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Abstract

Methionine is a sulfur-containing residue found in most proteins which are particularly susceptible to oxidation. Although methionine oxidation causes protein damage, it can in some cases activate protein function. Enzymatic systems reducing oxidized methionine have evolved in most bacterial species and methionine oxidation proves to be a reversible post-translational modification regulating protein activity. In this review, we inspect recent examples of methionine oxidation provoking protein loss and gain of function. We further speculate on the role of methionine oxidation as a multilayer endogenous antioxidant system and consider its potential consequences for bacterial virulence.

KEYWORDS
antioxidant system, bacteria, methionine sulfoxide, methionine sulfoxide reductases, protein oxidation

1 | INTRODUCTION

Oxidation of the sulfur atom in the side chain of methionine (Met) generates a sulfoxide (Figure 1a). This reaction converts Met into methionine sulfoxide (Met-O), which exists as two diastereoisomers: Met-R-O and Met-S-O (Lavine, 1947; Shechter et al., 1975). The intracellular proportion of protein-bound Met-R-O and Met-S-O is difficult to estimate: while steric hindrance resulting from protein structure can partly determine this ratio, it is generally accepted to be racemic (Davies, 2005; Tsvetkov et al., 2005). Further oxidation of Met-O irreversibly produces methionine sulfone (Nlelsen et al., 1985), although this reaction is unlikely to occur under physiological conditions.

Met-O can be reduced back to Met by the enzymatic Methionine Sulfoxide Reductase (Msr) system which is found in the three domains of life and in most bacterial species (Delaye et al., 2007). Msr enzymes catalyze the reduction of Met-O either by a thiol-based mechanism (MsrA and MsrB) or a molybdopterin-based reaction (MsrP) (Boschi-Muller & Branlant, 2014; Brokx et al., 2005; Gennaris et al., 2015; Juillan-Binard et al., 2017). While MsrA and MsrB are stereospecific, reducing protein-bound Met-S-O and Met-R-O respectively, MsrP reduces Met-O regardless of its stereochemistry (Brot et al., 1981; Gennaris et al., 2015; Grimaud et al., 2001; Tarrago et al., 2018). MsrA and MsrB share little sequence identity but exhibit a similar catalytic site structure, probably resulting from convergent evolution (Gladyshev, 2002). MsrA and MsrB are often found in the cytoplasm of bacterial cells, but in several species, including *Neisseria*, *Streptococci* and *Haemophilus*, an extracytoplasmic bifunctional MsrAB protein fusion has evolved (Han et al., 2016; Kappler et al., 2019). MsrP is a periplasmic reductase broadly conserved in Gram-negative bacteria (Gennaris et al., 2015). We refer readers to the literature on the biochemistry, regulation and conservation of bacterial Msr systems (Achilli et al., 2015; Aussel & Ezraty, 2021; Delaye et al., 2007; Ezraty et al., 2017; Kappler et al., 2019).

Met residues are sensitive to different oxidants: Met oxidation can be initiated by radical and non-radical derivatives of oxygen (Vogt, 1995), reactive chlorine species (Gray et al., 2013) and

(a)

(b)
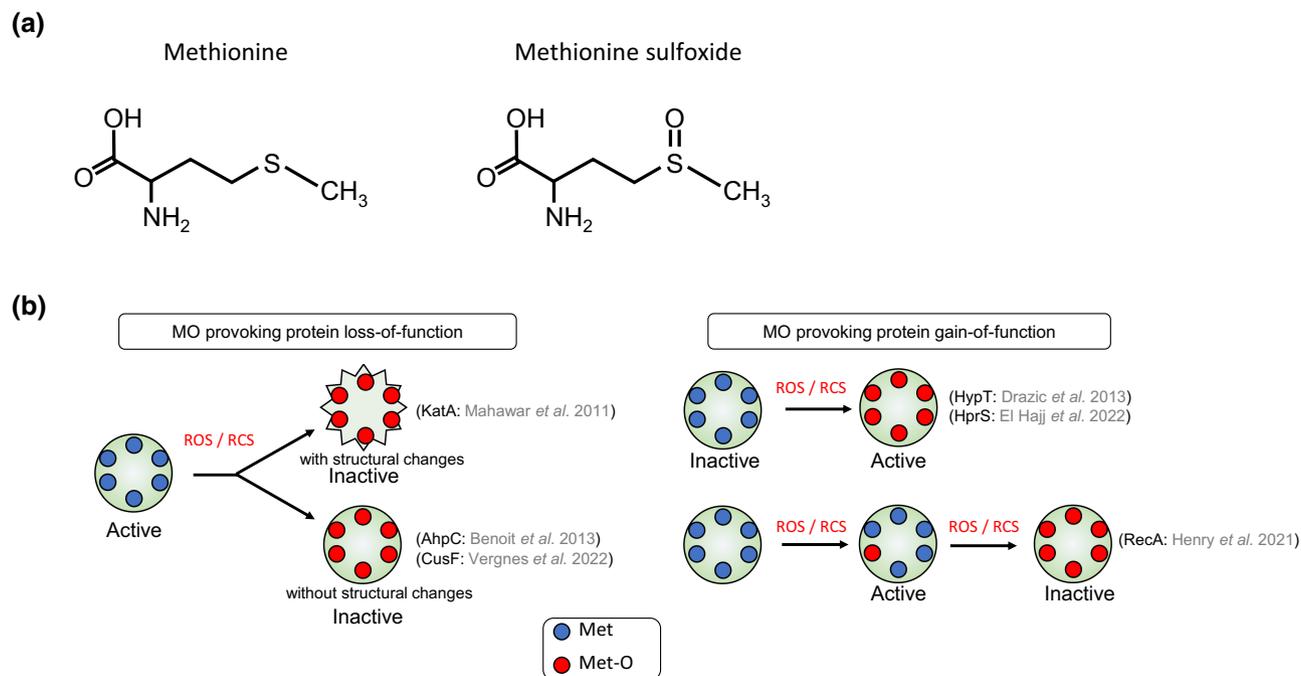
FIGURE 1 Consequence of methionine oxidation on protein function. (a) Structures of methionine and methionine sulfoxide. (b) The oxidation of methionine residues to methionine sulfoxide can lead to the inactivation or the activation of protein functions. Blue dots indicate Met residues, red dots indicate Met-O residues. Reactive Oxygen Species (ROS), Reactive Chlorine Species (RCS).

reactive nitrogen species (John et al., 2001). In particular, both hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) can oxidize Met residues, although the latter exhibits a faster reaction rate (Davies, 2005; Pattison & Davies, 2001; Winterbourn et al., 2016). H_2O_2 and HOCl are major oxidants produced by phagocytic cells to combat bacterial infections (Klebanoff et al., 2013), therefore, Met-O homeostasis plays an important role in host-pathogen interactions.

Whether oxidation of protein-bound Met should be classified as a post-translational modification (PTM) is subject to debate. On the one hand, this process triggers a chemical modification of a protein following its biosynthesis. On the other hand, the term PTM generally applies to modifications that are mediated by enzymes, and most examples of bacterial Met oxidation arise, to date, from stochastic chemical reactions. Nonetheless, recent reports suggesting that protein-bound Met-O residues are also generated enzymatically in human cells (Manta & Gladyshev, 2017), open the door to a new definition of Met oxidation that has led us to classify it as a PTM in this review.

Whereas Met oxidation in proteins was initially seen as being exclusively damaging, it is now clear that this process has implications for gene expression, damage sensing and modulation of enzymatic activity in most biological systems (Valverde et al., 2019). Here, we explore the different consequences of Met oxidation for bacterial proteins.

2 | METHIONINE OXIDATION AS A DELETERIOUS PTM

Met-O is more hydrophilic than Met (Black & Mould, 1991), therefore, oxidation of Met residues within proteins may trigger major structural changes, misfolding or aggregation, which ultimately impairs protein function (Chao et al., 1997). In principle, systemic failures can arise from the oxidation of a few Met residues in a single protein. For instance, protein trafficking through the cytoplasmic membrane is altered by Met oxidation of Ffh (Ezraty et al., 2004), a ubiquitous component of the Signal Recognition Particle (SRP) required for membrane insertion of transmembrane proteins (Ezraty et al., 2004; Steinberg et al., 2018). Although the Msr system prevents global damage inflicted by Met oxidation, the reduction of protein-bound Met-O is not always sufficient to restore protein activity. Upon HOCl exposure, Met residues of the *Helicobacter pylori* catalase, KatA, are oxidized, which triggers a conformational change in the enzyme and loss of catalytic activity (Mahawar et al., 2011) (Figure 1b). In this case, the Msr enzymes need to work in concert with the molecular chaperone GroEL to refold and repair the damaged protein (Alamuri & Maier, 2004; Mahawar et al., 2011). Considering that Met residues of GroEL are susceptible to oxidation by HOCl, rendering the chaperone inactive in *E. coli* (Khor et al., 2004), Met oxidation appears to be a severe obstacle to the regeneration of protein activity. This hypothesis is strengthened by further reports highlighting that (i) repair of Met-O is essential for maintaining protein stability (Ezraty et al., 2004; Kim et al., 2001) and (ii) another molecular chaperone, the periplasmic protein SurA, undergoes Met oxidation-mediated loss of activity (Gennaris et al., 2015).

Alternatively, Met oxidation of a protein can impair its function without substantially altering its structure (Figure 1b). The alkyl hydroperoxide reductase AhpC of *H. pylori* and the metallochaperone CusF of *E. coli* both exhibits Met oxidation after exposure to HOCl and H_2O_2 respectively (Benoit et al., 2013;

Vergnes et al., 2022). Consequently, Met oxidation significantly decreases the catalytic activities of AhpC and CusF but has little effect on conformational changes and their oligomeric states (Benoit et al., 2013; Vergnes et al., 2022). Similarly, the heat shock protein Hsp16.3 of *Mycobacterium tuberculosis* contains three Met that are oxidized upon treatment with H_2O_2 (Abulimiti et al., 2003). Sulfoxidation of these Met residues increases the tendency of oligomeric dissociation but does not significantly affect the secondary structure of Hsp16.3 (Abulimiti et al., 2003). How Met oxidation impairs protein function in the absence of structural changes is not always clear. In bacteria, Met oxidation has been shown to prevent proper protein-substrate interaction. For instance, CusF carries two Met residues within its active site whose oxidation is critical for its copper-binding capacity (Vergnes et al., 2022). In multicellular organisms, Met oxidation may inhibit other PTMs of nearby residues and thereby modify protein features, as reported for kinase substrate proteins of Arabidopsis (Hardin et al., 2009). Within the human proteome, the existence of a crosstalk between phosphorylation and Met oxidation has been suggested based on evidence that Met residues located in the vicinity of phosphorylation sites are preferentially oxidized in vivo (Veredas et al., 2017).

In general, whether Met oxidation results in protein loss-of-function is difficult to predict. Location, orientation and neighbouring residues of Met residues prone to oxidation are likely to be major factors in this process (Ghesquière et al., 2011). Although new computational approaches now enable prediction of Met oxidation sites within peptides and proteins (Delmar et al., 2021), accurate methods to predict functional changes caused by Met oxidation remain to be developed. In contrast, recent tools for protein structure prediction (Jumper et al., 2021) open possibilities for determining conformational changes subsequent to Met oxidation; notably relying on in silico glutamine substitutions, a Met-O mimicking residue (Drazic et al., 2013; Henry et al., 2021).

3 | METHIONINE OXIDATION AS A REGULATORY PTM

Whereas oxidation of Met residues in proteins is usually regarded as being detrimental, growing evidence suggests that Met oxidation allows regulation of protein activity and furthermore modulates key cellular functions. This vision is now commonly shared for most living organisms, including humans (Moskovitz & Smith, 2021). In bacteria, the first study to report that Met oxidation controls protein activity revealed that HypT, the *E. coli* hypochlorite-responsive transcription factor, is activated upon oxidation of its Met residues (Drazic et al., 2013) (Figure 1b). More precisely, sulfoxidation of three Met residues was sufficient to change the HypT oligomeric state and to promote its DNA-binding capacity, resulting in higher HOCl tolerance (Drazic et al., 2013, 2014). According to the current model, Met oxidation of HypT has a dual effect. Firstly, it restores oxidized metabolites as the activated form of HypT up-regulates

genes involved in Cys and Met biosynthesis and sulfur metabolism. Secondly, it helps to decrease the level of reactive oxygen species (ROS) because HypT in its activated form down-regulates genes involved in iron acquisition, thereby preventing further oxidative damage generated by the Fenton reaction (Drazic et al., 2013; Gebendorfer et al., 2012; Imlay, 2008). Inactivation of HypT occurs when the cytoplasmic Msr system reduces HypT Met-O residues back to Met (Drazic et al., 2013), probably leading to a shutdown of the HypT response. Met oxidation-induced HypT activation is conserved in *Salmonella*, suggesting an evolutionary benefit of such a mechanism (Jo et al., 2019).

More recently, the two-component system HprSR has been proposed to be activated upon Met oxidation of the transmembrane HprS sensor (El Hajj et al., 2022) (Figure 1b). HOCl exposure oxidizes Met residues located in the periplasmic loop of HprS, which then activates the response regulator HprR. Similarly to HypT, the Met oxidation-mediated activation of HprS and HprR provides protection against further HOCl damage. HprR notably up-regulates the expression of *msrP*, which is known to reduce periplasmic Met-O (Gennaris et al., 2015). Interestingly, oxidized Met of HprS is reduced by MsrP (El Hajj et al., 2022). This observation suggests a model in which Met residues of HprS might gauge the periplasmic HOCl level and control the production of MsrP through a negative feedback mechanism (El Hajj et al., 2022).

Proteins bearing multiple Met that are susceptible to oxidation can exhibit different degrees of Met oxidation. One can thus wonder whether differential Met oxidation results in different functional outcomes. The *E. coli* recombinase RecA carries two Met residues which are particularly susceptible to oxidation: Met164 and Met35 (Henry et al., 2021) (Figure 1b). By relying on a combination of RecA variants whose Met are substituted by glutamine or alanine, Henry et al. demonstrated that while oxidation of Met164 activated RecA-dependent SOS induction, oxidation of Met35 altered RecA function leading to both loss of SOS induction and loss of recombinase activity (Henry et al., 2021). Because Met164 is more prone to oxidation than Met35, Met164 could act as a frontline sensor for intracellular oxidative stress. According to this hypothesis, partial oxidation of RecA would result in the DNA-damage stress response being triggered and RecA being replenished through a Met oxidation-based positive feedback activation (Henry et al., 2021). Together with HypT and HprS, RecA illustrates that Met oxidation contributes to redox sensing in bacteria.

4 | METHIONINE OXIDATION AS AN ANTIOXIDANT DEFENSE MECHANISM

Besides its regulatory aspect, Met oxidation has been suggested to act as an endogenous antioxidant system. This theory, first elaborated in the late 90's by the group of Earl R. Stadtman, proposes that surface-exposed Met (that are readily accessible to oxidants) essentially serve as oxidant scavengers shielding key residues from oxidative damage. Pioneering observations showing that the oxidation of

the glutamine synthetase's surface-exposed Met had little effect on its enzymatic activity (Levine et al., 1996) incited further investigation into the role of Met in antioxidant systems. By replacing 40% of protein-bound Met residues in *Escherichia coli* with norleucine (the non-oxidizable carbon analogue of Met), Luo and Levine found that cells containing protein-bound norleucine were more susceptible to oxidative stress than cells containing protein-bound Met, which led the authors to conclude that Met residues in proteins act as an antioxidant defense mechanism (Luo & Levine, 2009). It has since been reported that certain bacterial proteins use their Met as antioxidant baits (Alamuri & Maier, 2006; Melkani et al., 2006; Schmalstig et al., 2018). In *H. pylori*, the deletion of the genes encoding the urease UreAB results in high sensitivity to HOCl. However, inactivation of the urease function does not result in a defect in HOCl resistance, suggesting an antioxidant role independent of its catalytic activity. The UreAB complex consists of 25 Met of which 11 are oxidation-prone and repaired by the Msr system (Schmalstig et al., 2018). These results suggest that the cyclic oxidation–reduction of Met residues in proteins lowers the cellular level of oxidants.

In their study, Rosen et al. noticed that oxidation of Met residues in the outer membrane and periplasmic proteins had little impact on bacterial viability, in contrast to the inner membrane and cytoplasmic proteins (Rosen et al., 2009). This observation raises the question of whether the Met antioxidant system works on a multilayer basis. Arguably, Met residues located in the cell envelope act as the first line of defence against exogenous oxidative stress, mopping up oxidants and preventing their diffusion inside the cell (Figure 2). Once in the cell, oxidants encounter Met-rich proteins (MRPs), such as the periplasmic MrpX protein whose role is to scavenge oxidants (Melnyk et al., 2015). MRPs exhibit local or global enrichment of Met in their primary sequence (the average Met content in *E. coli* proteins is ~2.9% [McCaldon & Argos, 1988; Maisonneuve et al., 2008; Liang et al., 2012]), are broadly conserved (Liang et al., 2012) and

potentially connected to the Msr system. For instance, in *Azospira suillum*, *mrpX* is part of the same operon as *msrP* (Melnyk et al., 2015). Phylogenetic analyses reveal that, in most Enterobacteriaceae, an MRP named YeaC (Met content in *E. coli* ~9%) is encoded by a gene that lies immediately downstream of *msrB*. In addition to MRPs, the cytoplasmic pool of free Met is also known to scavenge oxidants (Spero et al., 2022) and enzymatic systems have evolved to reduce free Met-O (Dhouib et al., 2016; Ezraty et al., 2005; Lin et al., 2007). As mentioned above, at the protein-level, surface-exposed Met residues shield catalytic sites from oxidative damage (Levine et al., 1996). Similarly to biofilms where peripheral cells prevent the diffusion of oxidants towards the interior of cell clusters (Stewart et al., 2016), envelope-located Met, MRPs, surface-exposed protein-bound Met and free Met residues could hamper the penetration of oxidants into the interior of cells and proteins. This "millefeuille" model is made even more efficient, as Met-O residues of most of these components are known to be rescued by the Msr system.

5 | INVOLVEMENT OF METHIONINE OXIDATION IN BACTERIAL VIRULENCE AND INFECTION

Met oxidation is an important determinant of bacterial virulence. Most evidence comes from the characterization of *msr* mutants from various species that show impaired growth or diminished infection capabilities within host cells. For instance, *msr* mutants of *F. tularensis*, *S. typhimurium*, *H. pylori*, *M. smegmatis* and *S. aureus* exhibit decreased survival rates in phagocytic cells (Denkel et al., 2011; Douglas et al., 2004; Mahawar et al., 2011; Saha et al., 2017; Singh et al., 2015). In addition, the deletion of *msr* genes attenuates the ability of pathogenic species to colonize infectious models, including mammals, insects and plants (Denkel et al., 2011; Hassouni
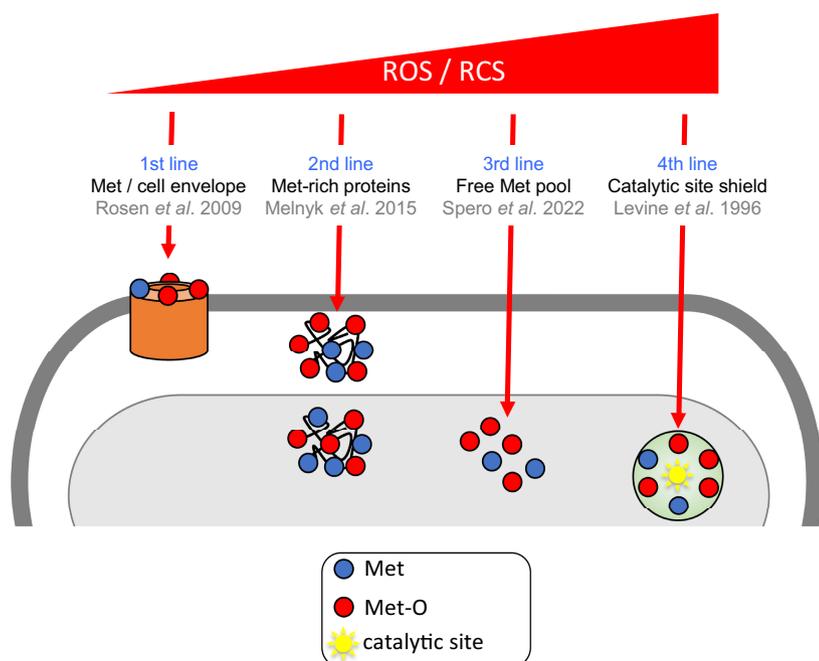
FIGURE 2 The "millefeuille" model. In this speculative model, peripheral Met act as a shield against oxidative damage. Exogenous oxidants, such as Reactive Oxygen Species (ROS) and Reactive Chlorine Species (RCS) first encounter Met of outer-membrane proteins. Other lines of defense are made up of periplasmic and cytoplasmic Met-rich proteins and free cytoplasmic Met that scavenge oxidants. Within proteins, surface-exposed Met prevent critical oxidative damage of catalytic sites. Blue dots indicate Met residues, red dots indicate Met-O residues.

et al., 1999; Hitchcock et al., 2010; Romsang et al., 2013; Saha et al., 2017; Zhao et al., 2010).

Recent findings suggest that Met oxidation of bacterial proteins could modulate human host cell responses to infection by the pathobiont *Haemophilus influenza* (Nasreen et al., 2020). Loss of the MsrAB fusion-protein decreases survival in mice but also changes the expression of host genes encoding proteins with bactericidal functions (Nasreen et al., 2020). Interestingly, in *H. influenza* MsrAB is located in the periplasm and repairs cell envelope proteins, including different adhesins that had undergone Met oxidation damage (Nasreen et al., 2022; Skaar et al., 2002). It thus is possible that Met oxidation of bacterial cell surface proteins affects host-pathogen interactions. In this regard, *msr* genes have been shown to preserve adherence of bacterial cells (Lei et al., 2011; Wizemann et al., 1996). In *Fusobacterium nucleatum*, MsrAB is essential for attachment to multiple tissues (Scheible et al., 2022), and in several human colonizers, the absence of *msr* genes reduces biofilm formation (Jalal & Lee, 2020; Nasreen et al., 2020).

The majority of studies linking virulence and Met oxidation suggests that bacterial Met residues are amongst the primary targets of host-derived oxidants. Indeed, HOCl and H_2O_2 are produced as oxidative bursts by macrophages and neutrophils as a defense mechanism (Gaut et al., 2001; John et al., 2001; Mastroeni et al., 2000; Vriesema et al., 2000). The bactericidal effect of HOCl linearly correlates with the degree of Met oxidation of inner membrane and cytosolic *E. coli* proteins (Rosen et al., 2009) and, consistently, host cells lacking enzymatic systems producing HOCl or H_2O_2 are less efficient at killing microbes (Rosen et al., 2009). Overall, these reports highlight the importance of the Msr enzymes in invading host cells. This suggests that bacteria's ability to repair Met-O is a priority for in-host survival and further effective colonization. It is thus not surprising that the MsrAB protein has been considered a candidate for a vaccine against *Neisseria gonorrhoeae* (Jen et al., 2019). One should however note that MsrA/B are conserved in humans, which could potentially lead to cross-reactivity issues, however, the periplasmic reductase MsrP is restricted to bacteria, and could therefore be a promising target for novel therapeutics against Gram-negative bacteria.

Considering that bacterial stress responses are triggered by Met oxidation of distinct regulators upon exposure to oxidants generated by the innate immune system (e.g., HypT, RecA, HprS), the regulatory role of Met oxidation could have evolved as a means to protect invading cells against host attack.

6 | OUTLOOK

The last decade of investigations into protein-bound Met oxidation has led us to review our perception of this PTM. It is now clear this process participates in gene regulation and cell signalling. Moreover, the reversible nature of Met oxidation allows it to function as a modulator of signal transduction pathways, in a similar way to disulfide bond formation/reduction (Cremers & Jakob, 2013) and phosphorylation/dephosphorylation events (Dworkin, 2015).

Recent advances in the prediction (Aledo et al., 2017; Delmar et al., 2021; Sankar et al., 2018) and quantification of Met-O sites within proteins (Bettinger et al., 2020; Ghesquière & Gevaert, 2014) will help uncover which parts of the bacterial proteome undergo Met oxidation. In live cells, genetically encoded stereospecific fluorescent sensors of Met-O allow the assessment of average cellular Met-O levels (Tarrago et al., 2015). Nonetheless, despite intense research and technical advances, the dynamics of Met-O biogenesis and repair remain hard to estimate due to the difficulty of monitoring nascent Met oxidation events in vivo.

Consequently, the fraction of spontaneously arising Met-O residues in bacteria remains to be elucidated. The observation that Msr enzymes are required for protein stability in aerobic and unstressed growth conditions (Ezraty et al., 2004), suggests that a basal level of Met-O exists in the absence of exogenous oxidative stress. Yet, questions remain including: (i) whether this fraction participates in regulating cellular functions, (ii) how much of this fraction is actively repaired by the Msr system and (iii) whether such spontaneous events are associated with specific subcellular locations.

Finally, the recent observation that Met-O residues are generated in response to chlorate stress in different bacterial species (Loiseau et al., 2022; Spero et al., 2022) paves the way for new investigations into the biogenesis of Met-O in the absence of oxygen.

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