Mechanism of oxygen detoxification by the surprisingly oxygen-tolerant hyperthermophilic archaeon, *Pyrococcus furiosus*

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The anaerobic archaeon *Pyrococcus furiosus* grows by fermenting carbohydrates producing H_2, CO_2, and acetate. We show here that it is surprisingly tolerant to oxygen, growing well in the presence of 8% (vol/vol) O_2. Although cell growth and acetate production were not significantly affected by O_2, H_2 production was reduced by 50% (using 8% O_2). The amount of H_2 produced decreased in a linear manner with increasing concentrations of O_2 over the range 2–12% (vol/vol), and for each mole of O_2 consumed, the amount of H_2 produced decreased by approximately 2 mol. The recycling of H_2 by the two cytoplasmic hydrogenases appeared not to play a role in O_2 resistance because a mutant strain lacking both enzymes was not more sensitive to O_2 than the parent strain. Decreased H_2 production was also not due to inactivation of the H_2-producing, ferredoxin-dependent membrane-bound hydrogenase because its activity was unaffected by O_2 exposure. Electrons from carbohydrate oxidation must therefore be diverted to relieve O_2 stress at the level of reduced ferredoxin before H_2 production. Deletion strains lacking superoxide reductase (SOR) and putative flavodiiron protein A showed increased sensitivity to O_2, indicating that these enzymes play primary roles in resisting O_2. However, a mutant strain lacking the proposed electron donor to SOR, rubredoxin, was unaffected in response to O_2. Hence, electrons from sugar oxidation normally used to produce H_2 are diverted to O_2 detoxification by SOR and putative flavodiiron protein A, but the electron flow pathway from ferredoxin does not necessarily involve rubredoxin.

The anaerobic archaeon *Pyrococcus furiosus* grows optimally near 100 °C by fermenting various carbohydrates and peptides to form organic acids, CO_2, and H_2, or, in the presence of elemental sulfur (S^0), H_2S (1). In the metabolism of sugars to acetate via a modified Embden-Meyerhof pathway, the two oxidation steps are catalyzed by glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) and pyruvate ferredoxin oxidoreductase (POR), both of which use oxidized ferredoxin as an electron acceptor rather than nicotinamide–adenine dinucleotide or NADP (2, 3). In the absence of S^0, the reduced ferredoxin is oxidized by a membrane-bound [NiFe]-hydrogenase (MBH) that conserves energy in the form of an ion gradient that can be used to generate ATP via a membrane-bound ATP synthase (4). It is estimated that for each glucose molecule oxidized, the formation of H_2 by MBH is able to add 1.2 ATP to the energy pool. Substrate level phosphorylation from the conversion of phosphoenolpyruvate to pyruvate and the conversion of acetyl-CoA to acetate is responsible for 2.0 ATP per glucose molecule for a total of 3.2 ATP/glucose (4).

In addition to MBH, *P. furiosus* has two cytoplasmic [NiFe]-hydrogenases termed soluble hydrogenases I and II (SHI and SHII). Both enzymes are extremely active in vitro using H_2 to reduce NADP to NADPH; these are assumed to be the physiological reactions (5). Support for this comes from the related hyperthermophile, *Thermococcus kodakarensis,* in which a mutant strain lacking the homolog of *P. furiosus* SHI produced higher levels of H_2. It was proposed that *T. kodakarensis* recycles H_2 via SHI to reduce NADP in the cell (6, 7).

In general, anaerobic organisms lack the classical defense mechanism against reactive oxygen species (ROS) found in aerobic organisms, which includes superoxide dismutase and catalase, although there are some exceptions (8–10). This is the case for *P. furiosus* because its genome contains no homologs of either of these enzymes (11). In contrast, anaerobes have been proposed to contain a superoxide-reducing system based on superoxide reductase (SOR), an enzyme first identified in *P. furiosus* (12). An SOR homolog from *Desulfoarculus baarsii* was first implicated in dealing with superoxide (O_2^-) when it was able to suppress the phenotypes of an *E. coli* mutant lacking both superoxide dismutase enzymes (13). It was later shown that SOR reduces O_2^- rather than dismutating it (12). Other proteins in *P. furiosus* have been implicated in an oxidative stress response to ROS, including the peroxidase rubrerythrin (Rbr (14)), the electron carrier rubredoxin (Rd (15)), and NADP rubredoxin oxidoreductase (NROR (16), Fig. 1). The entire reduction pathway has been reconstituted in vitro using NADPH as the ultimate reductant for O_2^- reduction (17). The mononuclear iron site of SOR is reduced by Rd that is in turn reduced by the flavoprotein NROR, which uses NAD(P)H as the electron donor. The same pathway has been shown in vitro to reduce Rbr, which can reduce H_2O_2 rather than dismutating it like catalase (14). One particular advantage to reduction rather than dismutation of O_2^- is that O_2 is not generated, which could contribute to additional toxicity.

Another family of proteins important in the protection from ROS in anaerobes is flavodiiron proteins (FDPs). Two activities have been reported for these proteins: the reduction of O_2 and the reduction of NO (18). The FDP of *Desulfovibrio gigas* is termed rubredoxin oxygen oxidoreductase (ROO) and it reduces O_2 in vitro using Rd as the electron donor (19). In vivo evidence for a role of FDP in O_2 detoxification was found in *Desulfovibrio vulgaris*. A strain lacking ROO was shown to be more sensitive than its parent when exposed to air (20). *P. furiosus* contains two FDPs, putative FdpA encoded by PF0751 and FdpB encoded by PF0694, but their physiological roles are not known. In this study, we investigated the O_2 sensitivity of *P. furiosus*, the possible mechanisms that it uses for protection, and the relationship between O_2 resistance and H_2 metabolism. The organism was reported to be a strict anaerobe unable to grow in the presence of O_2 (1).

Results

Growing *P. furiosus* Cells Are O_2-Resistant. The sensitivity of *P. furiosus* to O_2 exposure was investigated in sealed 100-mL culture bottles

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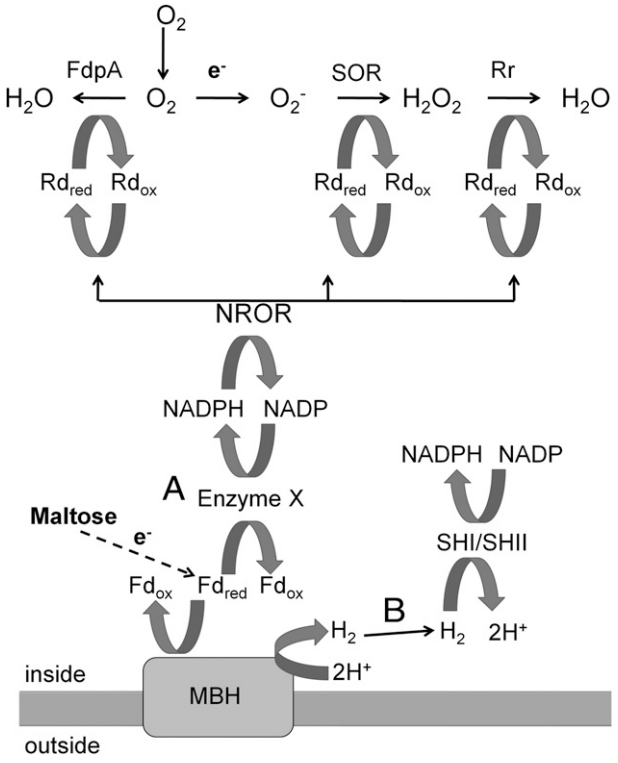

Fig. 1. Model of electron flow in the oxidative stress defense system of *P. furiosus*. The oxidation of maltose results in the reduction of ferredoxin, which is used to produce H_2 by the MBH. Two different routes are proposed to connect the reduced ferredoxin (Fd) pool to the NADPH pool, which is the proposed electron source for the oxidative stress defense enzymes FdpA, SOR, and Rbr. Route A: An unknown enzyme (X) directly transfers electrons from reduced Fd to NADP. Route B: The H_2 that is produced by MBH is recycled by oxidation by the soluble hydrogenases SHI and SHII forming NADPH. NROR and Rd are proposed to transfer electrons from NADPH to SOR, Rbr, and FdpA.

containing 40-mL cultures by injecting increasing amounts of pure O_2 to the headspace at different times during the growth cycle (Fig. 2). The organism was surprisingly resilient to O_2 when O_2 was added in midlog phase, with concentrations close to 20% (vol/vol in headspace) required to show a 50% decrease in cell yield (after 8 h; Fig. 2*A*). However, if O_2 was present before inoculation or was added during the lag phase of growth (before 2 h), 8% O_2 caused a decrease in cell yield by almost 70% (Fig. 2*B*). To study the effects of O_2 exposure in *P. furiosus*, the experimental model used an 8% (vol/vol) concentration of O_2 added ~4 h after inoculation when the cell density reached 1×10^7 cells/mL. As shown in Fig. 3, this partially but not completely inhibited the growth of the parent strain, designated COM1c2, making it possible to compare strains harboring various gene deletions.

Exposure to O_2 Decreases H_2 Production by *P. furiosus*. The effects of 8% O_2 on the growth of COM1c2 at 95 °C were investigated using maltose as the carbon source. Throughout growth, samples were taken to monitor growth (measured by protein production), acetate, and H_2. As shown in Fig. 3, the O_2 challenge had a greater effect on H_2 production than it did on cell growth. After 9 h, the O_2 challenge caused a 9% decrease in growth but a 50% decrease in H_2 yield. The specific H_2 production curve (Fig. 3*C*) demonstrates the decrease in specific H_2 production throughout the course of the O_2 challenge experiment. Unlike H_2 production, the production of acetate, the main organic acid by-product of *P. furiosus* growth, was not specifically decreased compared with protein production (Fig. 3*D*).

Quantitation of O_2 Effect on H_2 Production. To quantify the effect of O_2 on H_2 production, various concentrations of O_2 (2–12%, or 0.8–4.9 mM in the gas phase) were added to actively growing *P. furiosus* cultures, and protein, H_2 production, and O_2 consumption was measured at various intervals (Fig. S1*A*). As shown in Fig. 4, the amount of H_2 produced decreased in a linear manner with increasing concentrations of O_2. At the highest O_2 concentration used (4.9 mM in the gas phase), the amount of O_2 remaining after 6 h exposure was almost zero (Fig. S1*B*). Moreover, the slope of a plot of H_2 concentrations versus O_2 concentrations measured 6 h after O_2 addition was −2.1 (Fig. 4), indicating that for each mole of O_2 consumed, approximately 2 mol of H_2 were used. This agrees with the expected stoichiometry, in which electrons that would otherwise be used to reduce protons to H_2 (a two-electron process) are used for the reduction of O_2 to H_2O (a four-electron process). At the 2- and 4-h time points (Fig. 4) when the O_2 was not completely removed from the cultures, the slopes of the O_2 versus H_2 concentration plots were −0.8 and −1.6, respectively. Hence, H_2 production continues in the presence of O_2, but at a diminished rate in proportion to concentration of the O_2 present.

Working Models of O_2 Detoxification in *P. furiosus*. There are several mechanisms by which O_2 stress could specifically affect H_2 production by growing *P. furiosus* cells. The H_2-generating hydrogenase MBH could be inactivated as a result of the O_2 stress, thereby impairing H_2 production. Conversely, the flow of electrons from carbohydrate oxidation via ferredoxin could be diverted from MBH and used to reduce and detoxify O_2, which would result in less H_2 produced. In other words, O_2 detoxification and H_2 production would be competing processes in *P. furiosus*. A third possibility is that the H_2 already produced by *P. furiosus* is recycled by one or both of the cytoplasmic hydrogenases, SH1 and SHII, to form NADPH, which in turn provides electrons for O_2 reduction. The working model shown in Fig. 1 illustrates the proposed roles of the enzymes involved in how *P. furiosus* resists oxidative stress and how they are interconnected. In pathway A, the reduced ferredoxin and NADPH pools are connected by an unknown enzyme (X) thereby connecting H_2 production and O_2 detoxification. Pathway B shows how H_2 could be recycled to generate NADPH for O_2 detoxification. Next we describe experiments designed to differentiate between these possibilities and determine how H_2 production and O_2 detoxification are linked.

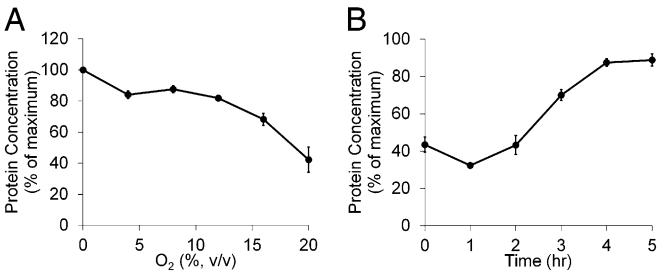

Fig. 2. Exposure of *P. furiosus* cultures to different concentrations of O_2 at different points of growth. Cultures (40 mL) in 100-mL bottles were grown at 95 °C in medium containing 5 g/L maltose, 0.5 g/L yeast extract, and no sulfide on a shaking platform (150 rpm). Growth was monitored near the end of log phase (8 h) using total cell protein. (*A*) Cultures were exposed to 8% O_2 at various times in the growth cycle. (*B*) Cultures were exposed to increasing amounts of O_2 after 4 h of growth (about 20% grown). Values are % protein produced compared with a culture not challenged with O_2.

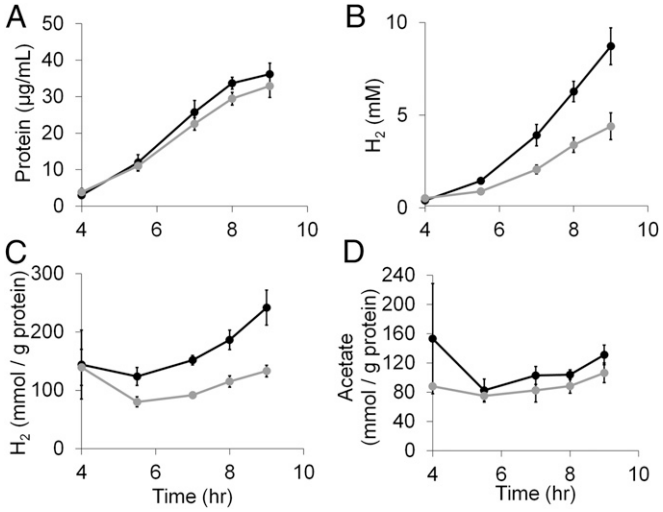

Fig. 3. Oxygen challenge of *P. furiosus*. Cultures (40 mL) in 100-mL bottles were grown at 95 °C in medium containing 5 g/L maltose, 0.5 g/L yeast extract, and no sulfide on a shaking platform (150 rpm). Cultures were grown anoxically (filled circles) or were exposed to 8% O_2 (vol/vol in headspace) after 4 h of growth (gray circles). (*A*) Growth was monitored using total cell protein. (*B*) Total H_2 production. (*C*) Specific H_2 production where the amount of H_2 produced was divided by the protein concentration of the culture. (*D*) Specific acetate production where the amount of acetate produced was divided by the protein concentration of the culture.

H_2 Recycling Does Not Play a Major Role in O_2 Detoxification in *P. furiosus*. It has been proposed that the homolog of SHI in the related organism, *T. kodakarensis,* can function as an uptake hydrogenase oxidizing H_2 and reducing NADP (6, 7). If H_2 can be recycled in *P. furiosus* by either of the cytosolic hydrogenases SHI or SHII, this could explain the decreased H_2 production seen after O_2 exposure. If H_2 recycling plays a role in O_2 defense, a strain lacking both SHI and SHII would be unable to oxidize the H_2 that is produced and therefore might be more sensitive to O_2. In addition, the exogenous addition of H_2 to the headspace of the growing cultures might protect *P. furiosus* from O_2 stress by providing additional reductant for O_2 reduction. However, as shown in Fig. S2, when exposed to 8% O_2, the double-mutant SHI–SHIIc strain behaved in a similar fashion to the COM1c2 strain, and both had similar rates of H_2 production during growth (Fig. S2*A*). Hence, SHI and SHII do not appear to play a role in O_2 resistance. This was confirmed by exposing the COM1c2 strain to 12.5% O_2 with varying amounts of H_2 (up to 15%, vol/vol) present in the headspace. The increased concentration of O_2 (to 12.5%) was used to accentuate the growth phenotype because H_2 production could not be accurately measured when adding high concentrations of H_2 to the headspace. Growth of the COM1c2 strain was inhibited by O_2, but increasing the concentrations of H_2 up to 15% (vol/vol) in the presence of O_2 had no significant effect on growth (Fig. S2*B*). If H_2 metabolism and O_2 metabolism are connected by electron flow, pathway B shown in Fig. 1 appears to represent the pathway of electron flow, at least from sugar oxidation to NADPH. This raises the questions of whether the lower yield of H_2 in the presence of O_2 is due to the partial inactivation of MBH by O_2.

Decrease in H_2 Production on O_2 Exposure Is not Caused by Direct Damage of MBH. To determine if the O_2 growth challenge resulted in direct damage to the MBH, the H_2 production activity of MBH was measured in cell-free extracts using ferredoxin reduced by POR as the electron donor. Extracts were prepared from cells that were and were not challenged with 8% O_2 for 30 min before harvesting. The activities of the two types of extracts were similar: 0.22 ± 0.01 units/mg for unexposed cells and 0.21 ± 0.01 units/mg protein for O_2-challenged cells. The lack of difference in MBH activity indicates that the 50% decrease in the yield of H_2 seen with the O_2 challenge is not due to inactivation of MBH (or of POR, which generates the reduced ferredoxin). Therefore, we propose that, as shown in Fig. 1, reductant from sugar oxidation is diverted away from MBH at the level of reduced ferredoxin, which is used instead to generate NADPH. This then raises the issue of how the apparent increased amount of NADPH generated in cells in the presence of O_2 is used to confer resistance to O_2.

Response of Mutants Lacking SOR, FdpA, and Rd to O_2 Challenge. Strains were constructed that lacked one or more of the genes that encode the enzymes that are proposed to be involved in the oxidative stress response of *P. furiosus* (Table 1). These included SOR, FdpA, and Rd. The strains were tested for O_2 sensitivity by exposure to 8% O_2 after 4 h of growth at 95 °C. H_2 production was measured over time and the results are shown in Fig. 5. As with the COM1c2 parental strain, all of the mutant strains when exposed to O_2 exhibited a lag and decrease in H_2 production. However, although the mutant lacking Rd was similarly sensitive to O_2, the mutants lacking SOR1 or FdpA1, and the double-mutant SOR FdpA1, were much more sensitive to O_2 than their respective parent strains. In fact, the FdpA1 and double mutant SOR FdpA1 strains were much more sensitive to O_2 than the SOR1 strain and displayed no H_2 production after the O_2 challenge. Both strains lacking FdpA did not grow after O_2 exposure, explaining their lack of H_2 production (Fig. S3).

Discussion

P. furiosus is regarded as a fastidious anaerobe (1) and so one of the most surprising results of the current study was its tolerance to exposure to O_2. It can withstand atmospheric levels of O_2 (21%) when exposed during growth. A critical aspect is that cells must be actively growing, however, because the final cell yield decreased dramatically (30% of the control culture) when a culture was inoculated into the standard medium with O_2 at 8% (vol/vol) present. Other organisms that were once considered to be strictly anaerobic have also been shown to be relatively aerotolerant and able to survive exposure to air (21% O_2 vol/vol), including some of the sulfate-reducing *Desulfovibrio* species (20, 21) and methanogens from the genus *Methanosarcina* (22). On

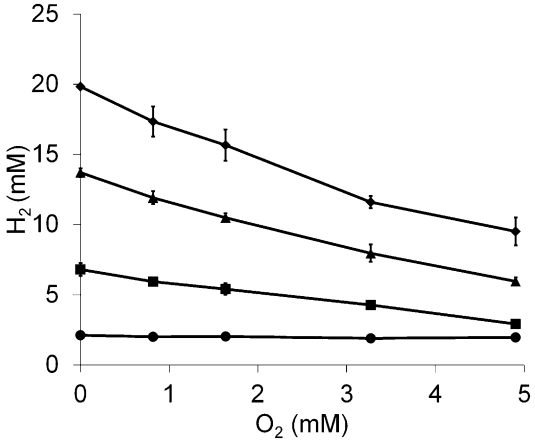

Fig. 4. Exposure of *P. furiosus* cultures to O_2 decreases H_2 production in a linear fashion. Cultures (40 mL) in 100-mL bottles were grown at 95 °C in medium containing 5 g/L maltose, 0.5 g/L yeast extract but no added sulfide on a shaking platform (150 rpm). After 6 h of growth, cultures were exposed to a range of O_2 concentrations (0.8–4.9 mM in the gas phase) and the amount of H_2 produced was measured at 0 (circles), 2 (squares), 4 (triangles), and 6 h (diamonds) after O_2 exposure.

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Table 1. *P. furiosus* strains constructed and/or used in this study

Strain	Genotype	Deleted ORF(s)	Source
COM1 (MW0002)*	Δ*pyrF*	PF1114	(41)
COM1c2 (MW0004)	Δ*pyrF*::P_{gdh}*pyrF*	None, restored	This work
SOR1 (MW0017)	Δ*pyrF* Δ*sor*	PF1114, PF1281	This work
Rd1 (MW0019)	Δ*pyrF* Δ*rd*::P_{gdh}*pyrF*	PF1282	This work
FdpA1 (MW0020)	Δ*pyrF* Δ*fprA*::P_{gdh}*pyrF*	PF0751	This work
SOR FdpA1 (MW0021)	Δ*pyrF* Δ*sor* Δ*fprA*::P_{gdh}*pyrF*	PF1281, PF0751	This work
Δ*shI* Δ*shII* (MW0015)	Δ*pyrF* Δ*shI* Δ*shII*	PF1114, PF0891–PF0894, PF1329–PF1332	(41)
SHI SHIIc (MW0016)	Δ*pyrF*::P_{gdh}*pyrF* Δ*shI* Δ*shII*	PF0891–PF0894, PF1329–PF1332	This work

*MW strain codes in parentheses are laboratory strain designations.

the other hand, the hyperthermophilic bacterium *Thermotoga maritima* could grow in the presence of only 0.5% (vol/vol) O_2 in the gas phase when exposed at the beginning of growth (23). It has been proposed that some *Desulfovibrio* species can even respire O_2, resulting in proton translocation (21, 24). Whole-genome transcriptional analysis has shown that in the response of *P. furiosus* to peroxide stress, the genes encoding the proposed oxidative defense system of *P. furiosus* shown in Fig. 1 are not upregulated. Rather, these genes are constitutively expressed, indicating that *P. furiosus* is always prepared for exposure to ROS (25). This notion is consistent with the idea that in their natural environments, hyperthermophilic organisms such as *P. furiosus* are exposed to O_2 suddenly when cool, oxygenated seawater mixes with hydrothermal vent fluid (26, 27).

There was a dramatic reduction (50%) in the amount of H_2 produced by growing cells when O_2 was present (Fig. 3). Theoretically, 1.2 ATP of the total 3.2 ATP gained from converting one glucose molecule to acetate is derived from the production of H_2 by MBH, so the amount of ATP lost from the decreased H_2 production can be estimated to be around 19%. The presence of O_2 resulted in a decrease in cell protein yield of about 9%, indicating that ATP production is not limiting growth under these conditions. Therefore, although the effect on H_2 production is dramatic, the cells are able to compensate and continue to grow despite the loss of ATP. This also suggests that O_2 is not directly damaging cells to a large extent and that metabolism persists despite O_2 exposure. This is consistent with the fact that the H_2-producing hydrogenase of *P. furiosus,* MBH, was unaffected in terms of its activity in cells exposed to O_2. We also show here that the 50% reduction in H_2 production as a result of O_2 exposure is not due to an increase in the amount of H_2 recycled by the cytoplasmic hydrogenases SHI and SHII. In fact, there appeared to be no connection between these enzymes and O_2 metabolism.

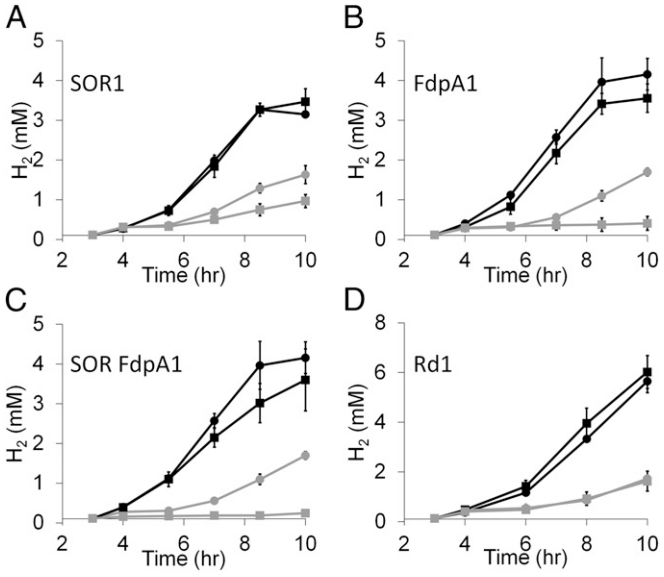

Fig. 5. Exposure of *P. furiosus* mutants lacking SOR, FdpA, and Rd to O_2 challenge. Cultures (40 mL) in 100-mL bottles were grown at 95 °C in medium containing 5 g/L maltose, 0.5 g/L yeast extract, and no sulfide on a shaking platform (150 rpm). Cultures parent (circles) or mutant (squares) were grown anoxically (black) or were exposed to 8% O_2 (vol/vol in headspace) after 4 h of growth (gray). Total H_2 production was measured throughout growth. (*A*) SOR1, (*B*) FdpA1, (*C*) SOR FdpA1, and (*D*) Rd1.

Quantitation of the effect O_2 exposure had on H_2 production revealed that each mole of O_2 added to the culture resulted in 2 mol less H_2 produced. This ratio is consistent with the proposed model in which electrons from sugar fermentation that are normally used to generate H_2 via MBH are diverted to reduce O_2 when the cells are exposed to O_2 stress. This reduction in H_2 yield also means that less energy (ATP) is conserved, but this also does not affect cell growth. Hence, there must be a mechanism to transfer electrons from reduced ferredoxin to NADP (Fig. 1). One possibility is that *P. furiosus* can reduce NADP directly during glycolysis using the enzyme nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN), which generates 3-phosphoglycerate and reduces NADP, bypassing the ferredoxin-reducing enzyme GAPOR (28, 29). Interestingly, strains of the related organism *T. kodakarensis* lacking either GAPN or GAPOR were unable to grow by glucose fermentation, suggesting that GAPN does play an essential role. In addition, the two reductant pools of reduced ferredoxin and NADPH could be connected by cytoplasmic oxidoreductase I (CORI) (PF1327 and PF1328). The true physiological function of CORI is not known because in vitro it catalyzes several reactions, including the reduction of NADP with reduced *P. furiosus* ferredoxin (30). CORI can also use NADPH to both reduce Rd and reduce polysulfide to H_2S (31). Interestingly, the genome of *P. furiosus* also contains a homolog of CORI termed CORII (PF1910 and PF1911) and they are reciprocally regulated during growth on sugars and peptides (32). Determining the pathways of electron flow between the two major electron pools in *P. furiosus* is a focus of future research.

Surprisingly the *P. furiosus* mutant lacking the intermediate electron transfer protein Rd did not have an obvious growth phenotype in the presence of O_2. However, O_2-sensitive growth phenotypes were seen for the deletion strains lacking FdpA and SOR. These data support the activities shown in Fig. 1, in which these two enzymes serve as the terminal reductases and reduce O_2 and O_2^-, respectively. Such a role for FdpA is in agreement with what has been proposed in *Desulfovibrio* sp. in which FdpA is termed ROO (19, 20), although the biochemical activity of FdpA from *P. furiosus* was not investigated here. Interestingly, the *fdpA* mutant in *P. furiosus* was more sensitive to O_2 than the *sor* mutant. Because O_2^- is derived in vivo from adventitious electron transfer to O_2 (33, 34), the presence of FdpA in the *sor*

mutant could limit the amount of O_2^- produced, but in the *fdpA* strain, the first line of O_2 stress defense is missing. Nevertheless, these data demonstrate that both FdpA and SOR play roles in the oxidative stress response of *P. furiosus*.

The results presented here therefore led us to conclude that FdpA is the primary enzyme in removing O_2, whereas SOR removes O_2^- when *P. furiosus* is exposed. The working model for how these enzymes obtain reductant is shown in Fig. 1. We have established that the response to O_2 is independent of H_2 and the hydrogenases MBH, SHI, and SHII, and that reductant is diverted from MBH to the enzymes involved in the oxidative stress response. How electrons are provided for removal of ROS still remains to be established, especially given that a mutant lacking Rd was not more sensitive to O_2 than the parent strain. A potential explanation is that ferredoxin or another redox protein in *P. furiosus* can directly reduce both FdpA and SOR in the absence of Rd. The role of Rd in vivo is still not clear. For example, *Escherichia coli* contains an FdpA homolog termed flavorubredoxin that contains an Rd domain, in addition to the flavo and di-iron domains, and it catalyzes NO reduction (35). A mutant strain lacking flavorubredoxin had increased sensitivity to NO (36). However, the mutant was successfully complemented by FprA from *Moorella thermoacetica* and ROO from *D. gigas* and *D. vulgaris,* even though these proteins do not contain an Rd domain and no other Rd-like protein is present in *E. coli* (37–39).

The surprising discovery that FdpA, SOR, and Rbr are enzymes that deal with oxidative stress in anaerobes has been followed by much debate as to why anaerobes use the strategy of reduction to detoxify ROS rather than using dismutation like most aerobic organisms (40). Another side to the story, however, is the effects that using reduction, rather than dismutation, to detoxify ROS have on the metabolism of anaerobic organisms. The in vivo evidence presented here indicates that under certain conditions, the metabolic strain of using reduction to detoxify O_2 can result in detectable phenotypes, such as decreased H_2 production. This observation can be used to analyze both the oxidative stress defense system and the flow of electrons in anaerobic organisms such as *P. furiosus*; such studies are under way.

Materials and Methods

Strains and Strain Construction. *P. furiosus* strains used in this study are listed in Table 1. All strains were constructed using the methodology previously described (41). The marker-replaced deletions of *fdpA* and *rd* were constructed by transforming linear DNA products made by overlap PCR in which 500 bp immediately upstream and downstream of the target gene were overlapped flanking the P_{gdh}*pyrF* cassette with terminator (41) into the COM1 strain and/or the SOR1 strain. Transformants were selected on solid defined media for uracil prototrophy. To construct the markerless deletion of *sor* (SOR1), 1-kb flanking regions of the target gene were cloned into the pGLW015 plasmid (41), which contains the P_{gdh}*pyrF* cassette for prototrophic selection. The plasmid was transformed into *P. furiosus* COM1 (Δ*pyrF*) selecting uracil prototrophy on solid defined medium and counterselected for plasmid loss using 5-fluoroorotic acid resistance as previously described (41). All deletion strains constructed were screened for deletion by PCR amplification of the locus using primers outside the homologous flanking regions. Isolates containing the deletions were colony-purified by serial passage on solid defined medium. Further strain conformation was obtained by PCR sequencing of the PCR amplifications and by quantitative PCR analysis using primers internal to the deleted gene in question.

The COM1c2 and SHI SHIIC strains in which the *pyrF* gene was reintroduced were constructed by transforming their parent strains with a construct containing the P_{gdh}*pyrF* cassette flanked by a 1-kb sequence surrounding the original *pyrF* locus (Table 1). A markerless deletion of *sor* (SOR1) and marker-replaced deletions of *fdpA* (FdpA1) and *rd* (Rd1) were constructed from the COM1 background strain (Δ*pyrF*). In addition, a marker-replaced deletion of *fdpA* was made in the SOR1 background stain forming the Δ*sor* Δ*fdpA* (SOR FdpA1) double-mutant strain. All strains were confirmed using PCR and sequence analysis. In addition, the quantitative PCR products of deleted genes could not be detected in any of the newly constructed strains.

Growth Conditions. All strains were grown as previously reported (42) in complex growth medium using maltose as the carbon source and containing yeast extract (0.5 g/L), but no sulfide was added to prevent chemical interference in the O_2 challenge experiments. Unless otherwise stated, growth experiments were carried out in triplicate in 40-mL cultures at 95 °C on a shaking incubator (150 rpm). To add O_2 or H_2 to the cultures, separate sealed bottles at room temperature were filled with 100% of the indicated gas and a syringe was used to transfer known amounts to growth temperature cultures. Percentages of O_2 and H_2 reported are calculated vol/vol in the headspace of the culture bottle. The solubility of O_2 at 95 °C in a brine solution with similar salt concentration to the growth medium used was measured at 25 μM (43).

Analyses for Protein, H_2, O_2, and Acetate. Protein assays were used to monitor growth throughout the course of this study. The Bradford method (44) was used to quantitate cell protein using BSA as the standard. To monitor H_2 production and O_2, 1-mL samples from the headspace of cultures at the growth temperature were transferred to argon filled sealed 5-mL vials at room temperature. From the secondary vials, 1 mL was injected into a Shimadzu GC-8A gas chromatograph for H_2 and O_2 measurements. Acetate production was measured from 1-mL culture samples as previously described (45). Error bars were calculated as the SD of measurements taken from independent cultures grown in triplicate, and slopes were calculated using linear regression trend lines.

Isolation of RNA and Quantitative PCR Analyses. Acid-phenol was used to extract total RNA from *P. furiosus* cells (46). A second acid-phenol isolation followed by Turbo DNase (Ambion) treatment (30 min, 37 °C) and Absolutely RNA cleanup kit (Agilent Technologies) was performed to further purify the RNA. cDNA was made with the AffinityScrpt QPCR cDNA Synthesis Kit (Agilent). Quantitative PCR experiments were carried out in technical duplicate using an Mx300P instrument (Agilent) and the Brilliant SyBR green QPCR master mix (Agilent) out to 40 cycles. The absence of signal in the deletion mutant strains compared with the positive control of the parent or COM1 strain was used to confirm the deletion mutants.

Hydrogenase Assays. Cultures (300 mL) were grown in triplicate at 95 °C on a shaking incubator (150 rpm) to midlog phase (5 h) before cultures were challenged or not with 8% O_2 for 30 min. Cells were harvested and whole cell extracts were prepared anaerobically and were measured for protein content. Assays were performed anaerobically in 5-mL sealed vials at 85 °C and were started by addition of whole cell extract. Assay mixtures contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM $MgCl_2$, 10 mM pyruvate, 0.5 mM coenzyme A, and 7 μM ferredoxin. The H_2 produced was measured using a Shimadzu GC-8A gas chromatograph. Hydrogenase activity is reported in units where 1 unit is equal to 1 μmol of H_2 produced per minute.

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