Review

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Thiol-based redox switches in the major pathogen

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Abstract: Staphylococcus aureus is a major human pathogen, which encounters reactive oxygen, nitrogen, chlorine, electrophile and sulfur species (ROS, RNS, RCS, RES and RSS) by the host immune system, during cellular metabolism or antibiotics treatments. To defend against redox active species and antibiotics, S. aureus is equipped with redox sensing regulators that often use thiol switches to control the expression of specific detoxification pathways. In addition, the maintenance of the redox balance is crucial for survival of S. aureus under redox stress during infections, which is accomplished by the low molecular weight (LMW) thiol bacillithiol (BSH) and the associated bacilliredoxin (Brx)/BSH/bacillithiol disulfide reductase (YpdA)/NADPH pathway. Here, we present an overview of thiol-based redox sensors, its associated enzymatic detoxification systems and BSH-related regulatory mechanisms in S. aureus, which are important for the defense under redox stress conditions. Application of the novel Brx-roGFP2 biosensor provides new insights on the impact of these systems on the BSH redox potential. These thiol switches of S. aureus function in protection against redox active desinfectants and antimicrobials, including HOCl, the AGXX® antimicrobial surface coating, allicin from garlic and the naphthoquinone lapachol. Thus, thiol switches could be novel drug targets for the development of alternative redox-based therapies to combat multi-drug resistant S. aureus isolates.

Keywords: bacillithiol; electrophiles; HOCl; ROS; Staphylococcus aureus; thiol switches.
In addition, specific transcriptional regulators are involved in redox sensing of reactive species, which control defense mechanisms to restore the reduced state of the cytoplasm (Antelmann and Helmann 2011; Chen et al. 2011; Hillion and Antelmann 2015). Redox sensing transcription factors often utilize conserved cysteine residues to sense and respond to redox stress conditions via post-translational thiol modifications, including thiol-disulfide switches as the most common theme (Antelmann and Helmann 2011; Chen et al. 2011; Hillion and Antelmann 2015; Vazquez-Torres 2012). Through innovative mass spectrometry-based redox proteomics approaches, many new thiol switches have been discovered over the past years, which are present in metabolic and redox enzymes, transcription and translation factors. Thiol switches are important regulatory devices from bacteria to men, which alter structure and functions of proteins, often leading to their inactivation and reprogramming of cellular metabolism. Thiol oxidation of redox sensing transcription factors controls specific regulons involved in detoxification pathways or regeneration of redox homeostasis (Antelmann and Helmann 2011; Hillion and Antelmann 2015). Here, we provide an overview of thiol-based redox sensing regulators, the associated detoxification pathways and BSH-related thiol-disulfide reducing systems in the human pathogen S. aureus. These defense mechanisms provide resistance and enable survival and efficient adaptation to the host environment contributing to the virulence of this major pathogen.

Sources and thiol chemistry of reactive oxygen, electrophile, chlorine, nitrogen and sulfur species (ROS, RES, RCS, RNS and RSS)

In all organisms, the reduced state of protein thiols in the cytoplasm is maintained by LMW thiols and enzymatic thiol-disulfide reducing systems (Van Laer et al. 2013). S. aureus encounters various redox active species during infections by the host immune defense, antibiotic treatment or cellular metabolism, which affects the cellular redox balance, including ROS, RES, RNS, RCS and RSS (Hillion and Antelmann 2015). The most frequent source of ROS is the aerobic respiratory chain, when O$_2$ is incompletely reduced by stepwise one-electron transfer reactions, generating superoxide anion (O$_2$•$^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH•) (Figure 1) (Imlay, 2003, 2008). Furthermore, autoxidation of flavin cofactors in redox enzymes contributes to ROS production. In the Fenton reaction, Fe$^{2+}$ and H$_2$O$_2$ produce the highly toxic OH•, which can oxidize proteins, lipids and DNA (Forman and Torres 2001; Imlay, 2003, 2008).

Figure 1: Generation of reactive oxygen, chlorine, nitrogen and electrophile species (ROS, RCS, RNS, RES) during aerobic respiration, infections and antibiotics treatment in Staphylococcus aureus. ROS are generated in the respiratory chain by one-electron transfer to O$_2$, including O$_2$•$^-$ and H$_2$O$_2$. The OH• is produced by reaction of H$_2$O$_2$ and Fe$^{2+}$ in the Fenton reaction (Imlay 2003). In activated neutrophils, NOX produces O$_2$•$^-$, which is dismutated by SOD to H$_2$O$_2$. MPO converts H$_2$O$_2$ and Cl$^-$ to the bactericidal HOCl (Winterbourn and Kettle 2013). In neutrophils, iNOS synthesizes NO• from arginine. RES include quinones, such as benzoquinone (BQ), which are electron carriers of the respiratory chain (menaquinone) and structural elements of catechol-type siderophores and antibiotics (e.g. pyocyanin, naphthoquinone and ciprofloxacin). Methylglyoxal is produced as toxic aldehyde in the glycolysis. Allicin is an organosulfur compound that acts as antimicrobial.
In addition, pathogens encounter ROS, RCS and RNS generated during the oxidative burst of activated macrophages and neutrophils as the first line defense of the innate immune system, including $O_2^{•−}$, $H_2O_2$, nitric oxide (NO), and hypochlorous acid (HOCl) (Winterbourn and Kettle 2013; Winterbourn et al. 2016; Ulfig and Leichert 2020). While $O_2^{•−}$ is produced by the membrane-bound NADPH oxidase (NOX) in neutrophils, superoxide dismutase (SOD) dismutates $O_2^{•−}$ to $H_2O_2$. The myeloperoxidase (MPO) produces the strong oxidant HOCl from $H_2O_2$ and chloride anion (Cl$^−$) in neutrophils after degranulation of azurophilic granules (Figure 1) (Winterbourn and Kettle 2013; Winterbourn et al. 2016; Ulfig and Leichert 2020). While low concentrations of 25 µM $O_2^{•−}$ and 2 µM $H_2O_2$ are produced in the neutrophil phagosome, MPO generates millimolar levels of HOCl, which are released during infections to kill pathogens (Winterbourn et al. 2006).

ROS react with Cys thiols to form unstable sulfenic acids (R-SOH), which are further oxidized to intramolecular or intermolecular protein disulfides or S-thiolations of proteins with LMW thiols, such as S-bacillithiolations or S-CoAlations in S. aureus (Figure 2) (Antelmann and Helmann 2011; Imber et al. 2019; Tsuchiya et al. 2018). In the absence of proximal thiols, ROS can irreversibly oxidize Cys thiols to sulfenic (R-SO$_2$H) or sulfonic acids (R-SO$_3$H) (Antelmann and Helmann 2011). While $H_2O_2$ reacts slowly with biological thiols according to second-order rate constants in the range of $k = 18–26$ M$^{-1}$s$^{-1}$ (Winterbourn and Metodiiewa 1999), the reaction of HOCl with thiols is several orders of magnitude faster and occurs with rate constants of $k > 10^9$ M$^{-1}$s$^{-1}$ (Storkey et al. 2014). HOCl chlorinates Cys thiols to generate unstable sulfenylchloride (R-SCI), which is further oxidized to form reversible or irreversible thiol oxidation products (Davies 2011; Hawkins et al. 2003). Apart from Cys thiols, HOCl reacts with methionine residues to methionine sulfoxides (Davies 2011; Rosen et al. 2009). The reaction of HOCl with primary amines leads to chloramine formation (Green et al. 2017). Chloramines formed with free amino acids can further decompose to electrophiles, such as glyoxal, acrolein and $p$-hydroxyphenylacetdehyde, which react with thiols via S-alkylation (Beavers and Skaar 2016; Hazen et al. 1998). HOCl can modify proteins by N-chlorination as another reversible modification, which activates chaperone functions of proteins (Davies 2011; Hawkins et al. 2003; Müller et al. 2014; Ulfig and Leichert 2020). In S. aureus and Bacillus species, we have shown that HOCl causes widespread S-bacillithiolations of redox sensing regulators and metabolic enzymes, which function in redox regulation of protein activities and protection against overoxidation to Cys sulfonic acids (Chi et al., 2011,2013; Imber et al. 2019).

Moreover, neutrophils use the inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO$^•$) from arginine as bactericidal RNS. In bacteria, endogenous NO$^•$ is generated by the nitrate reductase from nitrate, which is used as anaerobic electron acceptor (Foster et al. 2003; Stomerski et al. 2019). NO$^•$ is a weak oxidant and can further react with $O_2^{•−}$ to generate peroxynitrite (ONOO$^−$) as highly reactive intermediate between ROS and RNS (Rady 2018). NO$^•$ was shown to interact with $H_2O_2$ to form nitroxyl (HNO) (Peng et al. 2017a; Walsh and Giedroc 2020). Thus, this crosstalk between ROS, RNS and RSS yields new reactive species that could partly explain the broad specificity of certain redox sensors. RNS can lead to post-translational thiol modifications of proteins and LMW thiols resulting in S-nitrosylation (Cys-SNO), which occurs via different chemical routes (Fernando et al. 2019; Foster et al. 2003; Stomerski et al. 2019). NO$^•$ autoxidation by oxygen yields dinitrogen oxide and trioxide ($N_2O$ and $N_2O_3$).
N$_2$O$_3$. N$_2$O activates Cys thiols to thyl radicals (Cys-S·), which react with NO· to Cys-SNO (Figure 2). N$_2$O$_3$ directly reacts with Cys thiolates to form Cys-SNO (Fernando et al. 2019; Foster et al. 2003; Stomberski et al. 2019). In addition, the NO group can be transferred from metal-NO complexes or LMW-SNOs to protein Cys thiolates via trans-S-nitrosylation (Stomberski et al. 2019). Thus, S. aureus must be equipped with effective protection systems against ROS, RCS and RNS encountered endogenously and during interaction with host immune cells.

Additionally, S. aureus is exposed to antibiotics, xenosiderophores and metabolites, which have electron-deficient centers and are termed as reactive electrophilic species (RES). In S. aureus, endogenous RES are present as menaquinones in the electron chain, catechol-type siderophores and glycolytic aldehydes, such as methylglyoxal. Host-derived electrophiles are generated during macrophage and neutrophil infections by RNS and ROS as secondary reactive metabolites from oxidation of amino acids, unsaturated fatty acids and carbohydrates (Marnett et al. 2003). Electrophilic fatty acids accumulate especially during inflammation and can exert anti-inflammatory signaling effects (Delmastro-Greenwood et al. 2014). Electrophilic quinones have two modes of actions, an oxidative and an alkylating mode (Monks et al. 1992; O’Brien 1991). In the oxidative mode, quinones are reduced by one-electron transfer reactions to the highly reactive semiquinone anion ($\cdot$S$\equiv$) (Figure 2). In the alkylating mode, quinones react irreversibly with thiol groups via thiol-S-alkylation, resulting in protein aggregation and thiol depletion in the proteome and thiol metabolome (O’Brien, 1991; Monks et al., 1992; Liebeke et al., 2008). In general, the ability of quinones to alkylate protein thiols decreases with the number of substitutions of the quinone ring adjacent to the keto groups (O’Brien 1991; Smith 1985). Unsubstituted quinones, such as benziquinones have a high ability to alkylate and aggregate protein thiols (Brunmark and Cadenas 1989; Liebeke et al. 2008). However, the fully substituted naphthoquinone lapachol was recently shown to act only via the oxidative mode in S. aureus (Linzner et al. 2020). In addition, the electrophile methylglyoxal is produced as toxic byproduct during glycolysis from dihydroxyacetone phosphate in many organisms (Booth et al. 2003; Ferguson et al. 1998; Kalapos 2008). Methylglyoxal conjugates nucleophilic thiol and amino groups of amino acids, DNA and RNA bases, leading to glycation end products (Kalapos 2008).

In addition, host and bacterial cells produce reactive sulfur species (RSS), such as H$_2$S, HS$^-$ and S$^{2-}$, which have important signaling functions and act as gasotransmitters (Walsh and Giedroc 2020). RSS can lead to persulfoxidations of protein thiols and LMW thiols (RSSH). Persulfides have nucleophilic and electrophilic properties (Benchoad et al., 2020). While host phagocytes generate ROS and HOCl as microbicidal killing agents, RSS have beneficial properties as antioxidants to provide protection of bacterial pathogens against oxidative stress and antibiotics during infections (Shatalin et al. 2011; Walsh and Giedroc 2020). H$_2$S has been shown to confer tolerance to antibiotics by counteracting ROS production in many bacteria (Mironov et al. 2017; Shatalin et al. 2011). Mechanistically, H$_2$S has been proposed to sequester free iron to protect bacteria against oxidative stress elicited by antibiotics by inhibition of the Fenton reaction (Mironov et al. 2017). In S. aureus, endogenous H$_2$S is generated by the enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) in the transsulfuration pathway from cysteine and homocysteine (Walsh and Giedroc 2020). The S. aureus cbs/cblc mutant, deficient in H$_2$S biogenesis, was impaired in survival under macrophage and neutrophil infections, indicating that RSS are important antioxidants and defense mechanisms during host-pathogen interactions (Toliver-Kinsky et al. 2019; Walsh and Giedroc 2020). H$^+$ reacts with Cys-SOH or protein disulfides leading to widespread persulfoxidations of proteins in the proteome of S. aureus (Figure 2). Persulfoxidation of the major virulence regulator MgrA inhibits its DNA binding activity resulting in lower secretion of cytotoxins in the secretome (Peng et al. 2017b; Peng et al. 2017). Furthermore, S. aureus encounters RSS in the form of the antimicrobial organosulfur compound and common foodstuff allicin from garlic, which is composed of the diallylthiosulfinate (Borlinghaus et al. 2014). In S. aureus, allicin reacts with redox sensitive protein thiols and LMW thiols via S-thiaoallylation, leading to inactivation of protein functions (Loi et al. 2019).

**Defense mechanisms of S. aureus against ROS, RES, RCS, RNS and RSS**

**Enzymatic detoxification systems**

**Superoxide anion and H$_2$O$_2$ detoxification**

S. aureus encodes several antioxidant enzymes, including superoxide dismutases (SODs), catalases and peroxiredoxins, which are involved in detoxification of O$_2$·$^-$ and H$_2$O$_2$ and provide protection under infection conditions (Imlay 2008; Mishra and Imlay 2012). Two superoxide dismutases (SodA and SodM) are present in S. aureus that
catalyze the dismutation of $\text{O}_2^\cdot{}$ to $\text{H}_2\text{O}_2$ (Clements et al. 1999; Valderas and Hart 2001; Valderas et al. 2002). While other bacteria possess Fe$^{2+}$- and Mn$^{2+}$-containing SODs, SodA and SodM of $\textit{S. aureus}$ are both Mn-dependent enzymes. Both SODs are induced by the redox cycling agents paraquat and methyl viologen as well as during the post-exponential growth phase in $\textit{S. aureus}$ (Clements et al. 1999; Karavolos et al. 2003; Valderas and Hart 2001; Valderas et al. 2002). We have recently shown that transcription of $\text{sodA}$ and $\text{sodM}$ is strongly enhanced under lapachol stress, which exerts its toxicity via redox cycling in $\textit{S. aureus}$, leading to $\text{O}_2^\cdot{}$ and $\text{H}_2\text{O}_2$ production (Linzner et al. 2020). In addition, SodM expression was upregulated in $\textit{S. aureus}$ in airway environments of cystic fibrosis patients (Treffon et al. 2018).

Both SodA and SodM are important for virulence in a murine abscess model and required for nutritional immunity in $\textit{S. aureus}$, when Mn$^{2+}$ is restricted in the host due to sequestering by calprotectin during infections (Garcia et al. 2017; Gaupp et al. 2012; Karavolos et al. 2003; Kehl-Fie et al. 2011). SodA is required for oxidative stress resistance and virulence in a murine infection model in the presence of Mn$^{2+}$, while SodM is more important under Mn$^{2+}$-starvation and provides resistance to nutritional immunity (Garcia et al. 2017). The cambialistic nature of SodM has been demonstrated in vitro, showing similar activity with Mn$^{2+}$ and Fe$^{2+}$ as metal cofactor. Thus, SodA is strictly Mn-dependent and SodM can switch from a Mn$^{2+}$- to a Fe$^{2+}$-dependent enzyme under conditions of Mn$^{2+}$ depletion during infections (Garcia et al. 2017). Interestingly, expression of $\text{sodA}$ is regulated by the MntR-dependent small non-coding RNA Rsac, which is induced under Mn$^{2+}$-starvation and basepairs with the $\text{sodA}$ mRNA, thereby inhibiting translation of SodA under conditions of nutritional immunity, when expression of the cambialistic SodM is favored to combat oxidative stress (Lalouama et al. 2019). The altered metal specificity of SodM is caused by two mutations, which evolved in close proximity to the active site, but without direct contacts of the sidechains to the metal or metal-coordinating ligands (Barwinski-Sendra et al. 2020).

Catalases (Kat) and peroxiredoxins (Prx) are the primary antioxidant enzymes for detoxification of $\text{H}_2\text{O}_2$ in most aerobic bacteria (Imlay, 2003, 2008; Mishra and Imlay 2012). While catalases operate at high $\text{H}_2\text{O}_2$ levels under oxidative stress, peroxiredoxins scavenge physiological levels of $\text{H}_2\text{O}_2$ produced during aerobic respiration (Imlay, 2003, 2008; Mishra and Imlay 2012). $\text{H}_2\text{O}_2$ detoxification by the major vegetative catalase involves heme iron for disproportionation of $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and $\text{O}_2$. In contrast, peroxiredoxins use a peroxidative Cys (Cp) for reduction of $\text{H}_2\text{O}_2$, leading to Prx-SOH and subsequent disulfide formation between Cp and the resolving Cys (Cr) (Poole et al. 2011). Recycling of oxidized Prx requires electron donors, such as the Trx/Trxr/NADPH pathway (Mishra and Imlay 2012).

$\textit{S. aureus}$ exhibits a remarkable $\text{H}_2\text{O}_2$ resistance and can survive 100 mM $\text{H}_2\text{O}_2$ (Weber et al. 2004), which depends on the major catalase KatA. KatA is peroxide-inducible, mediates resistance to oxidative stress and is controlled by the metalloregulatory Fur-family PerR repressor (Horsburgh et al. 2001a,b). While PerR was required for full virulence in a murine skin abscess model of infection, KatA was not essential for pathogenicity (Horsburgh et al. 2001a). However, KatA is important for survival under glucose starvation and desiccation conditions and promotes nasal colonization in $\textit{S. aureus}$ (Cosgrove et al. 2007; Horsburgh et al. 2001a). In addition, transcription of katA was strongly enhanced under oxidative and electrophile stress in $\textit{S. aureus}$, including HOCI, AGXX®, MHQ, allicin and lapachol (Fritsch et al. 2019; Loi et al., 2018a,b, 2019; Linzner et al. 2020). Moreover, the katA mutant was sensitive to lapachol-induced ROS generation, indicating that KatA provides protection under quinone stress (Linzner et al. 2020).

The peroxiredoxins of $\textit{S. aureus}$ include the alkyl hydroperoxide reductase (AhpCF), the thiol peroxidase (Txp), the bacterioferritin comigratory protein (Bcp) and two thiol-dependent peroxidases (GpxA1 and GpxA2). As a typical 2-Cys peroxiredoxin, bacterial AhpC reacts with peroxides to the CP-SOH, resulting in intersubunit disulfide formation between Cp and Cr in the AhpC dimer and oligomerization to an inactive AhpC decamer complex (Parsonage et al., 2008,2015). Oxidized AhpC is regenerated by the flavin disulfide oxidoreductase AhpF, which uses NADPH or NADH as electron donors (Mishra and Imlay 2012; Poole and Ellis 1996). In $\textit{S. aureus}$, the ahpCF and bcp peroxiredoxins are both PerR-controlled and $\text{H}_2\text{O}_2$-inducible (Horsburgh et al. 2001a). KatA and AhpCF have compensatory roles in $\text{H}_2\text{O}_2$ resistance, persistence and nasal colonization (Cosgrove et al. 2007). In the katA mutant, the ahpCF operon showed increased expression, contributing to $\text{H}_2\text{O}_2$ detoxification. Vice versa, expression of katA was elevated in the ahpCF mutant, providing $\text{H}_2\text{O}_2$ resistance (Cosgrove et al. 2007). The katA ahpCF double mutant exhibits growth defects during aerobic cultivations due to elevated endogenous ROS levels leading to DNA damage and cell death (Cosgrove et al. 2007).

In addition, $\textit{S. aureus}$ encodes two homologs of the thiol-dependent peroxidase Ohr, which are involved in detoxification of organic hydroperoxides, originating from lipid peroxidation (Dubbs and Mongkolsuk 2007; Mongkolsuk and Helmann 2002). The Ohr paralogs are redox-controlled by the thiol-based MrrR/Ohr-R-family repressors MgrA and SarZ as well as by MsaB in $\textit{S. aureus}$ (Chen et al.
However, many other peroxiredoxins respond to oxidative stress, such as Tpx, Bcp or GpxA1 and GpxA2, which remain to be, characterized in future studies.

**HOCl detoxification**

While the functions of KatA and AhpCF in peroxide detoxification are widely studied, the enzymatic pathways for removal of HOCl are only beginning to emerge. We have recently identified the NADPH-dependent flavin disulfide oxidoreductase MerA, which responds most strongly to HOCl and is controlled by the Rrf2-family HypR repressor in *S. aureus* (Loi et al., 2018b). MerA conferred protection under oxidative stress provoked by HOCl, AGXX® and allicin in *S. aureus*. In addition, the merA mutant was impaired in survival under macrophage infections, indicating important roles of MerA in the virulence of *S. aureus* (Loi et al., 2018). Thus, we hypothesize that the physiological function of MerA could be the reduction of strong oxidants, such as HOCl or OH•, which are generated under infection, aerobic growth or by ROS-producing antimicrobials.

The MerA flavin disulfide reductase harbors a C4gXXC4g active site, which forms a disulfide during catalysis to transfer electrons from NADPH via a FAD cofactor to the substrate, such as allicin and possibly HOCl (Argyrou and Blanchard 2004). Accordingly, Cys43 was required for survival under HOCl stress and infections in *S. aureus* (Loi et al., 2018). Moreover, MerA is a homolog of the RclA flavin disulfide reductase of *Escherichia coli* and *Salmonella* Typhimurium, which is controlled by the HOCl specific RclR regulator and was shown to promote survival of *S. Typhimurium* inside the phagosome (Baek et al., 2020; Parker et al. 2013). The crystal structure of RclA of *S. Typhimurium* revealed the typical flavin disulfide reductase-fold with two Cys in the active site and a FAD cofactor (Baek et al. 2020). Interestingly, Cu2+ enhanced the RclA-mediated oxidation of NADH, resulting in decreased oxygen levels, which might inhibit the oxidative burst of macrophages (Baek et al. 2020). In *Pseudomonas aeruginosa*, an alkyl hydroperoxidase D-like protein PA0565 (RcsA) was shown to reduce HOCl via its catalytic C60XXC63 motif and provides resistance under HOCl and infections (Nontaleerak et al. 2020). Thus, AhpD-like peroxiredoxins, MerA and RclA flavoenzymes could be responsible for HOCl removal in different bacterial pathogens.

**Quinone and aldehyde detoxification**

Furthermore, *S. aureus* encodes enzymes for detoxification of quinones, including quinone reductases (Frp, AzOR1), a nitroreductase (YodC), an FMN-linked monooxygenase, thiol-dependent dioxygenases (CatE, CatE2, MhqE) and a phospholipase/carboxylesterase (MhqD) (Fritsch et al. 2019; Ji et al. 2013). The quinone and nitroreductases catalyze reduction of quinones to hydroquinones. The thiol-dependent dioxygenases function in ring cleavage of quinones for their detoxification (Leelakriangsak et al. 2008). These quinone detoxification enzymes confer resistance to quinones and quinone-like antimicrobials and are controlled by the quinone-sensing MarR-type repressors QsrR and MhqR in *S. aureus* (Fritsch et al. 2019; Ji et al. 2013).

In addition, several uncharacterized thiol-dependent glyoxalases and aldehyde dehydrogenases are present in *S. aureus*, which might be involved in the detoxification of methylglyoxal and other aldehyde substrates. We have recently characterized the aldehyde dehydrogenase AldA, which showed broad-spectrum activity for oxidation of various aldehyde substrates, including methylglyoxal (Imber et al. 2018b). However, AldA was shown to confer resistance to HOCl stress and could be involved in methylglyoxal detoxification, which is elevated under HOCl stress (Imber et al. 2018b). In *E. coli*, methylglyoxal detoxification involved the GSH-dependent glyoxalase pathway, converting methylglyoxal to lactate (Booth et al. 2003; Ferguson et al. 1998). While there are structural and biochemical studies on putative glyoxalase enzymes in *S. aureus* (Chirgadze et al. 2018; Kim et al. 2017), a functional glyoxalase pathway has not been characterized. Of particular interest for future studies should be the DJ-1/Thj/PpfI superfamily protein HchA, which is induced under oxidative and electrophile stress conditions in *S. aureus*, such as AGXX®, HOCl, MHQ, allicin and lapa-chol (Fritsch et al. 2019; Loi et al., 2018a,b, 2019; Linzner et al. 2020). Purified HchA showed glyoxalase III and chaperone holdase activity in biochemical assays in vitro (Kim et al. 2017). However, the role of HchA in *S. aureus* cells under various stress conditions remains to be investigated.

**H2S and NO• detoxification**

*S. aureus* cells possess detoxification enzymes for removal of toxic H2S, which are encoded by the CstR-controlled cstAB-sqr operon and provide protection against RSS (Luebke et al. 2014). The sulfide:quinone oxidoreductase SQR catalyzes the two-electron oxidation of HS−, leading to formation of LMW persulfides. CstA functions as sulfur-transferase reacting with inorganic polysulfides and persulfides of the cysteine desulfurase SufS. The persulfide dioxygenase CstB oxidizes LMW persulfides, such as
bacillithiol persulfides (BSSH) and coenzyme A persulfides (CoASSH) to produce thiosulfate and LMW thiols (Higgins et al. 2015; Shen et al., 2015, 2016; Walsh and Giedroc 2020).

*S. aureus* adapts to NO− stress by expression of the lactate dehydrogenases *ddh*, *ldh1* and *lqo* and two terminal oxidases *qoxABCD* and *cydAB* for maintenance of the redox balance (Fuller et al. 2011). The flavohemoglobin Hmp catalyzes the oxidation of NO− to NO3−, representing the main enzymatic mechanism of RNS detoxification in *S. aureus* (Goncalves et al. 2006). NO− leads to activation of the SrAB two-component system, which controls expression of *hmp*, *qoxABCD* and *cydAB* (Grosser et al. 2016; Kinkel et al. 2013). Apart from Hmp, the flavodiiron NO− reductase Nor contributes to NO− detoxification, which is induced under anaerobic conditions and important for the virulence in *S. aureus* (Favazzo et al. 2019; Lewis et al. 2015).

**Functions of bacillithiol in detoxification and antibiotics resistance**

LMW thiols are non-proteinogenous thiol compounds that are present in millimolar concentrations in the bacterial cytoplasm and function in maintenance of redox homeostasis (Chandrangsu et al. 2018; Loi et al. 2015; Van Laer et al. 2013). While the tripeptide glutathione (GSH) is utilized as LMW thiol by most Gram-negative bacteria, *Staphylococcus* species and other Firmicutes produce bacillithiol (BSH) as alternative LMW thiol, which functions as GSH surrogate to maintain redox homeostasis (Chandrangsu et al. 2018). BSH derivatives are present in *Staphylococcus* species and other Gram-positive bacteria, *Staphylococcus* lacticus, *Clostridium* and *Clostridium* difficile, representing the redox active antibiotics fosfomycin, allicin, lapachol and AGXX® in *S. aureus*. (1) ROS, HOCl and AGXX® oxidize BSH to bacillithiol disulfide (BSSB) as shown in *vitro* and *in vivo* in *S. aureus* (Dickerhof et al. 2020). At higher levels, HOCl leads to BSH sulfonamide formation and overoxidation to BSH sulfonic acids (Dickerhof et al. 2020). The reaction of HOCl with BSH is very fast and occurs with second order rate constants of 6 × 107 M−1 s−1 (Dickerhof et al. 2020).

The reduced state of BSH is maintained by the NADPH-dependent flavin disulfide reductase YpdA in *S. aureus*, which functions as a BSSH reductase (Figure 3) (Linzner et al. 2019; Mikheyeva et al. 2019). YpdA acts in the Brx/BSH/YpdA/NADPH reductase pathway to regenerate *S*. bacillithiolated proteins under oxidative stress in *S. aureus* as outlined in the next sections (Linzner et al. 2019).

Importantly, BSH was identified as virulence factor in clinical MRSA isolates, such as COL and the highly virulent USA300 strain. BSH promotes survival inside murine macrophages and neutrophils in human whole-blood infection assays (Posada et al. 2014; Pöther et al. 2013; Rajkarnikar et al. 2013). Moreover, *S. aureus* isolates of the NCTC8325-4 lineage evolved mutations in the bshC gene, leading to the lack of BSH synthesis (Newton et al. 2012;...

![Figure 3: Bacillithiol functions in detoxification, antibiotics resistance and virulence in *S. aureus*. BSH is involved in detoxification of ROS, RES, HOCl, RSS and confers resistance to the redox active antibiotics fosfomycin, allicin, lapachol and AGXX® in *S. aureus*.](image-url)
Pöther et al. 2013). Thus, the *S. aureus* strain SH1000 of the NCTC8325-4 lineage was impaired in survival in infection assays compared to the SH1000 *bshC* repaired strain (Pöther et al. 2013). Interestingly, the *S. aureus bsha*, *ypdA* and *brxAB* mutants showed similar defects in survival inside *J774.1* macrophages and neutrophils, indicating that the Brx/Bsh/YpdA/NADPH system is essential under infections to regenerate BSH and protein thiols (Linzner et al. 2019; Mikheyeva et al. 2019; Pöther et al. 2013; Posada et al. 2014). Future studies should investigate the physiological role of BSH and the Brx/Bsh/YpdA/NADPH redox pathway in *S. aureus* in murine infection models with defects in the NADPH oxidoase and myeloperoxidase to elucidate if BSH homeostasis provides protection against the oxidative burst of neutrophils and macrophages.

Phenotype analyses of BSH-deficient mutants revealed important functions of BSH in the defense against ROS, HOCl, electrophiles, metals, xenobiotics, fosfomycin, rifampicin, AGXX®, allicin and lapachol in *S. aureus* (Figure 3) (Chandrangsu et al. 2018; Linzner et al. 2020; Loi et al., 2018a, 2019).

BSH is an important cofactor for the thiol-S-transferase FosB, which confers fosfomycin resistance. FosB catalyzes the ring cleavage of fosfomycin leading to formation of the BS-fosfomycin-conjugate (Figure 3) (Chandrangsu et al. 2018; Roberts et al. 2013). Both *fosB* and *bsha* mutants were similarly susceptible under fosfomycin stress in *S. aureus*, supporting the function of FosB as a BSH-dependent thiol-S-transferase (Posada et al. 2014; Rajkarnikar et al. 2013). In addition, the BSH-dependent S-transferase BstA was shown to conjugate BSH to electrophiles, such as 1-chloro-2,4-dinitrobenzene, monobromobimane and the antibiotic cerulenin in *S. aureus* (Newton et al., 2011, 2012; Perera et al. 2019). BstA belongs to the widely conserved DinB/YBT superfamily of S-transferases in BSH-, MSH- and GSH-producing bacteria (Newton et al. 2011; Perera et al. 2014). The resulting BS-electrophile conjugates are cleaved by the BSH S-conjugate amidase BshB to GlcN-Mal and mercapturic acids (CysSR), which are subsequently exported (Figure 3) (Newton et al. 2011). Thus, the thiol-S-transferases FosB and BstA are important determinants of antibiotics resistance in *S. aureus*.

Additionally, BSH and the Brx/Bsh/YpdA/NADPH pathway were shown to confer protection against the naphthoquinone lapachol in *S. aureus* (Linzner et al. 2020). Lapachol acts via the oxidative mode, resulting in ROS formation, which leads to increased thiol oxidations and an oxidative shift in the *E*$_{\text{BSH}}$ in *S. aureus*. Thus, the *bsha*, *brxAB* and *ypdA* mutants were more sensitive in growth and survival assays under lapachol treatment, indicating that the Brx/Bsh/YpdA/NADPH pathway is essential to restore thiol homeostasis under lapachol stress (Linzner et al. 2020).

Similarly, BSH was shown to promote resistance to the antimicrobial surface coating AGXX® (Largentech GmbH, Berlin), which is composed of Ag' and Ru' ions and can be electroplated on medical devices and implants (Clauss-Lendzian et al. 2018; Gupta et al. 1999; Guridi et al. 2015; Heiss et al. 2017; Loi et al. 2018a; Vaishampayan et al. 2018). The Ag' and Ru' ions form a micro-galvanic cell, resulting in H$_2$O$_2$ and OH• generation due to the electric field. In support of ROS production, AGXX® caused strong oxidative, quinone and metal stress responses in the *S. aureus* transcriptome (Loi et al. 2018a). AGXX® induced an oxidative shift in the *E*$_{\text{BSH}}$ and protein S-bacillithiolation of GapDH. The *bsha* mutant was strongly impaired in growth and survival under AGXX® stress, indicating an important role of BSH in protection against ROS generated by AGXX® (Loi et al. 2018a). Thus, BSH is a beneficial antioxidant in *S. aureus*, to ensure survival during host-pathogen interactions and under ROS-producing antimicrobials.

Furthermore, BSH functions in detoxification of RSS and RNS in *S. aureus*, such as H$_2$S and nitroxyln (HNO), the latter being an intermediate of NO• and H$_2$S (Peng et al. 2017a,b). H$_2$S caused widespread persulfidations in the proteome and thiol metabolome of *S. aureus* as revealed by elevated amounts of BSH persulfide (BSSSH), CoASH persulfide (CoASSH) and cysteine persulfide (CysSSH) after exposure to Na$_2$S and HNO (Figure 3) (Peng et al. 2017a,b). Among the targets for persulfidations are the glycolytic GapDH and the major virulence regulator MgrA, which both were redox-controlled and inactivated by persulfidations of the active site Cys residues. The reversal of persulfidations was controlled by the novel thioredoxins TrxP and TrxQ (Peng et al. 2017b; Peng et al. 2017).

In addition, BSH is crucial for resistance towards alliin and polysulfanes from garlic in *S. aureus* and *Bacillus subtilis* (Borlinghaus et al. 2014; Chi et al. 2019; Loi et al. 2019). The diallylthiosulfinate alliin decomposes upon heating to diallyl polysulfanes with up to seven sulfur chains (Münchberg et al. 2007; Tocmo et al. 2017). Allicin and diallyl tetrasulfane (DAS4) caused a strong oxidative and sulfur stress response in the transcriptome and widespread S-thioallylations of redox sensitive enzymes and transcriptional regulators in the proteome (Figure 2) (Chi et al. 2019; Loi et al. 2019). BSH-deficient mutants of *S. aureus* and *B. subtilis* are strongly impaired in growth and survival under allicin and DAS4 treatment (Arbach et al. 2019; Chi et al. 2019; Loi et al. 2019). In *S. aureus*, allicin caused a strong oxidative shift in the *E*$_{\text{BSH}}$, which is caused by BSH depletion and formation of S-allylmercaptobacillithiol (BSSA) (Figure 3). BSSA was identified as...
Redox regulation of proteins by protein S-bacillithiolation

During infections, *S. aureus* encounters the highly bacill-driven HOCl as part of the oxidative burst by activated neutrophils (Winterbourn and Kettle 2013; Winterbourn et al. 2016). HOCl stress leads to formation of BSH mixed disulfides, termed as protein S-bacillithiolations, which are widespread across *Bacillus* and *Staphylococcus* species (Chi et al., 2011, 2013; Lee et al. 2007). These S-bacillithiolated proteins include metabolic and antioxidant enzymes, translation factors and redox sensing regulators, which respond to oxidative stress (Figure 4) (Chi et al., 2011, 2013; Imber et al., 2018a, 2019). S-bacillithiolation was proposed to function in redox regulation of HOCl-specific transcription factors and to prevent irreversible overoxidation of vulnerable Cys thiols to sulfonic acids (Imber et al. 2019). In *B. subtilis*, the OhrR repressor was shown to sense HOCl and organic hydroperoxides (OHP) by S-bacillithiolation at its lone Cys15 residue, resulting in expression of the OhrA peroxidoredoxin, which confers OHP and HOCl resistance (Chi et al. 2011; Lee et al. 2007).

In *S. aureus*, two OhrR homologs SarZ and MgrA are important virulence and antibiotics resistance regulators and share the conserved N-terminal Cys residue of the OhrR family, which senses oxidative stress via thiol oxidation (Figure 4) (Chen et al. 2011; Hillion and Antelmann 2015). The crystal structure of SarZ was resolved with Cys13 in the reduced, Cys-SOH and S-thiolated form with benzene thiol (Poor et al. 2009). Only the S-thiolated SarZ resulted in conformational changes in the DNA-binding helix-turn-helix motif, resulting in dissociation of SarZ from the target gene promoter (Poor et al. 2009). Increased thiol oxidation of MgrA and SarZ was found under HOCl stress in the redox proteome of *S. aureus*, supporting the hypothesis that both might be redox-controlled by S-bacillithiolation in vivo (Imber et al. 2018a). Furthermore, MgrA was regulated by persulfidation under sulfide stress, which impacts virulence factor secretion in the secretome of *S. aureus* (Peng et al. 2017b; Peng et al. 2017).

Additionally, conserved S-bacillithiolated proteins were identified as metabolic enzymes with redox active catalytic centers, such as the methionine synthase MetE, the inosine monophosphate (IMP) dehydrogenase GuaB, the glyceraldehyde 3-phosphate dehydrogenase GapDH and the aldehyde dehydrogenase AldA (Figure 4) (Chi et al. 2011; Imber et al., 2018a, 2019). MetE represents the most abundant S-bacillithiolated protein in *B. subtilis* cells, which forms the BSH mixed disulfide at Cys730 in the active site Zn\(^{2+}\) center, resulting in inactivation of the enzyme and methionine auxotrophy under HOCl stress. Moreover, the enzymes SerA, PpaC, MetI and YxjG were S-bacillithiolated under HOCl stress, which act in the same pathway as MetE (Chi et al. 2011; Imber et al., 2018a, 2019).

The glycolytic GapDH displayed 29% increased thiol oxidation under HOCl stress and was the most abundant S-bacillithiolated protein in *S. aureus* cells, representing 49% of the total Cys proteome (Imber et al., 2018a). GapDH S-bacillithiolation occurs under oxidative stress at the active site Cys151, resulting in enzyme inactivation and metabolic switching from glycolysis to the pentose phosphate...
Interestingly, GapDH inactivation by pathway (Deng et al. 2013; Imber et al. 2018a). Interestingly, GapDH inactivation by S-bacillithiolation proceeds faster compared to its inhibition by overoxidation in the absence of BSH in vitro (Imber et al. 2018a). Thus, S-bacillithiolation functions in redox regulation of GapDH activity and can prevent overoxidation of the active site (Imber et al. 2018a). Using molecular docking, BSH was modeled into the Cys151 active site of the apo- and holoenzymes. Interestingly, BSH occupies two different positions in the GapDH active site depending on the NAD⁺ cofactor. While the BSH moiety is located in the active site with Cys151 in the attacking state position in the holoenzyme, S-bacillithiolation of the apoenzyme occurs with Cys151 in the resting state in the absence of the NAD⁺ cofactor (Imber et al., 2018a,2019). However, S-bacillithiolation of GapDH did not cause major structural changes.

Similarly, the NAD⁺-dependent aldehyde dehydrogenase AldA showed 29% increased thiol oxidation at its active site Cys279 and was inactivated by S-bacillithiolation under HOCl stress in S. aureus (Imber et al. 2018a,b). Computational chemistry revealed similar attacking and resting state positions of Cys279 upon S-bacillithiolation of the AldA holo- and apoenzymes, respectively, as shown for GapDH. In addition, S-bacillithiolation of AldA elicits no major structural and conformational changes (Imber et al., 2018b,2019). AldA was shown to catalyze oxidation of formaldehyde, methylglyoxal, acetaldehyde and glycolaldehyde in vitro, but its physiological substrate in vivo is unknown. Transcription of aldA is elevated under oxidative and electrophile stress, such as formaldehyde, methylglyoxal, HOCl, allicin and AGXX®, and depends on an unknown redox regulator. Phenotype analyses revealed that AldA is required for survival under HOCl stress, but dispensable under aldehyde stress. Thus, the physiological role of AldA under HOCl stress and its regulation remain to be elucidated in future studies (Imber et al. 2018b).

Apart from GapDH and AldA, GuaB is another NAD⁺-containing enzyme with a highly conserved Cys308 active site that forms an adduct with the IMP substrate. GuaB is the most conserved target for S-glutathionylation, S-bacillithiolation and S-mycotothiolation across prokaryotes and eukaryotes (Imber et al. 2019; Loi et al. 2015). Based on the different conformations of Cys308 in the substrate bound enzyme or in the apoenzyme structures, the BSH moiety may adopt a similar position in the GuaB active site with Cys308 in the attacking or resting state, respectively, as shown for GapDH and AldA (Imber et al., 2018a,b,2019). Altogether, S-bacillithiolations were shown to occur at accessible active site Cys residues of redox sensitive metabolic enzymes or transcriptional regulators. While S-bacillithiolation of GapDH and AldA does not lead to conformational changes, major structural rearrangements in the DNA binding HTH motif are required to accommodate redox regulation of the 1-Cys type OhrR-family repressors.

**Reversal of protein S-bacillithiolation by the Brx/BSH/YpdA/NADPH pathway**

The bacilliredoxins BrxA, BrxB and BrxC were previously identified as S-bacillithiolated in B. subtilis and Staphylococcus carnosus under HOCl stress, which suggested their role in the reversal of protein S-bacillithiolations (Chi et al. 2013).
BrxA and BrxB are paralogs of the DUF1094 family with a Trx fold and a CGC active site motif, which co-occur together with the BSH synthesis enzymes in BSH-producing bacteria (Gaballa et al. 2014). However, the redox potential of BrxA is relatively positive with −130 mV, and rather in the range of disulfide isomerases (Derewenda et al. 2009). BrxC has a TCPIS active site motif suggesting its possible function as monothiol Brx.

The functions of BrxA and BrxB as bacilliredoxins in reduction of S-bacillithiolated proteins have been first demonstrated for the S-bacillithiolated OhrR and MetE proteins in B. subtilis (Gaballa et al. 2014). DNA binding assays revealed the reactivation of OhrR after de-bacillithiolation of OhrR-SSB with the BrxB resolving Cys mutant protein, but not with the BrxB active site mutant. The removal of BSH from S-bacillithiolated MetE in B. subtilis cell extracts was shown with both BrxA and BrxB using BSH specific Western blots and mass spectrometry (Gaballa et al. 2014). In S. aureus, Brx activity assays were conducted with purified GapDH as most abundant S-bacillithiolated protein in vivo (Imber et al. 2018a). The S. aureus BrxA homolog catalyzed the de-bacillithiolation of GapDH, as shown by reactivation of the glycolytic activity of GapDH to oxidize the glyeraldehyde 3-phosphate substrate to 1,3-bis-phosphoglycerate with generation of NADH. While the BrxA resolving Cys mutant could restore GapDH activity, the BrxA active site mutant did not. The de-bacillithiolation reaction could be monitored using non-reducing BSH specific Western blots (Imber et al. 2018a). These results established the function of BrxA and BrxB in reversal of S-bacillithiolations in vitro.

Furthermore, the flavin disulfide reductase YpdA was phylogenetically associated with the BSH biosynthesis enzymes and bacilliredoxins (Gaballa et al. 2010). Transcriptome analyses revealed increased transcription of ypdA, brxA, brxB, bshA, bshB and bshC under oxidative and electrophile stress, such as H2O2, HOCl, diamide, AGXX®, lapachol, allcin and azuerothil granule proteins in S. aureus (Linzner et al. 2020; Loi et al., 2018a,b,2019; Palazzolo-Ballance et al. 2008). These data provide evidence for a functional Brx/BSH/YpdA/NADPH redox cycle in S. aureus (Figure 3), which is co-regulated under redox stress conditions. YpdA was characterized as a NADPH-dependent BSSB reductase in S. aureus to regenerate BSH homeostasis during recovery from oxidative stress and under infections (Linzner et al. 2019; Mikheyeva et al. 2019). Moreover, the S. aureus brxA, bshA and ypdA mutants were more sensitive in growth and survival under HOCl stress and in macrophage infection assays in vivo (Linzner et al. 2019; Mikheyeva et al. 2019; Pöther et al. 2013; Posada et al. 2014). In addition, the ypdA mutant showed a 2-fold increased basal level of BSSB compared to the wild type (WT). Overproduction of YpdA, in turn, resulted in higher BSH levels (Mikheyeva et al. 2019). While the basal BSH levels of the S. aureus COL WT and the ypdA mutant were determined in a similar range of 1.5–1.9 µmol/g raw dry weight (rdw), the basal BSSB levels were measured as −0.05 µmol/g and −0.09 µg rdw in WT and ypdA mutant cells, respectively (Linzner et al. 2019). Thus, the BSH/BSSB ratio was determined as 35:1 for the WT and decreased to 17:1 in the ypdA mutant under non-stress conditions. Based on the high BSH levels in both WT and ypdA mutant strains, the basal $E_{BSH}$ was not affected under non-stress conditions along the growth curve as measured using the Brx-roGFP2 biosensor, which is described in more detail in the following section (Linzner et al. 2019). However, due to the increased BSSB levels, the ypdA mutant was impaired to regenerate the reduced $E_{BSH}$ upon recovery from oxidative stress, supporting the function of YpdA as BSSB reductase especially under oxidative stress in S. aureus (Linzner et al. 2019; Mikheyeva et al. 2019).

These results confirm previous findings, which revealed unchanged cytosolic GSH levels, but increased GSSG amounts in a yeast mutant deficient for the glutathione di-sulfide reductase Glr (Morgan et al., 2011,2013). Grx-roGFP2 biosensor measurements showed that the basal GSH redox potential ($E_{GSH}$) was only slightly increased by −20 mV in the yeast grl mutant, whereas the H2O2 response was much stronger in the grl mutant with an impaired recovery of the reduced state (Morgan et al. 2011). It was further shown that Gsx2 and Trx2 can compensate for GSSG reduction in the absence of Glr (Morgan et al. 2013). However, in S. aureus BrxA did not show BSSB reductase activity in vitro and the BSSB levels were not affected in the brxA mutant, indicating that BrxA has been described as a novel active site for ypdA in regeneration of BSH (Linzner et al. 2019; Mikheyeva et al. 2019).

YpdA is a flavin disulfide reductase, which contains a conserved Cys14 residue in the glycine-rich Rossmann-fold NADPH binding domain (GGPGC14G) (Bragg et al. 1997; Mikheyeva et al. 2019). Using NADPH coupled electron transfer assays, we demonstrated that YpdA consumes NADPH in the presence of BSSB, but not with other LMW thiol disulfides, such as GSSG, cystine or CoAS2, confirming its function as BSSB reductase in S. aureus in vitro (Linzner et al. 2019). The BSSB reductase activity of YpdA was dependent on the conserved Cys14, which is unique in YpdA homologs and represents a novel active site. In support of its active site function, Cys14 of YpdA was identified as HOCl-sensitive with >10% increased oxidation using the redox proteomics approach OxiCAT in S. aureus (Imber et al. 2018a). Thus, Cys14 might be S-bacillithiolated by BSSB, and regenerated by electron transfer from NADPH.
via the FAD co-factor. Moreover, YpdA works in concert with Brx and BSH to establish a functional Brx/BSH/YpdA/NADPH redox pathway for reduction of S-bacillithiolated proteins in \textit{S. aureus}. In biochemical assays with the complete Brx/BSH/YpdA/NADPH pathway, Brx was sufficient for the complete de-bacillithiolation of GapDH. In contrast, YpdA cannot regenerate S-bacillithiolated proteins, but functions instead in BSSB reduction to complete the Brx/BSH/YpdA/NADPH cycle (Linzner et al. 2019). In this redox cycle, the Brx active site Cys attacks the S-bacillithiolated protein, resulting in Brx-SSB formation (Gaballa et al. 2014). Brx-SSB is resolved with BSH, leading to BSSB formation, which is recycled by YpdA to BSH (Linzner et al. 2019). However, structural analyses are required to resolve the detailed catalytic mechanism of YpdA in BSSB reduction. Future studies should be directed to identify the substrates of the Brx/BSH/YpdA/NADPH pathway under infection conditions inside host cells, which promote virulence, survival and persistence of \textit{S. aureus} inside the host.

\section*{Monitoring the changes in the $E_{\text{BSH}}$ using the Brx-roGFP2 biosensor}

To monitor $E_{\text{BSH}}$ changes inside \textit{S. aureus}, BrxA were fused to the redox sensitive green fluorescent protein (roGFP2), generating a genetically encoded Brx-roGFP2 biosensor (Loi et al. 2017; Loi and Antelmann 2020). Upon oxidation, the two Cys residues in roGFP2 form a disulfide, influencing the spectral properties of roGFP2 (Meyer and Dick 2010; Schwarzländer et al. 2016). Specifically, the 488 nm excitation maximum is decreased and the 405 nm maximum increased, resulting in ratiometric changes in the 405/488 excitation ratio, which is quantified as oxidation degree (OxD). The fusion of Brx to roGFP2 facilitates the equilibration of roGFP2 with the BSH/BSSB redox pair enabling specific measurements of the $E_{\text{BSH}}$ in \textit{S. aureus} (Loi and Antelmann 2020; Loi et al. 2017; Meyer and Dick 2010; Schwarzländer et al. 2016). ROS exposure causes oxidation of cellular BSH to BSSB, which reacts with the Brx active site Cys of the Brx-roGFP2 probe, leading to Brx-SSB formation. The BSH moiety of Brx-SSB is transferred to the coupled roGFP2, which rearranges to the roGFP2 disulfide resulting in ratiometric changes of the excitation spectrum (Figure 5). Assuming that roGFP2 and the BSH/BSSB pair are in equilibrium, $E_{\text{BSH}}$ is equal to the calculated $E_{\text{roGFP2}}$. Consequently, $E_{\text{BSH}}$ can be calculated based on the OxD values of the Brx-roGFP2 biosensor and the previously determined $E_{\text{roGFP2}}$ of $-280$ mV (Dooley et al. 2004), according to the Nernst equation as described (Loi et al. 2017; Morgan et al. 2011).

We have used the Brx-roGFP2 biosensor to monitor the changes in the $E_{\text{BSH}}$ during the growth, under oxidative stress and antibiotics treatments in \textit{S. aureus} isolates and in different mutant backgrounds (Loi and Antelmann 2020; Loi et al. 2017). First, purified Brx-roGFP2 was shown to respond fast and specific to low levels of 10–100 $\mu$M BSSB, but not to other LMW disulfides \textit{in vitro}. The changes in $E_{\text{BSH}}$ were determined in \textit{S. aureus} COL bshA, brxAB and ypdA mutants along the growth and under oxidative stress to investigate the impact of the Brx/BSH/YpdA/NADPH pathway on the basal $E_{\text{BSH}}$ and in response to oxidative stress (Linzner et al. 2019; Loi et al. 2017). Brx-roGFP2 measurements along the growth revealed a highly reducing basal level $E_{\text{BSH}}$ in the range from $-282$ to $-295$ mV in the \textit{S. aureus} COL WT, which was similar in the ypdA and brxAB mutants (Linzner et al. 2019; Loi et al. 2017). However, in the bshA mutant the biosensor was fully oxidized (Figure 5) (Loi et al. 2017). This indicates an impaired redox balance in the BSH-deficient strain, which might be caused by ROS increase. Interestingly, the ypdA mutant showed a strong Brx-roGFP2 oxidation after exposure to HOCI and H$_2$O$_2$, but was unable to regenerate the reduced state of $E_{\text{BSH}}$ during recovery from oxidative stress due to its higher BSSB levels (Figure 5) (Linzner et al. 2019). In addition, the ypdA mutant was impaired in H$_2$O$_2$ detoxification as measured using the Tpx-roGFP2 biosensor (Linzner et al. 2019). These results confirmed the function of YpdA to regenerate reduced BSH after oxidative stress.

In addition, Brx-roGFP2 oxidation was analyzed after treatment of \textit{S. aureus} COL WT with oxidants, different antibiotics and ROS-producing antimicrobials to investigate $E_{\text{BSH}}$ changes (Loi et al., 2017,2018a, 2019). Brx-roGFP2 responds very fast to low levels of 50–100 $\mu$M HOCI, resulting in complete oxidation and slow recovery of the reduced $E_{\text{BSH}}$ in \textit{S. aureus} COL. Due to the high level of H$_2$O$_2$ resistance, Brx-roGFP2 was less reactive to 100 mM H$_2$O$_2$ and the \textit{S. aureus} cells were able to recover faster their reduced $E_{\text{BSH}}$ (Loi et al. 2017). Determination of the $E_{\text{BSH}}$ changes of \textit{S. aureus} COL after infection inside THP-1 macrophages revealed about 87% oxidation after 1 h, supporting that \textit{S. aureus} experiences the oxidative burst (Loi et al. 2017). However, Brx-roGFP2 did not respond to sub-lethal concentrations of different antibiotics classes, such as rifampicin, fosfomycin, ampicillin, oxacillin, vancomycin, aminoglycosides and fluoroquinolones in \textit{S. aureus} log phase cells (Loi et al. 2017).

In contrast, the Brx-roGFP2 biosensor was highly oxidized by the redox active antimicrobials AGXX®, allicin and lapachol resulting in an oxidative shift of $E_{\text{BSH}}$ in \textit{S. aureus} (Loi et al., 2018a, 2019, Linzner et al. 2020). Exposure of \textit{S. aureus} COL to sub-lethal concentrations of
AGXX® leads to strong biosensor oxidation, which is caused by OH· and other ROS generated by AGXX® (Clauss-Lendzian et al. 2018; Loi et al. 2018a). Allicin causes S-thio-allylations of BSH, resulting in BSSA formation, which oxidizes Brx-roGFP2 similar as BSSB (Loi et al. 2019). Thus, 50–100 µM allicin lead to fast and complete biosensor oxidation within 10 min in S. aureus, which was reversible with DTT, supporting the strong thiol reactivity of allicin (Figure 5) (Loi et al. 2019). The response of the Brx-roGFP2 biosensor was different for the naphthoquinone lapachol, which acts via the redox cycling mode in S. aureus (Linzner et al. 2020). Upon exposure to 100 µM lapachol, Brx-roGFP2 oxidation was slowly increased within 2 h with no recovery of reduced E\textsubscript{BSH} (Linzner et al. 2020). This indicates constant generation of H₂O₂ by lapachol as measured with the Tpx-roGFP2 biosensor. In conclusion, Brx-roGFP2 is a valuable tool to screen for ROS-production and intracellular redox potential changes by redox active antibiotics in S. aureus.

**Redox regulation of proteins by protein S-CoAlation**

S. aureus produces millimolar CoASH and encodes a CoASH disulfide reductase Cdr, which reduces CoASH disulfide (CoAS₂) back to CoASH in vitro (delCardayre and Davies 1998,1998). Thus, before the discovery of BSH, CoASH was proposed to function as LMW thiol in S. aureus (delCardayre and Davies 1998; Tsuchiya et al. 2018). CoASH is an important thiol cofactor, which activates sugar metabolites of the central carbon catabolism by formation of energy-rich CoA thioesters, such as acetyl-CoA and succinyl-CoA to ensure ATP generation (Jackowski and Rock 1986). Recently, evidence was provided that CoASH can function in redox modification of proteins via S-CoAlations, which may substitute for S-bacillithiolation in the absence of BSH in S. aureus (Gout 2019; Tsuchiya et al. 2018). S-CoAlated proteins were enriched using pulldown with monoclonal anti-CoASH antibodies and identified by mass spectrometry (Gout 2019; Tsuchiya et al. 2018). In S. aureus, about 356 S-CoAlated proteins were identified under diamide stress, including conserved redox sensitive metabolic and antioxidant enzymes (GapDH, AldA, GuaB, Trx, AhpC, Tpx) and transcriptional regulators (SarR, CtsR, AgrA, PerR, SarS), which harbor active site Cys residues and overlap with S-bacillithiolated proteins under HOCl stress (Gout 2019; Tsuchiya et al. 2018).

Furthermore, protein S-CoAlation of the active site Cys151 of GapDH resulted in enzyme inactivation, which was reversible by DTT reduction (Tsuchiya et al. 2018). Computational chemistry proposed that the ADP moiety of CoASH occupies the NAD⁺ binding pocket in the apoenzyme, facilitating S-CoAlation of Cys151 of GapDH (Tsuchiya et al. 2018). However, the physiological role of S-CoAlation in relation to sugar catabolism remains to be elucidated in S. aureus. Based on its metabolic role, enzymes involved in the activation of pyruvate and succinate by CoASH, such as pyruvate dehydrogenase (Pdh) and
Thiol-based and other redox switches in *S. aureus*  

**PerR as Fur-family metal-based peroxide sensor**

The peroxide specific PerR repressor belongs to the Fur family of metalloregulators and was characterized in *B. subtilis* and *S. aureus* (Faulkner and Helmann 2011; Pinochet-Barros and Helmann 2018). PerR negatively controls the adaptive response to H₂O₂ and confers H₂O₂ resistance in both bacteria (Faulkner and Helmann 2011; Horsburgh et al. 2001a; Ji et al. 2015; Mongkolsuk and Helmann 2002). The members of the PerR regulon function in ROS detoxification and iron storage, including genes for catalase, peroxiredoxins and thioredoxin reductase (*katA, ahpCF, bcp, trxB*), the iron storage ferritin and miniferritin (*ftnA, dps*), the ferric uptake (*fur*) repressor and the FeS cluster machinery (*sufCDSUB*) in *S. aureus* (Figure 6) (Horsburgh et al. 2001a). PerR binds to a conserved inverted repeat sequence ATTATAATTATAAT in the promoter region of the PerR regulon genes (Horsburgh et al. 2001a). In *S. aureus*, PerR is required for virulence in murine skin abscess and *Caenorhabditis elegans* infection models. In contrast, the catalase KatA is dispensable for pathogenicity (Ji et al. 2015; Horsburgh et al. 2001a).

The structures of *B. subtilis* and *S. aureus* PerR proteins contain two metal binding sites each, a structural Zn²⁺ site coordinated by four Cys residues and the regulatory Fe²⁺ or Mn²⁺ site, which includes His and Asp residues (Faulkner and Helmann 2011; Pinochet-Barros and Helmann 2018). The PerR repressors sense H₂O₂ by metal-catalyzed histidine oxidation in the regulatory Fe²⁺ binding sites, leading to their inactivation and derepression of transcription of the PerR regulons in *B. subtilis* and *S. aureus* (Ji et al. 2015; Lee and Helmann 2006; Pinochet-Barros and Helmann 2018). While the His and Asp residues in the regulatory site can bind both Fe²⁺ or Mn²⁺ as corepressors, only the Fe²⁺ bound PerR can sense H₂O₂ by iron-catalyzed His oxidation. Specifically, Fe²⁺ in the regulatory site is oxidized by H₂O₂ in a Fenton reaction to generate OH⁺, leading to oxidation of His37 or His91 to form 2-oxo-histidine in the

**Figure 6:** Redox regulation of the PerR repressor by iron-catalyzed His oxidation in response to H₂O₂ and by a putative thiol switch under HOCl stress in *S. aureus*. PerR senses H₂O₂ by Fe²⁺ catalyzed oxidation in the regulatory Asp/His site, leading to HO• formation and His37/His91 oxidation to 2-oxo-histidines, resulting in derepression of the PerR regulon (Ji et al. 2015; Lee and Helmann 2006). HOCl treatment leads most likely to PerR inactivation by a thiol switch in the structural Zn²⁺ site as identified in *Bacillus subtilis* cells using mass spectrometry *in vivo* (Chi et al. 2011). PerR controls genes encoding catalase, peroxiredoxins and thioredoxin reductase (*katA, ahpCF, bcp, trxB*), the ferric uptake (*fur*) repressor and the FeS cluster machinery (*sufCDSUB*) and the ferric uptake (*fur*) repressor in *S. aureus*. This figure is adapted from (Hilion and Antelmann 2015).
stress resistance, including the Multiple gene regulator MgrA, SarZ and the Staphylococcal accessory regulator SarA (Ballal et al. 2009; Chen et al. 2009; Kaito et al. 2006; Poor et al. 2009; Truong-Bolduc et al. 2005, 2008). MgrA and SarZ belong to the MarR/OhrR-family, which sense ROS by thiol switch mechanisms via a conserved single Cys residue (Chen et al. 2011). MgrA is a global virulence regulator, which controls expression of >300 genes, involved in virulence, autolysis, antibiotics resistance, capsule and biofilm formation (Luong et al. 2006). Among the MgrA regulon genes are α-toxin (hla), coagulase (coa), protein A (spa), large surface proteins (ebp, sarP and sasG), fibrinogen-binding protein (fnb), extracellular protease, nuclease (nuc), autolysins (lytM and lytN), multidrug efflux pumps (norA, norB and tetAB), virulence regulators (agr, lytRS, arlRS, sarS and sarV) and capsule biosynthesis (cap5) (Ingavale et al. 2005; Kaatz et al. 2005; Luong et al. 2006; Truong-Bolduc et al. 2005, 2008). In DNease-I footprinting experiments, purified MgrA protein was shown to protect the conserved nucleotide sequence (A/T)GGTGT, which was repeated thrice upstream of the sarV promoter (Crosby et al. 2016; Manna et al. 2004). MgrA is important for virulence of S. aureus as shown in several infection models, including murine abscess, septic arthritis, sepsis, murine bacteremia and endocarditis models (Chen et al. 2006; Jonsson et al. 2008; Li et al. 2019).

The second MarR/OhrR-type regulator SarZ regulates a large regulon involved in virulence, antibiotic resistance, cellular metabolism and the ohr peroxiredoxin as main target gene (Kaito et al. 2006). Purified SarZ was shown to bind rather non-specific to the promoter regions of hla, asp23 and agr, since a specific SarZ recognition motif could not be identified (Kaito et al. 2006). Both MgrA and SarZ sense oxidative stress by thiol switches, which involve the conserved redox sensing Cys13 residues. Thiol oxidation of MgrA and SarZ by organic hydroperoxides and H₂O₂ leads to formation of the Cys13-SOH (Chen et al., 2006, 2009; Poor et al. 2009; Truong-Bolduc et al. 2005, 2008). Further oxidation of SarZ with the synthetic redox sensor of HOCl stress

HypR as Rrf2-family redox sensor of HOCl stress

The Rrf2-family HypR repressor was identified as a novel redox sensor of HOCl stress in S. aureus, which uses a thiol switch mechanism (Loi et al. 2018b). Rrf2-family transcription factors are widespread in prokaryotes and regulate diverse functions, such as FeS cluster biogenesis (IscR), cysteine biosynthesis (CymR) and NO+ detoxification (NsrR, RsrR) (Karlinsey et al. 2012; Mettert and Kiley 2015; Nakano et al. 2014; Partridge et al. 2009; Remes et al. 2015; Soutourina et al. 2009, 2010). HypR senses strong oxidants, such as HOCl, diamide, AGXX® and allicin stress (Loi et al. 2018a,b, 2019). HypR negatively controls expression of the hypR-merA operon, which was most strongly upregulated under HOCl stress in the transcriptome (Loi et al. 2018b) (Figure 7). In addition, the hypR-merA operon was highly induced in S. aureus during phagocytosis assays with neutrophils (Voyich et al. 2005), suggesting important functions as HOCl defense mechanism. The HypR repressor was shown to bind to a 12-3-12 bp inverted repeat sequence TAATGTGACTA-TATAAT in the hypR-merA promoter region, which is conserved across staphylococci.

HypR controls the NADPH-dependent flavin disulfide reductase MerA, which confers resistance towards HOCl, allicin and AGXX® stress as well as under infection conditions inside murine macrophages J774A.1 (Loi et al. 2018a,b, 2019). We hypothesize that MerA could be involved in HOCl reduction to promote survival under infections inside macrophages and neutrophils. In addition, the coupled transcription in the hypR-merA operon suggests that MerA could be the redox partner of HypR to recycle oxidized HypR during recovery from oxidative stress. The detailed functional analyses of MerA in terms of HOCl resistance are important goals of our current research.
The HypR structure contains three Cys residues, including Cys33, Cys99 and Cys142, but only Cys99 is conserved in Rpf2 homologs (Loi et al. 2018b). While Cys33 is essential for redox sensing of HOCl stress, Cys99 is required for DNA binding activity of HypR in S. aureus. Under HOCl stress, HypR is oxidized to intersubunit disulfides between Cys33 and Cys99′ of opposing subunits in the HypR dimer, resulting in inhibition of its repressor activity and derepression of transcription of the hypR-merA operon (Loi et al. 2018b). Thus, the thiol switch model of HypR resembles that of a typical 2-Cys type redox sensing regulator. Future studies are directed to elucidate the structural changes of HypR upon oxidation, leading to its inactivation.

**The MarR-family regulators QsrR and MhqR sense quinones**

S. aureus and B. subtilis both encode two quinone-sensing MarR-type repressors, including the redox sensing MarR/DUF24-family regulator QsrR (or YodB) and the MarR-type repressor MhqR (Figure 8) (Chi et al. 2010; Fritsch et al. 2019; Ji et al. 2013; Leelakriangsak et al. 2008; Töwe et al. 2007). QsrR controls genes involved in the detoxification of quinones, such as ring-cleavage dioxygenases (catE, catE2), quinone and nitro reductases (azoRI, frp, and yodC) (Fritsch et al. 2019; Ji et al. 2013). A conserved inverted repeat with the consensus GTATAN5TATAC was identified as the QsrR operator in the promoter region of the QsrR regulon genes (Ji et al. 2013). The MhqR repressor negatively controls transcription of the mhqRED operon, encoding the dioxygenase MhqE and the phospholipase/carboxylesterase MhqD. MhqR was shown to bind to a 9-9 bp inverted repeat sequence TATCTCGAA-aTCGAaATA in the promoter region upstream of mhqR (Fritsch et al. 2019). The quinone and nitroreductases (AzoRI, Frp, YodC) function in quinone reduction to redox stable hydroquinones (Figure 8) (Antelmann et al. 2008). Since benzoquinone alkylates protein and LMW thiols (Liebeke et al. 2008), the dioxygenases CatE, CatE2 and MhqE might catalyze ring cleavage of hydroquinones and quinone-S-adducts (Tam le et al. 2006).

In S. aureus and B. subtilis, the MhqR and QsrR regulons are most strongly upregulated by methylhydroquinone (MHQ) in the transcriptomes and contribute independently to quinone resistance (Fritsch et al. 2019; Ji et al. 2013; Leelakriangsak et al. 2008; Töwe et al. 2007). Interestingly, the MhqR and QsrR regulons of S. aureus confer resistance to antimicrobials with quinone elements, such as pyocyanin, rifampicin and ciprofloxacin (Fritsch et al. 2019; Ji et al. 2013). Pyocyanin is a toxic pigment produced by P. aeruginosa. Thus, MhqR and QsrR provide resistance of S. aureus towards pyocyanin to survive respiratory co-infections with P. aeruginosa in cystic fibrosis patients (Noto et al. 2017).

Furthermore, the MhqR regulon conferred tolerance to lethal H$_2$O$_2$ stress and was important for long-term survival of S. aureus inside macrophages (Fritsch et al. 2019). Similarly, the QsrR regulon mediates resistance to killing by macrophages in infection assays, indicating a crucial role of quinone detoxification pathways for virulence and survival of S. aureus inside host cells (Ji et al. 2013). During infections, the QsrR and MhqR regulons could be involved in detoxification of host-derived catecholamines, which are produced in macrophages and neutrophils to enhance the inflammatory response (Flierl et al. 2007). Infection-relevant electrophilic quinones could further arise in neutrophils from MPO-catalyzed serotonin oxidation to tryptamine-4,5-dione, which might be detoxified by the QsrR and MhqR regulons to enhance survival of S. aureus (Ximenes et al. 2009). Phenotype analyses further revealed an increased respiratory chain activity and higher ATP levels in the S. aureus mhqR mutant, pointing to the physiological role of the MhqR regulon to maintain the respiratory menaquinones in their reduced state (Fritsch...
et al. 2019). In support of this notion, the MhqR regulon of B. subtilis was important for the formation of cell-wall free L-forms, which requires reduced quinones for continued respiration and contributes to survival and resistance (Kawai et al. 2015).

The MhqR repressors of B. subtilis and S. aureus do not use thiol-based mechanisms for redox sensing of quinones (Fritsch et al. 2019; Töwe et al. 2007). Since many MarR-type regulators harbor conserved ligand binding pockets, MhqR might sense quinones by direct binding to a ligand pocket, leading to derepression of the mhqRED operon in S. aureus (Grove 2017; Perera and Grove 2010; Wilkinson and Grove 2006).

In contrast to MhqR, QsrR contains a conserved redox sensing Cys5 and two non-conserved Cys30 and Cys33 residues (Ji et al. 2013). The redox sensing mechanism of QsrR was shown to involve thiol-S-alkylation of the Cys5 residue, resulting in QsrR inactivation (Figure 8) (Ji et al. 2013). The detailed mechanism and structural changes of QsrR upon S-alkylation have been resolved for reduced and menadione-bound QsrR. QsrR alkylation leads to movement and rotation of the α4 and α4′ DNA binding helices from 106 to 117°, which is incompatible with DNA binding and causes dissociation of QsrR from the promoter (Ji et al. 2013). However, this Cys-alkylation model has been resolved with the QsrR Cys30,33 double mutant protein. The B. subtilis YodB repressor was previously shown to sense diamide and quinones via intersubunit disulfide bond formation between the conserved Cys6 and the C-terminal Cys101′ or Cys108′ of opposing subunits in the YodB dimer in vivo (Chi et al. 2010).

Recent crystal structure analyses have resolved two mechanisms for YodB inactivation, the S-alkylation of Cys6 by methyl-p-benzoquinone and the thiol switch between Cys6 and Cys101′ under diamide stress (Lee et al. 2016). Quinones cause S-alkylation at Cys6 with minor structural changes by 3 Å rotations of the α4 and α4′ domains, similar as shown for QsrR (Lee et al. 2016). In contrast, diamide leads to Cys6-Cys101′ intersubunit disulfide formation with large structural rearrangement, leading to complete dissociation from the DNA (Lee et al. 2016). Transcriptome analyses revealed that the QsrR regulon is strongly upregulated by quinones and other redox active compounds, such as MHQ, HOCl, AGXX®, allicin and lapachol in S. aureus (Fritsch et al. 2019; Linzner et al. 2020; Loi et al. 2018a,b, 2019). Thus, the two Cys QsrR repressor most likely senses quinones and strong oxidants by S-alkylation and disulfide formation, respectively, which remains to be elucidated.

It is possible that QsrR/YodB and MhqR sense different quinone-related redox signals, controlling the detoxification/reduction of oxidized quinones and ROS by QsrR/YodB as overarching thiol-based regulator, while MhqR could be additionally involved in trapping and regulation of ring cleavage of reduced and oxidized quinones. QsrR/YodB uses a thiol switch mechanism to respond to ROS,
which oxidize respiratory menaquinones and require quinone detoxification/reduction pathways to restore respiration. MhqR could rather sense accumulating quinones to induce additional quinone detoxification pathways when the QsrR/YodB regulon is overwhelmed. The crosstalk of both systems was confirmed by lower expression of QsrR/YodB regulons in the mhqR mutants and vice versa in *B. subtilis* and *S. aureus* (Fritsch et al. 2019; Lee-lakriangsak et al. 2008; Töwe et al. 2007). The investigation of the kinetics, specificities, crosstalks and reversibilities of both systems are interesting goals of future mechanistic studies.

### The thiol-based redox sensor Spx and its YjbH adapter

The thiol-based redox sensor Spx belongs to the arsene reductase (ArsC) family, representing an unusual transcriptional activator lacking the HTH motif (Nakano et al. 2003, 2005; Zuber 2009). In *S. aureus*, transcription of *spx* is positively regulated by the ArlRS two-component system (Crosby et al. 2020). Spx is activated in response to oxidative stress by thiol oxidation of its CXXC redox switch motif to an intramolecular disulfide bond (Crosby et al. 2020). Spx is activated in response to oxidative stress by thiol oxidation of its CXXC redox switch motif to an intramolecular disulfide bond (Nakano et al. 2005). The Spx disulfide interacts with the α-C-terminal domain of the RNA polymerase and activates transcription of the Spx regulon, which is involved in the thiol redox homeostasis and oxidative stress resistance, including thioredoxin and thioredoxin reductase (*trxA, trxB*) (Figure 9) (Nakano et al. 2003, 2005; Zuber 2004, 2009). Thus, the spx mutant shows increased susceptibility to oxidative, heat and salt stress in *S. aureus* (Pamp et al. 2006; Wang et al. 2010). Furthermore, Spx is involved in *pia*-dependent biofilm formation and controls expression of the ica*ABCD* operon. Spx represses *cspA* transcription, which inhibits the activation of SigB leading indirectly to downregulation of aureolysin (*aur*) and staphyloxanthin biosynthesis (*crtOPQMN* operon) (Austin et al. 2019; Donegan et al. 2019).

The post-translational control involves the proteolytic degradation of Spx by the ClpXP system with the help of the YjbH adapter. Under control conditions, YjbH binds Spx, which is targeted to the ClpXP machinery for proteolytic degradation (Engman et al. 2012; Garg et al. 2009; Pamp et al. 2006). Oxidative stress causes YjbH self-aggregation possibly due to thiol oxidation of its Zn$^{2+}$ redox switch motif leading to Spx stabilization and increased Spx protein levels (Figure 9) (Engman and von Wachenfeldt 2015). Apart from proteolysis, YjbH controls antibiotic resistance in *S. aureus*. The *yjbH* mutant was resistant to β-lactam antibiotics due to increased PBP4 expression and peptidoglycan cross-linking (Gohring et al. 2011). Spx controls the mecA homolog *trfA* in *S. aureus*. While the *trfA* mutant was susceptible to oxacillin and glycopeptide antibiotics, *trfA* upregulation in the *yjbH* mutant conferred antibiotics resistance (Jousselin et al. 2013). In addition, Spx activates the MazEF toxin-antitoxin system, which promotes dormancy and antibiotic tolerance (Panasenko et al. 2020). The lack of the extracellular proteases, such as aureolysin in the *yjbH* mutant led to hypervirulence in a systemic mouse infection model and enhanced colonization of the kidney and the spleen in a murine sepsis model (Austin et al. 2019; Kolar et al. 2013).

### The TetR-family regulator and electrophile sensor GbaA

The TetR-family regulator GbaA (Glucose-induced biofilm accessory protein A) was characterized as a novel thiol switch and monothiol electrophile sensor (Ray et al. 2020). GbaA controls glucose-induced biofilm formation by the polysaccharide intracellular adhesion (PIA), which is composed of poly-N-acetyl glucosamine (PNAG) as biofilm matrix in *S. aureus* (Ray et al. 2020; You et al. 2014; Yu et al. 2018).
GbaA negatively controls two operons of unknown functions, encoding a putative glyoxalase, the NAD-dependent epimerase/dehydratase NmrA, the short chain dehydrogenase/oxidoreductase GbaB, an amidohydrolase and an α, β-fold hydrolase (Figure 10) (Ray et al. 2020; You et al. 2014; Yu et al. 2017). The GbaA repressor was shown to bind to a palindromic sequence AAACGGAGAGTTATCCGTTT in the upstream promoter region of gbaA. The gbaA mutant showed strongly enhanced biofilm formation in a super-biofilm elaborating clinical isolate S. aureus TF2758 (Yu et al. 2017), and biofilm formation was dependent on GbaB (Ray et al. 2020). GbaB was suggested to catalyze the oxidation of an alcohol to a sugar aldehyde in the PNAG biosynthesis pathway, but the physiological role and the inducer of the GbaA regulon are unknown (You et al. 2014; Yu et al. 2017).

Interestingly, the GbaA regulon was induced by different redox active compounds and antibiotics, including RNS, AGXX®, allicin, erythromycin, colistin, vancomycin and most strongly by the electrophiles methylglyoxal and N-ethylmaleimide (NEM) (Loi et al. 2018a, 2019; Mäder et al. 2016; Ray et al. 2020; Schlag et al. 2007). GbaA was shown to function as a thiol switch and an electrophile sensor in vitro, which involves the conserved Cys55 and Cys104 residues in the DNA binding and regulatory domains (Ray et al. 2020). GbaA is oxidized to an intramolecular disulfide in response to diamide, BSSB, GSSG and GSNO in vitro, which does not affect its DNA binding activity (Figure 10). However, treatment of the single Cys55A or Cys104A mutant proteins with NEM and BSSB resulted in NEM-alkylation and S-bacillithiolation of the remaining Cys leading to inactivation of the GbaA repressor (Figure 10) (Ray et al. 2020). In addition, Cys104 was found more reactive and accessible towards electrophiles, indicating that Cys104 is the redox sensing Cys and most likely modified by electrophiles or BSSB in vivo (Ray et al. 2020). Thus, the redox sensing mechanism of GbaA differs from other electrophile sensors, such as YodB, which senses disamide and quinones via thiol switch and S-alkylation mechanisms, both leading to structural changes and inhibition of its DNA binding activity (Chi et al. 2010; Lee et al. 2016; Leelakriangsak et al. 2008). While GbaA resembles a 2-Cys type regulator, only S-thiolation or S-alkylation causes structural changes (Ray et al. 2020). However, the physiological electrophile and functions of the GbaA regulon members under oxidative and electrophile stress and biofilm formation are unknown.

Since the GbaA regulon is required for glucose-induced biofilm formation, host-derived oxidation products of glucose, such as the α,β-dicarbonyls glucosone, glyoxal, methylglyoxal, glycolaldehydes, or other reactive glycolytic intermediates could be physiological electrophiles sensed by GbaA. These glucose oxidation products accumulate in human tissues, blood and activated neutrophils causing glycation of lysine, arginine and cysteine in proteins (Vetter 2015). In neutrophils, the MPO reaction product HOCl causes formation of chloramines, which are degraded to acrolein and p-hydroxyphenylacetaldehyde as further infection-related electrophiles (Hazen et al. 1998). Reactive aldehydes might increase in S. aureus during the switch to biofilm formation and anaerobic fermentation, which relies on high glycolytic activity for ATP production. In the next section, we discuss the SrrAB system, which senses oxygen levels and controls the switch to anaerobiosis and biofilm formation via the SrrB redox switch (Tiwari et al. 2020; Yarwood and Schlievert 2000). Thus, GbaA and SrrAB might be responsible to sense different signals during biofilm formation and the switch from aerobic to anaerobic conditions to allow biofilm matrix synthesis, removal of toxic aldehydes and metabolic adaptation.

Another infection-relevant electrophile could be the major immunoregulatory metabolite itaconate, which inhibits the succinate dehydrogenase resulting in metabolic rewiring during inflammatory macrophage activation (Lampropoulou et al. 2016). Itaconate reacts as electrophile with GSH and induces electrophile stress in host cells (Bambouskova et al. 2018). In P. aeruginosa lung infections, host-derived itaconate reprograms bacterial metabolism and induces biofilm formation via enhanced exopolysaccharide matrix biosynthesis (Riquelme et al. 2017).
Thus, itaconate could be a candidate electrophile to inactivate the GbaA repressor to promote PNAG synthesis and biofilm formation in *S. aureus*, which remains to be investigated.

Furthermore, GbaA belongs to the TetR-family regulators, which are often inactivated by direct binding of antibiotics as ligands to the C-terminal regulatory domain (Cuthbertson and Nodwell 2013). Since GbaA responds to various antibiotics and allicin (Loi et al. 2019; Mäder et al. 2016), a direct binding of antibiotics and electrophiles coupled with ROS-induced S-thiolation or S-alkylation of Cys104 could possibly inactivate GbaA in vivo. In summary, *S. aureus* can be exposed to a plethora of oxidants and electrophiles present during host-pathogen interactions that might be sensed by GbaA and other redox sensors, which enable host adaptation. Thus, future studies should shed light on the signals and redox chemistry of host-pathogen interactions.

### The SrrAB two-component system as redox sensor of oxygen levels

The *Staphylococcus* respiratory regulator (SrrAB) two-component system consists of the sensor histidine kinase SrrB and the DNA-binding response regulator SrrA (Yarwood and Schlievert 2000). The SrrAB regulon is implicated in long-term biofilm stability, anaerobic growth, oxidative and nitrosative stress resistance and contributes to the virulence and protection against neutrophil killing in *S. aureus* (Kinkel et al. 2013; Mashruwala and Boyd 2017; Pragman et al. 2004; Ulrich et al. 2007). SrrAB regulon members include genes for the biogenesis of cytochromes and terminal oxidases (*ctaB*, *cydAB*, *qoxABCD*), heme biosynthesis (*hemACDX*), anaerobic fermentation (*pfAB*, *adhE*, *nrdDG*), and RNS resistance (*hmp*, *scdA*) (Figure 11) (Kinkel et al. 2013; Mashruwala and Boyd 2017; Oogai et al. 2016; Yarwood and Schlievert 2000). In addition, the SrrA response regulator binds to the *agr*, *spa*, *srrAB*, *icaA*, RNAIII and *tst* promoters and is involved in virulence regulation, directly and indirectly via Agr (Pragman et al. 2004; Ulrich et al. 2007). However, no consensus sequence of a potential SrrA recognition motif could be identified in the promoter regions of the target genes. Furthermore, SrrAB positively influences expression of *dps*, *scdA*, *ahpCF* and *katA*, which function in iron storage, FeS-cluster repair and H$_2$O$_2$ detoxification leading to an oxidative stress resistance (Mashruwala and Boyd 2017). The crosstalk between Nos and SrrAB mediates the switch from aerobic to anaerobic energy metabolism, since SrrAB co-regulates *narG* and *nirB*, which are involved in nitrate respiration and nitrite transport (James et al. 2019). *S. aureus* utilizes SrrAB for adaptation to lower oxygen availability during anaerobiosis and changes in the respiratory flux (Yarwood and Schlievert 2000). SrrB senses the reduced state of the respiratory menaquinone pool and acts as kinase and phosphatase via the PAS domain to regulate the phosphorylation state of the response regulator SrrA (Kinkel et al. 2013; Mashruwala et al. 2017; Tiwari et al. 2020). Two cysteines (Cys464 and Cys501) are present in SrrB, directly or indirectly via a PAS domain/ligand complex, leading to a 40% increase in autophosphorylation of SrrB, resulting in SrrB inactivation. During anaerobiosis, the two Cys residues in SrrB are reduced directly by reduced menaquinones or indirectly by a ligand binding to the PAS domain of SrrB. Reduced SrrB is active as kinase, leading to autophosphorylation and phosphorylation of the response regulator SrrA. SrrA-P positively controls biofilm formation via the *icaABCD* operon and represses *tst* gene transcription. Anaerobic respiration and fermentation are activated by SrrAB, while virulence factors are repressed as indicated.

![Sensing of oxygen by the SrrAB two-component system](image)
aerobic growth and induces the oxidative stress response. Thus, SrrAB contributes to the protection of FeS cluster enzymes, such as aconitase and facilitates the growth under aerobic conditions in S. aureus (Mashruwala and Boyd 2017). In support of this notion, the SrrB disulfide under aerobic conditions was important for pathogenesis of S. aureus in an endocarditis model of infection (Tiwari et al. 2020).

**Outlook for future research**

In this review, we present an overview of thiol-based redox switches in the pathogen S. aureus, which sense different reactive species via conserved Cys residues and control specific enzymatic detoxification pathways. We provide new insights into the role of BSH and the Brx/BSH/YpdA/NADPH pathway for redox homeostasis under oxidative stress, infection conditions and redox active antimicrobials in S. aureus. Significant progress occurred in the physiological and biochemical characterization of the NADPH-dependent flavin disulfide reductase YpdA as a BSSB reductase, which established a functional Brx/BSH/YpdA/NADPH electron pathway for regeneration of S-bacillithiolated proteins. BSH and the Brx/BSH/YpdA/NADPH redox pathway are crucial for redox homeostasis in S. aureus during host-pathogen interactions and contribute to antibiotic resistance towards redox active antimicrobials. The novel Brx-roGFP2 biosensor revealed the impact of BSH and YpdA on redox homeostasis and the oxidative mode of action of the antimicrobials allicin, lapachol and AGXX®.

However, the functions of BSH as cofactor of redox enzymes for detoxification of electrophiles and oxidants are only beginning to emerge. While BSH-dependent and BSH-independent glyoxalases for methylglyoxal removal have been characterized in B. subtilis (Chandrangsu et al. 2014), nothing is known about related mechanisms in S. aureus, although methylglyoxal is the most conserved toxic electrophile in all organisms. In addition, S. aureus encodes so many peroxiredoxins, such as Tpx, Bcp, GpxA1 and GpxA2, but their functions, substrates and the roles of the Brx/BSH/YpdA/NADPH and Trx/TrxR/NADPH pathways for their recycling are completely unknown. These peroxiredoxins might be particularly important to prevent accumulation of host-derived fatty acid electrophiles during infections.

While BSH plays an important role in FeS cluster assembly in S. aureus, the detailed mechanism awaits further studies. Similarly, the role of BSH in metal storage or buffering under conditions of nutritional immunity, such as Fe³⁺, Cu²⁺ or Zn²⁺ starvation are important subjects in terms of host-pathogen interactions in S. aureus. Through mass spectrometry-based redox proteomics approaches, many new thiol switches, including S-bacillithiolated and S-CoAAlated proteins have been discovered recently. While the role of S-bacillithiolation in thiol protection and redox regulation has been studied for GapDH in S. aureus, the implication of this redox modification and its reversal by the Brx/BSH/YpdA/NADPH pathway for cellular physiology during S. aureus infections are important tasks of future research. Furthermore, the physiological role of S-CoAAlation and its reversal in S. aureus remain further subjects of investigations.

The described thiol-based redox regulators of S. aureus cover a wide spectrum of bacterial transcription factor families, including Fur (PerR), MarR (SarZ/MgrA/SarA/QsrR/MhqR), Rnf2 (HypR), ArsC (Spx), TetR (GbaA) and two-component systems (SrrAB). These redox regulators sense oxygen, ROS, HOCl, NEM, quinones, methylglyoxal or antibiotics and often promote survival during infections. The thiol switch mechanisms can be divided into 1-Cys type and 2-Cys type models, including intra- or intersubunit disulfide formation of the 2-Cys type regulators (e.g. QsrR, HypR, Spx, SrrAB) and S-thiolation, S-alkylation or S-phosphorylation of the 1-Cys type redox sensors (e.g. SarZ, MgrA, SarA, GbaA). Specific redox regulators may employ different regulatory mechanisms to sense different reactive species (e.g. PerR, QsrR, GbaA), which has yet to be explored mechanistically and for cellular physiology or pathophysiology.

Finally, future studies should be directed to investigate structural determinants of the specificity (or nonspecificity) of regulatory thiol switches towards sensing of ROS, RES or HOCl. While there is evidence for an extensive cross reactivity of thiol switches towards various ROS, RES or HOCl (e.g. QsrR, MhqR, HypR, Spx, GbaA), the structural changes might be similar for some, but different for other redox sensors depending on the signals. The challenge is to find the physiological important signal and to dissect whether primary or secondary signals lead to inactivation or activation of the redox regulator. The functions of the specific regulon members might help to deduce the specific signals important for physiology or pathophysiology. For example, the HypR repressor responds to neutrophil infections, HOCl and the redox active antimicrobials AGXX®, allicin and lapachol by intersubunit disulfide formation leading to upregulation of the disulfide reductase MerA. However, in terms of pathophysiology, HOCl is the most important signal, since MerA provides protection during infections. Similar cross reactivities to RES and antimicrobials are observed for QsrR/YodB and MhqR, but their functions in quinone detoxification have been clearly established. Thus, the specificity
of the primary or secondary redox signal has to be revealed in concert with the functions of the regulon members in the physiology or pathophysiology of *S. aureus*. The discovery and characterization of thiol switches, which sense novel biochemical signals during host-pathogen interactions, remain exciting future challenges in the field of redox biology in microbial pathogens.

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