Free Radical Biology and Medicine 128 (2018) 84–96

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Review Article

Application of genetically encoded redox biosensors to measure dynamic changes in the glutathione, bacillithiol and mycothiol redox potentials in pathogenic bacteria

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ARTICLE INFO

Keywords:
Listeria monocytogenes
Salmonella Typhimurium
Staphylococcus aureus
Mycobacterium tuberculosis
Glutathione
Bacillithiol
Mycothiol
roGFP2/redox biosensors

ABSTRACT

Gram-negative bacteria utilize glutathione (GSH) as their major LMW thiol. However, most Gram-positive bacteria do not encode enzymes for GSH biosynthesis and produce instead alternative LMW thiols, such as bacillithiol (BSH) and mycothiol (MSH). BSH is utilized by *Firmicutes* and MSH is the major LMW thiol of *Actinomycetes*. LMW thiols are required to maintain the reduced state of the cytoplasm, but are also involved in virulence mechanisms in human pathogens, such as *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Salmonella enterica* subsp. Typhimurium and *Listeria monocytogenes*. Infection conditions often cause perturbations of the intrabacterial redox balance in pathogens, which is further affected under antibiotics treatments. During the last years, novel glutaredoxin-fused roGFP2 biosensors have been engineered in many eukaryotic organisms, including parasites, yeast, plants and human cells for dynamic live-imaging of the GSH redox potential in different compartments. Likewise bacterial roGFP2-based biosensors are now available to measure the dynamic changes in the GSH, BSH and MSH redox potentials in model and pathogenic Gram-negative and Gram-positive bacteria.

In this review, we present an overview of novel functions of the bacterial LMW thiols GSH, MSH and BSH in pathogenic bacteria in virulence regulation. Moreover, recent results about the application of genetically encoded redox biosensors are summarized to study the mechanisms of host-pathogen interactions, persistence and antibiotics resistance. In particularly, we highlight recent biosensor results on the redox changes in the intracellular food-borne pathogen *Salmonella* Typhimurium as well as in the Gram-positive pathogens *S. aureus* and *M. tuberculosis* during infection conditions and under antibiotics treatments. These studies established a link between ROS and antibiotics resistance with the intracellular LMW thiol-redox potential. Future applications should be directed to compare the redox potentials among different clinical isolates of these pathogens in relation to their antibiotics resistance and to screen for new ROS-producing drugs as promising strategy to combat antimicrobial resistance.

1. Functions of low molecular weight thiols in pathogenic bacteria

1.1. Functions of glutathione in virulence and protein S-glutathionylation in pathogenic bacteria

Low molecular weight (LMW) thiols play important roles to maintain the reduced state of the cytoplasm in all organisms [1,2]. Glutathione (GSH) functions as major LMW thiol in Gram-negative bacteria and in few Gram-positives, such as *Streptococci, Listeria, Lactobacilli* and *Clostridia* (Fig. 1). However, some Gram-positive pathogens also use ABC transporters to import GSH either from host cells or from the growth medium, as shown for *Streptococcus pneumoniae* and *Listeria monocytogenes* [3,4]. The biosynthesis and functions of GSH have been widely studied in *Escherichia coli,* which produces millimolar concentrations of GSH [2,5]. GSH maintains protein thiols in its reduced state, functions as a storage form of cysteine and is resistant to metal-catalyzed autooxidation [2]. GSH undergoes autooxidation 7 times slower compared to free Cys. Under oxidative stress, GSH is oxidized to glutathione disulfide (GSSG) which is reduced by the glutathione reductase (Gor) on expense of NADPH (Fig. 2). The GSH/GSSG ratio ranges from 30:1 to 100:1 and the standard thiol-disulfide redox potential of GSH was determined as $E^{0'}$(GSSG/GSH) = − 240 mV at physiological pH values in the cytoplasm of *E. coli* [1,6]. Many detoxification functions of GSH have been studied in *E. coli*. GSH is important

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https://doi.org/10.1016/j.freeradbiomed.2018.02.018
Received 24 December 2017; Received in revised form 8 February 2018; Accepted 13 February 2018
Available online 15 February 2018

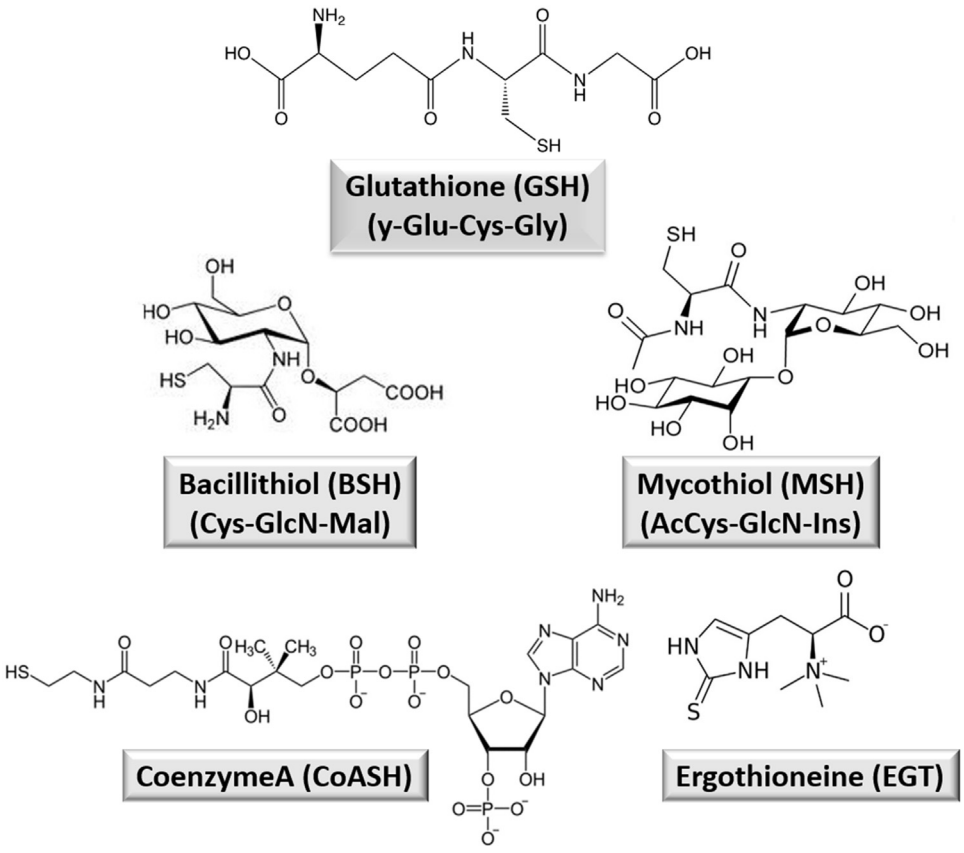

Fig. 1. Structures of major bacterial low molecular weight (LMW) thiols. The major LMW thiols are glutathione (GSH) present in Gram-negative bacteria and few Gram-positive bacteria. Bacillithiol (BSH) is the major LMW thiol in *Firmicutes*, such as *Bacillus* and *Staphylococcus* species. Mycothiol (MSH) is utilized in all *Actinomycetes*, including mycobacteria, corynebacteria and streptomycetes. Coenzyme A (CoASH) also serves as alternative LMW thiol-redox buffer in *S. aureus* and *B. anthracis*. Ergothioneine (EGT) is a histidine-derived alternative LMW thiol in mycobacteria.

for the defense against redox active compounds, xenobiotics, antibiotics, toxic metals and metalloids as reviewed previously [5]. Of note, GSH is an important cofactor of glyoxalases involved in detoxification of the toxic electrophile methyglyoxal as natural byproduct of the glycolysis in *E. coli* [7–10].

Apart from its well-studied detoxification functions, GSH contributes to the virulence of important human pathogens. The involvement of GSH in virulence has been studied in the extracellular facultative anaerobic pathogen *S. pneumoniae* as well as for the intracellular food-borne pathogens *L. monocytogenes* and *Salmonella enterica* subsp. Typhimurium (*S.* Typhimurium) [3,4,11–14]. The glutathione reductase Gor and the GSH-uptake system GshT protect *S. pneumoniae* against oxidative stress and toxic metal ions and are required for colonization and invasion in a mice model of infection [3]. *L. monocytogenes* is a facultative intracellular pathogen that has a saprophytic lifestyle in the soil and a parasitic in the host [15]. Specific evasion strategies enable to escape the phagolysosome and to proliferate inside the host cell cytosol. *L. monocytogenes* utilizes host-derived GSH, but can also synthesize bacterial GSH *via* the GshF fusion protein [14]. Bacterial and host-derived GSH are both important for virulence and expression of virulence factors in *L. monocytogenes*. The virulence mechanism involves activation of the positive regulatory factor A (PrfA) by allerosteric binding of GSH as cofactor to PrfA [13,14] **(**Fig. 3**)**. PrfA is a member of the CRP/FNR family and the master regulator for many virulence factors including the actin assembly factor ActA. ActA mediates actin polymerization and is essential for intracellular spread of the pathogen across host cells [15]. The structure of the PrfA-GSH complex has been recently determined to investigate the mechanisms for activation of PrfA upon GSH binding. GSH binding to a specific tunnel site of PrfA induces conformational changes in the tunnel site of PrfA that stabilizes the helix-turn-helix (HTH) motifs and primes PrfA for binding to the operator DNA [13]. Another structural study of the PrfA-GSH complex suggested that GSH binding induces local conformational changes in PrfA, allowing DNA binding and activation of gene transcription [16]. The GSH level and the reduced cytosol of the host cells further influence the virulence of *L. monocytogenes* [17]. Bacteria cultivated under reducing growth conditions in minimal medium with GSH had a higher PrfA activation state and virulence factor expression resulting in higher virulence in a murine infection model [17]. PrfA controls also listeriolysin O (LLO) as cholesterol-dependent cytolysin (CDC) required for host-cell lysis [15]. Interestingly, LLO was shown to be regulated by *S*-glutathionylation at a conserved Cys residue by host and bacterial derived GSH which inhibits its hemolytic activity to lyse red blood cells [18]. These two examples of PrfA and LLO highlight the important roles of GSH in activation of virulence factors expression and redox regulation in an important intracellular pathogen.

The intracellular pathogen *S.* Typhimurium, which causes gastroenteritis, resides inside a *Salmonella*-containing vacuole (SCV) and injects *Salmonella* pathogenicity island 2 effectors (SP-2) *via* a type-III-secretion system (T3SS) directly into the host cell. *S.* Typhimurium encounters oxidative stress by the phagocyte NADPH oxidase (Nox) that produces Reactive Oxygen Species (ROS) as oxidative burst. Reactive Nitrogen Species (RNS) are generated by the inducible NO synthase (iNOS) inside macrophages and neutrophils **(**Fig. 3**)**. In *S.* Typhimurium, GSH-deficient mutants displayed an increased sensitivity to ROS and RNS and were attenuated in an acute model of salmonellosis in $NRAMP^{R}$ mice that produces a high NO level [11]. Thus, GSH is important for the defense against ROS and RNS produced by Nox and iNOS as shown in the model of salmonellosis [11]. In addition, GSH was shown to be required for efficient transcription of the Spi-2 targets under NO stress. The Spi-2 system interferes with lysosomal trafficking and promotes intracellular replication inside the SCV [19,20]. Spi-2 reduces the contact between *Salmonella*-containing vacuoles (SCV) and NADPH phagocyte oxidase vesicles. Thus, Spi-2 protects *S.* Typhimurium against the oxidative burst inside macrophages by maintaining

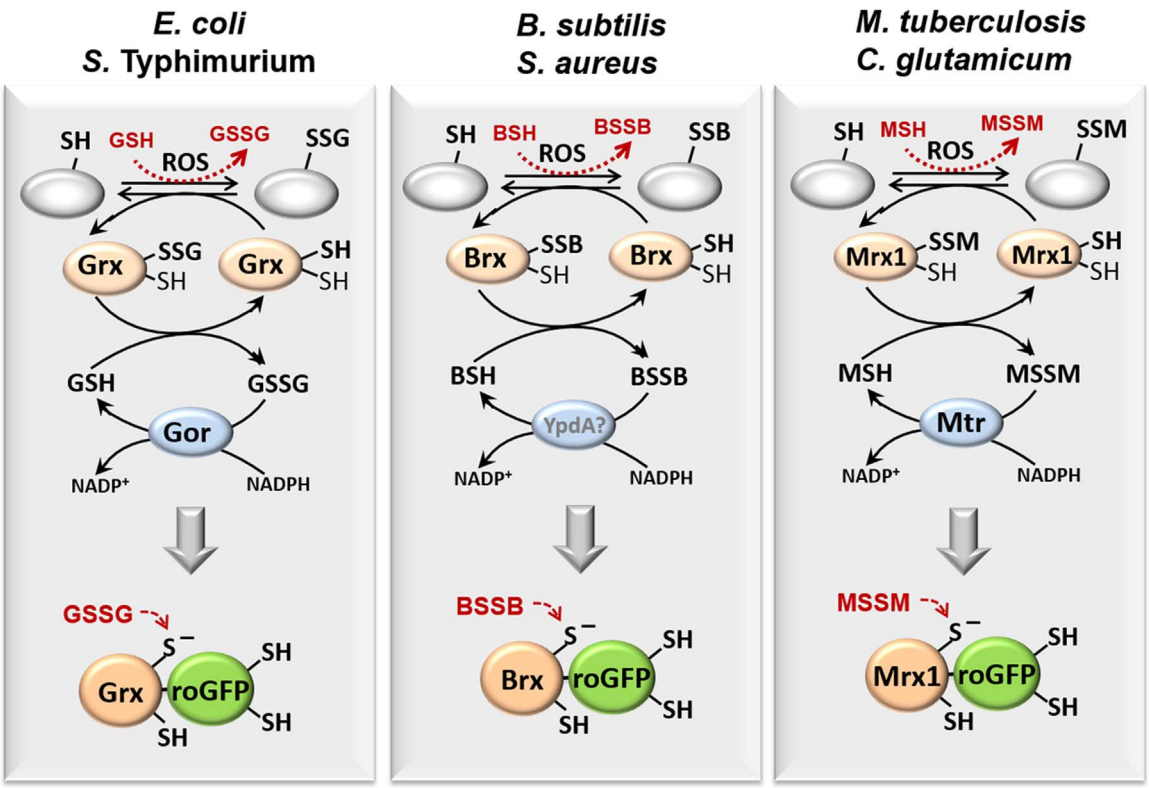

Fig. 2. Reduction of *S*-glutathionylations, *S*-bacillithiolations and *S*-mycothiolations by glutaredoxin, bacilliredoxin and mycoredoxin pathways and design of genetically encoded Grx1-roGFP2, Brx-roGFP2 and Mrx1-roGFP2 biosensors. The *S*-glutathionylated proteins are reduced by glutaredoxins (Grx) leading to a Grx-SSG intermediate that is reduced by GSH and the NADPH-dependent GSSG reductase (Gor). These pathways for reduction of *S*-glutathionylated proteins are present in *E. coli*, *S.* Typhimurium and other Gram-negative bacteria. Analogous bacilliredoxin and mycoredoxin pathways are present in BSH- and MSH-producing Gram-positive bacteria, such as *S. aureus* and *B. subtilis* as BSH producer and *M. tuberculosis* and *C. glutamicum* that utilize MSH. The *S*-bacillithiolated proteins are reduced by bacilliredoxins (Brx) leading to Brx-SSB formation. The regeneration of Brx-SSB could require BSH and perhaps the NADPH-dependent pyridine nucleotide oxidoreductase YpdA. In *Actinomycetes*, mycoredoxin1 (Mrx1) catalyzes reduction of *S*-mycothiolated proteins leading to Mrx1-SSM generation that is recycled by MSH and the NADPH-dependent MSSM reductase Mtr. The genetically-encoded biosensors were used to measure the dynamic changes of the intracellular redox potentials in eukaryotes and Gram-negative bacteria, such as *E. coli* and *S.* Typhimurium (Grx1-roGFP2) as well as in the Gram-positive bacteria *S. aureus* (Brx-roGFP2) and *M. tuberculosis* (Mrx1-roGFP2), respectively.

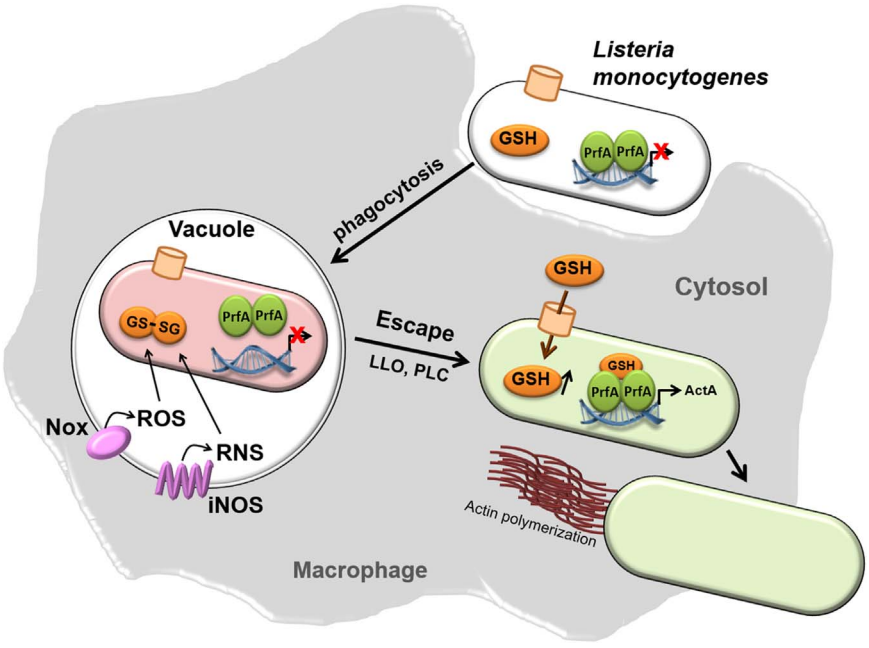

Fig. 3. Functions of GSH in PrfA activation for virulence factor expression in the intracellular pathogen *Listeria monocytogenes*. After phagocytosis by macrophages, the intercellular pathogen *L. monocytogenes* resides in an oxidizing vacuole (red), containing ROS and RNS that are produced by Nox and iNOS. *L. monocytogenes* has the ability to synthesize GSH, but can utilize GSH from host cells. In the oxidizing vacuole, GSH produced by *L. monocytogenes* is oxidized to GSSG, which does not bind the PrfA transcription factor [14]. *L. monocytogenes* escapes into the reducing host cell cytosol, leading to GSH regeneration and uptake of GSH from host cells. PrfA binds GSH and activates transcription of PrfA regulon genes, such as *actA*. ActA expression leads to Actin polymerization that allows movement of *L. monocytogenes* through host cells.
This figure is adapted from Ref. [14].

the intracellular thiol-redox balance [12,21]. The importance of this T3SS Spi-2 for ROS evasion was demonstrated using the roGFP2 biosensor as outlined in the biosensor section [12].

In *Yersinia pestis*, host-derived GSH functions in *S*-glutathionylation of the T3SS effector protein LcrV. *Y. pestis* causes bubonic plaques as extraordinary virulence mechanism and employs a T3SS for secretion of Yop effectors directly into the host cell cytoplasm [22]. These effectors function in pathogen evasion and neutralization of the host immune defense. The T3SS first secretes the LcrV protein, a plaque-protecting antigen that forms the needle cap protein of the T3SS and is essential for plaque pathogenesis [23,24]. LcrV is *S*-glutathionylated at Cys273 by host-derived GSH after its translocation and *S*-glutathionylation of LcrV is important for virulence of *Y. pestis* [25]. *S*-glutathionylated LcrV binds to host ribosomal protein S3 (RPS3), promotes effector secretion and macrophage killing. In addition, *S*-glutathionylation of LcrV contributes to bubonic plague pathogenesis in mice and rat models of infections [25]. In conclusion, GSH was shown to control expression and modification of virulence factors that are secreted by the T3SS in bacterial pathogens. Moreover, GSH is essential for survival under infection conditions in different pathogens, such as *S. pneumoniae, L. monocytogenes* and *S.* Typhimurium.

1.2. Functions of bacillithiol in the virulence and protein S-bacillithiolation in Gram-positive Firmicutes

The Gram-positive *Firmicutes* bacteria, such as *Bacillus* and *Staphylococcus* species utilize bacillithiol (BSH, Cys-GlcN-malate) as their major LMW thiol (Fig. 1) [26,27]. In *B. subtilis* and *S. aureus*, BSH is important for detoxification of many redox-active compounds. BSH-deficient mutants showed growth and survival defects after treatment with ROS, electrophiles, HOCl, toxins, alkylating agents, heavy metals and redox-active antibiotics, such as fosfomycin and rifampicin [28–30]. BSH functions as cofactor for thiol-dependent detoxification enzymes, such as thiol-*S*-transferases (FosB) and glyoxalases (GlxA/B). These thiol-dependent enzymes conjugate BSH to toxic electrophiles, fosfomycin and methylglyoxal for its detoxification [28,31]. BSH has also an impact on metal homeostasis and functions in Zn^{2+}-storage, FeS cluster assembly and copper buffering [32–35]. The standard thiol-redox potential of BSH was calculated as $E^{0'}$(BSSB/BSH) = − 221 mV and the BSH/BSSB ratios were determined as 100:1–400:1 under control conditions in *B. subtilis* cells [35–37]. Under NaOCl stress, the BSSB level is increased indicating a more oxidized BSH redox potential [38]. The NADPH-dependent pyridine nucleotide disulfide reductase YpdA is supposed to functions as BSSB reductase (Fig. 2), but its role in regeneration of BSH has not been demonstrated.

Of note, BSH has an important role for virulence in the major pathogen *S. aureus*. BSH protects *S. aureus* under infection-like conditions in phagocytosis assays using human and murine macrophages [29,30]. The survival of BSH-minus clinical MRSA strains was strongly impaired in human whole-blood survival assays [29]. The exact protective role of BSH inside the host is unknown, but the yellow antioxidant pigment staphyloxanthin was present at lower amounts in the absence of BSH [29]. *S. aureus* isolates carry many mobile genetic elements, such as prophages, pathogenicity islands, transposons and plasmids explaining their high genome diversity. Due to a former transposon or other insertion element, *S. aureus* NCTC8325 derivatives (*e.g.* SH1000) are *bshC* mutants and do not produce BSH [29,30,39]. Thus, also *S. aureus* SH1000 was impaired in survival inside murine macrophages and human epithelial cells and the phenotype could be restored by complementation with plasmid-encoded *bshC* [29,30]. Thus, BSH functions as virulence mechanism in the defense against the host immune system in *S. aureus* clinical isolates. Macrophages and neutrophils produce large quantities of ROS and HOCl as well as bactericidal ammonium chloramines during the oxidative burst [40–42]. Thus, the defense mechanism of BSH could involve regulatory mechanisms by formation of BSH mixed protein disulfides (*S*-bacillithiolations) in *S. aureus* inside neutrophils and macrophages.

To get insights into the targets for *S*-bacillithiolations in *S. aureus* under infection-like conditions, we have studied the quantitative thiol-redox proteome of *S. aureus* USA300 under NaOCl stress using the OxICAT approach [43]. In total, 58 Cys residues with > 10% increased thiol-oxidation could be quantified under NaOCl stress. In addition, five *S*-bacillithiolated were identified in *S. aureus* under NaOCl stress by shotgun proteomics. These *S*-bacillithiolated proteins showed the highest oxidation increase of > 29% in the OxiCAT analysis. The glyceraldehyde-3-phosphate dehydrogenase Gap was identified as most abundant *S*-bacillithiolated protein representing 4% of the total Cys abundance in the proteome. Protein *S*-bacillithiolation functions in redox regulation and protects the active site Cys151 of *S. aureus* Gap under H_2O_2 and NaOCl stress against overoxidation *in vitro* [43]. Future studies should reveal whether *S*-bacillithiolation of Gap or other proteins could provide protection of *S. aureus* under infection conditions inside macrophages and neutrophils. This adaptation to infection conditions in *S. aureus* could involve the metabolic re-configuration of central carbon metabolism as shown in eukaryotic organisms [44,45]. In yeast cells, Gap oxidation has been linked to the re-direction of the glycolytic flux into the pentose phosphate pathway (PPP) to increase NADPH levels. NADPH is used as electron donor for thioredoxin and glutathione reductases to recover from oxidative stress [44,45]. Similar mechanisms could be relevant also for *S. aureus* to enhance survival under infection conditions.

Apart from BSH, *S. aureus* produces also coenzymeA (CoASH) as abundant alternative LMW thiols and essential cofactor in cellular metabolism. Moreover, a CoASH disulfide oxidoreductase (Cdr) is encoded in the genome of *S. aureus* that could be involved in reduction of CoAS disulfides [27]. However, the functions of CoASH and Cdr for the redox regulation of proteins by CoA-thiolations are unknown in *S. aureus*. Recently, CoA-thiolation was shown in mammalian cells as a widespread post-translational redox modification under oxidative stress [46]. Numerous Cys peptides with CoA-thiolation sites were detected in H_2O_2-treated heart cells and in the mitochondria of liver cells from starved rats [46]. The authors developed a monoclonal antibody for enrichment of CoA-thiolated proteins and identified 80 CoA mixed disulfides (58 proteins) in heart cells and 43 CoA-thiolated Cys peptides (33 proteins) in liver cells using mass spectrometry. Many CoA-thiolated proteins function in main metabolic pathways, like the TCA cycle and the beta-oxidation pathway of fatty acids. These pathways involve activated CoA-derivatives, such as acetyl-CoA indicating that CoA metabolism and CoA-thiolation are functionally connected. It was also demonstrated that CoA-thiolation can inactivate enzymes and function in redox regulation of the glycolytic GapDH, the isocitrate dehydrogenase IDH and other metabolic enzymes [46]. Thus, it will be interesting to reveal if GapDH and other *S*-bacillithiolated proteins are also targets for CoA-thiolation in *S. aureus* under NaOCl stress.

The reduction of *S*-bacillithiolated proteins is catalyzed by bacilliredoxins (BrxA and BrxB) that belong to DUF1094 family. Brx proteins possess an unusual CGC motif, but function similar like glutaredoxins in *B. subtilis* and *S. aureus* (Fig. 2) [43,47]. Thus, Brx of *S. aureus* has been used to construct the first Brx-roGFP2-fused biosensor to measure changes in the BSH redox potential in *S. aureus* under oxidative stress and infection conditions inside human macrophages as outlined in the biosensor section.

1.3. Functions of mycothiol in the virulence and protein S-mycothiolation in Actinomycetes

Mycothiol (MSH; NAc-Cys-GlcNAc-myoinositol) is the major LMW thiol in high-GC Gram-positive *Actinomycetes,* including *Streptomycetes*, *Mycobacterium* and *Corynebacterium* species (Fig. 1) [48,49]. Under oxidative stress, MSH is oxidized to MSH disulfide (MSSM) and maintained in a reduced state by the mycothiol disulfide reductase Mtr. MSH is involved in detoxification of numerous compounds, such as ROS,

RES, alkylating agents, toxins, antibiotics (erythromycin, vancomycin, rifampin, azithromycin), heavy metals and toxic metalloids, aromatic compounds, ethanol and glyphosate as studied in different *Actinomycetes* [48,50–53]. In *Streptomyces lincolnensis*, MSH participates in the biosynthesis of the sulfur-containing antibiotics lincomycin [54]. For more details of these many detoxification functions of MSH and MSH-dependent enzymes, the reader is referred to previous and recent reviews [28,55].

Under hypochlorite stress, MSH was shown to form mixed disulfides with protein thiols, termed as protein *S*-mycothiolation [56–58]. Protein *S*-mycothiolation protects protein thiols against the formation of sulfinic and sulfonic acids and regulates protein activities, as demonstrated in *Corynebacterium glutamicum, Corynebacterium diphtheriae* and *Mycobacterium smegmatis*. About 25 *S*-mycothiolated proteins were identified in *C. glutamicum* [56], 26 proteins in *C. diphtheriae* [58] and 58 in *M. smegmatis* under NaOCl stress [57]. Among the S-mycothiolated proteins, several are conserved *S*-thiolated at their active sites Cys residues in different Gram-positive bacteria, including thiol-peroxidases/peroxiredoxins (Tpx, AhpC), ribosomal proteins (RpsM, RplC), the IMP dehydrogenase (GuaB), the myo-inositol-1-phosphate synthase (Ino1), the methionine synthase (MetE) and the glycolytic GapDH [38,56]. The extend of protein *S*-mycothiolation correlates with the different MSH levels in corynebacteria and mycobacteria [59]. While *M. smegmatis* contains 6 μmol/g raw dry weight (rdw) MSH [57], only 0.3 μmol/g rdw were determined in *C. diphtheriae* [58]. Thus, corynebacteria most likely utilize also alternative LMW thiols which remains to be investigated.

Mycobacteria utilize the histidine-derivative ergothioneine (EGT) as another alternative LMW thiol. MSH and EGT are both required for full virulence and redox homeostasis of *Mycobacterium tuberculosis* (*Mtb*) [60,61]. Both LMW thiols contribute also to full peroxide resistance of *M. smegmatis* [62]. EGT levels are even increased in the *mshA* mutant confirming that EGT can compensate for the absence of MSH [63]. Our redox proteomics studies revealed an increased thiol-oxidation level in the *M. smegmatis mshC* mutant which could involve alternative *S*-ergothionylation which remains to be elucidated [57]. However, in contrast to MSH, EGT is actively secreted into the supernatant [62]. Future studies should be directed to study the role of EGT secretion in regulation of EGT levels, modulation of host ROS levels and *S*-thiolation of bacterial and host proteins during infections.

Protein *S*-mycothiolation is redox-regulated by both, the mycoredoxin and thioredoxin pathways as demonstrated for thiol peroxidases (Tpx, Mpx, AhpE), the methionine sulfoxide reductase (MsrA) and the glycolytic GapDH *in vitro* [56,58,64–66]. Reduction of *S*-mycothiolated GapDH occurred much faster by Mrx1 compared to Trx *in vitro* indicating that Mrx1 is probably the main de-mycothiolating enzyme *in vivo* [58]. In addition, *S*-mycothiolation of GapDH is faster compared to its overoxidation *in vitro*. The methionine synthase MetE was further protected by *S*-mycothiolation under acid stress conditions in *C. glutamicum* [67]. These results indicate that *S*-mycothiolation can efficiently protect the active site Cys residues against overoxidation to sulfinic or sulfonic acids and can be reversed by both, the Mrx1 and Trx pathways. Mrx1 was used to construct the first MSH specific genetically encoded biosensor Mrx1-roGFP2 to measure changes in the MSH redox potential.

Apart from *S*-mycothiolation, MSH plays also an important role for growth, survival and antibiotics resistance under infection conditions in the major pathogen *Mtb* [61,68]. *Mtb* is the etiologic agent of tuberculosis (TB) disease resulting in about 2 million human death each year [69]. Due to the slow intracellular growth of *Mtb* inside the phagosomes of macrophages, TB patients have to be treated with antibiotics for several months, resulting in multiple and extreme drug resistant *Mtb* isolates (MDR/XDR) as a major health burden. MSH is involved in the activation of the first-line anti-TB drug isoniazid (INH) in *Mtb* [70]. INH is a pro-drug that is activated by the catalase KatG and MSH resulting in a NAD-INH adduct that finally inhibits InhA of the mycolic acid biosynthesis pathway [71]. Thus, the evolved INH resistant *Mtb* isolates often carry spontaneous mutations in *katG*, *mshA* and in the target gene *inhA* [51]. This requires alternative drug developments to treat emerging resistant *Mtb* isolates. Since MSH is important for virulence of *Mtb*, inhibitors of MSH biosynthesis and recycling have been successfully applied in combination therapies that target MshB, MshC, Mtr and the MSH-*S*-conjugate amidase Mca as new anti-TB drugs [72]. Moreover, ROS-producing compounds have been designed and may have a great potential to tackle anti-tuberculosis drug resistance. In the later sections, we will highlight recent work in drug research showing the power of the genetically encoded Mrx1-roGFP2 biosensor to study the role of MSH in antibiotics resistance, to reveal the involvement of ROS in the killing mode of antibiotics under infection conditions and to develop new combination therapies involving ROS-producing compounds.

2. Dynamic redox potential measurements using roGFP2-based biosensors in pathogens

The development of redox-sensitive green fluorescent proteins (roGFPs) has enabled the ratiometric measurement of the cellular redox potential at high sensitivity and spatiotemporal resolution using live-imaging approaches [73–76]. For construction of roGFPs, two redox-active Cys residues (Cys147 and Cys204) were introduced in the GFP molecule that form a disulfide bond upon oxidation resulting in conformational changes of the chromophore and fluorescence changes [76]. The roGFP2 biosensor has two excitation maxima at 405 and 488 nm, which change upon oxidation resulting in a ratiomeric biosensor response [74,77]. The Cys pair in roGFPs has been shown to equilibrate with the GSH/GSSG redox couple and the probes are widely used to measure the changes in the GSH redox potential in living eukaryotic cells [76]. However, the equilibration of endogenously expressed roGFPs with the GSH/GSSG pair is too slow and limited by the Grx expression levels. The Grx levels vary also in different compartments and are rate-limiting factors in the thiol-disulfide exchange reactions between the probe and the GSH pool.

To facilitate the specific response of roGFP2 with the GSH/GSSG redox couple, human glutaredoxin was fused to roGFP2 to construct the Grx1-roGFP2 biosensor for real-time measurements of the dynamic changes in the GSH redox potential (E_{GSH}) in eukaryotic organisms [75]. The Grx1-roGFP2 biosensor responds much faster within seconds to nanomolar concentrations of GSSG compared to unfused roGFP2 [74,75]. Thus, the Grx1-roGFP2 probe is highly specific and detects small changes in the GSH redox potential in living eukaryotic cells. To date, roGFP2 and Grx1-roGFP2 biosensors have been applied in many eukaryotic organisms and pathogens to study intracellular redox changes in *Arabidopsis thaliana, Caenorhabditis elegans* [75,78,79], yeast cells and the malaria parasite *Plasmodium falciparum* [80]. In particularly, pathogens are well suited to analyze the effect of drugs on the cellular redox metabolism and hence, the biosensors can help to screen for novel ROS-producing drugs. In this part of the review, we will present an overview about the application of roGFP2 biosensors in major human pathogens, including the foodborne intracellular pathogen *S*. Typhimurium, the extracellular Gram-positive pathogen *S. aureus* and in the intracellular major pathogen *M. tuberculosis*. Altogether, the biosensor results have advanced our understanding of the mechanisms of survival and intracellular replication, ROS evasion and persistence as well as antibiotics resistance in many important human pathogens.

2.1. Dynamic roGFP2-based biosensors to measure redox changes in Gram-negative bacteria

The roGFP2 biosensors were first applied in Gram-negative bacteria to measure the redox changes during growth, under oxidant and antibiotics treatment as well as infection conditions. In *E. coli*, plasmid-

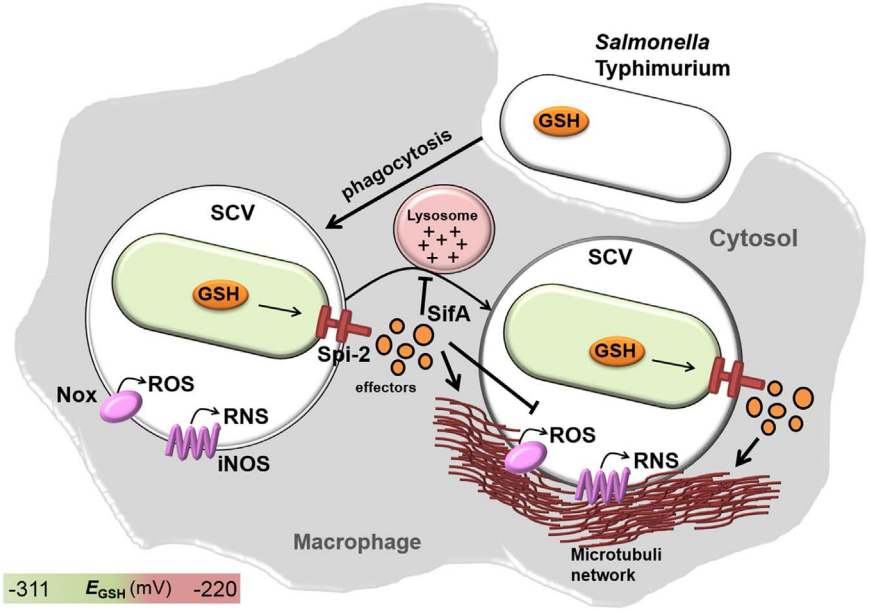

Fig. 4. Mechanisms of ROS evasion allowing intracellular replication of *Salmonella* Typhimurium inside the SCV to escape the host immune defense as revealed by the roGFP2 biosensor. The intracellular pathogen *S*. Typhimurium produces GSH and replicates inside macrophages in a *Salmonella*-containing vacuole (SCV). *S*. Typhimurium escapes ROS in the SCV by the type-III-secretion system Spi-2 that injects effectors directly into the host cell cytoplasm. GSH is required for transcription of the Spi-2 targets under NO stress. *S*. Typhimurium cells are highly reduced (green) inside in the SCV, while those that escape into host cells cytoplasm are oxidized [12]. The Spi-2 effector SifA affects co-localization of SCV and Nox vesicles and controls the vacuole integrity *via* microtubuli formation, which contributes to ROS evasion [12]. The Spi-2 effectors also interfere with lysosomal trafficking, promoting intracellular replication inside the SCV [19,20]. Thus, the Spi-2 system *via* its effector SifA functions in ROS evasion, controls vacuole integrity and maintains the intracellular redox balance of *S*. Typhimurium inside the SCV to allow intracellular replication [12].

encoded roGFP2 was used to observe cellular oxidation in response to different oxidants, toxic heavy metals and metalloids [81,82]. Toxic biocides, pollutants and metalloids are often found as environmental contaminants and originate from anthropogenic and natural sources. Thus, roGFP2 served as diagnostic tool to measure oxidative stress in *E. coli* by toxic environmental contaminants. Low levels of 0.1–1 mM H_2O_2 resulted in a rapid roGFP2 biosensor response. The roGFP2 biosensor showed also a fast response to heavy metals, such as Cd^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, arsenite and selenite as well as biocides and redox-cycling agents (menadione, naphthalene). However, quantification of the biosensor response using the microplate reader was not possible after exposure to toxic heavy metals or metalloids due to instability of the roGFP2 biosensor [81]. To increase roGFP2 stability, *E. coli* cells expressing the roGFP2 biosensor were immobilized in a transparent *k*-carrageenan (KC) matrix for further toxicity measurements [83]. The detection limit to measure a biosensor response was defined as 0.2 µg/l for arsenite and 5.8 ng/l for selenite. These immobilized roGFP2 expressing *E. coli* cells were applied to screen for bioavailability and toxic effects of pollutants [83].

2.1.1. The T3SS Spi-2 contributes to ROS evasion in S. Typhimurium

The first physiological studies in pathogenic Gram-negative bacteria using roGFP2 biosensors were performed in the intracellular pathogen *S*. Typhimurium that replicates inside the SCV [12]. *S*. Typhimurium escapes ROS by the T3SS Spi-2 that injects effectors directly into the host cell cytoplasm (Fig. 4). Thus, the biosensor was used to elucidate whether the T3SS Spi-2 contributes to evasion from the host innate immune defense to escape ROS and RNS. The intrabacterial redox changes were measured in *S*. Typhimurium after infection of HeLa cells and THP-1 cells that produce different ROS levels. In addition, the influences of the Spi-2 system and its effector SifA on ROS evasion strategies were investigated using *ssaR* and *sifA* mutants which are reviewed in this part.

S. Typhimurium encounters an acidic environment inside macrophages. Thus, it was first confirmed that the purified roGFP2 probe is not pH-sensitive *in vitro*. Next, the biosensor response inside *S*. Typhimurium cells was measured after treatment with H_2O_2 and the NO donor SpermineNONOate since *S*. Typhimurium has to cope with ROS and RNS that are produced by Nox and iNOS after phagocytosis. The roGFP2 biosensor responds very fast and reversible to 50–500 µM H_2O_2, but only high concentrations of 25 mM H_2O_2 lead to full oxidation of the probe inside *S*. Typhimurium. However, due to the detoxification by catalases and peroxidases, cells could quickly regenerate the reduced state even after treatment with high H_2O_2 levels. In contrast, exposure to 5–20 mM of the NO-donor resulted in a strongly increased biosensor oxidation with no recovery of the reduced state. These experiments verified that the probe detects intrabacterial redox changes under physiological micromolar ROS and RNS challenge.

To analyze the redox changes in *S*. Typhimurium after infection of host cells, epithelium-like HeLa cells and macrophages-like THP-1 cells were used. Interestingly, *S*. Typhimurium replicating inside THP-1 cells experienced higher levels of redox stress compared to bacteria infected in HeLa cells. The THP-1 cell line is known to produce higher ROS levels and is able to kill the majority of *S*. Typhimurium cells [12]. Moreover, redox stress heterogeneity was observed between different *S*. Typhimurium cells that maybe important to understand persistence and antibiotic resistance mechanisms.

In human and murine macrophages it was further shown that *S*. Typhimurium cells experience more redox stress in the cytosol compared to that residing in the SCV indicating that replication inside the vacuole contributes to ROS evasion. Thus, the role of the T3SS Spi-2 as ROS evasion strategy inside the SCV was investigated in the *ssaR* mutant that lacks the functional Spi-2 system (Fig. 4) [12]. The *ssaR* mutant displayed a higher oxidation level in THP-1 cells compared to the wild type indicating that the Spi-2 system contributes to ROS evasion. Previous studies revealed that Spi-2 effectors affect co-localization of SCV and phagocyte Nox vesicles, which contributes to ROS evasion [12,21]. Among the Spi-2 effectors, SifA was shown to control vacuole integrity as ROS evasion strategy. The biosensor measurements revealed that ROS evasion by the Spi-2 system requires an intact SCV since the *sifA* mutant experienced a higher redox stress [12]. Thus, the Spi-2 system functions *via* its effector SifA in ROS evasion to maintain the reduced state of the cytoplasm and to allow intracellular survival of *S*. Typhimurium [12].

2.1.2. Regulation of H_2O_2 detoxification and ROS-generation by antibiotics and toxic metals

Bacteria have evolved different antioxidant enzymes for ROS detoxification, such as catalases, thiol-dependent peroxidases, peroxiredoxins and superoxide dismutase [84]. The role of many H_2O_2 scavenging enzymes is often unknown in bacteria [85] and hence roGFP2 biosensors can contribute to study the dynamics and activity of ROS-degradation by the different bacterial enzymes. Thus, the roGFP2 biosensor was applied to measure redox changes and the ROS

detoxification capacity after treatment with H_2O_2, toxic heavy metals and antibiotics across different Gram-negative bacteria, including non-pathogenic and pathogenic *E. coli*, *Citrobacter rodentium*, *Yersinia pseudotuberculosis*, *Salmonella enterica* serovar Typhi and *S.* Typhimurium [86]. Using specific mutants in catalases and peroxidases, the kinetics of H_2O_2 detoxification was monitored for each antioxidant enzyme in different bacteria. Although the bacterial species were evolutionary related, the activities of their H_2O_2 detoxification enzymes showed strong variations. This enabled also to measure the ROS detoxification capacity of *S.* Typhimurium during priming with sub-lethal doses of 500 μM H_2O_2 and subsequent challenge with higher doses of 1 mM H_2O_2 compared to naïve cells. The primed bacteria could faster detoxify 1 mM H_2O_2 and recover to the reduced state compared to naïve bacteria [86].

In *S.* Typhimurium, the biosensor further allowed to measure endogenous ROS production in a catalase/peroxidase-negative *hpxf* mutant during different growth phases, media and temperatures. The endogenous ROS levels were highest during the later exponential growth at 37 °C in rich media compared to minimal medium. Thus, optimal growth conditions that allow a maximum growth rate correlate with high oxygen consumption and increased ROS generation. Similar as in the first *E. coli* roGFP2 approach [83], the toxicity of metals was assessed due to ROS production using the biosensor in *S.* Typhimurium [86]. While certain metal ions are required for H_2O_2 detoxification, exposure of *S.* Typhimurium to zinc and nickel contributed to ROS generation by inhibition of ROS detoxification enzymes (zinc) or spontaneous thiol-oxidation (nickel).

Next, biosensor measurements were performed under antibiotics treatment to validate whether ROS are involved in the killing mode of antibiotics, a continuous and controversial debate among microbiologists [87–89]. The oxidation-sensitive *S.* Typhimurium *hpxf* mutant was exposed to different antibiotics classes, including aminoglycosides, quinolones, cephalosporine and β-lactam antibiotics, but no increased biosensor oxidation could be monitored. This indicates that these antibiotics classes do not enhance endogenous ROS as killing mode in the *S.* Typhimurium *hpxf* mutant [86]. In contrast, Shukla and coworkers [90] showed that exposure to ampicillin, amikacin and ciprofloxacin leads to an impaired redox balance and increased biosensor oxidation in *E. coli*. Moreover, hydrogen persulfide (H_2S) was shown to protect *E. coli* against oxidative stress triggered by bactericidal antibiotics which is controlled by two mechanisms. H_2S mediated antibiotic tolerance involves rerouting of the electron flow from the energy-efficient cytochrome bo oxidase (Cyo) to the less-energy efficient cytochrome bd oxidase (CydBD) to maintain the respiratory flux and the redox balance. In addition, H_2S enhances the activities of the antioxidant enzymes catalase and superoxide dismutase which contributes to ROS detoxification under antibiotics treatments [90].

In *S.* Typhimurium, the roGFP2 biosensor was further applied to determine the real-time H_2O_2-influx [91]. The H_2O_2-influx was calculated by multiplication of the membrane permeability coefficient (P), the membrane surface area (A) and the difference between the inner and outer H_2O_2 concentrations (ΔC) as revealed by the degree of biosensor oxidation. The results showed that H_2O_2 first enters the cells by passive diffusion which is suddenly stopped, also termed as “switching point”. This stop in the H_2O_2 influx was caused by changes in the outer membrane permeability, as verified by spheroplasts lacking an outer membrane. The spheroplasts exhibited a significantly faster H_2O_2-influx without the “switching point”. The outer membrane proteins OmpA and OmpC were shown to regulate the H_2O_2 influx by opening and closing of their beta barrel structures [91].

Altogether, the roGFP2 biosensor has been widely used to measure the intrabacterial redox changes in several Gram-negative bacteria during the growth and under treatment with ROS and redox-active compounds, such as toxic metals and antibiotics as well as during infection and intracellular replication. The results revealed surprising differences in the H_2O_2 detoxification kinetics by antioxidant enzymes, such as catalases and peroxidases across closely related bacteria. Different antibiotics did not caused increased ROS-formation in a *S.* Typhimurium ROS-sensitive mutant [86], while Shukla and coworkers [90] revealed enhanced roGFP2 oxidation by antibiotics in *E. coli* cells. These different studies using the same roGFP2 biosensors further contribute to the controversial debate about the involvement of ROS in the killing mode of antibiotics. Moreover, roGFP2 biosensor measurements revealed that H_2O_2-influx is regulated by switching point due to OMPs that can open and close their beta-barrel. Of particular importance are further the roGFP2 biosensor measurements of *S.* Typhimurium inside the SCV. It was shown that the type-III-secretion system Spi-2 is required for ROS evasion and this depends on an intact vacuole. The bacteria were protected against ROS inside the SCV while bacteria that escaped into the host cell cytoplasm were more oxidized by ROS.

However, as critical remark, it has to be mentioned that the authors used only uncoupled roGFP2 for all measurements of the intrabacterial redox potential in *S.* Typhimurium. The unfused roGFP2 biosensor suffers from its low specificity for the GSH/GSSG redox couple and the limited availability of endogenous Grx. Thus, whether the roGFP2 probe specifically responds to GSH redox potential changes or other redox signals is not known. Future studies should be performed using the Grx1-roGFP2 biosensor which is highly specific to measure ratiometric changes in the GSH redox potential [75]. It will be also interesting to apply the Grx1-roGFP2 biosensor to study the mechanisms of ROS evasion in other GSH-utilizing intracellular pathogens, such as *L. monocytogenes* and *Legionella pneumophila*.

2.2. Dynamic measurement of the BSH redox potential (E_{BSH}) using the Brx-roGFP2 biosensor in the human pathogen S. aureus

We have recently fused bacilliredoxin (Brx) of *S. aureus* to roGFP2 to construct the first genetically encoded Brx-roGFP2 biosensor for dynamic measurement of the intracellular BSH redox potential (E_{BSH}) in *S. aureus* [92]. The BSH redox potential changes were determined during the growth, under ROS and NaOCl stress, during infection inside THP-1 macrophages and antibiotics treatments in two clinical MRSA isolates COL and USA300. In both MRSA strains, BSH enhances the survival during phagocytosis with human and murine macrophage-like cell lines [29,30]. Brx-roGFP2 is highly specific for physiological levels of 10–100 μM BSSB which depends on the Brx active site Cys *in vitro*. Thus, Brx-roGFP2 facilitates rapid equilibration of the biosensor with the BSH/BSSB couple to determine the changes in the BSH redox potential inside *S. aureus*.

First, an increased biosensor oxidation was measured in *S. aureus* COL and USA300 in rich medium during the stationary phase compared to the log phase. The dynamic range of Brx-roGFP2 was higher in COL compared to USA300, which may depends on their different BSH levels [29]. USA300 is a highly virulent CA-MRSA strain, which produces many unique virulence factors encoded on prophages, pathogenicity islands and other mobile genetic elements [93]. In addition, USA300 has a higher level of BSH compared to COL. Thus, the biosensor response of USA300 could be lower under diamide stress resulting in a lower dynamic range of fully reduced *versus* oxidized probes. In addition, strain USA300 could be less permeable or more resistant to diamide compared to COL, leading only to partial biosensor oxidation. Future studies should involve other strong oxidants, such as cumene hydroperoxide or redox cycling agents for full oxidation of the biosensor to increase the dynamic range in USA300.

Treatment of *S. aureus* COL with different oxidants resulted in a fast biosensor response, but at different oxidation degrees. While doses of 50–100 μM NaOCl stress lead to the fully oxidation of the biosensor, exposure of *S. aureus* to 1–10 mM H_2O_2 revealed only a slightly increased oxidation degree with rapid regeneration of the reduced state. This lower biosensor response under H_2O_2 stress might be due to the high H_2O_2 resistance of *S. aureus* which is able to survive up to 300 mM H_2O_2 [94]. We further measured the changes in BSH redox potential

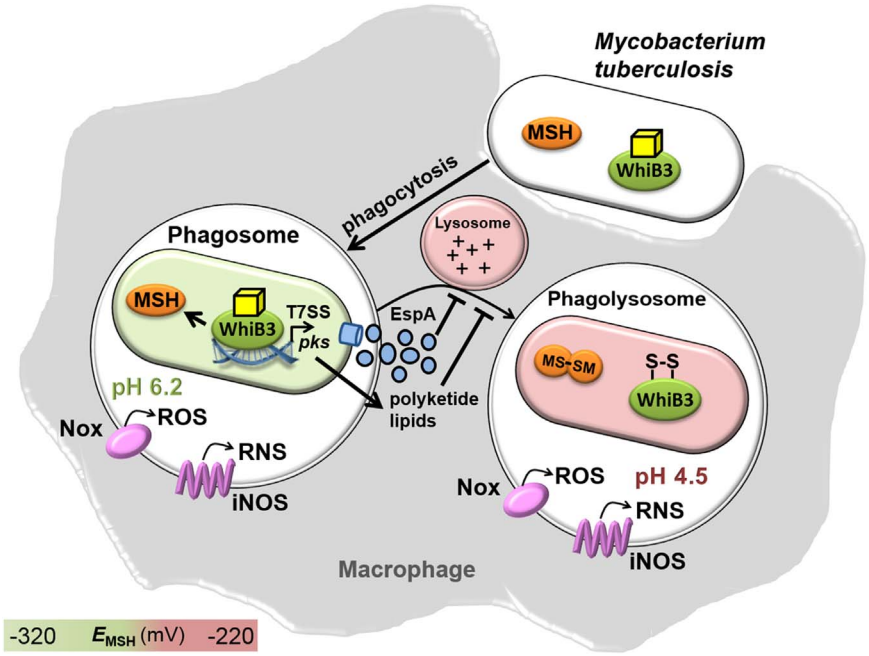

Fig. 5. The role of E_{MSH} and the WhiB3 transcription factor in *M. tuberculosis* persistence under acidic conditions during infection of macrophages as shown by the Mrx1-roGFP2 biosensor. *M. tuberculosis* is an intracellular pathogen that replicates inside the acidic phagosome of macrophages (pH ~ 6.2) preventing phagosomal maturation to phagolysosomes as survival mechanism. During immune activation of macrophages, phagosomes are fused with lysosomes resulting in further pH decrease to pH 4.5. The mild acidification in phagosomes causes a highly reduced E_{MSH} inside *M. tuberculosis*, while strong acidification leads to oxidized E_{MSH} as measured in phagolysosomes [96]. The WhiB3 transcription factor senses acidic conditions in the phagosome and activates transcription of WhiB3 regulon genes, such as type-VII-secretion system effectors (EspA) and polyketide lipids that inhibit phagosomal maturation. WhiB3 causes up-regulation of antioxidant systems (MSH, Trx) to restore the redox balance and to promote survival and persistence of *M. tuberculosis* inside the phagosome.
This figure is adapted from Ref. [96].

inside *S. aureus* COL after infection of THP-1 macrophages using flow cytometry. The Brx-roGFP2 biosensor was 87% oxidized in *S. aureus* COL inside macrophages indicating that *S. aureus* experiences oxidative stress after internalization. In future studies, the redox dynamics of persister cells inside macrophages should be investigated to reveal the BSH redox dynamics during internalization, which is often the cause of chronic *S. aureus* infections.

The biosensor response was also measured in *S. aureus* COL and USA300 *bshA* mutants and in RN4220, which is a natural *bshC* mutant of the NCTC8325-4 lineage. Brx-roGFP2 was fully oxidized in the BSH-deficient mutants indicating an impaired redox balance in the absence of BSH. In previous studies, a lower NADPH level was found in the *bshA* mutant perhaps explaining its impaired redox balance [29]. To clarify whether ROS generation contributes to the killing mode of antibiotics, *S. aureus* was exposed to sub-lethal doses of different antibiotics classes, including rifampicin, fosfomycin, ampicillin, oxacillin, vancomycin, aminoglycosides and fluoroquinolones. However, no increased oxidation degree of the Brx-roGFP2 biosensor was measured under antibiotics treatment, which confirms the findings in *S.* Typhimurium [86]. However, the biosensor responds fast to oxidants and could be a valuable tool in drug-research to screen for new ROS-generating antibiotics that affect the BSH redox potential in *S. aureus.* Future studies should be directed to measure the ROS detoxification capacity in mutants lacking antioxidant systems and in MRSA-isolates of various genetic lineages to unravel the link between ROS resistance and the BSH redox potential in *S. aureus*.

2.3. Dynamic measurements of the MSH redox potential (E_{MSH}) in *Mycobacterium tuberculosis* using the Mrx1-roGFP2 biosensor

In *Mtb,* an analogous Mrx1-roGFP2 biosensor was developed for dynamic measurements of the MSH redox potential (E_{MSH}) in drug-resistant isolates and inside the acidic phagosomes of macrophages [74,95,96]. The increasing prevalence of persistent and chronic relapsing *Mtb* infections as well as multiple and extreme drug-resistant (MDR/XDR) *Mtb* isolates are a major health burden. Thus, the development of new drugs against severe tuberculosis infections is an urgent need. The new biosensor was successfully applied to screen for ROS-generating anti-TB drugs and combination therapies (*e.g.* augmentin or isoniazid combinations) that affected E_{MSH} to study drug actions linked to the E_{MSH} to combat life-threatening TB infections [95,97–99]. It was revealed that the E_{MSH} inside infected macrophages is heterogeneous with sub-populations that have reduced, oxidized and basal levels of E_{MSH}. This redox heterogeneity depends on sub-vacuolar compartments inside macrophages and the cytoplasmic acidification that requires WhiB3 as central redox regulator [95,96]. These results using the Mrx1-roGFP2 biosensor have advanced the understanding how this major pathogen copes with anti-TB drug and persists inside macrophages. The major results obtained with Mrx1-roGFP2 are summarized in this part of the review.

After construction of the Mrx1-roGFP2 biosensor, it was demonstrated that the Mrx1-roGFP2 fusion is specific to measure MSSM, but does not respond to other LMW thiol-disulfides [95]. It was further controlled that overexpression of Mrx1-roGFP2 does not affect cellular metabolism, stress resistance and the basal level of E_{MSH} in *Mtb* [95]. Importantly, differences were observed in the biosensor response between slow growing *Mtb* strains and fast growing *M. smegmatis* resulting in a delayed response to H_2O_2 in *Mtb* and a rapid H_2O_2 response in *M. smegmatis* [95]. However, there was only little variation between the basal E_{MSH} in various drug-resistant (MDR/XDR) and drug-sensitive clinical *Mtb* isolates during laboratory growth, where the intracellular E_{MSH} was calculated as highly reduced with values of − 273 mV to − 280 mV [95]. However, in slow growing *Mtb* strains the E_{MSH} is more oxidizing compared to fast growing *M. smegmatis*. In *M. smegmatis*, a basal E_{MSH} of − 300 mV was calculated which is consistent with the higher MSH/MSSM ratio (200:1) in *M. smegmatis* compared to that in *Mtb* (50:1) [100].

2.3.1. E_{MSH} redox heterogeneity in Mtb sub-populations depends on specific vacuole compartments

In general, different *Mtb* strains did not show strong variations in their intracellular E_{MSH} when grown under *in vitro* conditions in growth media. However, this was completely different under *in vivo* infection conditions. Different *Mtb* sub-populations with reduced (− 300 mV), oxidized (− 240 mV) and basal E_{MSH} (− 270 mV) could be observed and quantified by flow cytometry under infection conditions inside THP-1 macrophages [95]. It was further shown that the reduced E_{MSH} sub-population is decreased and the oxidized E_{MSH} sub-population is increased at later time points of macrophage infections which correlates with a decreased MSH/MSSM ratio [95]. Thus, the intramacrophage

environment induces redox heterogeneity with different E_{MSH} sub-populations in *Mtb*. Of note, the sub-populations with reduced, oxidized and basal E_{MSH} were different during the time course of infections and also between various MDR/XDR *Mtb* isolates indicating a strongly varying redox balance between *Mtb* isolates. Immune activation further leads to an oxidative shift of *Mtb* sub-populations, which resulted from NO stress as part of host innate immune defense [95].

Mtb is an intracellular pathogen, that is engulfed by macrophages and trapped in an organelle, called the phagosome **(**Fig. 5**)**. Phagosomal maturation occurs by the interaction of phagosomes with endosomes and fusion with lysosomes to phagolysosomes, a highly acidic and microbicidal compartment that finally degrades invading bacteria [101]. However, *Mtb* successfully restricts phagosomal maturation by preventing fusion of phagosomes with lysosomes. This enables *Mtb* to persist and replicate inside the phagosome to cause chronic and relapsing *Mtb* infections [102,103]. It was suggested, that the different sub-vacuolar compartments might induce this E_{MSH} redox heterogeneity in *Mtb* [95]. The *Mtb* sub-populations were investigated in different vacuolar compartments including early endosomes, autophagosomes and lysosomes. Interestingly, the *Mtb* sub-population located in autophagosome showed almost oxidized E_{MSH}, while those residing in lysosomes were 58% oxidized and the sub-population in early endosomes showed mostly (54%) reduced E_{MSH}. Thus, the biosensor identified the sources of redox heterogeneity as the specific compartments in which *Mtb* resides inside macrophages.

2.3.2. Mechanisms of antibiotics-mediated ROS generation as strategy to combat drug resistance in Mtb

Due to the controversial debate about the role of ROS in antibiotic-mediated bacterial killing, the changes in intramycobacterial E_{MSH} were investigated after exposure to anti-TB drugs. In agreement with the biosensor responses under antibiotics stress in *S*. Typhimurium and *S. aureus* [86,92], no oxidative shift in E_{MSH} was reported in shake-flask experiments with *Mtb* populations that were exposed to sub-lethal anti-TB-drugs, *e.g.* isoniazide, ethambutol and rifampicin [95]. The only exception was the redox-cycling drug clofazimine, which caused an oxidative shift in E_{MSH} in *Mtb* shake-flask cultures. However, under macrophage infections, different antibiotics classes caused oxidative stress as shown by an oxidative shift in the E_{MSH} sub-populations, which was accompanied by increased killing of bacteria. Moreover, the redox heterogeneous sub-populations vary in their susceptibilities to antibiotics. The more oxidized population in autophagosomes and lysosomes was more susceptible to antibiotics killing, while the reduced population in endosomes displayed resistance to anti-TB drugs. Thus, immune activation inside macrophages potentiates drug killing while populations with reduced E_{MSH} promote antibiotics tolerance. Together these results showed important novel insights into the redox heterogeneity of *Mtb* sub-populations in different macrophage compartments, their susceptibility to antibiotics and the mechanisms of persistence [95].

In subsequent studies, several efforts were undertaken to understand the mechanisms of drug resistance and to develop new ROS-producing anti-TB drugs. These ROS-generating drug were used alone and in combination therapies as promising strategy to counteract the increasing problem of antimicrobial resistance and to combat XDR/MDR *Mtb* isolates [97–99]. First, hydroquinone-based antibiotics were synthesized, including ATD-3169 which was shown to cause superoxide production in *Mtb* isolates and increases the irreversible oxidized *Mtb* sub-population [99]. Next, combination therapies of isoniazid (INH) and inhibitors of antioxidant responses were found as promising strategy to threat drug resistant *Mtb* isolates [98]. Such inhibitors of antioxidant responses were ebselen, vancomycin and phenylarsine oxide that were highly effective in combination with INH to kill drug resistant Mtb isolates.

INH is a pro-drug that is activated by the catalase KatG and converted to a NAD-INH-adduct, that subsequently inhibits the enoyl-ACP reductase (InhA) in the mycolic acid biosynthesis pathway [98]. To identify the mechanisms of drug resistant *Mtb* strains, isoniazid resistance was studied in more detail in laboratory evolved INH-resistant *M. smegmatis* strains [98]. Genome sequencing revealed that INH resistant strains carried point mutations in genes for NADH dehydrogenase (*ndh*), catalase (*katG*) or the 3-dehydroquinate synthase (*aroB*). Transcriptomics identified antioxidant responses as dominating in the differentially transcribed genes in the INH resistant *M. smegmatis* strains. Moreover, the INH resistant strain was more sensitive to compounds that block antioxidant responses and disturb E_{MSH}. In agreement with this finding, the Mrx1-roGFP2 biosensor measurements revealed an oxidized shift in basal E_{MSH} and a higher sensitivity to oxidative stress by H_2O_2 in the INH-resistant *M. smegmatis* strain [98]. This higher ROS-sensitivity was not only observed in the INH-resistant *M. smegmatis* strain, but also in clinical MDR and XDR *Mtb* patient isolates. Thus, the evolution of drug resistance is associated with changes in the basal E_{MSH} and shifted to the oxidized redox state in multiple resistant *Mtb* isolates. Finally, it was shown that antibiotics that produce ROS or block antioxidant responses are in combination with INH more potent to induce oxidative shift in E_{MSH} during infections. These drugs should be promising strategies to tackle tuberculosis disease and to combat drug resistant isolates [98].

2.3.3. E_{MSH} regulates the redox state of WhiB4 mediating augmentin resistance and tolerance

In another study, the mode of action for combination therapy of β-lactam antibiotics (amoxicillin) with β-lactamase inhibitors (clavulanate), termed as augmentin, has been studied. The Mrx1-roGFP2 biosensor revealed a role of E_{MSH} and the WhiB4 redox sensor in augmentin resistance **(**Fig. 6**)** [97]. To study the mode of action of augmentin, a transcriptomics approach was used and identified cell wall and oxidative stress responses, respiration and carbon metabolism induced under augmentin treatment. Using biosensor measurements, an increase in the oxidized E_{MSH} sub-population was observed by augmentin over time during *Mtb* infections inside macrophages. Thus, augmentin effects the redox balance in *Mtb*, which potentiates its mycobactericidal effect and contributes to augmentin killing [97]. Furthermore, MSH was shown to protect *Mtb* from toxicity under augmentin treatment in survival assays. In further analysis, the FeS-cluster redox sensor WhiB4 was identified which regulates the shift to the oxidized E_{MSH} sub-population after augmentin treatment. Moreover, this oxidized shift modulates expression of the β-lactamase BlaC, which is regulated by WhiB4 in a redox-dependent manner. Specifically, BlaC is overexpressed in the *whiB4* mutant which increases resistance to β-lactam antibiotics **(**Fig. 6**)**. In contrast, overexpression of oxidized WhiB4 under augmentin treatment resulted in strong *blaC* repression and increased killing by β-lactams potentiating drug action. Thus, WhiB4 was identified as central regulator of β-lactam antibiotics resistance and the oxidative shift in E_{MSH} after augmentin combination therapy [97].

2.3.4. E_{MSH} regulates the redox state of WhiB3 mediating acid resistance and inhibition of phagosomal maturation

WhiB3 is another FeS cluster redox sensor that is also regulated by E_{MSH} and is essential for acid resistance of *Mtb* which allows survival of *Mtb* inside the acidic phagosome upon immune-stimulation [60,104,105]. WhiB3 was shown to play a protective role together with MSH under acidic stress conditions inside the phagosome of activated macrophages **(**Fig. 5**)** [96]. WhiB3 mediates acid resistance and inhibits phagosomal maturation, which is linked to changes in E_{MSH} under infection conditions. WhiB3 controls genes for lipid biosynthesis, secretion of the type-VII-secretion effectors as well as MSH biosynthesis and recycling under acidic stress. The limited decreased pH upon acidification of the phagosome (pH ~ 6.2) results in a reductive shift of E_{MSH} sub-populations and WhiB3 as well as MSH were found as key regulators for this reductive shift in E_{MSH}. WhiB3 was further required

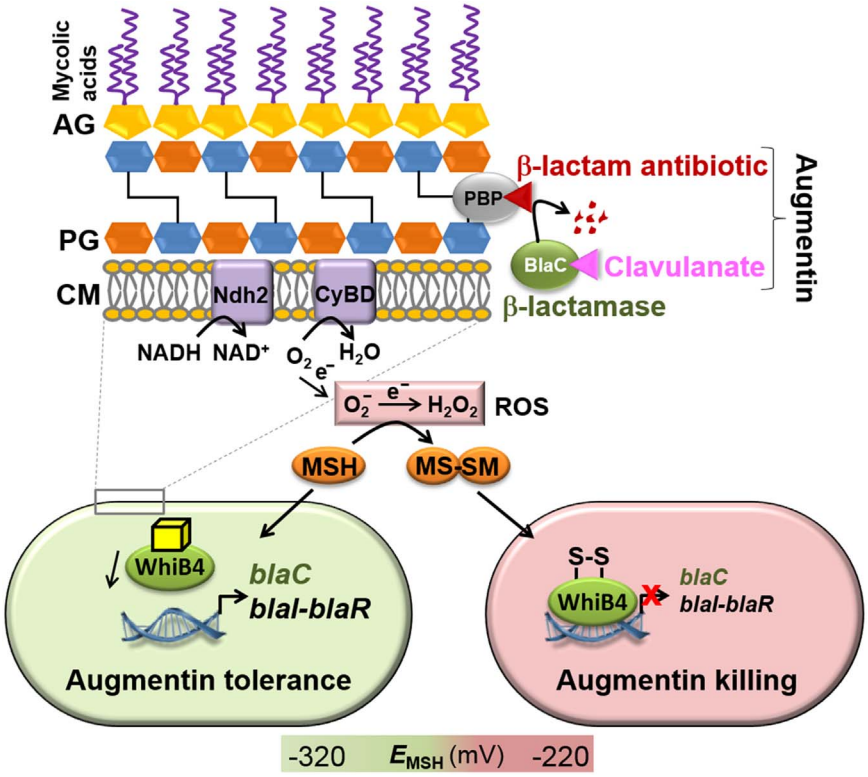

Fig. 6. The augmentin combination therapy of β-lactam antibiotics and β-lactamase inhibitor (clavulanate) causes ROS formation and changes in E_{MSH} in *Mtb* that affect WhiB4-mediated expression of β-lactamase expression. β-lactam antibiotics inhibit penicillin-binding proteins that cross-link the peptide side chains of the peptidoglycan (PG). Clavulanate inhibits the β-lactamase BlaC in *Mtb* that is controlled by the BlaI repressor and WhiB4. The combination therapy of β-lactam and Clavulanate (Augmentin) causes cell wall stress and ROS production in *Mtb* due to the re-direction of aerobic respiration *via* the Ndh2 and CyBD routes [97]. Increased ROS leads to the oxidative shift of E_{MSH} and oxidation of WhiB4 that represses transcription of *blaC* and the *blaI-blaR* operon resulting in down-regulation of the β-lactamase BlaC and killing by augmentin [97]. Tolerance to augmentin is induced by down-regulation or reduction of WhiB4 presumable in the reduced E_{MSH} sub-population resulting in derepression of the β-lactamase-encoding *blaC* gene directly or indirectly *via* derepression of the *blaIR* operon and proteolytic degradation of the BlaI repressor by the protease BlaR. This figure is adapted from Ref. [97]. Abbreviations: CM: cytoplasmic membrane, PG: peptidoglycan, Ndh2: NADH dehydrogenase 2, CyBD: cytochrome BD oxidase, PBP: penicillin-binding protein.

for survival under acidic conditions and protects *Mtb* from acid stress by controlling genes that restrict phagosomal maturation to subvert acidification and by down-regulation of the innate immune response. The *whiB3* mutant was also attenuated in the lung of guinea pigs. These results revealed a link between phagosome acidification, the reductive shift in E_{MSH} and virulence of *Mtb* that is controlled by WhiB3 mediating acid resistance and inhibiting phagosomal maturation as mechanism of persistent and chronic *Mtb* infections [96].

2.3.5. E_{MSH} is controlled by the sulfur assimilation pathway, the membrane SodA/DoxX/SseA complex and macrophage GSH production that are required for survival of Mtb

For the treatment of persistent *Mtb* infections, the sulfur assimilation pathway was selected as promising target that is required for biosynthesis of sulfur-containing amino acids and thiol-cofactors, such as cysteine and MSH [106]. The sulfur assimilation pathway, including the enzyme 5' adenosine phosphosulfate (APS) reductase (CysH), was especially important for virulence and survival of *Mtb* during chronic and persistent infections in mice and macrophage models [107,108]. Thus, a high-throughput drug screening approach was used to identify three inhibitors of the APS reductase as potent anti-TB compounds that decreased the levels of sulfur-containing metabolites, including MSH [106]. Using the Mrx1-roGFP2 biosensor, an oxidative shift in E_{MSH} was measured in response to these APS reductase inhibitors indicating the link between persistence, antibiotic tolerance and the sulfate assimilation pathway in *Mtb*.

In another study, the Mrx1-roGFP2 biosensor was used to identify the link between a novel membrane-associated oxidoreductase complex (MRC) and the MSH redox potential [109]. Using a Tn-seq approach, the authors screened for interactions of pathways required in *Mtb* for detoxification of radicals from the phagocyte oxidative burst. The superoxide dismutase (SodA), an integral membrane protein (DoxX) and the conserved thiol oxidoreductase SseA were identified as functionally linked MRC and the electron transfer was verified *in vivo*. Single mutants in each MRC component are similar sensitive to radical stress and exhibited an oxidized E_{MSH} as revealed by Mrx1-roGFP2 biosensor measurements. This study established a link between a novel oxidative stress resistance network with the E_{MSH} in *Mtb* to overcome the oxidative burst during infections [109].

An interaction between macrophage-derived GSH and E_{MSH} during *Mtb* infection has been revealed using the Mrx1-roGFP2 biosensor in a mice model of tuberculosis [110]. The GSH pool of macrophages depends on the xCT cystine-glutamate transporter, which is induced during *Mtb* infection. The deletion of xCT resulted in protection against TB and decreased pulmonary pathology in the mice lung. Mrx1-roGFP2 biosensor measurement revealed an oxidized E_{MSH} of *Mtb* in the infected mice xCT mutant. The increased E_{MSH} is caused by a decreased GSH production in the macrophages indicating a link between host GSH and bacterial MSH redox homeostasis. This study has further identified inhibitors of the xCT transporter as host-directed drugs for TB treatment [110].

Finally, the Mrx1-roGFP2 biosensor was applied in a mycobacterial biofilms under hypoxic conditions [111]. In the absence of oxygen as terminal electron acceptor, novel polyketide quinones were produced as alternative electron carriers in the respiratory chain to maintain bioenergetics and the membrane potential. About 70% of mycobacterial cells showed alterations in E_{MSH} under hypoxic biofilm conditions compared to planktonic cells, including 53% of cells with more reduced E_{MSH} and 16% with oxidative shift in E_{MSH}. Thus, the different oxygen levels across the biofilm affect the membrane potential and the MSH redox balance [111].

In summary, the Mrx1-roGFP2 biosensor was approved as valuable tool to study the mechanisms of redox heterogeneity, persistence and survival of *Mtb* under acidic conditions inside macrophage vacuolar compartments and the evolution and changes in E_{MSH} in drug resistant *Mtb* isolates. The biosensor has further contributed to elucidate novel ROS defense mechanisms in *Mtb*, such as the radical scavenging membrane MRC complex and the role of host GSH to regulate the MSH redox balance of *Mtb* inside macrophages. In drug research, the biosensor was used to study the regulation and mode of action of combination therapies (INH and augmentin) involving ROS-generating antibiotics as well as novel inhibitors of the sulfate-assimilation pathway as

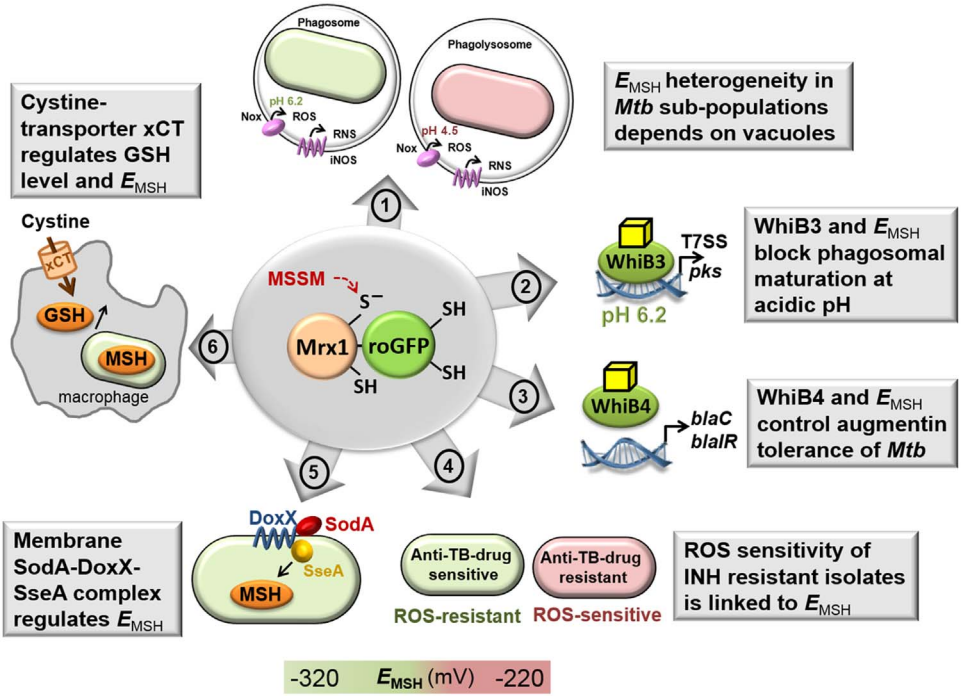

Fig. 7. Summary of the E_{MSH} changes in *Mtb* as measured using the Mrx1-roGFP2 biosensor. The genetically encoded Mrx1-roGFP2 biosensor contributed to a deeper understanding of pathogenicity, survival and anti-tuberculosis drug resistance mechanisms as follows: (1) E_{MSH} redox heterogeneity was shown in *Mtb* sub-populations that depends on the location in specific vacuole compartments [95]. (2) The WhiB3 sensor and E_{MSH} control induction of type-VII secretion systems and polyketide lipids under acid conditions in the phagosome to inhibit phagosomal maturation [96]. (3) The WhiB4 redox sensor and E_{MSH} control expression of β-lactamase to induce augmentin tolerance in the reduced *Mtb* population and augmentin killing in the oxidized *Mtb* population [97]. (4) Isoniazid (INH) resistant *Mtb* isolates have an oxidative E_{MSH} and are highly ROS-sensitive, while INH-sensitive strains are more resistant to ROS due to a more reduced E_{MSH} [98]. (5) The membrane-associated oxidoreductase complex (SodA-DoxX-SseA) regulates radical detoxification and MSH redox balance under infection conditions [109]. (6) The cystine-glutamate transporter xCT regulates cystine import into macrophages, resulting in increased host-GSH biosynthesis and a reduced E_{MSH} which contributes to TB disease in a mice infection model [110].

promising future anti-TB drugs to treat MDR/XDR, persistent and chronic *Mtb* infections. These main results revealed thus far using Mrx1-roGFP2 biosensor measurements in *Mtb* are summarized in the schematics of Fig. 7. Similar mechanisms might be relevant for other intracellular pathogens and persistent bacterial infections. As revealed in *Mtb* using the Mrx1-roGFP2 biosensor, redox heterogeneity of the intracellular pathogen *S*. Typhimurium could be also dependent on subvacuolar compartments. Inside the SCV, *S*. Typhimurium could be more tolerant to antibiotics due to a more reduced intrabacterial redox potential, which facilitates the persistent state. In contrast, cytosolic bacteria should have a more oxidized redox state and should be susceptible to clinical relevant antibiotics. The mechanisms of persistence and antibiotics resistance as result of redox heterogeneity remain interesting subject for future studies in redox infection biology.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (AN746/4-1 and AN746/4-2) within the SPP1710 on “Thiol-based Redox switches” and by the DFG Project C08 within the SFB973 to H.A. Further funding was provided by the DFG Research Training Group GRK1947, project (C1) and by an ERC Consolidator Grant (GA 615585) MYCOTHIOLOME to H.A.

Author disclosure statement

No competing financial interests exist.

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