Redox regulation by reversible protein S-thiolation in Gram-positive bacteria

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Abstract

Low molecular weight (LMW) thiols play an important role as thiol-cofactors for many enzymes and are crucial to maintain the reduced state of the cytoplasm. Most Gram-negative bacteria utilize glutathione (GSH) as major LMW thiol. However, in Gram-positive Actinomycetes and Firmicutes alternative LMW thiols, such as mycothiol (MSH) and bacillithiol (BSH) play related roles as GSH surrogates, respectively. Under conditions of hypochlorite stress, MSH and BSH are known to form mixed disulfides with protein thiols, termed as S-mycothiolation or S-bacillithiolation that function in thiol-protection and redox regulation. Protein S-thiolations are widespread redox-modifications discovered in different Gram-positive bacteria, such as Bacillus and Staphylococcus species, Mycobacterium smegmatis, Corynebacterium glutamicum and Corynebacterium diphtheriae. S-thiolated proteins are mainly involved in cellular metabolism, protein translation, redox regulation and antioxidant functions with some conserved targets across bacteria. The reduction of protein S-mycothiolations and S-bacillithiolations requires glutaredoxin-related mycoredoxin and bacilliredoxin pathways to regenerate protein functions.

In this review, we present an overview of the functions of mycothiol and bacillithiol and their physiological roles in protein S-bacillithiolations and S-mycothiolations in Gram-positive bacteria. Significant progress has been made to characterize the role of protein S-thiolation in redox-regulation and thiol protection of main metabolic and antioxidant enzymes. However, the physiological roles of the pathways for regeneration are only beginning to emerge as well as their interactions with other cellular redox systems. Future studies should be also directed to explore the roles of protein S-thiolations and their redox pathways in pathogenic bacteria under infection conditions to discover new drug targets and treatment options against multiple antibiotic resistant bacteria.

Keywords:
- Protein S-thiolation
- Bacillithiol
- Mycothiol
- Gram-positive bacteria

Abbreviations:
- Ac, acetyl
- AcCys, acetyl cysteine
- AhpE, membrane-associated peroxidase
- AldA, aldehyde dehydrogenase
- Bca, BSH S-conjugate amidase
- Bex, bacilliredoxin
- BSH, bacillithiol
- BshA, glycosyltransferase for GlcN-Mal biosynthesis
- BshB, deacetylase producing GlcN-Mal
- BshC, cysteine ligase for BSH biosynthesis
- GluB, isoinosine-5-monophosphate dehydrogenases
- GlcNAc, N-acetyl glucosamine
- GlxA/B, glyoxalases A and B
- Grx, glutaredoxin
- GSG, oxidized glutathione disulfide
- Gst, GSH-S-transferases
- H2O2, hydrogen peroxide
- HED, hydroxyethyl disulfide
- HTA, hemithioacetal
- INH, isoniazid
- Ins, myoinositol
- LC-MS/MS, liquid chromatography tandem mass spectrometry
- LMW, low molecular weight
- Mal, malate
- Mca, mycothiol-5-conjugate amidase
- Met, methionine
- MeT, methionine synthase
- MetSO, methionine sulfoxide
- MG, methylglyoxal
- MgsA, methylglyoxal synthase
- MRSB, methicillin-resistant Staphylococcus aureus
- MsrA, mycothiol oxidase
- MSH, mycothiol
- MSONH2, MSH sulfinamide
- MSNO, N-nitrosomycothiol
- MrxA/B, methionine sulfoxide reductase A/B
- MST, mycothiol-S-transferase
- Mtb, Mycobacterium tuberculosis
- NADPH, nicotinamide adenine dinucleotide phosphate
- NH, nicotinamide dinucleotide
- NOS, nitric oxide synthase
- PDB, Protein Data Bank
- PpaC, inorganic pyrophosphatase
- PpaD, protein Data Bank
- PpaE, protein Data Bank
- PPP, pentose phosphate pathway
- Protein-SSB, BSH protein mixed disulfide
- RES, reactive electrophile species
- RNS, reactive nitrogen species
- RSS, reactive sulfur species
- rGFP2, redox-sensitive green fluorescent protein
- ROS, reactive oxygen species
- SarZ, redox-sensing virulence regulator
- STRING, Search Tool for the Retrieval of Interacting Genes/Proteins
- Trx, thioredoxin
- TrxR, thioredoxin reductase
- YpdA, NADPH-dependent flavin oxidoreductase

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1. Introduction

Low molecular weight (LMW) thiols play an important role in many cellular processes in all organisms. They are crucial to maintain the reduced state of the cytoplasm and function as thiol-cofactors of enzymes involved in detoxification of reactive oxygen, electrophilic, chlorine, nitrogen and sulfur species (ROS, RES, RCS, RNS, RSS), toxins and antibiotics, in metal storage, buffering and transport, and sulfide homeostasis [1–3]. The well-studied LMW thiol glutathione (GSH) is produced in most eukaryotes, Gram-negative bacteria, and some Gram-positive bacteria, such as *Streptococci*, *Listeria*, *Lactobacilli* and *Clostridia* [2,4]. Most Gram-positive bacteria do not produce GSH and utilize instead alternative LMW thiols to cope with oxidative stress and redox regulation of metabolic enzymes. *Actinomycetes* utilize mycothiol (MSH) as their major LMW thiol (Fig. 1A) [5,6]. In *Firmicutes*, such as *Bacillus* and *Staphylococcus* species, bacillithiol (BSH) plays a related role like GSH to control cellular redox homeostasis under oxidative stress and infection conditions (Fig. 1B) [3,7]. There is also evidence that coenzyme A (CoASH) may substitute for the absence of BSH in some *Firmicutes*, such as *S. aureus* or *Bacillus megaterium* [2,8]. In addition, *S. aureus* and *Borrelia burgdorferi* both encode a CoAS disulfide reductase, further indicating that CoASH plays a role to cope with oxidative stress [8,9].

The question arises why different bacteria utilize different LMW thiols? Phylogenetic analyses revealed that the γ-glutamylcysteine synthase GshA, which catalyzes the first step of GSH biosynthesis, likely evolved in cyanobacteria and was distributed then to other bacteria and eukaryotes by lateral gene transfer [10,11]. Since the major function of GSH is the protection against oxygen toxicity, GSH biosynthesis was acquired with the evolution of oxygen by cyanobacteria. However, there was significant microbial diversity before the evolution of cyanobacteria, resulting in the evolution of alternative LMW thiols as protection mechanisms against increasing oxygen toxicity. Thus, it is not surprising that various prokaryotes utilize different LMW thiols [10].

LMW thiols are usually present in millimolar concentrations in the cytosol and are kept reduced by NADPH-dependent thiol-disulfide reductases [2,12]. Under oxidative and hypochlorite stress, redox-sensitive protein thiols are susceptible to various forms of thiol-oxidations, including reversible S-thiolations with LMW thiols as well as intra- or intermolecular protein disulfides (Fig. 2). In the absence of adjacent thiols, protein thiols can be also overoxidized to irreversible Cys sulfinic or sulfonic acids. Protein S-thiolations can have protective functions and control the activity of metabolic enzymes or transcription factors (Fig. 2). This review summarizes the current knowledge about the role of protein S-thiolations in cellular physiology and under oxidative stress.

![Fig. 1. Structures, distributions and chemical properties of mycothiol and bacillithiol.](image-url)
Fig. 2. Thiol-chemistry in response to H₂O₂ and HOCl stress. Thiol-oxidation of Cys residues by H₂O₂ generates an unstable Cys sulfenic acid intermediate (Cys-SOH) that can rapidly react with LMW thiols, such as mycothiol (MSH) and bacillithiol (BSH), resulting in S-mycothiolations (Cys-SSM) and S-bacillithiolations (Cys-SSB) respectively. Alternatively, Cys oxidation can lead to the formation of intermolecular or intramolecular disulfides in proteins. In the absence of LMW thiols, protein thiols can be overoxidized to irreversible Cys sulfonic acid (Cys-SO₂H) and sulfenic acid (Cys-SO₃H). HOCl reacts with protein thiols via chlorination, generating an unstable sulfenylchloride intermediate (Cys-SCI) that reacts further with MSH, BSH or protein-thiols to reversible S-mycothiolations, S-bacillithiolations or protein disulfides. In the absence of adjacent thiols, sulfenylchloride rapidly leads to overoxidation generating Cys sulfonic and sulfenic acids.

in Gram-positive bacteria, such as Bacillus subtilis, Staphylococcus aureus, Corynebacterium diphtheriae and Mycobacterium smegmatis. Molecular dynamics simulations have provided further insights into the structural protein changes upon S-thiolation, which are discussed for conserved S-bacillithiolated proteins at the molecular and mechanistic level.

2. Biosynthesis and function of mycothiol and bacillithiol in Gram-positive bacteria

2.1. The biosynthetic pathways and functions of mycothiol in Actinomycetes

The cysteinyl pseudo-disaccharide mycothiol (MSH; AcCys-GlcN-Ins) is composed of acetyl cysteine (AcCys), glucosamine (GlcN) and myo-inositol (Ins) and has a molecular weight of 486Da. MSH is the most major LMW thiol in all Actinomycetes, including Corynebacteria, Mycobacteria and Streptomyces (Fig. 1AC) [6,7,13]. The biosynthesis of MSH is catalyzed in five enzymatic steps, which involve MshA, MshA2, MshB, MshC and MshD (Fig. 3). The first step of the MSH biosynthesis is catalyzed by the glycosyltransferase MshA, which conjugates myo-inositol-1-phosphate (Ins-P) to UDP-N-acetyl glucosamine (UDP-GlcNAc) leading to the formation of N-acetyl glucosamine myo-inositol-1-phosphate (GlcNAc-Ins-P). In the second step, the phosphatase MshA2 catalyzes dephosphorylation of GlcNAc-Ins-P to N-acetyl glucosamine myo-inositol (GlcNac-Ins). The third step involves the metal-dependent deacetylase MshB for deacetylation of GlcNac-Ins leading to GlcN-Ins [6,14]. The MshB enzyme is a homolog of the MSH-conjugate amidase (Mca) that catalyzes the hydrolysis of MS-conjugates. In the fourth step of the MSH biosynthesis, the ATP-dependent ligase MshC ligates cysteine to GlcN-Ins leading to Cys-GlcN-Ins. The Cys ligase MshC is a homolog of the Cys-tRNA synthetase. The acetyltransferase MshD catalyzes the acetylation of the Cys amino group by acetyl-CoA as final step of MSH biosynthesis [6,13,15]. Under oxidative stress, MSH is oxidized to mycothiol disulfide (MSSM) which requires the NAD(P)H-dependent mycothiol disulfide reductase (Mtr) for NADPH-dependent reduction of MSSM to maintain a high MSH: MSSM redox ratio [13,16]. The levels of MSH vary strongly between different members of Actinomycetes with the highest levels of ~1–20 µmol/g dry weight (rdw) in Mycobacteria and much lower levels of 0.3 µmol/g rdw in C. diphtheriae [17,18].

The thiol pKₐ for MSH was determined as 8.76, which is only slightly more acidic (0.17 pH units) compared to the thiol pKₐ of GSH, and 0.4 pH units less acidic compared to the first microsopic thiol pKₐ value of free Cys (Fig. 1C) [19]. Since MSH is present at much higher levels compared to Cys in Actinomycetes, the mycothiolate anion is the most abundant LMW thiolate anion that reacts with oxidants and electrophiles in vivo. The thiol-redox potential of MSH is in the range of GSH and was calculated as E°⁰(MSNO/MSH) of ~ −230 mV (Fig. 1C). However, the thiol-redox potential of MSH calculated with biophysical methods showed discrepancies compared to previously measured E°⁰ values determined as ~ −300 mV using the Mtx1-reGFP2 biosensor [19,20]. This indicates technical challenges to measure the exact MSH/ MSSM ratio inside bacterial cells.

MSH plays an important role as thiol-cofactor for many enzymes that are involved in the detoxification of antibiotics, xenobiotics, ROS, RES, RNS, and other reactive species (Fig. 4) [2,6,7]. For a comprehensive overview of the detailed functions of MSH in different Actinomycetes the reader is referred to recent reviews which will be briefly outlined here and updated based on novel results [6,7,13].

MSH conjugates xenobiotics and antibiotics either spontaneously or enzyme-catalyzed by MSH S-transferases (MST), which belong to the DinB superfamily (Figs. 3 and 4) [21]. These MSH-S-conjugates are hydrolyzed by the mycothiol-S-conjugate amidase (Mca), releasing mercapturic acid derivatives (AcCys-R) and GlcN-Ins. The GlcN-Ins is recycled to MSH and the toxic mercapturic acid derivatives are exported from the cell. Mca was shown to be involved in detoxification of MSH-S-conjugates with the antibiotics cerulenin and rifamycin in Mycobacteria [13]. MSH and ergothioneine (EGT) were also shown to function as sulfur donors through S-glucosylation reactions to mediate amino sugar transfer, activation and modification during the biosynthesis of the lincomamide antibiotic lincomycin in Streptomyces lincolnensis [22,23]. This indicates a direct function of the LMW thiols in the biosynthesis and molecular assembly of sulfur-containing natural products [22,23].

MSH functions as thiol-cofactor for detoxification of formaldehyde, RNs, maleylpyruvate, methylglyoxal and arsenate and is required for activation of anti-mycobacterial prodrugs (Fig. 4). The MSH-dependent detoxification enzyme MscR is a dual function enzyme with N-nitrosothiol (MSNO) reductase and formaldehyde dehydrogenase activities [6,13]. Formaldehyde is conjugated to MSH leading to S-hydoxymethyl-MSH, which is further oxidized to an S-formyl thioester and formate. MSNO is converted by MscR to MSH sulfinamide [21]. MSH was shown as cofactor for the enzymatic isomerization of maleylpyruvate to fumarylpyruvate [24]. MSH is shown as a cofactor for the MSH-dependent arenolate reductases ArsCl/C2 in the detoxification of arsenate [25,26].

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M Sheffield further contributes to antibiotic resistance in Mycobacterium tuberculosis (MtB). MSH is required for activation of the pro-drugs isoniazid (INH) and nitrofuranylanaloides (NFs) which are potent anti-mycobacterial drugs [27,28]. INH is activated by the catalase KatG and MSH, leading to NAD-INH adduct formation and inhibition of the enoyl-ACP reductase (InhA) of the mycolic acid biosynthesis pathway [29]. INH resistant MtB isolates often carry spontaneous mutations in katG or mshA [14]. Novel antymycobacterial drugs, such as the nitrofurantoins derivatives NF and the thioureasymidine compound TP053 are activated by the MSH-dependent oxidoreductase Rv2466c, which was recently revealed as MSH-dependent nitroreductase to reduce nitro groups to amines [28,30]. Importantly, NF-resistant mutants were selected which had mutations in the gene encoding Rv2466c. This indicates that the MSH-dependent nitroreductase activity of Rv2466c is crucial for pro-drug activation [28]. Rv2466c has a DsbA-like structure and can also function as mycoredoxin-2 in demycothiolation with electrons from the MSH/Mtr pathway [30]. Rv2466c uses a monothio-diisulfide mechanism for reduction of S-mycocyanized proteins or intramolecular disulfides and a dithiol mechanism for prodrug activation (TP053) in vitro.

MSH further functions as reservoir of cysteine and is much less susceptible to auto-oxidation compared to cysteine [13]. In addition, MSH has important functions in the virulence and survival of the pathogen MtB under infection conditions [31]. An essential function of...
MSH for growth and viability was found in Mtb since the mshC mutant could be only generated in the presence of a second copy of mshC [31].

2.2. Shotgun proteomics identified widespread protein S-mycothiolations in Actinomycetes

Under HOCl stress, MSH was shown to form mixed disulfides with protein thiols, termed as protein S-mycothiolations. Protein S-mycothiolations are widespread redox-modifications and were identified in many Actinomycetes, such as C. glutamicum, C. diphtheriae and M. smegmatis [18,25,32]. However, the extent of protein S-mycothiolation under HOCl stress differs with 58, 25 and 26 proteins identified in M. smegmatis, C. glutamicum and C. diphtheriae, respectively (Table S1). This higher level of S-mycothiolated proteins in Mycobacteria might be correlated with the 20-fold higher MSH level compared to Corynebacteria [17]. Overall, S-mycothiolated proteins were shown to be involved in many metabolic pathways, including glycolysis, gluconeogenesis, glycogen and maltodextrin degradation, as well as in the biosynthetic pathways for fatty acids, amino acids and nucleotides, cofactors, and protein translation. Several conserved S-mycothiolated proteins have antioxidant functions, such as peroxiredoxins (Tpx, Mpx, AhpE, AhpC) and the methionine sulfoxide reductase MsrA [18,25,32,33].

In C. glutamicum, 25 proteins with S-mycothiolations were identified under HOCl stress and their level of oxidation was quantified using fluorescence-based thiol-redox proteomics [25] (Table S1). These S-mycothiolation targets function in glycolysis (Fba, Pta, XylB), glycolegen and maltodextrin degradation (MalP), in the amino acid biosynthesis pathways for serine, cysteine, methionine (MetE, SerA, Hom), nucleotides and thiamine cofactors (GuaB, PurL, ThiD1, ThiD2), antioxidant functions (Tpx, Mpx), methionine sulfoxide reduction (MsrA), heme degradation (HmuO), and protein translation (RpsF, RpsC, RpsM, Tuf).

Among these, Tuf, GuaB1, GuaB2, SerA and MetE are also conserved targets for S-thiolations across Gram-positive bacteria [25]. The most interesting S-mycothiolated metabolic enzyme in C. glutamicum was the maltodextrin phosphorylase (MalP), which is involved in glycogen degradation during the stationary phase [34]. The malP mutant was very sensitive under HOCl stress indicating an essential function of MalP under oxidative stress [25]. Furthermore, the glycogen content was not decreased in C. glutamicum wild type under HOCl stress despite drastically reduced glucose uptake rates. Thus, S-mycothiolation of MalP is suggested to inhibit its function in glycogen degradation to save the source of energy under oxidative stress [25].

Many antioxidant enzymes were S-mycothiolated at their active site Cys residues and it was further investigated if S-mycothiolation functions in redox-regulation of peroxiredoxins (Tpx, Mpx, AhpE) and methionine sulfoxide reductases (MsrA, MsrB) [25,33,35–38]. The thiol peroxidase (Tpx), an atypical 2-Cys peroxiredoxin was S-mycothiolated at its active and resolving Cys60 and Cys94 residues [25]. S-mycothiolation of Tpx in vitro inhibited its peroxidase activity. The MSH peroxidase (Mpx) was also S-mycothiolated at its peroxidatic Cys36 residue. The methionine sulfoxide reductase MsrA is involved in the repair of methionine sulfoxides and was S-mycothiolated at its conserved Cys91 residue. The cobalamin-independent methionine synthase (MetE) was S-mycothiolated at its Zn-binding active site Cys713 under HOCl stress and S-mycothiolation of MetE was shown to function in thiol-protection under acid stress [25,39]. The inosine-5-monophosphate (IMP) dehydrogenases GuaB1 and GuaB2 are conserved S-thiolated proteins across bacteria. GuaB1 and GuaB2 were S-mycothiolated at their active site Cys302 and Cys317 respectively, forming the thiol-midate intermediate [25].

In the pathogen C. diphtheriae, 26 S-mycothiolated proteins were identified under HOCl stress using shotgun liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis [18] (Table S1). These

![Fig. 3. The biosynthesis pathway of MSH and detoxification of electrophiles in Actinomycetes.](image-url)

The MSH glycosyltransferase MshA conjugates myo-inositol-1-phosphate (Ins-P) to UDP-N-acetyl glucosamine (UDP-GlcNAc) leading to N-acetyl glucosamine myo-inositol-1-phosphate (GlcNAc-Ins-P). Dephosphorylation and deacetylation of GlcNAc-Ins-P is catalyzed by the phosphatase MshA2 and the deacetylase MshB, respectively. MshC ligates cysteine to GlcN-Ins in an ATP-dependent reaction. MshD acetylates the Cys moiety of Cys-GlcN-Ins to produce MSH. The mycothiol-S-transferase (MST) catalyzes the MSH-dependent detoxification of toxic electrophiles (E), xenobiotics and antibiotics. The mycothiol-S-conjugate amidase (Mca) cleaves the amide bond to produce mercapturic acids (AcCys-R) and GlcN-Ins. Mercapturic acids are exported and GlcN-Ins is recycled to MSH. This figure is adapted from [93].
include five conserved targets for S-thiolations across Gram-positive bacteria, such as AhpC, the ribosomal proteins RplC and RpsM, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GapDH), and GuaB [18]. Other S-mycothiolated proteins are involved in energy metabolism, including the ribose S-phosphate isomerase DIP1796 and the NADH dehydrogenases Ndh, GlpD and DIP1726. Further targets for S-mycothiolation function in the biosynthesis of amino acids (LeuB, DapA, GlnA), purine (PurA), iron-sulfur-clusters (DIP1631) and cell wall metabolites (GlmS). The S-mycothiolated proteins contributed with 0.2–0.75% Cys abundance to the total Cys proteome of *C. diphtheriae* as revealed by shotgun proteomics [18]. Further biochemical studies of GapDH confirmed that protein S-mycothiolation functions in redox-regulation and thiol-protection against overoxidation as referred in the following section.

In *M. smegmatis*, we identified 58 S-mycothiolated proteins under HOCl stress [32] (Table S1). The conserved peroxiredoxins Tpx, AhpE and OsmC are S-mycothiolated at their active and/or resolving Cys residues, which are involved in redox-regulation and detoxification processes in *M. smegmatis*. The global transcriptional regulator for iron uptake of the DtxR-family (IdeR) was S-mycothiolated at Cys102 in its primary iron-binding site [32]. Many abundant enzymes of the energy metabolism were further identified as S-mycothiolated in *M. smegmatis*. These are involved in the glycolcatabolism, glycolysis, the glyoxalate shunt and gluconeogenesis. The glycolcine kinase GlpK3 and the glycolcine dehydrogenase Adh2 are abundant S-mycothiolated proteins since glycerol is used as sole source of carbon and energy in *M. smegmatis*. The generation of dihydroxyacetone phosphate (DHAP) involves GlpK3 and Adh2. Thus, S-mycothiolation could prevent glycerol degradation under HOCl stress to save the carbon and energy source. The isocitrate lyase AceA and the myo-inositol-1-phosphate synthase Ino1 were further identified as abundant S-mycothiolated proteins in *M. smegmatis*. AceA is the key enzyme of the glyoxylate bypass of the TCA cycle in *M. tuberculosis*, which enables the use of carbon for biomass production via gluconeogenesis during growth on fatty acids as sole source of carbon and energy [40]. Thus, S-mycothiolation could inhibit AceA under oxidative stress to stop gluconeogenesis [32]. Furthermore, abundant enzymes involved in the biosynthesis of fatty acids as precursors for mycolic acids were S-mycothiolated in *M. smegmatis*, including acetyl-CoA carboxylases (AccD5 and AccD6), the enoyl-CoA hydratase (EchA6), the methoxy mycoyl CoA synthase (UmaA), the acyl-CoA dehydrogenase (MSMEG_0531), and the acyl-CoA-thioesterase (MSMEG_6208). Other S-mycothiolated proteins of *M. smegmatis* are involved in the nucleotide biosynthesis, including GuaB and GuaB2, and biosynthesis enzymes for thiamine (CobN), as well as ribosomal proteins (RplC, RpsM, RpsR2) and amino acyl tRNA synthetases (GatC, GatD, GatE) for protein translation [32]. Overall, many detailed future studies are required to elucidate the physiological role of the widespread protein S-mycothiolations in redox regulation and/or thiol-protection in *Mycobacteria*. In particularly, it would be interesting if mycothiolation functions in redox regulation of transcriptional regulators under infection conditions in *M. tuberculosis*. Recent biochemical studies revealed the formation of S-mycothiolated NsrR, a FeS-cluster-based Rrf2-family regulator and NO-sensor in many Gram-positive bacteria [41].
Using mass spectrometry, the formation of several iron nitrosyl species and thiol-disulfide reducing pathways to restore the protein activity. The Mrx1/MSH/Mtr system is specific for the de-mycothiolation of MSH-mixed protein disulfides (Fig. 5). Mycoredoxin-1 (Mrx1) was characterized as a glutaredoxin-homolog in Actinomycetes [43]. Mrx1 has a typical Trx-like fold with a CGYC catalytic active site, composed of a four-stranded antiparallel β-sheet and surrounded by three α-helices. The CGYC motif is located at the N-terminus of the first α-helix. The conserved proline residue (Pro57) is located in cis-conformation opposite to the active site. The active site Cys14 is solvent exposed in both oxidized and reduced forms of Mrx1, while the C-terminal resolving Cys residues of Mrx1 are shown as spheres in (B) and as sticks in (C, D).

2.3. Protein S-mycothiolation is redox-controlled by the mycoredoxin-1 (Mrx1) and thioredoxin pathways

The redox regulation of protein S-mycothiolations requires specific thiol-disulfide reducing pathways to restore the protein activity. The Mrx1/MSH/Mtr system is specific for the de-mycothiolation of MSH-mixed protein disulfides (Fig. 5). Mycoredoxin-1 (Mrx1) was characterized as a glutaredoxin-homolog in Actinomycetes [43]. Mrx1 has a typical Trx-like fold with a CGYC catalytic active site, composed of a four-stranded antiparallel β-sheet and surrounded by three α-helices. The CGYC motif is located at the N-terminus of the first α-helix. The conserved proline residue (Pro57) is located in cis-conformation opposite to the active site. The active site Cys14 is solvent exposed in both oxidized and reduced forms of Mrx1, while the C-terminal resolving Cys17 is buried inside the protein [43]. Mrx1 has a negative redox potential of −218 mV that is in the same range as for glutaredoxins. Thus, Mrx1 can function as effective thiol disulfide reducing enzyme in Actinomycetes [44]. Mrx1 catalyzes the de-mycothiolation of MSH-mixed protein disulfides in a bimolecular nucleophilic substitution reaction analogous to the monothiol reaction mechanisms of glutaredoxins. During de-mycothiolation of the substrates, Mrx1 is S-mycothiolated at its active site Cys14. Oxidized Mrx1 is regenerated by MSH leading to formation of MSSM, which is reduced by Mtr to MSH with NADPH as electron donor. The electron transfer from the Mrx1/ MSH/Mtr electron pathway to the MSH mixed disulfide substrate was shown in a hydroxyethyl disulfide (HED) assay [43].

The peroxiredoxin Tpx of C. glutamicum was identified as first substrate for Mrx1 in vitro. S-mycothiolation of the peroxidatic Cys60 inhibited the peroxidase activity of Tpx, which could be restored by the Mrx1/MSH/Mtr electron pathway. Mass spectrometry confirmed the complete reduction of Tpx-SSM and MSH transfer to Mrx1, resulting in the formation of a transient Mrx1-SSM intermediate [25]. Another mycothiol peroxidase, Mpx of C. glutamicum controls intracellular ROS levels and was shown to be recycled by both, the Mrx1 and the thioredoxin/thioredoxin reductase (Trx/TrxR) pathways [35,38]. Reduction of the S-mycothiolated peroxidatic Cys36 of Mpx by Mrx1 proceeds via a monothiol mechanism as demonstrated by kinetic assays and MSH-specific Western blots [35]. Arsenate [Ars(V)] detoxification by the arsenate reductases ArsC1 and ArsC2 in C. glutamicum requires Mrx1 and the Mrx1/MSH/Mtr pathway. ArsC1/C2 catalyze conjugation of MSH to Ars(V) resulting in an arseno-MSH conjugate (As(V)-SM). Mrx1 reduces the As(V)-SM conjugate to arsenite [As(III)] via a monothiol mechanism (Fig. 4) [26].

In C. diphtheriae, the glycolytic GapDH was the most abundant S-mycothiolated protein contributing with 0.75% to the total Cys proteome [18]. GapDH uses the active site Cys for the nucleophilic attack at the aldehyde group of glyceraldehyde-3-phosphate (G3P) to catalyze its phosphorylation to 1,3-bisphosphoglycerate and thereby generating NADH [45]. GapDH is S-mycothiolated at the conserved catalytic active site Cys153 resulting in reversible inhibition of GapDH activity under HOCI stress [18]. It was previously shown that H2O2-stress leads to an inactivation of GapDH upon S-thiolation, resulting in a metabolic reconfiguration of central carbon metabolism [46,47]. The lack of glycolysis is compensated by an increased glycolytic flux through the
pentose-phosphate pathway (PPP) to provide increased levels of NADPH as cofactor for thiol-disulfide oxidoreductases. The active site Cys of GapDH is highly reactive and susceptible for various post-translational thiol-modifications in response to ROS and RNS, including S-glutathionylation, S-nitrosylation and sulfenic acid formation in many eukaryotes [48,49]. It was shown that the relatively high reactivity of the active site thiolate towards H2O2 depends on the stabilization of the eukaryotes [48,49]. It was shown that the relatively high reactivity of S-glutathionylation, Cys of GapDH is highly reactive and susceptible for various post-pentose-phosphate pathway (PPP) to provide increased levels of methionine sulfoxide reductase A (Mrx1) and Trx pathway. Reduction of S-mycothiolated GapDH occurred much faster by the Mrx1 pathway compared to Trx in vitro. Thus, Mrx1 plays probably the major role in demycothiolation of GapDH in vivo while Trx has only a minor role. These data are in agreement with the kinetics obtained for demycothiolation of Mpx by Mrx1, which was also faster compared to reduction by the Trx pathway [35].

MsrA and MsrB are further important redox enzymes which catalyze the reduction of two stereotypes of methionine sulfoxides (MetSO) to l-methionine [51]. While MsrA is specific for reduction of methionine-S-sulfoxide, MsrB shows activity for reduction of methionine-R-sulfoxides [52]. The Mrx1 and Trx pathway were shown to be involved in the recycling of the methionine sulfoxide reductase Cd-MsrA and Gc-MsrA during reduction of methionine sulfoxides in C. diphtheriae [36,37,53]. Two different redox relays operate in recycling of Cd-MsrA by the Trx and Mrx1 pathway upon MetSO reduction. Cd-MsrA has three Cys residues in positions 52, 206 and 215 with Cys52 as the active site. The disulfide relay which couples MetSO reduction by Cd-MsrA to the Trx pathway starts with formation of the Cys52-SOH upon attack of the MetSO substrate releasing reduced l-Met. Next, Cys52 is attacked by Cys206 or Cys216 resulting in intramolecular disulfides between Cys52 and either Cys206 or Cys215. This disulfide is reshuffled to a Cys206-Cys215 disulfide which is a substrate for Trx. In the MSH redox relay, Cys215-SOH forms an MSH mixed disulfide, followed by the subsequent transfer of MSH to Cys215 and Cys206. Cys206-SSM is finally the substrate that is recycled by the Mrx1 pathway. Thus, two different redox relays were discovered for Cd-MsrA upon MetSO reduction that require the Trx or Mrx1 pathway for regeneration [36]. Another study of Cg-MsrA’s catalytic mechanism in C. glutamicum revealed that Mrx1 reduces Cys56-SSM while Trx reduces the intramolecular disulfide between both C-terminal resolving Cys residues [53]. In addition, the Trx and Mrx1 pathways were required for reduction of Cg-MsrA under stress and the Trx pathway was found to be involved in Cg-MsrA regeneration under non-stress conditions. Apart from MsrA, the redox cycle of the methionine sulfoxide reductase MrxB of C. glutamicum was recently studied [37]. MrxB is specific for reduction of methionine-R-sulfoxides which requires the Trx pathway for recycling and was not important under oxidative stress in vivo [37].

The membrane-associated one-Cys peroxidase AhpE of M. tuberculosis was identified as S-mycothiolated at its active site Cys45 and was shown to be reduced by Mrx1 in vitro [33]. AhpE is S-mycothiolated during H2O2 detoxification. The reduction of AhpE-SSM was demonstrated in the presence of wild type Mrx1, indicating a dithiol mechanism. The peroxiredoxin AhpE comprises a molecular link between the peroxidase system and the Mrx1 redox pathway in Mycobacteria. Using NMR spectroscopy, the AhpE structures were resolved in the reduced, sulfenic acid, and sulfonic acid forms giving insights into the MSH-dependent reduction mechanism of AhpE [54].

In conclusion, recent studies on several identified S-mycothiolated antioxidant and metabolic enzymes (Tpx, Mpx, MsrA, and GapDH) revealed that both, the Mrx1 and Trx pathway are coupled to regeneration of their enzymatic activities under oxidative stress. However, Mrx1 was faster in demycothiolation of GapDH and Mpx compared to Trx indicating that Mrx1 is the primary de-mycothiolating enzyme under oxidative stress. In concert with MSH and Mtr, Mrx1 can undergo mono- and dithiol mechanisms to reduce protein MSH-mixed disulfides. It remains to be further investigated whether mycoredoxin-2 (Rv2466c) plays a related or alternative role in reduction of S-mycothiolated proteins.

2.4. The biosynthetic pathway and functions of bacillithiol in Firmicutes

Bacillithiol (BSH, Cys-GlcN-malate) is the α-anomeric glycoside of L-cysteinyl-D-glucosamine with L-malic acid and has an MW of 398 Da (Fig. 1BC). BSH functions as the major LMW thiol in many Firmicutes bacteria, including Bacillus and Staphylococcus species [5]. The biosynthesis of BSH occurs in three steps by the enzymes BshA, BshB and BshC. The glycosyltransferase BshA catalyzes the conjugation of UDP-N-acetylglucosamine (UDP-GlcNAc) to L-malate leading to GlcNAc-Mal (Fig. 6). GlcNAc-Mal is further deacylated by the deacetylase BshB to GlcN-Mal. The cysteine ligase BshC adds cysteine to GlcN-Mal as final step in the BSH biosynthesis [3,55]. BSH is oxidized to BSSB under oxidative stress and high BSH/BSSB ratios were calculated as 100:1–400:1 in B. subtilis cells [56]. The putative BSSB reductase was suggested to function as NADPH-dependent flavin oxidoreductase YpdA which co-occurs with the BSH biosynthesis enzymes in BSH-producing bacteria [55]. However, experimental evidence is lacking to demonstrate a role of YpdA as BSSB reductase [3]. The standard thiol redox potential of BSH was calculated as E°(BSSB/BSH) of −221 mV which is more positive compared to GSH (E°(GSSG/GSH) = −240 mV) (Fig. 1C) [56]. However, microscopic pK values of pKαH=7.97 and pKαH=9.55 were determined for the bacillithiol anion (with protonated and deprotonated Cys amino group, respectively), suggesting a more acidic BSH thiolate anion with enhanced reactivity (Fig. 1C) [56].

BSH functions in the detoxification of many thiol-reactive compounds, electrophiles, alkylating agents, toxic metals and antibiotics (Fig. 7) [3,55]. BSH-deficient mutants in B. subtilis showed increased sensitivities toward hypochlorite, diamide, methylglyoxal, ROS, osmotic and acidic stress, alkylating agents, and fosfomycin. For a comprehensive overview of the many functions of BSH, the reader is referred to a recent review, which will be summarized and updated here only briefly [3].

The BSH-dependent thiol-S-transferase FosB is involved in the detoxification of the antibiotic fosfomycin and cleaves the ring structure of the BS-fosfomycin-conjugate [57]. The fosB and bsh mutants showed equal sensitivities towards fosfomycin treatment in B. subtilis and S. aureus indicating that FosB is a BSH-dependent S-transferase for fosfomycin detoxification. Co-crystallization of S. aureus FosB with BSH resulted in the formation of a BSH-mixed disulfide at the active site Cys9 of FosB of S. aureus in vitro [55,58]. However, the electron density for the whole molecule of BSH was not complete in the FosB structure. The BSH S-transferase BstA catalyzes the conjugation of BSH to reactive electrophiles, such as chlorinated hydrocarbons and mono-bromobimane in vitro [21]. The resulting BS-electrophiles (BSR) are cleaved by the BSH conjugate amidas Bca or BshB2 into GlcNAc-Mal and mercapturic acids (CysSR). CysSR are exported from the cell by the potential efflux pumps encoded by the yflS and yflU genes [21].

BSH functions in detoxification of methylglyoxal as cofactor for the BSH-dependent glyoxalases GlxAB in B. subtilis [59]. Methylglyoxal...
reacts spontaneously with BSH to form BSH-hemithioacetal that is converted to S-lactoyl-BSH by GlxA. GlxB catalyzes the hydrolysis of S-lactoyl-BSH to lactate which is secreted. BSH is involved in detoxification of the toxic electrophile formaldehyde in the facultative methylotrophic bacterium *Bacillus methanolicus* which uses methanol as sole source of energy and carbon [60]. An unknown formaldehyde dehydrogenase is involved in oxidation of S-formyl-BSH in *Bacillus methanolicus* cells during growth on methanol, which is required for

Fig. 6. The biosynthesis pathway of bacillithiol in *Firmicutes*. The glycosyltransferase BshA catalyzes the conjugation of UDP-N-acetylglucosamine (UDP-GlcNAc) to L-malate leading to N-acetylglucosamine malate (GlcNAc-Mal). GlcNAc-Mal is deacetylated by the deacetylase BshB to GlcN-Mal. The cysteine ligase BshC ligates cysteine to GlcN-Mal. The detoxification of toxic electrophiles (E) requires conjugation by the bacillithiol-S-transferase (BST) leading to BSH-S-conjugates which are cleaved by the BSH-S-conjugate amidase (Bca). Mercapturic acids are exported and GlcN-Mal is recycled to BSH. This figure is adapted from [93].

Fig. 7. The many functions of bacillithiol in *Firmicutes*. BSH is involved in detoxification of ROS, RES, HOCI, RSS and antibiotics (fosfomycin, rifampicin) in *B. subtilis* and *S. aureus*. ROS lead to oxidation of BSH producing bacillithiol disulfide (BSSB). Under sulfide stress, BSH persulfides (BSHSSH) were determined, which can be reduced by thioredoxins (TrxP and TrxQ). Electrophiles (RX) are conjugated to BSH by the BSH S-transferase BstA to form BS-electrophiles (BSR). The BSH S-conjugate amidases Bca or BshB2 cleave BSR into GlcNAc-Mal and mercapturic acids (CysSR) that are exported. BSH is used as cofactor for the epoxide hydrolase FosB in fosfomycin detoxification and functions as a cofactor for the glyoxalases GlxA and GlxB in *B. subtilis* in methylglyoxal detoxification. GlxA converts BSH-hemithioacetal to S-lactoyl-BSH that is a substrate for GlxB producing D-lactate. BSH-dependent detoxification of formaldehyde might be catalyzed by the formaldehyde dehydrogenase (AdhA) in *B. subtilis*. BSH functions in metal homeostasis as Zn$^{2+}$ buffer and in FeS cluster assembly. In *S. aureus*, BSH is important for the virulence of *S. aureus* in macrophage infection assays. Under HOCI stress, proteins are S-bacillithiolated, which can be reversed by bacilliredoxins (Brx). This figure is adapted from [2].
BSH is further implicated in Fe2+ homeostasis and is suggested to play a role in FeS cluster assembly and transport in *S. aureus* and *B. subtilis* [63, 64]. Mutants with defects in BSH biosynthesis showed a delayed growth upon depletion of Fe2+ or branched chain amino acids in the growth medium. The activities of several FeS-cluster enzymes (e.g., LeuCD, IlvD) were also decreased in the BSH-deficient mutant. These phenotypes suggest a potential role of BSH in FeS cluster biogenesis [64, 65]. Further growth analyses with *nfu* and *sufA* mutants indicate that BSH may participate in the biogenesis of FeS cluster proteins independently of the SufA and Nfu carriers [64, 65]. Finally, BSH has been shown to play a role in transport of copper to the Cu+ chaperone CopZ and Cu+ buffering to avoid Cu+ toxicity [66]. During Cu+ transport by BSH, CopZ was identified as S-sbacillithiolated in *B. subtilis* [67].

BSH is also involved in virulence and survival of *S. aureus* under macrophage infection conditions as revealed by phenotype analysis of *bshA* mutants in clinical methicillin-resistant *S. aureus* (MRSA) isolates [60].
COL, USA300 and SH1000 [69–71]. *S. aureus* COL represents a hospital-acquired (HA) MRSA strain while USA300 was isolated as community-acquired (CA)-MRSA strain with strongly increased virulence due to many toxins encoded on prophages and other mobile genetic elements [72]. SH1000 is a natural *bsH* mutant belonging to the NCTC8325-4 lineage [73]. NCTC8325-4 is a laboratory model strain that was obtained from a sepsis isolate after removal of three resident prophages by UV. The survival of *S. aureus* COL and USA300 *bsH* mutants as well as of the natural *bsH* mutant of strain SH1000 were decreased in phagocytosis infections assays using murine macrophages and human whole-blood assays as compared to the wild type [69,71]. These results suggest a role of BSH in the defense against the host-immune system and in the survival of *S. aureus* under infection conditions [69–71]. However, it remains to be shown if BSH is required for protection of *S. aureus* inside neutrophils against the antimicrobial activity of the myeloperoxidase (MPO) which is involved in HOCI production.

### 2.5. Physiological role of protein S-bacillithiolations in Firmicutes

BSH plays an important role in post-translational modifications of proteins under oxidative stress in Gram-positive bacteria. In response to HOCI stress, protein thiols are oxidized to mixed disulfides with BSH, termed as protein S-bacillithiolation [74–76]. In total, we identified eight common and 29 unique S-bacillithiolated proteins using shotgun proteomics in *B. subtilis*, *B. amyloliquefaciens*, *Bacillus pumilus*, *Bacillus weihenstephanensis* and *Bacillus subtilis* C1498 [Table S2] [75,76]. Many of the identified S-bacillithiolated proteins are involved in important cellular processes, such as the biosynthesis of amino acids, cofactors and nucleotides, protein translation, and redox-signalling of ROS. In *B. subtilis*, the MarR-type repressor OhrR was S-bacillithiolated under HOCI and organic hydroperoxide (OHP) stress at its redox-sensing Cys151. S-bacillithiolation of OhrR at its lone Cys residue leads to inactivation of its repressor function and derepression of transcription of the ohrA gene encoding the OhrA peroxiredoxin which confers resistance under HOCI and OHP stress [Fig. 8A] [74,75]. MetE was identified as the most abundant S-bacillithiolated protein in *B. subtilis*, which was S-bacillithiolated at the active site Cys730 and at the non-conserved Cys719 [75]. S-bacillithiolation of MetE under HOCI stress resulted in a methionine auxotrophy phenotype perhaps to stop translation during recovery from oxidative stress (Fig. 8B). In addition, other enzymes involved in the Cys and Met biosynthesis pathways were identified as targets for S-bacillithiolation, including SerA, PpaC, MetL and YxG. The translation elongation factor EF-Tu (TuA) and GuaB were also identified as abundant and essential S-bacillithiolated proteins under HOCI stress [25,75,76]. The bacilliredoxins BxRα, BxRβ and BxRγ were identified as S-bacillithiolated in *B. subtilis* and *S. carnosus*, which could be intermediates of the bacilliredox redox pathway [76].

In *S. aureus* we determined the redox state of 228 Cys residues using OxICAT. We identified 58 HOCI-sensitive Cys residues that showed >10% increased oxidation levels under HOCI stress. Among these HOCl-sensitive thiol-switches, 19 showed 20–30% increased oxidation that included the S-bacillithiolated proteins GapDH, AldA, GuaB and RpmJ. GapDH is S-bacillithiolated at the active site Cys151 and represents the most abundant S-bacillithiolated protein contributing with 4% to the total Cys proteome of *S. aureus* (Fig. 8C) [42]. In kinetic assays, GapDH of *S. aureus* was faster inactivated by H₂O₂ and HOCI stress in the presence of BSH due to S-bacillithiolation compared to overoxidation with the oxidants alone in vitro [42]. These experiments provide evidence that S-bacillithiolation is involved in redox regulation and thiol-protection of Cys151 of GapDH against overoxidation in *S. aureus* [42]. The oxidative inactivation of GapDH in *S. aureus* may induce metabolic re-configuration of carbon metabolism and redirection into the PPP as shown in eukaryotic organisms [47,49].

The aldehyde dehydrogenase AldA was identified as another target for S-bacillithiolation under HOCI stress in *S. aureus* (Fig. 8C) [77]. Aldehyde dehydrogenases (ALDHs) catalyze the NAD⁺-dependent oxidation of aldehydes to their carboxylic acids in four steps (Fig. 9) [78]. In the first step, a tetrahedral hemithioacetal-enzyme complex is formed. NAD⁺ binding repositions the catalytic cysteine, allowing the nucleophilic attack on the carbonyl carbon of an aldehyde substrate. The nucleophilicity of the catalytic cysteine is achieved by deprotonation through an adjacent conserved glutamate residue. In the second step, hybrid transfer from the hemithioacetal intermediate to NAD⁺ generates a covalent thioester intermediate. In the third step, NADH is released from the enzyme allowing hydrolysis of the thioester intermediate. Therefore, nucleophilic water attacks the thioester intermediate to generate a second tetrahedral intermediate. In the final step, hydrolysis of the thioacetyl-enzyme complex releases a carboxylic acid product [78].

GapDH and AldA displayed both 29% increased oxidation under HOCI stress as revealed by the OxICAT approach in vivo [18,77]. Treatment of AldA with H₂O₂ in the presence of BSH resulted in reversible S-bacillithiolation and loss of activity, which could be restored upon DTT reduction [77]. The purified AldA enzyme showed a broad substrate spectrum, catalyzing the oxidation of various aldehydes, including formaldehyde, methylglyoxal, acetaldehyde and glycolaldehyde. Thus, the question of the natural substrate for AldA remains unresolved. Transcriptional analysis further revealed an increased *aldA* transcription under formaldehyde, methylglyoxal and HOCI stress in a SigA-independent manner. Thus, our results point to an unknown redox-sensing regulator controlling expression of *aldA* under thiol-stress conditions. However, in survival phenotype assays, the *aldA* mutant was more sensitive to HOCI stress, but not to aldehydes. This suggests that unknown aldehyde substrates might be generated under HOCI stress as potential substrate for AldA [77].

### 2.6. Structural insights into the active sites of targets for S-bacillithiolations

We were further interested in the structural changes of redox-sensitive proteins after S-bacillithiolation of their active sites. Molecular docking and molecular dynamic simulations were used to model the
Fig. 10. Structural insights into conserved targets for S-bacillithiolation. (A, B) The S-bacillithiolated active site pocket of *S. aureus* GapDH in the apo- (A) and holo-enzyme (B). (C) The superposition of the GapDH active site of the apo- (blue) and holo-enzyme (green). The molecular docking of BSH was performed on the GapDH apo- (PDB ID: 3LC7) and holo-enzyme structures (PDB ID: 3LVF) (D, E) The S-bacillithiolated active site pocket of *S. aureus* AldA in the apo- (blue) (D) and holo-enzyme (green) (E). Molecular docking of BSH was performed on the AldA structure (PDB ID: 3TY7). (F) The active site of the *B. anthracis* GuaB in the apo-enzyme (blue) (PDB ID: 3TSB) and substrate-bound structure (green) (PDB ID: 3USB), the substrate is not shown. (G, H) The active site of the *S. mutans* MetE in the oxidized (PDB ID: 3L7R) (G) and in the zinc-replete structure (PDB ID: 3T0C) (H). In the oxidized structure, C632 and C715 form an intramolecular disulfide bond. The asterisk indicates a glutamate residue that was modelled as alanine due to the lack of the electron density at this position. (I) The superposition of the active site of the MetE oxidized (blue) and zinc-replete (green) structures. The zinc cation is shown as gray sphere. (J) The active site of the *S. aureus* PpaC (PDB ID: 4RPA). The active site residues and the neighboring cysteine residues are shown as sticks and coloured by atom type. The colour of the carbon atoms allows to distinguish between active site residues (purple) and neighboring cysteine residues that were found S-bacillithiolated (dark red) or were not S-bacillithiolated (light orange). The manganese cations are shown as pink spheres. (K, L) The redox-active cysteine residue of *S. aureus* SarZ. (K) Superposition of the reduced (blue) (PDB ID: 3HSE) and oxidized form (green) (PDB ID: 3HRM) of SarZ. (L) Superposition of the reduced (blue) and benzene thiol-bound SarZ in chain C (magenta) and in chain D (light pink) (PDB ID: 3HSR). One monomer of the SarZ dimer is shown in blue (reduced SarZ), green (oxidized SarZ), magenta (benzene thiol-bound SarZ chain C) or light pink (benzene thiol-bound SarZ chain D), whereas the other monomer is shown in shades of gray. All the important residues in all panels are shown as sticks and coloured by atom types (oxygen-red, nitrogen-blue, sulfur-yellow, phosphor-orange, carbon-variable) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).
S-bacillithiolated active sites C151 of GapDH (PDB ID: 3L7C, 3LVF) [79] and C279 of AldA (PDB ID: 3TY7) of S. aureus in the apo- and holo-enzyme structures, respectively (Fig. 10A-E) [42,77]. Both Cys residues act as a nucleophile in catalysis, leading to the formation of a covalent intermediate [79,80]. Interestingly, in aldehyde dehydrogenases, the Cys residue adopts a “resting” conformation in the apo-enzyme and an “attacking” conformation in the holo-enzyme [80]. The molecular docking of BSH into the AldA active site resulted in two different positions of the BSH depending on C279 activation state (Fig. 10D, E). While C279 is in “resting” position in the absence of the NAD+ cofactor, S-bacillithiolation and their available crystal structures. Table 1.

<table>
<thead>
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<th>Protein</th>
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<th>Related structures (PDB ID)</th>
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<th>Function of the cysteine residue</th>
<th>Confirmed post-translational modification</th>
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<td>C279 C96 C151</td>
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<td>C279 C96</td>
<td>active site residue</td>
<td>S-bacillithiolation, overoxidation</td>
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<tr>
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<td>- 3T73</td>
<td>C307 C326 C158</td>
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<tr>
<td>GuaB</td>
<td>Bacillus anthracis</td>
<td>3TSB 3USB 3TSD</td>
<td>C308 C326 C158</td>
<td>active site</td>
<td>S-bacillithiolation</td>
</tr>
<tr>
<td>MetE</td>
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<td>- 3L7R 3T0C</td>
<td>C632 C715 C308</td>
<td>zinc-binding site</td>
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<td>3HBM 3HSE 3HSR</td>
<td>C13</td>
<td>redox-active</td>
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</table>

* Also confirmed in other species.
non-conserved Cys residues, such as C96 of *S. aureus* GapDH that was neither S-bacillithiolated nor overoxidized and is buried in the structure like C118 in PpaC (Fig. 11B) [42]. In contrast, in GuaB of *B. anthracis*, the non-conserved C326 close to the active site was identified as S-bacillithiolated because it is accessible for BSH (Fig. 11C) [77].

The most interesting targets for S-bacillithiolation are redox-sensing regulators, such as the thiol-based OhrR repressor which was S-bacillithiolated at the conserved C15 under HOCI and CHP stress [74,75]. In *S. aureus*, the OhrR-homolog SarZ was identified as redox-sensitive repressor and the structural changes were investigated during thiol-oxidation of the single C13, including S-thiolation with the synthetic benzene thiol [75,82,87,88]. The crystal structures of SarZ in reduced (PDB ID: 3HSE), sulfenic acid (PDB ID: 3HMR), and mixed disulfide form (PDB ID: 3HSR) [82] provide the explanation for inhibition of SarZ repressor activity. Upon C13 oxidation, several α-helices of the DNA-binding helix-turn-helix motif change their conformations leading to dissociation of SarZ from the promoter DNA. Inhibition of SarZ repressor activity results in derepression of transcription of a large SarZ regulon, including the *ohr* peroxiredoxin gene and genes involved in cellular metabolism, virulence, and antibiotic resistance [87]. Interestingly, oxidation of SarZ to the C13 sulfenic acid does not lead to inactivation and structural changes of SarZ (Fig. 10K). Only further overoxidations of C13 to sulfonic acid or S-thiolation causes SarZ inactivation [82]. The structure of the S-thiolated C13-benzene thiol complex provides insights into possible binding sites for LMW thiols. Benzene thiol occupies different positions in different SarZ monomers present in the asymmetric unit of the S-thiolated SarZ structure. In monomer B and C, benzene thiol is oriented toward the surface of the protein, whereas in monomer D, it is buried in a hydrophobic cavity in the dimer interface (Fig. 10L). Benzene thiol might enter the cavity with C13 and the formation of the disulfide bond occurs at a position seen in monomer B, C. Afterwards, C13 changes the conformation yielding another orientation of benzene thiol, as in monomer D [82]. In the case of the BSH binding, the location of BSH in the hydrophobic pocket is less probable because BSH contains several charged groups.

In general, all discussed potential BSH-binding site are composed of positively or negatively charged residues that can interact with the amine, hydroxyl and carboxyl groups of the BSH. Our structural analysis shows that the S-bacillithiolated Cys residues in different proteins are easily accessible for BSH due to their location either in the active sites of proteins or at the protein surface. The Cys residues buried in protein structure, e.g. C96 of GapDH and C118 of GuaB, do not undergo S-bacillithiolation. In the majority of targets, S-bacillithiolation occurs at the catalytic active sites (C151 of GapDH, C279 of AldA, C308 of GuaB, C715 of MetE) or redox-sensing Cys residues of thiol-based redox sensors, such as C13 of SarZ or C15 of OhrR. S-thiolation of SarZ leads to conformational changes, which is the mechanism of derepression of SarZ controlled target genes [82]. The models of *S. bacillithiolated* GapDH and AldA do not support structural changes. This is in agreement with the catalytic reactions of substrate conversion in GapDH and AldA that are not accompanied by significant structural alterations. In contrast, the inactivation of SarZ requires structural changes in the DNA binding domains as redox-regulatory mechanism. Therefore, it is tempting to speculate that S-thiolation of enzymes in most cases does not lead to structural changes, although exceptions are possible.

2.7. Protein S-bacillithiolation is redox controlled by bacilliredoxins (Brx)

The reduction of S-bacillithiolated proteins in * Firmicutes* is catalyzed by the bacilliredoxin redox pathway (Fig. 12). The bacilliredoxins BrxA and BrxB were identified as paralogs of the DUF1094 family in *B. subtilis*, which shared 53% sequence identity [89,90]. Phylogenomic profiling identified BrxA and BrxB as Trx-like proteins with unusual CGC active sites. The crystal structure of BrxA revealed overall structural similarities to thioredoxins. However, the redox potential was determined as ~130 mV, which is much more positive compared to that of thioredoxin proteins [91]. Furthermore, BrxC was identified as candidate for a monothiol Brx which has a TCPIS active motif and is similar to monothiol Grx. However, the function of BrxC in de-bacillithiolation remains to be elucidated [92]. The flavin disulfide reductase YpdA was suggested as putative BSSB reductase in the STRING search due to its phylogenetic co-occurrence together with the BSH biosynthesis enzymes [58]. However, the catalytic activity of YpdA in BSSB reduction could not be demonstrated yet.

Bacilliredoxins function analogous to glutaredoxins by the attack of
the active site Cys on BSH-mixed protein disulfides, resulting in the transfer of BSH to the active site Cys of Brx (Fig. 12) [89]. In B. subtilis, the S-bacillithiolated OhrR repressor and MetE were identified as natural substrates for BrxA and BrxB in vitro. The DNA-binding activity of the OhrR repressor could be recovered after de-bacillithiolation by the BrxB-SSB mutant protein. The de-bacillithiolation of MetE-SSB was demonstrated by BSH-specific Western blot analysis and mass spectrometry, but MetE reactivation could not be shown in vitro. While de-bacillithiolation of OhrR-SSB is catalyzed mainly by BrxB, reduction of MetE-SSB can be catalyzed by both BrxA and BrxB [89].

In S. aureus, the glycolytic GapDH was demonstrated as specific substrate for de-bacillithiolation by BrxA (SAUSA300_1321) during recovery from oxidative stress [42]. Using in vitro activity assays, the glycolytic GapDH activity could be restored after de-bacillithiolation by BrxA and the BrxCGA-resolving Cys mutant, but not by the BrxAGC-active site Cys mutant. BSH-specific Western blot analysis supported the reduction of GapDH-SSB in vitro [42]. However, in vivo evidence for the functions of Brx and YpdA in the oxidative stress response is still missing in Firmicutes.

3. Outlook for future research

In this review, we report an update in the research of the functions and properties of the LMW thioles mycothiol and bacillithiol, which are dominant scavengers of ROS and other reactive species in Actinomycetes and Firmicutes. MSH and BSH were also shown to contribute to the pathogenicity and antibiotic resistance mechanisms of major human pathogens, such as M. tuberculosis and S. aureus indicating the importance of LMW thioles in the defense against the host innate immune system during infections. Significant progress has been made in the structural and mechanistic characterization of many MSH and BSH-dependent detoxification enzymes, as well as MSH and BSH biosynthesis enzymes. However, the catalytic mechanism of the putative BshC ligase and the NADPH-dependent flavin disulfide reductase YpdA are still unknown. Similarly, other unknown MSH- or BSH-dependent formaldehyde dehydrogenases, quinone reductases, and peroxidases might play important roles in detoxification of reactive compounds. There is also significant progress for the role of BSH in FeS cluster assembly, Zn²⁺ and Cu⁺ homeostasis, but related roles of MSH have not been investigated and may be important to maintain metal homeostasis under infection conditions. Recent work also showed an involvement of BSH in sulfide homeostasis and sulfhydrations that are implicated in redox regulation of metabolic enzymes and virulence regulators. Finally, over the last years many targets for protein S-mycothiolation and S-bacillithiolation have been discovered through shotgun proteomics and quantitative thiol-redox proteomics. For some antioxidant enzymes (AhpE, Mpx, MsrA) and metabolic enzymes (GapDH), the roles of MSH and BSH in thiol-protection and redox-regulation under oxidative stress have been clearly shown. Molecular docking of BSH into the active sites of GapDH and AldA of S. aureus provided first insights that S-bacillithiolation does not require structural changes. Moreover, BSH can occupy two different positions in the active sites with the active site Cys in the attacking or resting state, depending on the presence of the NAD⁺ cofactor. Our structural comparison here revealed that these mechanisms could be relevant also for many other conserved targets for S-thiolations, such as GuaB. However, the physiological roles of these protein S-thiolations in cellular physiology in Actinomycetes and Firmicutes requires much more detailed future work. In addition, the functions of bacilliredoxins under oxidative stress remain to be elucidated. Thus, future research should be directed to elucidate the physiological roles of the LMW thioles BSH and MSH and the many targets for protein S-bacillithiolation and S-mycothiolation in bacterial physiology under stress and infection conditions.

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Author disclosure statement

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.redox.2018.08.017.

References

[31] D. Shenton, C.M. Grant, Protein S-thiolation targets glycolysis and protein synthesis


