Life inside and out: making and breaking protein disulfide bonds in *Chlamydia*

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Professor Martin AC FAA is Director of the Griffith Institute for Drug Discovery (GRIDD). GRIDD is home to Australia's compound collection (Compounds Australia) and holds the country's largest collection of Australian biota processed for drug discovery (NatureBank). GRIDD has unique resources for bioaffinity screening and high throughput screening/high content imaging. Professor Martin is a renowned structural biologist who has made pioneering discoveries in redox biology and drug discovery. She reported the first crystal structure of a DsbA oxidative protein folding catalyst and identified the evolutionary link between thioredoxins and bacterial DsbAs. She has reported the structures of more than 130 proteins and protein:inhibitor complexes. Prof Martin is President of the Asian Crystallography Association, and a member of the Executive Committee of the International Union of Crystallography. She was elected a Fellow of the Australian Academy of Science in 2017. In 2018 she was awarded the highest civilian honour in Australia, Companion (AC) in the General Division of the Order of Australia "*For eminent service to science, and to scientific research, particularly in the field of biochemistry and protein crystallography applied to drug-resistant bacteria, as a role model, and as an advocate for gender equality in science.*"

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REVIEW ARTICLE

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Life inside and out: making and breaking protein disulfide bonds in *Chlamydia*

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ABSTRACT

Disulphide bonds are widely used among all domains of life to provide structural stability to proteins and to regulate enzyme activity. *Chlamydia* spp. are obligate intracellular bacteria that are especially dependent on the formation and degradation of protein disulphide bonds. Members of the genus *Chlamydia* have a unique biphasic developmental cycle alternating between two distinct cell types; the extracellular infectious elementary body (EB) and the intracellular replicating reticulate body. The proteins in the envelope of the EB are heavily cross-linked with disulphides and this is known to be critical for this infectious phase. In this review, we provide a comprehensive summary of what is known about the redox state of chlamydial envelope proteins throughout the developmental cycle. We focus especially on the factors responsible for degradation and formation of disulphide bonds in *Chlamydia* and how this system compares with redox regulation in other organisms. Focussing on the unique biology of *Chlamydia* enables us to provide important insights into how specialized suites of disulphide bond (Dsb) proteins cater for specific bacterial environments and lifecycles.

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Introduction

Disulphide bonds between protein cysteine sulphur atoms are found in all domains of life, as well as in viruses, and provide thermal and structural stability for many secreted and membrane proteins. The function of many Gram-positive and Gram-negative bacteria virulence factors are dependent on the formation of correct disulphide bonds, including those important in; adhesion to a host cell (Jacob-Dubuisson et al. 1994; Bouwman et al. 2003), spread and growth (Yu et al. 2000; Hiniker and Bardwell 2004), and host cell manipulation (Watarai et al. 1995; Jackson and Plano 1999; Pugsley et al. 2001; Miki et al. 2004; Lin et al. 2008; Miki et al. 2008; Ren G et al. 2014; Mariano et al. 2018). Consequently, incorrect protein folding or disruption of disulphide bond (Dsb) formation can attenuate bacterial virulence, although without affecting viability (Ireland et al. 2014). Thus, the disulphide oxidative pathway, responsible for the introduction of disulphide bonds into bacterial proteins, has been validated as a target for the development of anti-virulence antimicrobial drugs (Fruh et al. 2010; Adams et al. 2015; Halili et al. 2015; Landeta et al. 2015; Smith et al. 2016; Bocian-Ostrzycka et al. 2017).

Members of the *Chlamydia* genus are Gram-negative obligate intracellular bacteria with a unique biphasic developmental cycle correlating with Dsb formation and degradation. In the developmental cycle *Chlamydia* alternates between two different morphological forms; the infectious extracellular elementary body (EB) and the intracellular replicating reticulate body (RB). As a result of an extensive network of disulphide cross-linked proteins in the chlamydial outer membrane complex (COMC), the EB envelope is extremely rigid making it resistant to the harsh extracellular environment (Hackstadt et al. 1985; Hatch et al. 1986; Newhall 1987; Hatch 1996). Disrupting the disulphide cross-linking network in EBs has severe consequences for infectivity (Hackstadt et al. 1985). Moreover, the redox state of the cysteines in the outer membrane proteins is critical in each step of *Chlamydia* development (Stirling et al. 1983; Hatch et al. 1984; Hackstadt et al. 1985; Sardinia et al. 1988; Raulston et al. 2002). Despite this body of evidence on the importance of Dsb regulation in the outer membrane proteins,

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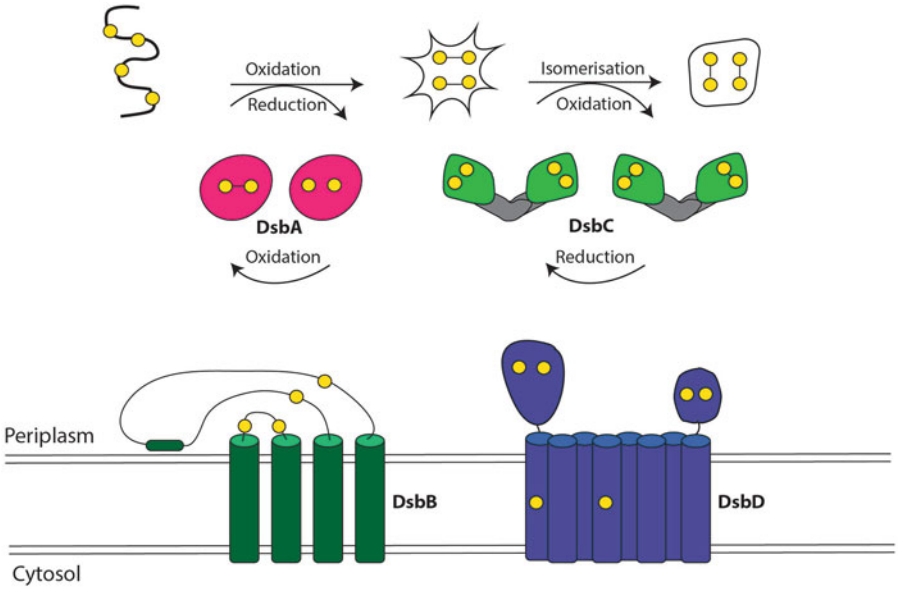

Figure 1. Disulphide bond formation in *E. coli*. In the disulphide oxidative pathway of *E. coli*, disulphide bonds are contributed to a reduced substrate by DsbA. As a result, DsbA is reduced and is subsequently oxidized by the membrane protein DsbB. Incorrectly formed disulphides are corrected by the dimeric oxidoreductase DsbC, which is then returned to the reduced, active state by the membrane protein DsbD. Cysteines are shown as yellow circles and disulphide bonds between two cysteines are indicated by a black line.

relatively little is known about the factors responsible for controlling the redox protein status.

This review focuses on disulphide bonds in the chlamydial envelope, with particular attention to (i) the changes in the disulphide-bonded network that accompany progression of the chlamydial developmental cycle, (ii) factors responsible for formation and degradation of disulphides in *Chlamydia,* and (iii) how Dsb formation in *Chlamydia* compares with that in other bacteria.

Dsb oxidation in bacteria

The bacterial machinery that introduces disulphide bonds into proteins was first identified and characterized in the model organism *E. coli K-12* and comprises the two dithiol oxidoreductases DsbA and DsbB; DsbA is a periplasmic enzyme consisting of a thioredoxin fold with an embedded helical domain. The thioredoxin fold contains the catalytic site comprising a Cys-X-X-Cys (where X is any amino acid) motif and a proline (near in space, but distant in sequence to the Cys-X-X-Cys motif) that adopts a *cis* conformation (Martin et al. 1993). DsbA introduces disulphide bonds to unfolded substrate proteins by accepting an electron pair from cysteines in the substrate thereby forming a covalent bond. DsbB is responsible for recycling DsbA to the oxidized, active state and does so by accepting the electron pair from DsbA and transferring them to a quinone cofactor and ultimately molecular oxygen via the electron transport pathway (Kobayashi et al. 1997; Bader et al. 1999). DsbB is localized to the cytoplasmic membrane and has four membrane-spanning helices and two periplasmic loops. Each periplasmic loop contains a cysteine pair essential for oxidation of DsbA (Bardwell et al. 1991). DsbA can introduce non-native disulphide bonds and these need to be corrected. This is the function of the disulphide isomerase pathway comprising DsbC and DsbD (Berkmen et al. 2005) (Figure 1). DsbC is a V-shaped homodimer, with a thioredoxin domain in each of the two protomers, and is responsible for shuffling incorrect disulphide bonds (Berkmen et al. 2005). It is recycled back to the reduced, active state by the membrane protein redox partner DsbD (Rozhkova et al. 2004; Gruber et al. 2006; Stirnimann et al. 2006). DsbD is also responsible for reducing the disulphide isomerase DsbG, that protect single cysteine residues from oxidation (Depuydt et al. 2009). DsbG is distantly related to DsbC and, like DsbC, forms a V-shaped homodimer (Heras et al. 2004).

A bioinformatic screen identified DsbA homologues in 265 of 421 screened bacterial genomes (Heras et al. 2009). All DsbA enzymes contain a thioredoxin domain and a Cys-X-X-Cys (where X is any amino acid) catalytic motif, but structural and biochemical analysis of more than 25 DsbA enzymes shows that they are diverse in surface properties and redox potential (for extensive reviews of DsbA enzymes see Heras et al. 2009; Shouldice et al. 2011; McMahon et al. 2014; Landeta et al. 2018). Even though the *E. coli* Cys-Pro-His-Cys catalytic motif is well-conserved, up to 10 other

combinations of residues in the dipeptide separating the two cysteines have been identified (Heras et al. 2009). The catalytic dipeptide and the polar residues proximal to the N-terminal Cys residue are the main contributors to the diverse redox characteristics observed amongst disulphide oxidoreductases (Charbonnier et al. 1999; Lafaye et al. 2009; Ren G et al. 2009; Rinaldi et al. 2009).

Most Gram-negative bacteria encode disulphide machinery resembling the oxidative and isomerase pathways in *E. coli*. However, some exhibit an extended suite of Dsb proteins. Some enterobacteria (Grimshaw et al. 2008; Totsika et al. 2009) (Lin et al. 2009) harbour a DsbL/DsbI redox pair that is homologous to the DsbA/DsbB redox pair (Figure 2(A)), but with a more narrow substrate range that is not able to compensate for lack of DsbA/DsbB activity (Grimshaw et al. 2008; Lin et al. 2009; Totsika et al. 2009). Other proteobacteria, such as the alpha-proteobacterium *Wolbachia pipientis* (Walden et al. 2013), the beta-proteobacterium *Neisseria meningitidis* (Sinha et al. 2004; Tinsley et al. 2004) and the gamma-proteobacterial *S. enterica* Typhimurium (Heras et al. 2009; 2010), and *Pseudomonas aeruginosa* (Arts et al. 2013) encode multiple DsbA homologues that vary in redox properties, substrate specificity and membrane association. For example, two of the three DsbAs in *N. meningitidis* are anchored to the membrane, whereas the third is soluble in the periplasm (Figure 2(B)). In addition to the two DsbA enzymes, *P. aeruginosa* also has two DsbB homologues, each of which can interact with both DsbA1 and DsbA2 (Arts et al. 2013) (Figure 2(C)). Epsilon-proteobacteria also encodes an extended suite of Dsb proteins. For example, members of the *Campylobacter* genus have two DsbA homologs with different substrate specificity as well as two DsbB homologs, DsbB and DsbI (not related to DsbI mentioned above) (Bocian-Ostrzycka et al. 2015) of which only DsbB interacts with the two DsbAs (Raczko et al. 2005) (Figure 2(D)). *Helicobacter pylori* also has a DsbI homologue as well as a homodimeric oxidoreductase known as DsbK that has oxidase as well as isomerase activity (Yoon et al. 2011; Roszczenko et al. 2012; Lester et al. 2015).

Many intracellular Gram-negative bacteria such as *Francisella tularensis*, *Legionella pneumophila*, and *Rickettsia* harbour homodimeric DsbA homologues with reductase as well as oxidase activity that are capable of oxidizing and isomerizing disulphide bonds *in vitro* (Kpadeh et al. 2013; Ren G et al. 2014) (Figure 2(E)).

Although Gram-positive bacteria do not have a periplasm, the peptidoglycan-techoic/mycolic-acid layer is suggested to form a periplasmic-like space in which disulphide bonds can be formed (Bayan et al. 2003; Matias and Beveridge 2005). Relative to the *E. coli* set of Dsb proteins (DsbA/B/C/D), many non-Gram-negative bacteria have a markedly different suite of Dsb proteins. For example, the firmicutes *Staphylococcus aureus* and *Listeria monocytogenes,* both harbour a DsbA homologue, but no DsbB homologue (Figure 2(F)) (Dumoulin et al. 2005; Heras et al. 2009). Another firmicute, *Bacillus subtilis,* encodes two DsbA-like proteins, BdbA, and BdbD and two DsbB-like proteins, BdbB and BdbC, forming two functional redox pairs, BdbA-BdbB and BdbC-BdbD (Figure 2(G)) (Bolhuis et al. 1999; Kouwen et al. 2007). In the oxidative pathway of all aerobic cyanobacteria and some actinobacteria and Gram-negative deltaproteobacteria DsbA is oxidized by the DsbB analogue VKOR (vitamin K epoxide reductase) in a mechanism similar to the DsbA/DsbB pathway (Dutton et al. 2008; Li et al. 2010; Wang et al. 2011) (Figure 2(H)).

Extremophiles also use disulphide bonds to stabilize cytoplasmic and secreted proteins, as evidenced by even number of cysteines (Mallick et al. 2002; Beeby et al. 2005; Ladenstein and Ren 2008; Jorda and Yeates 2011), and by observation of disulphide bonds in the crystal structure of thermophilic cytoplasmic proteins (Toth et al. 2000; Cacciapuoti et al. 2004; Karlstrom et al. 2005; Boutz et al. 2007; Guelorget et al. 2010). A putative extra-cytoplasmic DsbA-like enzyme with a thioredoxin fold and a Cys-Pro-His-Cys catalytic motif was first identified in *Pyrococcus horikoshii* (PhDsb) and is conserved in the Archaeal kingdom (Kuroita et al. 2007). PhDsb has functional properties similar to *E. coli* DsbA, but with significantly higher thermostability (Kuroita et al. 2007). A cytoplasmic protein disulphide oxidoreductase (PDO) with two thioredoxin domains, each containing a Cys-X-X-Cys motif, has been identified in several hyperthermophiles, such as *Sulpholobus solfataricus* (Guagliardi et al. 1994), *Methanococcus jannaschii* (Mcfarlan et al. 1992) and *Pyrococcus furiosus* (Ren B et al. 1998). The finding that PDO has oxidase and reductase activity and is capable of isomerizing disulphide bonds *in vitro* (Pedone et al. 2004; D'Ambrosio et al. 2006; Pedone et al. 2006) supports the theory that PDO is involved in Dsb formation in the cytoplasm of extremophiles. In *Crenarchaea* two membrane-bound VKOR homologues have been identified; in one the active site faces the cytoplasm and in the other it faces the extra-cytoplasmic side (Hatahet and Ruddock 2013; Hibender et al. 2017). Combined with the finding of oxidoreductases in both the cytoplasm and the extra-cytoplasmic space, this might suggest two distinctive pathways for Dsb formation in the cytoplasm and the extra-cytoplasmic space (Figure 2(I)).

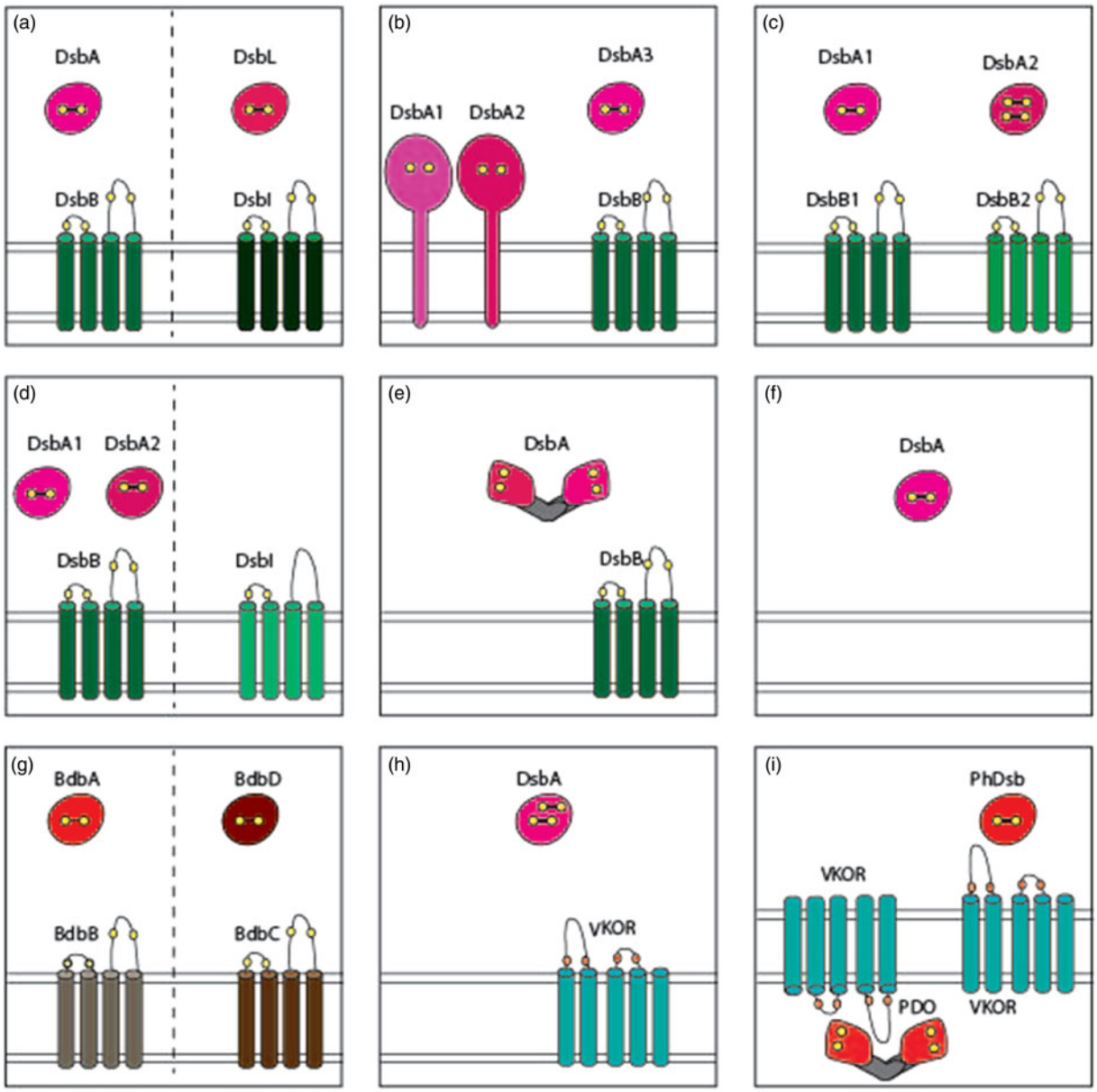

Figure 2. Selected alternative Dsb systems in bacteria. (A) Some Gram-negative bacteria, including uropathogenic *E. coli, Salmonella, Shewanella* have a specialized redox-pair, DsbL/DsbI in addition to the standard DsbA/DsbB redox pair (the two redox pairs are separated by a dotted line). (B) *N. meningitidis* and *S. enterica* Typhimurium encode three DsbA homologs and one DsbB homolog. Two of the DsbA homologs in *N. meningitidis*, DsbA1 and DsbA2, are lipoproteins, associated with the inner membrane. (C) *P. aeruginosa* encodes two DsbA homologs as well as two DsbB homologs. Both DsbB homologs are able to oxidize both DsbA homologs. (D) *C. jenuni* encodes two DsbA homologs and two DsbB homologues, DsbB and DsbI, of which only DsbB interacts with DsbA. (E) *F. tularensis* has a dimeric DsbA homolog with oxidase as well as isomerase activity and a DsbB homolog. (F) Some Gram-positive bacteria, such as *S. aureus*, encode only a DsbA homolog, and no DsbB homolog. (G) *B. subtilis* has two DsbA-like proteins, BdbA and BdbD as well as two DsbB-like proteins, BdbB and BdbC. BdbD and BdbC form a redox relay responsible for introduction of disulphide bonds (the two redox pairs are separated by a dotted line). (H) In Actinobacteria, including *M. tuberculosis*, disulphide bonds are introduced by a redox pair consisting of DsbA and the DsbB analog, VKOR. (I) A DsbA-like extra-cytoplasmic oxidoreductase, phDsb, has been identified in some thermophiles. Many thermophiles also encode a cytoplasmic dimeric oxidoreductase, PDO. Two VKOR homologues have been identified in *Crenarchaea*. The active site faces the cytoplasm in one, whereas it faces the extra-cytoplamic space in the other. Cysteines are shown as yellow circles and disulphide bonds between two cysteines are indicated by a black line.

It is evident from our brief introduction to Dsb proteins that disulphide bonds are ubiquitous in bacteria, but that the pathways vary between different bacterial classes (for more thorough reviews refer to Kadokura and Beckwith 2010; Berkmen 2012; Cho and Collet 2013; Landeta et al. 2018). The differences in structural

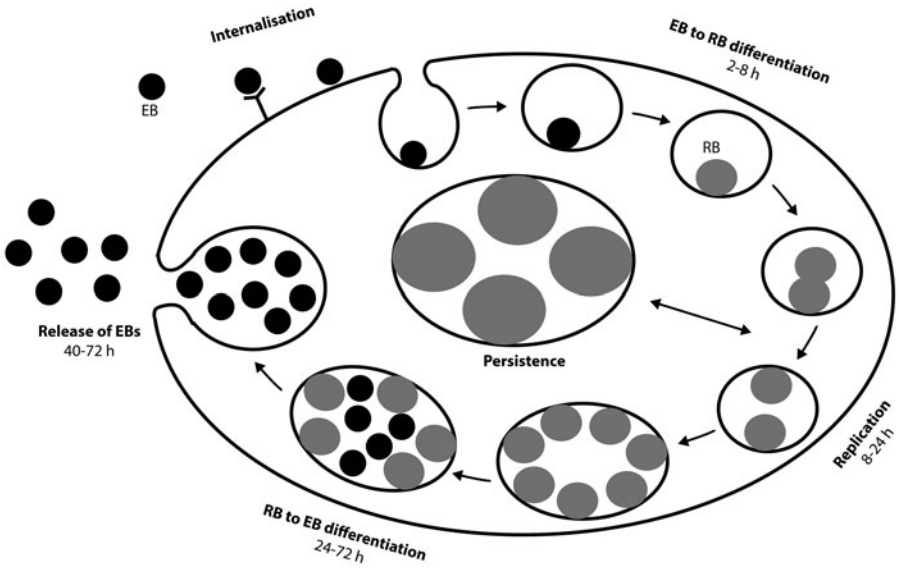

Figure 3. *Chlamydia spp* developmental cycle. Infection is initiated when the infectious EB attaches to host cell heparan sulphate proteoglycan and host cell receptors at the host cell surface. The EB is internalized into a host membrane-derived inclusion. Within the inclusion the EB differentiates into RB that replicates via binary fission. Late in the developmental cycle, RBs differentiate to EBs in an asynchronous manner and new infectious progeny are released via host cell lysis or extrusion of the inclusion. Alternatively, in response to cellular stress the RB can enter a reversible state of persistence.

and redox properties of the primary bacterial oxidase DsbA, combined with the observed diversity in Dsb repertoire and specificity among the bacterial classes suggests that different morphologies, ecological niches, and lifestyles (even in extreme conditions) have differing requirements for protein Dsb formation.

The extracellular and intracellular life of *Chlamydia*

In the extracellular environment outside the host, *Chlamydia* exists as a spore-like non-replicative form called the EB. In addition to the rigid disulphide cross-linked envelope, the EB is distinguished from the intracellular RBs by being smaller and having highly compact DNA (Shaw et al. 2000). Chlamydial infections are initiated by attachment of the EB to the host cell and the EBs are subsequently internalized to a host-cell derived vesicle, called an inclusion vacuole (Nans et al. 2014) (Figure 3).

Following internalization, the EB differentiates to RB (Figure 3). The differentiation process includes reduction of the disulphide bonds in COMC proteins and transcription of early genes, facilitating host-pathogen interactions (Belland et al. 2003). The RBs then undergo at least six rounds of binary fission at 8–20 h post-infection (Lee et al. 2018). Commencing approximately 24 h post-infection, RBs differentiate to EBs in an asynchronous manner controlled by reduction in RB size (Lee et al. 2018). The new infectious progeny are released by one of two mechanisms: host cell lysis or extrusion of the inclusion (Hybiske and Stephens 2007; Kerr et al. 2017).

As a response to stress caused by, for example, nutrient deprivation or β-lactam antibiotics, the RB can enter a distinct morphological and metabolic form referred to as aberrant bodies or "persistence" (Beatty et al. 1993; 1994a; 1994b) (Figure 3). During the persistent state, an RB transitions to an enlarged aberrant body that ceases to divide but remains viable and chromosome replication continues (Koehler et al. 1997; Gerard et al. 2001). This form can remain inside the cell indefinitely, and upon removal of the stress the aberrant bodies revert back to RBs and continue the developmental cycle (Hatch 1975; Harper et al. 2000; Wyrick 2010; Bavoil 2014).

The chlamydial outer membrane complex

The rigid nature of the chlamydial EB is a consequence of substantial Dsb cross-linking within and between proteins associated with the outer membrane. This is known as the *Chlamydia* Outer Membrane Complex (COMC). Proteins associated with the COMC include the Major Outer Membrane Protein (MOMP), two cysteine-rich proteins (OmcA and OmcB), the polymorphic membrane proteins (Pmps) (except PmpD), PorB, OprB, Pal, OMP85, CTL0887, CTL0541, CTL0645 and the type III secretion system components CdsC, CdsD, and CdsF, (Mygind et al. 2000; Tanzer and Hatch 2001; Vandahl

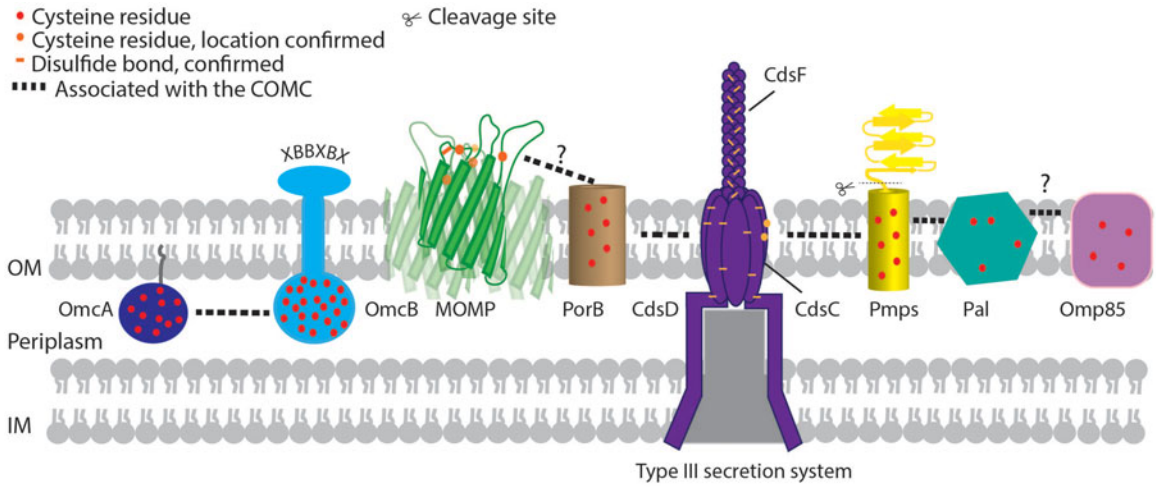

Figure 4. Composition of the COMC. The COMC consists of cysteine-rich outer membrane proteins and periplasmic proteins associated with the outer membrane. In this schematic, the presence of cysteine residues and disulphide bonds with experimentally validated locations are shown in orange, whereas other possible cysteines are shown in red. A dashed line indicates association of proteins with COMC. The outer membrane and inner membrane are marked OM and IM, respectively. The structure of MOMP (green) is based on the model of Atanu et al. (2013). MOMP is inserted into the membrane as a trimer with all cysteines located in predicted extracellular loops. For clarity, two of the three monomers in the MOMP trimer shown here are semi-transparent, and their loops and disulphides are not shown. It is unclear if MOMP is disulphide-linked to other COMC proteins. OmcA has a cysteine content of ~15% and is associated with the membrane via a lipid anchor. OmcB has a cysteine content of 4–6% with no cysteines in the membrane-spanning region. At least one heparin-binding motif (XBBXBX) is exposed on the surface. All Pmps from C. trachomatis are translocated to the surface and with the exception of PmpD, all Pmps in *C. trachomatis* are COMC-associated. Pmps are autotransporters inserted in the membrane as a β-barrel. There is increasing evidence that Pmps mature by undergoing proteolytic cleavage (scissors). Pmp6 and Pmp21 both have cleavage sites between the predicted N-terminal β-helix and the predicted C-terminal β-barrel . PorB and Pal also form part of the COMC, though it is uncertain if Omp85 is COMC-associated. The T3SS CdsF protein forms homomeric intermolecular disulphide bonds, whereas CdsC forms both homomeric intermolecular and heteromeric intermolecular disulphide bonds with CdsD. CdsC may also form intermolecular disulphide bonds with the COMC.

et al. 2004; Birkelund et al. 2009; Tan et al. 2009; Liu et al. 2010) (Figure 4).

The most well-characterized constituents of the COMC are the MOMP and the two cysteine-rich proteins OmcA and OmcB. MOMP constitutes 60% of the outer membrane protein mass of *Chlamydia* (Caldwell et al. 1981). MOMP adopts a 14- or 16-strand β-barrel (Wyllie et al. 1998; Rodriguez-Maranon et al. 2002; Findlay et al. 2005; Sun et al. 2007; Atanu et al. 2013; Feher et al. 2013), that, in the outer membrane, forms trimers independent of intra- or intermolecular disulphide bonds (Bavoil P et al. 1984; Findlay et al. 2005; Sun et al. 2007). Depending on species and serovar MOMP contains between 7 and 10 cysteine residues, of which seven are conserved. Four of these are located in predicted external loops and are expected to form intramolecular disulphide bonds (Yen et al. 2005). This is in agreement with the finding that MOMP forms high order homomeric oligos in the outer membrane (Hackstadt et al. 1985). There is disagreement as to whether MOMP is cross-linked by disulphides to other protein in the COMC (Sun et al. 2007; Liu et al. 2010).

The second most abundant protein in the *Chlamydia psittaci* envelope is OmcA (also referred to as Omp3 or small cysteine-rich protein), that with a cysteine content of ~15% (depending on species and serovar) is the most cysteine-rich chlamydial protein. OmcA is mainly hydrophilic with no apparent membrane spanning sections (Everett and Hatch 1991), but is associated with the outer membrane through a lipid anchor (Allen et al. 1990). OmcB (also referred to as Omp2 or large cysteine-rich protein) has a cysteine content of 4–6%, (again dependent on species and serovar) and is conserved amongst chlamydial species (70–80%). OmcB contains hydrophobic and amphiphilic segments that are potentially capable of spanning the outer membrane, but the subcellular localization of OmcB is the subject to some debate; most recent studies suggest that OmcB is partly surface exposed and partly periplasmically located (Fadel and Eley 2007, 2008; Moelleken and Hegemann 2008). This is consistent with the finding that OmcB binds to Glycosaminoglycans (GAGs) on the host cell surface and heparan sulphate on the host cell surface is required for infection with *C.*

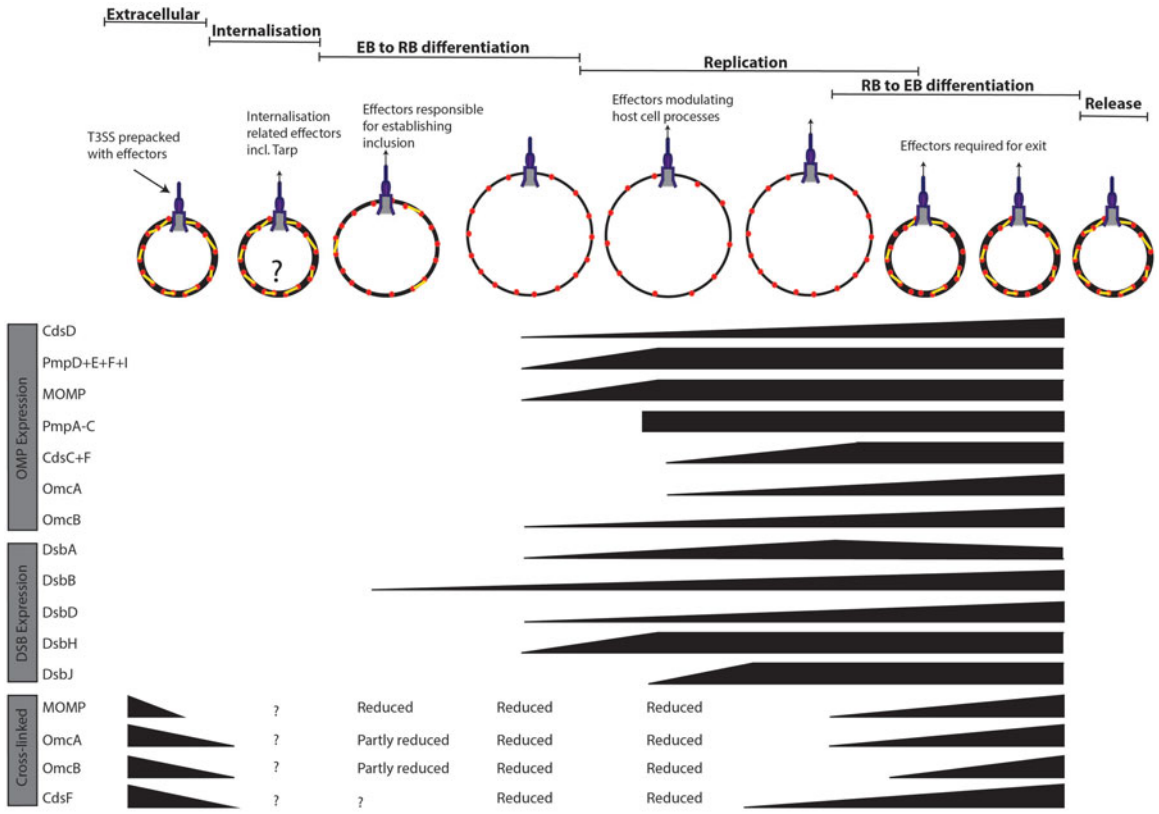

Figure 5. Expression and disulphide cross-linked state of COMC proteins during the developmental cycle. MOMP, OmcA, OmcB and CdsF form part of the COMC and are all oxidized in the extracellular EB. EB contains functional T3SS pre-packed with effectors synthesized late in the previous infection. During or immediately after internalization the disulphides in MOMP and CdsF are reduced and the disulphides in OmcA and OmcB are partly reduced. Internalization related effectors such as Tarp are secreted by the T3SS possibly facilitated by reduction of disulphides within and between the T3SS components. Early after internalization, effectors involved in establishing the inclusion are secreted by T3SS. During the replicative phase the T3SS secretes Inc proteins responsible for recruiting host cell proteins, nutrient acquisition and maintenance of the inclusion. The constituents of the COMC, apart from OmcA and OmcB, are synthesized during the early stages of replication and synthesis continues until host cell lysis. The same is true for the Dsb enzymes, except for DsbJ. OmcA, OmcB and DsbJ synthesis is initiated late in the replication stage or early in the RB to EB differentiation. Shortly thereafter the cysteines in MOMP, CdsF, OmcA and OmcB are oxidized. Late in the cycle, effectors are secreted that prepare the inclusion for exit from the host. At this stage effector proteins required during attachment and internalization in the next cycle are synthesized. Red dots represent cysteine residues and yellow lines represent disulphide bonds. T3SS is shown at the top.

trachomatis and *Chlamydia pneumoniae* (Fechtner et al. 2013).

The type III secretion system (T3SS) in *Chlamydia* resembles the T3SS in other Gram-negative pathogens, despite many of the essential components sharing very limited sequence homology (Betts et al. 2008). Interestingly, several components of the chlamydial T3SS contain a higher proportion of cysteines compared with equivalent components in other bacteria (Betts-Hampikian and Fields 2011). The cysteine residues in the chlamydial T3SS components CdsC, CdsD, and CdsF, contribute to Dsb cross-linking of the EB membrane. The two conserved cysteine residues in the needle subunit proteins, CdsF, engage in a homogenic intermolecular disulphide (Figure 4). The cross-linking of the needle subunits might be responsible for maintaining secretion-competent needles in the extracellular EBs. Disulphide bonds are also formed between CdsF and CdsD that connects the inner and outer rings in the T3SS (Figure 4). CdsC contributes to the COMC by engaging in disulphide bonds with other COMC proteins, although the identity of these proteins is unknown (Betts-Hampikian and Fields 2011) (Figure 4).

Making and breaking disulphides in the chlamydial envelope

As described above, the COMC proteins are heavily disulphide cross-linked in extracellular EBs, whereas the same cysteines are reduced in RBs (Hatch et al. 1986; Newhall 1987; Betts-Hampikian and Fields 2011; Wang et al. 2014) (Figure 5). In this section, we review what is

known about the relationship between the developmental cycle and the expression and redox state of COMC proteins. In early studies, protein expression levels of MOMP, OmcA, and OmcB were determined by isotopic labelling using ^{35}S-cysteine and SDS-PAGE analysis of infected HeLa cells (Hatch et al. 1986; Newhall 1987). More recent studies used mRNA expression levels determined by microarray (Belland et al. 2003; Nicholson et al. 2003). The more sensitive mRNA microarray data found that the onset of expression of these proteins occurs earlier than observed subsequently in studies by Newhall (1987) and Hatch et al. (1986). Transcriptional profiling of the *C. trachomatis* genome by Belland et al. classified the proteins as immediate-early genes (0–3 h post-infection), early genes (3–8 h post-infection), mid-cycle genes (8–24 h post-infection), and late genes (24–48 h post-infection) (Belland et al. 2003).

Attachment and internalization

Several COMC proteins are involved in host cell internalization of *Chlamydia*. Attachment is mediated by OmcB interacting with heparan sulphate-like GAGs on the host cell, although it is not known whether the disulphide bonds in OmcB are reduced at this stage or remain oxidized (Zhang and Stephens 1992; Fadel and Eley 2007; Fechtner et al. 2013). Oxidized MOMP also participates in attachment of EB to the host cell by functioning as a cytoadhesin (Su et al. 1988; 1990; 1996). The Pmps in *C. trachomatis* and *C. pneumoniae* are likewise important in the early stages of infection; three different Pmp subtypes have been identified as adhesins, but their redox state during internalization is unknown (Wehrl et al. 2004; Crane et al. 2006; Molleken et al. 2010; Becker and Hegemann 2014). The T3SS promotes internalization by secreting effector proteins into the host cell (Clifton et al. 2004) (Figure 5).

EB to RB differentiation

Following entry into the host cell, EBs differentiate into RBs (Newhall 1987). An important step in EB to RB differentiation is reduction of disulphides in the outer membrane proteins. Notably, disulphide reduction is not sufficient for complete EB to RB differentiation (Hackstadt et al. 1985),

Reduction of the COMC during internalization is crucial for infection (Raulston et al. 2002), although this does not facilitate complete EB to RB differentiation (Hackstadt et al. 1985). MOMP and the T3SS components are reduced within 1–2 h of infection (Hatch et al. 1986; Betts-Hampikian and Fields 2011; Wang et al. 2014) and OmcA and OmcB are at least partly reduced by then (Hatch et al. 1986) (Figure 5). Reduction of the disulphide bonds in the T3SS might facilitate secretion of effector proteins (Betts-Hampikian and Fields 2011) such as Incs, responsible for modification of the inclusion body (Damiani et al. 2014; Gambarte Tudela et al. 2015).

Replication

During the replicative stage, the majority of the chlamydial proteins are synthesized (mid-cycle genes), including the COMC proteins. There is broad consensus that expression of MOMP, the T3SS component CdsF, as well as PmpE and PmpF, is initiated at the early stages of replication and is continuously expressed throughout the developmental cycle. The remaining Pmps as well as CdsC and CdsD start expressing shortly thereafter (Figure 5). Small amounts of OmcA and OmcB are synthesized from 12 h post-infection and are further upregulated in the late stages of replication and early stages of RB to EB differentiation (Figure 5) (Hatch et al. 1986; Newhall 1987; Shaw et al. 2000; Belland et al. 2003; Nicholson et al. 2003; Betts-Hampikian and Fields 2011).

RB to EB differentiation

According to the study by Hatch et al. (1986) MOMP remains reduced until host cell lysis and is only oxidized upon exposure to the extracellular environment. However, studies by Newhall (1987) and Wang et al. (2014) show that MOMPs with cysteines in the reduced thiol form are inserted in the outer membrane of developing EBs and are subsequently oxidized during RB to EB differentiation (Figure 5). In contrast, the cysteine residues in OmcA and OmcB are oxidized concurrently with expression (Hatch et al. 1986; Newhall 1987; Mukhopadhyay et al. 2006; Wang et al. 2014). Immunoblot analysis of *C. trachomatis* infected HeLa cells suggests that cysteines in the type III secretion system are reduced in the RB and that CdsF cysteines are oxidized to disulphides in the early stages of RB to EB differentiation (Hatch et al. 1986; Newhall 1987; Betts-Hampikian and Fields 2011). Late in the infection cycle T3SS effectors, secreted during invasion of the host cell in the following cycle, are synthesized. Late-cycle effectors are expressed and secreted by the T3SS presumably involved in exit from the host cell (Valdivia 2008).

Dsb formation is important for infectivity and progression through the developmental cycle

Apart from contributing to extracellular EB resistance to the harsh extracellular environment, the disulphide cross-linking network is also important for chlamydial infectivity, evident from the observation that disruption of the COMC Dsb cross-links significantly reduces EB infectivity (Hackstadt et al. 1985; Raulston et al. 2002). This suggests that the disulphide-dependent integrity of the EB state is pivotal for infection. However, reduction of the disulphide cross-linking network is required for successful internalization (Bavoil P et al. 1984; Hackstadt et al. 1985; Peeling et al. 1989; Raulston et al. 2002; Lazarev et al. 2010). In turn, failure to reestablish the Dsb network of the COMC disrupts RB to EB differentiation (Stirling et al. 1983; Hatch et al. 1984).

Dsb formation between COMC proteins might regulate protein function

The effector protein Tarp, as well as the T3SS components CdsF, CdsC, and CdsD are mid-cycle genes (Belland et al. 2003), but are required for internalization. This has led to the theory that T3SS and effector proteins synthesized mid to late in the previous infection cycle persist in the outer membrane of the extracellular EB in the subsequent infection cycle (Betts-Hampikian and Fields 2011; Saka et al. 2011). Upon host cell entry, T3SSs expressed in the previous infection cycle, are thought to secrete effector proteins into the host cell where they initiate EB invasion, disarm the immune response, and delay maturation of EB-containing endosomes ultimately leading to development of the inclusion vacuole (Valdivia 2008). To avoid premature secretion of effectors, regulation of chlamydial T3SS activity is pivotal. In addition to regulatory factors common to other T3SS (Silva-Herzog et al. 2011; Plano and Schesser 2013; Ferrell and Fields 2016), it has been suggested that chlamydial T3SS is regulated via a disulphide-dependent mechanism (Ferrell and Fields 2016). Although it is evident that reduction of disulphides in T3SS correlates with its secretion activity, it is not yet known whether reduction of the cross-linking network is a prerequisite for secretion, or if secretion by T3SS is required for reduction of the disulphides in other components of the COMC (Betts-Hampikian and Fields 2011).

The porin function of MOMP has been suggested to be regulated by redox state. This hypothesis was based on findings that MOMP-containing outer membrane vesicles reconstituted in phosphatidylcholine and dicetylphosphate showed reduced channel size and permeability when disulphide bonds were present compared to reduced (Bavoil P et al. 1984). However, an *in vitro* study revealed no effect on channel properties upon treatment with the oxidizing agents hydrogen peroxide, oxidized glutathione, or Cu^{2+}-phenanthroline (Wyllie et al. 1998). In a later study of the channel properties of MOMP, no difference was observed upon treatment with the reducing agent DTT, although the MOMP redox state was not determined in this study (Sun et al. 2007). Like porins from the *Vibrio photobacterium* group, MOMP has a long external loop made up of residues close to the C-terminus (Wang et al. 2006). In other porins, this loop regulates channel diffusion size by folding back into the barrel (Nikaido 2003; Siritapetawee et al. 2004). As Cys337 in the loop is predicted to form a Dsb with Cys26 located inside the barrel (Wang et al. 2006) this offers a molecular explanation for the potential MOMP redox regulation.

Reduction of disulphide bonds in *Chlamydia*

Despite the association between the redox state of COMC proteins and chlamydial development, the processes involved in COMC redox regulation are not well known. One of the strongest pieces of evidence that protein disulphide redox state is critical for the chlamydial developmental cycle relates to a protein from infected host cells. Host cell protein disulphide isomerase (PDI) is required for both attachment and internalization of *C. trachomatis* L2/434/Bu and *C. psittaci* PF6 BC in a hamster cell model, but plays distinct roles in the two processes (Conant and Stephens 2007; Abromaitis and Stephens 2009). First, host PDI facilitates attachment in a manner independent of PDI oxidoreductase activity (Abromaitis and Stephens 2009). Even though PDI plays a purely structural role in attachment there is no evidence that the EB binds directly to PDI, rather the EB binds to an as-yet-unidentified receptor that is associated with PDI (Abromaitis and Stephens 2009). Second, internalization of *Chlamydia* into host cells requires PDI mediated disulphide reduction, although it is unclear whether the PDI substrate in this case is a host cell or a chlamydial protein (Abromaitis and Stephens 2009). However, it has been suggested that PDI-mediated disulphide reduction of T3SS, and particularly CdsF, activates secretion of effectors (Abromaitis and Stephens 2009). The PDI-dependent internalization process of *Chlamydia* is especially interesting as it shares similarities with the PDI-dependent internalization of many viruses (Ryser et al. 1994; Fenouillet et al. 2001; Gallina et al. 2002; Barbouche et al. 2003; Markovic et al. 2004; Wehrl et al. 2004; Ou and Silver 2006).

Intracellular glutathione also plays a role in the early stages of *Chlamydia* infection, although the mechanism remains to be elucidated (Lazarev et al. 2010). Treatment of *C. trachomatis* D/UW-3/Cx with a glutathione precursor increases the inclusion size upon infection of HeLA cells, whereas inhibition of glutathione synthesis and oxidation of glutathione led to four- to sixfold inhibition of infectivity (Lazarev et al. 2010). In *C. psittaci* chlamydial protein synthesis is required for reduction of MOMP (Hatch et al. 1986), but this observation has not been confirmed and no chlamydial protein has, yet, been associated with COMC disulphide reduction in the early stages of infectivity.

Extra-cytoplasmic oxidoreductases in *Chlamydia*

It is not yet known if any chlamydial enzymes are required for COMC protein disulphide reduction or oxidizing COMC protein thiols during differentiation into EBs. However, functional gene assignment of the *C. pneumoniae* and *C. trachomatis* genomes identified five putative extra-cytoplasmic oxidoreductases of the Dsb family (Belland et al. 2003; Mac et al. 2008). The gene products identified included homologs of the well-characterized oxidoreductase redox relay system consisting of DsbA and DsbB responsible for introduction of disulphide bonds to many excreted bacterial proteins as described above, a DsbD homologue of the ScsB family and two Dsb proteins unique to *Chlamydia*, DsbH, and DsbJ.

Despite the identification of Dsb proteins in *C. trachomatis* and *C. pneumoniae*, few of these proteins have been characterized leaving the annotation of the Dsb proteins questionable. We have performed a bioinformatic analysis showing that DsbA, DsbB, DsbD, DsbJ, and DsbH are highly conserved in the *Chlamydiaceas* (Table 1). Further, sequence identities with the *E. coli* Dsb homologues of ~20% for DsbA, DsbB, and DsbD supports the annotation of these as chlamydial Dsb proteins.

According to a transcriptional study DsbA (referred to as DsbG in the study), DsbB and DsbD are all mid-cycle genes with the onset of synthesis 8 h post-infection (Belland et al. 2003) (Figure 5). However, a more recent study reporting on DsbA protein levels reveals that no DsbA is present in replicating RBs 18 h post-infection but that it is present in EBs 44 h post-infection just after exit from the host cell. Consequently, Figure 5 shows DsbA expressed later than DsbB and DsbD.

DsbH and DsbJ are also mid-cycle genes, although DsbH synthesis is not observed until 16 h post-infection (Belland et al. 2003). Even though DsbJ synthesis is observed at 8 h post-infection transcription is highly up-regulated 24 h post-infection and might part of a heavily regulated cluster of mid-cycle genes (Nicholson et al. 2003).

Even though one study refers to CTL0429 as DsbG, we have identified the enzyme as a DsbA enzyme a previous study characterizing the structure and biochemical properties of the enzyme (Christensen et al. 2016). The structure of *C. trachomatis* DsbA shares the characteristic features of DsbA enzymes with a thioredoxin domain, containing the active site cysteines, and an inserted helical domain (Christensen et al. 2016). The structure of *C. trachomatis* DsbA is more closely related to the DsbA from the Gram-positive bacterium *S. aureus*, the acid-fast bacterium *M. tuberculosis* and the alpha-proteobacterium *W. pipentis* than the DsbA from the Gram-negative beta- and gamma-proteobacteria. Like the DsbAs from *M. tuberculosis, P. aeruginosa* and *W. pipientis, C. trachomatis* DsbA has an additional disulphide between helix 2 and helix 5 Unique to *C. trachomatis* DsbA is an additional, unpaired cysteine residue and an unusual catalytic motif with a dipeptide consisting of two small uncharged amino acids (Cys-Ser-Ala-Cys). In accordance with the finding that the dipeptide is an important determinant of the redox potential (Chivers et al. 1997; Huber-Wunderlich and Glockshuber 1998; Quan et al. 2007) the unusual dipeptide contributes to the exceptionally low redox potential (−229 mV) of *C. trachomatis* DsbA which is the least oxidizing DsbA characterized to date. No substrates have been identified for *C. trachomatis* DsbA, hence it is not known whether it is involved in the oxidation of COMC proteins.

Curiously, even though a DsbD homologue has been identified in *C. pneumoniae* and *C. trachomatis* (Mac et al. 2008) no obvious DsbC disulphide isomerase protein is evident in *Chlamydia* genomes. The *Chlamydia* spp DsbD enzymes are predicted to be closely related to the ScsB (Suppressor of Copper Sensitivity protein B) family (Cho et al. 2012), that *Caulobacter crescentus* uses to recycle ScsC to its reduced state. In *Caulobacter crescentus* (Cho et al. 2012), *S. typhimurium* (Gupta et al. 1997; Shepherd et al. 2013), and *proteus mirabilis* (Furlong et al. 2017) the ScsA-D proteins are involved in peroxide reduction and protection against copper ion toxicity. Scs proteins comprising a four-gene cassette in many Gram-negative bacteria) (Altschul et al. 1997) and the *P. mirabilis* ScsB is required for isomerase activity of the soluble periplasmic ScsC (Furlong et al. 2018). However, a BLAST search against *Chlamydia* spp using *S. enterica* Typhimurium ScsA, ScsB, ScsC or ScsD as

Table 1. Chlamydial Dsb proteins were identified by a BLASTp 2.8.0+ search using standard parameters (Altschul et al. 1997) against the protein from *C. trachomatis*.

	Conserved in Chlamydiacea	Seq. similarity to *C. trachomatis* (%)	Seq. similarity to *E. coli*
DsbA	*C. trachomatis*	99	19%
	C. muridarum	89	19%
	C. suis	86	19%
	C. felis	75	21%
	C. abortus	74	21%
	C. psittaci	73	21%
	C. cavie	72	21%
	C. pneumoniae	72	20%
	C. gallinacean	68	20%
	C. avium	68	19%
DsbB	*C. trachomatis*	100	22%
	C. muridarum	86	20%
	C. suis	85	22%
	C. gallinacea	75	19%
	C. psittaci	74	19%
	C. abortus	73	20%
	C. pneumoniae	73	20%
	C. cavie	73	20%
	C. avium	72	19%
	C. felis	69	19%
DsbD	*C. trachomatis*	99–100	22%
	C. suis	80	20%
	C. muridarum	78	21%
	C. psittaci	56	24%
	C. felis	55	24%
	C. abortus	54	24%
	C. cavie	54	24%
	C. pneumoniae	54	24%
	C. gallinacea	52	24%
	C. avium	50	23%
DsbH	*C. trachomatis*	99–100	Not in *E. coli*
	C. muridarum	79	Not in *E. coli*
	C. suis	75	Not in *E. coli*
	C. pneumoniae	51	Not in *E. coli*
	C. psittaci	51	Not in *E. coli*
	C. abortus	51	Not in *E. coli*
	C. felis	50	Not in *E. coli*
	C. cavie	48	Not in *E. coli*
	C. gallinacea	47	Not in *E. coli*
DsbJ	*C. trachomatis*	99–100	Not in *E. coli*
	C. suis	85	Not in *E. coli*
	C. muridarum	81	Not in *E. coli*
	C. ibidis	58	Not in *E. coli*
	C. abortus	55	Not in *E. coli*
	C. felis	55	Not in *E. coli*
	C. caviae	54	Not in *E. coli*
	C. pecorum	54	Not in *E. coli*
	C. pneumoniae	54	Not in *E. coli*
	C. gallinacea	53	Not in *E. coli*
	C. psittaci	51	Not in *E. coli*

search terms only identifies a ScsB homolog in *Chlamydia* spp (Table S1A-D). This is consistent with the reported lack of an ScsC homolog in *Chlamydiales* (Cho et al. 2012). But this leaves open the questions of which family of oxidoreductases this protein belongs to and which proteins it potentially interacts with. The DsbD homologues in the *Chlamydiacea* are generally more closely related to *S. typhimurium* ScsB (25–27% sequence identity, data not shown) than to *E. coli* DsbD (~24%, Table 1). Although not conclusive, this supports the theory that the chlamydial DsbD belongs to the ScsB family

A BLAST search of *C. pneumoniae* TW-183 DsbH revealed that, apart from being highly conserved amongst members of the *Chlamydia* genus, DsbH is primarily found in anaerobic or acidophilic Gram-negative bacteria (Table S1E). Similar to *Chlamydia,* anaerobic Gram-negative bacteria have a high number of secreted and transmembrane proteins with an odd number of cysteines (57% compared to 39% in *E. coli*) (Dutton et al. 2008; Mac et al. 2008).

DsbH is thought to be responsible for maintaining a reducing environment in the chlamydial periplasm to prevent unpaired cysteines forming non-native

intermolecular disulphide bonds (Mac et al. 2008). This is interesting in light of recent findings that the *C. trachomatis* periplasm is reducing in the early phases of the developmental cycle and increasingly oxidizing as infection proceeds. This is in contrast to the cytoplasm, endoplasmic reticulum, and mitochondria where the redox environment is unchanged until the cell dies. (Wang et al. 2014). Structural and biochemical characterization of DsbH revealed that DsbH is a periplasmic disulphide reductase with functional properties that resemble those of the reductase thioredoxin and the C-terminal domain of *E. coli* DsbD (DsbDγ) and with a redox potential (−269 mV) approaching that of *E. coli* thioredoxin (−270 mV) (Mac et al. 2008). In accordance with this DsbH adopts a thioredoxin fold with topology identical to thioredoxin and similar to DsbDγ. DsbH is distinguished from thioredoxin by strand β5 being only loosely connected to the central β-sheet and it has been suggested that this change might be responsible for the different roles of thioredoxin and DsbH. As DsbD in *E. coli* is responsible for the reduction of DsbC and DsbE, two proteins with disulphide reductase activity, in *Chlamydia* DsbD is a possible redox partner for the disulphide reductase DsbH.

DsbJ is highly conserved within the *Chlamydia* genus, but not found outside the *Chlamydiae* phylum (Table S1F). This suggests that DsbJ has a unique and specialized role in *Chlamydia*. Although distantly related to other thioredoxin-fold proteins, DsbJ lacks a catalytic CXXC motif. It has previously been suggested that DsbJ has a regulatory effect on DsbH, although a mechanism behind a possible regulation has not been described (Wang et al. 2014). As DsbJ is a late gene (expressed 16 h post-infection) (Belland et al. 2003) one possibility is that DsbJ negatively regulates DsbH reductase activity, thereby allowing Dsb formation in the late stages of chlamydial development. This would be an important role in the switch from a reducing to an oxidizing *Chlamydia* periplasm. As *Chlamydia* spp, to the best of our knowledge, are the only bacteria that exhibit a shift in the redox environment of the periplasm, such a role would be consistent with DsbJ being found only in *Chlamydia*.

Conclusions and future directions

In this review, we have summarized how the redox state of cysteine-rich proteins in the chlamydial envelope accompanies progression of the chlamydial developmental cycle. An important question that remains to be answered is the interplay between different extracytoplasmic oxidoreductases and how the correct redox state of COMC is secured during the developmental cycle.

Chlamydia, a Gram-negative intracellular parasite, encodes – in common with most other Gram-negative bacteria – the DsbA, DsbB, and DsbD enzymes. In addition, *Chlamydia* share the rare oxidoreductase DsbH with anaerobic and acidophilic Gram-negative bacteria. This variation in Dsb repertoire among bacteria emphasizes the important and ubiquitous role that protein disulphide bonds, and their regulation, play in bacterial lifestyle. Unpicking the specific roles of the Dsb proteins and their mutual interaction is impaired by *Chlamydia* being refractory to routine genetic manipulations. However, in the light of recent advances in genetic manipulation of *Chlamydia* (Bastidas and Valdivia 2016; Ouellette 2018), we might soon be able to investigate the role of Dsb proteins to gain a deeper insight into the Dsb formation and degradation in the chlamydial developmental cycle.

Disulphide-dependent infection and development are unique to *Chlamydia* among bacteria, even though the suite of Dsb proteins in *Chlamydia* is, with the exception of DsbJ, present in other bacteria. Homologs of the canonical oxidative pathway in bacteria, DsbA and DsbB, are widely found across *Chlamydia* suggesting that the Dsb formation machinery operates in a similar manner in *Chlamydia*. DsbH seems to play a specialized role in Gram-negative bacteria with a high proportion of secreted and membrane proteins with an odd number of cysteines i.e. *Chlamydia* and anaerobic bacteria (Dutton et al. 2008; Mac et al. 2008). The proposed DsbJ-mediated regulation of DsbH suggests that, although the suite of Dsb protein in Chlamydia is not markedly different from other Gram-negative bacteria, the redox processes might be subject to tighter regulation. This supports the hypothesis that these intracellular bacteria utilize specialized Dsb proteins to meet the specific needs of the intra- and extracellular phases. Hence, for *Chlamydia* at least, life is indeed different on the inside.

Disclosure statement

The authors report no conflict of interest.

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