Review

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Redox regulation in host-pathogen interactions: thiol switches and beyond

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Abstract: Our organism is exposed to pathogens on a daily basis. Owing to this age-old interaction, both pathogen and host evolved strategies to cope with these encounters. Here, we focus on the consequences of the direct encounter of cells of the innate immune system with bacteria. First, we will discuss the bacterial strategies to counteract powerful reactive species. Our emphasis lies on the effects of hypochlorous acid (HOCl), arguably the most powerful oxidant produced inside the phagolysosome of professional phagocytes. We will highlight individual examples of proteins in gram-negative bacteria activated by HOCl via thiol-disulfide switches, methionine sulfoxidation, and N-chlorination of basic amino acid side chains. Second, we will discuss the effects of HOCl on proteins of the host. Recent studies have shown that both host and bacteria address failing protein homeostasis by activation of chaperone-like holdases through N-chlorination. After discussing the role of individual proteins in the HOCl-defense, we will turn our attention to the examination of effects on host and pathogen on a systemic level. Recent studies using genetically encoded redox probes and redox proteomics highlight differences in redox homeostasis in host and pathogen and give first hints at potential cellular HOCl signaling beyond thiol-disulfide switch mechanisms.

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Introduction

Bacteria, and especially pathogenic bacteria, have to be able to effectively counter a number of different obstacles in order to survive. The most challenging one is perhaps the interaction with the host organism. The human host organism has evolved various strategies to effectively fight pathogens. During infection with bacteria, specialized phagocytes, such as neutrophils and macrophages, can engulf the pathogens into a membrane-derived vesicle, the phagosome, where, upon formation of the phagolysosome they bombard bacteria and other invaders with a complex mixture of reactive oxygen (ROS), nitrogen (RNS), and chlorine species (RCS). This process is called “oxidative burst” and is driven by an enzyme crucial for effective pathogen killing: the NADPH oxidase complex (NOX2). NOX2 is assembled at the phagosomal membrane and produces partially reduced oxygen by the addition of one electron, derived from NADPH, to molecular oxygen, yielding the ROS superoxide anion ($O_2^{\cdot -}$). Subsequently, $O_2^{\cdot -}$ is transformed into H_2O_2 and then the enzyme myeloperoxidase (MPO) turns H_2O_2 into one of the most potent oxidants produced *in vivo* – hypochlorous acid (HOCl) (for comprehensive reviews see Mortaz et al. 2018; Winterbourn et al. 2016). This formation of HOCl by MPO seems to be pH-dependent and less effective at higher pH (Atosuo and Suominen 2019). HOCl can cause irreversible damage to virtually all biomacromolecules (Albrich et al. 1981) and, especially, to proteins. Protein unfolding and aggregation are some of the common consequences of HOCl exposure (Hawkins and Davies 1999; Müller et al. 2014; Winter et al. 2008).

Therefore, it is not surprising that bacteria have developed counterstrategies to survive the “oxidative burst”. Some pathogens have the ability to manipulate phagocytic cells to decrease their antimicrobial activity. For instance, neutrophils infected with *Francisella tularensis* do not efficiently generate ROS, because the bacteria

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disrupt NOX2 assembly in the host. *F. tularensis* also impairs neutrophil activation by heterologous stimuli such as phorbol esters and opsonized zymosan particles. *F. tularensis* is then able to escape the phagosome and has been found to invade the neutrophil's cytosol (McCaffrey and Allen 2006). *Mycobacterium tuberculosis* can also manipulate host signaling pathways, ensuring that the phagocytic process stops at the early endosome stage and phagosome-lysosome fusion does not occur. *Mycobacteria* can then eventually escape from the phagosomes and translocate into the cytosol of macrophages (Ehrt and Schnappinger 2009; Jamwal et al. 2016; Meena and Rajni 2010).

But in bacteria lacking the capabilities to directly manipulate the host cells, counterstrategies typically involve the genetic upregulation of the expression and/or activation of specific protective proteins. The activation of the latter as well as the sensing to induce the former is typically accomplished by post-translational modification of protein side chains. In the case of sensing these highly reactive oxidant species this usually involves the oxidation of specific amino acids leading to a conformational change of the protein, allowing the activated protein to fulfill new functions (Hillion and Antelmann 2015). The most prominent modifications found under these conditions are thiol-disulfide switches, found for example in OxyR, RclR, and Hsp33 (Jakob et al. 1999; Parker et al. 2013; Zheng et al. 1998). Further modifications involve other thiol-derived modifications such as a sulfenamide switch (NemR) (Gray et al. 2013b) or a switch based on the sulfoxidation of methionine (HypT) (Drazic et al. 2013a). Additionally, under conditions of hypochlorite stress, some bacteria, such as *Bacillus* and *Staphylococcus* species, also use S-thiolation (formation of mixed disulfides between low molecular weight thiols, such as mycothiol and bacillithiol, and protein thiols) as an essential molecular mechanism that function in thiol protection and redox regulation (Imber et al. 2019; Ulrich and Jakob 2019).

In the case of individual proteins involved in redox sensing in bacteria, we will address two main protein groups: transcription factors and molecular chaperones. Transcriptional factors control HOCl-stress responses on the genetic level by upregulating the expression of survival genes and therefore enabling the bacteria to withstand HOCl and the accompanying oxidative stress. Overall, the genetic control of the response to HOCl and other reactive chlorine species is still not fully understood (Gray et al. 2013a). High HOCl concentrations in the cell do affect not only gene expression but also cause severe ATP depletion. Therefore, conventional ATP-dependent chaperon systems, such as GroEL/GroES or DnaK/DnaJ/GrpE systems fail to work. This is particularly dire, since HOCl, as mentioned, is by itself causing protein aggregation. Thus, a key strategy in overcoming HOCl stress is the prevention of the formation of cytotoxic protein aggregates, as those contribute to cellular stress and eventually cell death. For this purpose, bacteria have developed chaperone-like holdases (Hsp33 (Jakob et al. 1999), RidA (Müller et al. 2014), CnoX (Goemans et al. 2018b)), which are activated under oxidative and specifically HOCl stress conditions. These can bind and protect proteins that unfold upon HOCl-stress and prevent their inactivation and eventual aggregation or degradation. Hsp33, as some of the aforementioned transcription factors, is activated by a thiol-disulfide switch, whereas RidA and CnoX are activated by N-chlorination, a novel HOCl-specific switch mechanism. Once HOCl is removed from the cell, the ATP pool is restored. This allows the ATP-dependent foldases to pick up the holdases' client proteins and to refold them (recently reviewed in Goemans and Collet, 2019; Sultana et al. 2020).

Not only the above-mentioned individual proteins, but the bacterial cell as a whole is affected by the oxidative stress that occurs during host-pathogen interactions. Thus, global -omics are an important tool in the study of host-pathogen interactions. The fact that the transcription factors NemR, HypT, and RclR react towards hypochlorous acid was in part deduced by transcriptomics studies (Barth et al. 2009; Gray et al. 2013b; Parker et al. 2013). However, determining the overall redox state of bacteria and/or host cells during their interaction brings its own challenges. The use of redox-specific genetically encoded sensors, such as roGFP2, facilitated the quantitative measurement of the redox-state of bacteria and host cells with microscopic and fluorescence spectrometric methods and has advanced our understanding of oxidative stress and the role of HOCl in phagocytosis (Degrossoli et al. 2018; Van Der Heijden et al. 2015; Xie et al. 2019). Redox-proteomics experiments, guided by the roGFP2-based probes revealed a complete breakdown in the homeostasis of *E.coli*'s thiol proteome in phagocytized bacteria (Leichert et al. 2008; Xie et al. 2019).

But not only the bacterial cell is susceptible to hypochlorite-stress, very often the host organism is also affected by the consequences of HOCl production in phagocytes (Davies and Hawkins 2020). HOCl and other reactive species might "leak" from the phagolysosome into the host cytoplasm or even outside of the cell. We did indeed observe a shift in the cytoplasmic redox-homeostasis of phagocytizing neutrophils. Furthermore, HOCl (intracellular or extracellular) could serve as a signaling molecule; however, it is usually scavenged quickly through reaction with proteins. In the extracellular environment this transforms plasma proteins into HOCl-activated chaperone-like holdases, which protect host proteins. Those HOCl-activated

plasma proteins also serve as modulators of immune cell activity at the sites of infection (Gorudko et al. 2014; Ulfig et al. 2019; Ulfig and Leichert 2020).

In this review, we will discuss the role of redox switches in host-pathogen interaction, both from the viewpoint of pathogen and host. Our review focusses on the role of HOCl in these processes. First, we will highlight the responses of bacterial cells (with an emphasis on the gram-negative model organism *E.coli*) to reactive species produced by immune cells of the host organism, and we will provide an overview over well-studied stress-activated transcription regulators that could become activated under these conditions. Then we will review HOCl-activated chaperone-like holdases, a class of proteins that seems to play an important role in the response to severe HOCl-induced oxidative stress. Then, we will switch from individual proteins to an overview of the changes in the cellular redox-homeostasis of bacterial cells during host-pathogen interactions. Here we discuss the use of genetically encoded redox probes as well as redox-proteomic studies in bacteria exposed to immune cells. Finally, we will provide an overview of the effect of HOCl on the host organism in the vicinity of infection and the host cell's response to the elevated concentrations of HOCl during host-pathogen interaction.

HOCl-sensing in bacteria: thiol-based switches and beyond

First, we would like to highlight the bacterial response strategies towards HOCl and other so-called reactive chlorine species (RCS), such as monochloramines. Although sensor proteins that detect reactive oxygen (ROS) and reactive nitrogen species (RNS) and their regulatory networks have been well-characterized, less is known about the genetic responses to RCS (Gray et al. 2013a; Vázquez-Torres 2012). HOCl, which is produced within the phagolysosome of neutrophils, reacts with various amino acids inside the engulfed pathogen, leading to their oxidation. However, cysteine and methionine side-chains are, due to the exceptionally high second-order rate constants of their reaction, particularly prone to HOCl oxidation when compared to other amino acid residues (Pattison and Davies 2001; Winterbourn et al. 2016). Since reactions of HOCl with these residues often lead to the same products as their reaction with other reactive species (such as H_2O_2), HOCl was found to be able to efficiently activate redox regulators that are also involved in the response towards ROS, RNS or reactive electrophilic species (Gray et al. 2013a). Therefore, it is often difficult to distinguish between sensors, which react exclusively towards RCS and those, which evolved primarily to react towards other reactive species, and which react to RCS simply due to the fact that HOCl is so highly reactive. Nevertheless, recent evidence of RCS-specific transcription factors, such as RclR and HypT, has been provided (Drazic et al. 2013a; Parker et al. 2013). RclR senses HOCl via a thiol-disulfide switch. Nevertheless, even high concentrations of H_2O_2 were not able to fully activate RclR as a transcription factor, making it specific for HOCl (Parker et al. 2013). HypT is an especially interesting example, because it senses HOCl via methionine sulfoxidation, a reaction that occurs at high velocities in the presence of HOCl (second-order rate constant of the reaction $k = 3.4 \times 10^7\ M^{-1} s^{-1}$) but is quite slow in the presence of H_2O_2 (second-order rate constant of the reaction $k = 2 \times 10^{-2} M^{-1} s^{-1}$ (Davies 2016; Pattison and Davies 2001; Storkey et al. 2014). We will discuss the transcription factors that regulate stress-genes, which promote cell survival under oxidative stress conditions, predominantly in response to RCS, but also to other reactive species. Although the detection of the stressors by the transcription factors occurs fast, expression of the regulated genes, and protein synthesis may take up valuable time, which might be needed in face of a life-threatening oxidant as HOCl: without swift counteraction, HOCl causes protein aggregation and unfolding (Hawkins and Davies 1999). Therefore, bacteria have developed strategies to immediately protect protein homeostasis in response to HOCl stress. This is done via post-translational modification and activation of chaperone-like holdases. Prominent examples are the redox-regulated chaperones Hsp33, RidA, and CnoX (Goemans et al. 2018b; Jakob et al. 1999; Müller et al. 2014). Hsp33 is activated by oxidation of its cysteines and concomitant unfolding conditions. This could be high concentrations of H_2O_2 at elevated temperatures or in the presence of bile salts, or, HOCl, which by itself is a strong denaturing agent (Voth and Jakob 2017). Then again, RidA and CnoX are exclusively activated by RCS via an N-chlorination-based mechanism. In pathogenic bacteria, these redox-sensing regulators and chaperones often play a role as virulence factors and are used for adaptation to the host immune defense, establishing their important role in host-pathogen interactions (Dahl et al. 2015; Hillion and Antelmann 2015).

Gene regulation in response to oxidative stress in bacteria

OxyR is one of the best-characterized redox-sensitive regulators activated by a thiol-disulfide switch mechanism. As a LysR family repressor, it controls mainly the response to H_2O_2

in many gram-negative bacteria (Imlay 2008). OxyR oxidation caused by H_2O_2 occurs at the peroxidatic Cys199 that is oxidized to a sulfenic acid and subsequently forms an intramolecular disulfide bond with the resolving Cys208 in each of the four subunits of the OxyR tetramer (Lee et al. 2004; Pedre et al. 2018; Zheng et al. 1998). In *Escherichia coli*, oxidized OxyR positively regulates genes for cellular antioxidant systems, such as peroxidases, catalases, disulfide reductases (thioredoxin, glutaredoxins), Dps, and metal transporters (Imlay 2008) (Figure 1). Nevertheless, HOCl, as a potent oxidant of thiols (Hawkins et al. 2003), can also oxidize the cysteines in the OxyR molecule and activate the transcription of OxyR-regulated genes (Gebendorfer et al. 2012; Small et al. 2007). HOCl can be generated by immune cells, and indeed, we found cysteines of OxyR significantly more oxidized in phagocytized *E. coli*, when compared to non-phagocytized *E. coli* (Xie et al. 2019). Transcriptomic analysis of *E. coli* strains treated with HOCl revealed that, while the expression level of *oxyR* itself did not change after the oxidant treatment, the genes regulated by OxyR were moderately upregulated (Wang et al. 2009). In another experiment, Gundlach and Winter showed that an *E. coli* cell population, evolved in the lab to be resistant to high HOCl levels, contained partially oxidized OxyR and a constitutively upregulated OxyR regulon (Gundlach and Winter 2014). *In vitro,* oxidized OxyR could be reduced by both the thioredoxin and glutaredoxin systems. Studies with *E. coli* strains defective in either the thioredoxin or glutaredoxin systems indicate that OxyR is preferably reduced by glutaredoxin *in vivo* (Åslund et al. 1999), while *Pseudomonas aeruginosa* seems to use the thioredoxin system (Wei et al. 2012).

To summarize, OxyR, while primarily a sensor for hydrogen peroxide, is also a significant factor in response to HOCl. As such, it plays a crucial role in host-pathogen interactions, corroborated by the fact that OxyR was found to contribute to the virulence of *E. coli* strain (O17:K+:H18) in animal models of urinary tract infection (Johnson et al. 2013). Moreover, OxyR was also shown to be important for the virulence of other bacteria (such as *P. aeruginosa* (Lau et al. 2005), *Xanthomonas oryzae* (Yu et al. 2016), *Vibrio parahaemolyticus* (Chung et al. 2016), *Ralstonia solanacearum* (Flores-Cruz and Allen 2011), etc.). In the next sections we will cover more HOCl-specific regulators.

HOCl-sensing by the formation of a sulfenamide bond in NemR

NemR is an example of a redox-regulated transcription factor specific for both HOCl and reactive electrophilic species (Gray et al. 2013b; Umezawa et al. 2008). *E. coli* NemR is a transcription regulator from the TetR family. It acts as a repressor for the *nemRA* operon and the *gloA* gene. Under non-stress conditions, the expression of these genes is repressed by NemR binding firmly to the *nemRA* and *gloA* promoter regions (Gray et al. 2013b). NemA (N-ethylmaleimide reductase) acts as an oxidoreductase and can reduce electrophiles, such as glyoxal, methylglyoxal, oxidized quinones, and *N*-ethylmaleimide. GloA (glyoxalase 1) is one of the essential proteins in the cellular detoxification of glyoxal and methylglyoxal (Lee et al. 2013). Both NemA and GloA are required for survival in the presence of HOCl, due to the high production of a variety of toxic electrophiles *in vivo* under these conditions (Gray et al. 2013b). NemR is sensitive towards and is selectively inactivated by quinones and aldehydes, as well as hypochlorous acid and chloramines. The presence of these oxidants leads to the dissociation of NemR from the DNA, leading to the expression of the *nemRA* operon and *gloA* (Gray et al. 2013b). The underlying mechanism is a sulfenamide-based thiol switch, which forms after oxidation of Cys106, presumably to a sulfenic acid. This sulfenic acid then reacts with the neighboring ε-amino group of Lys175, forming a sulfenamide linkage, which induces a conformational change (Gray et al. 2015) (Figure 1). Additionally, RCS-mediated chlorination leads to an oxidative cascade, oxidizing and chlorinating other cysteine residues in NemR (Gray et al. 2013b; Hillion and Antelmann 2015).

HOCl-specific disulfide-bond formation in the regulator RclR

Another example of an HOCl-specific redox regulator is RclR from *E.coli.* RclR is specific for chlorine species, such as HOCl, but does not respond to the treatment with other ROS, electrophiles, or thiol-reactive compounds. RclR is a redox-sensing transcriptional activator of the AraC-family, which also uses a thiol-based oxidation mechanism to sense HOCl. Upon treatment with HOCl, RclR's Cys21 is oxidized, presumably to a sulfenyl-chloride, which hydrolyzes to a sulfenic acid. This leads to a partial activation of the protein, followed by the formation of an intramolecular disulfide bond between Cys21 and Cys89. In this oxidized form, RclR is stabilized and can regulate the expression of three genes essential for bacterial survival of RCS-stress (*rclA*, *rclB*, and *rclC*) (Figure 1). The deletion of any of these genes increased RCS stress sensitivity, indicating that the respective gene products play an essential role in surviving RCS treatment (Parker et al. 2013). Recently, an insight into

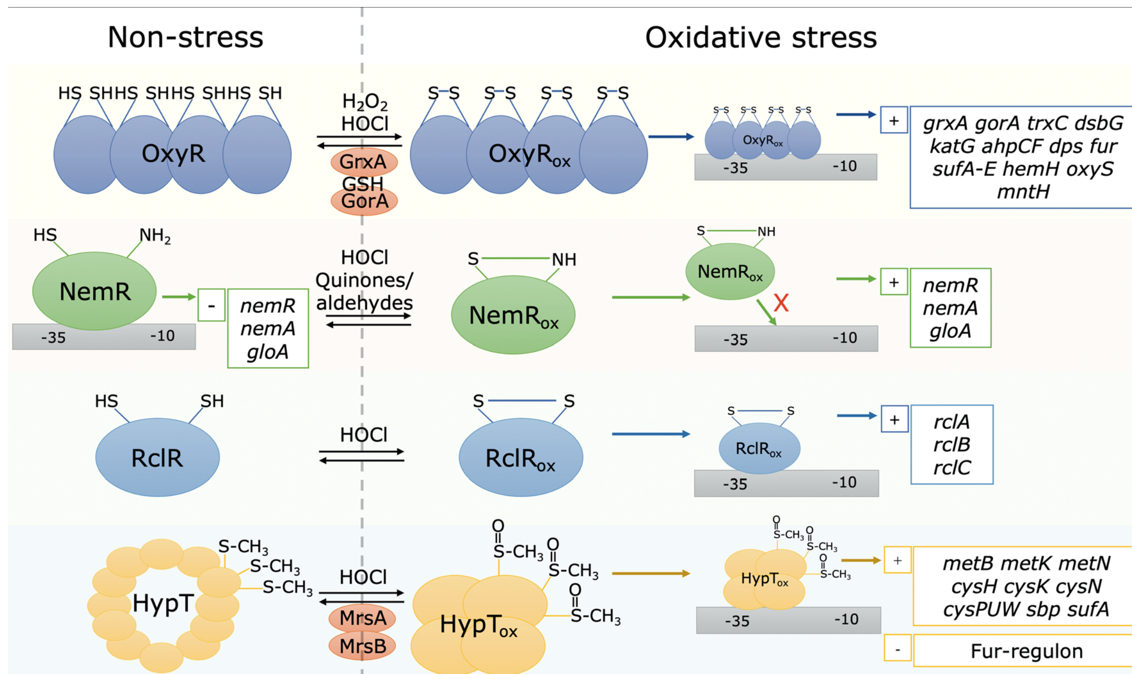

Figure 1: HOCl-regulated transcription factors in *E. coli*.
Upon exposure to H_2O_2 or HOCl, cysteines in the OxyR molecule are oxidized and form disulfide bridges. In this form OxyR can bind to the promoter regions of its target genes. OxyR positively regulates genes of peroxide detoxification (*katG*, *ahpCF*), the Fe-storage protein miniferritin (*dps*), glutaredoxin (*grxA*), thioredoxin (*trxC*), glutathione reductase (*gorA*), the periplasmic sulfenic acid oxidoreductase (*dsbG*), the ferric uptake regulator (*fur*), the Fe-S-cluster assembly machinery (*sufABCDE*), ferrochelatase (*hemH*), manganese import (*mntH*), and the small RNA *oxyS*. Once the stress subsides, OxyR is reduced by thiol reducing systems such as the GrxA/GSH/Gor system. Under non-stress conditions, NemR binds firmly to the promoter region of the *nemRA* operon and the *gloA* gene, repressing their expression. NemR is oxidized by HOCl, leading to the formation of a sulfenamide linkage between a cysteine and a lysine residue in NemR. Once NemR is oxidized, it can no longer bind to the DNA and the expression of *nemRA* and *gloA* is induced. Products of these genes detoxify glyoxal, methylglyoxal, and other reactive species in the cell. RclR is activated by HOCl by a thiol-disulfide switch mechanism. Upon oxidation it activates the expression of three genes involved in RCS-stress survival (*rclA*, *rclB*, and *rclC*). In the absence of HOCl, thiol-reducing systems within the cell can presumably remove the disulfide bond, inactivating RclR. In its reduced form HypT forms a dodecamer, however, after oxidation with HOCl, it forms a tetramer. HOCl oxidizes three methionines in every HypT subunit, leading to its activation. In its active form HypT positively regulates genes for sulfur, cysteine and methionine biosynthesis (*metB*, *metK*, *metN*, *cysH*, *cysK*, *cysN*, *cysPUW*, *sbp*, *sufA*) and negatively regulates genes of the Fur-regulon that regulate iron uptake. HypT can be inactivated by methionine sulfoxide reductases MsrA and MsrB, once the stress subsides.

the function of the RclA protein was obtained. Accelerated by Cu^{2+} ions, RclA can oxidize NADH, consuming oxygen and, therefore, leading to a lowering of oxygen levels *in vitro*. This could contribute to the inhibition of the oxidative burst capacity in the phagosomes of macrophages (Baek et al. 2020; Derke et al. 2019). Nevertheless, the exact functions of the RclB and RclC proteins for HOCl stress protection remain unknown (Parker et al. 2013).

HOCl activates HypT through methionine sulfoxidation

HypT is an HOCl-specific redox sensor and transcriptional activator (Gebendorfer et al. 2012). HypT belongs, like OxyR, to the LysR-family of transcriptional regulators. But unlike OxyR, which has a tetrameric structure, HypT was shown to form a dodecameric ring-like structure (Drazic et al. 2014). These HypT-dodecamers dissociate into dimers and tetramers in the presence of DNA, which then act as the DNA-binding species. These tetramers were found *in vivo* in HOCl-treated *E. coli* cells (Drazic et al. 2014; Gebendorfer et al. 2012). HypT contributes to HOCl resistance in *E. coli* by upregulating genes involved in the biosynthesis of sulfur, methionine, and cysteine (*metB, metK, metN, cysH, cysK, cysN, cysPUW, sbp, sufA*), while downregulating genes promoting iron acquisition (genes of the Fur-regulon: *entC, entH, fecABCDE, fecR, fepCD, ryhB, tonB, yncE*). The upregulation of genes involved in cysteine and methionine biosynthesis enables the cell to restore the pool of reduced cysteine and methionine, which are both rapidly oxidized

by HOCl. Downregulation of the iron acquisition machinery might prove useful in the presence of HOCl, as *E. coli fur* mutants, which have higher intracellular iron levels, were shown to be more sensitive to HOCl stress, likely due to increased ROS generation via the Fenton reaction (Dukan and Touati 1996; Gebendorfer et al. 2012). Interestingly, instead of a thiol-based switch, HypT uses the reversible oxidation of methionine to methionine sulfoxide (Met-SO) as the activating event. The cysteine residues of HypT seem to only play a role in protein stability and oligomerization (Drazic et al. 2013b). Biochemical and mutational studies have shown that three methionine residues Met123, Met206, and Met230 are involved in the regulation of HypT activity. These are oxidized to Met-SO after treatment of HypT with HOCl *in vitro* (Drazic et al. 2013a, 2014). Individual substitution of these methionine residues by isoleucine in HypT abolished the HOCl resistance normally conferred by HypT to bacterial cells, whereas the substitution by glutamine, mimicking the Met-SO form of HypT, resulted in constitutively active HypT (Drazic et al. 2013a). As mentioned above methionine oxidation by H_2O_2 is nine orders of magnitude slower (second-order rate constant $k = 2 \times 10^{-2}\ M^{-1}s^{-1}$) than oxidation by HOCl (second-order rate constant $k = 3.4 \times 10^{7}\ M^{-1}\ s^{-1}$) (Davies 2016; Pattison and Davies 2001; Storkey et al. 2014). Therefore, the use of methionine oxidation as switching mechanism allows for a specific sensing of HOCl by HypT, in contrast to OxyR and NemR, which can be both activated by other thiol-modifying stimuli. Similar to the aforementioned regulators, HOCl-oxidized and activated HypT can be reduced within the cell upon return to non-stress conditions by cellular antioxidant systems: MsrA and MsrB, *E. coli*'s methionine sulfoxide reductases (Figure 1) (Drazic et al. 2013a).

Other transcription factors have also been shown to play a part in overcoming HOCl-stress. One example is the ArcAB two-component system, which, under aerobic conditions, has been identified as a potential HOCl- and H_2O_2-responsive regulator in *E. coli*, *Salmonella enterica* and other pathogenic species (Loui et al. 2009; Lu et al. 2002; Pardo-Esté et al. 2018). The response regulator ArcA negatively regulates the expression of two porin genes, *ompD* and *ompW*, in response to both H_2O_2 and HOCl (Calderón et al. 2011; Morales et al. 2012), thereby reducing the influx of these oxidants into phagocytized bacteria. Nevertheless, it is currently unknown by which mechanism ArcAB senses HOCl. As a regulator that responds to oxidative species produced by immune cells it is required for virulence in *Haemophilus influenza*, *Vibrio cholerae*, *Actinobacillus pleuropneumoniae*, and *S. enterica* ser. Typhimurium (Buettner et al. 2008; Morales et al. 2013; Sengupta et al. 2003; De Souza-Hart et al. 2003; Wong et al. 2007).

Another example is SoxR. The oxidation of its Fe-S cluster upon exposure to superoxide or presumably HOCl activates the expression of genes encoding SOD, efflux proteins, and nitroreductases and the repression of porin expression. However, it is unclear if any of these upregulated proteins actually take part in cell protection against RCS-stress (Crack et al. 2012; Gray et al. 2013a).

While we focus on transcriptional regulators in gram-negative bacteria in this review, it should be noted that gram-positive bacteria possess transcriptional regulators that can be activated by RCS-stress as well, including OhrR, Spx, and PerR. Like OxyR, those are known to also react with oxidants other than HOCl (Gray et al. 2013a; Hillion and Antelmann 2015). Additionally, recently two new transcriptional regulators that are specific to HOCl has been identified: HypR in *Staphylococcus aureus* and HypS in *Mycobacterium smegmatis* (Loi et al. 2018; Tung et al. 2020).

Protecting bacterial protein homeostasis under HOCl stress: activation of Hsp33 by disulfide bond formation

As we mentioned above, two important factors are necessary to counteract HOCl-stress. First is upregulation of protective proteins, which is achieved by activation of transcription regulators. Second is maintenance of protein homeostasis and the prevention of protein unfolding by HOCl. The latter is done by the activation of redox-regulated chaperones, such as Hsp33. Transcriptional analysis revealed that Hsp33 is constitutively expressed in the cell at a low level; however, the expression levels are drastically increased under rpoH (σ^{32}) control, once bacteria experience stress conditions causing protein unfolding (Chuang and Blattner 1993; Dahl et al. 2015; Jakob et al. 1999; Nonaka et al. 2006). In reduced Hsp33, four highly conserved cysteines in the C-terminal redox switch domain are engaged in the high-affinity binding of one Zn^{2+} ion. The Zn^{2+} stabilizes the cysteine thiols in their highly reactive thiolate anion form (Jakob et al. 2000). Hsp33 can be activated by a combination of slow-acting oxidants, such as H_2O_2, together with protein unfolding conditions (elevated temperatures or bile salts), or by exposure to the protein unfolding oxidant HOCl (Cremers et al. 2014; Jakob et al. 1999; Krewing et al. 2019; Winter et al. 2008). Under

these stress conditions, the cysteines form two intramolecular disulfide bonds, resulting in the release of zinc and unfolding of the C-terminal region of the protein. This unfolding destabilizes the upstream linker region, which connects the protein's N-terminus with the C-terminal redox switch domain, leading to the dimerization and activation of Hsp33 as a chaperone (Cremers et al. 2010). Furthermore, strong oxidants such as HOCl could potentially lead to even higher oligomer formation of oxidized Hsp33 (Graumann et al. 2001), potentially increasing the client binding surface (Reichmann et al. 2012). Upon return to non-stress conditions, oxidized Hsp33 dimers can be reduced by the cellular glutaredoxin and thioredoxin systems (Hoffmann et al. 2004; Winter et al. 2005); however, Hsp33's client proteins remain bound. Hsp33 can then pass its client proteins to the DnaK/DnaJ/GrpE chaperone system, which allows for the refolding of the client proteins to their native state (Dahl et al. 2015) (Figure 2). Hsp33's importance for the detoxification of HOCl-induced toxic protein aggregates has been further supported by the fact that *E. coli* strains lacking Hsp33 are more sensitive towards HOCl treatment in comparison to the wild type (Winter et al. 2005). Additionally, because Hsp33 is highly conserved in prokaryotes, but not present in higher eukaryotes, it is a potentially promising drug target, as its lack diminishes the virulence of pathogenic bacteria such as pathogenic *E. coli* variants or *V. cholerae* (Dahl et al. 2015).

N-chlorination, a novel mechanism to activate the chaperone-like holdase RidA

If exposed to high doses of HOCl, the ε-amino group of lysine residues, the guanidinium group of arginines, and the terminal amino groups of proteins can undergo reversible N-chlorination. Previously, it was reported that the formation of N-chloramines has a damaging effect on proteins, causing their inactivation, unfolding, and aggregation (Hawkins and Davies 1999). However, more recent evidence emerged that numerous bacterial and nonbacterial proteins can become activated upon HOCl- and chloramine treatment (Goemans et al. 2018b; Müller et al. 2014; Ulfig et al. 2019). These proteins typically turn into holdase-like chaperones.

The first example of protein activation by HOCl via N-chlorination is RidA from *E. coli*. RidA belongs to the highly conserved, but functionally diverse YjgF/YER057c/UK114 protein family. Under non-stress conditions, *E. coli* RidA serves as an enamine/imine deaminase, which accelerates the IlvA-catalyzed deamination of threonine into 2-ketobutyrate (Lambrecht et al. 2012). RidA is also involved in the nitrosative stress response. Reaction with reactive nitrogen species leads to S-nitrosylation and thiol overoxidation of RidA's sole cysteine C107 to sulfinic and sulfonic acid. Upon the overoxidation of C107, the enamine/imine deaminase activity of RidA is lost (Lindemann et al. 2013).

However, in the presence of HOCl or monochloramines, RidA does not only lose its enamine/imine deaminase activity but also inhibits the activity of IlvA, forming a stable complex with this protein. This indicated that RidA might act as a redox-regulated chaperone-like holdase under HOCl-stress conditions, which was then further confirmed by aggregation studies using a variety of different client proteins, including unfolded IlvA. Untreated RidA, however, does not exhibit this chaperone activity. Notably, HOCl and other RCS, such as monochloramines, activate RidA, whereas incubation with other oxidants, such as hydrogen peroxide or diamide, had no activating effect. A 10-fold molar excess of HOCl or monochloramines over RidA is enough to activate RidA and maximize its chaperone-like holdase activity (Müller et al. 2014).

Moreover, activation of RidA is cysteine-independent, and a cysteine-free variant is fully active after exposure to HOCl (Müller et al. 2014). Subsequent structural studies revealed that RidA activation is connected with an increase in surface hydrophobicity of the protein, a hallmark of binding sites in chaperones (Frydman 2001), and potentially the formation of higher oligomers. The RCS treatment decreases free amino group content of RidA and leads to the reversible addition of up to 10 chlorine atoms. These results led to the conclusion that the N-chlorination of lysine and/or arginine residues is likely responsible for the RCS-induced activation of RidA's chaperone activity. RidA's activation was shown to be fully reversible, and treatment with DTT, ascorbic acid, or other physiological reductants, such as GSH and thioredoxin, can reverse RidA's chaperone activity *in vitro* (Figure 2). The inactivation of the N-chlorinated RidA by thiol-based reductants is based on the ability of N-chloramines to oxidize thiols, presumably through S-chlorination and subsequent hydrolysis of the sulfenyl chloride to a sulfenic acid (Peskin and Winterbourn 2001). Analysis of RidA's client proteins from *E. coli* in HOCl treated cell lysates revealed an enrichment of cytosolic proteins of the primary metabolism in line with their cellular abundance, which points towards an unspecific binding preference of N-chlorinated RidA to unfolded proteins *in vivo*. Numerous cysteine-free client proteins were identified, demonstrating that binding of RidA to its client proteins does not depend on disulfide bond formation. Moreover, in the presence of 1–2 mM HOCl

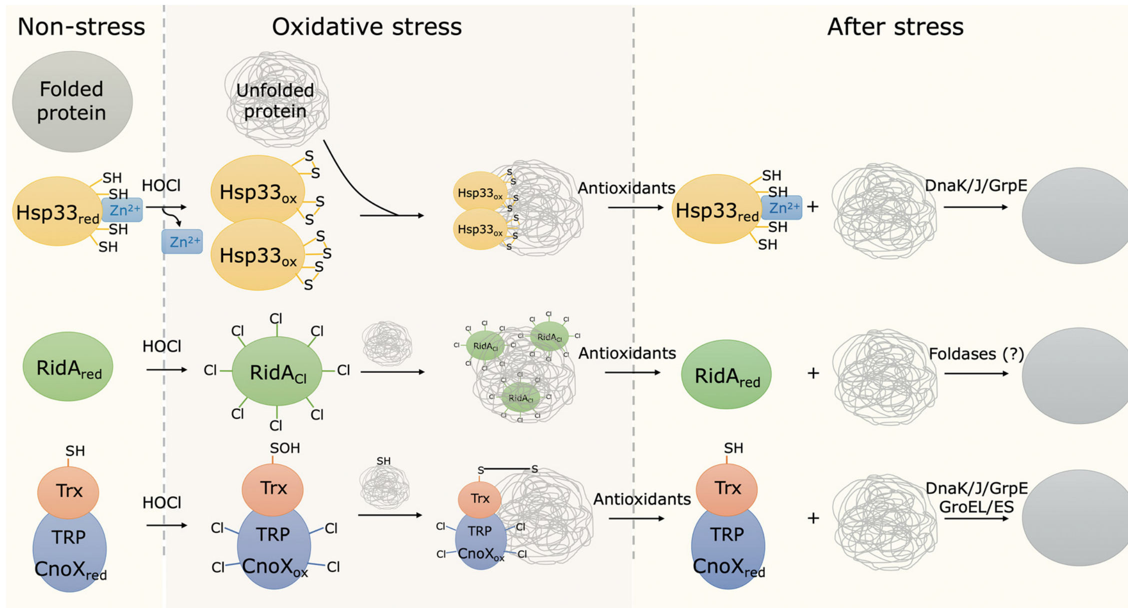

Figure 2: HOCl-activated chaperone-like holdases.
HOCl causes oxidation and unfolding of a number of cytoplasmic proteins. Under these conditions, however, the chaperone-like holdases Hsp33, RidA and CnoX are activated. Once Hsp33 is oxidized, two disulfide bonds in its molecule are formed and zinc ion is released, which allows Hsp33 to dimerize and bind unfolded proteins. RidA is activated by HOCl via N-chlorination and in its chlorinated form it can bind unfolded client proteins. CnoX has two protein domains: a tetratricopeptide repeats domain (TRP), which is N-chlorinated, and a thioredoxin (Trx) domain with a single cysteine residue in its active site. This cysteine is oxidized by HOCl to a sulfenic acid. The oxidized cysteine in the Trx domain can then form a reversible disulfide bond with the bound client protein, protecting its cysteines from overoxidation, while the TRP domain binds the unfolded regions of the client protein. Once the stress subsides, the chaperone-like holdases are inactivated by cellular antioxidant systems such as thioredoxin or glutathione and the client proteins are transferred to ATP-dependent chaperones such as DnaK/J/GrpE or GroEL/ES for refolding.

a Δ*ridA* strain showed a significantly prolonged lag phase when compared to the wild-type strain, indicating that RidA has a role in protecting *E. coli* cells against the consequences of HOCl-mediated stress conditions (Müller et al. 2014).

Protection from unfolding and cysteine oxidation by one protein: the “chaperedoxin” CnoX

Recently, another bacterial protein, CnoX, was found to be activated by a mechanism similar to RidA’s. CnoX is inactive under non-stress conditions, whereas upon exposure to HOCl, it turns into an effective chaperone-like holdase. Chlorinated residues were found to be located in four atypical tetratricopeptide repeats (TPR) at the C-terminus of CnoX (Goemans et al. 2018b). These TPR motifs are often present in holdases and folding factors and mediate protein-protein interaction (Kenneth and Ratajczak 2011). Chaperone activity of CnoX is tightly connected to the chlorination of the TRP domain, as the *E. coli* strain lacking the TRP domain was not able to complement the growth phenotype of a *cnoX*-deficient strain upon exposure to HOCl *in vitro* (Goemans et al. 2018b). Remarkably, after stress, CnoX was not only able to transfer its substrate to the DnaK/DnaJ/GrpE foldase system (like Hsp33) but also was shown to successfully cooperate with GroEL/ES system. Therefore, CnoX is the first known holdase that has been shown to use the GroEL/ES to assist with protein refolding (Goemans and Collet 2019). Moreover, the TRP motif in CnoX is connected to an N-terminal thioredoxin (Trx) fold, typically found in oxidoreductases. Thus, CnoX is not only a chaperone-like holdase that can bind a variety of substrate proteins and prevent their aggregation but, thanks to the thioredoxin domain, CnoX can also protect its clients from thiol overoxidation by forming mixed disulfides with their cysteines (Goemans et al. 2018a) (Figure 2). Similarly to the Δ*ridA* strain, Δ*cnoX* strain was highly sensitive to 2 mM HOCl, but the growth was fully restored by expression of wild-type CnoX from a plasmid, indicating that the sensitivity of this deletion strain to HOCl is a consequence of the loss of the corresponding protein

(Goemans et al. 2018b). The ability to create mixed disulfides, protecting the proteins from overoxidation, in combination with chaperone activity, classified CnoX to a first member of the newly formed class of proteins termed chaperedoxins (Goemans et al. 2018b). As such CnoX kills two birds with one stone: it protects its clients against HOCl-induced aggregation and overoxidation of its thiol residues.

HOCl sensing in the host

As outlined in the previous chapters, *E. coli* and other bacteria have evolved several systems to sense HOCl produced by the host and to adequately react towards its presence. To our current knowledge, the sensing of HOCl in transcription factors is based on the reversible modification of sulfur-containing amino acids, such as cysteine oxidation to disulfides or sulfenamides, or methionine sulfoxidation. Regulation by N-chlorination has not yet been observed in these cases, but a new class of systems involved in protein homeostasis has been shown to prevent protein unfolding when N-chlorinated. We now want to turn our attention towards the host.

Extracellular HOCl: locally confined to sites of inflammation

At first glance, dedicated signaling pathways that sense the presence of extracellular HOCl seem unnecessary in the host, as a systemic elevation of the HOCl-concentration is not very likely, with HOCl production being mostly confined to immune cells and especially to neutrophils. Nevertheless, it has been estimated that neutrophils can produce substantial amounts of HOCl that leak into the extracellular space: they have been reported to generate HOCl at concentrations of up to 25–50 mM per hour (Summers et al. 2008; Weiss et al. 1982). Thus, in the confined space of the interstitial fluid of inflamed tissues, HOCl accumulates at levels that could conceivably lead to changes in protein and cell activity. Consequently, a number of effects of extracellular HOCl on cells has been described, such as the activation of NF-κB in T-lymphocytes (Schoonbroodt et al. 1997) and the activation of p53 in fibroblasts (Vile et al. 1998). It has been shown that HOCl stimulates the MAP kinase pathway and enhances cell survival (Midwinter et al. 2001), and, using an enzymatic HOCl-generating model, HOCl has been found to affect iron metabolism (Mütze et al. 2003). HOCl can also control the activity of matrix metalloprotease through oxidative inactivation (Fu et al. 2004). Further effects of HOCl on cells that have been described are the induction of apoptosis by extracellular HOCl in transformed cells in the so-called HOCl-signaling pathway. However, the action of hypochlorous acid in this pathway is indirect: HOCl needs to further react with extracellular superoxide to generate the actual apoptosis-inducing hydroxyl radical (Bechtel and Bauer 2009). The typically low levels of these oxidants in the extracellular space might limit this reaction under physiological conditions. It has also been shown that the presence of exogenous HOCl affects insulin resistance through the phosphorylation state of protein kinase Cθ, but this regulation is mediated through peroxynitrite (Zhou et al. 2015). The indirect nature of these extracellular “HOCl-sensing” signaling pathways suggests that direct HOCl-mediated signaling is most likely confined to close spatiotemporal vicinity of its production, in line with its high reactivity and low stability in a biological system. This close spatiotemporal vicinity is probably achieved at the location of acute inflammatory processes. Here, extracellular proteins can be exposed to substantial concentrations of HOCl, when close to producing cells (Lampert and Weiss 1983). As mentioned before, a major effect of HOCl is the induction of protein aggregation (Winter et al. 2008). Thus, most likely, the maintenance of protein homeostasis under elevated HOCl concentrations plays a crucial role in the host as well. And indeed, alpha-2-macroglobulin, a holdase-type chaperone found in blood plasma, shows elevated activity, when exposed to HOCl (Wyatt et al. 2014). Other plasma proteins have been shown to be transformed into so-called advanced oxidation protein products (“AOPPs”) upon HOCl exposure (Witko-Sarsat et al. 1996). These AOPPs are then able to bind other proteins (Gorudko et al. 2014; Witko-Sarsat et al. 2003). The mechanism, by which these proteins were turned into chaperone-like holdases remained unclear. Due to the fact that extracellular proteins typically undergo oxidative folding in the ER and most of their cysteines are already engaged in disulfide bonds, further regulative oxidation by HOCl can be mostly excluded in those cases. However, we could recently show that N-chlorination is the mechanism that enhances alpha-2-macroglobulin activity and turns the majority of plasma proteins (based on abundance) into effective chaperone-like holdases (Ulfig et al. 2019). We could furthermore demonstrate that N-chlorination of plasma proteins is a major factor that turns them into potent activators of immune cells. These N-chlorinated plasma proteins are also pro-survival factors for immune cells, hinting at potential HOCl-sensing mechanisms that utilize plasma proteins as mediators.

HOCl as intracellular messenger

The consideration that direct HOCl-mediated signaling needs to happen in close spatial and temporal vicinity of its production is probably even more true within the cytosol, with its high abundance of thiols and other antioxidants that would inactivate HOCl. Nevertheless, there is evidence that HOCl is indeed a required messenger for effective NETosis in neutrophils, the cell type that produces the highest amounts of HOCl: neutrophils of patients lacking MPO, the HOCl-generating enzyme, cannot form NETs effectively (Metzler et al. 2011). Experiments showed that low levels of HOCl regulate NET release by neutrophils (Palmer et al. 2012). Nevertheless, extracellular HOCl cannot rescue the lack of cellular MPO in neutrophils stimulated with PMA (Björnsdottir et al. 2015), further corroborating the requirement of spatiotemporal vicinity for effective HOCl signaling. The exact nature of the involvement of HOCl in NETosis-signaling is not understood. Two major pathways are involved in NET-formation: One dependent on protein kinase C (PKC), the other on phosphoinositide-3-kinase (PI3K). While the former is involved in PMA-dependent NET formation, the other is involved in the formation of NETs in neutrophils exposed to *E. coli*. Nevertheless, NET formation in both cases (PMA-or *E. coli*-induced) can be abrogated by inhibition of MPO (Xie et al. 2020), suggesting that HOCl either interacts with both pathways, or a third, as of yet unknown, pathway.

Global studies addressing redox-regulation in host-pathogen interactions: pathogen side

So far, we have reviewed studies on individual proteins and pathways that are redox-regulated in a host-pathogen setting. These have been mainly found through biochemical experiments, on a case by case basis. We now want to review global methodologies to address and quantify redox regulation in bacteria during host-pathogen interactions.

Fluorescent redox probes can be used to monitor the oxidants that pathogens are exposed to in the phagolysosome. Redox sensitive dyes such as $DCFH_2$ or the pH and HOCl-sensing cHOClate can be attached to yeast particles to monitor the kinetics of ROS production in the phagolysosome (Thekkan et al. 2019; Tlili et al. 2011). Other probes specific for HOCl, such as RS-19, have also been successfully used to monitor the production of hypohalous acids within the phagolysosome (Albrett et al. 2018). Nevertheless, these probes measure the reactive species within the phagolysosome, not necessarily within the pathogen. Here, genetically encoded fluorescent redox probes based on roGFP2 proved useful: roGFP2 is a variant of GFP, which contains two engineered cysteine residues that can form a disulfide bond upon oxidation (Hanson et al. 2004). This disulfide bond formation changes its fluorescence properties, increasing the excitation maximum at 405 nm while simultaneously lowering the excitation maximum at 488 nm. This shift allows for the ratiometric quantitation of the probe's redox state (see Morgan et al., 2011 for a comprehensive review). In bacteria, these probes can be used to measure the redox state in general and in mutant strains under constitutive oxidative stress (Reuter et al. 2019). Expressed in *Salmonella*, roGFP2 was used to directly measure the oxidative stress dynamics in bacteria during macrophage infection (Van Der Heijden et al. 2015). As roGFP2 by itself is not very reactive toward glutathione or hydrogen peroxide, fusion probes with GRX1 and Orp1 have been created (Gutscher et al. 2008, 2009). These attached redox proteins are highly susceptible to oxidation by either oxidized glutathione (GRX1) or hydrogen peroxide (Orp1) and transfer the thiol oxidation to the fused roGFP2, effectively equilibrating the probe with the surrounding oxidants. We expressed these probes in *E. coli* and saw that the redox homeostasis broke down completely in bacteria phagocytized by neutrophils and all three probes were oxidized indiscriminately (Degrossoli et al. 2018) (Figure 3A). Combined with our data showing that roGFP2-based probes are highly susceptible toward hypochlorous acid (Müller et al. 2017a, b), we concluded that HOCl is the oxidant causing the (virtually complete) oxidation of the probes in phagocytized bacteria, which could be confirmed by pharmacological inhibition of myeloperoxidase (Degrossoli et al. 2018). While direct oxidation of roGFP2-based probes by HOCl has been demonstrated *in vitro*, parts of the *in vivo* oxidation by HOCl could potentially be caused by indirect means. The thiols of GSH and glutaredoxins are prone to oxidation by HOCl as well and thus could contribute to the probe`s oxidation. Other fusion proteins with roGFP2 have been recently developed, including those fused to redox proteins from *Plasmodium falciparum* (roGFP2 fused to the peroxiredoxin *Pf*AOP) (Staudacher et al. 2018) and low molecular redoxins from several bacteria, such as *S. aureus*, *Corynebacterium glutamicum* and *M. tuberculosis* (Loi et al. 2017; Tung et al. 2018, 2019). Additionally, rationally designed probes sensitive enough to measure metabolic base-line levels of oxidants were created by fusing roGFP2 to peroxiredoxin mutants lacking the resolving cysteine, such as $Tsa2\Delta C_R$

from yeast and human Prx2(C172A) (Morgan et al. 2016; Pastor-Flores et al. 2020). These probes expand our toolkit for monitoring the cellular redox state in host-pathogen interactions.

In order to elucidate the effects of the massive oxidative stress we saw reflected in the probe's redox states on *E. coli*'s "own" thiol residues, we need to turn to global -omics technologies. Traditional methodologies for the global assessment of regulatory processes in bacteria include transcriptomics and proteomics. In this way, the regulons of the aforementioned regulators OxyR, and HypT could be defined and NemR's susceptibility towards bleach was uncovered (Gebendorfer et al. 2012; Gray et al. 2013b; Zheng et al. 2001) and the broad client spectrum of HOCl-treated RidA was discovered (Müller et al. 2014). These methods can determine the abundance of transcripts and proteins in organisms or cell lysates; however, they usually provide only limited information on oxidative modifications. To identify, and more importantly, quantify the oxidative modification of protein thiols, redox proteomics methods need to be employed. Those use thiol-specific, MS-detectable probes that can be attached to cysteine residues in a differential trapping approach. First, all *in vivo* reduced cysteines are chemically blocked with this probe. Then, reversibly oxidized thiols are reduced *in vitro* and labeled with an isotopically different, but otherwise identical version of the probe. We established one of the first of these methods, which we termed OxICAT, because it detects the oxidation state of the thiol proteome using ICAT chemistry (isotope coded affinity tags) (Leichert et al. 2008). Methodologies based on other, sometimes more capable or highly chemoselective probes, such as iodoTMT or dimedone derivatives, have been developed more recently (Pan et al. 2014; Paulsen et al. 2012 and see Nietzel et al. 2017; Yang et al. 2016 for recent, comprehensive reviews). Either in the primary MS or in the pattern of the fragment spectra, those isotopically different forms of the probe can be quantified and then those signals can be assigned to the oxidized or reduced quantities of a cysteine-containing peptide (Thamsen and Jakob 2011). In

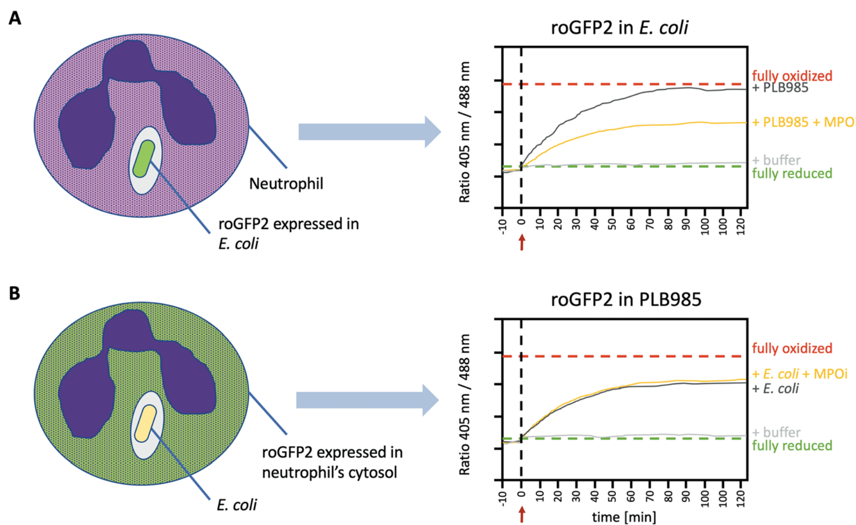

Figure 3: Schematic drawing of the redox state of *E. coli* and neutrophil-like PLB-985 cells upon phagocytosis as monitored by roGFP2 fluorescence.

In a co-incubation assay, *E. coli* cells were mixed with differentiated neutrophil-like PLB-985 cells at an MOI (multiplicity of infection) of 10:1 (*E. coli* : PLB-985). Then, the redox state of roGFP2 was tracked by measuring fluorescence intensities at 510 nm at the excitation wavelengths 488 and 405 nm. The fluorescence excitation ratio (405/488 nm) was used as measurement of probe oxidation and all values could be compared to the values of a chemically oxidized (red dashed line) and reduced (green dashed line) probe. (A) When roGFP2-expressing *E. coli* were mixed with differentiated PLB-985 cells, the redox-state of roGFP2 in the *E. coli* cells increased in a time-dependent manner, reaching its (virtually fully oxidized) maximum after approximately 80 min (dark grey line), while buffer treatment had a negligible effect on the probe's oxidation (light grey line). Pre-treatment of neutrophil-like cells with a myeloperoxidase inhibitor (MPOi) resulted in a significant attenuation of the probes' response in phagocytized *E. coli* cells (orange line) (schematic drawing, adapted from data from (Degrossoli et al. 2018)). (B) When roGFP2 was expressed in PLB-985 cells that were mixed with *E. coli* cells in a similar manner, the oxidation of roGFP2 in the neutrophils' cytoplasm also increased in a time-dependent manner (dark gray line). However, the probe was not fully oxidized and the neutrophils were seemingly able to maintain a shifted redox homeostasis. MPOi did not affect the shifted redox homeostasis of the neutrophils' cytoplasm in response to phagocytosis (orange line) (schematic drawing, adapted from data from (Xie et al. 2020)).

this way, we were able to identify thiols that react towards hydrogen peroxide, hypochlorous acid, peroxynitrite and other exogenous and endogenous stress conditions in bacteria and yeast (Brandes et al. 2011; Leichert et al. 2008; Lindemann et al. 2013). Those proteomic methods have been performed in individual organisms for a long time. When performing and/or evaluating these *in vitro* experiments, it should be mentioned that reactive species, by their very nature, react with media components. Depending on the choice of medium, HOCl can, for example, rapidly lose its bactericidal properties due to reactions with media components. In some commonly used bacterial and cell culture media such as LB, TSB (Tryptone Soy Broth), MEM (Minimal Essential Medium Alpha), or DMEM (Dulbecco's Modified Eagle Medium), RCS could no longer be detected after 15 min of incubation of 200 μM HOCl, presumably due to the high content of amino acids and other HOCl-quenching substances in these formulations. In contrast, RCS were much more stable in minimal media and buffers such as RPMI (Roswell Park Memorial 1640 Medium), MOPS, PBS (Phosphate Buffered Saline), or HBSS (Hank's Balanced Salt Solution) (Ashby et al. 2020).

However, globally probing the bacterial proteome in a host-pathogen setting brings certain challenges. Essentially, in those experiments the researcher is dealing with two organisms, one of which, the bacterium, is typically present in much lower abundance: owing to the much smaller size of the bacterium, there are far fewer bacterial proteins in a sample consisting of phagocytized bacteria in immune cells. One workaround is, as mentioned, the use of probes. This could also include antibodies for immunohistochemistry or hybridization probes. However, those measure only the oxidation state of the probe itself, or the abundance of individual gene products. In order to get meaningful insight into the proteome of phagocytized bacteria, it is necessary to separate the organisms by partial lysis combined with fractional centrifugation. Most bacteria have a comparatively strong cell wall, so a mild lysis of the host cell will keep bacteria intact. This then facilitates proteomic experiments on separated bacteria (see Yang et al. 2015 for a comprehensive review). Performing redox proteomics brings additional challenges, as sample preparation due to the separation of bacterial cells from the immune cells is time consuming. Bacteria are quick to restore their thiol redox homeostasis in the absence of an external stressor (Leichert et al. 2003; Müller et al. 2013). Thus, swift work and the selection of a good control are essential. In a study probing the redox proteome of bacteria phagocytized by a neutrophil-like cell line we chose bacteria not yet associated with the immune cells as an internal control (Xie et al. 2019). These bacterial cells were in the same co-incubation/infection assay but could be separated from the immune cells by centrifugation at low g-forces (Figure 4). During the following sample preparation, we treated those control bacteria in a manner identical to the phagocytized bacteria, which we still needed to separate from the host cell. i.e. we performed a mock host cell lysis and the very same subsequent centrifugation and washing steps that we performed with the actual sample (Figure 4). By comparing the redox proteome of the bacteria not associated with the immune cells to the redox proteome of phagocytized bacteria, we were able to account for thiol oxidation and/or reduction events that occurred during sample preparation. What we found was a total breakdown of the thiol redox state of bacteria associated with immune cells: the large majority of cysteine residues in phagocytized bacteria changed their oxidation state by 30% or more. While the largest portion of bacterial protein-thiol residues was oxidized between 0 and 10% in bacteria not associated with immune cells, thiols with such a low amount of oxidation all but disappeared in phagocytized bacteria (Xie et al. 2019) (Figure 4).

Global redox state of the host cytosol

As mentioned above, redox signaling is implied in regulatory processes within immune cells. A prime example is the seeming HOCl-dependence of NETosis in neutrophils. As neutrophils produce large quantities of HOCl in their phagolysosome and HOCl could potentially permeate the membrane, it could be argued that phagocytosis of pathogens and the subsequent oxidative burst should have an effect on the host cells' thiol redox homeostasis as well. Expressing roGFP2 in the cytosol of a neutrophil-like cell line, we saw that this was indeed the case. Bacterial phagocytosis was associated with a rise in the oxidation state of the probe (Xie et al. 2020) (Figure 3B). Interestingly, however, pharmacological inhibition of MPO, the HOCl-producing enzyme, did not affect this elevation in the cellular thiol oxidation state. This was in contrast to the oxidation state of the probe in bacteria under the same conditions, which was significantly lower in immune cells that were treated with an MPO inhibitor (Degrossoli et al. 2018). Another difference was the extent of probe oxidation. While the probe was virtually fully oxidized in phagocytized bacteria, immune cells, which phagocytized these very bacteria, could maintain their redox homeostasis, albeit at a new steady state that was shifted by a little more than +50 mV. We thus argued that immune cells must

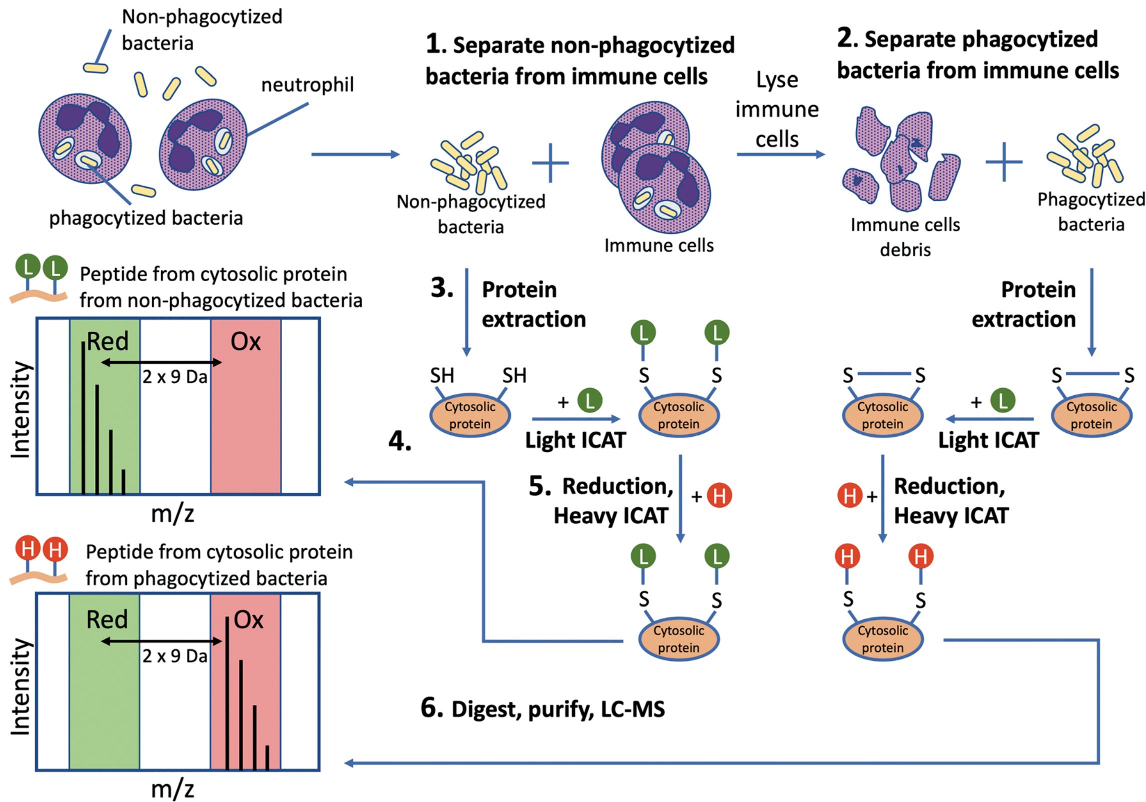

Figure 4: Analysis of the thiol redox proteome of phagocytized *E. coli* using OxICAT as performed in Xie et al. (2019). In a co-incubation assay, *E. coli* bacteria were mixed with neutrophil-like PLB-985 cells at an MOI (multiplicity of infection) of 10:1. After incubation, non-phagocytized bacteria were separated from the neutrophils by centrifugation (1). Subsequently, the immune cell fraction was lysed, releasing the bacteria. Phagocytized bacteria were then separated from the immune cell debris by centrifugation (2). Then the protein was extracted from phagocytized and non-phagocytized bacteria. Here, one hypothetical, cytosolic protein, containing 2 cysteines prone to oxidation upon phagocytosis, is highlighted (3). Free, reduced thiols were labeled with the isotopically light ICAT reagent (Light ICAT, green circle) (4). Steps (3) and (4) were performed concurrently, i.e. cells were lysed, and proteins labeled simultaneously in a denaturing buffer containing sufficient amounts of the ICAT reagent, preventing oxidation and/or thiol-disulfide exchange reactions during cell-lysis. Then, reversibly oxidized cysteines were reduced using Tris(2-carboxyethyl)phosphine (TCEP) and labeled with the isotopically heavy version of the ICAT reagent (9x^{13}C-ICAT) (Heavy ICAT, red circle) (5). Both differentially labeled protein lysates were then digested by trypsin and the ICAT-labeled, cysteine-containing peptides were purified using the ICAT's biotin tag. Finally, both peptide mixtures were analyzed using mass spectrometry (6). The reduced cysteines from the hypothetical cytosolic protein derived from the non-phagocytized bacteria was labeled with Light ICAT, while the same hypothetical peptide, oxidized in the cytosol of phagocytized bacteria was labeled with Heavy ICAT. The oxidation state can then be deduced from the MS plot, based on the characteristic mass difference of 9 Da between the Light and Heavy ICAT.

have effective means keeping HOCl contained in the phagolysosome (Xie et al. 2020), potentially by reaction with phagolysosomal proteins to create non-permeable N-chloraminated proteins. Although the change in the cytosolic redox state of the immune cells was not dependent on HOCl, it was not sufficient to induce effective NETosis: Neutrophils treated with an MPO-inhibitor and activated with PMA or *E. coli* did still experience a change in their cellular redox potential, but were significantly less effective in NET formation. This hints at an HOCl-specific pathway, potentially triggered in close vicinity to the phagolysosome. Given the fact that thiols are highly reactive towards HOCl (second-order rate constant of this reaction $k = 3.6 \times 10^8$ (Storkey et al. 2014) and the overall change in the thiol-redox homeostasis is neither HOCl-dependent nor sufficient for the effective induction of NETosis, it is tempting to speculate that this signaling pathways could involve non-thiol-based switches as well.

Conclusion

HOCl, a highly biocidal oxidant, plays a major role in the interaction between neutrophils and pathogens. Bacteria have evolved several HOCl-responsive transcription factors that control antioxidant genes, essential for surviving

HOCl exposure and oxidative stress. To counter the HOCl-induced protein aggregation, they possess chaperone-like holdases that can maintain protein homeostasis in the absence of ATP-dependent refolding. However, in phagocytized bacteria, the sheer amount of hypochlorous acid overwhelms these systems and leads to the complete breakdown of the cellular thiol-redox homeostasis. HOCl is thus an important weapon in the bacteria-killing arsenal of neutrophils. But it is a double-edged sword: the host, too, has to be able to cope with elevated levels of hypochlorous acid. And to some extent, it can. Our data implies that neutrophils can contain HOCl, at least for a while, in the phagolysosome. If, however, HOCl escapes from immune cells into the extracellular space, the major proteins of plasma and interstitial fluid are turned into effective chaperone-like holdases by N-chlorination in a mechanism similar to the one activating HOCl-protective proteins found in bacteria. Those N-chlorinated plasma proteins are also effective modulators of immune cells. Within the cytoplasm of host immune cells, HOCl seems to act as a signaling molecule as well, as neutrophils devoid of the HOCl-producing MPO are not able to effectively enter NETosis. Interestingly, this signaling seems to be independent of the shift in thiol homeostasis that is concomitant with the phagocytosis of bacteria, suggesting that HOCl-based cellular signaling might, too, go beyond thiol-based switch mechanisms.

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