contain a chemical energy source such as ATP and there is no proton gradient across the outer membrane (2). Thus all processes that take place in the periplasm must be independent of external energy sources or be energised by inner membrane proteins utilising cytoplasmic ATP. The periplasmic environment is extremely sensitive to the conditions in the external milieu as porins allow the diffusion of all water soluble molecules under 600 Da in size into the periplasm. This combination of changeable conditions and the lack of an external energy source makes the periplasm a challenging environment for proteins to function in. In addition to proteins, the periplasm contains the peptidoglycan layer. This layer consists of polymeric chains of alternating units of the glycans N-acetylglucosamine (GlcNac) and N-acetylmuramic acid (MurNac) which are cross-linked via peptides attached to the MurNac units (8). Peptidoglycan forms a rigid layer that has an important role in maintaining cellular shape and protects bacteria from lysis when they are exposed to osmotic pressure (1).

The Gram-negative inner membrane

The periplasm is separated from the cytoplasm by the inner membrane. Unlike the outer membrane, the inner membrane is a conventional phospholipid bilayer. This bilayer does not contain porins and functions as a semi-permeable barrier, allowing bacteria to maintain different conditions in their cytoplasm than in the external environment. For example, in an acidic environment the cytoplasmic pH can be kept at an acceptable level by the utilisation of protons for the decarboxylation of arginine,

lysine or glutamate (9). Like the outer membrane, the inner membrane contains periplasmic lipoproteins and integral membrane proteins. Integral inner membrane proteins (IMPs) differ from OMPs in that they do not form membrane-spanning β-barrel structures, but contain α-helical transmembrane segments. IMPs perform a large variety of crucial cellular functions, e.g. energy production, the transport of nutrients into the cytoplasm and the translocation of OMPs into the periplasm (10).

The biogenesis of the Gram-negative cell envelope

LPS, phospholipids and peptidoglycan

The biogenesis of the Gram-negative cell envelope is a rather complex process, as the required components are all synthesised in the cytoplasm or inner membrane and thus must be post-synthetically transported to their correct location and correctly assembled once they reach it (Fig. 2).

LPS synthesis takes place at the inner membrane. The lipid A-core module is synthesised at the inner leaflet of this membrane, flipped across the bilayer by the MsbA translocase and, when applicable, synthesis is completed by the addition of the O-antigen in the outer leaflet of the inner membrane (4). Subsequently, the hydrophobic LPS molecules must be transported across the periplasm and inserted into the outer membrane. This is achieved by the Lpt system. LPS is released from the inner membrane and transferred to the IMP LptC, a process that is energised by ATP hydrolysis by an inner membrane ABC-transporter composed of LptB, LptF and LptG (6). Next, LPS is transferred to the soluble periplasmic protein LptA which is thought to form a bridge across the periplasm (11). The transfer of LPS from LptC to LptA has recently been shown to depend on ATP hydrolysis by LptBFG (12). LPS is transported to the outer membrane along the LptA bridge and is inserted into the outer leaflet of the outer membrane by an outer membrane protein complex consisting of the OMP LptD and the lipoprotein LptE (4). The exact mechanism by which this complex inserts LPS specifically into the outer leaflet of the membrane is as yet poorly understood.

Little is known about the mechanism by which the phospholipids that make up the inner leaflet of the outer membrane are transported across the periplasm. These lipids are synthesised at the inner leaflet of the inner membrane and can subsequently be flipped across the bilayer by MsbA or spontaneously flip across the membrane in the presence of transmembrane α-helices (6). It is unknown how the hydrophobic lipids are transported across the periplasm, though possibilities include transport at contact sites between the two membranes or the action of specific lipid transporter proteins.

The synthesis of the peptidoglycan layer is a complex multi-step process. UDP-MurNac-peptide precursors are synthesised in the cytoplasm, followed by their linkage to a lipid moiety and anchoring to the inner membrane, the addition of GlcNac units at the inner leaflet of the inner membrane and the translocation of the formed molecules to the outer leaflet of the inner membrane by a flippase (8). Subsequently, polymerization of the GlcNac-MurNac units and cross-linking through their peptides is achieved by a class of proteins known as penicillin-binding proteins (8).

Protein translocation and insertion at the inner membrane

As the synthesis of all bacterial proteins takes place in the cytoplasm, it is imperative that proteins that function in the cell envelope are correctly sorted and transported to their correct location. The first obstacle all cell envelope proteins encounter during transportation is the inner membrane. IMPs must be inserted into this membrane with their correct topology and soluble periplasmic proteins, OMPs and lipoproteins must be translocated across it. For the vast majority of cell envelope proteins this is

achieved with the assistance of the Sec translocon. The core of the Sec translocon is a trimeric protein complex composed of SecY, SecE and SecG (13). This complex forms a gated membrane channel that allows unfolded polypeptide chains to be translocated across or inserted into the inner membrane (13).

The Sec translocon can function co-or post-translationally, with the latter being the preferred route for the translocation of OMPs, periplasmic proteins and lipoproteins and the former being preferred for the insertion of IMPs. IMPs have an N-terminal signal peptide of high hydrophobicity that is recognised by the signal recognition particle (SRP) as the nascent polypeptide chain appears from the ribosome (14). The resulting complex is targeted to the Sec translocon by the SRP receptor FtsY (14). The nascent chain is then inserted into the Sec translocon's pore and hydrophobic segments insert into the membrane through the channel's gate, in some cases assisted by the YidC insertase (10). A couple of small IMPs, e.g. subunit c of the F_1F_0-ATPase, have been shown to insert into the membrane in a Sec-independent manner, only requiring the YidC insertase (10). Unlike the insertion of IMPs, the translocation of soluble periplasmic proteins, OMPs and lipoproteins generally occurs post-translationally. After translation these proteins are prevented from folding by the cytoplasmic chaperone SecB (6). The proteins contain an N-terminal signal sequence that is recognised by SecA, an ATPase that pushes the unfolded polypeptide chains through the Sec translocon's pore (13). SecA is also required for the translocation of large periplasmic domains of IMPs (14). Some periplasmic proteins are translocated Sec-independently by the Tat pathway. Three proteins are involved in this pathway: TatA, which forms oligomeric membrane pores, and TatB and TatC, which are thought to play a part in recognising substrates (14). Unlike proteins that utilise the Sec translocon, proteins that are translocated by the Tat machinery fold in the cytoplasm and are translocated as natively folded proteins (14).

Assembly of OMPs at the outer membrane

Once proteins have been translocated across the inner membrane their signal sequences are cleaved off. Soluble periplasmic proteins are now free to fold into their native conformation. OMPs, however, must be transported across the periplasm to the outer membrane and assembled into the membrane once they reach it. To prevent the unfolded OMPs from misfolding or aggregating in the aqueous environment of the periplasm their amphipathic β-strand regions must be shielded by periplasmic chaperones, e.g. SurA, Skp or DegP (2, 6). The function and mode of action of these chaperones will be discussed in detail later.

To reach the outer membrane OMPs must cross the peptidoglycan cell wall. The peptidoglycan layer contains holes large enough for globular proteins of up to approximately 50 kDa to pass through them (15). Chaperone-OMP complexes may be able to pass through these holes. However, it is also possible that local degradation of the peptidoglycan layer to create larger holes could be required for the transport of some proteins. *E. coli* contains many enzymes that can degrade peptidoglycan and many trans-envelope protein machines have been shown to require the formation and maintenance of holes in the peptidoglycan layer for their assembly (16, 17). Little is known however, about the importance of peptidoglycan modifications for OMP transport.

Protected by chaperones, the OMPs are transported across the periplasm to the outer membrane, where they are assembled into the membrane by the Bam machinery. Though OMPs can spontaneously fold and insert into phospholipid bilayers *in vitro*, the Bam machinery is essential for their correct assembly into the outer membrane *in vivo* (5, 6). In *E. coli* the Bam machinery consists of one OMP, BamA, and four lipoproteins, BamB, BamC, BamD and BamE (6). BamA is an essential

protein that consists of a membrane spanning β-barrel and a large N-terminal periplasmic moiety consisting of five POTRA (polypeptide translocation associated) domains (P1-5) (6). It is thought that the β-barrel domain is responsible for the membrane insertion of OMPs while the POTRA domains play a role in interactions with substrates, periplasmic chaperones and the Bam lipoproteins (6). The role of the Bam lipoproteins is unclear. It has been shown that BamC, D and E form a complex that interacts with the POTRA domain closest to BamA's C-terminal β-barrel domain (P5) and that BamB interacts with BamA independently (18). The BamA-BamB interaction is unaffected by the deletion of the most N-terminal POTRA domain (P1), but the deletion of P1 and P2 is sufficient to break the interaction (18). Of the lipoproteins, only BamD is essential, with the depletion of BamB causing major defects in OMP assembly, but not lethal ones, and BamC or BamE depletion causing only minor defects in OMP assembly (6). The Bam machinery recognises its substrates through a C-terminal signal motif that has been shown to be essential for the recognition of OMPs by the Bam machinery *in vitro* and the incorporation of OMPs into the outer membrane *in vivo* (19, 20). SurA, a periplasmic chaperone which plays a major role in OMP biogenesis, has been shown to physically interact with BamA, probably through the P1 domain, suggesting a possible role for SurA in delivering OMP substrates to the Bam machinery (21, 22). The exact mechanism by which the Bam machinery achieves the incorporation of OMPs into the outer membrane is poorly understood. BamA has been shown to form closable membrane channels which are opened in the presence of OMPs or peptides corresponding to their C-terminal signal peptides (20). These pores may allow the membrane insertion of OMPs. It is unclear whether OMPs adopt their β-barrel structure prior to or during the membrane insertion process (23).

Sorting and transport of periplasmic lipoproteins

Once lipoproteins have been translocated into the periplasm they are acylated by the formation of a thioester diglyceride at a cysteine residue located directly behind their signal sequence (2). Following this their signal sequence is removed and the N-terminal amino group of the cysteine residue is also acylated (2). The added lipid moiety anchors the lipoproteins in the outer leaflet of the inner membrane. Subsequently, lipoproteins must be transported to the outer membrane or retained in the inner membrane. Transport of lipoproteins to the outer membrane is performed by the Lol system. An ABC-transporter composed of the proteins LolC, LolD and LolE removes lipoproteins from the inner membrane and transfers them to the soluble lipoprotein carrier chaperone LolA that shields their hydrophobic lipid moieties from the aqueous periplasm (1). LolA crosses the periplasm and transfers the lipoproteins to the outer membrane lipoprotein LolB which inserts them into the outer membrane (1). Lipoproteins destined for the inner membrane have a Lol avoidance signal, usually an aspartate residue directly behind the N-terminal cysteine residue, that prevents them from entering the Lol system and thus allows them to be retained in the inner membrane (2).

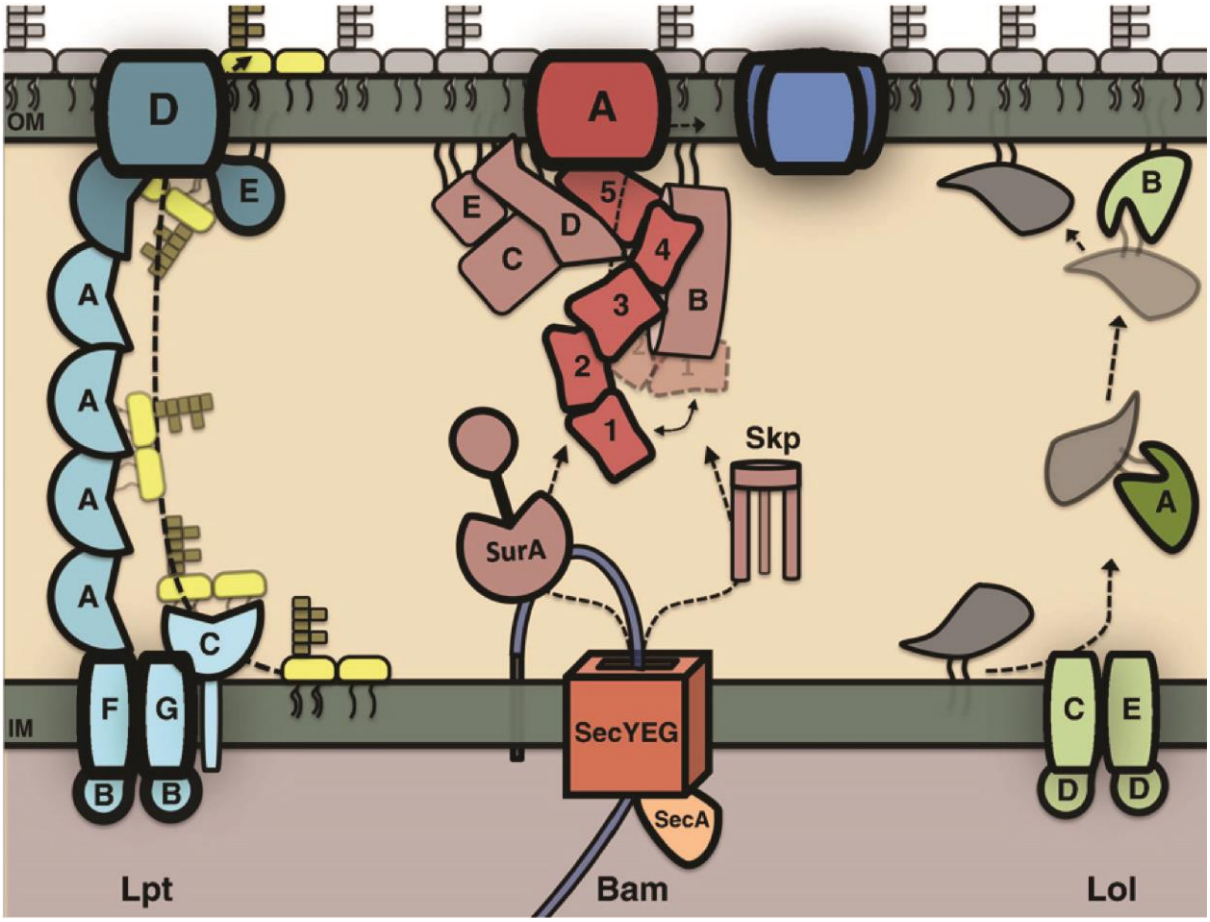

Figure 2: The systems involved in the transport of outer membrane components across the periplasm in *E. coli*. LPS is transported by the Lpt system. The ABC-transporter composed of LptB, LptF and LptG energises the release of LPS from the inner membrane to the IMP LptC. Subsequently, LPS is transferred to LptA and transported to the outer membrane assembly machinery composed of LptD and LptE via a trans-periplasm LptA bridge. OMPs are translocated across the inner membrane by the Sec translocon. The ATPase SecA pushes them through the pore formed by SecY, SecE and SecG. Periplasmic chaperones such as Skp and SurA protect OMPs from aggregation or misfolding in the periplasm and transport them to the Bam machinery which assembles them into the outer membrane. BamA is the most important member of this complex. Its flexible POTRA moiety [1-5] is likely responsible for the binding of substrate proteins, with its β-barrel domain being responsible for their insertion into the membrane. The role of the lipoproteins BamB-E is unclear. Lipoproteins destined for the outer membrane are transferred from the inner membrane to the lipoprotein carrier LolA by the ABC-transporter composed of LolC, LolD and LolE. LolA shields their lipid moiety as they are transported across the periplasm to the outer membrane lipoprotein LolB which is responsible for their insertion into the outer membrane. Figure taken from (6).

Folding factors: molecular chaperones and folding catalysts

Classes of folding factors

The correct folding of proteins into their native conformation is a crucial process in all organisms. Though many proteins can spontaneously fold into their correct conformation *in vitro*, as the native state is the lowest energy conformation, the process of protein folding *in vivo* is fraught with obstacles. During the folding process regions containing many hydrophobic amino acids, which would normally be buried within a protein's globular structure or in a membrane, are solvent exposed. In the crowded cellular environment such exposed hydrophobic regions can easily interact with similar regions from other proteins leading to the formation of toxic protein aggregates (24). In addition, proteins may be trapped in metastable, incorrectly folded states which must be partially unfolded in order to allow the protein to adopt its native conformation (24). To ensure effective protein folding and prevent the accumulation of toxic aggregates all organisms contain proteins that assist other proteins in the folding process: folding factors.

Molecular chaperones

Molecular chaperones are general folding factors that assist in the protein folding process by recognising and binding unfolded proteins, thus shielding aggregation-prone protein regions from the aqueous cellular environment. The major chaperones that assist in protein folding in the cytoplasm of *E. coli* have been extensively studied. DnaK (Hsp70) prevents protein aggregation by binding and releasing hydrophobic protein stretches in an ATP-dependent cycle (25). GroEL (Hsp60) works together with a co-chaperone, GroES (Hsp10), and forms a ring-shaped cavity into which proteins exposing hydrophobic regions are sequestered, allowing them to fold in a shielded environment (25). GroEL's substrate binding and release cycle is also ATP-driven (25). Hsp90 binds and releases its substrates in an ATP-dependent cycle, though the mechanism by which it performs its chaperone function is as yet poorly understood (25). Similar chaperone systems are found in almost all organisms (24). A number of *in vitro* assays can be used to determine whether a protein displays chaperone activity. They include the luciferase refolding assay, where the ability of a protein to assist the refolding of denatured luciferase is evaluated, and the citrate synthase aggregation assay, where the ability of a protein to prevent the aggregation of denatured citrate synthase is determined (26, 27).

Peptidyl-prolyl isomerases

As well as by molecular chaperones, protein folding is assisted by folding catalysts, enzymes that catalyse a specific step in the protein folding process. One such step is the cis-trans isomerisation of the peptide bond at the N-terminal side of proline residues. Almost all of the peptide bonds formed during translation are in the energetically favoured trans conformation and this is the conformation that is found for almost all peptide bonds in native protein structures. This does not hold true for the peptide bond at the N-terminal side of proline residues, approximately 6 percent of these X-Pro peptide bonds are in the cis conformation (28). The isomerisation of these bonds from the trans to the cis conformation can form a major hurdle in protein folding and is often the rate-limiting step (29). Peptidyl-prolyl isomerases (PPIases) are proteins that are able to catalytically accelerate the cis-trans isomerisation of these peptide bonds and thus can act as folding factors that accelerate the folding of proteins containing cis-prolines (29, 30). Most PPIases belong to one of three families of homologues: FK-506 binding proteins (FKBPs), cyclophilins or parvulins (29). The members of each family share

a common architecture of their catalytic domain. The simplest PPIases consist of a single PPIase domain, but many contain additional domains, e.g. protein binding domains that provide the PPIases with substrate specificity (30). PPIases may also contain multiple PPIase domains from the same family or even PPIase domains from two different families (30). Many PPIases display PPIase-independent chaperone activity and in some cases this may be their main function *in vivo* (30). The periplasmic chaperone SurA is an example of a PPIase that displays chaperone activity and it has been demonstrated that this chaperone activity is more important for its function *in vivo* than its PPIase activity (31).

Disulphide forming enzymes and disulphide isomerases

Another factor that influences protein folding is the correct formation of disulphide bonds between cysteine residues. These bonds are often crucial in enabling a protein to adopt its correct fold. Disulphide bonds are formed by disulphide forming enzymes, which oxidise two cysteine residues in a substrate protein by reducing one of their own disulphide bonds (32). However, this process is usually unspecific and incorrect disulphide bonds may be formed in proteins containing more than two cysteine residues, leading to incorrect folding of the protein. This is corrected for by the disulphide isomerases. These isomerases recognise proteins with incorrect disulphide bonds and break the disulphide bond through the formation of an intermolecular disulphide bond between a reduced cysteine residue within the isomerase and one of the cysteine residues from the original disulphide bond (32). Subsequently, a new intramolecular disulphide bridge is formed, either in the substrate or in the isomerase itself (32). This leads to the formation of a new disulphide bond in the substrate or to the release of a reduced form of the substrate in which new disulphide bonds can be formed by disulphide forming enzymes (32). Some disulphide isomerases, e.g. DsbC from *E. coli*, display chaperone activity which is independent of their ability to isomerase disulphide bonds (33).

Protein folding in the periplasm

Chaperones and folding catalysts in the periplasm

The periplasm contains a number of chaperones and folding catalysts that play important roles in ensuring that cell envelope proteins can adopt and maintain their native conformations. As OMPs contain aggregation-prone amphipathic β-strand regions the action of chaperones is indispensable for their transport across the periplasm. The periplasmic proteins Skp, DegP and SurA have been implicated in the process of OMP biogenesis in *E. coli* (21). Soluble periplasmic proteins and the periplasmic domains of membrane proteins may also require the assistance of chaperones during the folding process. The sensitivity of the periplasm to external conditions means that periplasmic proteins are more readily exposed to stress conditions, e.g. pH changes, than their cytoplasmic counterparts. These stress conditions can cause proteins to denature and aggregate, but the damage can be limited by the action of chaperones (7). The mode of action of periplasmic chaperones must differ from that of their cytoplasmic counterparts, as the periplasm does not contain ATP to drive an ATP-dependent substrate binding and release cycle. Thus substrate binding and release must be independent of external chemical energy sources or powered by the hydrolysis of cytoplasmic ATP by inner membrane proteins.

The periplasmic unfolded protein stress responses

The accumulation of unfolded or mislocalised proteins in the periplasm can initiate a stress response that helps restore cell envelope function. *E. coli* has two periplasmic stress response systems: the σ^E

response and the Cpx response. The accumulation of OMPs in the periplasmic space leads to the induction of the σ^E response (34). The central player in this system is the transcription factor σ^E. Under non-stress conditions this transcription factor is bound to the cytoplasmic part of an IMP known as RseA (35). A periplasmic protein, RseB binds to the periplasmic part of RseA and prevents it from being cleaved by the DegS protease, a protease that is anchored to the inner membrane with its proteolytic domain located in the periplasm (35, 36). Upon the accumulation of OMPs in the periplasm their C-terminal signal sequences bind to and activate the DegS protease (37). This leads to DegS cleaving RseA in its periplasmic domain, though RseB must also be released from RseA to allow cleavage and it is unclear what triggers this release (34). After cleavage by DegS, RseA is cleaved in its transmembrane domain by the protease RseP (38). This leads to the release of σ^E bound to the cytoplasmic portion of RseA into the cytosol, where the remainder of RseA is degraded by the protease ClpXP (39). σ^E is now free to promote the transcription of the genes in its regulon which include genes encoding periplasmic chaperones involved in OMP biogenesis, e.g. Skp and DegP, members of the Bam complex and genes involved in lipopolysaccharide synthesis (40). σ^E also initiates the transcription of various sRNAs which block the translation of a number of OMPs, preventing the further accumulation of OMPs in the periplasm (41). The Cpx response is induced upon the accumulation of unfolded proteins in the periplasm (7). In the presence of unfolded proteins CpxP, a periplasmic protein, is released from the inner membrane kinase CpxA (7). This leads to the autophosphorylation of CpxA followed by the phosphorylation of the cytoplasmic protein CpxR by CpxA (7). This phosphorylation activates CpxR, leading to the transcription of the genes of the Cpx regulon. This regulon includes the genes encoding DegP, the periplasmic PPIase PpiA, and the disulphide forming enzyme DsbA (42).

In the remainder of this thesis the function and mode of action of the periplasmic chaperones and folding catalysts identified so far will be discussed. These include the chaperones involved in OMP biogenesis, a number of periplasmic PPIases and enzymes involved in the formation and isomerisation of disulphide bonds. The main focus will be on the chaperones and folding catalysts from *E. coli* as this is by far the most extensively studied member of the Gram-negative bacteria. However, the situation in other Gram-negative bacteria will also be briefly summarised.

Periplasmic folding factors in *E. coli*

Chaperones involved in OMP biogenesis

SurA

SurA is a periplasmic chaperone that plays a major role in the biogenesis of OMPs in *E .coli*. The *surA* gene was first identified in a screen for genes required for survival in the stationary growth phase (43). Some six years after this discovery SurA was implicated in the biogenesis of OMPs. *surA* mutants display sensitivity to a number of substances known to be markers for outer membrane deficiencies, e.g. vancomycin, bile salts and SDS, and the overexpression of SurA suppresses the phenotypical effects of mutations in genes involved in outer membrane biogenesis (44-46). *surA* mutants display defective folding of the OMPs LamB, OmpA and OmpF, while the folding of periplasmic proteins is unaffected, suggesting that SurA may be involved in the folding or assembly of OMPs (44, 45). *surA* is essential for pilus biogenesis as it is required for the assembly of the OMPs PapC and FimD, which function as ushers in the assembly of P and type 1 pili respectively (47, 48). A proteomics study showed that the absence of SurA leads to a significant decrease in the abundance of 8 out of the 23 OMPs studied (49). Disruption of *surA* also activates the σ^E stress response, another sign that SurA is involved in periplasmic protein folding (44).

The SurA protein consists of two parvulin-like PPIase domains (P1 and P2) flanked by an N-terminal domain (N) and a C-terminal tail (C) (44). The full length protein displays PPIase activity, but only the P2 domain is catalytically active (31, 44, 46). PPIase activity is not required for SurA's function *in vivo*, as catalytically inactive SurA variants are able to restore the phenotypes of *surA* mutants (31). In fact, even a mutant lacking both PPIase domains is functional *in vivo* (31, 50). In addition to its PPIase activity SurA can act as a molecular chaperone *in vitro,* with only the N and C domains being required for this activity (31). SurA preferentially binds unfolded OMPs over other proteins, whether folded or unfolded, and interacts with peptides with aromatic-rich sequence motifs that occur frequently in OMPs (31, 51-54). In combination, these data point to SurA functioning as an OMP chaperone, binding unfolded OMPs in the periplasm and preventing them from aggregating or misfolding. SurA interacts with BamA's P1 domain, suggesting that it can transfer OMPs to the Bam machinery which is responsible for their assembly at the outer membrane (21, 22).

The crystal structure of the full length SurA protein revealed that the N, P1 and C domains fold into a core module, with the P2 domain linked to it through two polypeptide linkers 25-30 Å in length (Fig. 3A) (55). The N domain contains a 50 Å long crevice into which short peptide stretches from neighbouring molecules were bound, suggesting it may be SurA's substrate binding site (55). The idea that substrates bind to the N-terminal domain is consistent with the observation that an N-terminal fragment of SurA is able to bind peptides and that the N+C module has chaperone activity and is functional *in vivo* (31, 54). However, experiments with peptides that were selected as high affinity SurA binders by phage display revealed that these peptides bind to the P1 domain (56). The crystal structure of one of these peptides bound to a SurA mutant lacking its P2 domain revealed SurA to have a significantly different conformation than that observed previously (Fig. 3B) (56). SurA was shown to form dimers, with the P1 domains releasing from the N+C core module and forming a sandwich around the peptide and the two monomers interacting through their N and C domains (56). Thus it seems that SurA has multiple peptide binding sites and may be able to adapt its conformation to bind different substrates. It has been suggested that the binding site in the N domain may be

responsible for chaperone activity, with the P1 binding site being responsible for the recognition of OMPs (56). However, as SurA lacking both PPIase domains is functional *in vivo* the binding site in the P1domain is not strictly required for SurA's function. It is unclear whether the N domain peptide binding site has heightened affinity for motifs present in OMPs. It would be interesting to determine whether the N+C fragment of SurA displays a preference for binding OMPs. If not, then the general chaperone activity present in this fragment may be sufficient to allow it to bind aggregation-prone regions of OMPs and prevent them from misfolding or aggregating in the periplasm.

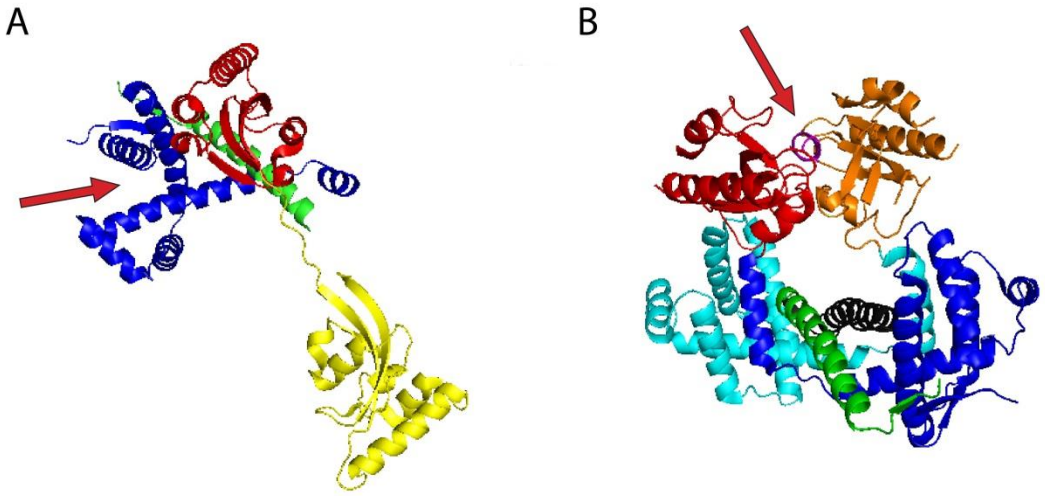

Figure 3: (A): Crystal structure of substrate-free SurA (PDB code 1M5Y (55)). The N domain is shown in blue, the P1 domain in red, the P2 domain in yellow and the C domain in green. The arrow indicates the position of the peptide binding groove in the N domain. Flexible linker regions between the N domain and the P1 domain and between the P2 domain and the C domain are not shown as they were not defined in the PDB file. (B) Crystal structure of peptide-bound dimers of SurA mutants lacking the P2 domain (PDB code 2PV3 (56)). One monomer is coloured as in (A). In the other monomer the N domain is shown in cyan, the P1 domain in orange and the C domain in black. The arrow indicates the position of the peptide binding cleft, with the bound peptide shown in pink. A flexible linker region between the P1 domain and the C domain and a loop within the P1 domain are not shown as they were not defined in the PDB file.

Skp

Another protein that plays a role as an OMP chaperone in the periplasm is Skp. *E. coli* Skp was originally identified in 1988 and was shown to bind DNA (57). Later Skp was implicated in the translocation of proteins into inverted plasma membrane vesicles (58). After it was discovered that Skp is localised to the periplasm, it was suggested that it may play a role at a late step of protein translocation across the inner membrane or in the sorting of proteins after translocation (59). Skp was implicated in OMP biogenesis when it was discovered that it can bind to OMPs and that a *skp* deletion mutant displays lowered OMP levels (60). Mutations in the *skp* gene induce the σ^E stress response, providing evidence for an involvement in protein folding in the periplasm (46). Deletion of the *skp* and *surA* genes is synthetically lethal, suggesting they function in similar processes (61).

Skp interacts with OMPs during their translocation by the Sec machinery and enables their release into the periplasm by forming soluble Skp-OMP complexes (62, 63). *In vitro,* Skp accelerates the insertion of OMPs into negatively charged phospholipid bilayers in the presence of LPS, but it inhibits their insertion in the absence of LPS (64, 65). Thus it appears that the formation of Skp-OMP complexes prevents OMPs from inserting into membranes and that LPS can trigger the release of OMPs from these complexes, leading to their insertion into membranes. Skp interacts with

phospholipid bilayers and this interaction leads to conformational changes which render it protease insensitive, this may allow Skp to interact with the inner membrane enabling it to access OMPs as they appear from the translocation machinery (66). Thus it appears Skp binds OMPs as they are translocated across the inner membrane and forms soluble periplasmic complexes with them, in which the OMPs are maintained in a folding-competent state.

Skp forms trimers with a jellyfish-like shape (Fig. 4A) (67, 68). Each monomer contributes 4 β-strands to a β-barrel that forms a core trimerisation module and three double α-helical tentacles extend roughly 60 Å from this core module, with each monomer contributing one tentacle (67, 68). The three tentacles form a cavity with many hydrophobic residues on the inside of the cavity and positively charged residues at the tips and on the exterior surface of the tentacles (67, 68). Skp trimers form 1:1 complexes with OMPs, binding the entire β-barrel domain of unfolded OMPs, including extracellular loops and periplasmic turns, in their hydrophobic cavity (69-71). Interestingly, the soluble periplasmic domain of OmpA was shown to reside outside the Skp cavity and to adopt a folded conformation in Skp-OmpA complexes (Fig. 4B) (70). OMPs enter Skp's cavity at the bottom and "climb" up it, with their N-terminus entering first (72).

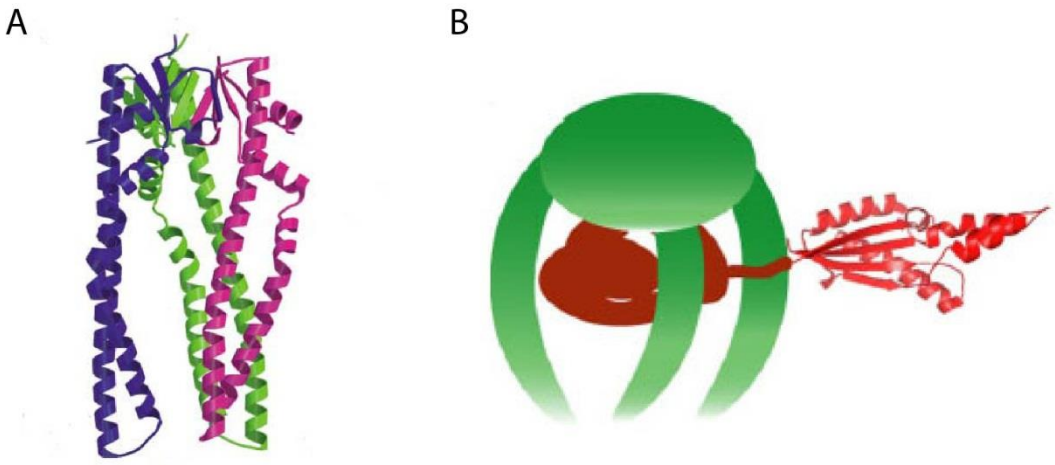

Figure 4: (A): Structural model of a Skp trimer based on its crystal structure. Skp has a jellyfish-like structure with 3 α-helical tentacles extending from a trimerisation module. The individual monomers are coloured blue, green and pink. (B): Schematic model of a Skp-OmpA complex. Skp is coloured green and OmpA is red. OmpA's unfolded β-barrel domain binds in the hydrophobic cavity formed by Skp's tentacles while its soluble periplasmic domain resides outside of the cavity and is fully folded. (A) was taken from (68) and (B) from (70).

Binding of LPS was shown to lead to conformational changes in OmpA-Skp complexes, causing OmpA's extracellular loops to become more solvent exposed (69). This, in combination with the fact that negative phospholipids and LPS are required for Skp to release substrates into phospholipid bilayers, implies that electrostatic interactions of the positively charged Skp with negatively charged molecules are important for the release of OMPs from OMP-Skp complexes, possibly by causing electrostatic interactions between Skp and negatively charged (regions of) OMPs to be broken. The relevance of this finding *in vivo* is unclear, as it is believed that the insertion of OMPs into the outer membrane is dependent on the Bam machinery. It has been noted that the POTRA domains of BamA have negatively charged regions and it has been postulated that these regions could interact with the positively charged regions of Skp, triggering the transfer of OMPs to the Bam machinery (65, 69). However, Skp has not been shown to interact with any member of the Bam machinery and it has been

suggested that Skp may not be involved in transferring OMPs to the Bam machinery, but may pass substrates on to SurA which can then transfer them to the Bam machinery (2).

Though Skp has mainly been studied in the context of its function as an OMP chaperone, evidence exists that suggests Skp also functions as a more general periplasmic chaperone. Co-expression of Skp has been shown to enhance the yield of a number of antibody fragments when they were expressed in *E. coli* and targeted to the periplasm and Skp can prevent the aggregation of lysozyme and a number of soluble antibody fragments *in vitro* (68, 73). Additionally, a proteomics-based search for *in vivo* interaction partners of Skp identified a number of soluble periplasmic proteins (74). Thus Skp may play a part in assisting the folding of soluble periplasmic proteins or in preventing their aggregation under stress conditions, as well as functioning as an OMP chaperone.

DegP

DegP is a periplasmic protease that is essential for *E. coli* growth at 42 ^{0}C and is responsible for degrading misfolded periplasmic proteins (75-77). It is a member of the HtrA family of serine proteases. Consistent with its role as a quality control factor DegP can only degrade proteins when they are in an unfolded conformation (78). In addition to its protease activity DegP displays chaperone activity and it switches from chaperone to protease activity as the temperature rises (79). A protease-deficient DegP mutant, in which the active site serine is mutated to an alanine (S210A), is sufficient to rescue the temperature sensitive phenotype of a *degP* mutant, suggesting DegP's chaperone activity is important for its function *in vivo* (79). DegP has been implicated in OMP biogenesis as it is regulated by the σ^{E} stress response, *surA* and *degP* deletion is synthetically lethal and a *degP* deletion mutant displays lowered levels of OMPs (40, 61, 80). DegP depletion is lethal in cells expressing assembly-deficient variants of OmpC or OmpF (81, 82). Interestingly, expression of $DegP_{S210A}$ rescues this lethal phenotype, though the mutant OMPs are not assembled into the outer membrane (81, 82). This suggests that $DegP_{S210A}$ can capture these mutant OMPs and prevent them from aggregating or clogging up OMP assembly pathways.

DegP consists of a protease domain and two PDZ domains (PDZ1 and PDZ2), which are often involved in protein-protein interactions. DegP's protease domain is sufficient for *in vitro* chaperone activity, but the PDZ1 domain is required for efficient protease activity, with binding of substrates to the PDZ1 domain leading to allosteric activation of the protease domain (79, 83-85).

The crystal structure of substrate-free DegP revealed that it assembles into hexamers composed of two trimers that interact through loops that extend from the protease domains of one trimer into the other trimer and block the formation of the active conformation of its active sites (86). Upon an increase in temperature, conformational changes occur in these loops, suggesting a possible mechanism for DegP's temperature-dependent switch in activity (87). Upon substrate binding DegP forms large 12-mer or 24-mer cage-like structures, built of trimers identical to those in the hexameric structure, but contacting each other through interactions between their PDZ domains (Fig. 5) (80, 88). The active sites of the protease domains have their active conformations and face the interior of the cage, suggesting cages may be formed upon substrate binding, allowing substrate degradation, which is followed by the dissociation of the DegP cages (80, 88). The cages interact with liposomes through positively charged regions on the cage surface and it has been suggested DegP cages could span the periplasm, allowing OMPs to travel from the inner to the outer membrane in a shielded environment (80). In addition to the formation of soluble cage structures, DegP can assemble into membrane-associated bowl-like structures and substrates can be bound within these bowls (89). However, the importance of these multi-trimeric structures for DegP's function *in vivo* is unclear. DegP mutants that

only form trimers retain chaperone and protease activity *in vitro* and can rescue the temperature sensitive phenotype of *degP* mutants *in vivo*, suggesting the formation of multi-trimeric complexes is not required for DegP's function *in vivo* (90).

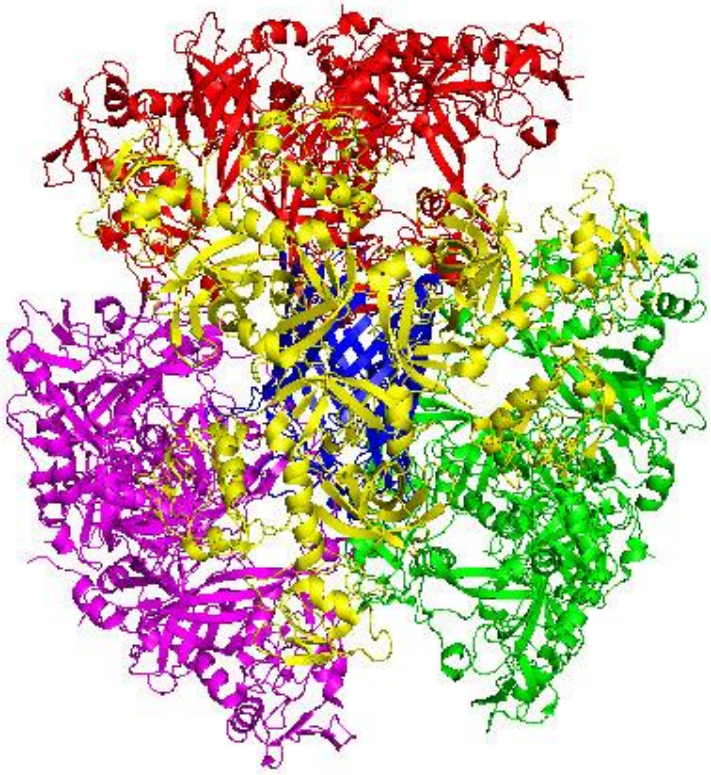

Figure 5: Structural model of a dodecameric DegP cage with a folded OmpC β-barrel in its cavity based on cryo-electron microscopy pictures and the crystal structures of DegP trimers and OmpC (PDB code 2ZLE (80)). The four DegP trimers are shown in yellow, red, pink and green and OmpC is shown in blue.

The OMP chaperone network

As SurA, Skp and DegP have all been implicated in OMP biogenesis the question arises as to how they cooperate to ensure the correct assembly of OMPs *in vivo*. A number of possible mechanisms have been proposed (Fig. 6). Skp and SurA may work sequentially, with Skp binding OMPs at the inner membrane and facilitating their release from the Sec translocon and SurA working downstream of Skp, transferring OMPs to the Bam machinery that inserts them into the membrane (2). This model is supported by the fact that Skp interacts with OMPs during their translocation across the inner membrane and that SurA interacts with the Bam complex, suggesting it may act at a later stage (21, 62). An alternative model is that SurA is responsible for the transport of the majority of OMPs across the periplasm, with Skp and DegP forming a back-up pathway through which OMPs that fall off the SurA pathway can be rescued or degraded (21). This view is supported by the fact that deletion of *surA* and *skp* or *surA* and *degP* is synthetically lethal, while the deletion of *skp* and *degP* is tolerated (21, 61). There is also no evidence that Skp and SurA interact, which would be required for the transfer of OMPs from Skp to SurA. Proteomics studies have shown that *surA* deletion leads to significant drops in the levels of a subset of OMPs in the outer membrane, while *skp* deletion does not

lead to significant changes in the levels of any OMPs (49, 91). Depletion of SurA from a *skp* deletion mutant leads to a drop in the levels of almost all OMPs, providing further evidence that SurA is the major OMP chaperone, with Skp playing a secondary role (49). Based on the observations that Skp and SurA bind unfolded OMPs with quicker kinetics, but more weakly than DegP and that OMPs are transferred to DegP after binding by Skp or SurA *in vitro*, a new model has been proposed where Skp and SurA bind OMPs immediately after their release from the Sec translocon and then transfer them to DegP, which is responsible for their further processing (92). However, it is possible that the slow transfer of unfolded OMPs from Skp and SurA to DegP reflects the fact that DegP is responsible for the degradation or safe sequestration of OMPs when their assembly into the outer membrane is deficient.

Periplasmic PPIases

In addition to SurA, the periplasm of *E. coli* contains three other PPIases: the FKBP FkpA, the cyclophilin PpiA and the parvulin-like PPIase PpiD. Though these proteins are all PPIases, and thus might be expected to function similarly, they appear to have distinct functions in periplasmic protein folding.

PpiD

Like SurA, PpiD is a parvulin-like PPIase whose main function is believed to be that of a molecular chaperone. The *ppiD* gene was originally identified as a multicopy suppressor of the phenotype of *surA* mutants and deletion of *ppiD* and *surA* was reported to be synthetically lethal (93). However, later studies showed that a *ppiD surA* mutant can be created and that its phenotype is not distinguishable from that of a *surA* mutant (47, 94). Overexpression of PpiD cannot restore the phenotype of a *surA* mutant, but does rescue the synthetic lethality of SurA depletion in a *skp* mutant and leads to somewhat higher levels of OMPs in this mutant (94). *ppiD* is regulated by both the periplasmic Cpx stress response and the cytoplasmic σ^{32} heat shock response, indicating that it is involved in protein quality control (93).

PpiD is anchored to the inner membrane by an N-terminal transmembrane helix and has a large periplasmic moiety consisting of an N-terminal domain that displays sequence similarity to the N-terminal domain of SurA, a catalytically inactive parvulin-like PPIase domain, which is structurally very similar to the P1 domain of SurA, and a C-terminal domain of unknown function (95). Like SurA, PpiD displays chaperone activity that is independent of its parvulin domain and binds peptides, though PpiD displays broader substrate specificity than SurA (94, 96). PpiD binds substrates during their translocation across the inner membrane and its membrane localisation is required for its function *in vivo* (94, 97). Based on these observations it has been proposed that PpiD plays a role in the early folding steps of a wide range of periplasmic proteins (94).

The role of PpiD in rescuing the lethality of the deletion of *skp* and *surA* is interesting, as it suggests that OMPs can be transported to the outer membrane independently of both Skp and SurA. It has been proposed that in the absence of both chaperones PpiD can perform the function that is usually performed by Skp in the Skp/DegP pathway, functioning in the early stages of OMP maturation, with DegP functioning at a later stage (94). In this context it is also interesting to note that the periplasmic moiety of BamA can adopt an extended conformation of up to 140 Å in length (98). This is only 30-40 Å shorter than the estimated thickness of the periplasm, which raises the intriguing possibility that BamA may be able to interact directly with an inner membrane-anchored protein like PpiD.

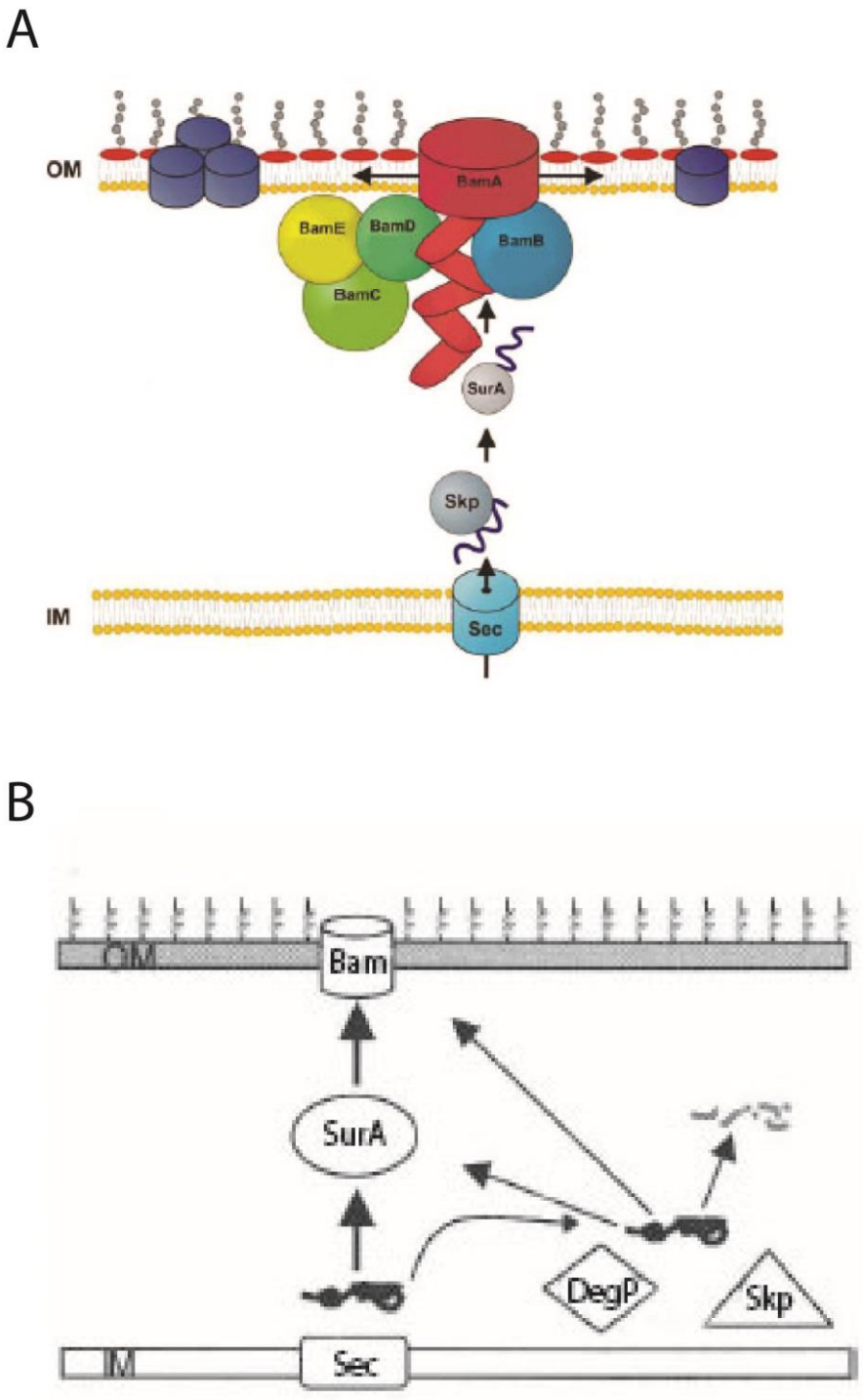

Figure 6: Proposed pathways for the transport of OMPs across the periplasm. (A): the sequential pathway: OMPs are bound by Skp as they are translocated across the inner membrane. Skp then transfers the OMPs to SurA. SurA interacts with the Bam machinery and transfers the OMPs to it, allowing their incorporation into the outer membrane. DegP is responsible for degrading any OMPs that fall off the transport pathway (B): the parallel pathways. SurA is solely responsible for the transport of most OMPs from the Sec machinery to the Bam machinery. Skp and DegP work together to process any OMPs that fall off the SurA pathway. OMPs may be directed back to SurA or directly to the Bam machinery by Skp/DegP. OMPs that cannot be rescued are degraded by DegP. (A) was adapted from (99) and (B) was adapted from (21).

FkpA

The FkpA PPIase was first implicated in periplasmic protein folding when it was identified in an overexpression screen for genes that decrease the activity of the σ^E response in *E. coli* strains with defects in outer membrane biogenesis (46). Inactivation of *fkpA* induces the σ^E response, but does not cause any further phenotype (46, 47). It does, however, lead to increased temperature sensitivity of a *degP* mutant (100). The *fkpA* gene is regulated by the σ^E response (40). FkpA assists the folding of soluble periplasmic proteins through its PPIase-independent chaperone activity (100-102). This may be its main function *in vivo*, as catalytically inactive FkpA can decrease the induction of the σ^E stress response in a *fkpA degP* mutant almost as well as wild type FkpA (100).

FkpA consists of two domains, a C-terminal FKBP PPIase domain and an N-terminal dimerisation domain. It forms V-shaped dimers, with the N-terminal domains forming a core module (103). Two α-helical arms extend away from this core module, ending in the FKBP domains (103). It is unclear what part of FkpA is responsible for its chaperone activity. Both individual domains display chaperone activity *in vitro*, but when expressed individually neither domain can supress the protein folding stress caused by *fkpA* deletion in cells lacking DegP, suggesting the entire protein is required for proper chaperone activity *in vivo* (100, 103, 104). An NMR-study suggested that substrates mainly bind to the FKBP domains and that the α-helical linkers are flexible, allowing the dimer to adapt its shape to accommodate differently sized substrates (105).

PpiA

Little is known about the fourth periplasmic PPIase, PpiA. It consists of a single PPIase domain of the cyclophilin type and displays PPIase activity *in vitro* (106, 107). The *ppiA* gene is part of the Cpx regulon, suggesting PpiA plays a part in periplasmic protein folding (42). *ppiA* mutants have no phenotype, though the inactivation of *ppiA* did cause slight growth defects in *fkpA ppiD, ppiD surA* and *fkpA ppiD surA* backgrounds (47, 108). PpiA's exact cellular function is unclear.

The disulphide bond pathway

Unlike the cytoplasm, the periplasm is an oxidising environment, meaning periplasmic proteins can contain disulphide bonds. The formation of the correct disulphide bonds in the periplasm of *E. coli* is achieved by a group of proteins collectively known as the Dsb proteins.

DsbA

The main disulphide forming protein in *E. coli* is DsbA. It was first implicated in disulphide bond formation when it was discovered that inactivation of the *dsbA* gene leads to defective disulphide bond formation and protein folding in the periplasm (109, 110). DsbA is a disulphide oxidoreductase consisting of a thioredoxin domain with the characteristic C-X-X-C catalytic motif and an α-helical cap domain (111). A disulphide bond can be formed between the two cysteine residues of the active site (C30 and C33). This disulphide bond is unstable, as the reduced form of DsbA is stabilised by the fact that C30 is deprotonated to a thiolate anion under physiological conditions, leading to the formation of a stabilising hydrogen bonding network (112, 113). DsbA's unstable disulphide bond is easily transferred to its substrates, which are thought to bind to DsbA in the area between its thioredoxin domain and α-helical cap domain (114). In agreement with its role as a disulphide forming enzyme DsbA is found in its oxidised state *in vivo* and is maintained in this state by the inner membrane protein DsbB (115). Various cysteine-containing cell envelope proteins have been shown to be DsbA substrates *in vivo*, including OmpA, DegP and RNaseI (116, 117).

DsbC

Another protein involved in disulphide bond formation in *E. coli* is DsbC. This protein was first implicated in disulphide bond formation when it was found that *dsbC* mutants have a similar phenotype to *dsbA* mutants, with increased sensitivity to DTT and benzylpenicillin and an inability to grow on minimal media lacking cysteine, and that overexpression of DsbC rescues the phenotype of a *dsbA* mutant and vice versa (118, 119). *dsbC* mutants display delayed disulphide bond formation in proteins containing multiple disulphide bonds, but not in proteins containing a single disulphide bond (120). DsbC contains a C-X-X-C catalytic motif that can form an unstable disulphide bond, but is found exclusively in the reduced form *in vivo* and is maintained in this form by the inner membrane protein DsbD (121, 122). DsbC stimulates the folding of proteins containing incorrect disulphide bonds more effectively than DsbA, but is less effective than DsbA at oxidising fully reduced proteins (121, 122). Based on these observations DsbC was proposed to act as a disulphide isomerase, catalysing the rearrangement of any incorrect disulphide bonds formed by DsbA. DsbC forms an intermolecular disulphide bond between a cysteine in its active site and one of its substrate's cysteine residues involved in the incorrect disulphide bond. Following this a new disulphide bond can be formed within the substrate or the substrate can be released in its reduced form. It has been suggested that DsbA co-translocationally forms consecutive disulphide bonds and that DsbC is required for their isomerisation to non-consecutive disulphide bonds (122, 123). All the proteins identified as DsbC substrates *in vivo* (RNaseI, MepA, AppA and LptD) contain a non-consecutive disulphide bond (116, 122-125). However, DsbA can correctly fold RNaseI *in vivo*, suggesting it does not exclusively form consecutive disulphide bonds (126). DsbC is able to function as a back-up disulphide bond forming enzyme in the absence of DsbA, possibly utilising disulphide bonds formed after the reduction of incorrect bonds to form new bonds in substrate proteins (127). It also displays chaperone activity that is independent of its catalytic activity, preventing the aggregation and assisting the refolding of denatured GADPH and lysozyme *in vitro* (33, 128). However, catalytically inactive DsbC variants were not able to rescue a *dsbC* mutant's phenotype, proving that its disulphide isomerase activity is more important for its function *in vivo* than its chaperone activity (118).

DsbC forms V-shaped dimers, with the C-terminal catalytic thioredoxin domains at the ends of two α-helical arms that extend away from an N-terminal dimerisation module (129). The inside of the V forms a hydrophobic cleft, providing a likely binding site for misfolded protein substrates (129). The presence of the N-terminal dimerisation domains, which allow the formation of this hydrophobic stretch, is required for chaperone and isomerase activity (130). Conformational changes in the α-helices connecting the thioredoxin domains to the dimerisation domains may allow DsbC dimers to adapt their shape, enabling them to bind differently sized substrates (129). Thus it appears DsbC functions by recognising misfolded proteins with incorrect disulphide bonds by binding them in its hydrophobic cleft and subsequently reducing their disulphide bonds leading to the formation of the correct disulphide bond in the substrate or the release of a reduced form of the substrate.

DsbG

There is a third disulphide oxidoreductase in the periplasm of *E. coli*: DsbG. DsbG was first identified as a disulphide oxidoreductase when it was discovered that *dsbG* mutants display heightened sensitivity to DTT and that overexpression of DsbG can partially restore the phenotypes of *dsbC* and *dsbA* mutants (131). Overexpression of DsbG increases the yield of heterologous proteins with multiple disulphide bonds expressed in a *dsbC* mutant and it is maintained in its reduced form by DsbD *in vivo* (132). In addition, DsbG is similar to DsbC in that it displays chaperone activity that is independent of its catalytic activity (133). This suggests that DsbG may be functionally similar to

DsbC and thus function as a disulphide isomerase. However, unlike DsbC, DsbG does not display disulphide isomerase activity *in vitro* and attempts to find proteins dependent on DsbG for their folding *in vivo* failed to identify any (116, 127, 134). Instead, DsbG interacts with proteins containing a single cysteine residue that is essential for their activity and maintains this cysteine residue in its reduced form, preventing its oxidation to a sulfenic acid (135). Thus it appears DsbG is responsible for keeping these proteins in their active form. DsbC can also perform this function, though less efficiently than DsbG (135).

DsbG forms V-shaped dimers similar to those formed by DsbC (136). DsbG dimers differ from DsbC dimers in the size and properties of their substrate binding cleft (Fig. 7). The DsbG cleft is larger than that of DsbC and the surface of the cleft displays negatively charged patches (136). This is consistent with the hypothesis that DsbC binds unfolded proteins in its smaller, hydrophobic cleft, whereas DsbG's larger, negatively charged cleft allows it to interact with folded protein substrates.

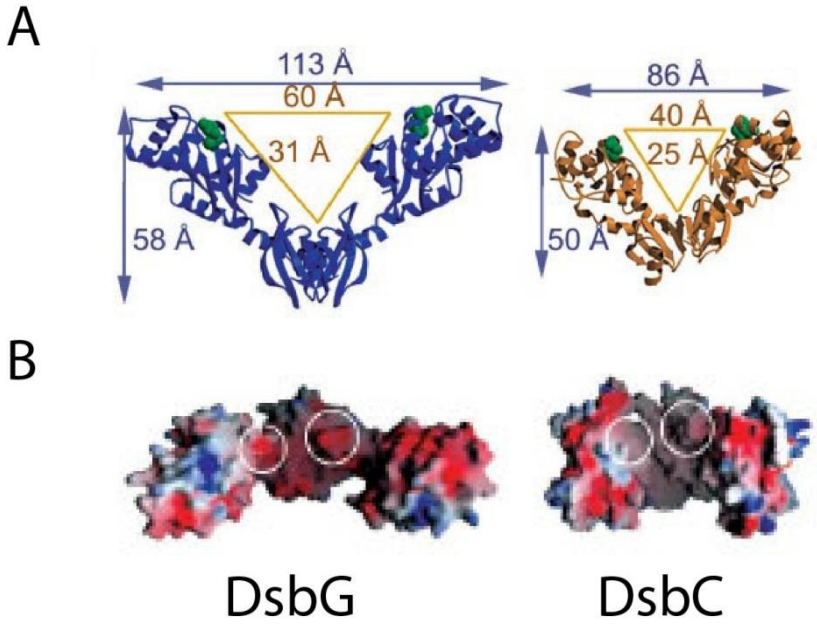

Figure 7: Structural comparison of DsbC and DsbG. (A): side view of the V-like structures showing the dimensions of the substrate binding clefts. (B): top view of the V-like structures with the surface electrostatic potential shown (red=negative, blue=positive). The circles indicate highly charged patches in DsbG that are uncharged in DsbC. Taken from (136).

Spy

The chaperone Spy was only recently identified as a player in periplasmic protein folding. Overexpression of Spy was found to rescue *E. coli* cells made dependent on the correct folding of an unstable variant of the protein Im7 (137). The *spy* gene is regulated by the Cpx stress response, suggesting a function in protein quality control (42). Spy displays chaperone activity *in vitro*, preventing the aggregation and assisting the folding of various model substrates and preventing the formation of amyloid fibres of the curli protein CsgA (137, 138). Spy forms dimeric α-helical cradle-like structures and substrate binding studies suggested substrates may bind to large areas of Spy, suggesting Spy may be able to form a coat around aggregation-prone protein regions (137).

The lipoprotein chaperone LolA

The N-terminal lipid moiety of lipoproteins makes them poorly soluble in water and thus this moiety must be shielded from the aqueous periplasm during transport to the outer membrane. In *E. coli* the essential protein LolA is responsible for the release of lipoproteins from the inner membrane by forming soluble LolA-lipoprotein complexes and their specific transfer to the outer membrane lipoprotein LolB, which is responsible for anchoring them in the outer membrane (139-141). LolA forms a partial β-barrel, with 3 α-helices forming a lid that shields a hydrophobic cavity on the inside of the barrel from the aqueous environment (142). This lid can adopt an open conformation, exposing the hydrophobic cavity and allowing the binding of lipoprotein's lipid moieties within it, but in the absence of lipoproteins the lid is closed (143).

Lipoproteins destined for the outer membrane are bound by the inner membrane ABC-transporter composed of LolC, LolD and LolE and are released from the membrane utilising energy from the hydrolysis of cytoplasmic ATP by LolD (144). Then the lipoproteins are transferred to LolA, which opens its lid to allow lipoprotein binding (Fig. 8) (144). LolA shields the lipid moieties of the lipoproteins during transport across the periplasm. At the outer membrane LolA transfers the lipoproteins to LolB. LolB is structurally similar to LolA in that it forms a partial β-barrel containing a hydrophobic cavity with 3 α-helices at the open side (142). NMR studies revealed that LolA and LolB interact by the binding of the inside of LolA's β-barrel to the outside of LolB's and that this interaction triggers conformational changes in the entire LolA molecule (145). It has been proposed that LolA and LolB form a tunnel-like structure through which the lipoproteins can be transferred from LolA to LolB (145). Transfer of lipoproteins is likely driven by the fact that LolB-lipoprotein complexes are more stable than LolA-lipoprotein complexes, due to differences in the properties of their hydrophobic cavities and the stabilisation of LolA's closed conformation by hydrogen bonds between an arginine residue on the inside of the β-barrel and residues in the α-helical lid (142).

The acid stress chaperones HdeA and HdeB

HdeA and HdeB are periplasmic chaperones that protect *E. coli* from the effects of acid stress. Upon exposure to an external milieu with a pH of 1-3, e.g. the stomach of mammalian organisms, *E. coli* can maintain its cytoplasmic pH at an acceptable level (approximately 4.5) by the activation of amino acid decarboxylase systems (9). Amino acid decarboxylases utilise protons to decarboxylate glutamate, lysine or arginine, preventing the cytoplasmic pH from dropping too far (9). The periplasmic pH, however, is identical to that of the external milieu. At low pH values proteins are liable to unfold and aggregate and this must be prevented in order for *E. coli* to survive. The acid-activated chaperones HdeA and HdeB are responsible for accomplishing this.

HdeA and HdeB both form ordered dimers at neutral pH which dissociate into disordered monomers at low pH (146-148). Only the disordered monomers display chaperone activity, preventing the acid-induced aggregation of periplasmic proteins (146-148). The two chaperones display different pH optima, with HdeA being most effective at pH 2 and HdeB being most effective at pH 3, probably due to the HdeB dimers dissociating at a higher pH than the HdeA dimers (148). However, both chaperones are required for effective acid resistance at either pH *in vivo* (148).

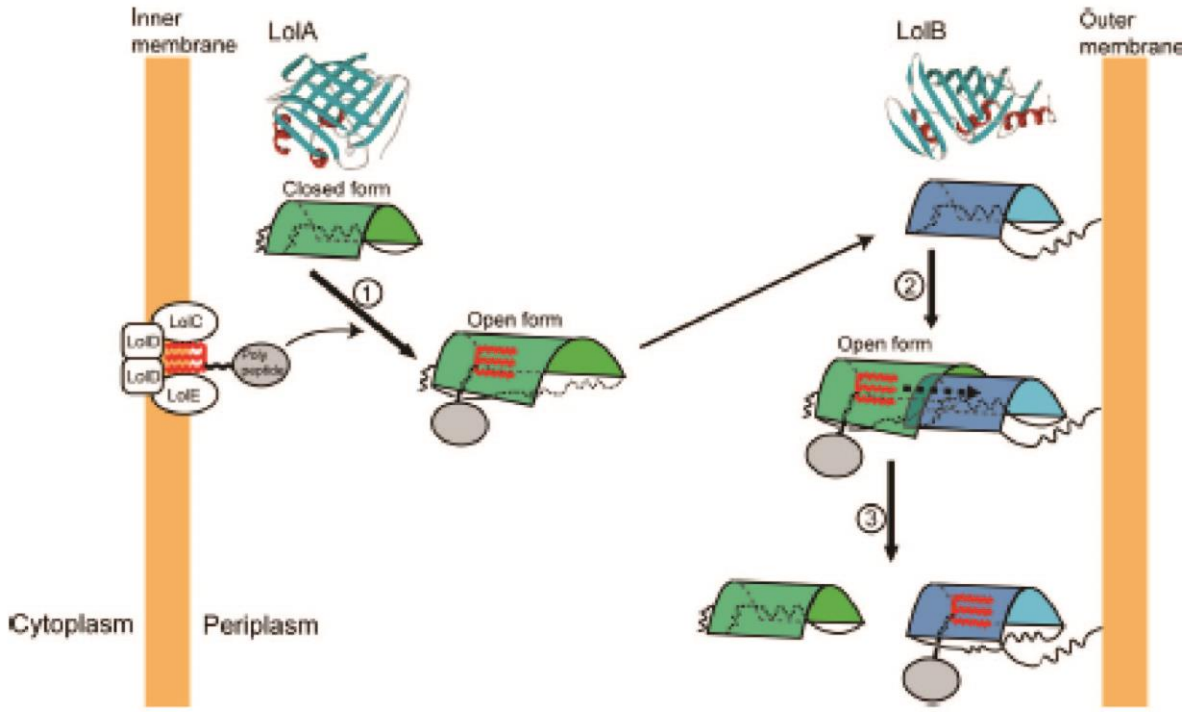

Figure 8: Schematic representation of the transport of lipoproteins across the periplasm by the Lol system. In substrate-free LolA an α-helical lid shields its hydrophobic cavity from the environment. Upon release of a lipoprotein from the inner membrane by LolCDE this lid opens, allowing the lipid moiety of the lipoprotein to bind to LolA [1]. LolA shields the lipid moiety from the aqueous environment during transport across the periplasm to the outer membrane lipoprotein LolB. LolA binds to LolB, triggering conformational changes in LolA and leading to the transfer of the lipoprotein from LolA to LolB [2]. LolB is responsible for inserting the lipoprotein into the outer membrane, while LolA is released in its closed form [3]. Taken from (145).

At neutral pH HdeA folds into compact α-helical monomers that form dimers with a highly hydrophobic dimer interface (147). Upon acid-induced dissociation of the dimers this hydrophobic region becomes exposed and can bind to hydrophobic regions of unfolded proteins, preventing their aggregation (146, 149). HdeA's disordered conformation at low pH enables it to adapt its structure to bind a variety of different substrates (150). Its N-and C-termini are positively charged at low pH and play an important role in maintaining the solubility of HdeA-substrate complexes (149). Acid-induced dissociation of HdeA dimers is a very fast process, enabling HdeA to bind unfolding substrates almost immediately after a drop in pH (151). Upon neutralisation of the pH substrates are released in their non-native state (151). This release is relatively slow, preventing the accumulation of high concentrations of aggregation-prone unfolded proteins and enabling the released substrates to refold, possibly assisted by other periplasmic chaperones (151, 152). Cross-linking studies revealed that a large number of periplasmic proteins, including SurA, DegP and FkpA, are HdeA substrates *in vivo* (152).

HdeB also forms dimers with a buried hydrophobic interface at neutral pH that dissociate to disordered monomers at low pH values, suggesting HdeB's mode of action is similar to that of HdeA (153). HdeB monomers adopt a very similar fold to HdeA monomers, but the relative orientation of the monomers within the dimer is different (153). Intermolecular salt bridges stabilise the HdeB dimer at neutral pH, but are broken at low pH, suggesting a possible mechanism for the pH-dependent dissociation of the dimers (153). An intramolecular salt bridge is also broken at low pH, possibly contributing to the heightened disorder in the monomers at low pH (153). HdeA dimers contain no

intermolecular salt bridges at neutral pH and it is unclear what triggers their dissociation at low pH. It is also unclear why both chaperones are required for efficient acid resistance at pH 2 and 3 *in vivo*, a possible explanation could be that the proteins act on a different set of substrates.

Periplasmic folding factors in other Gram-negative bacteria

Periplasmic folding factors in Gram-negative bacteria other than *E. coli*

Though the periplasmic chaperones and folding catalysts present in *E. coli* have been studied extensively, far less is known about the situation in other Gram-negative bacteria. Homologues of periplasmic chaperones have been shown to be involved in periplasmic protein folding in various Gram-negative bacteria. A SurA homologue has been shown to be involved in OMP biogenesis in *Salmonella enterica,* with deletion of the *surA* gene leading to lowered levels of various OMPs (154). Deletion of the gene encoding for a DegP homologue did not affect OMP levels (154). In *Shigella flexneri* Skp, SurA and DegP homologues are required for the surface presentation of the autotransporter IscA (155). Skp is thought to assist the folding of the soluble extracellular domain of IscA in the periplasm, while the exact contributions of SurA and DegP are unknown (156). In *Dickeya dadantii* Skp and SurA homologues are involved in OMP biogenesis and SurA is required for the transport to the outer membrane of PnlH, a protein that is translocated into the periplasm by the Tat machinery and is subsequently anchored in the outer membrane by its uncleaved Tat signal sequence (157).

Relatively recently, two species, *Bordetella pertussis* and *Campylobacter jejuni*, have been shown to contain similar chaperones that do not have a clear *E. coli* homologue, named Par27 and PEB4 respectively (158, 159). These chaperones display certain similarities to SurA. They are both PPIases of the parvulin type and consist of a single PPIase domain flanked by N-and C-terminal domains (158, 159). Both Par27 and PEB4 display chaperone and PPIase activity *in vitro* (158, 159). Unlike SurA, substrate-free Par27 and PEB4 form U-shaped dimers (Fig. 9) (159, 160). The bottom of the U is formed by the N and C domains that fold into two modules that are structurally highly similar to the N+C fragment of SurA (159, 160). The formation of these modules is dependent upon dimerisation as

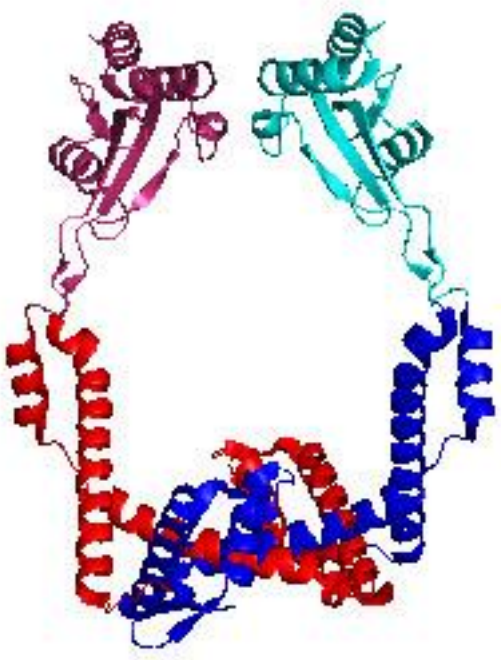

Figure 9: Crystal structure of PEB4. The structure of a single PEB4 dimer is shown (PDB code 3FRW (159)). One monomer has its N-and C-terminal domains coloured red and its PPIase domain coloured pink. The other monomer has its N-and C-terminal domain coloured blue and its PPIase domain coloured cyan.

both monomers contribute to both of the modules. The PPIase domains are separated from the dimerisation moiety by α-helical arms (159, 160). *C. jejuni peb4* mutants display an altered OMP profile and Par27 preferentially binds OMPs, suggesting these proteins may act as periplasmic OMP chaperones (158, 161). The exact role these chaperones play in OMP biogenesis is unclear and both species also contain a SurA homologue. However, the *C. jejuni* SurA homologue, which only has a single PPIase domain, did not display any chaperone activity *in vitro* and its function *in vivo* is unclear (159).

Disulphide bond forming enzymes have been identified and studied in various bacteria (162). Many of the systems in other bacteria differ from that of *E. coli*. For example, *Neisseria meningitidis*, the bacterium that causes meningitis, contains three homologues of DsbA, single homologues of DsbB, DsbC and DsbD, and no homologue of DsbG (163). Two of the three DsbA homologues are inner membrane lipoproteins, with the third being a soluble periplasmic protein (164). They appear to have distinct functions *in vivo*, possibly due to differences in their substrate specificity (163, 164).

The Lol system for lipoprotein transport is quite widely conserved, with homology searches revealing that over 300 of the 529 bacteria species studied encode LolC/E and LolA homologues (165). Only 153 species were found to encode a LolB homologue, suggesting the mechanism of lipoprotein insertion into the outer membrane may vary more than the mechanisms of release from the inner membrane and transport across the periplasm (165). Some bacteria also contain lipoproteins that are anchored to the outer leaflet of the outer membrane and it is unclear how these lipoproteins are translocated across the membrane. *Pseudomonas aeruginosa* is the only Gram-negative bacterium other than *E. coli* in which the Lol system has been biochemically characterised. Homologues of all five Lol proteins are present in *P. aeruginosa* and they perform the same functions as in *E. coli* (166). The *P. aeruginosa* Lol system does differ from that of *E. coli* in that the *P. aeruginosa* LolCDE ABC-transporter recognises other inner membrane retention signals in addition to the Asp2 Lol avoidance signal. Proteins with a lysine residue at position 3 or a serine residue at position 4 are not released from the inner membrane by *P. aeruginosa* LolCDE, but are released by *E. coli* LolCDE (166, 167).

In addition to *E. coli*, *S. flexneri* and *Brucella abortus* have been shown to contain HdeA homologues that are important for acid resistance, with mutations in the *hdeA* gene leading to significant reductions in the acid resistance of both species (168, 169).

A bioinformatics search for potential periplasmic chaperones in *N. meningitidis*

The periplasm of *N. meningitidis* contains homologues of Skp and SurA, and a single HtrA-family protease, which is most similar to *E. coli* DegQ, an HtrA-family protease that can compensate for the loss of DegP when overexpressed (170). As in *C. jejuni*, the *N. meningitidis* SurA homologue only has one PPIase domain (170). The function of these proteins in OMP biogenesis seems to differ from that of their *E. coli* counterparts. The deletion of *skp* and *surA* or *degQ* and *surA* is not synthetically lethal in *N. meningitidis* and the deletion of *surA* does not cause any changes in OMP profile (170). *Skp* mutants do display lowered porin levels, but this is caused by a drop in porin expression levels rather than defective porin assembly and various more minor OMPs are not affected (170). Thus it appears that SurA does not play a significant role in OMP biogenesis in *N. meningitidis* or that *N. meningitidis* contains additional chaperones that can compensate for the loss of SurA. Skp appears to play a role in the biogenesis of porins, but not of other OMPs. This suggests that other periplasmic chaperones must be involved in the transport of OMPs across the periplasm in *N. meningitidis*. To identify potential candidates for this function a bioinformatics search was performed to find *N. meningitidis* homologues of the other periplasmic folding factors identified so far.

BLAST searches using the amino acid sequences of periplasmic chaperones and folding catalysts from *E. coli* revealed that *N. meningitidis* strain MC58 contains homologues of the periplasmic PPIases PpiD, FkpA and PpiA. The gene product of the gene NMB1238 was identified as a PpiD homologue. This gene encodes for a protein predicted to be 512 amino acids in length. As predicted using the NCBI conserved domain search engine, it has a putative PPIase domain (aa 230-372) and an N-terminal domain belonging to the SurA N-terminal domain superfamily (aa 1-140). It has a predicted transmembrane helix (aa 12-31), suggesting that, like PpiD, it spans the inner membrane (as predicted using the TMHMM 2.0 server). The gene product of NMB1567 was identified as a homologue of *E. coli* FkpA. This gene is predicted to encode a 272 amino acid protein containing a FKBP PPIase domain (aa 162-250) and a domain similar to the N-terminal domain of FkpA (aa 43-155). It has a predicted signal sequence (aa 1-22), suggesting it is a periplasmic protein (as predicted using the SignalP 4.1 server). A BLAST search using the sequence of *E. coli* PpiA identified two possible homologues, encoded for by NMB1262 and NMB0791. Like PpiA, both proteins are predicted to be composed of a single PPIase domain of the cyclophilin family. However, NMB1262 contains a predicted signal sequence (aa 1-22), whereas NMB0791 does not. Thus it appears that the NMB1262 gene product is the *N. meningitidis* PpiA homologue, with the NMB0791 gene product being a cytoplasmic cyclophilin-like PPIase. BLAST searches using the sequences of *E. coli* Spy, HdeA and HdeB failed to identify any homologous proteins in *N. meningitidis.*

As Par27 and PEB4 may function as OMP chaperones in *B. pertussis* and *C. jejuni* respectively BLAST searches were performed to identify potential homologues of these proteins in *N. meningitidis*. Both searches identified the gene product of NMB0345 as a potential homologue. Both proteins also had regions of sequence similarity to the *N. meningitidis* SurA homologue. The predicted NMB0345 gene product is a 288 amino acid protein containing a parvulin-like PPIase domain (aa 133-259) and a predicted signal sequence (aa 1-20). A BLAST search using the NMB0345 protein sequence failed to identify any homologues of this protein in *E. coli*.

To identify other potential SurA-like PPIases in *N. meningitidis*, BLAST searches were performed using the sequences of SurA's PPIase domains. Searching with the sequence of the P2 domain, the gene product of NMB0346 was found to contain a parvulin-like PPIase domain. This 252 amino acid predicted protein has its predicted PPIase domain near its C-terminus (aa 112-226) and has an N-terminal signal sequence (aa 1-23). The NMB0346 gene is located directly downstream from the NMB0345 gene and the two proteins display some sequence similarity (22% identical, 43% similar). Neither protein has a homologue in *E. coli*. However, BLAST searches revealed that homologues of both proteins are present in many species within the *Neisseria* genus and in other β-proteobacteria, particularly in members of the orders *Burkholderiales* and *Neisseriales*.

The lipoprotein transport system of *N. meningitidis* has not been characterised. To identify potential members of this system BLAST searches were performed using the amino acid sequences of the members of the *E. coli* Lol system. BLAST searches using the sequence of *E. coli* LolA identified the gene product of NMB0622 as a potential homologue. This predicted protein is 207 amino acids long, has a predicted signal sequence (aa 1-25) and contains a single domain belonging to the LolA family (aa 36-165). BLAST searches using the sequence of *E. coli* LolB identified the NMB0873 gene product as a potential homologue. NMB0873 encodes for a predicted protein of 193 amino acids. This protein has a predicted cleavage site for signal peptidase II, the signal peptidase that cleaves off the signal sequences of lipoproteins, directly before Cys16 of the full sequence (as predicted by the LipoP 1.0 server). The residue at position 17 of the full sequence is an alanine, suggesting this protein is an outer membrane lipoprotein. BLAST searches using the sequences of LolC and LolE revealed that *N. meningitidis* contains a single protein that is highly homologous to these two *E. coli* proteins: the gene

product of NMB1235. This 415 amino acid protein is predicted to have four transmembrane helices and its topological organisation is predicted to be very similar to that of LolC and LolE. *N. meningitidis* also contains a LolD homologue. A BLAST search using the sequence of *E. coli* LolD identified a large number of potential homologues in *N. meningitidis*, indicative of the presence of many different ABC-transporters in this organism. The highest scoring homologue was the gene product of NMB1234, a 231 amino acid protein. The NMB1234 gene is located directly downstream from the NMB1235 gene. This genomic location is similar to that of the *lolD* gene in *E. coli*, which is located in between the *lolC* and *lolE* genes. The fact that only one LolC/E homologue was found in *N. meningitidis* suggests that the transmembrane moiety of its lipoprotein releasing ABC-transporter may be composed of a homodimer of this protein.

Table 1: Potential periplasmic folding factors identified in *N. meningitidis* MC58 by homology searches.

Characterised homologue	*N. meningitidis* MC58 locus tag
PpiD (*E. coli*, YP_488733)	NMB1238
FkpA (*E. coli*, YP_492085)	NMB1567
PpiA (*E. coli*, YP_492068)	NMB1262
Par27 (*B. pertussis*, NP_882074)/PEB4 (*C. jejuni*, YP_001482134)	NMB0345
None, but contains a parvulin-like PPIase domain	NMB0346
Spy (*E. coli*, YP_490004)	No homologue found
HdeA (*E. coli*, YP_491925)	No homologue found
HdeB (*E. coli*, YP_491926)	No homologue found
LolA (*E. coli*, YP_489163)	NMB0622
LolB (*E. coli* YP_489476)	NMB0873
LolC/LolE (*E. coli*, YP_489384/YP_489386)	NMB1235
LolD (*E. coli*, YP_489385)	NMB1234

In summary, various potential periplasmic folding factors have been identified in *N. meningitidis* by homology searches (Table 1). The gene products of NMB1238, NMB1567 and NMB1262 were identified as homologues of the *E. coli* PPIases PpiD, FkpA and PpiA respectively. NMB0345 was found to be homologous to *B. pertussis* Par27 and *C. jejuni* PEB4. NMB0346 appears to be a periplasmic parvulin-like PPIase with some sequence similarity to NMB0345. These potential periplasmic folding factors may provide a starting point for further investigations into the periplasmic aspect of OMP biogenesis in *N. meningitidis.* Especially NMB0345 may be of interest in this context as Par27 and PEB4 have been postulated to be involved in OMP biogenesis in *B. pertussis* and *C. jejuni* respectively. NMB1238 is also interesting as PpiD over-expression can rescue the synthetic lethality of *surA* and *skp* deletion in *E. coli*. SurA may also play a part, albeit a more minor one than in *E. coli*, with other periplasmic chaperones being able to fully compensate for its absence. NMB0346 is also an interesting candidate as it is not strongly homologous to any of the periplasmic chaperones characterised so far. *N. meningitidis* contains homologues of all members of the Lol system, though it only contains a single LolC/E homologue (NMB1235) suggesting the transmembrane moiety of the *N. meningitidis* Lol system's ABC-transporter may be a homodimer. It seems likely that this system functions in a similar manner to that of *E. coli*. One interesting aspect of outer membrane lipoprotein biogenesis in *N. meningitidis* is that, unlike *E. coli*, it contains lipoproteins that are anchored to the outer leaflet of the outer membrane (2). The mechanism by which these lipoproteins are transported across the outer membrane is unclear and identifying the proteins involved in this process could be an interesting future research line.

Novel methods in chaperone research

Traditionally, novel chaperones have been identified based on their deletion phenotypes indicative of defective protein folding, induction by stress response systems or homology to known chaperones. Their chaperone activity could then be confirmed and studied using a variety of *in vitro* biochemical assays. These methods have their limitations. Certain minor chaperones may not display a discernible deletion phenotype, particularly under non-stress conditions. The *in vivo* substrate specificity of chaperones was often determined by studying the expression levels or folding status of a small number of potential substrate proteins, e.g. by Western blotting. This does not yield a comprehensive picture of a chaperone's set of substrates *in vivo*. In the last 10 years or so a number of methods have been developed that have the potential to solve these issues in the future.

A technology that has the potential to be used extensively in chaperone research in the future is mass spectrometry based proteomics. This technology can be used to evaluate the *in vivo* substrate specificities of chaperones in a more comprehensive manner than is possible using traditional methods. Proteomics can be used to evaluate the effect of the depletion of chaperones on the abundance of many of the proteins in a cell or subcellular compartment, e.g. the bacterial cell envelope. For example, this method has been used to determine the effect of the deletion of the *surA* or *skp* genes on the outer membrane proteome (49, 91). Mass spectrometry can also be combined with pull-down or *in vivo* cross-linking experiments to identify the binding partners of a chaperone. The binding partners of Skp, DsbA and DsbG have been identified in this manner (74, 117, 135). In future, proteomics methods will greatly facilitate the evaluation of the *in vivo* substrate specificity of newly identified chaperones.

Another novel method that has great potential for future research into periplasmic folding factors is the use of protein reporters to evaluate the folding status of a protein *in vivo*. Using this method the folding factors that are important for the folding of a certain target protein can be identified. The basic concept of this method is that a fusion protein is created consisting of the target protein fused to a reporter protein that is only activated when the target protein is correctly folded (171). Possible reporter systems include fluorescent proteins and proteins that provide resistance to antibiotics or other toxic molecules (171). An example is the split-GFP system, where a single β-strand of GFP is fused to the target protein and the remainder of GFP is expressed separately (Fig. 10) (172). The single β-strand can structurally complement the rest of the GFP molecule, leading to the formation of fluorescent GFP, but only if the target protein is correctly folded (172). A somewhat different approach is the use of sandwich fusion proteins. These fusion proteins are constructed by inserting the sequence of the target protein into the sequence of the reporter protein. The reporter protein can only fold into its native conformation if the target protein is folded correctly. If the chosen reporter protein confers resistance to certain toxic molecules, screening for strains with altered sensitivity to those molecules can identify strains where the folding of the target protein is improved or impaired. This strategy was used in the study that identified the Spy chaperone. An unstable variant of the Im7 protein was inserted into two different reporter proteins: DsbA, which is important for cadmium resistance in *E. coli*, and β-lactamase, which confers resistance to penicillin (137). After random mutagenesis strains with increased resistance to cadmium and penicillin were isolated and found to have increased levels of the Im7 variant due to the increased expression of the periplasmic chaperone Spy (137). Target-reporter fusion systems have good high-throughput potential and thus can be used

in genetic screens or random mutagenesis experiments designed to identify novel chaperones or to evaluate which chaperones are required for the correct folding of a certain target protein.

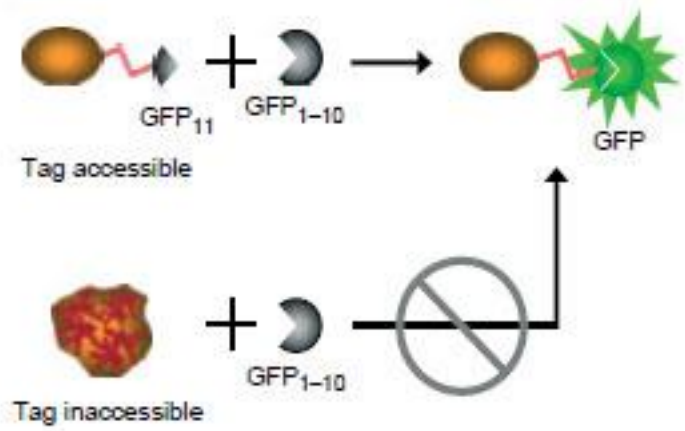

Figure 10: Schematic representation of the principle behind the split-GFP reporter system. A single β-strand of GFP (tag) is fused to a target protein and the remainder of GFP is expressed separately. If the target protein is correctly folded the GFP tag can structurally complement the remainder of GFP, yielding a fluorescence signal. If the target protein misfolds or aggregates the tag becomes inaccessible and can no longer complement GFP, leading to the absence of a fluorescence signal. Taken from (172).

A relatively new technology that could be used for the identification of novel periplasmic folding factors is the use of phenotype microarrays. This technology utilises 96-wells plates to determine the respiratory activity of a bacterial strain under many different growth conditions (173). Each well of the 96-wells plates contains a dried layer of chemicals at the bottom of the well, which forms the bacterial growth medium when rehydrated (173). The composition of this layer is different in every well, allowing a different growth condition to be tested in each well. The bacterial strain that is to be studied is added to the 96-wells plates as a cell suspension. This suspension contains a redox dye that turns purple when it is reduced (173). During incubation the appearance of this purple dye is used as a readout for the respiratory activity of the bacteria. This technique allows the effect of many different growth conditions on the respiratory activity of a bacterial strain to be evaluated in a high-throughput manner. Phenotype microarrays could be used for the phenotypical characterisation of strains where the genes encoding potential periplasmic folding factors have been knocked out. The simultaneous evaluation of many phenotypical traits yields a higher sensitivity than evaluating a small number of phenotypical traits. For example, deletion strains that display minor sensitivity to various markers for outer membrane biogenesis defects could easily be identified using this technique, even if the individual phenotypes are too minor to be declared significant using classical techniques, where the sensitivity to a small number of markers is tested.

Summarising Discussion

Over the last 25 years or so a lot of research has focused on the identification and characterisation of the factors involved in protein folding in the periplasm of *E. coli*. These efforts have led to a greater understanding of the function and mode of action of many of these factors. However, a number of critical questions remain unanswered.

One of the most extensively studied processes involving periplasmic folding factors is the transport of OMPs across the periplasm to the outer membrane. SurA, Skp and DegP have been shown to be involved in this transport process, but it is still unclear how these proteins work together to achieve the efficient transport of OMPs to the outer membrane. The recent *in vitro* reconstitution of a functional Bam complex provides a great framework to study the molecular mechanisms by which SurA, Skp and DegP interact with each other and with the Bam machinery to ensure that OMPs are efficiently incorporated into the outer membrane (174). It would also be interesting to evaluate the mechanism by which the overexpression of the inner membrane-anchored chaperone PpiD can rescue the lethality of the simultaneous deletion of *skp* and *surA* (94).

The Dsb proteins are responsible for the formation and maintenance of the correct disulphide bonds in the periplasm. DsbA is primarily responsible for the formation of disulphide bonds, DsbC for the isomerisation of incorrect disulphide bonds and DsbG for keeping free cysteine residues in their reduced form. Though the functions of these proteins are relatively well understood, questions still remain as to the mechanisms by which they recognise substrates and how this recognition is coupled to their catalytic activity.

The Lol system is responsible for the transport of outer membrane lipoproteins across the periplasm. Lipoproteins are released from the inner membrane by LolCDE, transported across the periplasm by LolA and inserted into the membrane by LolB. The functions of the various components of the Lol system have been determined, but questions still remain regarding the mechanisms by which they perform these functions. It is unclear how LolB assists the insertion of lipoproteins into the outer membrane. The molecular mechanism by which LolCDE recognises the inner membrane retention signals of inner membrane lipoproteins is another interesting aspect that is as yet poorly understood.

Surprisingly little is known about the role periplasmic folding factors play in the quality control of soluble periplasmic proteins. Folding factors may assist the folding of soluble periplasmic proteins by preventing the aggregation of folding intermediates. They are probably also required to prevent soluble periplasmic proteins from aggregating or misfolding under certain stress conditions. For example, the HdeA/B chaperones are responsible for preventing periplasmic proteins from aggregating upon a drop in the periplasmic pH. It is unclear why both of these chaperones are required for effective acid resistance at pH 2 and 3, despite their similar mode of action. The molecular mechanism of the acid-induced dissociation of HdeA dimers is also still unclear.

In addition to the acid stress-specific chaperones HdeA and HdeB, FkpA and Spy are the only folding factors identified so far whose main function appears to be assisting the folding or preventing the aggregation of soluble periplasmic proteins. Many other periplasmic folding factors, including SurA, Skp, DegP, PpiD, DsbC and DsbG, have been shown to display general chaperone activity *in vitro* and may assist the folding of soluble periplasmic proteins or help prevent their aggregation under stress conditions. Determining the exact roles of the folding factors identified so far in assisting the

folding of soluble periplasmic proteins and protecting them from aggregation under stress conditions will be a challenge for the future, as will the identification of novel folding factors involved in these processes. Systems that utilise target-reporter fusion proteins to report on the *in vivo* folding status of a target protein have great potential to facilitate the identification of such novel folding factors.

Though the periplasmic folding factors of *E. coli* have been extensively studied, far less is known about those of other Gram-negative bacteria. Though many Gram-negative bacteria may contain systems that are similar to those of *E. coli*, evidence exists that suggests the situation in some species differs substantially from that in *E. coli*. For example *B. pertussis* and *C. jejuni* have been shown to contain chaperones that are not present in *E. coli* yet play a part in OMP biogenesis in those organisms. *N. meningitidis* is an example of a Gram-negative bacterium where homologues of many of the periplasmic folding factors identified in *E. coli* are present, but where many of them appear to function slightly differently. In *N. meningitidis* SurA and Skp do not appear to play the crucial roles in OMP biogenesis they do in *E. coli* and three homologues of DsbA are present, which appear to have different substrate specificities. Elucidating the mechanisms of periplasmic protein folding in Gram-negative bacteria other than *E. coli*, particularly in those that are of interest due to their pathogenic properties, will provide many research opportunities in future. Evaluating the role of the potential folding factors identified in the homology searches presented here could provide a starting point for further research into the periplasmic protein folding systems of *N. meningitidis*.

Further research into the factors involved in periplasmic protein folding will not only lead to a better understanding of this fascinating process, but may have implications for biotechnology and medicine. Periplasmic folding factors may be of importance for the biotechnological production of recombinant proteins, preventing the periplasmic misfolding or aggregation of recombinant proteins targeted to the periplasm or destined for secretion. Periplasmic folding factors are also potential drug targets as they play important roles in bacterial cellular function. However, the transport of drugs across the outer membrane could pose a problem and the relatively unspecific substrate binding properties of most periplasmic folding factors could make designing small molecule inhibitors very challenging.

References

1. Silhavy, T.J., Kahne, D., and Walker, S. (2010) The bacterial cell envelope. *Cold Spring Harbor perspectives in biology.* **2,** a000414

2. Bos, M.P., Robert, V., and Tommassen, J. (2007) Biogenesis of the gram-negative bacterial outer membrane. *Annual Review of Microbiology.* **61,** 191-214

3. Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews.* **67,** 593-656

4. Sperandeo, P., Dehò, G., and Polissi, A. (2009) The lipopolysaccharide transport system of Gram-negative bacteria. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids.* **1791,** 594-602

5. Wimley, W.C. (2003) The versatile β-barrel membrane protein. *Current Opinion in Structural Biology.* **13,** 404-411

6. Ricci, D.P., and Silhavy, T.J. (2012) The Bam machine: A molecular cooper. *Biochimica et Biophysica Acta - Biomembranes.* **1818,** 1067-1084

7. Merdanovic, M., Clausen, T., Kaiser, M., Huber, R., and Ehrmann, M. (2011) Protein quality control in the bacterial periplasm. *Annual Review of Microbiology.* **65,** 149-168

8. Lovering, A.L., Safadi, S.S., and Strynadka, N.C.J. (2012) Structural perspective of peptidoglycan biosynthesis and assembly. *Annual Review of Biochemistry.* **81,** 451-478

9. Hong, W., Wu, Y.E., Fu, X., and Chang, Z. (2012) Chaperone-dependent mechanisms for acid resistance in enteric bacteria. *Trends in Microbiology.* **20,** 328-335

10. Luirink, J., Yu, Z., Wagner, S., and De Gier, J.-. (2012) Biogenesis of inner membrane proteins in *Escherichia coli.* *Biochimica et Biophysica Acta - Bioenergetics.* **1817,** 965-976

11. Chng, S.-., Gronenberg, L.S., and Kahne, D. (2010) Proteins required for lipopolysaccharide assembly in *Escherichia coli* form a transenvelope complex. *Biochemistry (N.Y.).* **49,** 4565-4567

12. Okuda, S., Freinkman, E., and Kahne, D. (2012) Cytoplasmic ATP hydrolysis powers transport of lipopolysaccharide across the periplasm in *E. coli*. *Science.* **338,** 1214-1217

13. Du Plessis, D.J.F., Nouwen, N., and Driessen, A.J.M. (2011) The Sec translocase. *Biochimica et Biophysica Acta - Biomembranes.* **1808,** 851-865

14. Dalbey, R.E., and Kuhn, A. (2012) Protein traffic in Gram-negative bacteria - how exported and secreted proteins find their way. *FEMS Microbiology Reviews.* **36,** 1023-1045

15. Dijkstra, A.J., and Keck, W. (1996) Peptidoglycan as a barrier to transenvelope transport. *Journal of Bacteriology.* **178,** 5555-5562

16. Van Heijenoort, J. (2011) Peptidoglycan hydrolases of *Escherichia coli*. *Microbiology and Molecular Biology Reviews.* **75,** 636-663

17. Scheurwater, E.M., and Burrows, L.L. (2011) Maintaining network security: How macromolecular structures cross the peptidoglycan layer. *FEMS Microbiology Letters.* **318,** 1-9

18. Kim, S., Malinverni, J.C., Sliz, P., Silhavy, T.J., Harrison, S.C., and Kahne, D. (2007) Structure and function of an essential component of the outer membrane protein assembly machine. *Science.* **317,** 961-964

19. Jansen, C., Heutink, M., Tommassen, J., and De Cock, H. (2000) The assembly pathway of outer membrane protein PhoE of *Escherichia coli*. *European Journal of Biochemistry.* **267,** 3792-3800

20. Robert, V., Volokhina, E.B., Senf, F., Bos, M.P., Van Gelder, P., and Tommassen, J. (2006) Assembly factor Omp85 recognizes its outer membrane protein substrates by a species-specific C-terminal motif. *PLoS biology.* **4,** e377

21. Sklar, J.G., Wu, T., Kahne, D., and Silhavy, T.J. (2007) Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. *Genes and Development.* **21,** 2473-2484

22. Bennion, D., Charlson, E.S., Coon, E., and Misra, R. (2010) Dissection of β-barrel outer membrane protein assembly pathways through characterizing BamA POTRA 1 mutants of *Escherichia coli*. *Molecular Microbiology.* **77,** 1153-1171

23. Knowles, T.J., Scott-Tucker, A., Overduin, M., and Henderson, I.R. (2009) Membrane protein architects: The role of the BAM complex in outer membrane protein assembly. *Nature Reviews Microbiology.* **7,** 206-214

24. Hartl, F.U., Bracher, A., and Hayer-Hartl, M. (2011) Molecular chaperones in protein folding and proteostasis. *Nature.* **475,** 324-332

25. Mayer, M.P. (2010) Gymnastics of molecular chaperones. *Molecular Cell.* **39,** 321-331

26. Buchner, J., Grallert, H., and Jakob, U. (1998) Analysis of chaperone function using citrate synthase as nonnative substrate protein. *Methods in Enzymology.* **290,** 323-338

27. Schroder, H., Langer, T., Hartl, F.-., and Bukau, B. (1993) DnaK, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. *EMBO Journal.* **12,** 4137-4144

28. Pal, D., and Chakrabarti, P. (1999) Cis peptide bonds in proteins: Residues involved, their conformations, interactions and locations. *Journal of Molecular Biology.* **294,** 271-288

29. Göthel, S.F., and Marahiel, M.A. (1999) Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. *Cellular and Molecular Life Sciences.* **55,** 423-436

30. Barik, S. (2006) Immunophilins: For the love of proteins. *Cellular and Molecular Life Sciences.* **63,** 2889-2900

31. Behrens, S., Maier, R., De Cock, H., Schmid, F.X., and Gross, C.A. (2001) The SurA periplasmic PPIase lacking its parvulin domains functions *in vivo* and has chaperone activity. *EMBO Journal.* **20,** 285-294

32. Messens, J., and Collet, J.-. (2006) Pathways of disulfide bond formation in *Escherichia coli*. *International Journal of Biochemistry and Cell Biology.* **38,** 1050-1062

33. Chen, J., Song, J.-., Zhang, S., Wang, Y., Cui, D.-., and Wang, C.-. (1999) Chaperone activity of DsbC. *Journal of Biological Chemistry.* **274,** 19601-19605

34. Ades, S.E. (2008) Regulation by destruction: Design of the σ^E envelope stress response. *Current Opinion in Microbiology.* **11,** 535-540

35. De Las Peñas, A., Connolly, L., and Gross, C.A. (1997) The σ^E-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of σ^E. *Molecular Microbiology.* **24,** 373-385

36. Cezairliyan, B.O., and Sauer, R.T. (2007) Inhibition of regulated proteolysis by RseB. *Proceedings of the National Academy of Sciences of the U.S.A.* **104,** 3771-3776

37. Walsh, N.P., Alba, B.M., Bose, B., Gross, C.A., and Sauer, R.T. (2003) OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. *Cell.* **113,** 61-71

38. Alba, B.M., Leeds, J.A., Onufryk, C., Lu, C.Z., and Gross, C.A. (2002) DegS and YaeL participate sequentially in the cleavage of RseA to activate the σ^E-dependent extracytoplasmic stress response. *Genes and Development.* **16,** 2156-2168

39. Flynn, J.M., Levchenko, I., Sauer, R.T., and Baker, T.A. (2004) Modulating substrate choice: The SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation. *Genes and Development.* **18,** 2292-2301

40. Rhodius, V.A., Suh, W.C., Nonaka, G., West, J., and Gross, C.A. (2006) Conserved and variable functions of the σ^E stress response in related genomes. *PLoS Biology.* **4,** e2

41. Valentin-Hansen, P., Johansen, J., and Rasmussen, A.A. (2007) Small RNAs controlling outer membrane porins. *Current Opinion in Microbiology.* **10,** 152-155

42. Price, N.L., and Raivio, T.L. (2009) Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. *Journal of Bacteriology.* **191,** 1798-1815

43. Tormo, A., Almiron, M., and Kolter, R. (1990) *surA*, an *Escherichia coli* gene essential for survival in stationary phase. *Journal of Bacteriology.* **172,** 4339-4347

44. Rouvière, P.E., and Gross, C.A. (1996) SurA, a periplasmic protein with peptidyl-prolyl isomerase activity, participates in the assembly of outer membrane porins. *Genes and Development.* **10,** 3170-3182

45. Lazar, S.W., and Kolter, R. (1996) SurA assists the folding of *Escherichia coli* outer membrane proteins. *Journal of Bacteriology.* **178,** 1770-1773

46. Missiakas, D., Betton, J.-., and Raina, S. (1996) New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. *Molecular Microbiology.* **21,** 871-884

47. Justice, S.S., Hunstad, D.A., Harper, J.R., Duguay, A.R., Pinkner, J.S., Bann, J., Frieden, C., Silhavy, T.J., and Hultgren, S.J. (2005) Periplasmic peptidyl prolyl cis-trans isomerases are not essential for viability, but SurA is required for pilus biogenesis in *Escherichia coli. Journal of Bacteriology.* **187,** 7680-7686

48. Palomino, C., Marín, E., and Fernández, L.Á. (2011) The fimbrial usher FimD follows the SurA-BamB pathway for its assembly in the outer membrane of *Escherichia coli. Journal of Bacteriology.* **193,** 5222-5230

49. Vertommen, D., Ruiz, N., Leverrier, P., Silhavy, T.J., and Collet, J.-. (2009) Characterization of the role of the *Escherichia coli* periplasmic chaperone SurA using differential proteomics. *Proteomics.* **9,** 2432-2443

50. Watts, K.M., and Hunstad, D.A. (2008) Components of SurA required for outer membrane biogenesis in uropathogenic *Escherichia coli. PLoS ONE.* **3,** e3359

51. Bitto, E., and McKay, D.B. (2003) The periplasmic molecular chaperone protein SurA binds a peptide motif that is characteristic of integral outer membrane proteins. *Journal of Biological Chemistry.* **278,** 49316-49322

52. Bitto, E., and McKay, D.B. (2004) Binding of phage-display-selected peptides to the periplasmic chaperone protein SurA mimics binding of unfolded outer membrane proteins. *FEBS Letters.* **568,** 94-98

53. Hennecke, G., Nolte, J., Volkmer-Engert, R., Schneider-Mergener, J., and Behrens, S. (2005) The periplasmic chaperone SurA exploits two features characteristic of integral outer membrane proteins for selective substrate recognition. *Journal of Biological Chemistry.* **280,** 23540-23548

54. Webb, H.M., Ruddock, L.W., Marchant, R.J., Jonas, K., and Klappa, P. (2001) Interaction of the periplasmic peptidylprolyl cis-trans isomerase SurA with model peptides: The N-terminal region of SurA is essential and sufficient for peptide binding. *Journal of Biological Chemistry.* **276,** 45622-45627

55. Bitto, E., and McKay, D.B. (2002) Crystallographic structure of SurA, a molecular chaperone that facilitates folding of outer membrane porins. *Structure.* **10,** 1489-1498

56. Xu, X., Wang, S., Hu, Y.-., and McKay, D.B. (2007) The periplasmic bacterial molecular chaperone SurA adapts its structure to bind peptides in different conformations to assert a sequence preference for aromatic residues. *Journal of Molecular Biology.* **373,** 367-381

57. Aasland, R., Coleman, J., Holck, A.L., Smith, C.L., Raetz, C.R., and Kleppe, K. (1988) Identity of the 17-kilodalton protein, a DNA-binding protein from *Escherichia coli*, and the *firA* gene product. *Journal of Bacteriology.* **170,** 5916-5918

58. Thome, B.M., Hoffschulte, H.K., Schiltz, E., and Muller, M. (1990) A protein with sequence identity to Skp (FirA) supports protein translocation into plasma membrane vesicles of *Escherichia coli. FEBS Letters.* **269,** 113-116

59. Thome, B.M., and Muller, M. (1991) Skp is a periplasmic *Escherichia coli* protein requiring SecA and SecY for export. *Molecular Microbiology.* **5,** 2815-2821

60. Chen, R., and Henning, U. (1996) A periplasmic protein (Skp) of *Escherichia coli* selectively binds a class of outer membrane proteins. *Molecular Microbiology.* **19,** 1287-1294

61. Rizzitello, A.E., Harper, J.R., and Silhavy, T.J. (2001) Genetic evidence for parallel pathways of chaperone activity in the periplasm of *Escherichia coli. Journal of Bacteriology.* **183,** 6794-6800

62. Harms, N., Koningstein, G., Dontje, W., Muller, M., Oudega, B., Luirink, J., and De Cock, H. (2001) The early interaction of the outer membrane protein PhoE with the periplasmic chaperone Skp occurs at the cytoplasmic membrane. *Journal of Biological Chemistry.* **276,** 18804-18811

63. Schäfer, U., Beck, K., and Müller, M. (1999) Skp, a molecular chaperone of Gram-negative bacteria, is required for the formation of soluble periplasmic intermediates of outer membrane proteins. *Journal of Biological Chemistry.* **274,** 24567-24574

64. Bulieris, P.V., Behrens, S., Holst, O., and Kleinschmidt, J.H. (2003) Folding and insertion of the outer membrane protein OmpA is assisted by the chaperone Skp and by lipopolysaccharide. *Journal of Biological Chemistry.* **278,** 9092-9099

65. Patel, G.J., Behrens-Kneip, S., Holst, O., and Kleinschmidt, J.H. (2009) The periplasmic chaperone Skp facilitates targeting, insertion, and folding of OmpA into lipid membranes with a negative membrane surface potential. *Biochemistry (N.Y.).* **48,** 10235-10245

66. De Cock, H., Schäfer, U., Potgeter, M., Demel, R., Müller, M., and Tommassen, J. (1999) Affinity of the periplasmic chaperone Skp of *Escherichia coli* for phospholipids, lipopolysaccharides and non-native outer membrane proteins. Role of Skp in the biogenesis of outer membrane protein. *European Journal of Biochemistry.* **259,** 96-103

67. Korndörfer, I.P., Dommel, M.K., and Skerra, A. (2004) Structure of the periplasmic chaperone Skp suggests functional similarity with cytosolic chaperones despite differing architecture. *Nature Structural and Molecular Biology.* **11,** 1015-1020

68. Walton, T.A., and Sousa, M.C. (2004) Crystal structure of Skp, a prefoldin-like chaperone that protects soluble and membrane proteins from aggregation. *Molecular Cell.* **15,** 367-374

69. Qu, J., Behrens-Kneip, S., Holst, O., and Kleinschmidt, J.H. (2009) Binding regions of outer membrane protein A in complexes with the periplasmic chaperone Skp. A site-directed fluorescence study. *Biochemistry (N.Y.).* **48,** 4926-4936

70. Walton, T.A., Sandoval, C.M., Fowler, C.A., Pardi, A., and Sousa, M.C. (2009) The cavity-chaperone Skp protects its substrate from aggregation but allows independent folding of substrate domains. *Proceedings of the National Academy of Sciences of the U.S.A.* **106,** 1772-1777

71. Qu, J., Mayer, C., Behrens, S., Holst, O., and Kleinschmidt, J.H. (2007) The trimeric periplasmic chaperone Skp of *Escherichia coli* Forms 1:1 complexes with outer membrane proteins via hydrophobic and electrostatic interactions. *Journal of Molecular Biology.* **374,** 91-105

72. Lyu, Z.-., Shao, Q., Gao, Y.Q., and Zhao, X.S. (2012) Direct observation of the uptake of outer membrane proteins by the periplasmic chaperone Skp. *PLoS ONE.* **7,** e46068

73. Entzminger, K.C., Chang, C., Myhre, R.O., McCallum, K.C., and Maynard, J.A. (2012) The Skp chaperone helps fold soluble proteins *in vitro* by inhibiting aggregation. *Biochemistry (N.Y.).* **51,** 4822-4834

74. Jarchow, S., Lück, C., Görg, A., and Skerra, A. (2008) Identification of potential substrate proteins for the periplasmic *Escherichia coli* chaperone Skp. *Proteomics.* **8,** 4987-4994

75. Lipinska, B., Fayet, O., Baird, L., and Georgopoulos, C. (1989) Identification, characterization, and mapping of the *Escherichia coli htrA* gene, whose product is essential for bacterial growth only at elevated temperatures. *Journal of Bacteriology.* **171,** 1574-1584

76. Lipinska, B., Zylicz, M., and Georgopoulos, C. (1990) The HtrA (DegP) protein, essential for *Escherichia coli* survival at high temperatures, is an endopeptidase. *Journal of Bacteriology.* **172,** 1791-1797

77. Strauch, K.L., Johnson, K., and Beckwith, J. (1989) Characterization of *degP*, a gene required for proteolysis in the cell envelope and essential for growth of *Escherichia coli* at high temperature. *Journal of Bacteriology.* **171,** 2689-2696

78. Kim, K.I., Park, S.-., Kang, S.H., Cheong, G.-., and Chung, C.H. (1999) Selective degradation of unfolded proteins by the self-compartmentalizing HtrA protease, a periplasmic heat shock protein in *Escherichia coli*. *Journal of Molecular Biology.* **294,** 1363-1374

79. Spiess, C., Beil, A., and Ehrmann, M. (1999) A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell.* **97,** 339-347

80. Krojer, T., Sawa, J., Schäfer, E., Saibil, H.R., Ehrmann, M., and Clausen, T. (2008) Structural basis for the regulated protease and chaperone function of DegP. *Nature.* **453,** 885-890

81. Misra, R., Castillokeller, M., and Deng, M. (2000) Overexpression of protease-deficient DegP(S210A) rescues the lethal phenotype of *Escherichia coli* OmpF assembly mutants in a *degP* background. *Journal of Bacteriology.* **182,** 4882-4888

82. CastilloKeller, M., and Misra, R. (2003) Protease-deficient DegP suppresses lethal effects of a mutant OmpC protein by its capture. *Journal of Bacteriology.* **185,** 148-154

83. Iwanczyk, J., Damjanovic, D., Kooistra, J., Leong, V., Jomaa, A., Ghirlando, R., and Ortega, J. (2007) Role of the PDZ domains in *Escherichia coli* DegP protein. *Journal of Bacteriology.* **189,** 3176-3186

84. Meltzer, M., Hasenbein, S., Hauske, P., Kucz, N., Merdanovic, M., Grau, S., Beil, A., Jones, D., Krojer, T., Clausen, T., Ehrmann, M., and Kaiser, M. (2008) Allosteric activation of HtrA protease DegP by stress signals during bacterial protein quality control. *Angewandte Chemie - International Edition.* **47,** 1332-1334

85. Krojer, T., Pangerl, K., Kurt, J., Sawa, J., Stingl, C., Mechtler, K., Huber, R., Ehrmann, M., and Clausen, T. (2008) Interplay of PDZ and protease domain of DegP ensures efficient elimination of misfolded proteins. *Proceedings of the National Academy of Sciences of the U.S.A.* **105,** 7702-7707

86. Krojer, T., Garrido-Franco, M., Huber, R., Ehrmann, M., and Clausen, T. (2002) Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine. *Nature.* **416,** 455-459

87. Sobiecka-Szkatula, A., Polit, A., Scire, A., Gieldon, A., Tanfani, F., Szkarlat, Z., Ciarkowski, J., Zurawa-Janicka, D., Skorko-Glonek, J., and Lipinska, B. (2009) Temperature-induced conformational changes within the regulatory loops L1-L2-LA of the HtrA heat-shock protease from *Escherichia coli*. *Biochimica et Biophysica Acta - Proteins and Proteomics.* **1794,** 1573-1582

88. Jiang, J., Zhang, X., Chen, Y., Wu, Y., Zhou, Z.H., Chang, Z., and Sui, S.-. (2008) Activation of DegP chaperone-protease via formation of large cage-like oligomers upon binding to substrate proteins. *Proceedings of the National Academy of Sciences of the U.S.A.* **105,** 11939-11944

89. Shen, Q.-., Bai, X.-., Chang, L.-., Wu, Y., Wang, H.-., and Sui, S.-. (2009) Bowl-shaped oligomeric structures on membranes as DegP's new functional forms in protein quality control. *Proceedings of the National Academy of Sciences of the U.S.A.* **106,** 4858-4863

90. Kim, S., and Sauer, R.T. (2012) Cage assembly of DegP protease is not required for substrate-dependent regulation of proteolytic activity or high-temperature cell survival. *Proceedings of the National Academy of Sciences of the U.S.A.* **109,** 7263-7268

91. Denoncin, K., Schwalm, J., Vertommen, D., Silhavy, T.J., and Collet, J.-. (2012) Dissecting the *Escherichia coli* periplasmic chaperone network using differential proteomics. *Proteomics.* **12,** 1391-1401

92. Wu, S., Ge, X., Lv, Z., Zhi, Z., Chang, Z., and Zhao, X.S. (2011) Interaction between bacterial outer membrane proteins and periplasmic quality control factors: A kinetic partitioning mechanism. *Biochemical Journal.* **438,** 505-511

93. Dartigalongue, C., and Raina, S. (1998) A new heat-shock gene, *ppiD*, encodes a peptidyl-prolyl isomerase required for folding of outer membrane proteins in *Escherichia coli*. *EMBO Journal.* **17,** 3968-3980

94. Matern, Y., Barion, B., and Behrens-Kneip, S. (2010) PpiD is a player in the network of periplasmic chaperones in *Escherichia coli. BMC Microbiology.* **10,** 251

95. Weininger, U., Jakob, R.P., Kovermann, M., Balbach, J., and Schmid, F.X. (2010) The prolyl isomerase domain of PpiD from *Escherichia coli* shows a parvulin fold but is devoid of catalytic activity. *Protein Science.* **19,** 6-18

96. Stymest, K.H., and Klappa, P. (2008) The periplasmic peptidyl prolyl cis-trans isomerases PpiD and SurA have partially overlapping substrate specificities. *FEBS Journal.* **275,** 3470-3479

97. Antonoaea, R., Fürst, M., Nishiyama, K.-., and Müller, M. (2008) The periplasmic chaperone PpiD interacts with secretory proteins exiting from the SecYEG translocon. *Biochemistry (N.Y.).* **47,** 5649-5656

98. Gatzeva-Topalova, P.Z., Warner, L.R., Pardi, A., and Sousa, M.C. (2010) Structure and flexibility of the complete periplasmic domain of BamA: The protein insertion machine of the outer membrane. *Structure.* **18,** 1492-1501

99. Walther, D.M., Rapaport, D., and Tommassen, J. (2009) Biogenesis of β-barrel membrane proteins in bacteria and eukaryotes: Evolutionary conservation and divergence. *Cellular and Molecular Life Sciences.* **66,** 2789-2804

100. Arié, J.-., Sassoon, N., and Betton, J.-. (2001) Chaperone function of FkpA, a heat shock prolyl isomerase, in the periplasm of *Escherichia coli. Molecular Microbiology.* **39,** 199-210

101. Bothmann, H., and Plückthun, A. (2000) The periplasmic *Escherichia coli* peptidylprolyl cis,trans-isomerase FkpA: I. Increased functional expression of antibody fragments with and without cis-prolines. *Journal of Biological Chemistry.* **275,** 17100-17105

102. Ramm, K., and Plückthun, A. (2000) The periplasmic *Escherichia coli* peptidylprolyl cis,trans-isomerase FkpA: II. Isomerase-independent chaperone activity *in vitro. Journal of Biological Chemistry.* **275,** 17106-17113

103. Saul, F.A., Arié, J.-., Vulliez-le Normand, B., Kahn, R., Betton, J.-., and Bentley, G.A. (2004) Structural and functional studies of FkpA from *Escherichia coli*, a cis/trans peptidyl-prolyl isomerase with chaperone activity. *Journal of Molecular Biology.* **335,** 595-608

104. Ramm, K., and Plückthun, A. (2001) High enzymatic activity and chaperone function are mechanistically related features of the dimeric *E. coli* peptidyl-prolyl-isomerase FkpA. *Journal of Molecular Biology.* **310,** 485-498

105. Hu, K., Galius, V., and Pervushin, K. (2006) Structural plasticity of peptidyl-prolyl isomerase sFkpA is a key to its chaperone function as revealed by solution NMR. *Biochemistry (N.Y.).* **45,** 11983-11991

106. Liu, J., and Walsh, C.T. (1990) Peptidyl-prolyl cis-trans-isomerase from *Escherichia coli*: A periplasmic homolog of cyclophilin that is not inhibited by cyclosporin A. *Proceedings of the National Academy of Sciences of the U.S.A.* **87,** 4028-4032

107. Wagner, G. (1994) Three-dimensional solution structure of *Escherichia coli* periplasmic cyclophilin. *Biochemistry (N.Y.).* **33,** 2761-2772

108. Kleerebezem, M., Heutink, M., and Tommassen, J. (1995) Characterization of an *Escherichia coli rotA* mutant, affected in periplasmic peptidyl-prolyl cis/trans isomerase. *Molecular Microbiology.* **18,** 313-320

109. Bardwell, J.C.A., McGovern, K., and Beckwith, J. (1991) Identification of a protein required for disulfide bond formation *in vivo. Cell.* **67,** 581-589

110. Kamitani, S., Akiyama, Y., and Ito, K. (1992) Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme. *EMBO Journal.* **11,** 57-62

111. Martin, J.L., Bardwell, J.C.A., and Kuriyan, J. (1993) Crystal structure of the DsbA protein required for disulphide bond formation *in vivo. Nature.* **365,** 464-468

112. Zapun, A., Bardwell, J.C.A., and Creighton, T.E. (1993) The reactive and destabilizing disulfide bond of DsbA, a protein required for protein disulfide bond formation *in vivo. Biochemistry (N.Y.).* **32,** 5083-5092

113. Guddat, L.W., Bardwell, J.C.A., and Martin, J.L. (1998) Crystal structures of reduced and oxidized DsbA: Investigation of domain motion and thiolate stabilization. *Structure.* **6,** 757-767

114. Paxman, J.J., Borg, N.A., Horne, J., Thompson, P.E., Chin, Y., Sharma, P., Simpson, J.S., Wielens, J., Piek, S., Kahler, C.M., Sakellaris, H., Pearce, M., Bottomley, S.P., Rossjohn, J., and Scanlon, M.J. (2009) The structure of the bacterial oxidoreductase enzyme DsbA in complex with a peptide reveals a basis for substrate specificity in the catalytic cycle of DsbA enzymes. *Journal of Biological Chemistry.* **284,** 17835-17845

115. Kishigami, S., Akiyama, Y., and Ito, K. (1995) Redox states of DsbA in the periplasm of *Escherichia coli. FEBS Letters.* **364,** 55-58

116. Hiniker, A., and Bardwell, J.C.A. (2004) *In vivo* substrate specificity of periplasmic disulfide oxidoreductases. *Journal of Biological Chemistry.* **279,** 12967-12973

117. Kadokura, H., Tian, H., Zander, T., Bardwell, J.C.A., and Beckwith, J. (2004) Snapshots of DsbA in action: Detection of proteins in the process of oxidative folding. *Science.* **303,** 534-537

118. Missiakas, D., Georgopoulos, C., and Raina, S. (1994) The *Escherichia coli dsbC* (*xprA*) gene encodes a periplasmic protein involved in disulfide bond formation. *EMBO Journal.* **13,** 2013-2020

119. Shevchik, V.E., Condemine, G., and Robert-Baudouy, J. (1994) Characterization of DsbC, a periplasmic protein of *Erwinia chrysanthemi* and *Escherichia coli* with disulfide isomerase activity. *EMBO Journal.* **13,** 2007-2012

120. Rietsch, A., Belin, D., Martin, N., and Beckwith, J. (1996) An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli. Proceedings of the National Academy of Sciences of the U.S.A.* **93,** 13048-13053

121. Zapun, A. (1995) Structural and functional characterization of DsbC, a protein involved in disulfide bond formation in *Escherichia coli. Biochemistry (N.Y.).* **34,** 5075-5089

122. Joly, J.C., and Swartz, J.R. (1997) *In vitro* and *in vivo* redox states of the *Escherichia coli* periplasmic oxidoreductases DsbA and DsbC. *Biochemistry (N.Y.).* **36,** 10067-10072

123. Berkmen, M., Boyd, D., and Beckwith, J. (2005) The nonconsecutive disulfide bond of *Escherichia coli* phytase (AppA) renders it dependent on the protein-disulfide isomerase, DsbC. *Journal of Biological Chemistry.* **280,** 11387-11394

124. Denoncin, K., Vertommen, D., Paek, E., and Collet, J.-. (2010) The protein-disulfide isomerase DsbC cooperates with SurA and DsbA in the assembly of the essential β-barrel protein LptD. *Journal of Biological Chemistry.* **285,** 29425-29433

125. Ruiz, N., Chng, S.-., Hinikera, A., Kahne, D., and Silhavy, T.J. (2010) Nonconsecutive disulfide bond formation in an essential integral outer membrane protein. *Proceedings of the National Academy of Sciences of the U.S.A.* **107,** 12245-12250

126. Messens, J., Collet, J.-., Van Belle, K., Brosens, E., Loris, R., and Wyns, L. (2007) The oxidase DsbA folds a protein with a nonconsecutive disulfide. *Journal of Biological Chemistry.* **282,** 31302-31307

127. Vertommen, D., Depuydt, M., Pan, J., Leverrier, P., Knoops, L., Szikora, J.-., Messens, J., Bardwell, J.C.A., and Collet, J.-. (2008) The disulphide isomerase DsbC cooperates with the oxidase DsbA in a DsbD-independent manner. *Molecular Microbiology.* **67,** 336-349

128. Liu, X.-., and Wang, C.-. (2001) Disulfide-dependent folding and export of *Escherichia coli* DsbC. *Journal of Biological Chemistry.* **276,** 1146-1151

129. McCarthy, A.A., Haebel, P.W., Törrönen, A., Rybin, V., Baker, E.N., and Metcalf, P. (2000) Crystal structure of the protein disulfide bond isomerase, DsbC, from *Escherichia coli*. *Nature Structural Biology.* **7,** 196-199

130. Sun, X.-., and Wang, C.-. (2000) The N-terminal sequence (residues 1-65) is essential for dimerization, activities, and peptide binding of *Escherichia coli* DsbC. *Journal of Biological Chemistry.* **275,** 22743-22749

131. Andersen, C.L., Matthey-Dupraz, A., Missiakas, D., and Raina, S. (1997) A new *Escherichia coli* gene, *dsbG*, encodes a periplasmic protein involved in disulphide bond formation, required for recycling DsbA/DsbB and DsbC redox proteins. *Molecular Microbiology.* **26,** 121-132

132. Bessette, P.H., Cotto, J.J., Gilbert, H.F., and Georgieou, G. (1999) *In vivo* and *in vitro* function of the *Escherichia coli* periplasmic cysteine oxidoreductase DsbG. *Journal of Biological Chemistry.* **274,** 7784-7792

133. Shao, F., Bader, M.W., Jakob, U., and Bardwell, J.C.A. (2000) DsbG, a protein disulfide isomerase with chaperone activity. *Journal of Biological Chemistry.* **275,** 13349-13352

134. Van Straaten, M., Missiakas, D., Raina, S., and Darby, N.J. (1998) The functional properties of DsbG, a thiol-disulfide oxidoreductase from the periplasm of *Escherichia coli. FEBS Letters.* **428,** 255-258

135. Depuydt, M., Leonard, S.E., Vertommen, D., Denoncin, K., Morsomme, P., Wahni, K., Messens, J., Carroll, K.S., and Collet, J.-. (2009) A periplasmic reducing system protects single cysteine residues from oxidation. *Science.* **326,** 1109-1111

136. Heras, B., Edeling, M.A., Schirra, H.J., Raina, S., and Martin, J.L. (2004) Crystal structures of the DsbG disulfide isomerase reveal an unstable disulfide. *Proceedings of the National Academy of Sciences of the U.S.A.* **101,** 8876-8881

137. Quan, S., Koldewey, P., Tapley, T., Kirsch, N., Ruane, K.M., Pfizenmaier, J., Shi, R., Hofmann, S., Foit, L., Ren, G., Jakob, U., Xu, Z., Cygler, M., and Bardwell, J.C.A. (2011) Genetic selection designed to stabilize proteins uncovers a chaperone called Spy. *Nature Structural and Molecular Biology.* **18,** 262-269

138. Evans, M.L., Schmidt, J.C., Ilbert, M., Doyle, S.M., Quan, S., Bardwell, J.C.A., Jakob, U., Wickner, S., and Chapman, M.R. (2011) *E. coli* chaperones DnaK, Hsp33 and Spy inhibit bacterial functional amyloid assembly. *Prion.* **5,** 323-334

139. Matsuyama -i, S., Tajima, T., and Tokuda, H. (1995) A novel periplasmic carrier protein involved in the sorting and transport of *Escherichia coli* lipoproteins destined for the outer membrane. *EMBO Journal.* **14,** 3365-3372

140. Yokota, N., Kuroda, T., Matsuyama, S.-., and Tokuda, H. (1999) Characterization of the LolA-LolB system as the general lipoprotein localization mechanism of *Escherichia coli. Journal of Biological Chemistry.* **274,** 30995-30999

141. Tajima, T., Yokota, N., Matsuyama, S.-., and Tokuda, H. (1998) Genetic analyses of the *in vivo* function of LolA, a periplasmic chaperone involved in the outer membrane localization of *Escherichia coli* lipoproteins. *FEBS Letters.* **439,** 51-54

142. Takeda, K., Miyatake, H., Yokota, N., Matsuyama, S.-., Tokuda, H., and Miki, K. (2003) Crystal structures of bacterial lipoprotein localization factors, LolA and LolB. *EMBO Journal.* **22,** 3199-3209

143. Oguchi, Y., Takeda, K., Watanabe, S., Yokota, N., Miki, K., and Tokuda, H. (2008) Opening and closing of the hydrophobic cavity of LolA coupled to lipoprotein binding and release. *Journal of Biological Chemistry.* **283,** 25414-25420

144. Taniguchi, N., and Tokuda, H. (2008) Molecular events involved in a single cycle of ligand transfer from an ATP binding cassette transporter, LolCDE, to a molecular chaperone, LolA. *Journal of Biological Chemistry.* **283,** 8538-8544

145. Nakada, S., Sakakura, M., Takahashi, H., Okuda, S., Tokuda, H., and Shimada, I. (2009) Structural investigation of the interaction between LolA and LolB using NMR. *Journal of Biological Chemistry.* **284,** 24634-24643

146. Hong, W., Jiao, W., Hu, J., Zhang, J., Liu, C., Fu, X., Shen, D., Xia, B., and Chang, Z. (2005) Periplasmic protein HdeA exhibits chaperone-like activity exclusively within stomach pH range by transforming into disordered conformation. *Journal of Biological Chemistry.* **280,** 27029-27034

147. Gajiwala, K.S., and Burley, S.K. (2000) HdeA, a periplasmic protein that supports acid resistance in pathogenic enteric bacteria. *Journal of Molecular Biology.* **295,** 605-612

148. Kern, R., Malki, A., Abdallah, J., Tagourti, J., and Richarme, G. (2007) *Escherichia coli* HdeB is an acid stress chaperone. *Journal of Bacteriology.* **189,** 603-610

149. Wu, Y.E., Hong, W., Liu, C., Zhang, L., and Chang, Z. (2008) Conserved amphiphilic feature is essential for periplasmic chaperone HdeA to support acid resistance in enteric bacteria. *Biochemical Journal.* **412,** 389-397

150. Tapley, T.L., Körnera, J.L., Barge, M.T., Hupfeld, J., Schauerte, J.A., Gafni, A., Jakob, U., and Bardwell, J.C.A. (2009) Structural plasticity of an acid-activated chaperone allows promiscuous substrate binding. *Proceedings of the National Academy of Sciences of the U.S.A.* **106,** 5557-5562

151. Tapley, T.L., Franzmann, T.M., Chakraborty, S., Jakob, U., and Bardwell, J.C.A. (2010) Protein refolding by pH-triggered chaperone binding and release. *Proceedings of the National Academy of Sciences of the U.S.A.* **107,** 1071-1076

152. Zhang, M., Lin, S., Song, X., Liu, J., Fu, Y., Ge, X., Fu, X., Chang, Z., and Chen, P.R. (2011) A genetically incorporated crosslinker reveals chaperone cooperation in acid resistance. *Nature Chemical Biology.* **7,** 671-677

153. Wang, W., Rasmussen, T., Harding, A.J., Booth, N.A., Booth, I.R., and Naismith, J.H. (2012) Salt bridges regulate both dimer formation and monomeric flexibility in HdeB and may have a role in periplasmic chaperone function. *Journal of Molecular Biology.* **415,** 538-546

154. Fardini, Y., Trotereau, J., Bottreau, E., Souchard, C., Velge, P., and Virlogeux-Payant, I. (2009) Investigation of the role of the Bam complex and SurA chaperone in outer-membrane protein biogenesis and type III secretion system expression in *Salmonella. Microbiology.* **155,** 1613-1622

155. Purdy, G.E., Fisher, C.R., and Payne, S.M. (2007) IcsA surface presentation in *Shigella flexneri* requires the periplasmic chaperones DegP, Skp, and SurA. *Journal of Bacteriology.* **189,** 5566-5573

156. Wagner, J.K., Heindl, J.E., Gray, A.N., Jain, S., and Goldberg, M.B. (2009) Contribution of the periplasmic chaperone Skp to efficient presentation of the autotransporter IcsA on the surface of *Shigella flexneri. Journal of Bacteriology.* **191,** 815-821

157. Rondelet, A., and Condemine, G. (2012) SurA is involved in the targeting to the outer membrane of a tat signal sequence-anchored protein. *Journal of Bacteriology.* **194,** 6131-6142

158. Hodak, H., Wohlkönig, A., Smet-Nocca, C., Drobecq, H., Wieruszeski, J.-., Sénéchal, M., Landrieu, I., Locht, C., Jamin, M., and Jacob-Dubuisson, F. (2008) The peptidyl-prolyl isomerase and chaperone Par27 of *Bordetella pertussis* as the prototype for a new group of parvulins. *Journal of Molecular Biology.* **376,** 414-426

159. Kale, A., Phansopa, C., Suwannachart, C., Craven, C.J., Rafferty, J.B., and Kelly, D.J. (2011) The virulence factor PEB4 (Cj0596) and the periplasmic protein Cj1289 are two structurally related SurA-like chaperones in the human pathogen *Campylobacter jejuni. Journal of Biological Chemistry.* **286,** 21254-21265

160. Clantin, B., Leyrat, C., Wohlkönig, A., Hodak, H., Ribeiro Jr., E.d.A., Martinez, N., Baud, C., Smet-Nocca, C., Villeret, V., Jacob-Dubuisson, F., and Jamin, M. (2010) Structure and plasticity of the peptidyl-prolyl isomerase Par27 of *Bordetella pertussis* revealed by X-ray diffraction and small-angle X-ray scattering. *Journal of Structural Biology.* **169,** 253-265

161. Rathbun, K.M., and Thompson, S.A. (2009) Mutation of PEB4 alters the outer membrane protein profile of *Campylobacter jejuni. FEMS Microbiology Letters.* **300,** 188-194

162. Kadokura, H., and Beckwith, J. (2010) Mechanisms of oxidative protein folding in the bacterial cell envelope. *Antioxidants and Redox Signaling.* **13,** 1231-1246

163. Sinha, S., Langford, P.R., and Kroll, J.S. (2004) Functional diversity of three different DsbA proteins from *Neisseria meningitidis. Microbiology.* **150,** 2993-3000

164. Tinsley, C.R., Voulhoux, R., Beretti, J.-., Tommassen, J., and Nassif, X. (2004) Three homologues, including two membrane-bound proteins, of the disulfide oxidoreductase DsbA in *Neisseria meningitidis*: Effects on bacterial growth and biogenesis of functional type IV pili. *Journal of Biological Chemistry.* **279,** 27078-27087

165. Okuda, S., and Tokuda, H. (2011) Lipoprotein sorting in bacteria. *Annual Review of Microbiology.* **65,** 239-259

166. Tanaka, S.-., Narita, S.-., and Tokuda, H. (2007) Characterization of the *Pseudomonas aeruginosa* Lol system as a lipoprotein sorting mechanism. *Journal of Biological Chemistry.* **282,** 13379-13384

167. Narita, S.-., and Tokuda, H. (2007) Amino acids at positions 3 and 4 determine the membrane specificity of *Pseudomonas aeruginosa* lipoproteins. *Journal of Biological Chemistry.* **282,** 13372-13378

168. Waterman, S.R., and Small, P.L.C. (1996) Identification of σ^S-dependent genes associated with the stationary-phase acid-resistance phenotype of *Shigella flexneri. Molecular Microbiology.* **21,** 925-940

169. Valderas, M.W., Alcantara, R.B., Baumgartner, J.E., Bellaire, B.H., Robertson, G.T., Ng, W.-., Richardson, J.M., Winkler, M.E., and Roop II, R.M. (2005) Role of HdeA in acid resistance and virulence in *Brucella abortus* 2308. *Veterinary Microbiology.* **107,** 307-312

170. Volokhina, E.B., Grijpstra, J., Stork, M., Schilders, I., Tommassen, J., and Bos, M.P. (2011) Role of the periplasmic chaperones Skp, SurA, and DegQ in outer membrane protein biogenesis in *Neisseria meningitidis*. *Journal of Bacteriology.* **193,** 1612-1621

171. Quan, S., and Bardwell, J.C.A. (2012) Chaperone discovery. *Bioessays.* **34,** 973-981

172. Cabantous, S., Terwilliger, T.C., and Waldo, G.S. (2005) Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. *Nature Biotechnology.* **23,** 102-107

173. Bochner, B.R. (2009) Global phenotypic characterization of bacteria. *FEMS Microbiology Reviews.* **33,** 191-205

174. Hagan, C.L., Kim, S., and Kahne, D. (2010) Reconstitution of outer membrane protein assembly from purified components. *Science.* **328,** 890-892