Glutathione and Catalase Provide Overlapping Defenses for Protection against Respiration-Generated Hydrogen Peroxide in *Haemophilus influenzae*

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Cellular metabolism of molecular oxygen produces reactive and potentially toxic oxygen species such as superoxide radicals, hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (5). *Escherichia coli* generates ca. 14 μM H$_2$O$_2$ per s when it grows exponentially in air in minimal glucose medium (36). Recently, Seaver and Imlay showed that *E. coli* mutants completely defective in H$_2$O$_2$ scavenging activity grow poorly in air and that growth often stopped entirely when these mutant cells were repeatedly subcultured (36). This implies that aerobically grown *E. coli* generates enough H$_2$O$_2$ to poison scavengerless cells. Indeed, H$_2$O$_2$ itself can potentially damage enzymes by oxidizing sulphydryl and iron-sulfur moieties and, upon conversion to a hydroxyl radical, it produces mutagenic and lethal lesions (4, 21).

*E. coli* mainly expresses two systems to destroy H$_2$O$_2$. The first system is embodied by two types of catalase enzymes, a bifunctional catalase/peroxidase (HPI), encoded by *katG* (39), and a monofunctional catalase (HPII), encoded by *katE* (45), which are both heme-containing enzymes involved in the dismutation of H$_2$O$_2$ into O$_2$ and H$_2$O. Clear evidence shows that the two catalase genes are regulated differently in terms of growth phase and responsiveness to oxidative stress (reviewed in reference 22). HPII is transcriptionally induced during logarithmic growth in response to low micromolar concentrations of H$_2$O$_2$. This induction requires the positive transcriptional activator OxyR, which directly senses oxidative stress. On the other hand, HPII is not peroxide inducible and its gene, *katE*, is transcribed at the transition from the exponential growth phase to the stationary growth phase by RNA polymerase containing the alternative sigma subunit σ$^s$, the product of the *rpoS* (or *katF*) gene.

Next to these catalases, *E. coli* expresses a two-enzyme H$_2$O$_2$-scavenging system, the alkyl hydroperoxide reductase (AhpR) system, which was initially characterized as rapidly reducing diverse organic hydroperoxides such as cumene- and t-butylhydroperoxide (18). Later, Niimura et al. (26) showed that the specific activities of the *Salmonella enterica* serovar Typhimurium AhpR homolog for the reduction of cumene hydroperoxide and H$_2$O$_2$ are within the same order of magnitude. One component of the AhpR system, AhpF, is a flavoprotein that shuttles reducing equivalents from NAD(P)H to AhpC, the actual peroxidase and a member of the peroxiredoxin family of thiol peroxidases. The *ahpC* and *ahpF* genes are organized in a two-gene operon, and transcription is OxyR controlled (38).

In *E. coli*, AhpR and HPI have discrete roles in scavenging H$_2$O$_2$. AhpR is the primary scavenger of endogenous H$_2$O$_2$. HPPI contributes little when H$_2$O$_2$ concentrations are low, but it becomes the more effective scavenger when H$_2$O$_2$ concentrations are high or, presumably, when the absence of a carbon source depletes the cell of the NADH necessary for AhpR activity (36).

Analogously, catalases are believed to be indispensable in eukaryotic cells to protect compartments subjected to high H$_2$O$_2$ concentrations, e.g., the peroxisomal matrix and membrane (6), whereas the H$_2$O$_2$ resulting from the cellular metabolism of molecular oxygen is primarily scavenged by thioldependent peroxidases (13, 29). In animals, these peroxidases are fueled by either the glutathione or the thioredoxin redox cycle. The tripeptide-reduced glutathione (GSH; γ-L-glutamyl-L-cysteinyl glycine) is an abundant and ubiquitous low-molecular-weight thiol with a presumed role in many cellular processes, ranging from oxidative stress defense to cysteine...
storage (reviewed in reference 31). During these processes, GSH is often converted into its symmetrical disulfide form, oxidized glutathione (GSSG), and regeneration occurs via the NADPH-dependent action of glutathione reductase. Glutathione deficiency in higher eukaryotes is at the basis of several diseases caused by the degradation of mitochondria (8, 19), an observation which allowed us to conclude that H$_2$O$_2$ generated through respiration is scavenged inside the mitochondria by GSH peroxidase (GPx). Furthermore, the finding that glutathione-deficient yeast strains are hypersensitive to H$_2$O$_2$ points to the importance of glutathione-based peroxide removal in eukaryotes (10, 17).

*Haemophilus influenzae* is a strictly human commensal organism. As unencapsulated species, the bacterium is found in the upper respiratory tracts of up to 80% of healthy adults and, as encapsulated species, in 3 to 5% of normal individuals (25). Encapsulated strains are capable of invasive infections, including meningitis, pneumonia, and epiglottitis.

An acatalasemic *H. influenzae* Rd mutant was constructed by transposon-mediated inactivation of one structural gene, *hktE*. Consequently, *hktE* accounts for total catalase activity in *H. influenzae* cells (3). The *hktE* gene product, HktE, is strongly homologous to *E. coli* KatE. However, the *H. influenzae* catalase is downregulated in the stationary growth phase and is upregulated by exposure to H$_2$O$_2$. As such, *hktE* regulation is similar to that of *katG* (3). Consistent with findings in other bacteria, disruption of catalase production in *H. influenzae* causes hypersensitivity to high H$_2$O$_2$ concentrations (2). On the other hand, deletion of the *hktE* gene produces only a modest reduction in the ability to cause lethal sepsis after parenteral challenge and causes no change in the ability to colonize after intranasal inoculation in the infant rat model of infection (2). Consequently, the *H. influenzae* *hktE* gene is not of major importance for the process of colonization and invasive infection.

Because *H. influenzae* catalase mutants do not show any growth disadvantage either in vitro in rich medium or in vivo after intranasal inoculation in infant rats, it is anticipated that at least one other H$_2$O$_2$-scavenging system supports peroxide removal in *H. influenzae*. AhpR is not involved because neither an *ahpC* nor an *ahpF* homolog is apparent from the *H. influenzae* Rd genome sequence (7).

Recently, we reported that *H. influenzae* is naturally deficient in glutathione biosynthesis (42) and showed that *H. influenzae* instead imports glutathione from the growth medium. In this way, extracellular glutathione (supplemented as GSSG to prevent chemical reactions with the oxidizing test chemicals) protects cultures against methylglyoxal, S-nitrosoglutathione, and t-butylhydroperoxide toxicity. Interestingly, an open reading frame (H10572) was isolated that encodes glutathione-based t-butylhydroperoxide removal. On the other hand, the presence of GSSG in the growth medium of *H. influenzae* NCTC 8143 cultures does not influence the sensitivity to exogenous H$_2$O$_2$ stress (42). We show here, however, that glutathione is a crucial component in the metabolism of H$_2$O$_2$ endogenously generated during aerobic growth.

**MATERIALS AND METHODS**

**Media.** Mueller-Hinton broth (MH broth) was prepared from a dehydrate (Fluka at Sigma-Aldrich, St. Louis, Mo.) and autoclaved. MH medium broth was MH broth supplemented with *Haemophilus* test medium supplement (Oxoid, Hampshire, United Kingdom), containing V-factor (NAD) and X-factor (hemin), according to the manufacturers’ instructions.

*H. influenzae* specific minimal medium (MC medium) was prepared essentially as described by Herrriott et al. (15). L-cystine (Sigma-Aldrich) was added to a final concentration of 50 μM, and *Haemophilus* test medium supplement was added according to the manufacturers’ instructions. GSSG-supplemented MC medium [MC(GSSG)] was prepared by replacing l-cystine with GSSG (Sigma-Aldrich) at the same final concentration. Both l-cystine and GSSG were added from a sterile concentrated stock solution. For anaerobic growth, the recipe for the preparation of Mc minimal medium was adjusted as follows: (i) sodium lactate was replaced by disodium fumarate (final concentration, 5 mM), and (ii) the final glycerol concentration was raised from 0.3 to 0.5%. This medium is referred to as anMC or anMC(GSSG) medium throughout the text, depending on whether l-cystine or GSSG was added, respectively; 1.8% agar was added to prepare anMC and anMC(GSSG) agar plates.

**Bacterial strains and growth conditions.** Wild-type strain *H. influenzae* Rd was purchased from the American Type Culture Collection (Manassas, Va.). The construction of the *H. influenzae* catalase-negative strain AB2593 (Rd hktE::mini-Tn10Cm) was reported previously (2). Strain AB2593 was kindly provided by William R. Bishai (Department of Medicine, Division of Infectious Diseases, Johns Hopkins University School of Medicine).

Cultures were routinely grown at 37°C under anaerobic conditions in anMC medium. Anaerobic cultures were prepared in a Coy chamber (Coy Laboratory Products, Inc.) under an atmosphere of 85% N$_2$–10% H$_2$–5% CO$_2$ in culture tubes that fit tightly into the cuvette holder of a Shimadzu 1240 Mini Single-Beam UV-VIS spectrophotometer (Shimadzu, Duisburg, Germany). The system enables cell density measurements without the need to transfer culture samples into regular cuvettes. The tubes were closed with a silicone stopper and were shaken outside the Coy chamber. As such, anaerobiosis was preserved, a finding confirmed in preliminary experiments by adding 0.002% resazurine to the growth medium (resazurine is a redox-sensitive dye added to media as a simple, qualitative indicator of the redox conditions of the media). Tn10-linked mutations in *hktE* were confirmed by assaying catalase activity in whole cells as described earlier (12).

For aerobic shift experiments, precultures grown anaerobically overnight were diluted 1:100 in anMC or anMC(GSSG) medium to an optical density at 600 nm (OD$_{600}$) of ~0.005 and then grown anaerobically to an OD$_{600}$ of ~0.15. The silicone stopper was then removed in order to shift the cells into air. In the case of aerobic-shift experiments to monitor growth in the presence of nonynemine H$_2$O$_2$ scavenging, pyruvate was added to the anMC or anMC(GSSG) cultures from a buffered sterile stock solution to a final concentration of 0.75%. In the case of aerobic-shift experiments to evaluate catalase activity in response to aerobiciosis, cell extracts were prepared, and catalase activity was measured, as described previously (42), 20 min after the removal of the silicone stopper. These catalase activities were then compared to those derived from continuously anaerobically grown counterparts.

Heat-killed bacteria were prepared by incubating suspensions at 60°C for 1 h.

**Disk diffusion.** Overnight anaerobically grown precultures were diluted 1:100 times in anMC or anMC(GSSG) medium to an OD$_{600}$ of ~0.005 and then grown anaerobically to an OD$_{600}$ of 0.5 (late exponential phase). The following manipulations were performed inside the Coy chamber. Using a sterile cotton swab, cells were inoculated onto the entire surface of anMC or anMC(GSSG) plates. Round sterile filters (5.5-mm diameter) were placed in the center of the plates and spotted with either 5 μl of 3% H$_2$O$_2$ or 5 μl of 500 mM t-butyldihydroperoxide. The plates were placed in an anoxic jar and incubated for 2 days at 37°C. The diameter of the zone of complete inhibition was recorded in millimeters. The experiment was performed in triplicate; mean values are reported.

**H$_2$O$_2$ detection.** The Amplex red hydrogen peroxide/peroxidase assay kit (Molecular Probes, Eugene, Ore.) was used to detect H$_2$O$_2$ according to the manufacturer’s instructions. This assay can be performed either fluorometrically or spectrophotometrically. We chose the latter approach because the extinction coefficient of the Amplex red reagent oxidation product (resorufin) is high (54,000 cm$^{-1}$ M$^{-1}$), resulting in a detection limit of ca. 0.2 μM. The latter value is low enough to complete the experiments described here. Absorbance was measured at 563 nm by using a Shimadzu 1240 Mini Single-Beam UV-VIS spectrophotometer. The assay procedure was performed in a total volume of 400 μl. For each assay, no-H$_2$O$_2$ controls were monitored.

**H$_2$O$_2$ scavenging by whole cells.** Overnight anaerobically grown precultures were diluted 1:100 times in anMC or anMC(GSSG) medium to an OD$_{600}$ of ~0.005 and grown anaerobically to an OD$_{600}$ of 0.15. Cells were pelleted in a microcentrifuge, washed twice, and resuspended in room temperature phos-
RESULTS

H. influenzae Rd catalase null mutant strain AB2593 exhibits a severe aerobic growth defect in minimal medium. The H. influenzae strains examined here, the HktE-negative strain AB2593, and the wild-type isogenic parent strain Rd are identical to those studied by Bishai et al. (2), who used them to characterize catalase mutants of H. influenzae in terms of colonization and invasion abilities. This primary report illustrates that these strains share identical growth characteristics when grown aerobically in rich medium. We found that the growth curve of strain AB2593, grown in rich medium (MHs), is indeed similar to the growth curve obtained with wild-type cultures. No variation was observed when AB2593 cells were repeatedly subcultured in MHs medium. Also, no aberrations were detected for aerobic growth in minimal medium (MiC) inoculated with a stationary-phase MHs medium culture. However, these latter MiC cultures appeared to have lost one or more protective factors for surviving aerobic conditions. Indeed, second-generation AB2593 MiC subcultures showed severe growth defects when grown aerobically (Fig. 1), whereas growth in the absence of oxygen proceeded at wild-type levels (data not shown). Growth even stopped entirely in third-generation AB2593 MiC subcultures (Fig. 1).

A clear representation of the aerobic growth defect of catalase-negative AB2593 cells could be obtained by shifting cultures, after repeated anaerobic subculturing in anMiC medium, from anaerobic to aerobic conditions (Fig. 2). Whereas Rd cultures immediately responded to the shift by the expected growth acceleration due to the initiation of aerobic respiration, AB2593 cultures responded by no more than a slight increase of the doubling time as a step toward complete growth arrest and death.

As mentioned before, catalases are ubiquitous among aerobic organisms, where they serve to detoxify the H$_2$O$_2$ that arises from exposure to oxygen. So, we first studied whether it is this H$_2$O$_2$ that poisons AB2593 grown aerobically in minimal medium. Therefore, we repeated the aerobic-shift experiments in the presence of sodium pyruvate, a nonenzyme scavenger of peroxides (41). We observed that catalase-negative H. influenzae cells grown in pyruvate-supplemented anMiC medium doubled at wild-type rates after the aerobic shift (Fig. 2). A question arises as to why endogenously generated H$_2$O$_2$ kills catalase-negative H. influenzae cells in MiC medium but fails to do so in rich MHs medium. Note that we here refer to endogenously generated H$_2$O$_2$. We indeed tested anMiC medium and anMiC(GSSG) medium for the nonenzymatic production of H$_2$O$_2$ and found that concentrations were below the detection limit (ca. 0.2 M). For comparison, Luria-Bertani medium chemically generates ca. 1.2 M H$_2$O$_2$ per min (14).

Exogenous glutathione conceals the aerobic growth defect in minimal medium of H. influenzae AB2593. The fact that even fairly dense cultures of AB2593 died as a result of an aerobic shift (Fig. 2) rules out the possibility that a dilution effect causes growth inhibition in minimal medium. More likely, the aerobic growth defect is the result of the different composition of the growth media. One particular component that is not present in (an)MiC medium compared to rich medium is glutathione. When supplied to the growth medium in oxidized form, this thiol tripeptide was recently shown to strengthen the oxidative defense of naturally glutathione-deficient H. influenzae (42). We therefore repeated the aerobic-shift experiments in the presence of GSSG (Fig. 2) and observed that AB2593 cultures grown in glutathione-supplemented minimal medium are completely relieved from defective growth under aerobic conditions.

Effects of intracellular glutathione-based peroxide reduction on the metabolism of H$_2$O$_2$ produced during aerobic respiration. In order to elucidate whether glutathione-deficient AB2593 cells indeed exhibit problems in detoxifying the H$_2$O$_2$ that is produced during aerobic respiration, we repeated our aerobic-shift experiment and monitored the H$_2$O$_2$ concentrations in the medium (Fig. 3). For all conditions tested, we measured higher H$_2$O$_2$ concentrations immediately after the aerobic shift. This most likely represents the adaptation time required to build up adequate antioxidant activity. Table 1 illustrates that the shift to oxidative metabolism caused a substantial 6.6-fold increase in catalase activity in glutathione-
deficient *H. influenzae* Rd cells, indeed suggesting that, in response to the shift to air, H₂O₂ is generated at a rate greater than what could be metabolized to a steady-state level (i.e., \( \frac{1}{100} \) M) by the antioxidant machinery present in anaerobically growing cells. This hypothesis, however, appears not to be valid for glutathione-supplemented cells. Note that the experiment illustrated in Table 1 only focuses on catalase activity, which, as described below, appears to be less efficient compared to glutathione-based peroxide removal. Thus, in response to the aerobic shift, induction of the latter system may be sufficient to alleviate the toxicity of respiratory-generated H₂O₂ to levels too low to induce the synthesis of catalase.

Twenty minutes after the shift to air, the growth of glutathione-supplemented AB2593 cultures continued, metabolizing H₂O₂ to concentrations below the detection limit. On the other hand, glutathione-deficient catalase-negative cells clearly experienced problems in controlling H₂O₂ production, as noticed by the steady H₂O₂ accumulation in the growth medium. Once we had determined that both glutathione-based H₂O₂ removal and catalase activity are important during aerobic growth, we wondered whether these two systems represent the entire H₂O₂-scavenging activity of *H. influenzae*. Therefore, we set up a time-response experiment exemplifying the in vivo H₂O₂-scavenging activities of whole metabolically active cells (Fig. 4). We observed that, whereas glutathione-supplemented AB2593 scavenged 1.5 M H₂O₂ as well as did its wild-type parent, the glutathione-deficient *hktE* mutant exhibited virtually no H₂O₂-scavenging activity. Also, heat-killed glutathione-supplemented AB2593 cells failed to remove H₂O₂ from solution. Thus, *H. influenzae* relies on two systems for the destruction of H₂O₂ generated through respiration: a glutathione-based system that requires metabolically active cells on the one hand and catalase on the other.

**Table 1.** Effect of shift to oxidative metabolism on catalase activity monitored in *H. influenzae* Rd extracts derived from cultures grown in the presence or absence of GSSG

<table>
<thead>
<tr>
<th>Growth medium (growth condition)</th>
<th>Mean catalase (HktE) activity (( \mu )mol of H₂O₂ decomposed/mg protein) ± SEM</th>
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</thead>
<tbody>
<tr>
<td>anMic(GSSG) (anaerobiosis)</td>
<td>34.0 ± 2.3</td>
</tr>
<tr>
<td>anMic(GSSG) (aerobiosis)</td>
<td>33.6 ± 4.5</td>
</tr>
<tr>
<td>anMic (anaerobiosis)</td>
<td>34.4 ± 1.2</td>
</tr>
<tr>
<td>anMic (aerobiosis)</td>
<td>228 ± 22</td>
</tr>
</tbody>
</table>

See Materials and Methods for experimental details.

Results are expressed as the mean values from three independent experiments.
were not saturated by the highest H$_2$O$_2$ concentrations tested, contrast, HktE-expressing cells (glutathione-de viscosity) reached a maximum initial velocity with increasing H$_2$O$_2$ concentration (Fig. 4) and the fact that the glutathione-based peroxidatic system was significantly more efficient than that propelled by catalase.

Calculation of endogenous H$_2$O$_2$ production during aerobic growth. As shown in Fig. 3, substantial H$_2$O$_2$ accumulated in the medium of glutathione-deficient AB2593 cultures. These cultures were shown to be completely deficient in H$_2$O$_2$ scavenging at low micromolar concentrations, so that virtually all of the H$_2$O$_2$ that enters or is formed within these cells diffuses out without being scavenged (36, 37). For this reason, the measurement of excreted H$_2$O$_2$ is a valid proxy for measurement of endogenous H$_2$O$_2$ formation. Between 20 and 30 min after the shift to air, glutathione-deficient AB2593 cultures at an OD$_{600}$ of 0.15 accumulated 1.05 μM H$_2$O$_2$ (mean of three independent experiments). Using the relation that 1 ml of bacteria at an OD$_{600}$ of 1.0 contains 0.94 μl of cytoplasmic volume (see Materials and Methods), we calculated that aerobically grown log-phase H. influenzae forms H$_2$O$_2$ at a rate of ~12.4 μM per s.

DISCUSSION

In 1983, Meister and Anderson (23) proposed that glutathione protects mammalian cells from oxidative damage. Two decades and several studies later, it has become definite that glutathione is indispensable for eukaryotic cells to deal with reactive oxygen species generated by aerobic respiration. For the most part, this results from the fact that GSH functions as a cofactor for peroxidatic H$_2$O$_2$ removal (16, 35). In contrast, Greenberg and Demple (11) reported that a glutathione-deficient mutant of E. coli K-12 has normal resistance to H$_2$O$_2$ and cumene hydroperoxide. Thus, even though intracellular GSH can efficiently scavenge free radicals nonenzymatically and can reach millimolar levels, it is not essential for protecting E. coli from oxidative damage. Moreover, no growth defects were reported for mutant acatalasemic E. coli genetically inactivated for glutathione biosynthesis (32, 44).

We demonstrated here that the acatalasemic H. influenzae mutant strain AB2593 requires glutathione in its growth medium in order to survive endogenous H$_2$O$_2$ stress associated with aerobic growth. In a previous study (42), we showed that H. influenzae, although it possesses glutathione metabolizing enzymes, is naturally glutathione deficient and that glutathione is acquired by importing the thiol tripeptide (either as GSH or as reducible symmetrical and asymmetrical [mixed] disulfide forms of GSH) from the growth medium. Therefore, since H. influenzae AB2593 is deficient in catalase, the restoration of wild-type growth rates in glutathione-supplemented (an)Mlc medium could be the result of pure glutathione-based nonenzymatic H$_2$O$_2$ scavenging inside the cell (28). However, the fact that glutathione-supplemented AB2593 scavenges H$_2$O$_2$ at wild-type rates (Fig. 4) and the fact that the glutathione-based H$_2$O$_2$ removal attains a maximum initial velocity with increasing H$_2$O$_2$ concentrations (Fig. 5) indicate that intracellular GSH donates electrons to a powerful peroxidase.

Glutathione-dependent peroxidatic H$_2$O$_2$ removal requires deterioration of whole glutathione-deficient wild-type cells at high extracellular H$_2$O$_2$ concentrations were much greater than those for whole cells that only relied on the glutathione-based peroxidatic system. On the other hand, at extracellular H$_2$O$_2$ concentrations of up to 5 μM, glutathione-based scavenging was significantly more efficient than that propelled by catalase.

To verify this result in terms of kinetic behavior of the HktE and glutathione-based peroxidatic systems, we set up a concentration-response experiment exemplifying the rate of H$_2$O$_2$ decomposition achieved by a steady number of metabolically active whole cells (Fig. 5). The peroxidatic activity inside glutathione-supplemented AB2593 cells became saturated when extracellular H$_2$O$_2$ concentrations exceeded 25 μM. In contrast, HktE-expressing cells (glutathione-deficient wild type) were not saturated by the highest H$_2$O$_2$ concentrations tested, a finding consistent with the millimolar $K_m$ values reported for other catalases (1, 20, 33). Furthermore, the H$_2$O$_2$ decom-

![Graph](https://via.placeholder.com/150)

**FIG. 4.** Glutathione-supplemented acatalasemic H. influenzae and wild-type H. influenzae Rd remove low micromolar concentrations of extracellular H$_2$O$_2$ at similar rates. Cultures of Rd and AB2593 were grown anaerobically in anMlc(GSSG) medium (Rd, □; AB2593, △) and in anMlc medium (Rd, ▲; AB2593, ▽), and resuspended in PBS at an OD$_{600}$ of 0.075. Heat-killed bacteria (◇) were prepared by incubating the PBS suspension at 60°C for 1 h. H$_2$O$_2$ was added to a final concentration of 1.5 μM. The H$_2$O$_2$ concentration was measured at various time points after addition of H$_2$O$_2$ as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Strain (medium)</th>
<th>Zone of inhibition (mm)</th>
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<tbody>
<tr>
<td>Rd (Mlc)</td>
<td>26</td>
</tr>
<tr>
<td>AB2593 (Mlc)</td>
<td>21</td>
</tr>
<tr>
<td>AB2593 (Mlc(GSSG))</td>
<td>46</td>
</tr>
<tr>
<td>AB2593 (Mlc(GSSG))</td>
<td>33</td>
</tr>
</tbody>
</table>

* Each assay was done by disk diffusion of 5 μl of 3% H$_2$O$_2$. See Materials and Methods for experimental details.
Prx/Grx peroxidase. We observed that H$_2$O$_2$ binds very efficiently through the characterization of the Prx/Grx protein, which shares 63% sequence identity with the Prx/Grx enzyme in Chromatium gracile (30). Because the Prx/Grx protein encodes a gene (ORF HI0572) encoding a novel GSH-dependent peroxidase, as can be demonstrated by deducing the $K_m$ value from an empirical fit to the data given by the concentration-response curve shown in Fig. 5; note that the abscissa displays extracellular H$_2$O$_2$ concentrations and, therefore, the deduced $K_m$ value cannot be interpreted as real Michaelis constant. The extracellular concentration at half-maximal rate was ca. 5.8 $\mu$M, which is very similar to the Michaelis constant (2.29 $\mu$M) obtained via in vitro kinetics of the Prx/Grx peroxidase (30).

As observed for E. coli (36), our results strongly suggest that aerobically grown log-phase H. influenzae scavenges the majority of endogenous H$_2$O$_2$ through one catalase and one peroxidase. Indeed, as reported for catalase and AhpR-deleted E. coli (36), glutathione-deficient H. influenae lacking catalase is almost completely devoid of H$_2$O$_2$-scavenging activity (Fig. 4). Notably, in contrast to the fact that H$_2$O$_2$ scavengerless E. coli grows, albeit poorly, in rich and minimal media in air (36), we observed that glutathione-deficient AB2593 is completely unable to grow aerobically. So, the continual accumulation of H$_2$O$_2$ inside scavengerless aerobically growing cells appears to be more damaging to H. influenzae than to E. coli. In growing cells, the steady-state concentration of H$_2$O$_2$ depends on the rates of its formation and of its dissipation. Given that H$_2$O$_2$ removal is essentially absent in both strains, the growth difference could be the result of differences in H$_2$O$_2$ formation rates. We observed rates (~12.4 $\mu$M per s) that are 1 order of magnitude higher than those reported by Gonzales-Flecha and Demple (ca. 1 to 2 $\mu$M per s) for E. coli (9). However, Seaver and Imlay (36) reported rates of H$_2$O$_2$ production in exponentially growing E. coli (~14 $\mu$M) similar to those reported here for H. influenzae. This inconsistency is the result of differences in experimental procedure to measure H$_2$O$_2$ formation rates (36). Since our procedure is very similar to the one used by Seaver and Imlay (i.e., actual H$_2$O$_2$ formation rates are measured by using H$_2$O$_2$ scavengerless log-phase cultures grown at a continuous flow of GSH. Glutathione reductase is the central enzyme in glutathione redox cycling, and this reductase exclusively exploits NADPH as a reductant. Heat-killed glutathione-supplemented AB2593 is not able to generate reducing power. This probably explains our observation that these cells were totally ineffective in H$_2$O$_2$ scavenging (Fig. 4). Consequently, this may well explain why exogenous H$_2$O$_2$ stress results in a twofold increase in glucose-6-phosphate dehydrogenase (G6PD) activity (42), whereas G6PD levels remain unaltered and for both lower and higher eukaryotes G6PD expression is rate-limiting enzyme of the pentose phosphate pathway, and this reductase exclusively exploits NADPH as a reductant. Heat-killed glutathione-peroxidase-depleted E. coli, NADH is the preferred reductant for AhpR-dependent peroxidatic activity (26) and, it therefore appears that no G6PD induction is required in response to H$_2$O$_2$ stress.

Recently, we reported that the H. influenzae genome encloses a gene (ORF HI0572) encoding a novel GSH-dependent t-butylhydroperoxide reductase (Prx/Grx) (42). Naturally glutathione-peroxidase-deficient E. coli expresses a vast amount of GSH-dependent t-butylhydroperoxide reductase activity when transformed with a multicopy plasmid carrying the HI0572 locus. The Prx/Grx protein shares 63% sequence identity with the garA gene product of Chromatium gracile (30). Because the C. gracile peroxidase reduces both t-butylhydroperoxide and H$_2$O$_2$ at high rates (43), we anticipated that Prx/Grx might be the major peroxidase involved in H$_2$O$_2$ metabolism of H. influenzae. To examine this premise, we are currently generating both an H. influenzae HI0572 mutant and an H. influenzae HI0572 hktE double mutant. In the meantime, we recently obtained supportive biochemical evidence through the characterization of the H. influenzae Rd Prx/Grx peroxidase. We observed that H$_2$O$_2$ binds very efficiently to the GSH-dependent peroxidase, as can be demonstrated by deducing the $K_m$ value from an empirical fit to the data given by the concentration-response curve shown in Fig. 5. Since our procedure is very similar to the one used by Seaver and Imlay (i.e., actual H$_2$O$_2$ formation rates are measured by using H$_2$O$_2$ scavengerless log-phase cultures grown at

![FIG. 5. Dependence of scavenging rate on extracellular H$_2$O$_2$ concentration. Rates of H$_2$O$_2$ decomposition were measured in dilute suspensions (0.075 OD$_{600}$) of H. influenzae Rd grown in anMlc medium (■) and of AB2593 (hktE) grown in anMlc(GSSG) medium (△). Rates were normalized to a value of 1.0 OD$_{600}$.](http://jb.asm.org/)
37°C), we suggest that H. influenzae and E. coli cells generate equal amounts of H₂O₂ per s. Thus, the growth difference between H₂O₂ scavengerless H. influenzae and E. coli cannot be explained in terms of differences in H₂O₂ formation rates. Since GSH is a potent nonenzyme scavenger of reactive oxygen species and since it also functions as a cofactor for enzymes involved in oxidative stress defense, such as glutaredoxins and glutathione-S-transferases, deprivation of intracellular GSH not only blocks GSH-dependent H₂O₂ removal but also prevents all other processes that require GSH in the H. influenzae cytoplasm. As such, glutathione-deficient AB2593 is subjected to additional, e.g., thiol-disulfide, stress (32) compared to H₂O₂ scavengerless E. coli, which explains why glutathione-deficient AB2593 is not viable under aerobic conditions.

For E. coli, it was proposed that AhpR and HPI have discrete roles in scavenging H₂O₂. AhpR scavenges low levels of H₂O₂, and HPI scavenges high levels of H₂O₂ (36). We obtained analogous results showing that glutathione-based peroxidatic activity is more effective at scavenging very low concentrations of H₂O₂, whereas HktE is the more effective enzyme at higher concentrations. Omitting GSSG from the media of H. influenzae NCTC 8143 cultures results in a twofold increase in catalase activity (42). Moreover, the catalase activity of glutathione-deficient H. influenzae NCTC 8143 is similar compared to the activity of glutathione-supplemented counterparts stressed with 50 μM H₂O₂ for 1 h. This implies that glutathione deficiency and micromolar H₂O₂ stress affect hktE gene expression to a similar extent (42). Moreover, Table 1 illustrates that aerobiosis caused a drastic 6.6-fold increase in catalase activity in glutathione-deficient H. influenzae Rd cells. We and others observed no such regulation of catalase for E. coli deficient in glutathione biosynthesis compared to the isogenic parent (28; data not shown). Thus, in H. influenzae, debilitation of the glutathione-based peroxidatic system causes catalase induction, which may be a compensatory response to provide enough, though less efficient, H₂O₂-scavenging activity. This compensatory response may well explain our observation illustrated in Fig. 4 that either catalase or glutathione-based peroxidase activities alone give the same rate of H₂O₂ scavenging by whole cells as when both are present. Compensatory interactions between catalase and peroxidase (AhpR) synthesis have been observed previously in a wide range of bacteria. In E. coli (36), Xanthomonas campestris (24), Bacteroides fragilis (34), and Pseudomonas aeruginosa (27), mutations in the peroxidatic system, AhpR, cause catalase induction.

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glutathione in regulation of hydroperoxidase I in growing Escherichia coli.


