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Review

Reducing systems protecting the bacterial cell envelope from oxidative damage

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ABSTRACT

Exposure of cells to elevated levels of reactive oxygen species (ROS) damages DNA, membrane lipids and proteins, which can potentially lead to cell death. In proteins, the sulfur-containing residues cysteine and methionine are particularly sensitive to oxidation, forming sulfenic acids and methionine sulfoxides, respectively. The presence of protection mechanisms to scavenge ROS and repair damaged cellular components is therefore essential for cell survival. The bacterial cell envelope, which constitutes the first protection barrier from the extracellular environment, is particularly exposed to the oxidizing molecules generated by the host cells to kill invading microorganisms. Therefore, the presence of oxidative stress defense mechanisms in that compartment is crucial for cell survival. Here, we review recent findings that led to the identification of several reducing pathways protecting the cell envelope from oxidative damage. We focus in particular on the mechanisms that repair envelope proteins with oxidized cysteine and methionine residues and we discuss the major questions that remain to be solved.

1. Introduction

Inadvertent one-electron transfer reactions between intracellular molecular oxygen and redox enzymes such as those of the respiratory chain generate partially reduced, reactive oxygen species (ROS). These include the superoxide anion (O_2^-, +1 e^-), hydrogen peroxide (H_2O_2, +2 e^-), and the hydroxyl radical ($HO^\cdot$, +3 e^-) [1,2]. In addition to this production as by-products of aerobic metabolism, ROS can also be actively generated by enzymes such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [3,4], cytochrome P450 [4] or Ero1, a protein involved in oxidative protein folding in the endoplasmic reticulum [5]. In the case of bacteria, these microorganisms also encounter high levels of ROS generated, for instance, in metal-rich environments or released by competing microbes and host cells [2].

Elevated levels of ROS can damage DNA, proteins and membrane lipids, which can potentially lead to cell death. Therefore, most living organisms contain enzymatic systems that help them to cope with oxidative stress. Proteins like catalases, peroxiredoxins and superoxide dismutases (SODs) are on the front lines and directly react with harmful ROS to convert them to innocuous products: catalases and peroxiredoxins reduce peroxides while SODs catalyze the dismutation of the superoxide anion. In addition, oxidoreductases such as thioredoxins and glutaredoxins not only supply certain of these ROS scavengers with the reducing equivalents they need to fight the redox battle but are also actively involved in the repair of oxidatively damaged proteins. The bacterial defense mechanisms against oxidative stress have been extensively studied in the cytoplasm. In contrast, how the bacterial cell envelope is protected from oxidative damage is much less understood. In this review, we summarize the recent findings that led

Abbreviations: BCP, bacterioferritin-comigratory protein; e^-, electron; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; IM, inner membrane; LPS, lipopolysaccharide; M, membrane; Met, methionine; Met-O, methionine sulfoxide; Met-O_2, methionine sulfone; Msr, methionine sulfoxide reductase; NADPH, nicotinamide adenine dinucleotide phosphate; O_2, molecular oxygen; O_2^-, superoxide anion; OM, outer membrane; PG, peptidoglycan; Prx, peroxiredoxin; ROS, reactive oxygen species; RNS, reactive nitrogen species; RCS, reactive chlorine species; SOD, superoxide dismutase; SOH, sulfenic acid; SO_2H, sulfinic acid; SO_3H, sulfonic acid; SRP, signal recognition particle; Tpx, thiol peroxidase; TR, thioredoxin reductase; Trx, thioredoxin

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to the identification of a multi-layered cellular network scavenging ROS and rescuing proteins from oxidation in bacterial extracytoplasmic compartments. Although we mostly focus here on the Gram-negative bacterium *Escherichia coli*, we also discuss recent data on the protection of proteins targeted to the envelope of other microorganisms.

2. The periplasm is an oxidizing compartment where disulfide bond formation occurs

The envelope of *E. coli* and of other Gram-negative bacteria is a structural and permeability barrier, which is essential for cell shape and growth. It is composed of two membranes: the inner membrane (IM), which is in direct contact with the cytoplasm, and the outer membrane (OM), which constitutes the interface between the cell and the external environment [6]. The IM and the OM differ in terms of structure and composition [6]. While the IM is a classical phospholipid bilayer, the OM is asymmetric and composed of phospholipids and lipopolysaccharides (LPS) in the inner and outer leaflet, respectively [6]. The IM and the OM are separated by the periplasm, a viscous compartment that contains a thin layer of peptidoglycan and represents 10–20% of the total cell volume [7,8]. In *E. coli*, about 400 proteins are targeted to the periplasm [9], where they perform a variety of important physiological functions, such as assisting the folding of nascent proteins, mediating the uptake and transport of nutrients or detoxifying toxic compounds.

With a redox potential higher than that of the cytoplasm (−165 mV *vs* −260/−280 mV in *E. coli*, respectively) [10–13], the periplasmic space is considered as an oxidizing compartment. Consistently, the majority of cysteine residues present in periplasmic proteins are oxidized to disulfides. These disulfides, which are important for protein stability, are introduced in periplasmic proteins by the soluble oxidoreductase DsbA (Fig. 1), a thioredoxin-fold protein [14] with a CXXC catalytic site [15]. The cysteine residues of this conserved motif form a very unstable disulfide, which is transferred to newly synthesized proteins as they enter the periplasm, releasing DsbA in the reduced state [16,17]. DsbA is then recycled back to the oxidized state by the IM protein DsbB (Fig. 1), which generates disulfide bonds *de novo* from quinone reduction [18–20]. DsbA preferentially introduces disulfides into proteins entering the periplasm by oxidizing cysteine residues that are consecutive in the protein sequence [21]. Therefore, when proteins require disulfides to be formed between non-consecutive cysteines, DsbA often introduces incorrect disulfides. These non-native disulfides are corrected by DsbC (Fig. 1), a periplasmic V-shaped homodimeric protein [22–24]. Each subunit of DsbC contains a catalytic CXXC motif, located within a thioredoxin-fold. In contrast to DsbA, the catalytic cysteines of DsbC are kept reduced in the periplasm, allowing this protein to function as an isomerase or a reductase [23]. While dozens of proteins have been identified as DsbA substrates [9,25–28], only a handful of proteins with multiple cysteine residues depend on DsbC for folding. They include the periplasmic enzymes MepA, AppA, RNase I and End1 [25,28,29] as well as the OM-localized ß-barrel protein LptD [30] and the stress sensor lipoprotein RcsF [31,32]. The protein that maintains the CXXC motif of DsbC in the reduced state is DsbD (Fig. 1), an IM protein that transfers reducing power across the membrane by passing electrons received from cytoplasmic thioredoxin (Trx) to a variety of reducing pathways that function in the cell envelope [23,33–36]. Thioredoxin is a ubiquitous oxidoreductase that is kept reduced by thioredoxin reductase (TR) at the expense of NADPH (Fig. 1) [33–35].

3. ROS scavengers in the bacterial periplasm

Scavenging enzymes constitute the first line of defense against ROS. The spontaneous dismutation of O_2^- to O_2 and H_2O_2 not being sufficient to maintain low intracellular concentrations, Gram-negative bacteria, like most living organisms, commonly synthesize SODs to catalyze this reaction. As superoxide cannot easily cross biological membranes [37,38], it is not surprising that SODs are commonly targeted to the different compartments of the cell that are exposed to superoxide anions to reduce them in situ. In *E. coli* for instance, SodA and SodB function in the cytoplasm [39,40], while SodC, whose folding and activity require the formation of a disulfide bond [41,42], is targeted to the periplasm (Fig. 2) [43]. The physiological importance of SodC, a protein synthesized by cells in stationary phase [43], remains unclear, even though the enzyme most likely serves to detoxify the superoxide anions released in the periplasm by the respiratory complexes (Fig. 2) [44]. In pathogenic bacteria, periplasmic SODs have been implicated in virulence, probably by scavenging the superoxide anions released by the host macrophages during the oxidative burst [45].

In most organisms, H_2O_2 is reduced by peroxidases and catalases [2]. In *E. coli*, the primary scavenger is the cytoplasmic peroxiredoxin (Prx) AhpCF (Fig. 2), a two-component thiol-based peroxidase that transfers electrons from NADH to H_2O_2, thereby reducing it to water [46]. AhpCF is very efficient in reducing H_2O_2 generated at physiological concentration (μM) [46]. At least two additional Prxs, the thiol peroxidase (Tpx) and the bacterioferritin-comigratory protein (BCP) (Fig. 2), are active in the *E. coli* cytoplasm: Tpx is involved in the reduction of bulkier hydroperoxides [47], while BCP is able to reduce a broader range of substrates, but with lowered catalytic efficiencies [48]. In addition to Prxs, *E. coli* also encodes two cytoplasmic catalases, KatG and KatE (Fig. 2), which mostly serve to reduce peroxides when they accumulate at higher concentration (mM) [46,49,50]. KatG, which is only weakly expressed in cells growing in exponential phase, is induced under oxidative stress conditions [51], while KatE is strongly expressed in stationary-phase cells [52].

While peroxide scavengers are commonly found in the bacterial cytoplasm, they seem to be absent from the cell envelope (*E. coli* Tpx, which was initially described as a periplasmic Prx [53], is actually expressed in the cytoplasm [54]). This probably correlates with the fact that H_2O_2 is able to diffuse through biological membranes and suggests that the detoxification systems expressed in the cytoplasm are enough to protect the cell from peroxide-induced damages. However, a recent search for the substrates of the IM protein ScsB, a DsbD homolog present in the alpha-proteobacterium *Caulobacter crescentus*, led to the identification of the first peroxide reduction pathway active in the bacterial periplasm [55]. In this bacterium, ScsB was shown to deliver electrons to TlpA, a thioredoxin-like protein present in the periplasm, which in turn reduces a periplasmic Prx, PprX (Fig. 2) [55]. Characterization of PprX revealed that this enzyme is active against H_2O_2 and cumene hydroperoxide. Although the physiological importance of PprX in *C. crescentus* remains to be determined, this discovery indicates that, at least for certain bacteria, it is important to directly scavenge peroxides in the cell envelope before they reach the cytoplasm.

4. Repair of oxidatively damaged envelope proteins

Within proteins, the sulfur-containing amino acids cysteine and methionine are particularly vulnerable to oxidation by ROS. The first oxidation product of cysteine residues exposed to ROS is the sulfenic acid derivative (-SOH). Sulfenic acids are highly reactive

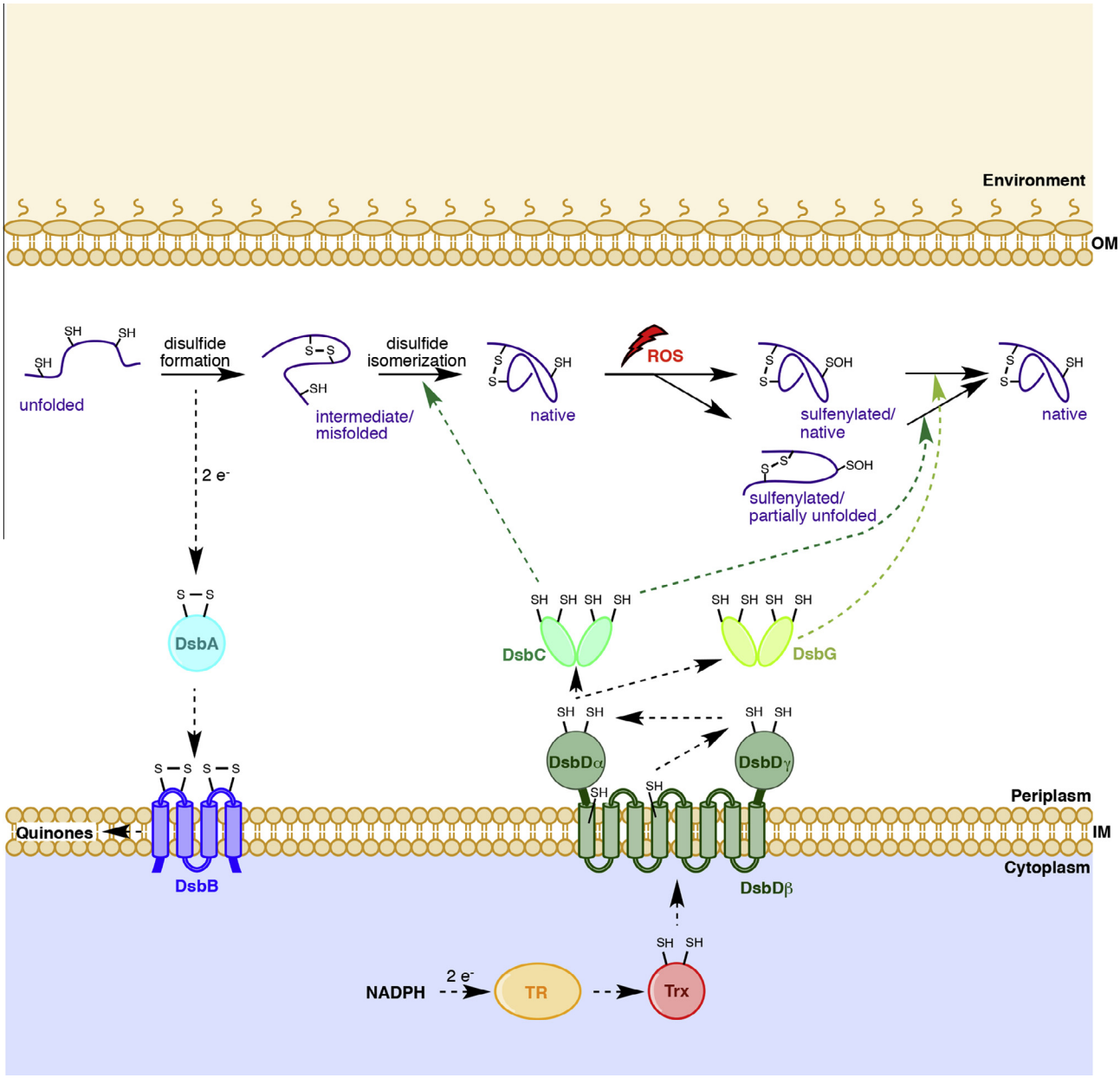

Fig. 1. Disulfide bond formation system in the *E. coli* periplasm. Disulfide bonds are introduced into newly translocated, unfolded proteins in the periplasm by DsbA. Electrons are then transferred to the IM protein DsbB and finally to quinones. DsbC corrects DsbA mistakes and is maintained reduced by DsbD, an IM protein which receives electrons from the cytoplasmic thioredoxin (Trx) system. DsbG, which rescues sulfenylated proteins in the periplasm, is also reduced by DsbD. In addition to its role as protein disulfide isomerase, DsbC cooperates with DsbG in the rescue of certain sulfenylated periplasmic proteins. We propose that DsbC preferentially interacts with (partially) unfolded sulfenylated proteins. In contrast, DsbG seems to be better designed to react with globular proteins presenting oxidized cysteine residues. Dashed arrows represent electron flows. TR = thioredoxin reductase, OM = outer membrane, IM = inner membrane.

intermediates that, unless stabilized within the protein microenvironment, react with another cysteine present in the vicinity to form a disulfide bond or are further oxidized to the irreversible sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) acid modifications. In the case of methionine residues, oxidation generates methionine sulfoxides (Met-O). If unrepaired, sulfenic acid and Met-O formation can lead to protein inactivation, misfolding or even degradation [1,56]. In order to prevent these deleterious effects, most organisms are equipped with enzymes catalyzing the repair of oxidized cysteine and methionine residues. For instance, certain proteins from the thioredoxin superfamily can either directly react with a sulfenic acid or reduce the disulfide bond resulting from its reaction with another cysteine residue (see below). Alternatively, sulfenic acids can first react with glutathione [57], which is often present at high intracellular concentrations [58], before the glutathionylated cysteine residue is reduced by glutaredoxins [59]. Regarding Met-O, they can be converted back to methionine by enzymes known as methionine sulfoxide reductases (Msrs). These protein repair mechanisms, which were initially described and characterized in the cytoplasm, were recently identified in the bacterial cell envelope and will be the focus of the following sections.

4.1. Repair of oxidized cysteine residues

As explained above, most cysteine residues present in periplasmic proteins are involved in disulfide bonds, which *de facto*

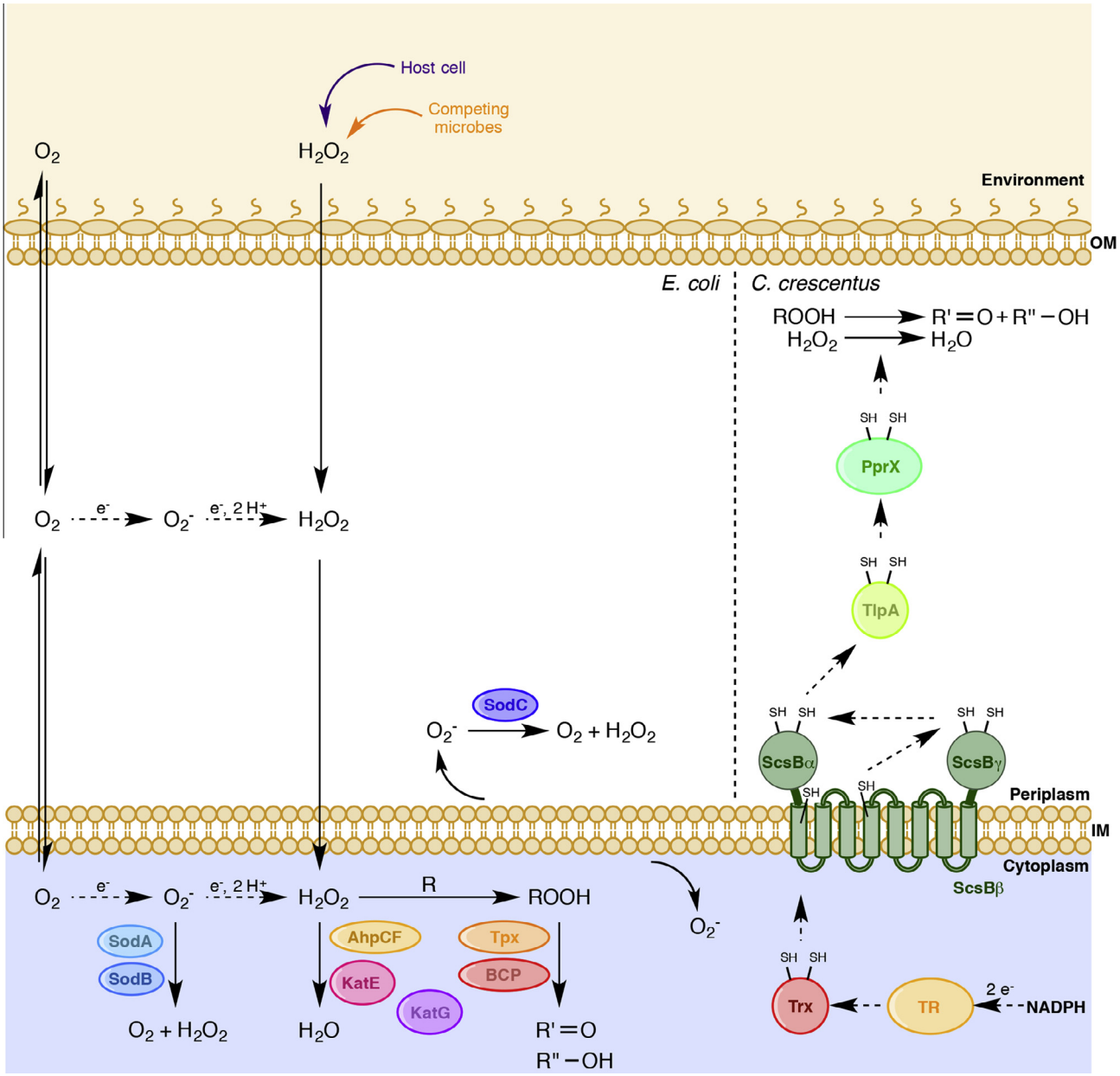

Fig. 2. ROS scavengers in bacteria. The superoxide anion, which cannot easily cross biological membranes, is dismutated into H_2O_2 and O_2 by SODs. SodA and SodB function in the cytoplasm, while SodC functions in the periplasm. H_2O_2, which can cross membranes, is reduced by peroxidases and catalases. In the *E. coli* cytoplasm, AhpCF reduces H_2O_2 very efficiently. Tpx reduces bulkier hydroperoxides (ROOH), while BCP is able to reduce a broader range of substrates. The *E. coli* cytoplasm also contains two catalases, KatG and KatE. In the periplasm of *C. crescentus*, H_2O_2 is reduced by PprX, a protein that receives electrons from TlpA. TlpA is in turn reduced by ScsB, a DsbD homolog. The arrows going from the IM to O_2^- indicate the O_2^- whose formation results from electron leakage from the respiratory chain complexes. Dashed arrows represent electron flows. TR = thioredoxin reductase, OM = outer membrane, IM = inner membrane.

protects them from unwanted oxidation. However, a small number of periplasmic proteins contain cysteine residues that remain in the reduced thiol state *in vivo* [60]. Because they are not involved in disulfide bonds, these cysteine residues are vulnerable to oxidation and can be damaged by the ROS present in the periplasm to form sulfenic acids. Using a biotinylable probe specifically reacting with sulfenic acids to covalently modify them [61], several *E. coli* periplasmic proteins were indeed found to be sulfenylated [62]. One of these proteins was identified as YbiS, an L,D-transpeptidase which catalyzes the covalent attachment of the lipoprotein Lpp, the numerically most abundant *E. coli* protein, to the peptidoglycan. Interestingly, YbiS contains a single cysteine and this residue is required for catalytic activity [63]. Thus, oxidizing YbiS inactivates the protein, impairing Lpp attachment. However, YbiS and other periplasmic enzymes whose activity also depends on a cysteine residue benefit from the protection of a reducing system controlling their redox state in the oxidizing environment of the periplasm [62]. The key player in this system is the soluble periplasmic protein DsbG (Fig. 1), a protein sharing 26% sequence identity with the protein disulfide isomerase DsbC. Like DsbC, DsbG is a V-shaped homodimeric protein with a thioredoxin-fold and a CXXC catalytic motif that is kept reduced by the IM protein DsbD (Fig. 1) [62]. As DsbD is recycled by thioredoxin, these findings indicate that electrons originating from the

pool of NADPH in the cytoplasm contribute in protecting periplasmic enzymes with unpaired cysteine residues from oxidative inactivation.

The role of DsbC as a protein disulfide isomerase is well established: proteins with non-consecutive disulfides do not fold correctly in Δ*dsbC* mutants [23] and DsbC efficiently catalyzes disulfide bond isomerization in heterologous proteins with multiple disulfides, such as bovine pancreatic trypsin inhibitor [64] or RNase A [25]. Moreover, having the inner surface of its cleft lined with hydrophobic residues [24], DsbC seems to be designed to interact with misfolded proteins presenting non-native disulfide bonds. In order to identify new DsbC substrates, we recently expressed a DsbC variant in which the CXXC motif was replaced by CXXS ($DsbC_{CXXS}$). In proteins belonging to the thioredoxin superfamily, the first cysteine of the CXXC motif performs a nucleophilic attack on an oxidized substrate, leading to the formation of a mixed-disulfide intermediate [14,65]. The role of the second cysteine of the CXXC motif is to resolve this mixed-disulfide in order to release the substrate. Mutation of this second residue, as in $DsbC_{CXXS}$, stabilizes the mixed-disulfide complex, enabling the trapping of the thioredoxin-like protein with its substrates. Using this approach, we found that a particularly abundant substrate of DsbC was the arabinose-binding protein AraF [66], a soluble protein whose function is to bind L-arabinose in the periplasm to deliver it to the IM protein complex AraH-AraG for cytoplasmic import [67–70]. Unexpectedly for a DsbC substrate, AraF possesses a sole cysteine residue, highly conserved among bacteria, suggesting that it does not interact with DsbC for disulfide isomerization. Using dimedone, a sulfenic acid specific reagent [71], the single cysteine of AraF was found to be sensitive to oxidation, forming a sulfenic acid both *in vitro* and *in vivo* [66]. Moreover, exposure of the protein to oxidative stress conditions leads to the formation of a disulfide-linked homodimer *in vivo*, most likely resulting from the reaction between the sulfenic acid of one AraF molecule and the thiol group of a second one. This dimer accumulates in strains lacking *dsbC*, confirming that DsbC is involved in the regulation of AraF redox state. As dimer formation prevents L-arabinose binding, DsbC therefore rescues AraF from inactivation. AraF seems to be a specific DsbC substrate, DsbG being inefficient in reducing the disulfide-linked AraF dimer *in vitro*. Thus, these experiments indicate that the protein disulfide isomerase DsbC cooperates with DsbG in the defense mechanisms against oxidative stress in the bacterial cell envelope. The hydrophobic character of DsbC inner surface suggests that this protein is involved in the protection of proteins whose oxidation correlates with major conformational changes and/or partial unfolding (Fig. 1). This is probably the case for AraF, a highly flexible protein, in which the cysteine residue is buried near the L-arabinose binding site and poorly accessible. In contrast, DsbG, whose cleft is larger and more hydrophilic, seems to be better designed to react with globular proteins presenting oxidized, more exposed, catalytic cysteine residues (Fig. 1), like the L,D-transpeptidase YbiS.

4.2. Repair of oxidized methionine residues

Like cysteines, methionine residues are also readily oxidized when exposed to ROS, forming methionine sulfoxides (Met-O) [56,72,73]. Oxidation of methionine produces two diastereoisomers of Met-O, referred to as *R* and *S*, owing to the asymmetric position of the sulfur atom in the lateral chain. In living cells, Met-O generation can be reversed by the action of Msrs (see below). Although Met-O is a rather stable product, it can be further oxidized to methionine sulfone (Met-O_2) by strong oxidants, but this reaction only occurs to a small extent [56]. Met-O_2 formation, as opposed to Met-O, is considered to be an irreversible modification. The pathways and processes that regulate the redox state of cysteine residues have been extensively studied, yielding numerous examples of proteins containing oxidatively modified cysteines that are either targeted for repair or involved in redox regulation (see the previous section and Refs. [1,58,74–76]). In comparison, the biological consequences of methionine oxidation, as well as the systems involved in its reduction, are much less characterized, even though important progress has been made in recent years.

4.2.1. Physiological consequences of methionine oxidation

Methionine oxidation renders this hydrophobic amino acid more hydrophilic, which can alter the biochemical and structural properties of a protein and potentially cause a loss of biological function. For example, oxidation of certain methionine residues located in a methionine-rich domain of Ffh, the protein component of the signal recognition particle (SRP), has been shown to perturb the targeting of membrane proteins in *E. coli* [77]. Consistently, *E. coli* Msrs are required for normal functioning of Ffh during aerobic growth and Δ*msr* mutants exhibit defects in membrane protein targeting. Interestingly, these findings are in line with a study that showed that the two main pathways responsible for the targeting of proteins to the bacterial envelope, the SRP-dependent pathway and the SecA-dependent pathway [78], are both susceptible to methionine oxidation-mediated inactivation following exposure to hypochlorous acid (HOCl) [79].

It is important to note that methionine oxidation does not necessarily lead to protein inactivation. A recently described example is the *E. coli* transcription factor HypT, which is specifically activated by HOCl, an oxidizing reagent, through the oxidation of three Met residues to Met-O [80]. Following oxidation, HypT protects bacterial cells against HOCl-induced oxidative stress by up-regulating genes involved in cysteine and methionine biosynthesis and down-regulating genes involved in iron homeostasis. Efficient inactivation of the transcription factor was shown to depend on the reduction of the Met-O residues by Msrs. Moreover, it has also been proposed that oxidation of certain surface-exposed methionine residues could also serve as an endogenous antioxidant mechanism by preventing the oxidation of other residues that are important for activity. For instance, in the case of *E. coli* glutamine synthetase, an enzyme with 16 methionine residues, exposure of the protein to increasing concentrations of H_2O_2 leads to the oxidation of up to 8 surface-exposed methionine residues but does not significantly affect the enzymatic activity [81]. Further supporting this ROS-quenching property of methionine residues, it has been shown that an *E. coli* methionine auxotroph mutant becomes more sensitive to oxidative stress when it is grown in a norleucine-enriched medium. Norleucine is a structural analog of methionine lacking the sulfur moiety and which can therefore not form a sulfoxide [82].

4.2.2. The role of methionine sulfoxide reductases in the cell envelope

The first Msrs to be discovered were *E. coli* MsrA and MsrB, two cytoplasmic enzymes that specifically reduce the *S* and *R* diastereoisomers of Met-O, respectively [83–85]. Whereas MsrA can efficiently reduce free and protein-bound *S*-Met-O, MsrB preferentially reduces the *R*-Met-O isoform in a protein context [84]. MsrA and MsrB are present in virtually all aerobic organisms where they contribute to the defense mechanisms against oxidative stress [86,87]. In bacterial pathogens, they are important for virulence [88–97] and for protecting the cells from the arsenal of toxic compounds produced in massive amounts by the host defenses, including reactive chlorine species (RCS), reactive nitrogen species (RNS) and ROS [84,88–94,98–103]. A third member of the Msr family, fRMsr (renamed here MsrC) was identified in *E. coli* in 2007 [104]. MsrC, which is only present

in bacteria and yeast, specifically reduces free *R*-Met-O, thus allowing the repair, together with MsrA and MsrB, of all the Met-O species that can be generated in the cytoplasm.

The three Msrs described above do not share any sequence or structural homology. However, they possess a similar catalytic mechanism involving a catalytic cysteine residue [85,105]. This residue performs a nucleophilic attack on the sulfoxide moiety of Met-O, which leads to the formation of a sulfenic acid intermediate and the concomitant release of methionine. Depending on the protein, the sulfenic acid will then either form an intramolecular disulfide bond, which can be rearranged into another one if additional resolving cysteines are present in the Msr, or be directly reduced if there is no resolving cysteine. In most cells, Msrs are recycled to the reduced state by the thioredoxin system [105,106]. Noteworthy, certain prokaryotic and eukaryotic MsrA and MsrB contain a catalytic selenocysteine residue instead of a cysteine. In this case, a selenenic acid intermediate is formed during catalysis [107].

While cytoplasmic Msrs have been relatively well studied, their extracytoplasmic counterparts have been identified and characterized only in a few bacteria (Fig. 3). This is particularly surprising given that proteins targeted to the cell envelope are particularly exposed to the ROS produced by the host cells and are therefore likely to require repair mechanisms for oxidatively damaged methionine residues. The first identified Msr functioning in the bacterial cell envelope is the protein PilB, encoded by *Neisseria gonorrhoeae* and *Neisseria meningitidis* (Fig. 3A). PilB, which is targeted to the OM but faces the periplasm, is made up of tandem MsrA and MsrB domains fused to an N-terminal thioredoxin domain [108,109]. The N-terminal domain, which contains the typical CXXC catalytic motif found in most thioredoxin proteins, provides the two Msr domains with the electrons they need for Met-O reduction [109–111]. These electrons originate from the IM protein DsbD and the cytoplasmic thioredoxin system. Although expression of PilB confers protection to *N. gonorrhoeae* against ROS [108], a link between this protein and the virulence of the pathogen has yet to be made. A second example of extracytoplasmic Msr is found in *Streptococcus pneumoniae* (Fig. 3B). This Gram-positive bacterium expresses the fusion protein *Sp*MsrAB2, which is anchored to the cytoplasmic membrane by a

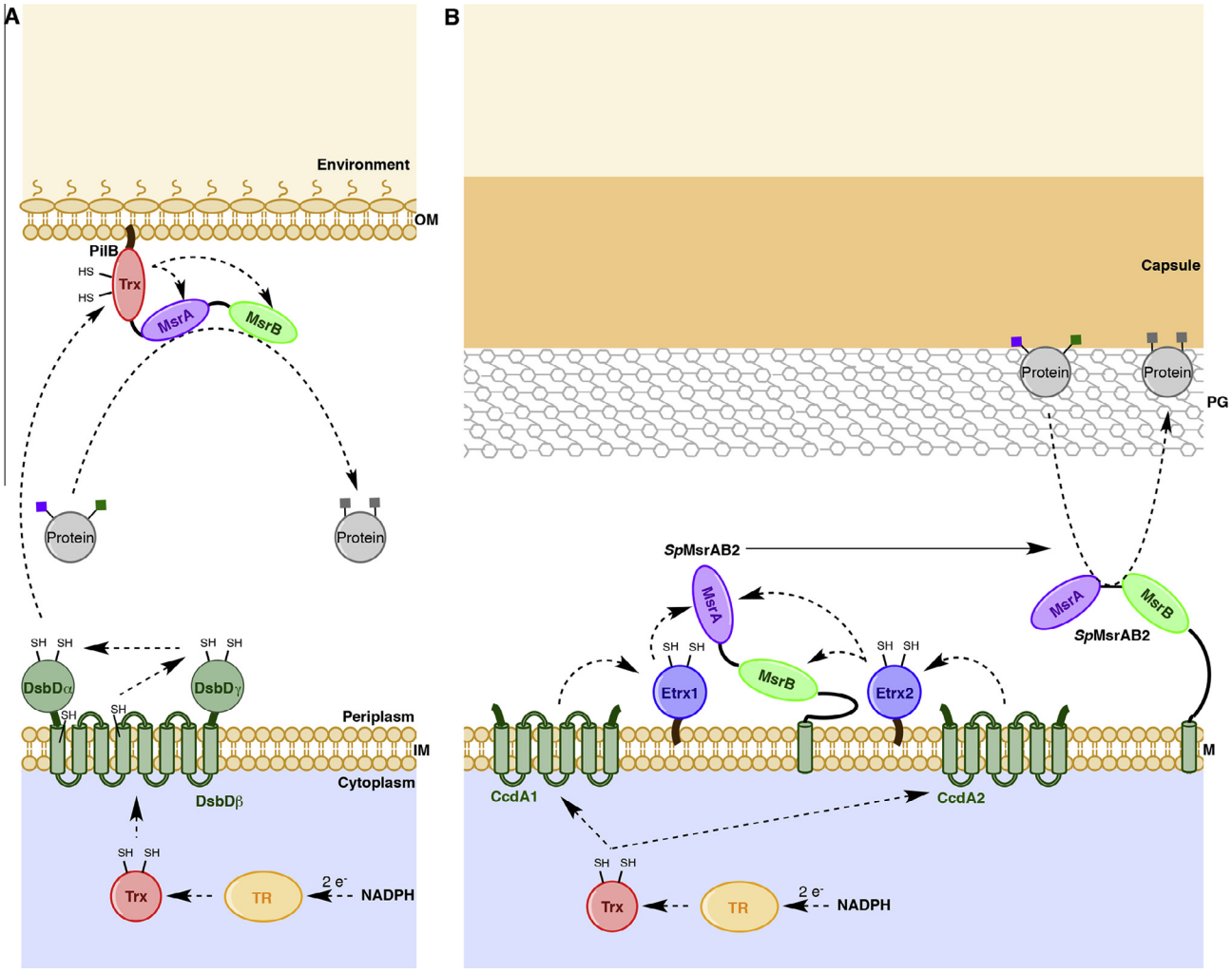

Fig. 3. Extracytoplasmic methionine sulfoxide (Met-O) reducing pathways. (A) Met-O reduction in the *Neisseria* envelope. The OM lipoprotein PilB is composed of two mirror MsrA and MsrB domains, reducing the *S* and the *R* isoforms of protein-bound Met-O, respectively. PilB contains an additional N-terminal thioredoxin (Trx) domain that keeps both Msr domains reduced using electrons provided by DsbD. The reducing power for Met-O reduction by PilB originates from the cytoplasmic Trx system. (B) Met-O reduction at the *S. pneumoniae* cell surface. The surface-exposed *Sp*MsrAB2 is anchored to the cell membrane by a transmembrane segment. *Sp*MsrAB2 is composed of fused MsrA and MsrB domains that are linked to the transmembrane segment by a long and flexible coiled coil region, which probably allows them to reach surface-exposed proteins in order to reduce the *S* and the *R* isoforms of Met-O, respectively. The surface-exposed lipoproteins Etrx1 and Etrx2 likely receive electrons from the cytoplasmic Trx system through the DsbD homologs CcdA1 and CcdA2, respectively. In turn, Etrx1 provides reducing power to the MsrA domain of *Sp*MsrAB2, while Etrx2 provides electrons to both Msr domains, although more efficiently to the MsrB domain. Dashed arrows represent electron flows. TR = thioredoxin reductase, OM = outer membrane, IM = inner membrane, M = membrane, PG = peptidoglycan. ■ = Met, ■ = *R*-Met-O, ■ = *S*-Met-O.

transmembrane segment and is surface exposed [112]. *Sp*MsrAB2 is expressed from an operon with a Trx-like protein (Etrx1) and with CcdA1, a protein with homology to the transmembrane domain of DsbD [112,113]. A second Trx-like protein (Etrx2), with homology to Etrx1, is expressed from an operon with a second DsbD homolog (CcdA2), but without an additional MsrAB [112]. The surface exposed lipoproteins Etrx1 and Etrx2 likely receive electrons from the cytoplasmic thioredoxin system through CcdA1 and CcdA2, respectively. In turn, Etrx1 provides reducing power to the MsrA domain of *Sp*MsrAB2, while Etrx2 can provide electrons to both Msr domains, although more efficiently to the MsrB domain [112]. Interestingly, deletion of *SpmsrAB2* renders the cells hypersensitive to exogenous H_2O_2 and attenuates the virulence and spread of the pneumococci from the nasopharynx to the lungs and blood. Deleting both *etrx1* and *etrx2* has a similar impact, suggesting that both CcdA-Etrx pathways are necessary for full *Sp*MsrAB2 reductase activity.

It is interesting to note that certain cytoplasmic Msrs may also indirectly contribute to the maintenance or assembly of extracellular bacterial structures by yet unknown mechanisms. For example, *E. coli* MsrA has been proposed to be involved in the maintenance of surface type I fimbriae [95], a structure important for the attachment to the host cell, and is also important for mature biofilm formation [114].

5. Conclusions

The findings discussed in this review highlight the importance of having multiple reducing pathways present in the oxidizing environment of the bacterial envelope. Not only do these pathways

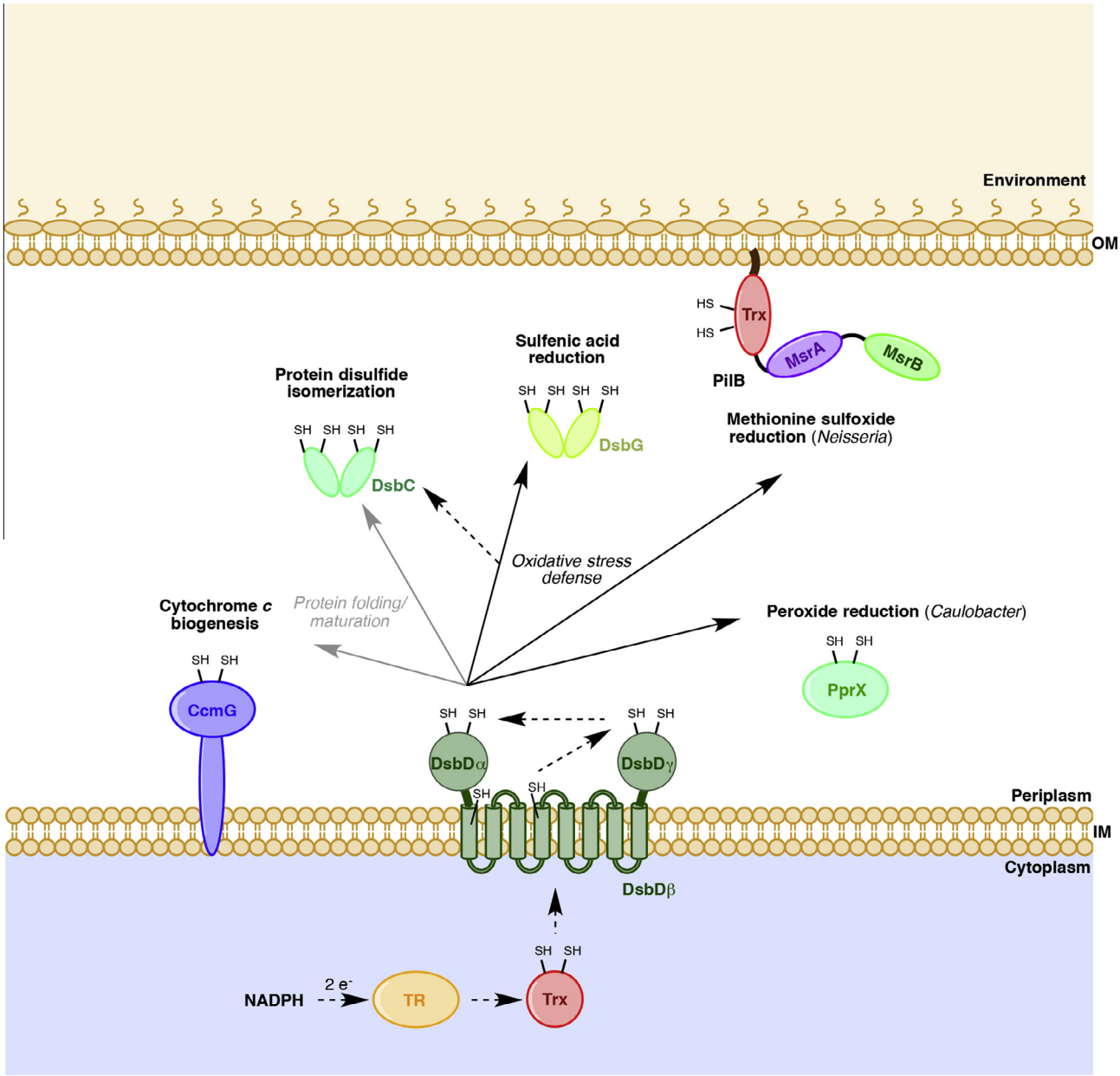

Fig. 4. DsbD is an electron hub that transfers electrons to several oxidoreductases present in the cell envelope. DsbD-like proteins provide reducing equivalents originating from the cytoplasmic thioredoxin system to periplasmic proteins involved in protein folding and maturation: DsbD reduces disulfide isomerases such as *E. coli* DsbC and oxidoreductases involved in the maturation of cytochrome *c*, such as *E. coli* CcmG. DsbD-like proteins also transfer electrons to proteins involved in the defense mechanisms against oxidative stress, such as *E. coli* DsbG (and DsbC), which controls the global sulfenic acid content of the periplasm, *Neisseria* PilB, a multidomain protein that exhibits Msr activity and *C. crescentus* PprX, a periplasmic peroxiredoxin. TR = thioredoxin reductase, OM = outer membrane, IM = inner membrane.

ensure correct protein folding in this compartment, but they also contribute to protect proteins from inactivation either by directly scavenging ROS before they reach their protein targets, or by ensuring protein repair following oxidative damage. Strikingly, the IM protein DsbD has emerged in recent years as a key actor in these pathways by serving as an electron hub dispatching reducing power originating from the cytoplasmic NADPH pool to the periplasm (Fig. 4). As such, DsbD allows protein disulfide isomerization by keeping protein disulfide isomerases like *E. coli* DsbC reduced and functional, thus enabling correct protein folding and stability. DsbD also provides electrons to CcmG, a thioredoxin-like protein involved in cytochrome *c* maturation in the periplasm [115–117]. More information on this important role of DsbD, which was not discussed above, can be found in the following reviews [36,118]. In addition to providing electrons for protein assembly and maturation in the cell envelope, DsbD also supplies several pathways involved in the oxidative stress defense in this compartment. As previously discussed, DsbD provides electrons for peroxide scavenging to PprX through TlpA. It also allows protein repair by reducing DsbG, thus enabling this reductase to cooperate with DsbC in rescuing proteins containing single sulfenylated cysteines. Finally, DsbD provides reducing power to PilB for the repair of oxidized methionine residues.

Despite the abundance of new data clarifying how reducing pathways function in the bacterial envelope, many questions remain to be addressed. For instance, the physiological importance of the recently discovered pathways such as those involved in sulfenic acid reduction and ROS scavenging need to be further clarified. It would be of particular interest to identify additional sulfenylated protein targets in the cell envelope and to determine if this modification is involved in the regulation of their biological function. The physiological consequences of the absence of superoxide and peroxide scavenging systems in the cytoplasm have been extensively studied. However, the role of their periplasmic counterparts, such as periplasmic SODs and PprX, remains unclear and requires further characterization. Finally, although methionine reduction pathways have been well studied in the cytoplasm, the existence of extracytoplasmic equivalents has only been very recently uncovered. PilB, which is present in a small subset of Gram-negative bacteria including *Neisseria* species, is so far the only described Msr in the bacterial envelope. It is therefore tempting to speculate that additional Met-O reduction systems exist in the envelope of bacteria that are devoid of PilB, in order to protect ROS-exposed methionine residues.

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