Escherichia coli cytochrome *c* peroxidase is a respiratory oxidase that enables the use of hydrogen peroxide as a terminal electron acceptor

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Microbial cytochrome *c* peroxidases (Ccp) have been studied for 75 years, but their physiological roles are unclear. Ccps are located in the periplasms of bacteria and the mitochondrial intermembrane spaces of fungi. In this study, Ccp is demonstrated to be a significant degrader of hydrogen peroxide in anoxic *Escherichia coli*. Intriguingly, *ccp* transcription requires both the presence of H_2O_2 and the absence of O_2. Experiments show that Ccp lacks enough activity to shield the cytoplasm from exogenous H_2O_2. However, it receives electrons from the quinone pool, and its flux rate approximates flow to other anaerobic electron acceptors. Indeed, Ccp enabled *E. coli* to grow on a nonfermentable carbon source when H_2O_2 was supplied. *Salmonella* behaved similarly. This role rationalizes *ccp* repression in oxic environments. We speculate that micromolar H_2O_2 is created both biologically and abiotically at natural oxic/anoxic interfaces. The OxyR response appears to exploit this H_2O_2 as a terminal oxidant while simultaneously defending the cell against its toxicity.

oxidative stress | anaerobic respiration | OxyR

The facultative anaerobe *Escherichia coli* lives adjacent to the epithelial layer of the mammalian gut, where it can respire by scavenging trace oxygen that diffuses into the lumen from the epithelial cells. When oxygen levels decline, *E. coli* can ferment, although it is less successful than the coresident obligate anaerobes. However, upon excretion, *E. coli* can thrive, whereas the obligate anaerobes enter a period of stasis.

In fully oxic habitats, *E. coli* must cope with reactive oxygen species that are generated internally through the adventitious oxidation of redox enzymes (1). Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are potentially toxic, because they oxidatively inactivate cytoplasmic enzymes that use exposed [4Fe–4S] clusters or ferrous iron atoms as prosthetic cofactors (2–6). H_2O_2 also oxidizes the intracellular pool of unincorporated iron through the Fenton reaction, thereby generating hydroxyl radicals that can damage DNA (7). To protect itself from these oxidants, *E. coli*—like virtually all organisms—routinely synthesizes superoxide dismutases to keep O_2^- levels low and catalase and NADH peroxidase to minimize H_2O_2. As a consequence, wild-type *E. coli* grows well in oxic environments.

In addition to these basal protections, most bacteria have the capacity to ramp up their defenses when they encounter extracellular H_2O_2 (8, 9). Exogenous H_2O_2 moves freely through porins into the periplasm, and because it is small and uncharged, it can gradually diffuse across the cytoplasmic membrane into the cell interior, where it poses a hazard (10). When H_2O_2 levels rise in *E. coli*, the H_2O_2 directly oxidizes the sensory cysteine residue of the OxyR transcription factor, triggering the formation of a disulfide bond that locks the protein into an activated conformation (11, 12). OxyR then activates the expression of defensive genes (13). Catalase and NADH peroxidase activities are boosted more than 10-fold. Suf proteins improve the repair of iron–sulfur clusters (14, 15), the manganese importer MntH allows Mn to replace ferrous iron in mononuclear enzymes (16, 17), and the miniferritin Dps protects DNA from Fenton chemistry by sequestering loose iron (18–20). When H_2O_2 levels later fall, glutaredoxins deactivate OxyR by reducing its disulfide bond (21).

Data indicate that ~200 nM steady-state intracellular H_2O_2 is sufficient to activate this response (10, 21). Because the cytoplasmic membrane is only semipermeable to H_2O_2 and the cytoplasm contains robust peroxidase and catalase activities, the internal H_2O_2 concentration is 5- to 10-fold lower than the external H_2O_2 concentration (10). Accordingly, measurements indicate that external H_2O_2 levels must exceed 2 μM to induce the intracellular OxyR response (21). Thus, one infers that despite dwelling in mostly hypoxic environments, *E. coli* must encounter micromolar levels of external H_2O_2 at some stage in its normal lifestyle. Strikingly, even obligate anaerobes manifest H_2O_2-inducible defenses, mediated either by OxyR or by PerR (22–24). The implication is that microbes may confront toxic doses of H_2O_2 even in low-oxygen environments.

Such a conclusion might resolve the conundrum of cytochrome *c* peroxidases. The biochemical activity of these enzymes was first described in 1940 (25), and although their reaction mechanism has been elaborated in detail (26), their physiological role continues to perplex. Notably, the bacterial enzymes are expressed only under anoxic conditions (27, 28). We now report that through YhjA, a cytochrome *c* peroxidase homolog, *E. coli* can exploit H_2O_2 as a terminal oxidant in a form of anaerobic respiration. This represents a surprising positive use of H_2O_2 in metabolism. YhjA is induced only when oxygen is scarce and H_2O_2 is present, implying that micromolar H_2O_2 can be found at oxic–anoxic interfaces. We

Significance

Hydrogen peroxide has been regarded exclusively as a hazard for bacteria; its sources and concentrations in natural habitats are uncertain. The cytochrome *c* peroxidase of *Escherichia coli* exhibits an expression pattern and flux rate that provides surprising insights into these issues. This periplasmic enzyme is induced only when H_2O_2 is present and molecular oxygen is absent. Intriguingly, it was ineffective as a defensive enzyme, but through its linkage to the quinone pool it did enable *E. coli* to respire using H_2O_2 as an anaerobic electron acceptor. We suggest that both chemical and biotic processes generate micromolar H_2O_2 at oxic/anoxic interfaces and that this scenario is common enough that microbes have evolved strategies to productively exploit the H_2O_2.

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suggest that YhjA be renamed Ccp, and we continue to use Ccp throughout this paper.

Results

Cytochrome *c* Peroxidase Is a Fourth Enzyme That Scavenges H_2O_2. *E. coli* has three enzymes that are known to degrade H_2O_2 in vivo: the KatG and KatE catalases, plus the NADH peroxidase AhpCF. For convenience, mutants that lack all three enzymes (*katG katE ahpCF*) are termed hydroperoxidase-deficient (Hpx^-) (29). When Hpx^- mutants are grown aerobically, they do not clear micromolar H_2O_2 from medium at a significant rate (Fig. 1). However, we were surprised to find that when the same strain was grown and exposed to H_2O_2 under anoxic conditions, it displayed substantial ability to degrade H_2O_2 (Fig. 1, rightmost bar).

The culture filtrate did not degrade H_2O_2 (Fig. S1), indicating that the scavenging activity resided within the cells. We considered the possibility that H_2O_2 might be eliminated through Fenton reactions with the cytoplasmic pool of unincorporated iron. However, our calculations indicated that the cell was unlikely to contain enough iron to support the observed rate of H_2O_2 clearance (*SI Materials and Methods*), and indeed the scavenging activity persisted when cells were perfused with dipyridyl, a chelator that fully blocks intracellular Fenton chemistry (7) (Fig. S1).

Therefore, we focused upon *E. coli* enzymes that have been reported to display peroxidase activities in vitro: thiol peroxidase (Tpx) (30), bacterioferritin comigratory protein (BCP) (31), a glutathione peroxidase homolog (BtuE) (32), a predicted cytochrome *c* peroxidase (YhjA, or Ccp) (28), and osmotically inducible peroxiredoxin (OsmC) (33). The physiological roles of these enzymes are uncertain, and they have not been reported to degrade H_2O_2 in vivo. We also considered the cytochrome *bd*-I, *bd*-II (AppBC), and *bo* terminal oxidases. These enzymes terminate aerobic respiration by transferring electrons from respiratory quinones to oxygen, but they also exhibit some peroxidase activity, because H_2O_2 is a formal intermediate in the oxygen-reduction process (34, 35). Each of the genes was deleted from the Hpx^- parent strain, and the rate of H_2O_2 scavenging was then remeasured under anoxic conditions. The rate was substantially diminished only in the *Δccp* mutant, and this effect was reversed when *ccp* was overexpressed from a plasmid (Fig. 2). The other mutations had no further impact even when they were introduced into the Hpx^- *Δccp* strain. We conclude that Ccp is an authentic scavenger, whereas the other proteins play little role in H_2O_2 clearance under these conditions. We do not know the source of the slight scavenging activity that persists in the Hpx^- *Δccp* strain, and it is not considered further here.

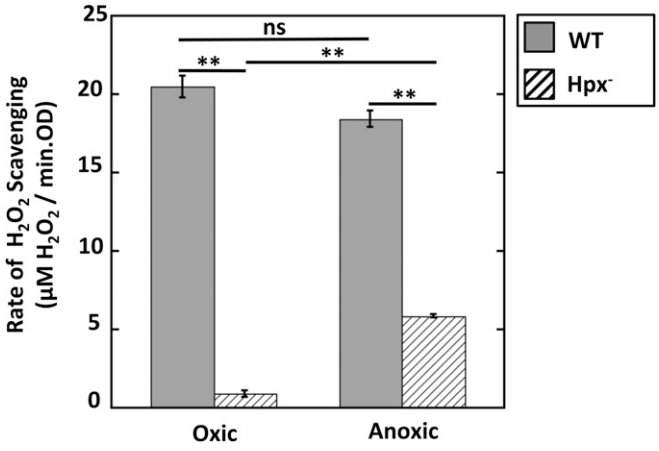

Fig. 1. Under anoxic conditions, a new H_2O_2-degrading activity appears. Wild-type (MG1655) and Hpx^- (LC106) cells were grown and assayed aerobically or anaerobically. Rates of H_2O_2 scavenging were measured as described. Error bars in this and subsequent figures represent SEM of three independent experiments. Asterisks represent statistical significance [*$P \le 0.05$; **$P \le 0.01$; ***$P \le 0.001$; not significant (ns), $P > 0.05$].

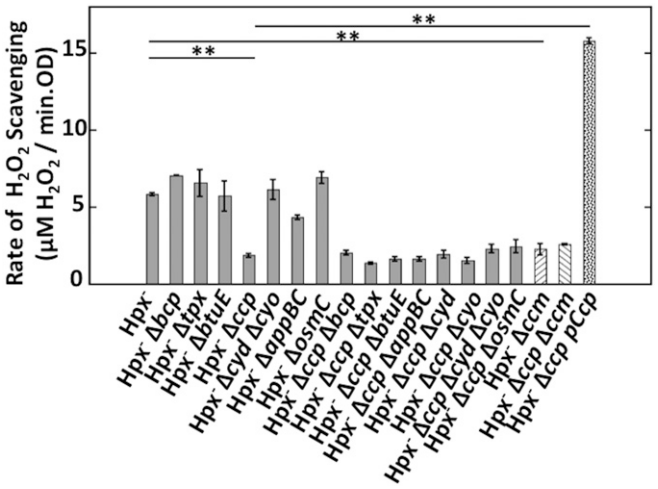

Fig. 2. Ccp scavenges H_2O_2 in anoxic Hpx^- cells. The Hpx^- parent strain and mutant derivatives were grown anaerobically, and the rate of H_2O_2 scavenging was measured. Cross-hatched bars: The cytochrome *c* maturation machinery is required for Ccp activity. Spotted bar, far right: the Hpx^- Δ*ccp* mutant was genetically complemented using pACYC184-*ccp* under its own promoter. Asterisks represent statistical significance (*$P \le 0.05$; **$P \le 0.01$; ***$P \le 0.001$; ns, $P > 0.05$). The single mutants other than Hpx^- Δ*ccp* and Hpx^- Δ*ccm* were not significantly different from Hpx^-. None of the double mutants were significantly different from Hpx^- Δ*ccp*. Strains used: Hpx^- (LC106), Hpx^- Δ*bcp* (MK150), Hpx^- Δ*tpx* (MK154), Hpx^- Δ*btuE* (MK158), Hpx^- Δ*ccp* (MK146), Hpx^- Δ*cyd* Δ*cyo* (SSK53), Hpx^- Δ*appBC* (MK180), Hpx^- Δ*osmC* (MK208), Hpx^- Δ*ccp* Δ*bcp* (MK172), Hpx^- Δ*ccp* Δ*tpx* (MK174), Hpx^- Δ*ccp* Δ*btuE* (MK176), Hpx^- Δ*ccp* Δ*appBC* (MK182), Hpx^- Δ*ccp* Δ*cyd* (MK164), Hpx^- Δ*ccp* Δ*cyo* (MK166), Hpx^- Δ*ccp* Δ*cyd* Δ*cyo* (MK170), Hpx^- Δ*ccp* Δ*osmC* (MK210), Hpx^- Δ*ccm* (MK198), Hpx^- Δ*ccp* Δ*ccm* (MK418), and Hpx^- Δ*ccp* pAcyc184-*ccp* (MK430).

Ccp Is an Authentic Peroxidase That Derives Electrons from the Quinone Pool. The *yhjA* (here, *ccp*) gene is annotated as a potential cytochrome c peroxidase. Well-studied Ccp enzymes contain two *c*-type hemes and localize in the mitochondrial intermembrane space or bacterial periplasm. In vitro, these enzymes can receive electrons from reduced respiratory cytochrome *c* protein; the Ccp can then transfer electrons to H_2O_2, reducing it to water (25, 36–38).

The *E. coli* respiratory chain does not use cytochrome *c*, and its Ccp belongs to a second class that has a third *c*-type heme, which serves as the electron entry port when artificial reductants are provided (26). *E. coli* has a single known pathway for synthesizing and exporting *c*-type heme to periplasmic proteins; this *ccmABCDEFGH* operon has been shown to provide *c*-type hemes to periplasmic nitrite reductase, which is encoded by the adjacent *nir* operon. We found that the Hpx^- Δ*ccmABCDEFGH* and the Hpx^- Δ*ccp* Δ*ccmABCDEFGH* mutants phenocopied the Hpx^- Δ*ccp* mutant, confirming that the Ccm pathway is also the source of the *c*-type heme that activates Ccp (Fig. 2).

Because the Fenton reaction is chemically simple, it can be catalyzed at modest rates by many enzymes that contain solvent-exposed iron cofactors. However, such adventitious chemistry often requires high concentrations of H_2O_2. For example, although the *bo* and *bd*-I terminal oxidases can reduce H_2O_2 to water (35, 39), they do so effectively only at millimolar H_2O_2 concentrations that vastly exceed the low-micromolar concentrations of H_2O_2 that are likely to occur in nature (Fig. S2). In contrast, by measuring

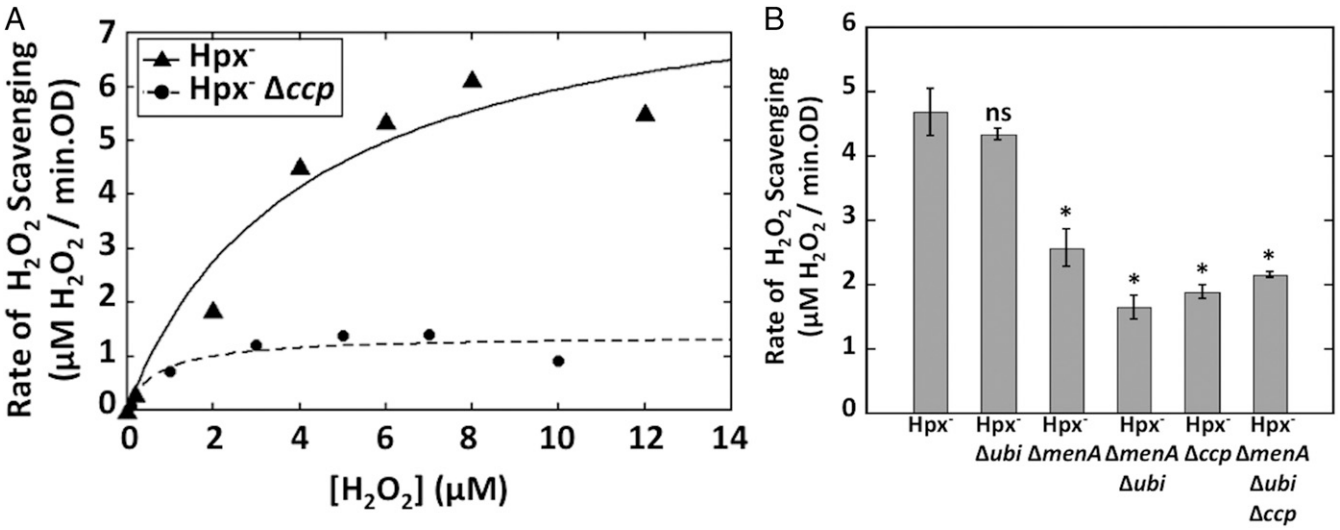

Fig. 3. (*A*) Ccp has a low effective K_m for H_2O_2. Rates of anoxic H_2O_2 scavenging were measured with Hpx⁻ (LC106) and Hpx⁻ Δ*ccp* (MK146) cells. Triplicate measurements determined K_m(app) to be 5.2 ± 0.6 μM. A single trial is shown here. (*B*) Respiratory quinones are required for Ccp function. The Hpx⁻ strain (LC106) and its derivatives lacking ubiquinone (LC148); menaquinone (SSK6); both (LC160); *ccp* (MK146); or ubiquinone, menaquinone, and *ccp* (MK184) were grown anaerobically in LB medium, and the rates of H_2O_2 scavenging were measured. Asterisks represent statistical significance compared with the Hpx⁻ strain (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ns, $P > 0.05$).

H_2O_2 clearance in an Hpx⁻ background, we determined that the apparent K_m of Ccp in intact cells was 5.2 ± 0.6 μM (Fig. 3*A*). We conclude that H_2O_2 is likely to be its physiological substrate.

Ccp has been predicted to be a single-pass enzyme that is tethered to the cytoplasmic membrane, with the bulk of its polypeptide in the periplasm (40). The electron transport chain in the cytoplasmic membrane is an available source of electrons for periplasmic redox enzymes. In vitro, some quinone analogs were found to be capable of reducing some bacterial Ccp enzymes (41–43), although the activity was not detected with the Ccp of *E. coli* (42). We observed that Hpx⁻ *ubi* mutants retained Ccp scavenging activity, but the activity was diminished in *men* mutants and was essentially absent from *ubi men* double mutants (Fig. 3*B*). Menaquinone is the primary quinone in membranes under the anoxic conditions of these experiments (44).

Periplasmic nitrite and nitrate reductases also acquire electrons from the quinone pool. In those cases, the structural genes for the catalytic enzymes sit alongside genes that encode bridging proteins that deliver electrons from the quinones to the catalytic enzymes. No analogous gene accompanies *ccp*. We verified that the Ccp scavenging activity persisted in mutants lacking the Nap and Nrf bridge proteins (Fig. S3); we infer that its association with the cytoplasmic membrane allows Ccp to receive electrons directly from the quinone pool.

Ccp enables anaerobic respiration with H_2O_2 as the terminal oxidant. Partridge et al. (28) demonstrated previously that *E. coli* expresses *ccp* (called *yhjA* in their study) only under anoxic conditions, and mutants lacking either the Fnr or OxyR transcription factors failed to do so. They identified Fnr and OxyR binding sites upstream of the transcriptional start site. In their experiments, the involvement of OxyR appeared not to require the presence of H_2O_2. We found that the addition of H_2O_2 stimulated Ccp activity by threefold (Fig. 4*A*). We generated a *ccp–lacZ* transcriptional fusion and reexamined this pattern. Anoxia alone

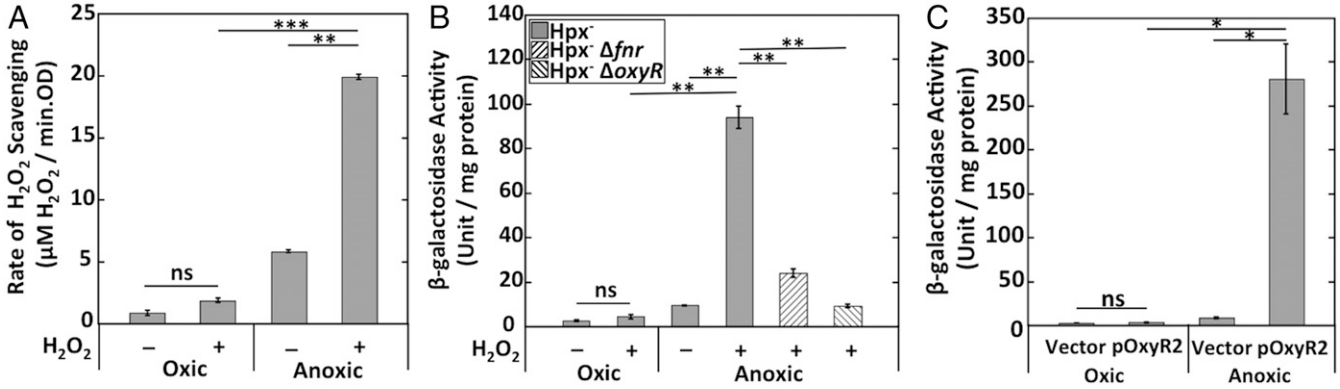

Fig. 4. Strong *ccp* expression requires both the absence of oxygen and the presence of H_2O_2. (*A*) The rates of H_2O_2 scavenging were measured in Hpx⁻ cells grown in oxic or anoxic conditions. Where indicated, cells were incubated with 40 μM H_2O_2 for 1 h before assay. (*B*) β-galactosidase activity from the transcriptional *ccp′–lacZ*⁺ reporter fusion was measured in Hpx⁻ (MK250), Hpx⁻ Δ*fnr* (MK274), and Hpx⁻ Δ*oxyR* (MK278) mutants grown in oxic or anoxic media. Where indicated, cells were incubated with 40 μM H_2O_2 for 1 h before harvesting. (*C*) β-galactosidase activity from the fusion was measured in wild-type cells containing either a pACYC184-*oxyR2* plasmid (expressing a constitutively active form of OxyR) (MK346) or empty vector (MK344), grown anaerobically. Asterisks represent statistical significance (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ns, $P > 0.05$).

was insufficient to promote full expression; H_2O_2 was also required (Fig. 4*B*). This pattern was most easily observed with Hpx$^-$ strains that do not rapidly clear H_2O_2 from the medium, but H_2O_2 stimulated expression in wild-type cells as well (Fig. S4). Neither *fnr* nor *oxyR* mutants showed induction (Fig. 4*B*). The requirement for H_2O_2 was relieved by expression of OxyR2, a mutant form of OxyR that remains activated even in the absence of H_2O_2 (Fig. 4*C*). We suspect that the contamination of LB by H_2O_2 (45) may previously (28) have given the misleading impression that H_2O_2 was unnecessary for *ccp* induction. Collectively, the dual regulation by the OxyR and Fnr transcription factors ensures that *E. coli* expresses *ccp* only if molecular oxygen is absent and H_2O_2 is present. The requirement for H_2O_2 confirmed our view that it is the natural substrate of the enzyme.

Molecular oxygen is an obligatory precursor in all known routes of H_2O_2 formation, so the fact that Ccp is synthesized only in its absence was perplexing. However, we and others have observed that both facultative and obligately anaerobic bacteria continue to express oxidative defenses even when oxygen is absent, suggesting that H_2O_2 can find its way into anoxic habitats (*Discussion*). Therefore, we considered the possibility that in anoxic environments Ccp might replace AhpCF as the primary scavenger of H_2O_2. However, an *ahpC–lacZ* fusion demonstrated that AhpCF continues to be synthesized at full force in anaerobic cells (Fig. S5*A*). In fact, AhpCF and catalase remained the major enzymes involved in clearing H_2O_2 from the bulk medium, because Hpx$^-$ strains were defective at this activity. Ccp was a minor contributor (Fig. S5*B*).

The established role of scavenging enzymes is to shield cells from the toxic effects of H_2O_2. It seemed plausible that Ccp might act as a new layer of defense that would degrade exogenous H_2O_2 before it could diffuse into the cytoplasm, where H_2O_2-sensitive enzymes are located. When wild-type cells and *ccp* mutants were challenged with millimolar H_2O_2, the rates of killing were indistinguishable (Fig. S6). However, that protocol might fail to elicit the protective effect of a peroxidase because the H_2O_2 dose grossly exceeds the effective K_m of the enzyme, such that it can scavenge only a minute fraction of the H_2O_2. An alternative is to challenge cells with low doses of exogenous H_2O_2 that just barely poison metabolism. To do so, we tested the vulnerability of *ccp* mutants to a gradient of exogenous H_2O_2 through zone-of-inhibition experiments using a minimal glucose medium (Fig. 5 *A* and *B*). In this situation, the poisoning of amino acid biosynthetic enzymes sets the H_2O_2 sensitivity of the cell at 0.5–1 μM intracellular H_2O_2 (46); we thought this arrangement offered the best chance to detect any defensive impact of Ccp. However, the *ccp* mutants were again no more sensitive than wild-type cells. Only in the Hpx$^-$ background did Ccp exert an impact, which we attribute to its providing the sole route of clearance of H_2O_2 from the bulk medium. These data resemble zone-of-inhibition results that were previously obtained with other bacteria (43, 47–50) and or fungi (51, 52), in which mutants showed either only a marginal increase in sensitivity, or none at all. In contrast, the Hpx$^-$ strain was extremely sensitive, showing that in *E. coli* the cytoplasmic peroxidase and catalase activities play a much greater role in protecting the cell from H_2O_2.

To understand why Ccp fails to shield the cytoplasm, we modeled H_2O_2 fluxes as H_2O_2 moves from the external environment into the periplasm. Outer membrane porins allow very rapid H_2O_2 movement into the periplasm (53), whereas the cytoplasmic membrane exhibits a membrane permeability coefficient that is lower by at least two orders of magnitude (10, 54). Calculations showed that the activity of Ccp could diminish the periplasmic concentration of H_2O_2 by only 0.1% in the face of its rapid exchange with the external environment (*SI Materials and Methods*). Accordingly, the rate of subsequent influx into the cytoplasm was effectively unchanged by Ccp (Fig. 5*C*). Thus, it

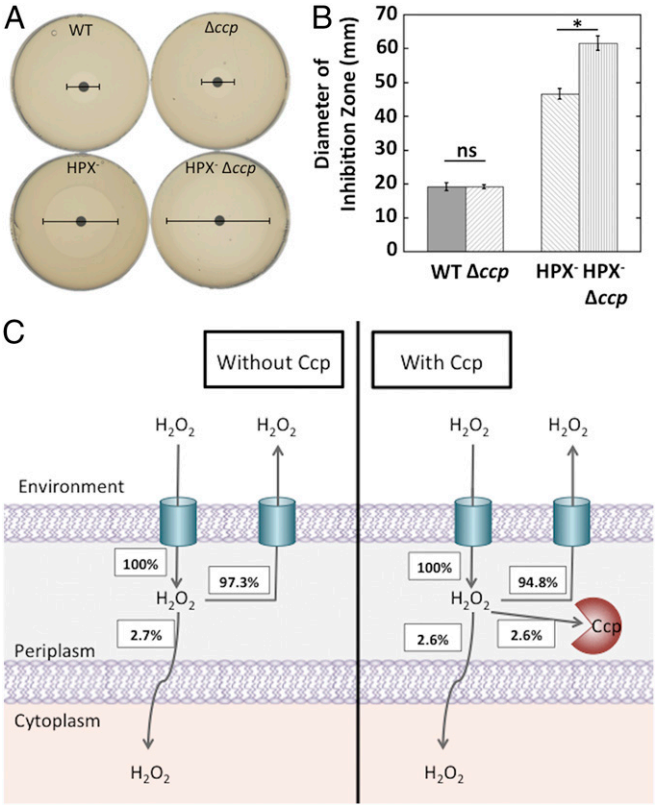

Fig. 5. Ccp cannot protect the cytoplasm from exogenous H_2O_2. (*A*) Wild-type (MG1655), Δ*ccp* (MK416), Hpx$^-$ (LC106), and Hpx$^-$ Δ*ccp* (MK146) mutant cells were grown to OD_{600} ~0.1 and spread on a plate. Disks soaked in H_2O_2 were put on the plate, and the diameter of zone of inhibition was measured after 24 h. (*B*) Data from *A* are shown as bar graphs. (*C*) Modeling (*SI Materials and Methods*) shows that H_2O_2 exchange between the external environment and the periplasm is too fast for Ccp to significantly diminish the periplasmic H_2O_2 level. Therefore, Ccp has minimal effect on H_2O_2 entry into the cytoplasm. Steady-state fluxes (in %) are calculated relative to the rate of H_2O_2 entry into the periplasm. Asterisks represent statistical significance (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ns, $P > 0.05$).

seems implausible that the main role of Ccp is to protect the cell from exogenous H_2O_2.

Because Ccp mediates electron flow from respiratory quinones to H_2O_2, its physiological role may be to exploit H_2O_2 as a terminal electron acceptor, an idea that was broached by Atack and Kelly (26). The regulatory data fit this model, as Ccp expression was blocked when oxygen, a superior acceptor, is available. Further, the presence of oxygen blocks Ccp turnover by competing for the electrons carried by the quinone pool (Fig. 6*A*). To probe further, we tested the effects upon *ccp* expression of added nitrate and fumarate, which are alternative anaerobic electron acceptors. Nitrate is a better respiratory substrate than fumarate, because nitrate reductase provides an additional coupling site in generating proton-motive force. Like oxygen, nitrate inhibited Ccp-mediated scavenging and *ccp–lacZ* expression (Fig. 6 *B* and *C*). *E. coli* has three transcription factors that respond to the presence of nitrate—NarL, NarP, and ArcA—but experiments failed to clarify which of these transcription factors might be involved in the repression (55) (Fig. S7).

Fluxes through various *E. coli* metabolic pathways vary over five orders of magnitude. If the role of Ccp were to enable anaerobic respiration, the flux through Ccp should approximate fluxes to other electron acceptors. The rate of respiration through Ccp to H_2O_2 was measured under fully induced conditions; it was elevated threefold compared with the noninduced

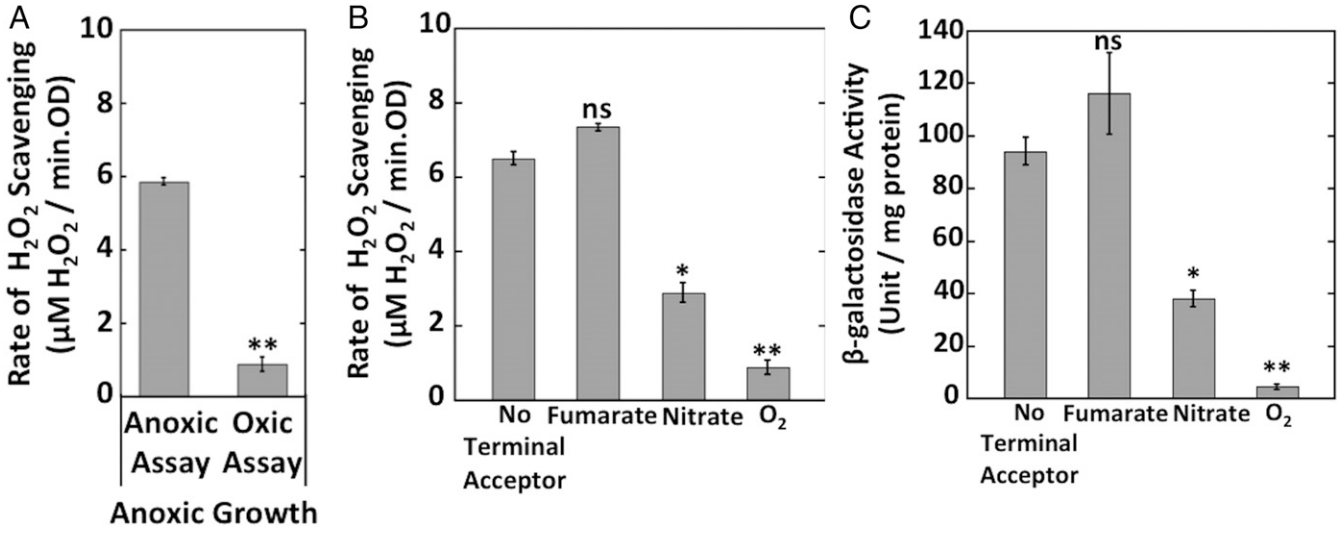

Fig. 6. Oxygen and nitrate block both Ccp activity and synthesis. (*A*) Hpx$^-$ (LC106) cells were grown anaerobically, and the rate of H_2O_2 scavenging was measured in absence or presence of oxygen. (*B*) Hpx$^-$ (LC106) cells were both grown and assayed for H_2O_2 scavenging either without an electron acceptor or in the presence of fumarate, nitrate, or oxygen. (*C*) Expression of the transcriptional *ccp′–lacZ*$^+$ reporter fusion in Hpx$^-$ (MK250) cells grown either without an electron acceptor or in the presence of fumarate, nitrate, or oxygen. Cells were incubated with 40 μM H_2O_2 for 1 h before harvesting. Asterisks represent statistical significance compared with anoxic assay in *A* and no terminal acceptor in *B* and *C* (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ns, $P > 0.05$).

conditions that had been sampled in Fig. 1 (Fig. 7). This rate was found to be somewhat less than the respiratory rates with molecular oxygen and nitrate but comparable to the respiratory rate with fumarate. Thus, although the Ccp flux is insufficient to shield the cell from H_2O_2, it is sufficient to provide a respiratory benefit.

To test this idea more directly, we monitored anaerobic growth with glycerol as sole carbon source. Glycerol catabolism depends upon the oxidation of α-glycerolphosphate by the respiratory GlpABC dehydrogenase complex, which transfers electrons to the menaquinone pool. Thus, respiration can proceed only if an exogenous terminal oxidant is available. We tested whether H_2O_2 could support growth. Unlike other respiratory substrates, which can be added in millimolar concentrations, H_2O_2 must be maintained at micromolar levels to avoid toxicity; therefore, 5 μM H_2O_2 was supplied, and the H_2O_2 levels in the medium were monitored and replenished periodically. Dilute cells were used so they would not exhaust the H_2O_2 too quickly, and growth was tracked by viable cell counts rather than optical density. After the preculture electron acceptor (fumarate) was removed, cells divided twice more and then stopped. Fig. 8 shows that H_2O_2 enabled continued growth. Growth did not occur if *ccp* was deleted, and it was restored in the complemented strain.

To rule out the possibility that the observed growth phenotype is specific to K12 strains, we confirmed that H_2O_2 also enabled the anaerobic respiration of an *E. coli* B strain and of *Salmonella typhimurium* (Fig. 9). In sum, the regulatory, flux, and growth data support the conclusion that the role of Ccp may be to exploit H_2O_2 as a respiratory substrate when better substrates are not available. Its location in the periplasm allows turnover even as AhpCF protects internal enzymes by keeping cytoplasmic H_2O_2 levels low.

Discussion

Cytochrome *c* peroxidases are widely distributed among microbes. Previous studies demonstrated their activity in vitro, using artificial electron donors; here, experiments confirm that they degrade micromolar H_2O_2 at substantial rates in vivo. Ccps have generally been assumed to be defensive enzymes, but our analysis does not support this view, at least for *E. coli* Ccp. The enzyme cannot provide enough activity for isolated cells to lower the periplasmic H_2O_2 level below that of the surrounding medium, so Ccp does not shield the cytoplasm from H_2O_2. Further, because *E. coli* is a minor member of the intestinal flora, it is unlikely to assume the responsibility for clearing H_2O_2 from its environment. We do recognize that, in principle, if *E. coli* grew in a clonal biofilm with only a slow H_2O_2 influx from the surrounding environment, the scavenging activity that Ccp provides might help lower the H_2O_2 level within the biofilm itself. However, the scavenging activity provided by Ccp does not significantly exceed that which is already provided by cytoplasmic enzymes. Further, for Ccp to deplete H_2O_2 within a biofilm, the biofilm would have to be dense enough to limit H_2O_2 entry, but without simultaneously blocking the influx of the carbon sources that provide the

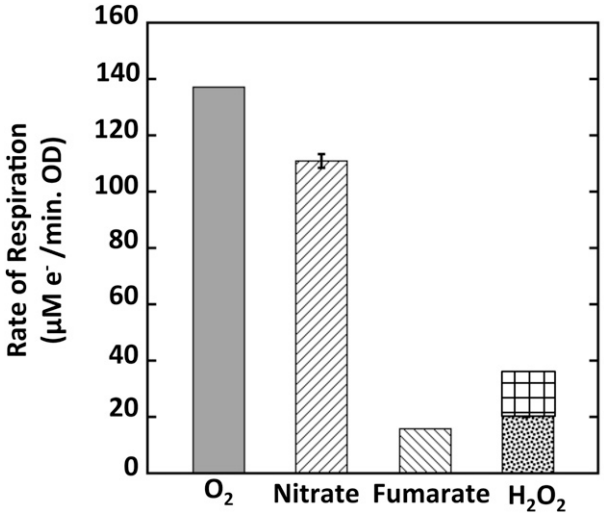

Fig. 7. Rate of respiration of H_2O_2 is comparable to that of fumarate. Comparison of respiration rates by wild-type cells with different electron acceptors in glycerol medium. The grid pattern extension of the H_2O_2 bar represents calculated V_{max}, because unlike the other acceptors, H_2O_2 was provided at a subsaturating (10 μM) concentration. See *Materials and Methods* for details. The rate of respiration by fumarate was calculated in *SI Materials and Methods*.

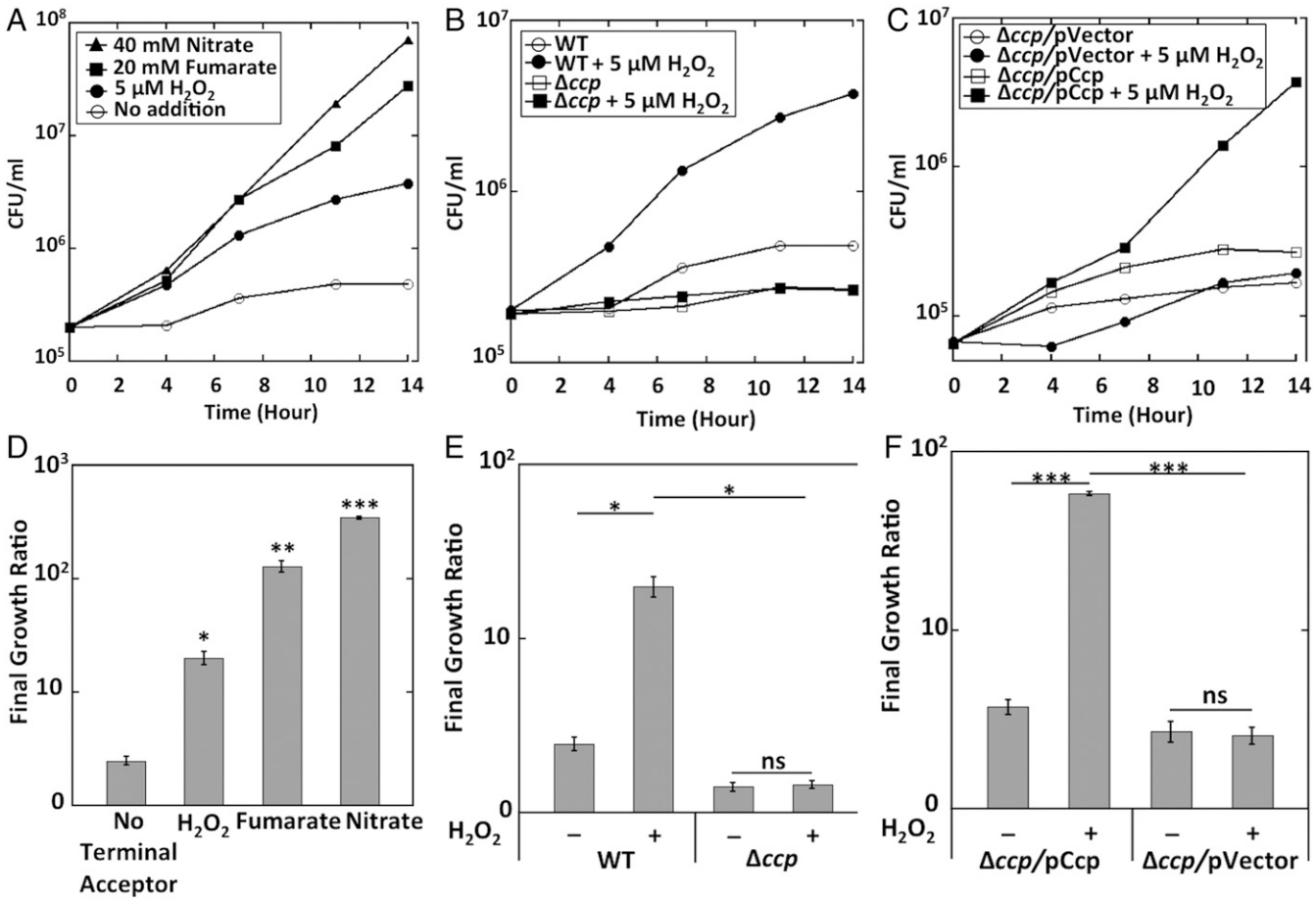

Fig. 8. Ccp allows respiratory growth using H_2O_2 as the final electron acceptor. (*A*) Wild-type cells were grown in anoxic glycerol medium without any electron acceptor (○) or in the presence of 40 mM nitrate (▲), 25 mM fumarate (■), or 5 μM H_2O_2 (●). Viable cells (cfus) were determined at different time points. Residual growth in the absence of H_2O_2 appears to be due to trace oxygen in the anaerobic chamber (Fig. S8). (*B*) WT and Δ*ccp* (MK416) strains were grown in anoxic glycerol medium in the presence or absence of 5 μM H_2O_2. (*C*) Δ*ccp* mutants were complemented with the *ccp* gene under its own promoter in a plasmid. Strains with pACYC184-*ccp* (MK436) and the empty vector (MK432) were grown in presence or absence of 5 μM H_2O_2. (*D*) The initial ($t = 0$) and the final (14-h) time points from three biological replicates of *A* were used to calculate the growth ratio for each growth condition. (*E*) The initial ($t = 0$) and the final (14-h) time points from three biological replicates of *B* were used to calculate the growth ratio for each growth condition. (*F*) The initial ($t = 0$) and the final (14-h) time points from three biological replicates of *C* were used to calculate the growth ratio for each growth condition. Asterisks represent statistical significance (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ns, $P > 0.05$).

respiratory electrons. Thus, Ccp could conceivably contribute as a defensive enzyme only in a very constrained set of circumstances.

However, our data do show that Ccp can allow *E. coli* to use H_2O_2 as a respiratory substrate. The regulatory controls upon *ccp* are more completely resolved for *E. coli* than for other bacterial or fungal Ccps, and they fit this model (Fig. 10). The transcriptional dependence upon OxyR dictates that the gene be transcribed only when H_2O_2 is present; Fnr collaborates to ensure that transcription only occurs when a better electron acceptor is not available. Apparent OxyR and Fnr binding sites have been identified (28). The details of promoter architecture and transcription factor interaction will be interesting, particularly because with other genes OxyR seems to operate independently of other transcription factors. This regulatory scheme is a nice demonstration that the two transcription factors are truly specific for their effector oxidants: in controlling *ccp*, Fnr is nonresponsive to H_2O_2, and OxyR is not activated by O_2 per se.

But the bigger picture presents an obvious question: In what circumstances might *E. coli* encounter H_2O_2 but not O_2? We suggest that at oxic–anoxic interfaces both abiotic and biotic processes may generate H_2O_2. Such interfaces occur along the margins of intestines and in static ground sediments, and their defining characteristic is that highly reducing environments containing reduced sulfur and metal species collide with an oxygenated one. In the intestinal lumen, sulfate-reducing bacteria generate millimolar amounts of sulfide (56, 57), which presumably diffuses to the margins where it will mix with oxygen. *E. coli* lives in those margins, as evidenced by the fact that to colonize a mouse intestine, it relies upon a specialized cytochrome oxidase with high oxygen affinity and the ability to function in the presence of sulfide (58–60). When reductants encounter oxygen, metal-catalyzed sulfide oxidation generates H_2O_2. Further, lactic acid bacteria commonly dwell in hypoxic environments. Although they are classic fermentative organisms, when oxygen becomes available many of them redirect substrate through pyruvate, lactate, or NADH oxidases. These enzymes produce H_2O_2 as a stoichiometric product (61, 62). Finally, *Bacteroides* species that predominate in the intestine generate substantial H_2O_2 when they encounter oxygen, presumably due to the adventitious autoxidation of their low-potential redox systems (63). Thus, at these interfaces, both chemical and biological processes are likely to diminish O_2 levels and send H_2O_2 diffusing into anoxic zones. We suspect that this is the source of the H_2O_2 that *E. coli* exploits. Indeed, *Campylobacter ccp* mutants exhibit colonization defects

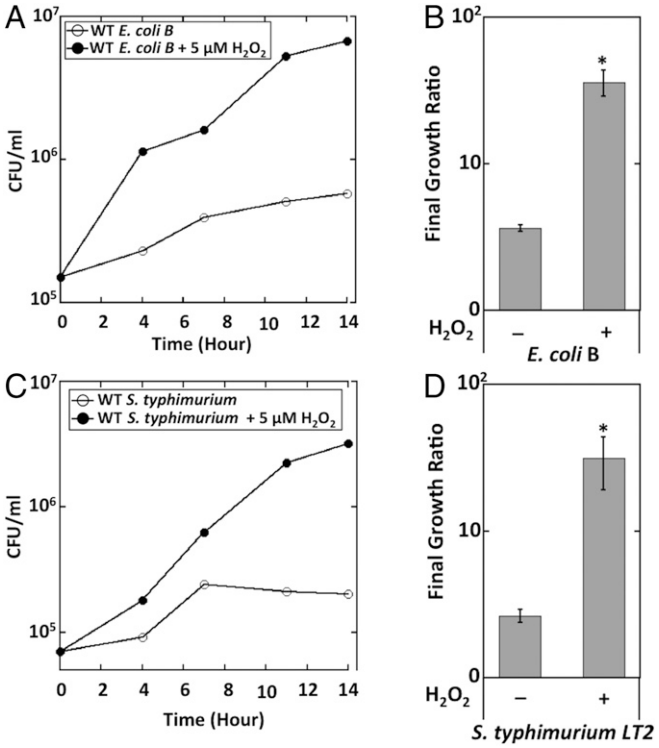

Fig. 9. *E. coli* B and *S. typhimurium* LT2 can grow using H_2O_2 as the final electron acceptor. (*A*) Wild-type *E. coli* B cells were grown in anoxic glycerol medium without any electron acceptor (○) or in the presence of 5 μM H_2O_2 (●). Viable cells (cfus) were determined at different time points. (*B*) The initial ($t = 0$) and the final (14-h) time points from three biological replicates of *A* were used to calculate the growth ratio for each growth condition. (*C*) Wild-type *S. typhimurium* LT2 was grown in anoxic glycerol medium without any electron acceptor (○) or in the presence of 5 μM H_2O_2 (●). Viable cells (cfus) were determined at different time points. (*D*) The initial ($t = 0$) and the final (14-h) time points from three biological replicates of *A* were used to calculate the growth ratio for each growth condition. Asterisks represent statistical significance compared with growth with no terminal acceptor (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ns, $P > 0.05$).

that are consistent with the notion that H_2O_2 is an important component of the intestinal environment (64).

Other bacteria also express Ccp only in hypoxic environments, suggesting that it serves a common purpose in most organisms. Seib et al. (65) noted that *Neisseria gonorrhea* encodes a Ccp enzyme, whereas its close relative *N. meningitidis* does not. The authors speculated that the key difference is that *N. meningitidis* dwells in the oxygen-rich nasopharynx, whereas *N. gonorrhea* lives in the urogenital tract where oxygen is scant and lactic-acid bacteria may excrete H_2O_2. Recently, Stacy et al. (66) noted a shift in the gene set used by *Aggregatibacter actinomycetemcomitans* when it was coinfected with *Streptococcus gordonii* in a murine thigh abscess model; they inferred that *Aggregatibacter* respiration was stimulated by a terminal oxidant that *S. gordonii* released. The data would fit the idea that *Aggregatibacter* Ccp was exploiting H_2O_2 that *S. gordonii* generates as a fermentation product.

The apparent K_m of *E. coli* Ccp is 5 μM, and the electron flux through Ccp would be enough to sustain growth only if H_2O_2 approached this level. We and others have modeled H_2O_2 flow through a porous outer membrane and a semipermeable inner membrane into a cytoplasm containing high titers of peroxidases and catalases (10, 54). The upshot is that micromolar H_2O_2 outside cells will generate an equivalent level in the periplasm but perhaps a 10-fold lower level in the cytoplasm. The set point of OxyR is submicromolar (10, 21), so this flux is enough to activate OxyR and to induce Ccp. This situation allows Ccp to use micromolar external H_2O_2 as a periplasmic oxidant while at the same time the threat to cytoplasmic biomolecules is minimal. The other activities comprising the OxyR response would presumably help to keep the cell fit even while it respired using H_2O_2.

Although catalase generates molecular oxygen when it degrades H_2O_2, the primary scavengers of H_2O_2 in enteric bacteria are NADH peroxidases (29, 67). Therefore, oxygen production by H_2O_2-fed cells was insufficient to enable cytochrome oxidase-dependent growth. In any case, in natural habitats any oxygen generated by cytoplasmic catalases would immediately escape the source bacterium before it could be employed for respiration, because oxygen crosses membranes at diffusion-limited rates.

The existence of H_2O_2 in microoxic environments may explain why many bacteria are programmed for robust anti-H_2O_2 responses—including obligate anaerobes, whose oxidative defenses otherwise seem counterintuitive. One might suppose that these systems are useful during the occasional entry of anaerobes into oxic environments—but some of their peroxidatic scavenging systems, including rubrerythrins, are ineffectual when oxygen levels are high enough to poison central metabolism (24). Implicitly, these peroxidases, like Ccp, must serve to degrade H_2O_2 in habitats that contain little oxygen. Interestingly, low-micromolar H_2O_2 levels are also predicted to obtain to the phagosomal vesicles. By chance, *Salmonellae*, which naturally move between intestinal and macrophage habitats, might be equipped by its OxyR regulon to handle both environments.

The employment of a toxin such as H_2O_2 as a respiratory substrate strikes an ironic note, but this evolutionary step recapitulates the adoption of O_2. Life emerged in an anoxic world; when O_2 accumulated 2 billion years later (68), cells were threatened by its propensity to deactivate enzymes that have radical or low-potential metal centers. Defensive enzymes arose. But the toxicity of oxygen did not preclude the simultaneous appearance of respiratory enzymes that exploit it as an electron acceptor. We infer that the story with H_2O_2 may be analogous.

Materials and Methods

Reagents. All antibiotics (ampicillin, chloramphenicol, kanamycin, tetracycline, and spectinomycin), ortho-nitrophenyl-β-galactoside, 2,2′-bipyridyl, horseradish peroxidase, 30% hydrogen peroxide, casein acid hydrolysate, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, and NADH were purchased from Sigma-Aldrich. Amplex UltraRed reagent was obtained from Life Technologies.

Strains and Bacterial Growth. Strains, primers, and plasmids used in this study are listed in Tables S1–S3. Deletion mutations were made using the λ-red recombinase (69) or ordered from the Keio collection (70). Mutant strains were assembled by P1 transduction (71), and the inheritance of mutant alleles was confirmed by PCR analysis. A *malE*::Tn*10* mutation was used to cotransduce the *ubiA420* allele; the *malE* mutation itself was inconsequential in these experiments because the media lacked maltose. Transcriptional *lacZ* fusions were created as described (72), and the fusions were recombined into the lambda attachment site so that the native alleles were retained.

Luria broth and base minimal A salts were of standard composition (71). Glucose–amino acid medium was minimal A salts supplemented with 0.2% casein acid hydrolysate, 0.2% glucose, 5 μg/mL thiamine, 0.02% $MgSO_4$, and 0.5 mM tryptophan. Where indicated, 40 mM nitrate or 25 mM fumarate was added. Uracil (1 mM) was added to the media in experiments that involved quinone biosynthetic mutants, because dihydroorotate dehydrogenase requires a quinone substrate.

β-galactosidase activity was measured as described (71), using cells that were grown to OD_{600} of 0.1 and then challenged with H_2O_2 for 1 h if indicated. Protein concentrations were measured using Bradford assay (Coomassie protein assay reagent; Thermo Scientific) using BSA as the standard. All anoxic growth and assays were done using anoxic buffers, media, and reaction components in a Coy anaerobic chamber (Coy Laboratory Products, Inc.) under 85% N_2, 10% H_2, and 5% CO_2.

Hydrogen Peroxide Scavenging Assay. Overnight cultures were diluted to OD_{600} of 0.01 into fresh glucose–amino acids medium, and cells were grown

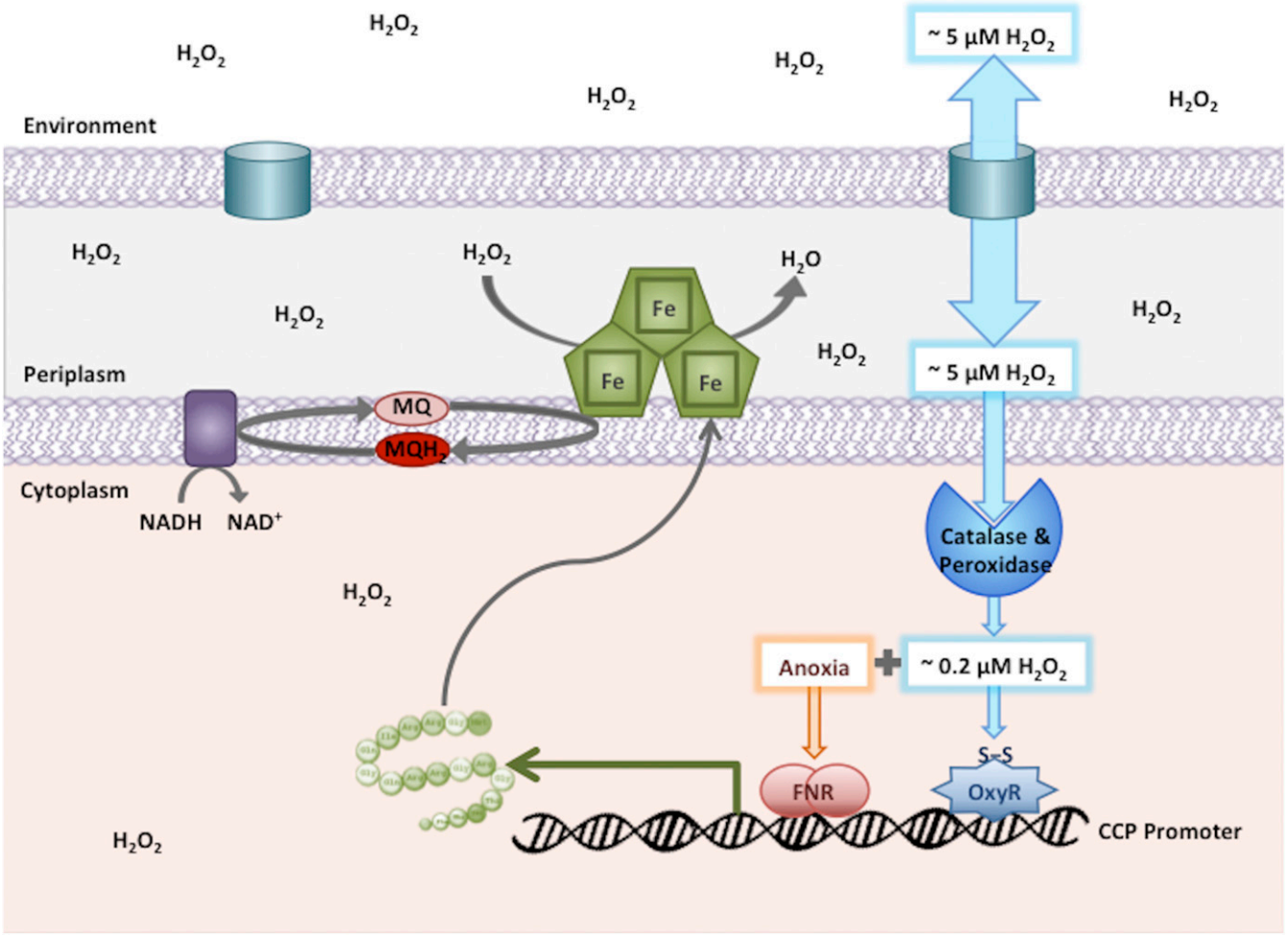

Fig. 10. Proposed model for Ccp expression and function. H_2O_2 rapidly enters the periplasm through porins but penetrates the inner membrane at a lower rate. Activation of OxyR stimulates synthesis of catalase and NADH peroxidase, which keep the cytoplasmic H_2O_2 concentration low. In anoxic conditions, FNR is also activated, and together it and OxyR induce *ccp*. Ccp enters the periplasm and allows the respiratory chain to reduce H_2O_2 to H_2O. In isolated cells, Ccp enables anaerobic respiration, but it does not degrade H_2O_2 quickly enough to reduce the periplasmic or cytoplasmic H_2O_2 concentrations.

to OD_{600} of 0.2. Cells were then diluted to OD_{600} of 0.02 in fresh medium containing 10 μM H_2O_2. The culture was maintained at 37 °C, and 1-mL samples were removed every 4 min for 20 min. The samples were centrifuged (1 min at 15,300 × *g*, 4 °C) and the supernatant was frozen on dry ice and stored at −80 °C freezer. The samples were subsequently thawed on ice, and the concentration of peroxide was measured using Amplex UltraRed/horseradish peroxidase (29) in a Shimadzu RF Mini-150 fluorometer.

Glycerol Growth Assays. Cells were grown anaerobically overnight in minimal A medium supplemented with 40 mM glycerol, 25 mM fumarate, amino acids (histidine, tyrosine, isoleucine, leucine, valine, and phenylalanine, each at 0.5 mM), 0.02% magnesium sulfate, and 5 μg/mL thiamine. The branched-chain and aromatic amino acids were provided to avoid growth disruptions due to the potential inactivation of iron enzymes in their biosynthetic pathways. *E. coli* cannot catabolize these amino acids to use them as energy sources. Fumarate was included to enable anaerobic respiration. Cells were then precultured in the same medium to OD_{600} of 0.1 and then washed four times with minimal A salts to remove fumarate. Bacteria were diluted to OD_{600} of 0.0001 in the fresh 37 °C medium without fumarate. Respiratory oxidants (40 mM nitrate, 25 mM fumarate, or 5 μM H_2O_2) were added as indicated. The residual H_2O_2 levels were periodically determined by Amplex UltraRed analysis, and H_2O_2 was replenished as needed to restore the concentration to 5 μM. Samples were periodically removed, diluted, and plated on LB agar. Colonies were counted the next day, and the colony-forming units in the original culture were calculated. Slight residual growth in the absence of electron acceptors was apparent only at very low cell densities and probably indicates trace oxygen in the media (Fig. S8).

NADH Oxidation Assay. Inverted membrane vesicles were prepared as described (73) from Δ*cyo* and Δ*cyd* mutants that had been grown aerobically in LB medium to 0.25 OD_{600}. The NADH oxidation reaction was performed using anoxic reagents in the Coy chamber with inverted membrane vesicles, 50 μM NADH, and different concentrations of H_2O_2. NADH oxidation was measured using the extinction coefficient for NADH of 6,220 $cm^{-1} \cdot M^{-1}$ at 340 nm.

Nitrate Reduction and Oxygen Consumption Assays. Wild-type cells were grown to 0.25 OD_{600} in glucose–amino acid medium supplemented with 40 mM nitrate. Cells were washed three times with minimal A salts to remove the residual nitrite and inoculated to 0.020 OD_{600} in glucose-amino acid medium. The concentration of nitrite was measured every 30 min using a modified version of the Griess assay. Sulfanilamide (250 μL of 2% wt/vol in 5% HCl), *N*-(1-naphthyl)ethylenediamine dihydrochloride (250 μL of 0.1% wt/vol in water) and 500 μL sample were mixed, and the absorbance at 540 nm was measured after 20 min of incubation in room temperature.

The rate of oxygen consumption was determined during aerobic growth in glycerol medium, using a Clark electrode (Micrometrix). Wild-type cells were grown anaerobically from 0.01 to 0.1 OD_{600}. Cells were moved to the electrode chamber, and the level of oxygen was recorded every 10 s for 5 min. The rate of respiration with oxygen was then normalized to OD_{600}.

The rate of anaerobic respiration using fumarate as an acceptor is calculated in *SI Materials and Methods*.

H_2O_2 Killing Assay. Cells were grown anaerobically to OD_{600} = 0.3 in LB, and 2.5 mM H_2O_2 was added. At different time points, samples were diluted and

plated on LB agar. Colonies were counted after a day, and percent survival was calculated based on colony-forming units.

Measuring Zones of Inhibition. All steps were performed in the anaerobic chamber with anoxic materials. Cells were grown in minimal glucose medium to $OD_{600} = 0.1$; they were then mixed with 4 mL top agar (0.8%, 50 °C) and spread on plates of the same composition. Sterile disks (6 mm) were soaked in 15 μL 100 mM H_2O_2 and placed in the middle of the plate. The diameter of the inhibition zone was measured after 24 h.

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1. Imlay JA (2013) The molecular mechanisms and physiological consequences of oxidative stress: Lessons from a model bacterium. *Nat Rev Microbiol* 11:443–454.
2. Kuo CF, Mashino T, Fridovich I (1987) α, β-Dihydroxyisovalerate dehydratase. A superoxide-sensitive enzyme. *J Biol Chem* 262:4724–4727.
3. Flint DH, Tuminello JF, Emptage MH (1993) The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *J Biol Chem* 268:22369–22376.
4. Jang S, Imlay JA (2007) Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. *J Biol Chem* 282:929–937.
5. Sobota JM, Imlay JA (2011) Iron enzyme ribulose-5-phosphate 3-epimerase in *Escherichia coli* is rapidly damaged by hydrogen peroxide but can be protected by manganese. *Proc Natl Acad Sci USA* 108:5402–5407.
6. Gu M, Imlay JA (2013) Superoxide poisons mononuclear iron enzymes by causing mismetallation. *Mol Microbiol* 89:123–134.
7. Imlay JA, Chin SM, Linn S (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science* 240:640–642.
8. Christman MF, Morgan RW, Jacobson FS, Ames BN (1985) Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* 41:753–762.
9. Lee JW, Helmann JD (2006) The PerR transcription factor senses H2O2 by metal-catalysed histidine oxidation. *Nature* 440:363–367.
10. Seaver LC, Imlay JA (2001) Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli*. *J Bacteriol* 183:7182–7189.
11. Zheng M, Aslund F, Storz G (1998) Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279:1718–1721.
12. Lee C, et al. (2004) Redox regulation of OxyR requires specific disulfide bond formation involving a rapid kinetic reaction path. *Nat Struct Mol Biol* 11:1179–1185.
13. Zheng M, et al. (2001) DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J Bacteriol* 183:4562–4570.
14. Lee JH, Yeo WS, Roe JH (2004) Induction of the *sufA* operon encoding Fe-S assembly proteins by superoxide generators and hydrogen peroxide: Involvement of OxyR, IHF and an unidentified oxidant-responsive factor. *Mol Microbiol* 51:1745–1755.
15. Jang S, Imlay JA (2010) Hydrogen peroxide inactivates the *Escherichia coli* Isc iron-sulphur assembly system, and OxyR induces the Suf system to compensate. *Mol Microbiol* 78:1448–1467.
16. Kehres DG, Janakiraman A, Slauch JM, Maguire ME (2002) Regulation of *Salmonella enterica* serovar Typhimurium *mntH* transcription by H_2O_2, Fe^{2+}, and Mn^{2+}. *J Bacteriol* 184:3151–3158.
17. Anjem A, Imlay JA (2012) Mononuclear iron enzymes are primary targets of hydrogen peroxide stress. *J Biol Chem* 287:15544–15556.
18. Altuvia S, Almirón M, Huisman G, Kolter R, Storz G (1994) The *dps* promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase. *Mol Microbiol* 13:265–272.
19. Ilari A, Ceci P, Ferrari D, Rossi GL, Chiancone E (2002) Iron incorporation into *Escherichia coli* Dps gives rise to a ferritin-like microcrystalline core. *J Biol Chem* 277:37619–37623.
20. Park S, You X, Imlay JA (2005) Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx$^-$ mutants of *Escherichia coli*. *Proc Natl Acad Sci USA* 102:9317–9322.
21. Aslund F, Zheng M, Beckwith J, Storz G (1999) Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc Natl Acad Sci USA* 96:6161–6165.
22. Rocha ER, Herren CD, Smalley DJ, Smith CJ (2003) The complex oxidative stress response of *Bacteroides fragilis*: The role of OxyR in control of gene expression. *Anaerobe* 9:165–173.
23. Strand KR, et al. (2010) Oxidative stress protection and the repair response to hydrogen peroxide in the hyperthermophilic archaeon *Pyrococcus furiosus* and in related species. *Arch Microbiol* 192:447–459.
24. Mishra S, Imlay JA (2013) An anaerobic bacterium, *Bacteroides thetaiotaomicron*, uses a consortium of enzymes to scavenge hydrogen peroxide. *Mol Microbiol* 90: 1356–1371.
25. Altschul AM, Abrams R, Hogness TR (1940) Cytochrome c peroxidase. *J Biol Chem* 136: 777–793.
26. Atack JM, Kelly DJ (2007) Structure, mechanism and physiological roles of bacterial cytochrome *c* peroxidases. *Adv Microb Physiol* 52:73–106.
27. Van Spanning RJM, et al. (1997) FnrP and NNR of *Paracoccus denitrificans* are both members of the FNR family of transcriptional activators but have distinct roles in respiratory adaptation in response to oxygen limitation. *Mol Microbiol* 23:893–907.
28. Partridge JD, Poole RK, Green J (2007) The *Escherichia coli yhjA* gene, encoding a predicted cytochrome *c* peroxidase, is regulated by FNR and OxyR. *Microbiology* 153: 1499–1507.
29. Seaver LC, Imlay JA (2001) Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J Bacteriol* 183:7173–7181.
30. Cha MK, Kim WC, Lim CJ, Kim K, Kim IH (2004) *Escherichia coli* periplasmic thiol peroxidase acts as lipid hydroperoxide peroxidase and the principal antioxidative function during anaerobic growth. *J Biol Chem* 279:8769–8778.
31. Jeong W, Cha MK, Kim IH (2000) Thioredoxin-dependent hydroperoxide peroxidase activity of bacterioferritin comigratory protein (BCP) as a new member of the thiol-specific antioxidant protein (TSA)/Alkyl hydroperoxide peroxidase C (AhpC) family. *J Biol Chem* 275:2924–2930.
32. Arenas FA, et al. (2010) The *Escherichia coli btuE* gene, encodes a glutathione peroxidase that is induced under oxidative stress conditions. *Biochem Biophys Res Commun* 398:690–694.
33. Lesniak J, Barton WA, Nikolov DB (2003) Structural and functional features of the *Escherichia coli* hydroperoxide resistance protein OsmC. *Protein Sci* 12:2838–2843.
34. Ma J, et al. (1999) Glutamate-89 in subunit II of cytochrome *bo*3 from *Escherichia coli* is required for the function of the heme-copper oxidase. *Biochemistry* 38:15150–15156.
35. Al-Attar S, et al. (2016) Cytochrome *bd* displays significant quinol peroxidase activity. *Sci Rep* 6:27631.
36. Verduyn C, van Wijngaarden CJ, Scheffers WA, van Dijken JP (1991) Hydrogen peroxide as an electron acceptor for mitochondrial respiration in the yeast *Hansenula polymorpha*. *Yeast* 7:137–146.
37. Richardson DJ, Ferguson SJ (1995) Competition between hydrogen peroxide and nitrate for electrons from the respiratory chains of *Thiosphaera pantotropha* and *Rhodobacter capsulatus*. *FEMS Microbiol Lett* 132(1-2):125–129.
38. Goodhew CF, elKurdi AB, Pettigrew GW (1988) The microaerophilic respiration of *Campylobacter mucosalis*. *Biochim Biophys Acta* 933:114–123.
39. Zaslavsky D, Gennis RB (2000) Proton pumping by cytochrome oxidase: Progress, problems and postulates. *Biochim Biophys Acta* 1458:164–179.
40. Lomize AL, Lomize MA, Krolicki SR, Pogozheva ID (2017) Membranome: A database for proteome-wide analysis of single-pass membrane proteins. *Nucleic Acids Res* 45: D250–D255.
41. Yamada H, Takashima E, Konishi K (2007) Molecular characterization of the membrane-bound quinol peroxidase functionally connected to the respiratory chain. *FEBS J* 274:853–866.
42. Takashima E, Yamada H, Yamashita T, Matsushita K, Konishi K (2010) Recombinant expression and redox properties of triheme *c* membrane-bound quinol peroxidase. *FEMS Microbiol Lett* 302:52–57.
43. Charoensuk K, et al. (2011) Physiological importance of cytochrome *c* peroxidase in ethanologenic thermotolerant *Zymomonas mobilis*. *J Mol Microbiol Biotechnol* 20: 70–82.
44. Wallace BJ, Young IG (1977) Role of quinones in electron transport to oxygen and nitrate in *Escherichia coli*. Studies with a *ubiA*$^-$ *menA*$^-$ double quinone mutant. *Biochim Biophys Acta* 461:84–100.
45. Ezraty B, Henry C, Hérisse M, Denamur E, Barras F (2014) Commercial Lysogeny Broth culture media and oxidative stress: A cautious tale. *Free Radic Biol Med* 74:245–251.
46. Sobota JM, Gu M, Imlay JA (2014) Intracellular hydrogen peroxide and superoxide poison 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase, the first committed enzyme in the aromatic biosynthetic pathway of *Escherichia coli*. *J Bacteriol* 196:1980–1991.
47. Herren CD, Rocha ER, Smith CJ (2003) Genetic analysis of an important oxidative stress locus in the anaerobe *Bacteroides fragilis*. *Gene* 316:167–175.
48. Turner S, Reid E, Smith H, Cole J (2003) A novel cytochrome *c* peroxidase from *Neisseria gonorrhoeae*: A lipoprotein from a Gram-negative bacterium. *Biochem J* 373:865–873.
49. Takashima E, Konishi K (2008) Characterization of a quinol peroxidase mutant in *Aggregatibacter actinomycetemcomitans*. *FEMS Microbiol Lett* 286:66–70.
50. Schütz B, Seidel J, Sturm G, Einsle O, Gescher J (2011) Investigation of the electron transport chain to and the catalytic activity of the diheme cytochrome *c* peroxidase CcpA of *Shewanella oneidensis*. *Appl Environ Microbiol* 77:6172–6180.
51. Charizanis C, Juhnke H, Krems B, Entian K-D (1999) The mitochondrial cytochrome *c* peroxidase Ccp1 of *Saccharomyces cerevisiae* is involved in conveying an oxidative stress signal to the transcription factor Pos9 (Skn7). *Mol Gen Genet* 262:437–447.
52. Giles SS, Perfect JR, Cox GM (2005) Cytochrome *c* peroxidase contributes to the antioxidant defense of *Cryptococcus neoformans*. *Fungal Genet Biol* 42:20–29.
53. Nikaido H, Rosenberg EY (1981) Effect on solute size on diffusion rates through the transmembrane pores of the outer membrane of *Escherichia coli*. *J Gen Physiol* 77: 121–135.
54. Winterbourn CC, Hampton MB, Livesey JH, Kettle AJ (2006) Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: Implications for microbial killing. *J Biol Chem* 281:39860–39869.
55. Unden G, Bongaerts J (1997) Alternative respiratory pathways of *Escherichia coli*: Energetics and transcriptional regulation in response to electron acceptors. *Biochim Biophys Acta* 1320:217–234.
56. Macfarlane GT, Gibson GR, Cummings JH (1992) Comparison of fermentation reactions in different regions of the human colon. *J Appl Bacteriol* 72:57–64.
57. Pitcher MCL, Beatty ER, Cummings JH (2000) The contribution of sulphate reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. *Gut* 46:64–72.
58. Jones SA, et al. (2007) Respiration of *Escherichia coli* in the mouse intestine. *Infect Immun* 75:4891–4899.
59. Korshunov S, Imlay KRC, Imlay JA (2016) The cytochrome *bd* oxidase of *Escherichia coli* prevents respiratory inhibition by endogenous and exogenous hydrogen sulfide. *Mol Microbiol* 101:62–77.

60. Forte E, et al. (2016) The terminal oxidase cytochrome bd promotes sulfide-resistant bacterial respiration and growth. *Scientific Reports* 6:23788.
61. Pericone CD, Park S, Imlay JA, Weiser JN (2003) Factors contributing to hydrogen peroxide resistance in *Streptococcus pneumoniae* include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction. *J Bacteriol* 185:6815–6825.
62. Seki M, Iida K, Saito M, Nakayama H, Yoshida S (2004) Hydrogen peroxide production in *Streptococcus pyogenes*: Involvement of lactate oxidase and coupling with aerobic utilization of lactate. *J Bacteriol* 186:2046–2051.
63. Lu Z, Imlay JA (2017) The fumarate reductase of *Bacteroides thetaiotaomicron*, unlike that of *Escherichia coli*, is configured so that it does not generate reactive oxygen species. *MBio* 8:e01873–e16.
64. Bingham-Ramos LK, Hendrixson DR (2008) Characterization of two putative cytochrome *c* peroxidases of *Campylobacter jejuni* involved in promoting commensal colonization of poultry. *Infect Immun* 76:1105–1114.
65. Seib KL, Tseng HJ, McEwan AG, Apicella MA, Jennings MP (2004) Defenses against oxidative stress in *Neisseria gonorrhoeae* and *Neisseria meningitidis*: Distinctive systems for different lifestyles. *J Infect Dis* 190:136–147.
66. Stacy A, Fleming D, Lamont RJ, Rumbaugh KP, Whiteley M (2016) A commensal bacterium promotes virulence of an opportunistic pathogen via cross-respiration. *MBio* 7:e00782–e00716.
67. Hébrard M, Viala JP, Méresse S, Barras F, Aussel L (2009) Redundant hydrogen peroxide scavengers contribute to Salmonella virulence and oxidative stress resistance. *J Bacteriol* 191:4605–4614.
68. Anbar AD (2008) Oceans. Elements and evolution. *Science* 322:1481–1483.
69. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640–6645.
70. Baba T, et al. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. Mol Syst Biol 2:2006.0008.
71. Miller JH (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
72. Haldimann A, Wanner BL (2001) Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. *J Bacteriol* 183:6384–6393.
73. Messner KR, Imlay JA (1999) The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. *J Biol Chem* 274:10119–10128.
74. Cherepanov PP, Wackernagel W (1995) Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158:9–14.
75. Kullik I, Stevens J, Toledano MB, Storz G (1995) Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for DNA binding and multimerization. *J Bacteriol* 177:1285–1291.
76. Park S, Imlay JA (2003) High levels of intracellular cysteine promote oxidative DNA damage by driving the Fenton reaction. *J Bacteriol* 185:1942–1950.
77. Woodmansee AN, Imlay JA (2002) Reduced flavins promote oxidative DNA damage in non-respiring *Escherichia coli* by delivering electrons to intracellular free iron. *J Biol Chem* 277:34055–34066.
78. Keyer K, Imlay JA (1996) Superoxide accelerates DNA damage by elevating free-iron levels. *Proc Natl Acad Sci USA* 93:13635–13640.
79. Imlay JA, Fridovich I (1991) Assay of metabolic superoxide production in *Escherichia coli*. *J Biol Chem* 266:6957–6965.
80. Ravindra Kumar S, Imlay JA (2013) How *Escherichia coli* tolerates profuse hydrogen peroxide formation by a catabolic pathway. *J Bacteriol* 195:4569–4579.
81. Tran QH, Bongaerts J, Vlad D, Unden G (1997) Requirement for the proton-pumping NADH dehydrogenase I of *Escherichia coli* in respiration of NADH to fumarate and its bioenergetic implications. *Eur J Biochem* 244:155–160.
82. Borisov VB, et al. (2013) Cytochrome *bd* oxidase from *Escherichia coli* displays high catalase activity: An additional defense against oxidative stress. *FEBS Lett* 587: 2214–2218.