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Disulfide bond formation in prokaryotes

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Interest in protein disulfide bond formation has recently increased because of the prominent role of disulfide bonds in bacterial virulence and survival. The first discovered pathway that introduces disulfide bonds into cell envelope proteins consists of *Escherichia coli* enzymes DsbA and DsbB. Since its discovery, variations on the DsbAB pathway have been found in bacteria and archaea, probably reflecting specific requirements for survival in their ecological niches. One variation found amongst Actinobacteria and Cyanobacteria is the replacement of DsbB by a homologue of human vitamin K epoxide reductase. Many Gram-positive bacteria express enzymes involved in disulfide bond formation that are similar, but non-homologous, to DsbAB. While bacterial pathways promote disulfide bond formation in the bacterial cell envelope, some archaeal extremophiles express proteins with disulfide bonds both in the cytoplasm and in the extra-cytoplasmic space, possibly to stabilize proteins in the face of extreme conditions, such as growth at high temperatures. Here, we summarize the diversity of disulfide-bond-catalysing systems across prokaryotic lineages, discuss examples for understanding the biological basis of such systems, and present perspectives on how such systems are enabling advances in biomedical engineering and drug development.

The formation of disulfide bonds (DSBs) in proteins is an oxidative process that generates a covalent bond linking the sulfur atoms of two cysteine residues. DSBs contribute to the activity of many proteins by stabilizing them in their active conformations. In bacteria and eukaryotes, structural DSBs are rarely, if at all, found in proteins of cytoplasmic compartments. Instead, disulfide-bonded proteins are located in more oxidizing environments, such as the bacterial cell envelope and the eukaryotic endoplasmic reticulum[1–3]. In bacteria, in addition to being localized in the cell envelope, proteins with DSBs are also secreted into the growth media and/or into host cells[1,4]. The enzymes responsible for DSB formation in prokaryotes are localized to the bacterial cell envelope themselves, either as soluble proteins or as proteins bound to the cytoplasmic membrane. Thus, the introduction of DSBs into proteins occurs as the polypeptides are translocated into the cell envelope[5]. While archaea also make proteins that are exported from the cytoplasm and contain DSBs, certain extremophile archaea are exceptions to these generalizations as they also make disulfide-bonded proteins in their cytoplasms[6,7].

For many bacteria, DSBs play a role in the folding and stability involved in important cellular processes such as cell division, transport of molecules into the cell, response to environmental threats, and assembly of the outer membrane of Gram-negative bacteria[8,9]. Furthermore, virulence proteins, which are exported from the cytoplasm to either the bacterial cell envelope or into host cells during pathogenesis, require the stability conferred by DSBs in the presence of protease-rich and other destabilizing environments[10–12].

A bioinformatics approach (Box 1) used to analyse the genomes of bacteria and archaea suggests that DSBs are present in the proteome of most bacteria and members of Crenarchaea, but absent in many obligate anaerobes or intracellular organisms[7,13] (Fig. 1). Enzymes required for DSB formation in all kingdoms of life show significant parallels[14]. First, DSB formation requires a thioredoxin-family protein (for example, DsbA in *Escherichia coli*), which acts as an oxidant to catalyse sulfur–sulfur bond formation between pairs of cysteines in substrate proteins. DsbA-like proteins are either soluble in the bacterial periplasm or attached to the cytoplasmic membrane, with the active side located outside that membrane. Most organisms also have a disulfide generator enzyme (for example, DsbB in *E. coli*) that reoxidizes the thioredoxin-like protein, restoring its ability to promote DSB formation[13,15]. In a few bacteria, the second enzyme appears to be absent. Among the disulfide generator enzymes known to date in prokaryotes are the DsbB and vitamin K 2,3-epoxide reductase (VKOR) families. There are structural and functional similarities between these two families of enzymes[16,17]. DsbB and VKOR families share no protein sequence homology but they exhibit similar structural features (Box 1) and both contain a cofactor to generate a DSB de novo. This cofactor is a quinone molecule[18,19] that can be either ubiquinone, menaquinone (vitamin K_2) or phylloquinone (vitamin K_1)[20,21]. Detailed mechanistic aspects of DSB formation in Gram-negative bacteria have been previously reviewed in refs [22–25].

In this Review Article, we focus on the recent advances and diversity in DSB formation pathways in a variety of Gram-negative and Gram-positive bacteria, and in archaea.

DSB formation in Gram-negative bacteria

DSB-forming systems are found in many Gram-negative bacteria, including the prototypical DsbAB system in *E. coli*.

The prototypical DsbAB system. The discovery of enzymes in a pathway leading to protein DSB formation arose out of genetic and physiological studies of *E. coli*[1,15,26,27]. These studies revealed two enzymes required for this process. The first enzyme discovered was DsbA, a thioredoxin homologue located in the bacterial periplasm (Fig. 2a). The two cysteines of the CysXXCys motif in DsbA are in the disulfide-bonded form and can catalyse the formation of DSBs by a thiol–disulfide exchange reaction. This involves removing electrons from pairs of cysteines in substrate proteins, leading to the formation of a covalent bond[1,28].

The second enzyme, DsbB, is required for the maintenance of an active DsbA. It does this by restoring the disulfide-bonded state of the active site of DsbA after the latter enzyme has acted on its substrates[15]. DsbB is a cytoplasmic membrane protein with six cysteines, four of which are essential for the reoxidation of DsbA[15,29]. In its active form, DsbB is oxidized and regenerated by the oxidative activity of quinones. This oxidation of DsbB depends largely on ubiquinones under aerobic growth conditions, and menaquinones

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Box 1 | Identifying DSB-forming systems: from bioinformatics to structures

A strong bias for even numbers of cysteines in bacterial cell envelope proteins has been correlated to DSB formation in *E. coli* and other bacteria[13]. Thus, using bioinformatic approaches to count cysteines in proteins belonging to a particular subcellular compartment can suggest the presence of DSB proteins[7]. As the oxidative systems that directly lead to the formation of protein DSBs include Dsb-like proteins, searches for DsbA, DsbB and VKOR homologues also support such suggestions[13]. Nevertheless, these approaches should be verified through biochemical assays in vitro using purified enzymes, by localizing disulfide-bonded proteins to the relevant subcellular compartment and by functional demonstration that the cognate DsbA is essential for the oxidation of these proteins in vivo. The latter analysis is necessary as the presence of numerous thioredoxins in a cell may lead to identification of multiple DsbA-like proteins. Indeed, forced export of *E. coli* cytoplasmic reductant thioredoxin-1 to the periplasm can rescue an *E. coli* DsbA mutant and restore efficient DSB formation[159].

Bioinformatic approaches have revealed some bacteria that may encode very few exported proteins with an even number of cysteines[13]. For example, *Bacteroides fragilis* was unable to oxidize known disulfide-bonded proteins, suggesting that *Bacteroides* may either have limited ability to make DSBs or lack the oxidative system. We note that organisms with similar properties to *Bacteroides* fall into the classes of obligate anaerobes or obligate intracellular organisms, suggesting that DSB formation may be unfavourable under generally reducing environments such as anaerobic sediments or the host cell cytoplasm[13].

Finally, the structures of Dsb and VKOR proteins have provided profound insight into the molecular mechanisms of DSB formation. First, crystallization studies revealed that DsbB and VKOR proteins have structural similarity, with both proteins containing four transmembrane domains (TMs) that form a four-helix bundle encircling the quinone[19]. However, the cysteine that interacts with quinone is located on the periplasmic side of TM2 in DsbB, and on the periplasmic side of TM4 in VKOR. On the other hand, the pair of cysteines that interact with DsbA are located between TM3–4 in DsbB, and TM1–2 in VKOR[18,19]. Second, crystallization of DsbA proteins from human pathogens indicated a degree of structural divergence from the *E. coli* DsbA, and a comparative analysis of 13 prokaryotic DsbA structures revealed two classes that differ in their β-sheet arrangements—dividing them into Gram-negative and Gram-positive DsbA homologues. Each class is comprised of two subclasses, based on their surface features surrounding the CysXXCys active site. In class Ia, DsbA proteins exhibit a bigger hydrophobic groove than class Ib, while class II DsbA proteins look topologically distinct from class I and have an additional DSB, which might play a regulatory role[160].

under anaerobic conditions[20,30,31]. The reduced quinones then transfer the electrons to terminal oxidases of the respiratory chain, and from there to the final electron acceptors[20] (Fig. 2a).

While the DsbAB pathway in *E. coli* is directly responsible for joining a protein's cysteines into DSBs, this oxidation process sometimes results in an array of non-native DSBs in substrate proteins. These 'errors' may be largely due to the finding that DsbA interacts with cysteines of proteins as they appear in the periplasm, initiating DSB formation during protein translocation. Thus, a protein's cysteines are joined into DSBs as they appear sequentially in the periplasm[5,32]. If the DSBs formed in this way are not the same as the native DSBs, a misfolded protein is the result and a second pathway is necessary to ensure that the fully translocated protein ultimately contains the correct DSBs. This pathway utilizes the periplasmic protein DsbC, in a homodimeric form, to shuffle the non-native DSBs of proteins by isomerization and allowing formation of the correct native bonds. Attack of non-native DSBs requires a reduced DsbC, especially in fully oxidized substrates that lack a free thiol to facilitate intramolecular disulfide rearrangement. The electrons required for maintaining DsbC as a reductant are transferred from the cytoplasmic membrane protein DsbD, which itself is reduced by cytoplasmic thioredoxins[32–37] (Fig. 2a).

Variations in the DsbAB pathways. Since the discovery of the *E. coli* DsbAB and DsbCD pathways, researchers have elaborated the mechanisms of action of the component proteins and identified other organisms that use the same pathways. Based on a bioinformatics approach (Box 1), most Proteobacteria are predicted to use DsbA- and DsbB-like proteins to form DSBs in exported proteins; the exceptions are obligate intracellular Proteobacteria, obligate anaerobic Proteobacteria and Deltaproteobacteria[13] (Fig. 1). However, DsbAB pathways display distinct variations across the various lineages. For example, while the prototypical *E. coli dsbA* and *dsbB* genes are distant from each other in the chromosome, some bacteria encode DsbAB proteins as an operon. For those *dsbA* and *dsbB* genes that are expressed from an operon, the DsbB protein exhibits a large enough difference in length (49 residues) that it has been renamed DsbI, although it can reoxidize reduced *E. coli* DsbA[38,39]. To distinguish this pair of proteins from the more common DsbAB pair, the name of the DsbA homologue has been changed to DsbL. Among those bacteria that exhibit the same *dsbLI* operon are *Geobacter metallireducens*, pathogenic *E. coli* species and some *Salmonella* species[13]. In the two latter examples, the operon includes a third gene (*assT*) encoding the periplasmic enzyme arylsulfate-sulfotransferase[39–42] (Fig. 2b), the dedicated substrate of DsbLI[41,42]. Some *dsbLI* operons outside the Proteobacteria are found in prophages or plasmids, suggesting that they may have been incorporated into other species through horizontal gene transfer[13].

Some bacteria encode two or more sets of DsbAB proteins that appear to play different roles, but can substitute for each other in oxidizing particular substrates. Where sufficient analysis is available, it is clear that there is one DsbA acting on the majority of substrates and its absence causes multiple phenotypes, such as decreased virulence[43–48]. Gram-negative bacteria with multiple Dsb proteins include *Salmonella enterica* serovar Typhimurium[12,49], *Neisseria meningitidis* and *Pseudomonas aeruginosa*[50] (Fig. 2c). For instance, *N. meningitidis* encodes three DsbA homologues[51,52]; DsbA1 and DsbA2 are lipoproteins anchored to the inner membrane, while DsbA3 is a soluble periplasmic protein (Fig. 2d). The absence of these three proteins causes a growth defect[51]. DsbA1 and DsbA2 are necessary for assembly of the outer-membrane secretin PilQ, and consequently for type IV pilus biogenesis[53].

Some Proteobacteria DsbAs can reduce as well as oxidize DSBs in vivo[54–56]. For instance, *Legionella pneumophila* harbours two DsbAs, two DsbBs and two DsbD homologues. *L. pneumophila* DsbA2 is essential for growth and serves as both an oxidase and an isomerase[48]. For this task, DsbA2 exists as a homodimer (similar to *E. coli* DsbC[57]) and is in a mixture of oxidized and reduced forms, maintained by the activity of both DsbB and DsbD[48,54,58] (Fig. 2e). This DsbA homologue is widely distributed among intracellular Gram-negative pathogens that lack DsbC homologues, such as *Legionella*, *Coxiella*, *Anaplasma*, *Rickettsia*, *Brucella*, *Agrobacterium*, *Bartonella* and *Ehrlicha*[48]. Similarly, *Francisella tularensis* lacks

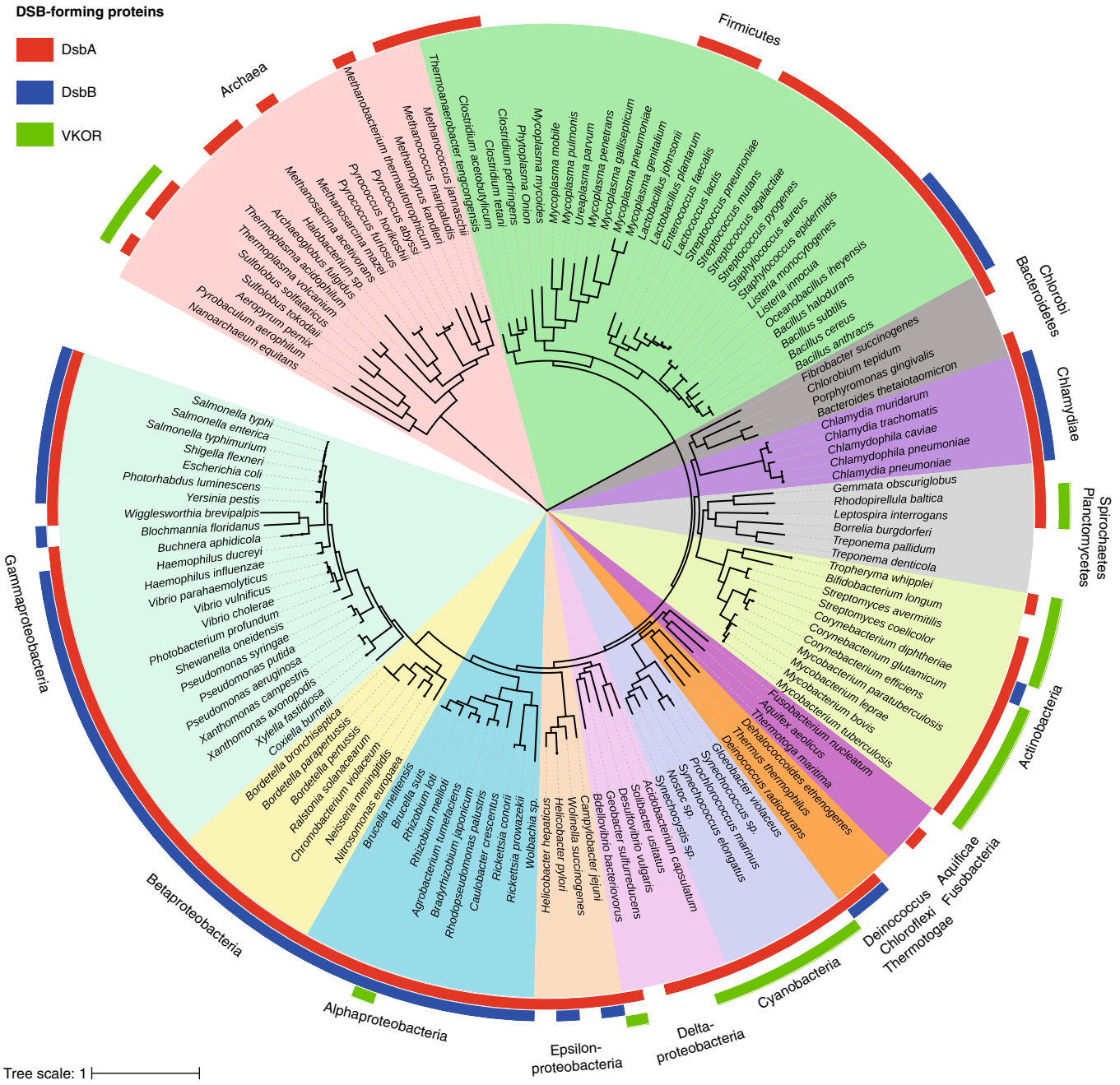

Fig. 1 | A 'Tree of Life' diagram showing the DsbAB, DsbA–VKOR and non-disulfide clades. Red, blue and green indicate the presence of DsbA, DsbB and VKOR homologues, respectively, in each species. Hidden Markov models for DsbA, DsbB and VKOR were obtained from Pfam 31.0 (https://pfam.xfam.org). Searches were run with HMMER v3.1b2 (obtained from http://hmmer.org). Additional models were constructed with hmmbuild, a HMMER program, by aligning homologues of experimentally characterized DSB-forming proteins, which were identified by BLAST (https://blast.ncbi.nlm.nih.gov/), aligned with Muscle v3.8.31 (https://www.drive5.com/) and manually trimmed. The phylogenetic tree was generated using the Interactive Tree of Life v3.5.4 web server (https://itol.embl.de) by modifying the default tree[158].

DsbCD homologues and its DsbA is also capable of reducing its substrates in vivo. Geographically distinct *Francisella* strains also have naturally occurring variations in the *cis*-proline motif adjacent to the CysXXCys site in the three-dimensional structure associated with altered isomerase activity and virulence[55,56].

Recent studies on DSB formation among Epsilonproteobacteria have revealed a diversity of Dsb proteins found in this subdivision (reviewed in ref. [59]). *Campylobacter jejuni* has two DsbB homologues and one, named DsbI (unrelated to our previous example of DsbLI of enteric bacteria), contains a β-propeller domain at the carboxy terminus with an unknown function. *C. jejuni* DsbB can complement the function of DsbI but not vice versa[60]. *C. jejuni* also harbours two DsbA proteins (oxidized by a single DsbB) that oxidize different substrates; DsbA2 oxidizes arylsulfotransferase, while DsbA1 oxidizes alkaline phosphatase and is involved in motility and autoagglutination[47]. *Helicobacter pylori* also has a DsbI homologue and a homodimeric DsbA-like protein named DsbK, that acts as an oxidase and is partly reoxidized by DsbI[61–64].

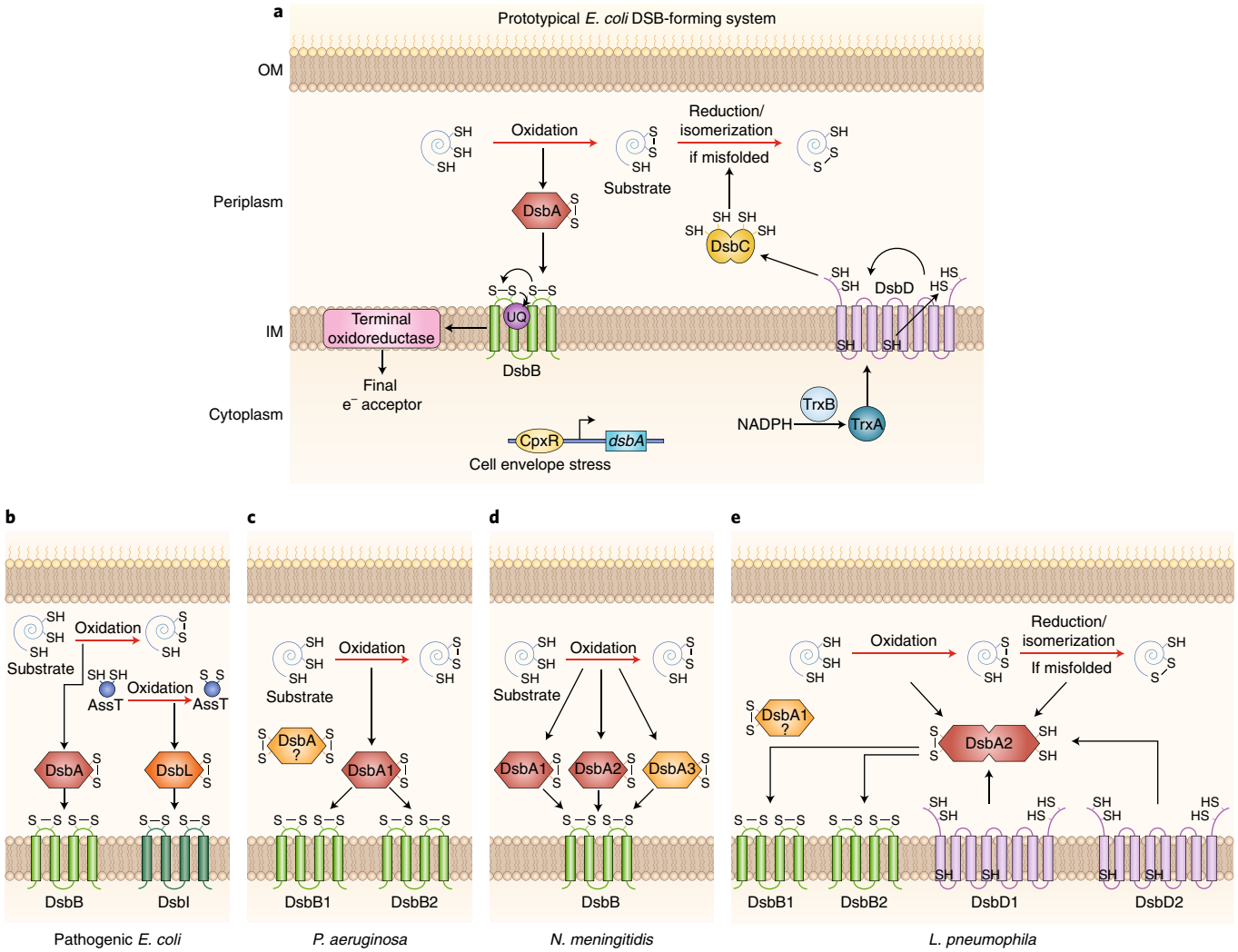

Fig. 2 | DsbAB pathways mediating disulfide bond formation. a, In *E. coli*, DsbA oxidizes a substrate and becomes reduced. To start a new catalytic cycle, DsbB reoxidizes DsbA by transferring the electrons to ubiquinone. If DsbA introduces incorrect disulfides in the substrate, DsbC reduces the non-native DSBs and allows the formation of native bonds. The electrons for this reduction process come from cytoplasmic thioredoxin to DsbD, and then to DsbC. **b**–**e**, Alternative permutations in the DSB formation pathway among Gram-negative bacteria include several DsbA or DsbB homologues such as pathogenic *E. coli* strains and *Salmonella enterica* sv. Typhimurium (**b**), *P. aeruginosa* (**c**) and *N. meningitidis* (**d**) and *L. pneumophila* (**e**). Black arrows indicate the flow of electrons. e⁻, electron; SH, thiol groups of cysteines; S–S, disulfide-bonded cysteines; UQ, ubiquinone; OM, outer membrane; IM, inner membrane; ?, unknown substrates.

Essentiality of DsbAB pathways. The viability of mutants lacking DSB-forming machinery varies from organism to organism. There could be as many as 300 disulfide-bonded proteins in the *E. coli* periplasm (when expressed) according to in silico predictions[13]. Nevertheless, no systematic attempt has been performed to validate such predictions, perhaps partly because many of the proteins may not be expressed under standard growth conditions[9,13]. Despite reduced stability and function of many proteins, *E. coli dsbA* and *dsbB* mutants are viable aerobically[1], but not anaerobically[65,66]. The reason that essentiality varies with growth conditions is that background oxidation of such proteins under aerobic conditions is sufficient to maintain a significant enough pool of active essential proteins to permit *E. coli* growth[66,67]. This background oxidation is much less efficient than DSB formation mediated by Dsb proteins and it varies widely among the numerous DsbA substrates[8,66,68]. From a list of ten essential *E. coli* proteins predicted to be exported and contain cysteines, only two are known to have DSBs—the cell division protein FtsN, and the lipopolysaccharide assembly protein LptD. A number of other Gram-negative bacteria either do not have LptD and FtsN proteins or they are non-essential, making it possible that DSB-deficient mutants of these species would be viable[69,70].

DSBs are also important for the stability of multiple virulence factors. Mutants lacking functional DSB formation display attenuated virulence in animal models. This was first noted in *Vibrio cholerae*, where a mutant inactivating a *dsbA* gene (named *tcpG* at the time) resulted in inability to colonize infant mice[71] and reduced cholera toxin activity[4,72]. DSB formation mutants also exhibit a variety of pathogenicity-related phenotypes, including altered adhesion, agglutination, motility, cell survival and spread (reviewed in refs [10–12]). The attenuation of virulence in mutants lacking Dsb proteins also occurs with other pathogenic bacteria, and searches for virulence factors have yielded Dsb homologues (Table 1).

Regulation of DSB formation. The regulation of *dsb* genes varies among organisms depending on the role that disulfide-bonded proteins play in the cell. In *E. coli*, the Cpx pathway induces synthesis of DsbA and other protein-folding catalysts during cell envelope stress. Such a stress may come into play when the cell experiences

Table 1 | DsbA and DsbB mutants that display attenuated virulence in infection models

Organism	Mutant	Infection model	Ref.
Vibrio cholerae	DsbA (TcpG)	Infant-mouse model for cholera	4,71b
Erwinia carotovora	DsbA	In planta potato tuber virulence assays	142
Pseudomonas syringae	DsbA	*Arabidopsis thaliana* and tomato infection	143b
E. coli K1	DsbA	Infant-rat model of invasive disease	144b
Haemophilus influenza	DsbB and DsbA	Infant-rat model of invasive disease and murine bacteraemia model	145,146b
Helicobacter pylori	DsbK	Mice gastric colonization	147,148
Salmonella enterica serovar Typhimurium	DsbA	Mice peritonitis infection model	43
Proteus mirabilis	DsbA	Mice model of urinary tract infection	149b
Helicobacter pylori	DsbI	Mice gastric infection	150
Pseudomonas aeruginosa	DsbA	Virulence in fruit fly *Drosophila melanogaster* and mice peritonitis model	45b
Xanthomonas campestris	DsbB	Black rot disease on pepper/radish plants	151b
Francisella tularensis subsp. *tularensis*	DsbB and DsbA (FipB)	Mice infection model for tularaemia	152,153
Uropathogenic E. coli CFT073	DsbAB[a]	Mice urinary tract infection model	39
Francisella tularensis subsp. *holarctica*	DsbA	Intraperitoneal and subcutaneous mice infections	154,155
Campylobacter jejuni	DsbBI[a]	Chicken intestinal tract colonization	46
Legionella pneumophila	DsbA2	*Acanthamoeba castellanii* amoeba infection	58
Aeromonas hydrophila	DsbA	*Dictyostelium* amoebae growth, rainbow trout infection and mice intraperitoneal infection	156
Burkholderia pseudomallei	DsbA	Mice peritonitis infection	157
Corynebacterium diphtheriae	DsbA	Guinea pig model of diphtheritic toxaemia	93

[a]Double mutants.[b]Screening studies to find virulence factors.

environmental conditions that interfere with protein folding, such as those that can be encountered by certain Enterobacteriaceae in host tissues during infection[73]. In *C. jejuni*, the expression of *dsb* genes is regulated by iron availability; the abundance of DsbA1 and DsbA2 decreases when iron becomes restricted[74]. Regulation of the Dsb system is also seen in *L. pneumophila*, an obligate intracellular pathogen of protozoa that alternates between an intracellular, vegetative, replicating form and a planktonic cyst-like form that is highly infectious. Interestingly, cyst germination appears to be restricted to intracellular environments due to a requirement for the environmentally scarce amino acid, cysteine. The transition to cyst occurs mostly outside the cytoplasm and at the expense of cell envelope remodelling. In this transition, an increase in *dsbA2* expression has been detected and is required for proper folding of the Dot/Icm type IV secretion system involved in delivery of several effector proteins into host cells[54,58].

DSB formation in Gram-positive organisms

As Gram-positive bacteria do not have a periplasm, proteins secreted from the cytoplasm are often either cell-wall-associated or extracellular. However, the layer of peptidoglycan–teichoic/mycolic-acid present is thought to create a periplasm-like space for Gram-positive bacteria[75–78] that provides a DSB-catalysing compartment, as seen in Gram-negative bacteria. Additionally, the DsbA–VKOR pathway in Actinobacteria provides a second pathway for DSB formation amongst both Gram-positive and -negative bacteria. To date, no DsbC-like disulfide-isomerization pathway has been identified in Gram-positive organisms. One explanation is that all the native disulfide-bonded proteins of these organisms have protein DSBs formed between cysteines that appear sequentially in the protein.

The prototypical DsbA–VKOR pathway. A bioinformatics approach using cysteine counting in proteins and homology searches (Box 1) indicates that bacterial groups such as the Actinobacteria, Cyanobacteria and aerobic Deltaproteobacteria contain proteins with DSBs and that they maintain DsbA oxidized not by DsbB, but by the protein VKOR—a homologue of human vitamin K epoxide reductase[13,79]. Bacterial VKOR was identified by the finding that its gene was either fused to a *dsbA*-like gene in *Salinibacter ruber*, *Leptospira interrogans, Bdellovibrio bacteriovorus, Synechocystis* sp. and *Synechococcus* sp.[13,19,79,80], or located directly adjacent to a *dsbA*-like gene in organisms such as *Mycobacterium tuberculosis*[13,81,82]. The role of VKOR in humans is to maintain the cellular pool of reduced vitamin K, a quinone cofactor, in the post-translational modification of blood coagulation proteins (reviewed in ref. [83]). The mechanism by which mycobacterial VKOR receives electrons from DsbA and transfers them to vitamin K (a menaquinone) is analogous to that by which bacterial DsbB receives electrons from DsbA and transfers them to quinones[81]. Thus, *E. coli* DsbB can replace VKOR in *Mycobacterium smegmatis* and, conversely, *M. tuberculosis* VKOR can replace DsbB in *E. coli*[84].

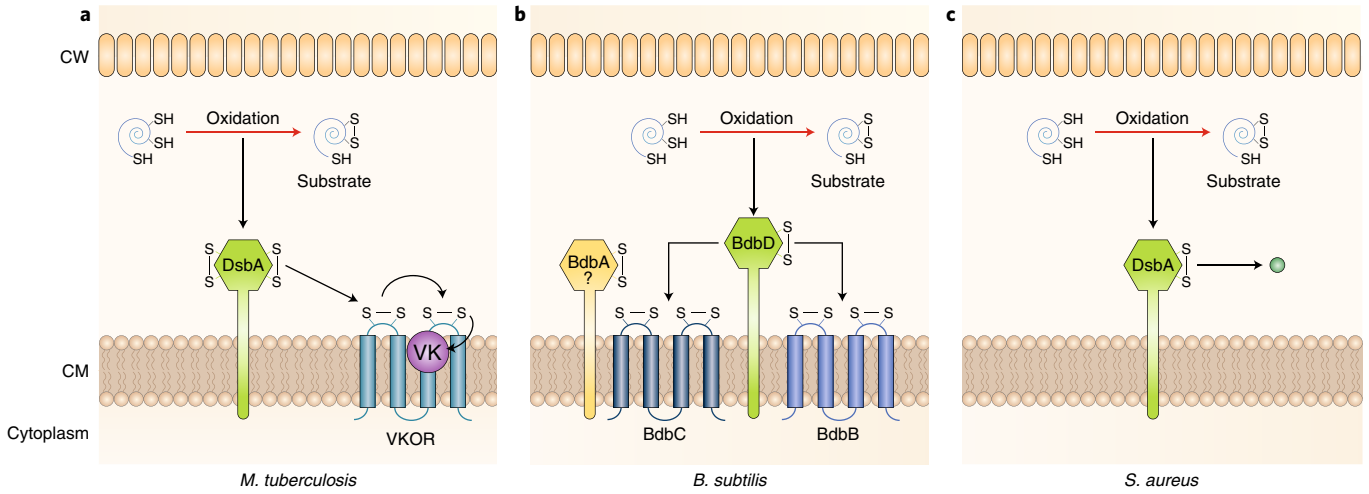

Fig. 3 | DSB formation in Gram-positive bacteria. a, Most Actinobacteria have a DsbA–VKOR pathway. For instance, *M. tuberculosis* uses VKOR instead of DsbB to reoxidize a DsbA-like protein. **b**, Alternative pathways observed in Firmicutes, such as those in *B. subtilis*, include Bdb proteins. BdbA and BdbD are DsbA-like, whereas BdbB and BdbC are DsbB-like proteins. **c**, Other variations include a single DsbA that can be re-oxidized by small molecule oxidants in *S. aureus*. Black arrows indicate the flow of electrons. SH, thiol groups of cysteines; S–S, disulfide-bonded cysteines; VK, vitamin K; CM, cytoplasmic membrane; CW, cell wall; ?, unknown substrates.

Even though DsbB and VKOR are not homologues and may have arisen by convergent evolution, an in silico analysis suggests that DsbB and VKOR may be highly divergent but have evolved from a common ancestor. That is, one of the two underwent a cyclic permutation followed by an ancient gene duplication/deletion event[17]. This analysis of 327 VKOR and 514 DsbB sequences revealed that VKOR shows a greater evolutionary diversity than DsbB, consistent with the existence of DsbB only in prokaryotes while VKOR is found in all major kingdoms of life[17]. Moreover, there are structural similarities between the two enzymes, including a highly conserved organization of transmembrane helical bundles despite a different functional ordering of helical pairs (Box 1).

DSB formation in Actinobacteria. Gram-positive Actinobacteria, including *Corynebacterium*, *Streptomyces* and *Mycobacterium*, secrete cell envelope proteins with even numbers of cysteines and encode predicted oxidoreductases and VKOR orthologues[13,85]. The *M. tuberculosis* DSB pathway consists of DsbA and VKOR. *M. tuberculosis* DsbA is a membrane-anchored protein that itself contains a structural DSB[82,86,87], shows oxidase activity in vitro and is able to interact in vitro, through cysteines, with a hexapeptide derived from the periplasmic loop of VKOR[82]. However, one group has reported that the mycobacterial DsbA exhibits isomerase but not oxidase activity in vitro[82,87]. Furthermore, wherever it is detected in bacteria, VKOR is encoded in an operon with DsbA or the two proteins are expressed as a fused protein. These properties are consistent with the suggestion that those two proteins are the analogues of DsbB and DsbA. However, there are two other oxidoreductases that display oxidase activity in vitro, DsbE and DsbF[88,89], that may also be involved in the DSB formation pathway. That VKOR and DsbA are essential for growth in *M. tuberculosis*, but neither DsbE nor DsbF are essential, suggests that both DsbA and VKOR are required to fold one or more essential substrates in mycobacteria[84,90] (Fig. 3a). However, more in vivo evidence is needed to confirm this.

Studies of the DSB-forming pathway of two additional Actinobacteria have revealed a VKOR homologue and a membrane-associated DsbA-like protein, MdbA (monoderm disulfide-bond forming protein A)[91,92]. *Actinomyces oris* MdbA is essential for growth and the MdbA–VKOR pair is required to introduce DSBs into proteins such as pilus shaft proteins[91]. Similarly, *Corynebacterium diphtheriae* has an MdbA protein that is required for proper growth, division, pilus assembly and diphtheria toxin oxidation[93]. Although the *C. diphtheriae* MdbA redox partner remains to be characterized, in silico predictions indicate the presence of a DsbB homologue in *C. diphtheriae* while *Corynebacterium efficiens, Corynebacterium glutamicum and Corynebacterium jeikeium* harbour a VKOR homologue instead[13] (Fig. 1).

DSB formation in Firmicutes. Gram-positive aerobic and facultative anaerobic Firmicutes, such as bacilli, *Listeria* and staphylococci, express DsbA-like and/or DsbB-like proteins that are involved in DSB formation. *Bacillus subtilis* has four Bdb proteins that share some similarity (~20% amino acid conservation) with *E. coli* Dsb proteins[94,95]. BdbA and BdbD are DsbA-like, while BdbB and BdbC are DsbB-like proteins (Fig. 3b). BdbD is membrane-bound with a calcium-ion binding site[96]. The BdbCD pair oxidizes Com proteins involved in DNA uptake and competence[97,98]. On the other hand, BdbAB is localized in the SPβ prophage locus, forming an operon with the genes for lantibiotic sublancin synthesis, a glycopeptide that requires two DSBs. However, only the BdbBC pair are required for sublancin production[99,100].

Staphylococcus aureus has a DsbA-like membrane-anchored lipoprotein that is able to generate DSBs without the presence of a disulfide generator enzyme, such as DsbB or VKOR[100,101] (Fig. 3c). While the reduced form of *E. coli* DsbA is more stable than the oxidized form, *S. aureus* DsbA lacks a destabilizing catalytic disulfide, and both oxidized and reduced *S. aureus* DsbA have identical thermodynamic stabilities[102]. This characteristic requires *E. coli* DsbA to be re-oxidized by DsbB, but may facilitate *S. aureus* DsbA re-oxidization efficiently by small molecule oxidants[102]. Despite the lack of growth phenotypes in the *S. aureus* DsbA mutant[101], its function has been implicated in forming DSBs for ComGC pseudopilin stability[103].

While bioinformatic analyses of anaerobic and some facultative anaerobic Firmicutes indicated the lack of *E. coli* DsbAB homologues[13,85], there have been recent efforts to find related oxidative pathways in the cell envelope of some members of Firmicutes, given that some of their proteins, such as tetanus toxin, botulinum toxin and streptococcal pyrogenic exotoxin A, require DSBs for activity[104–107]. A homology search for *B. subtilis* BdbD homologues revealed a DsbA-like disulfide oxidase in *Streptococcus gordonii* (named SdbA for *Streptococcus* disulfide bond protein A). This protein

is conserved in other members of anaerobic Firmicutes thought to lack DSB formation, such as *Clostridium tetani* and *Streptococcus pneumoniae*. SdbA is membrane-associated and involved in oxidation of autolysin in *S. gordonii*[107]. SdbA is also able to use only the C-terminal cysteine of the CysXXCys motif to oxidize proteins in vitro and in vivo[108]. Unlike other single-Cys-containing enzymes such as rhodanese PspE, which oxidizes proteins via a sulfenic acid derivative[109], SdbA can only use low-molecular-weight thiols to catalyse DSB formation[108]. The redox partner of SdbA, if one exists, remains to be discovered. As members of the genera Clostridia and Lactobacillales are obligate fermenting organisms and are generally thought to lack an electron transport chain[13], these organisms may have different ways to reoxidize SdbA.

Cytoplasmic DSBs in Archaea

The cysteines of cytoplasmic bacterial proteins are found mostly in the reduced state. The exceptions are enzymes in which DSBs are formed during their catalytic cycle and which, to be regenerated, are reduced by thioredoxins and glutaredoxins[107–109]. The reductive environment of the cytoplasm may play a role in the absence of cytoplasmic DSBs. However, an *E. coli* deleted for the reducing enzymes thioredoxin reductase and glutathione reductase, and combined with a weaker enzymatic substitute for glutathione reductase, accumulates oxidized thioredoxins in the cytoplasm. These thioredoxins are able to promote DSB formation in cell envelope proteins artificially localized to this normally reducing compartment[110–112]. Furthermore, results of studies on DSB formation pathways in extremophile archaea are consistent with the conclusion that most, if not all, proteins with structural DSBs require the presence of oxidative DSB-forming enzymes in their cellular compartment.

The proteomes of extremophiles were initially thought to exclude cysteines and lack DSBs, as it was assumed that cysteines would be oxidatively degraded by the high temperatures and harsh conditions under which these hyperthermophilic organisms grow[113]. However, in silico analyses revealed that the proteins of several thermophilic eubacteria and archaea, in particular *Pyrobaculum aerophilum* and *Aeropyrum pernix*, have a preference for even numbers of cysteines amongst both cytoplasmic and exported proteins and thus may contain DSBs in both compartments[7,114]. This prediction was confirmed by reports of cytoplasmic proteins that contain at least one DSB[115,116], suggesting that structural DSBs in cytoplasmic proteins are likely to be important in stabilizing proteins against thermal unfolding and denaturation.

The cytoplasmic protein disulfide oxidoreductase (PDO) is suggested to play a role in DSB formation in cytoplasmic proteins of hyperthermophiles[3,6,115,117] (Fig. 4). The PDO proteins purified from *Pyrococcus furiosus*, *Sulfolobus solfataricus* and *A. pernix* can oxidize, reduce and isomerize DSBs in vitro[118–120]. Further support for a cytoplasmic DSB pathway is the finding that Crenarchaea have two membrane-bound VKOR homologues, and that the active site of one is oriented towards the extra-cytoplasmic side of the cytoplasmic membrane, while the other faces the cytoplasmic side[112,117]. The organization of these two VKORs suggests that there may be two distinct pathways for DSB formation, with each VKOR catalysing the reoxidation of a putative oxidoreductase in its own compartment (Fig. 4). The two VKORs are presumed to use quinones for maintenance of their oxidized state. Quinone oxidation has also been observed in a different membrane protein (a sensor kinase, ArcB) with its cysteines facing the cytoplasm[121]. In agreement with this interpretation, when cloned into *E. coli*, the *A. pernix* VKOR protein that faces outward can reoxidize periplasmic DsbA. Meanwhile, the *A. pernix* VKOR active site facing the cytoplasm can reoxidize a DsbA engineered to be cytoplasmically localized[112], as well as the ApPDO protein[117]. However, not all hyperthermophilic organisms with predicted DSBs in cytoplasmic proteins have two VKOR proteins. Thus, for those organisms, the mechanisms of DSB formation remain to be investigated[117].

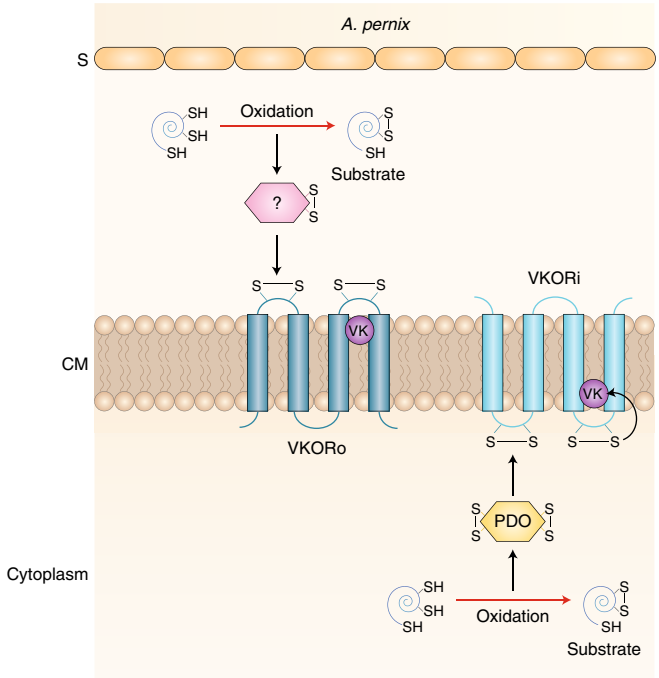

Fig. 4 | Cytoplasmic disulfide formation in Archaea. *A. pernix* has two pathways for DSB formation in each compartment that consists of two opposite-facing VKOR homologues (VKORo and VKORi), which may catalyse the reoxidation of a putative oxidoreductase in its own compartment. The cytoplasmic protein PDO is suggested to play a role in DSB formation in cytoplasmic proteins. Black arrows indicate the flow of electrons. SH, thiol groups of cysteines; S–S, disulfide-bonded cysteines; VK, vitamin K; CM, cytoplasmic membrane; S, surface layer; ?, unknown oxidoreductase.

DSBs in biotechnology and drug development

The study of DSB formation has led to research applications in biotechnology and drug development. In biotechnology for instance, co-expression of DsbA and DsbC has helped increase recombinant-protein-folding yields[122]. Additionally, the engineered *E. coli* strains that introduce DSBs in the cytoplasm allow production of correctly folded, disulfide-bonded proteins that are otherwise difficult to express[111,123], such as tissue plasminogen activator and biologically active antibodies[111,124]. The engineered *E. coli* strains have diminished cytoplasmic reductive pathways[110,125] and, in the case of the SHuffle strain, expression of DsbC in the cytoplasm allows formation of cytoplasmic DSBs[123]. Firmicutes such as *Lactobacillus* and *Lactococcus* spp. also have biotechnological value in fermentation and bacteriocin production[126]. The pediocin-like bacteriocins are a group of antimicrobial peptides produced by lactic acid bacteria and are reported to have DSBs to stabilize their structure[127–130]. The pre-bacteriocins genes often form an operon with an ABC transporter that cleaves off the peptide, an accessory protein (some with a CysXXCys motif) and its immunity protein. The cysteines of the CysXXCys motif of PedC are required to produce active pediocin with proper DSBs in *Lactobacillus sakei*, suggesting oxidase activity in these bacteriocin accessory proteins[130].

Given that DSB formation contributes substantially to the pathogenicity of bacteria, the Dsb proteins may represent attractive targets for development of inhibitors that can act as antibiotics or anti-virulence compounds. Inhibition of DSB formation would then result in simultaneous inactivation of several types of virulence factors. Additionally, such inhibitors may also shed light on the role of newly discovered DSB-forming systems in other organisms. For instance, *M. tuberculosis* VKOR inhibitors may trap the partner of

VKOR and also allow, by two-dimensional protein gel analysis[8,9], identification of the pathway substrates.

Studies have been initiated to find such drugs using screening approaches for inhibitors of DsbA, DsbB and VKOR proteins[65,131–135] (reviewed in ref. [136]). One such approach consisted of a nuclear magnetic resonance spectroscopy (NMR)-based fragment screen to find inhibitors of *E. coli* DsbB[131]; the inhibitors found competed with quinone or interfered with the affinity of DsbB for both quinone and DsbA. A second approach used cell- and target-based screening to identify inhibitors of the *E. coli* DsbB and *M. tuberculosis* VKOR enzymes[65]. This yielded classes of molecules inhibiting DsbB enzymes from *E. coli* and other Gram-negative bacteria. Two scaffold molecules found in these approaches were studied by structure–activity relationship (SAR) to improve potency and determine the mechanism of inhibition[65,134]. Both scaffolds affected the ability of DsbB to reoxidize DsbA, but one scaffold covalently bound to the catalytic cysteines of both DsbB and DsbA[134] while the other bound only to one catalytic cysteine of DsbB[65]. A third approach utilized an in silico screen and thermal shift, isothermal titration calorimetry, and substrate oxidation assays to identify peptides that inhibited *E. coli* DsbA by interfering with the DsbB-binding surface. These experiments yielded peptidomimetic fragments that bound covalently to an active-site cysteine of DsbA, as well as peptides that inhibited DSB formation through non-covalent binding[132,137]. Finally, a fourth screen used ligand-detected saturation transfer difference–NMR experiments to identify inhibitory fragments against *P. aeruginosa* DsbA1 (ref. [135]). Some of the hits bound non-covalently to *P. aeruginosa* DsbA1 on the face opposite to the active site surface[135].

Further SAR studies of existing inhibitors to improve potency may provide clinically valuable candidate leads. However, validation of these candidate inhibitors in bacteria presents a challenge. While some organisms have well-characterized DSB phenotypes (for example, in *E. coli*, DSB inhibition can be detected using DSB-sensitive β-galactosidase, anaerobic growth[65,67], or *lptD*[138]), validation of inhibitors in other bacteria, where the essentiality of Dsb proteins has not been established or where few disulfide-bonded substrates are known, may be more challenging. Better understanding of these pathways should help provide whole-cell assays for pursuing inhibition of DSB formation.

Finally, validation of DSB inhibitors in vivo during host infection will be required to identify the most effective and safe drug candidates for further preclinical development. On the issue of safety, some of the currently identified inhibitors do not show in vitro inhibition of human thioredoxin or protein disulfide isomerase enzymes[65,134,137], but others do have some reactivity with reduced glutathione[134]. Strong inhibition of DsbA or DsbB enzymes obtained in these screens correlated with covalent binding of inhibitors to the target enzyme[65,137,138]. Thus, one of the main challenges in developing selective drugs inhibiting thiol-redox enzymes is to demonstrate lack of human cross-reactivity through off-target profiling of the drug, as well as toxicity testing in eukaryotic cell lines and animals at early stages of drug development.

Conclusion

We have described the diversity observed in the DSB formation pathways among prokaryotes, but open questions remain. Why do bacteria encode such diversity? One possibility is that these differences reflect the variability in the ecological niche where the organisms evolved. For example, the obligate anaerobes that experience reducing environments, which may have a limited availability to make DSBs or lack the pathway. Others, such as the intracellular organism *Legionella*, produce DsbA during cyst germination, which is restricted to the availability of cysteine. Similarly, *Campylobacter* produces Dsb proteins depending on the availability of iron. Perhaps the abundance of Dsb proteins produced in the organism controls the amount of folded substrates (for example, virulence factors) that are required when the conditions are favourable to infect. Additionally, the redundancy of Dsb proteins may provide an advantage under conditions where many substrates need to be produced, or perhaps they have evolved a specialized activity for folding certain substrates that require priority under some conditions.

While identification of potential DsbA substrates has been undertaken through two-dimensional gel electrophoresis or substrate trapping, and some of these substrates have been confirmed (Supplementary Table 1), it remains challenging to characterize all substrates that are part of the pathway. Bioinformatics analyses have predicted the presence of DSBs in bacteria and archaea. However, there may be several limitations in these analyses that can obscure the presence of DSB formation pathways, especially if the system is dedicated to one or few substrates. As the bioinformatics approach requires identifying exported proteins, misassignment of protein localization in some organisms may occur due to the presence of a novel type of transport that does not use classical signal sequences or known export pathways. There are also organisms that use alternative covalent bonds to DSBs, such as the covalent bonds that occur between lysine and aspartic acid that stabilize cell surface proteins. This isopeptide bond is catalysed by a sortase and was found within the pilin subunit of *Streptococcus pyogenes* and *C. diphtheriae*[139,140]. These bonds are present in pilins and adhesins of Gram-positive bacteria and are thought to present parallels in function to DSBs found in Gram-negative organisms[139]. Similarly, another rare example of isopeptide bonds occurs between lysine and asparagine residues and confers stability to the capsid subunits of bacteriophage HK97 (ref. [141]).

In conclusion, DSB formation represents a key process in microbial life. Future work to identify disulfide-bonded substrates and understand the mechanism of DSB formation in recently discovered systems will allow us to identify the conditions that make DSB formation pathways essential in vivo, improve biotechnology applications as well as derive new antibacterials.

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C.L. and J.B. wrote the manuscript. D.B. performed the bioinformatic analysis.

Competing interests

The authors declare no competing interests.

Additional information

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