



# Haemophilus influenzae and oxidative stress

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*Haemophilus influenzae* is a commensal of the human upper respiratory tract. *H. influenzae* can, however, move out of its commensal niche and cause multiple respiratory tract diseases. Such diseases include otitis media in young children, as well as exacerbations of chronic obstructive pulmonary disease (COPD), sinusitis, conjunctivitis, and bronchitis. During the course of colonization and infection, *H. influenzae* must withstand oxidative stress generated by multiple reactive oxygen species produced endogenously, by other co-pathogens and by host cells. *H. influenzae* has, therefore, evolved multiple mechanisms that protect the cell against oxygen-generated stresses. In this review, we will describe these systems relative to the well-described systems in *Escherichia coli*. Moreover, we will compare how *H. influenzae* combats the effect of oxidative stress as a necessary phenotype for its roles as both a successful commensal and pathogen.

**Keywords:** *Haemophilus influenzae*, oxidative stress, catalase, superoxide

## INTRODUCTION

The sub-optimal metabolic reduction of molecular oxygen to water, during aerobic respiration, leads to the production of reactive oxygen species. These reactive oxygen species include superoxide, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and hydroxyl radicals. Bacteria have evolved to combat such potentially lethal insults. Common mechanisms include expression of the superoxide dismutases (SODs) that breakdown superoxide and catalases that decompose H<sub>2</sub>O<sub>2</sub>. Peroxides can also be decomposed by alkylhydroperoxides, bacterioferritin-co-migratory protein and periplasmic thiol peroxidases.

*Haemophilus influenzae* is a commensal of the upper respiratory tract in humans but can also cause respiratory tract diseases which include otitis media in young children, exacerbations of chronic obstructive pulmonary disease (COPD), sinusitis, conjunctivitis, and bronchitis. *H. influenzae* grows aerobically and as a facultative anaerobe. During aerobic growth, the organism experiences oxidative stress derived from its own metabolism. As both a commensal and a pathogen of the human upper respiratory tract, *H. influenzae* also endures oxidative stress derived from host defense cells (Craig et al., 2001; Naylor et al., 2007) and from co-pathogens [primarily *Streptococcus pneumoniae* (Pericone et al., 2000)]. *H. influenzae* has, therefore, evolved multiple overlapping defenses that allow the organism to survive in such a hostile environment.

In the field of oxidative stress in prokaryotes, a large body of exemplary work has been carried out using *Escherichia coli* as the model microorganism. This review will describe our current knowledge regarding the ability of *H. influenzae* to similarly cope with oxidative stress and discuss how these stress-resisting systems compare to the well-characterized *E. coli* systems.

## SUPEROXIDE AND H<sub>2</sub>O<sub>2</sub> TOXICITY

### SUPEROXIDE

The generation of superoxide radicals in an organism is an unavoidable by-product of aerobic respiration. Superoxide radicals can be generated by the univalent reduction of oxygen as the terminal acceptor of the electron transport chain (McCord and Fridovich, 1968; Fridovich, 1970; Imlay and Fridovich, 1991; Messner and Imlay, 1999). Superoxide radicals can also be generated enzymatically through the action of the NOX family of NADPH oxidases (Bedard and Krause, 2007). Superoxide oxidizes [4Fe-4S] clusters of dehydratases, which leads to the loss of iron from said clusters. Superoxide also inhibits transketolase which has a downstream effect on aromatic amino acid synthesis (Imlay, 2003). Due to the near ubiquity of superoxide generation, both eukaryotic and prokaryotic organisms possess SODs, metalloenzymes with roles in the detoxification of superoxide (McCord and Fridovich, 1969; Fridovich, 1995). Four categories of SOD have been identified, categorized by the metal cofactor at their active site (Grace, 1990; Abreu and Cabelli, 2010). Iron-containing SOD ([Fe]-SOD, encoded by *sodB*) and manganese containing SOD ([Mn]-SOD, encoded by *sodA*) are similar in both protein sequence and structure, differing primarily in the nature of their metal cofactor. Both [Fe]-SOD and [Mn]-SOD are found in bacteria, while the former are also found in plant cells and the latter are found in mitochondria and peroxisomes (Fridovich, 1995; Abreu and Cabelli, 2010). The third class of SOD is co-factored by nickel and appears to be restricted to *Streptomyces* spp. (Kim et al., 1996; Youn et al., 1996; Choudhury et al., 1999; Barondeau et al., 2004). The final class of SOD, encoded by *sodC*, is co-factored by both copper and zinc. This [Cu, Zn]-SOD is structurally unrelated to the single metal co-factored SODs. [Cu, Zn]-SOD was

originally thought to be present only in eukaryotic and plant cells but was subsequently identified in *E. coli* (Fridovich, 1995; Imlay and Imlay, 1996; Abreu and Cabelli, 2010). [Cu, Zn]-SOD homologues have also been identified in *Photobacter* spp., *Pseudomonas* spp., *Caulobacter crescentus*, and *Brucella abortus*, among others (Puget and Michelson, 1974; Martin and Fridovich, 1981; Bannister and Parker, 1985; Steinman, 1985; Beck et al., 1990; Steinman and Ely, 1990; Benov and Fridovich, 1994). [Cu, Zn]-SODs are transported to the periplasm. As superoxide generated within the bacterial cytosol cannot cross the inner membrane of the cell (Hassan and Fridovich, 1979), [Fe]-SOD and [Mn]-SOD must detoxify *endogenously* generated superoxide, whereas secreted [Cu, Zn]-SOD must have a role in detoxifying *exogenous* superoxide generated by the host (Schnell and Steinman, 1995; San Mateo et al., 1998).

### HYDROGEN PEROXIDE

The damage to the bacterial cell resulting from superoxide radicals is exacerbated by their involvement in the generation of H<sub>2</sub>O<sub>2</sub> (Gonzalez-Flecha and Demple, 1995, 1997). The dismutation of superoxide leads to the generation of dioxygen and H<sub>2</sub>O<sub>2</sub>. Thus *E. coli*, when grown exponentially in glucose-containing medium, produces approximately 14 μM H<sub>2</sub>O<sub>2</sub> per second in the cytosol (Seaver and Imlay, 2001b). As cell membranes are semi-permeable to H<sub>2</sub>O<sub>2</sub>, extracellular H<sub>2</sub>O<sub>2</sub> generated by host macrophages can also lead to an internal increase in H<sub>2</sub>O<sub>2</sub> in both the periplasm and cytosol of a bacterial cell (Seaver and Imlay, 2001b; Imlay, 2008). An increase in hydrogen peroxide in the periplasm could disrupt redox reactions critical for protein function (Kadokura et al., 2003; Heras et al., 2009). An increase in intracellular H<sub>2</sub>O<sub>2</sub> is detrimental to the cell through both the oxidation of [4Fe-4S] clusters of dehydratases as well as by protein carbonylation, which can lead to the inactivation of enzymes (Stadtman, 1993; Tamarit et al., 1998; Imlay, 2003). Critically, H<sub>2</sub>O<sub>2</sub> can also interact with intracellular free iron to generate toxic hydroxyl radicals, via the Fenton reaction (Imlay, 2003). If this iron is bound to DNA, then hydroxyl radicals generated via the Fenton reaction can damage the DNA (Imlay, 2003). Indeed, experiments that involved repeated passage of an *E. coli* strain that was unable to scavenge H<sub>2</sub>O<sub>2</sub>, under aerobic conditions, demonstrated DNA damage mediated by H<sub>2</sub>O<sub>2</sub> (Storz and Imlay, 1999; Park et al., 2005).

As opposed to SOD, which combats superoxide derived oxidative stress, bacteria have evolved multiple methods of combating H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. These methods are discussed in detail below.

## **Haemophilus influenzae's RESPONSE TO REACTIVE OXYGEN SPECIES**

### **SUPEROXIDE DISMUTASE AND THE RESPONSE TO SUPEROXIDE STRESS**

The first gene identified in *H. influenzae* that encodes a homologue of SOD was found fortuitously by Kroll et al. (1991). Characterization of capsulation genes in *H. influenzae* type b led to the identification of an adjacent ORE, *sodC*, which was predicted to encode a protein homologous to the [Cu, Zn]-SOD that was first identified in *Photobacterium leiognathi* (Steinman, 1987;

Kroll et al., 1991). However, no detectable [Cu, Zn]-SOD activity could be demonstrated in encapsulated *H. influenzae* strains. Analyses of the derived amino acid sequence of [Cu, Zn]-SOD from *H. influenzae* type b showed that the protein possessed only five of the six histidine residues that coordinate the metal ions in other [Cu, Zn]-SODs (Kroll et al., 1991). In contrast both a *sodC* homologue and functional [Cu, Zn]-SOD were found in 100% of *Haemophilus parainfluenzae* strains tested (Kroll et al., 1991) as well as other members of the *Haemophilus*, *Actinobacillus* and *Pasteurellaceae* (HAP) group (Langford et al., 1992; Kroll et al., 1995). Analyses of the derived [Cu, Zn]-SOD protein sequences from the HAP group showed they may share a critical property of prokaryotic [Cu, Zn]-SODs; they are exported to the periplasm where they are implicated in protection against exogenous, host-derived superoxide (Kroll et al., 1995; San Mateo et al., 1998). Finally, analysis of [Cu, Zn]-SOD proteins expressed by *H. parainfluenzae* and *Haemophilus ducreyi* provided insight into how [Cu, Zn]-SOD functions (Kroll et al., 1995; Forest et al., 2000). Specifically, [Cu, Zn]-SOD has an exposed, histidine-rich N-terminus that binds copper. Kinetically favorable binding of copper to the N-terminal region relative to the active site, but stronger binding of copper to the active site, relative to the N-terminal region suggests the bound copper binds the N-terminal region prior to transport to the protein's active site (Battistoni et al., 2001). The role of the histidine-rich N-terminal was demonstrated by expressing N-terminal truncated [Cu, Zn]-SOD from *H. parainfluenzae* in *E. coli*. Loss of the N-terminus, and presumably the ability to scavenge copper for the active site, had a significant impact on the mutated [Cu, Zn]-SOD's ability to protect *E. coli* from being killed in a phagocytosis assay (Battistoni et al., 2001).

It is now clear that *H. influenzae* lacks either a gene encoding a [Cu, Zn]-SOD, or the ability to synthesize functional [Cu, Zn]-SOD (Kroll et al., 1991). Further, Norskov-Lauritsen and co-workers showed that *sodC* was absent in all nontypeable *H. influenzae* (NTHi) strains investigated (Norskov-Lauritsen, 2009; Norskov-Lauritsen et al., 2009). Recently, it has been recognized that some strains previously thought to be NTHi are non-hemolytic *Haemophilus haemolyticus*. All NTHi isolates studied lacked both an identifiable homologue of *sodC* and [Cu, Zn]-SOD activity while the *H. haemolyticus* isolates possess a *sodC* gene (Fung et al., 2006). Finally, all of the genome sequences of NTHi currently deposited in Genbank lack an identifiable homologue of *sodC*.

The lack of SodC activity in *H. influenzae* raises an interesting question about how *H. influenzae* deals with exogenously generated superoxide as the immune response of the host likely subjects *H. influenzae* to superoxide generated by phagocytes. [Cu, Zn]-SODs are localized in the periplasm to detoxify exogenously generated superoxide (Hassan and Fridovich, 1979; Schnell and Steinman, 1995; San Mateo et al., 1998). However, *H. influenzae* can persist within the host yet lacks [Cu, Zn]-SOD. *Neisseria gonorrhoeae* similarly lacks an identifiable *sodC* homologue and detectable [Cu, Zn]-SOD activity (Tseng et al., 2001; Seib et al., 2004). *N. gonorrhoeae* however, can persist in purulent exudates, rich in polymorphonuclear leukocytes. When the sole SOD-encoding gene, *sodB*, was inactivated, *N. gonorrhoeae*

exhibited resistance to both endogenously and exogenously generated superoxide in a manganese-dependent manner. When the periplasmic-binding protein of an ABC transport system that takes up manganese was mutated, *N. gonorrhoeae* was sensitive to endogenously generated, superoxide-mediated oxidative stress. It was thus proposed that manganese scavenges superoxide non-enzymatically and thus protects cells from oxidative stress (Tseng et al., 2001; Seib et al., 2004). In contrast, work by the Imlay group suggested that, in *E. coli*, manganese has a more complex role as a substitute for iron in mononuclear metalloenzymes (Anjem et al., 2009). The gene that encodes the *E. coli* manganese importer, MntH, is regulated by OxyR and *mntH* expression is responsive to H<sub>2</sub>O<sub>2</sub> (Kehres et al., 2000). No homologue of *E. coli* *mntH* can be identified in *Haemophilus* spp. However, the manganese transporter, MntC, from *Synechocystis* is homologous to HfeA from NTHi strain 86-028NP. HfeA, annotated as a putative periplasmic chelated iron-binding protein (Harrison et al., 2005), is OxyR-regulated and responsive to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress (Harrison et al., 2007). Although there are no experimental data to suggest that HfeA has a role in manganese transport, it is possible that *Haemophilus* imports manganese via an oxidative stress-responsive transport system. This would suggest that NTHi, strain 86-028NP has a system akin to that in *E. coli*; imported manganese substituting for iron in metalloenzymes and thus protecting the proteins from damage induced by H<sub>2</sub>O<sub>2</sub>, one of the dismutation products of superoxide.

The role of [Mn]-SOD in *H. influenzae* is more clear. After the identification of a [Cu, Zn]-SOD, a [Mn]-SOD homolog was subsequently identified and characterized in *H. influenzae* type b. In contrast to [Cu, Zn]-SOD, this protein was active. Moreover, when expressed in *E. coli*, [Mn]-SOD from *H. influenzae* type b was regulated by the fumarate/nitrate reduction transcriptional regulator, Fnr and the ferric uptake regulator, Fur (Kroll et al., 1993). Using degenerate primers based on the sequences of *sodA* from *H. influenzae* strain Rd KW20 and NTHi strain 86-028NP, PCR analyses subsequently showed that homologues of *sodA* were present in all *Haemophilus* type strains tested (Cattoir et al., 2006).

To date, homologues of neither [Fe]-SOD or [Ni]-SOD have been found in any *Haemophilus* strain.

## THE OxyR AND Arc REGULATORS AND THE RESPONSE TO HYDROGEN PEROXIDE STRESS

### OxyR

The role of OxyR in protection against oxidative stress has been well-defined in *E. coli* (Gonzalez-Flecha and Demple, 1997). OxyR is activated by peroxide oxidation, which leads to the formation of an intra-molecular disulfide bond. Transcription of OxyR-regulated genes is activated by the binding of oxidized OxyR near the promoter of OxyR-regulated genes. The disulfide bond in activated OxyR can then be reduced by glutaredoxin or thioredoxin. OxyR is thus deactivated. Expression of *grxA*, the gene that encodes glutaredoxin in *E. coli*, is regulated by OxyR. Therefore, OxyR autoregulates its own activity (Zheng et al., 1998; Oktyabrsky et al., 2001). The OxyR regulon in *E. coli* is comprised of over 20 genes that encode proteins that include

the H<sub>2</sub>O<sub>2</sub> scavenging enzymes catalase and alkylhydroperoxide (AhpCF), the iron scavenging protein Dps, the ferric uptake regulator Fur and proteins involved in disulfide reduction [thioredoxin C, glutaredoxin A, glutathione reductase and a periplasmic reductase (Imlay, 2008)].

The first *H. influenzae* homologue of OxyR was identified in an NTHi strain while trying to identify a transferrin-binding protein (Maciver and Hansen, 1996). When *oxyR* mutants in *H. influenzae*, type b or a strain of NTHi were subsequently generated, the mutants were unable to grow when heme in the growth medium was replaced with protoporphyrin IX (PPIX). This PPIX-dependent loss of growth was reversed by the inclusion of either catalase or the peroxide scavenger sodium pyruvate in the growth medium. Possibly, the PPIX-containing medium was undergoing autooxidation to produce superoxide. The superoxide could then dismutate to generate H<sub>2</sub>O<sub>2</sub>. Consistent with this speculation, both the type b and the NTHi *oxyR* mutants were much more sensitive to the effects of H<sub>2</sub>O<sub>2</sub>, as compared to their respective parental strains. Surprisingly, the *H. influenzae* type b *oxyR* mutant exhibited similar levels of catalase activity as the parent strain. Sensitivity to H<sub>2</sub>O<sub>2</sub> could however be linked to the reduced catalase activity in the NTHi *oxyR* mutant (Maciver and Hansen, 1996). An *oxyR* mutant was generated in NTHi strain 86-028NP (Harrison et al., 2007). This NTHi *oxyR* mutant was more sensitive to H<sub>2</sub>O<sub>2</sub> than the parent at all concentrations tested. However, the strain 86-028NP *oxyR* mutant, although more sensitive to steady state treatment with 250 μM H<sub>2</sub>O<sub>2</sub> than the parent strain, was not killed by a 10 minutes treatment with 250 μM H<sub>2</sub>O<sub>2</sub>. Thus this mutant was used to define both genes whose expression was induced in the presence of 250 μM H<sub>2</sub>O<sub>2</sub>, as well as those genes whose expression was regulated by OxyR. H<sub>2</sub>O<sub>2</sub>-induced genes included those with roles in mitigating the effects of oxidative stress, as well as those genes that encode proteins that aid in the repair of proteins and DNA (Harrison et al., 2007).

Of great interest was the finding that expression of genes that encode members of the five major iron- or heme-uptake systems were also upregulated after H<sub>2</sub>O<sub>2</sub> treatment. A similar response in expression of genes with roles in iron uptake to hydrogen peroxide was also noted in *N. gonorrhoeae* (Stohl et al., 2005). Expression of genes with roles in iron uptake are classically regulated by Fur (Hantke, 2001). Moreover, OxyR regulates expression of Fur in *E. coli* (Zheng et al., 1999). This is not the case in NTHi strain 86-028NP; *fur* is not a member of the OxyR regulon. However, increased import of iron and iron-containing moieties into a cell that is subjected to H<sub>2</sub>O<sub>2</sub>-induced stress may lead to an increase in hydroxyl radical production through the Fenton reaction. We speculated that NTHi strain 86-028NP upregulates expression of iron import genes as a way of increasing available intracellular iron for the repair of damaged [4Fe-4S] clusters. Alternatively, upregulation of expression of iron import genes would increase the titer of iron-binding proteins that would act as iron sinks, preventing iron from being available to participate in the Fenton reaction. Oxidation of intracellular Fe<sup>2+</sup> to Fe<sup>3+</sup> through H<sub>2</sub>O<sub>2</sub> treatment may reduce the pool of Fe<sup>2+</sup> available for Fur. Lack of available iron for Fur will lead to derepression of expression of Fur-regulated genes, as observed



(Hantke, 2001; Harrison et al., 2007), a mechanism subsequently elucidated in *E. coli* (Varghese et al., 2007).

### Arc

The anoxic redox control (Arc) system is a two-component signal transduction system first identified in *E. coli* (Iuchi and Lin, 1988). The tripartite ArcB sensor is auto-phosphorylated under reducing conditions and transphosphorylates the response regulator ArcA. Auto-phosphorylation of ArcB and concomitant transphosphorylation of ArcA, via a phosphorelay, is increased in the presence of anaerobic metabolites, which include pyruvate and acetate (Georgellis et al., 1997, 1999). After oxidation, oxidized quinones prevent the autophosphorylation of ArcB (Georgellis et al., 2001a). ArcA is then dephosphorylated, via a reverse phosphorelay, which leads to altered rates of transcription of ArcA-regulated genes (Lynch and Lin, 1996; Oshima et al., 2002; Liu and De Wulf, 2004; Salmon et al., 2005).

The Arc system of *H. influenzae* strain Rd was found to be functionally homologous to that of *E. coli*, regulating gene expression based on the redox condition of the growth medium (Georgellis et al., 2001b). The Arc regulon of *H. influenzae* strain Rd, defined for cells grown under anaerobic conditions, was substantially smaller than that observed for *E. coli*. This is likely reflective of both the larger size of the *E. coli* genome, relative to that of *H. influenzae* strain Rd, the more restricted niche that *H. influenzae* occupies and the possibility that the conditions used in the *H. influenzae* experiment identified only a subset of Arc-regulated genes (Wong et al., 2007). Like ArcA in *E. coli*, ArcA in *H. influenzae* strain Rd represses expression of genes encoding proteins that function in both the respiratory chain and the tricarboxylic acid cycle. In *H. influenzae*, however, ArcA also increases expression of genes encoding proteins involved in polyamine metabolism and iron sequestration, including a Dps-like protein (Wong et al., 2007). In *E. coli*, Dps was shown to protect cells from DNA damage mediated by both iron and H<sub>2</sub>O<sub>2</sub> (Almiron et al., 1992; Chiancone and Ceci, 2010). Repression of expression of the former groups of genes will modulate respiratory activity and thus mitigate the production of reactive oxygen species while Dps and the products of the other ArcA-activated genes may protect against oxidative stress during anaerobiosis as well as when the cells transition from anaerobiosis to an aerobic lifestyle. Supporting this hypothesis was the finding that an anaerobically grown *arcA* mutant of *H. influenzae* strain Rd was very sensitive to H<sub>2</sub>O<sub>2</sub>. This H<sub>2</sub>O<sub>2</sub>-dependent effect was not as pronounced when cells were grown aerobically (Wong et al., 2007).

Unlike other membrane-bound sensor kinases, ArcB in *E. coli* has a short periplasmic bridge with no apparent role in signal transduction (Kwon et al., 2000). It was thus suggested that a Per-Arnt-Sim (PAS) domain, commonly found in sensory proteins, and lying between the transmembrane domain and the cytosolic domain, was necessary for anoxic signaling (Taylor and Zhulin, 1999; Matsushika and Mizuno, 2000; Henry and Crosson, 2011). ArcB homologues from *Salmonella enterica* serovar Typhimurium, *Yersinia pestis* and *Vibrio cholera* all possess PAS domains as well. However, despite the functional homology between the Arc system in *H. influenzae* strain Rd and *E. coli*, ArcB

from strain Rd lacks a PAS domain. ArcB from strain Rd must, therefore, sense anoxic condition in a PAS-independent manner (Georgellis et al., 2001b). Thus, it is possible that the absence of a PAS domain in ArcB from strain Rd is related to *H. influenzae* having a very restricted niche, the airway in humans that is their sole host.

De Souza-Hart and co-workers constructed an *arcA* mutant in a serotype b background. Survival of this mutant was impaired in both blood and serum, with complement possibly being the major bactericidal component. Survival of the type b *arcA* mutant was also impaired in a mouse model of virulence (De Souza-Hart et al., 2003). Similarly, a strain Rd *arcA* mutant demonstrated impaired survival in a mouse model of bacteremia. In contrast, however, the *arcA* mutant of strain Rd did not demonstrate an increased sensitivity to serum, suggesting strain-to-strain differences in the genes regulated by ArcA in *H. influenzae* (Wong et al., 2007, 2011). As ArcA modulated outer membrane protein expression in the type b strain, it was suggested that one or more outer membrane proteins might have been responsible for serum resistance (De Souza-Hart et al., 2003). Subsequently, the role of the Arc system in *H. influenzae*'s serum resistance was characterized in an NTHi strain (Wong et al., 2011). In NTHi, ArcA was shown to positively regulate expression of Lic2B, a glycosyltransferase that adds galactose to the outer core of the lipooligosaccharide. The promoter region of *lic2B* in this strain was found to have a putative ArcA-binding site; this putative binding site was also identified in additional NTHi strains (Wong et al., 2011). Supporting a more general role for ArcA in the regulation of *lic2B* was the identification of a putative ArcA-binding site in the promoter region of *lic2B* from a type b strain. Loss of ArcA led to increased deposition of iC3b, a cleavage product of complement component factor C3b on the bacterial surface (Wong et al., 2011). As Lic2B is important for NTHi survival in a mouse model of bacteremia, as well as for serum survival during anaerobic growth, it was suggested that ArcA senses changes in host environments due to alteration of redox conditions and thus can coordinately regulate expression of genes that are critical for immune evasion. Possibly, the Arc system is critical when *H. influenzae* residing within the low-oxygen environments found in venous blood (for invasive strains) or in mucosal biofilms that are suddenly subject to oxidative stress derived from host cells (Wong et al., 2011).

## CELLULAR DEFENSES AGAINST OXYGEN-MEDIATED CELL STRESS

### CATALASE

Catalase is one of the best-understood enzymes with a role in protection against oxidative stress. A number of subgroups of catalases have been identified, based on heterogeneities in sequence, heterogeneities in function or differences in metal cofactors (Zamocky and Koller, 1999). This review will, unless stated otherwise, refer to classic, heme co-factored catalase. Catalases are predominantly found expressed by species that utilize aerobic respiration and catalyzes the decomposition of H<sub>2</sub>O<sub>2</sub> to oxygen and water (Chelikani et al., 2004). There are two homologues of catalase in *E. coli*, K12; hydroperoxidase I and hydroperoxidase II, encoded by *katG* and *katE* respectively (Loewen et al., 1985;

Von Ossowski et al., 1991; Imlay, 2008). *katG* and *katE* are differentially regulated in *E. coli*. *katE* is expressed constitutively during exponential growth but, through regulation by RpoS, is increased in expression during stationary phase. *katE* expression is not inducible by H<sub>2</sub>O<sub>2</sub> treatment (Loewen et al., 1985; Vijayakumar et al., 2004). Conversely, *katG* is normally expressed at low levels and is induced upon peroxide stress. OxyR mediates this regulation of *katG* expression in *E. coli* and *S. typhimurium* (Christman et al., 1985, 1989).

Although catalase activity in *H. influenzae* was characterized in 1961 (Biberstein and Gills, 1961), an *H. influenzae* homologue of catalase was not identified until 1994. Bishai and co-workers screened an *H. influenzae* library for genes that complemented an *E. coli* strain that could not express *katE* in stationary phase (Bishai et al., 1994b) and identified *hktE*, which encodes a protein homologous to hydroperoxidase II from *E. coli*. HktE expression was, however, maximal during exponential growth and reduced during stationary phase, unlike hydroperoxidase II in *E. coli*. An *hktE* mutant of *H. influenzae* strain Rd grew similarly to the parent when cultured aerobically in rich medium. However, when the *hktE* mutant was cultured anaerobically in minimal medium, then shifted to an aerobic growth condition, it exhibited a growth defect, concomitant with a rise in H<sub>2</sub>O<sub>2</sub> in the medium. The parent exhibited no such growth defect. These growth defects (generated by the loss of catalase) could be prevented by the addition of a peroxide scavenger, sodium pyruvate, to the growth medium (Vergauwen et al., 2003a). The medium-specific killing of the *H. influenzae hktE* mutant was due to the absence of glutathione in the minimal medium. In fact, when *H. influenzae* strain Rd cells were starved for glutathione, the shift to aerobic respiration produced a ~7-fold increase in catalase activity. Moreover, when the *hktE* mutant was starved for glutathione and shifted to aerobic metabolism, there was a measurable accumulation of H<sub>2</sub>O<sub>2</sub> in the growth medium (Vergauwen et al., 2003a).

HktE's expression was inducible with H<sub>2</sub>O<sub>2</sub>, a characteristic of *E. coli* hydroperoxidase II (Bishai et al., 1994b; Harrison et al., 2007). When *hktE* mutations were generated in both Rd and type b *H. influenzae* strains, no catalase activity could be detected, even after peroxide-induced stress. Thus, HktE is the sole catalase in *H. influenzae*. Also, the *hktE* mutant strains were more sensitive to H<sub>2</sub>O<sub>2</sub>, as compared to their parent strains (Bishai et al., 1994a). *H. influenzae* type b *hktE* mutants were tested in an infant rat intraperitoneal model of virulence, as well as an infant rat intranasal model of colonization. In the intraperitoneal virulence model, when inoculated i.p. at lethal doses, the *hktE* mutant was impaired in its ability to cause sepsis, relative to the parent. However, when inoculated at sub-lethal doses, the *hktE* mutant was not impaired in its ability to cause persistent bacteremia, relative to the parent. Finally, in the colonization model, the *hktE* mutants exhibited similar levels of colonization as the parent (Bishai et al., 1994a; Vergauwen et al., 2006).

The regulation of expression of *hktE* in *H. influenzae* must be considered with respect to the niche *H. influenzae* occupies. As a commensal of the human nasopharynx and a bacterium capable of forming a biofilm, *H. influenzae* persists in stationary phase. Only after transit to other niches and growth does the organism

cause disease. As already discussed, *H. influenzae* has one catalase homologue, HktE, whose expression is down regulated during stationary phase. Also, *hktE* expression is induced when subject to peroxide stress. *H. influenzae* must be able to withstand the relatively high concentrations of oxygen in the nasopharynx. It must also be able to withstand peroxide stress likely encountered when *H. influenzae* has transitioned to a pathogen and is under assault from the host cells, particularly from the respiratory burst. However, an *hktE* mutant of *H. influenzae* type b, although slightly impaired in its ability to kill infant rats when administered at high doses, was not impaired in its ability to colonize or cause bacteremia in a nasal challenge infant rat model (Bishai et al., 1994a). This apparent absence of a role for catalase in virulence agrees with data from *Listeria monocytogenes*, *S. typhimurium*, *Shigella flexneri* and *Brucella abortus* (Fields et al., 1986; Gaillard et al., 1986; Franzon et al., 1990; Steele et al., 2010), but disagrees with data from *Xanthomonas campestris*, *P. aeruginosa* and *Staphylococcus aureus*, among others (Mandell, 1975; Lee et al., 2005; Jittawuttipoka et al., 2009). Also, a catalase mutant of *H. influenzae* was not significantly affected by the H<sub>2</sub>O<sub>2</sub> generated by *S. pneumoniae* (Pericone et al., 2000). With the caveat that the infant rat model of *H. influenzae* may not be ideal for showing host killing by oxidative stress (Bishai et al., 1994a), experimental data suggests that catalase appears to be the enzyme that is utilized primarily in the scavenging of high concentrations of endogenously generated, H<sub>2</sub>O<sub>2</sub> (Vergauwen et al., 2003a, 2006) while only being one part of a broader defense against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress.

#### GLUTATHIONE AND PEROXIREDOXIN/GLUTAREDOXIN

Glutathione is a cysteine-containing thiol found in all kingdoms of life. Glutathione has multiple roles within the bacterial cytosol that include control of redox potential, cell signaling and protection against multiple stresses such as oxidative stress. Critically, glutaredoxins can reduce disulfide bonds, utilizing the reducing power of glutathione (Fernandes and Holmgren, 2004; Dalle-Donne et al., 2009). This role for glutaredoxin and glutathione in the regulation of a protein's function was found in *E. coli*, where OxyR is regulated by glutaredoxin (Zheng et al., 1998; Aslund et al., 1999; Pomposiello and Demple, 2001). The role of glutathione in resistance to oxidative stress was also observed in yeast. When production of glutathione was prevented by a mutation in its synthesis pathway, yeast became more sensitive to oxidative stress induced by H<sub>2</sub>O<sub>2</sub> (Grant et al., 1996). However in *E. coli*, a mutation in glutathione synthetase failed to produce increased sensitivity to both H<sub>2</sub>O<sub>2</sub> and the organic hydroperoxide cumene hydroperoxide (CMH) (Greenberg and Demple, 1986). Greenberg and Demple speculated that *E. coli* exhibits an adaptive response to oxidative stress, in the absence of glutathione import. Other, overlapping means of protection must be present.

*H. influenzae* can import glutathione from the environment employing the ABC-like dipeptide transporter DppBCDF (Vergauwen et al., 2010). A periplasmic glutathione-binding protein was identified as a previously characterized heme-binding lipoprotein, HbpA, which was subsequently renamed GbpA. GbpA binds both oxidized and reduced glutathione. GbpA also

has a reduced affinity for conjugated or modified glutathione, preferentially importing useful forms of glutathione (Vergauwen et al., 2010). *H. influenzae* does not need glutathione for growth but imported glutathione has been shown to protect an NTHi strain against oxidative stress induced by H<sub>2</sub>O<sub>2</sub> or *tert*-butyl hydroperoxide (*t*-BOOH) (Pauwels et al., 2003, 2004; Vergauwen et al., 2003a).

This glutathione-based peroxidase activity was assayed in cell-free extracts from an NTHi strain and the activity was inhibited by iodoacetamide, an inhibitor of disulfide bond formation (Vergauwen et al., 2003b). As noted above, a catalase mutant of *H. influenzae* was not impaired in growth in rich medium, colonized infant rats at parental levels and caused similar levels of bacteremia (Bishai et al., 1994a). Based on these data, it was suggested that *H. influenzae* must have another method of scavenging peroxide, with glutathione being the electron donor for this peroxidase (Vergauwen et al., 2003a). This glutathione-mediated, disulfide bond-requiring protection against *t*-BOOH was subsequently found to be dependent on the peroxidase Prx/Grx (subsequently named PgdX). PgdX, a member of the thiol peroxidase family that can reduce H<sub>2</sub>O<sub>2</sub> and alkylhydroperoxides via glutathione redox cycling, is a chimeric protein with a peroxiredoxin domain and a glutaredoxin domain (Vergauwen et al., 2001). The peroxiredoxin domain is involved in detoxification of hydroperoxides while the glutaredoxin domain reduces and thus reactivates the peroxiredoxin domain (Pauwels et al., 2003). Subsequent BLAST analysis identified homologues of *H. influenzae* PgdX in a number of bacteria that are predominantly human pathogens. The homology was strongest over the peroxiredoxin domains (Pauwels et al., 2003). When the crystal structure of PgdX from *H. influenzae* was solved, it was found to exist as a homotetramer. Within the homotetramer, the peroxiredoxin domain of one monomer interacts with the glutaredoxin domain of the adjacent tetramer. Such a peroxiredoxin-glutaredoxin interaction would allow inter-monomer transfer of electrons between domains (Kim et al., 2003). A *pgdX* mutant generated in *H. influenzae* strain Rd exhibited a growth rate similar to the parent when cultured both aerobically and anaerobically in broth (Vergauwen et al., 2006). However, *pgdX* mutant cells were also more susceptible to *t*-BOOH and CMH, when compared to the parent (Pauwels et al., 2004). Of great interest was the observation that a *pgdX* mutant of *H. influenzae* strain Rd showed increased resistance to H<sub>2</sub>O<sub>2</sub>. The loss of PgdX was compensated by an increase in catalase expression (Pauwels et al., 2004). Also, if PgdX activity was reduced by decreasing the amount of glutathione available to the cell, catalase activity increased (Vergauwen et al., 2003a,b). PgdX has a kinetic preference for H<sub>2</sub>O<sub>2</sub>, as compared to alkylhydroperoxides. However, PgdX is optimized to scavenge lesser concentrations of H<sub>2</sub>O<sub>2</sub> than catalase. Thus, in the absence of PgdX, low levels of H<sub>2</sub>O<sub>2</sub> cannot be efficiently scavenged by catalase. The activity of OxyR is then induced and catalase activity is upregulated. Not unexpectedly, in the absence of both catalase and PgdX, *H. influenzae* strain Rd cannot decompose H<sub>2</sub>O<sub>2</sub> (Vergauwen et al., 2006). These data suggest that both catalase and PgdX scavenge metabolically generated H<sub>2</sub>O<sub>2</sub>, with PgdX being protective during stationary phase (Vergauwen et al., 2003b, 2006; Pauwels et al., 2004). Thus, due to the lower efficiency of PgdX in

scavenging H<sub>2</sub>O<sub>2</sub>, stationary phase cultures of *H. influenzae* are more susceptible to oxidative stress (Pauwels et al., 2004).

A mutant of *H. influenzae* strain Rd that lacks *pgdX* and *hktE* is unaffected during either aerobic growth, or after a shift to aerobic growth in rich medium (Vergauwen et al., 2006). This stands in contrast to a mutant strain of *E. coli* that lacks the ability to scavenge H<sub>2</sub>O<sub>2</sub> and exhibits decreased aerobic growth due to presumed DNA damage (Seaver and Imlay, 2001a). Conversely, when the same strain Rd mutant is grown in a chemically defined medium under aerobic conditions, all growth ceases due to H<sub>2</sub>O<sub>2</sub> accumulation leading to possible inactivation of amino acid or vitamin synthesis (Vergauwen et al., 2006). Aerobic respiration is required for some strains of *H. influenzae* to become invasive (Herbert et al., 2003). However, the ability of an *H. influenzae*, type b strain that lacks both catalase and PgdX to cause bacteremia in infant rats was unaffected. The authors speculate that the extent of the host response to *H. influenzae* invasion and/or the oxygen level in infant rat blood are not sufficient for the infant rat to prevent *H. influenzae* bacteremia, even in those strains which lacked catalase and PgdX (Vergauwen et al., 2006). A potentially similar situation occurs in *S. typhimurium*, wherein loss of catalase, a second peroxidase AhpC or OxyR has little effect on virulence (Buchmeier et al., 1995; Taylor et al., 1998). A *recA* mutant of *S. typhimurium* did, however, exhibit a large decrease in virulence in the mouse model (Buchmeier et al., 1995). The ability to repair damaged DNA thus appears to be more critical than the ability to detoxify H<sub>2</sub>O<sub>2</sub> and hydroxyl radicals. This is apparent in NTHi where both *recA* and *recN* expression were moderately induced by H<sub>2</sub>O<sub>2</sub> in NTHi strain 86-028NP (Harrison et al., 2007). This raises an important question. Catalase and PgdX are not essential for the virulence of *H. influenzae*, type b. Thus, what scavenging enzymes with roles in mitigating oxidative stress are critical for cell survival? Possibly, *H. influenzae* is more sensitive to superoxide *in vivo*, although a *sodA* mutation in *H. influenzae* type b only affects nasopharyngeal colonization, not invasive disease in an infant rat model of infection (D'Mello et al., 1997). Alternatively, the experimental conditions used to assess *H. influenzae*'s sensitivity to oxidative stress inducing compounds do not accurately recapitulate the conditions encountered *in vivo*.

Despite the apparent dispensability of PgdX for virulence in *H. influenzae*, PgdX is important in the ability of *H. influenzae* to form biofilms (Murphy et al., 2005). PgdX is expressed at a greater level in *H. influenzae* cells that form biofilms *in vitro*. In concert, when *pgdX* mutations were introduced into four *H. influenzae* strains isolated from patients with COPD, the strains' abilities to form biofilms were impaired, although the level of impairment varied between strains. COPD patients demonstrate elevated levels of H<sub>2</sub>O<sub>2</sub>, possibly derived from alveolar macrophages and systemic oxidative stress (Boots et al., 2003). The peroxide scavenging ability of PgdX may, therefore, be critical to the survival of *H. influenzae* in such environments.

#### THE ALKYLHYDROPEROXIDASE TsaA

The alkylhydroperoxidase AhpC was first identified in *S. typhimurium* during a search for enzymes that protected the cell against organic hydroperoxides, such as *t*-BOOH



and CMH (Jacobson et al., 1989). As well as this role in *S. typhimurium*, AhpC was shown to decompose both CMH and H<sub>2</sub>O<sub>2</sub> in *S. enterica* (Jacobson et al., 1989; Niimura et al., 1995; Seaver and Imlay, 2001a). AhpC is also the primary scavenger of low levels of endogenous H<sub>2</sub>O<sub>2</sub> in *E. coli*, while catalase scavenges high levels of H<sub>2</sub>O<sub>2</sub> (Seaver and Imlay, 2001a,b). After oxidation, AhpC is reduced using electrons from NADPH that are transferred via the flavoprotein AhpF (Jacobson et al., 1989). In *E. coli* AhpC and AhpF are encoded in an operon that is regulated by OxyR (Tartaglia et al., 1989). In discussions of the role of catalase in decomposition of H<sub>2</sub>O<sub>2</sub>, it was assumed, based on the genome sequence of *H. influenzae* strain Rd, that *H. influenzae* lacks AhpC (Vergauwen et al., 2006). Homologues of *ahpC* are present in the *Pasteurellaceae*, for example *tsaA* from *P. multocida*. A microarray-based genomic screen of genes present in NTHi strain 1885MEE subsequently identified an *H. influenzae* homologue of *tsaA*. A second *H. influenzae* homologue of *tsaA* was identified in NTHi strain 86-028NP (Munson et al., 2004; Harrison et al., 2005). A homologue of AhpF is not present in the NTHi strain 86-028NP genome (Harrison et al., 2005). Critically, expression of *tsaA* in NTHi strain 86-028NP was not inducible by H<sub>2</sub>O<sub>2</sub>. If functional, the unresponsiveness to H<sub>2</sub>O<sub>2</sub> suggests that TsaA in NTHi strain 86-028NP may have a role in detoxification of other reactive oxygen species (Harrison et al., 2007).

#### COMPARISONS BETWEEN THE ACTIVITIES OF CATALASE, PgdX AND TsaA IN *H. influenzae*

In *E. coli*, there are two peroxidases, catalase and alkylhydroperoxide (Seaver and Imlay, 2001a). Further, in *E. coli*, catalase, and AhpC have overlapping functions in the decomposition of H<sub>2</sub>O<sub>2</sub>. Catalase is the primary scavenger at high levels of H<sub>2</sub>O<sub>2</sub>, while AhpC is the primary scavenger at low levels of H<sub>2</sub>O<sub>2</sub> (Storz et al., 1989; Seaver and Imlay, 2001a,b). Loss of *katG*, *katE*, and *ahpCF* in *E. coli* results in a strain that can grow under anaerobic conditions but grows poorly under aerobic conditions. This *katG*, *katE*, *ahpCF* mutant strain exhibits increased endogenous hydroxyl radical formation and DNA damage (Park et al., 2005). The work of Vergauwen et al. (2003a) suggested that *H. influenzae* strain Rd uses two peroxidases, catalase and a glutathione-dependent peroxidase, possibly PgdX, to decompose H<sub>2</sub>O<sub>2</sub>. Non-enzymatic glutathione-dependent protection also occurs and loss of available glutathione leads to an increase in catalase activity. The AhpCF system is not present in *H. influenzae* strain Rd, although AhpC is present in some NTHi strains.

*E. coli* lacks a homologue of PgdX, but there are parallels between PgdX and AhpC that suggest they are functional analogues. In multiple species of bacteria, including *H. influenzae*, both PgdX and AhpC scavenge H<sub>2</sub>O<sub>2</sub> and *t*-BOOH with similar kinetics; they are both critical during exponential phase growth but not for virulence and they are both highly expressed (O'Toole et al., 1991; Seaver and Imlay, 2001a,b; Pauwels et al., 2003; Vergauwen et al., 2003a, 2006; Yoon et al., 2003; Murphy et al., 2005). Why then does NTHi strain 86-028NP possess both a homologue of AhpC (TsaA) and PgdX? Possibly NTHi strain 86-028NP has both enzymes as an important redundancy in the bacterium's ability to scavenge low concentrations of H<sub>2</sub>O<sub>2</sub>.

Alternatively, the non-responsiveness of TsaA to H<sub>2</sub>O<sub>2</sub> treatment in NTHi strain 86-028NP suggests that, in this bacterium, TsaA has an alternative function to that exhibited by its homologue, AhpC, in *E. coli*.

#### Dps

The ferritin-like protein, Dps, was first identified in *E. coli* as important for protection against conditions that arise during stationary phase, conditions that include protection from H<sub>2</sub>O<sub>2</sub> (Almiron et al., 1992). Expression of *dps* in *E. coli* is increased by exposure to H<sub>2</sub>O<sub>2</sub>, in an OxyR-dependent manner (Altuvia et al., 1994). Dps homologues with roles in protection against oxidative stress were subsequently identified in many gram negative and positive bacteria, including *S. enterica* serovar Typhimurium, *Campylobacter jejuni*, *B. subtilis*, *S. mutans* and *Listeria innocua* (Chen and Helmann, 1995; Yamamoto et al., 2000; Ishikawa et al., 2003; Halsey et al., 2004; Su et al., 2005). Dps binds iron, which is then oxidized to Fe<sup>3+</sup> by H<sub>2</sub>O<sub>2</sub>. Fe<sup>3+</sup> then forms a stable ferric oxide mineral core within a cavity in Dps. Thus iron is no longer available to contribute to the Fenton reaction and generate hydroxyl radicals (Park et al., 2005; Chiancone and Ceci, 2010). Critically Dps also binds DNA, which both protects the DNA from damage and slows initiation of chromosome replication. Slowing of replication will allow time for the DNA damage to be repaired (Martinez and Kolter, 1997; Grant et al., 1998; Chodavarapu et al., 2008).

Expression of *dps* in NTHi strain 86-028NP is induced by the presence of H<sub>2</sub>O<sub>2</sub>, in an OxyR-dependent manner (Harrison et al., 2007). Moreover, expression of *dps* in *H. influenzae* strain Rd is also induced by ArcA (Wong et al., 2007). A *dps* mutant of *H. influenzae* strain Rd, when grown anaerobically, is more sensitive to H<sub>2</sub>O<sub>2</sub>, as compared to the parent strain. Dps is thus protecting the cell preemptively against transitions from growth in low oxygen to aerobic stress. This H<sub>2</sub>O<sub>2</sub>-specific effect was mitigated when cells were grown aerobically (Wong et al., 2007). The reduced role of Dps in protection against H<sub>2</sub>O<sub>2</sub> in *H. influenzae* strain Rd cells grown aerobically suggests that the other major detoxification systems (catalase, SOD and PgdX) are more critical in aerobically growing cells (Wong et al., 2007).

#### CONCLUDING STATEMENTS

Compared to *E. coli*, *H. influenzae* is less well-equipped to combat oxidative stress. Of the three types of superoxide dismutases, a prototypic *H. influenzae* strain has only one functional member, [Mn]-SOD. Of the scavenging systems, a prototypic *H. influenzae* strain has a single catalase and a peroxiredoxin/glutaredoxin (PgdX). A prototypic *H. influenzae* strain lacks an alkylhydroperoxide system (AhpCF). However, the first sequenced strain of NTHi has a homologue of AhpC, although it lacks a cognate reductase encoded by *ahpF*. Of the non-enzymatic defenses, a prototypic *H. influenzae* strain lacks the ability to synthesize glutathione, but can import glutathione via GpbA. A prototypic *H. influenzae* also possesses a homologue of Dps that, in *E. coli* has a vital role in the mitigation of iron-dependent oxidative stress generated via the Fenton reaction. Critically, in *H. influenzae* there are overlapping and disconnected mechanisms of protection against oxidative stress. For a bacterium with a relatively

small genome, this retention of multiple genes, which encode proteins with similar protective abilities, indicates the importance of being able to protect itself against oxidative stress, as well as the need for a flexible and multifaceted system of defenses against such insults.

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