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Response of *Desulfovibrio vulgaris* Hildenborough to hydrogen peroxide: enzymatic and transcriptional analyses

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Abstract

We studied the effect of hydrogen peroxide (H_2O_2) stress on the anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. In a lactate/sulfate medium, growth was affected from 0.1 mM H_2O_2 and totally inhibited at 0.7 mM. Surprisingly, transcript analyses revealed that the PerR regulon exhibited opposite regulation in the presence of 0.1 and 0.3 mM H_2O_2. The variations in peroxidase- and superoxide dismutase-specific activities in the cell-free extracts of H_2O_2-stressed cultures were related to changes in the corresponding transcript abundance. Our data suggest that *sod*, *sor*, *ngr* and *tpx* genes, in addition to the PerR regulon, belong to the H_2O_2 stimulon.

Introduction

Desulfovibrio species belong to the sulfate-reducing bacteria (SRB) group, which are ubiquitous anaerobic microorganisms, exhibiting a large metabolic diversity. However, all members are unified by the use of sulfate as the terminal electron acceptor, which is reduced to hydrogen sulfide. Ecological studies show that, although classified as strict anaerobes, these microorganisms are able to deal with the temporary presence of oxygen in their natural habitats (marine surface waters, microbial mats, sewers, rice paddies and oil pipelines), and several *Desulfovibrio* species have been found to oxidize organic substrates under millimolar levels of oxygen (Dannenberg *et al.*, 1992). However, aerotolerant representatives of *Desulfovibrio* cannot utilize O_2 for growth (Cypionka, 2000).

Aerotolerance studies of anaerobic microorganisms are of great interest to understand oxidative stress responses and to determine new systems involved in the detoxification of reactive oxygen species (ROS). ROS derive from the sequential univalent reduction of dioxygen to a superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and a hydroxyl radical ($OH^{\bullet}$) (Imlay, 2002). In addition, the oxygen sensitivity of SRB is increased in the presence of sulfide, whose oxidation could generate ROS (Cypionka *et al.*, 1985). By spontaneous dismutation or during the course of its enzymatic detoxification by superoxide dismutase (SOD), superoxide is rapidly converted to H_2O_2. In addition to the oxidation of cysteinyl thiols and methionine residues (Imlay, 2002), one of the most deleterious effects of reactive oxidant H_2O_2 is its reaction with reduced iron ions to form $OH^{\bullet}$ through the Fenton reaction. The hydroxyl radical and other H_2O_2-derived ROS oxidize most cellular compounds at diffusion-limited rates, especially causing DNA damages and protein carbonylation, including inactivation of crucial enzymes in the pathways for lactate oxidation and sulfate reduction or involved in cell division (Imlay, 2003).

Studies have shown the presence of efficient complex enzymatic systems for scavenging of toxic ROS and for oxygen reduction in *Desulfovibrio* species. SOD and catalase, which are well-known enzymes to eliminate superoxide and H_2O_2 in aerobic organisms, have been characterized in some *Desulfovibrio* species (Dos Santos *et al.*, 2000; Davydova *et al.*, 2006). In addition to the aforementioned classical

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enzymes of ROS elimination, unique alternative systems of antioxidative defense involving a superoxide reductase (SOR) and NADH-dependent H_2O_2 peroxidases (rubrerythrins, nigerythrin) have also been found in *Desulfovibrio* species (Moura *et al.*, 1990; Coulter *et al.*, 1999; Brioukhanov, 2008). SOR and rubrerythrins reduce superoxide and H_2O_2 to water, respectively, without the regeneration of intracellular oxygen – an important feature of ROS detoxification pathways in cells of anaerobic microorganisms (Jenney *et al.*, 1999). SRB have thus developed different and complicated defense strategies to protect themselves against oxygen damages and exhibit aerotolerance (Lumppio *et al.*, 2001; Fournier *et al.*, 2003; Dolla *et al.*, 2006).

Genome sequencing of *Desulfovibrio vulgaris* Hildenborough (Heidelberg *et al.*, 2004) paved the way to functional genomics studies, and the effects of oxygen exposure have been studied at the transcriptome and proteome levels (Fournier *et al.*, 2006; Mukhopadhyay *et al.*, 2007; Pereira *et al.*, 2008). From the genome analysis, a PerR regulon that contained, in addition to the *perR* H_2O_2 sensor and response regulator (locus tag DVU3095), a set of genes, encoding proteins involved in peroxide reduction, has been proposed (Rodionov *et al.*, 2004). The *perR* regulator forms an operon with the *rbr1* (locus tag DVU3094) and *rdl* (locus tag DVU3093) genes that encode rubrerythrin 1 and rubredoxin-like protein, respectively (Lumppio *et al.*, 2001). The additional genes *ahpC* (encoding an alkyl hydroperoxide reductase) (locus tag DVU2247), *rbr2* (encoding rubrerythrin 2) (locus tag DVU2318) and *DVU0772* (encoding a conserved hypothetical protein) have been predicted to belong to the PerR regulon (Rodionov *et al.*, 2004). In addition to the PerR regulon members, *D. vulgaris* Hildenborough genome contains supplemental genes such as *ngr* (locus tag DVU0019) and *tpx* (locus tag DVU1228), encoding a nigerythrin and a thiol peroxidase (Heidelberg *et al.*, 2004), respectively, which could account for the total peroxidase activity *in vivo*.

While the antioxidative defense molecular mechanisms are well investigated in aerobic organisms including such classic models as *Escherichia coli* and *Bacillus subtilis*, relatively little experimental data are available on strict anaerobes. A better understanding of the specificity of complicated responses to oxidative stress in anaerobic microorganisms requires insights into the ways in which different oxidative conditions are toxic for the cells and also into the genes involved in the ROS cellular defense. Studies of how SRB cope with exposure to molecular oxygen and ROS provide important insights into the ecology of these bacteria as well as into their practical use in bioremediation. In this study, we report the effect of different H_2O_2 stresses on *D. vulgaris* Hildenborough growth. The expressions of key genes encoding ROS detoxification enzymes, including the PerR regulon, as well as corresponding global peroxidase and superoxide-scavenging enzymatic activities were followed as a function of H_2O_2 concentration and time of cell exposure.

Materials and methods

Strain, growth and stress-inducing conditions

The sulfate-reducing bacterium *D. vulgaris* strain Hildenborough (ATCC 29579/NCIMB 8303) was cultured in liquid lactate/sulfate medium C at 33 °C under gas exchange conditions in an anaerobic chamber (COY) under a 10% H_2–90% N_2 mixed gas atmosphere using 10% (v/v) inoculum (Fournier *et al.*, 2003). Growth was monitored by following the $OD_{600\,nm}$.

For H_2O_2 stress assays, cells were cultured under anaerobic conditions till $OD_{600\,nm}$ reached a value of about 0.35. At that time, a freshly prepared and filter-sterilized anaerobic solution of H_2O_2 was added to the cultures at final concentrations ranging from 0.05 to 0.7 mM and growth was further monitored.

For RNA quantification and enzymatic activity measurements, the cultures of *D. vulgaris* Hildenborough were grown under anaerobic conditions to the mid-exponential phase ($OD_{600\,nm}$ ~0.4). At that time, 0.1 or 0.3 mM H_2O_2 was added and aliquots were taken at 7, 30, 60, 90, 120 and 240 min. As a reference (untreated cells), cultures were performed under the same conditions without addition of H_2O_2, and aliquots were taken at the same time as for the H_2O_2-treated cells. All cultures were grown in triplicate. Equal volumes of each triplicate were mixed at each incubation time and cells were harvested by centrifugation (8000 ***g***, 15 min, 4 °C) for further experiments.

H_2O_2 quantification in the culture

Cells were cultured under anaerobic conditions to the mid-exponential phase ($OD_{600\,nm}$ ~0.4) as described above. At that time, 0.1 or 0.3 mM H_2O_2 was added. Aliquots (150 μL) were taken immediately after H_2O_2 addition and at 7, 30, 90 and 240 min. After centrifugation (12 000 ***g***, 3 min, room temperature) to pellet cells, H_2O_2 was quantified in the supernatant using the PeroXOquant Quantitative Peroxide Assay Kit from Pierce. As a control, to measure H_2O_2 decay in a cell-free medium, the culture was first centrifuged (12 000 ***g***, 3 min) to remove cells. The supernatant was transferred to a new tube and 0.1 mM H_2O_2 was added. The same procedure for H_2O_2 quantification as described above was performed. All steps were carried out under anaerobic conditions in a COY anaerobic chamber.

Measurements of specific activities of peroxidase and SOD

Cell pellets were resuspended in 10 mL of ice-cold 50 mM Tris-HCl buffer (pH 7.8). Cells were disrupted using a French press (Thermo Scientific) at 900 p.s.i. with cooling

in ice. Cell debris were removed by centrifugation (12 000 ***g***, 60 min, 4 °C) and supernatants, which corresponded to the cell-free extracts, were frozen in liquid N_2 and stored in aliquots at −80 °C for further measurements of enzymatic activities.

The peroxidase activity in cell-free extracts was determined spectrophotometrically at 25 °C (Kontron Instruments UVICON spectrophotometer) using 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) as a substrate (Gallati, 1979). The assay mixture in deionized water (1 mL of reaction volume) contained 96 mM potassium phosphate (pH 5.0), 8.7 mM ABTS diammonium salt (Sigma), 0.01% (w/w) H_2O_2, 0.004% (w/v) bovine serum albumin and 0.008% (v/v) Triton X-100. The oxidation of ABTS by H_2O_2 in the peroxidase reaction was recorded by a linear increase in the absorption at 405 nm for 2 min ($\varepsilon_{405} = 36.8\ M^{-1}\ cm^{-1}$). One unit of peroxidase activity is defined as the amount of enzyme required to oxidize 1 μmol of ABTS per 1 min.

SOD activity in the cell-free extracts was determined spectrophotometrically at 25 °C using the xanthine oxidase–cytochrome *c* method (McCord & Fridovich, 1969). The assay mixture in deionized water (1 mL of reaction volume) contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA disodium salt, 10 μM cytochrome *c* (Sigma), 50 μM xanthine (Sigma) and 1.7 mU xanthine oxidase (Sigma). The reduction of cytochrome *c* by the superoxide anion radical, generated from O_2 during the oxidation of xanthine in the xanthine oxidase reaction, was recorded by an increase in the absorption at 550 nm for 5 min. One unit of SOD activity is defined as the amount of enzyme required to inhibit the linear rate of reduction of cytochrome *c* by 50%.

Protein concentrations were determined using the Protein Assay Kit (Bio-Rad Laboratories).

RNA isolation and quantitative real-time PCR (qRT-PCR)

For total RNA isolation, cell pellets were rinsed three times with 10 mM Tris-HCl (pH 8.0) RNase-free buffer and finally resuspended in 200 μL of 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) RNase-free buffer. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics) according to the manufacturer's instructions with an extra DNase I digestion step in order to eliminate contaminating DNA. Extracted RNA (10 μg) was reverse transcribed using a random hexamer primer, dNTPs and Superscript II (Invitrogen) as described previously (Fournier *et al.*, 2006). cDNA was purified on a microcon YM-30 centrifugal filter unit (Millipore) and stored at −20 °C.

qRT-PCR was performed using the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit (Roche Diagnostics). cDNA was mixed with 0.5 μM of each primer and 2 μL of Master Mix in a 10 μL final volume. The pairs of oligonucleotide primers used to quantify the selected genes expression levels are shown in Supporting Information, Table S1. Real-time PCR runs were carried out on a LightCycler® Real-Time PCR System (Roche Diagnostics), with one cycle at 95 °C for 8 min, followed by up to 45 cycles at 95 °C for 12 s, 60 °C for 10 s and 72 °C for 20 s.

For each couple of primers, real-time PCRs were run in triplicate on each cDNA. RELATIVE EXPRESSION SOFTWARE TOOL (REST) was used to calculate the relative expression of each gene under each condition (Pfaffl *et al.*, 2002). The coefficients of variation of the determined crossing points for each set of replicates were lower than 0.46%. The 16S RNA gene was used as a reference for normalization.

Results

Influence of H_2O_2 on the growth of *D. vulgaris* Hildenborough

The influence of H_2O_2 on exponentially growing cells in a lactate/sulfate medium is shown in Fig. 1. While the addition of 0.05 mM H_2O_2 did not significantly perturb *D. vulgaris* Hildenborough growth, higher concentrations of H_2O_2 treatment induced both a lower growth rate and a lower final cell density. When 0.7 mM H_2O_2 was added, growth was completely inhibited (Fig. 1). In the presence of 0.1 and 0.3 mM H_2O_2, the growth rate was reduced by 20% and 37% and the final biomass was reduced by 9% and 23%, respectively. These H_2O_2 concentrations, which represent effective, but nonlethal concentrations, were selected for further experiments.

H_2O_2 decay in the culture was quantified. The data (Fig. 2) showed that H_2O_2 concentrations in the culture

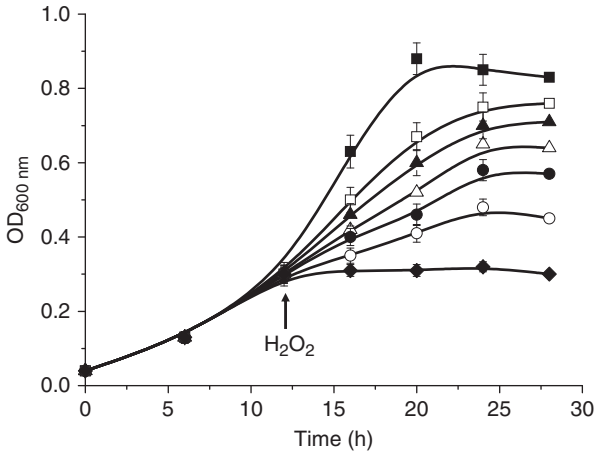

Fig. 1. Effect of H_2O_2 on the growth of *Desulfovibrio vulgaris* Hildenborough. The arrow indicates time of H_2O_2 addition. (■) No H_2O_2; (□) 0.1 mM H_2O_2; (▲) 0.2 mM H_2O_2; (△) 0.3 mM H_2O_2; (●) 0.4 mM H_2O_2; (○) 0.5 mM H_2O_2; (◆) 0.7 mM H_2O_2.

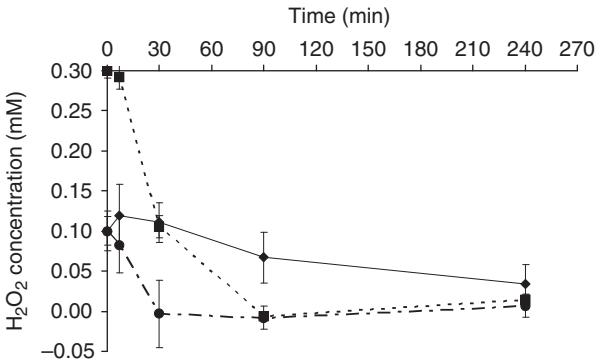

Fig. 2. H_2O_2 decay in a culture medium over time: 0.3 mM (■) and 0.1 mM (●) H_2O_2 was added to mid-exponentially grown cultures; (◆) 0.1 mM H_2O_2 was added to a cell-free medium.

decreased rapidly over time. When 0.1 mM H_2O_2 was added to the culture, no H_2O_2 could be detected in significant amounts after 30 min. In contrast, when 0.3 mM H_2O_2 was added, a similar decrease in concentration was observed, but after 30 min, about 0.11 mM H_2O_2 could be measured in the culture. After 90 min of incubation, H_2O_2 could no longer be detected in significant amounts. As a control, when 0.1 mM H_2O_2 was added to the cell-free medium, only a slight decrease in the H_2O_2 concentration was observed during the first 90 min. After 240 min, no H_2O_2 concentration could be significantly measured. This H_2O_2 decrease can be attributed to a chemical reduction because of the presence of hydrogen sulfide produced by the bacteria. However, under our conditions and during the first 90 min, this chemical reduction was negligible.

Genes expression variations

In order to observe the effect of H_2O_2 at the transcriptional level, the expression of genes, encoding proteins involved in ROS detoxification, was studied by qRT-PCR. Genes belonging to the predicted PerR regulon (*perR*, *rbr1*, *rbr2*, *ahpC* and *DVU0772*), *ngr* and *tpx*, which encode enzymes involved in H_2O_2 detoxification, as well as *sodB* (locus tag DVU2410) and *sor* (locus tag DVU3183), which encode enzymes participating in superoxide scavenging, were targeted. For the *DVU0772* gene, sequence analysis does not provide any information about the activity of the encoded hypothetical protein.

Addition of H_2O_2 at a final concentration of 0.3 mM significantly repressed the synthesis of mRNA of the PerR regulon members (from 2.8 to 4.3 times after 30 min) compared with the expression level of the same genes in untreated cells (Fig. 3a). The gene *ngr* was downregulated in the same order as PerR regulon members, while *tpx* was much less repressed after 30 min. In the same way, *sod* and *sor* genes were downregulated after 30 min. After longer

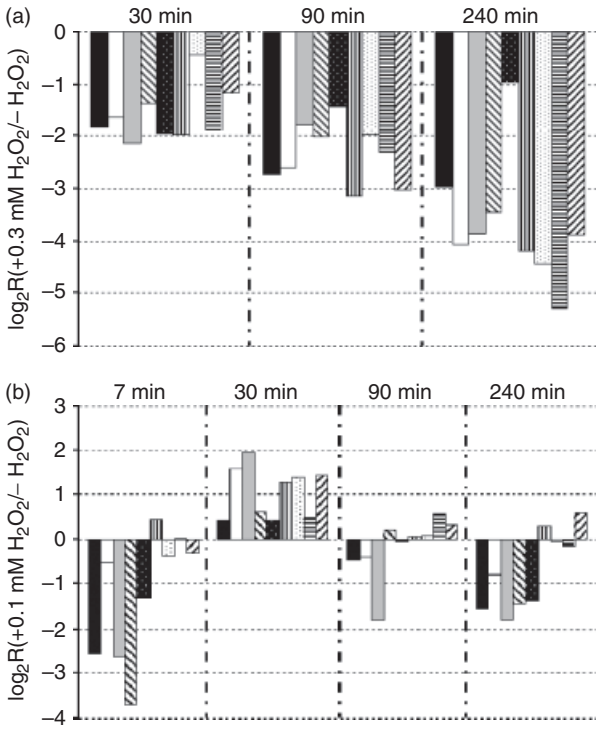

Fig. 3. Real-time PCR analysis of the effect of H_2O_2 on the expression levels of the following genes: ■, *perR*; □, *rbr1*; ▒, *rbr2*; ▨, *ahpC*; ▩, *DVU0772*; ▥, *ngr*; ⬚, *tpx*; ▤, *sodB*; ▨, *sor*. (a) Gene expression after exposure to 0.3 mM of H_2O_2 at different times. (b) Gene expression after exposure to 0.1 mM of H_2O_2 at different times. The first five bars correspond to the predicted members of the PerR regulon (Rodionov *et al.*, 2004).

exposure times, gene repression appeared to be stronger for all the tested genes.

In contrast, when H_2O_2 was added at a final concentration of 0.1 mM, gene expression levels varied depending on time (Fig. 3b): at the early time point (7 min), the PerR regulon was downregulated while the other genes did not show any significant expression changes (lower than 1.35-fold compared with untreated cells). At 30 min, all the tested genes were upregulated (up to 3.9 times), with the most significant changes in *rbr1*, *rbr2*, *ngr*, *tpx* and *sor* transcripts. However, after 90 min, the expressions of the tested genes were similar to those of untreated cells, except for *rbr2*, which was significantly downregulated. After 240 min, the genes belonging to the PerR regulon was strongly downregulated, while the expression levels of the other genes were not significantly modified compared with untreated cells (Fig. 3b).

Effects of peroxide stress on the specific activities of peroxidase and SOD

To study the H_2O_2 stress response of *D. vulgaris* Hildenborough at the biochemical level, the measurements of the

Table 1. Peroxidase- and SOD-specific activities

H_2O_2 exposure		Peroxidase activity (U mg^{-1}) of protein (± SD)	SOD activity (U mg^{-1}) of protein (± SD)
H_2O_2 (mM)	Time (min)		
0	30	5.25 ± 0.07	10.60 ± 0.15
0.1		7.71 ± 0.15	12.78 ± 0.22
0.3		4.82 ± 0.10	9.47 ± 0.11
0	60	5.43 ± 0.07	10.70 ± 0.12
0.1		6.52 ± 0.11	12.91 ± 0.19
0.3		4.66 ± 0.11	9.19 ± 0.15
0	90	5.64 ± 0.09	10.79 ± 0.15
0.1		6.20 ± 0.13	13.20 ± 0.23
0.3		4.62 ± 0.14	8.76 ± 0.12
0	120	6.25 ± 0.10	11.05 ± 0.16
0.1		5.57 ± 0.10	13.64 ± 0.22
0.3		4.20 ± 0.16	8.12 ± 0.17
0	240	6.56 ± 0.09	11.19 ± 0.15
0.1		5.50 ± 0.11	14.32 ± 0.27
0.3		3.47 ± 0.09	7.35 ± 0.15

specific activities of enzymes of antioxidative defense in cell-free extracts from cultures exposed to 0.1 and 0.3 mM H_2O_2 were performed at various times (30, 60, 90, 120 and 240 min). As a reference, peroxidase- and SOD-specific activities were measured in cell-free extracts from untreated cultures.

Upon addition of 0.1 mM H_2O_2, the specific peroxidase activity increased about 1.5-fold after 30 min, but reverted to almost its basic level after longer times of exposure (Table 1). It should be noted that these changes in specific peroxidase activity over time followed the same variation pattern of the PerR regulon, *ngr* and *tpx* gene expression (Fig. 2b).

In contrast, after the addition of 0.3 mM H_2O_2, the specific activity of peroxidase decreased by nearly 10% after 30 min. After 90 and 240 min, the peroxidase activity level was even lower, with 20% and 47% decreases, respectively, compared with untreated cells (Table 1). Specific peroxidase activity measurement is in agreement with the mRNA quantification, showing that in the presence of 0.3 mM H_2O_2, all genes encoding proteins related to peroxide scavenging (PerR regulon, *ngr*, *tpx*) were strongly down-regulated (Fig. 3a).

The low peroxide stress (0.1 mM H_2O_2) caused a 20–25% increase in SOD-specific activity during all exposure time intervals (Table 1). These data could be related to the fact that the number of *sor* and *sod* genes transcripts were more abundant in cells treated with 0.1 mM H_2O_2 than in untreated cells after 30 min (Fig. 3b). In contrast, exposure to 0.3 mM H_2O_2 (high-peroxide stress) induced a 10–35% decrease in SOD-specific activity depending on the exposure time from 30 to 240 min (Table 1), which is in agreement with the observed decrease in the corresponding mRNAs (Fig. 3a).

Discussion

The aerotolerance capabilities of anaerobic SRB make them suitable models to study the molecular systems involved in survival strategies. ROS detoxification is a key mechanism in the course of oxygen resistance. We have shown here that in a liquid lactate/sulfate medium, the growth of *D. vulgaris* Hildenborough is affected by as less as 0.1 mM of H_2O_2 and is totally inhibited in the presence of 0.7 mM, showing that under these cultivation conditions, H_2O_2 is a significant oxidative stress inducer.

Desulfovibrio vulgaris Hildenborough genome encodes several enzymatic systems to detoxify ROS (Heidelberg *et al.*, 2004) and a peroxide-sensing PerR regulon has been predicted to be involved in oxidative stress responses (Rodionov *et al.*, 2004). It was reported (Mukhopadhyay *et al.*, 2007) that the PerR regulon genes were upregulated when cells were exposed to 0.1% oxygen, but strongly downregulated together with a drastic reduction in cell viability consistent with time upon cell's exposure to air. Our work shows that the expression levels of the *D. vulgaris* Hildenborough PerR regulon genes are specific and strongly depend on the H_2O_2 concentration and time of cell's exposure (especially under low peroxide stress). Firstly, it demonstrates that all components of the PerR regulon are inducible by peroxide in the same way. Secondly, it shows that the expression of genes encoding other peroxidases such as the thiol peroxidase (*tpx*) or the nigerythrin (*ngr*) is also regulated by H_2O_2 and thus belongs to the H_2O_2 stimulon. In addition, we showed that that the PerR regulon and all members of the H_2O_2 stimulon defined above were inversely regulated in the presence of 0.1 and 0.3 mM H_2O_2. The response to low levels of H_2O_2 involves an increase in the gene expression of several proteins that alleviate the toxicity and damage of cell macromolecules caused by H_2O_2 stress.

H_2O_2 is a direct substrate for catalases and peroxidases. *Desulfovibrio vulgaris* Hildenborough genome encodes for a catalase, but the *katA* gene is located on a 202-kb megaplasmid with *nif* genes, which has been documented to be lost during growth in ammonium-containing media (Fournier *et al.*, 2003). Under these experimental conditions, peroxidases are thus the only enzymes responsible for H_2O_2 elimination. Peroxidase- and SOD-specific activity changes during the H_2O_2 stresses are in agreement with the transcriptional changes. Nevertheless, under normal anaerobic growth conditions, cells of *D. vulgaris* already contain relatively high levels of SOD and peroxidase activities required to respond to low oxidative stresses and to ensure survival.

During high-peroxide stress (0.3 mM H_2O_2), all tested genes that encoded metal-containing peroxidases (rubrerythrins and nigerythrin) SOD and SOR, were downregulated and global peroxidase- and SOD-specific activities were

significantly lower compared with those in H_2O_2-untreated cells. This decrease may represent a critical factor in causing the cell death of *D. vulgaris* upon strong oxidative stresses. It was demonstrated that the exposure of *D. vulgaris* Hildenborough to a high oxygen concentration induced the inactivation and degradation of metalloproteins particularly abundant in this bacterium (Pereira *et al.*, 2008). The release of metal cations from degraded proteins can contribute significantly to the production of further ROS (Dolla *et al.*, 2006). Hence, a global downregulation of the metalloproteins (including metal-containing ROS-scavenging enzymes) represents an effective strategy to limit the availability of free metals.

Under low-peroxide stress (0.1 mM H_2O_2), the increase of peroxidase (1.46-fold)- and SOD (1.2-fold)- specific activities after 30 min could be related to the upregulation of the corresponding genes at that time. Our data show that exposure of *D. vulgaris* to low-peroxide stress (0.1 mM H_2O_2) for 30 min causes *de novo* synthesis of the main enzymes of antioxidative defense.

We can thus propose that antioxidative defense systems of *D. vulgaris* Hildenborough can overcome the negative effects of low-peroxide stress, and so after an initial increase in the transcriptional responses, the gene expression levels revert to basic levels after elimination of H_2O_2. Because high-peroxide stresses are too deleterious for the cells, the corresponding genes (most of them encoding Fe-containing proteins) are downregulated to limit free-metal-induced damages and increase survival.

Rubrerythrins encoding genes (*rbr1* and *rbr2*) were the most upregulated members of the PerR regulon at the transcript level under low-peroxide stress (0.1 mM H_2O_2, 30 min). Previously, they have been identified as important enzymes for oxygen and other oxidative stresses (Fournier *et al.*, 2003). Interestingly, the *sor* gene was also strongly upregulated under such peroxide stress conditions, whereas no significant upregulation of this candidate was observed during 0.1% O_2 exposure (Mukhopadhyay *et al.*, 2007). Therefore, NADH-dependent H_2O_2 peroxidases (rubrerythrins, nigerythrin), together with thiol peroxidase and SOR, might play a major role in the H_2O_2 stress response.

Transcript analysis revealed that gene expression reverted to the same level as in untreated cells and even lower for a time period longer than 30 min (0.1 mM H_2O_2 stress), which can explain the continuing decrease in peroxidase-specific activity during the 60–240 min of exposure. H_2O_2 quantification revealed that H_2O_2 was rapidly consumed over time and no remaining H_2O_2 could be detected after 90 min when either 0.1 or 0.3 mM was added. It should be noted that oxidized compounds (for instance, polysulfide) could be formed due to the chemical reaction between H_2O_2 and hydrogen sulfide produced by *D. vulgaris* cells. It should be also noted that the presence of H_2S is the physiologic situation for these cells in their biotopes, and the addition of H_2O_2 (as ROS formed under temporary oxic conditions) to H_2S-producing cells can be considered as quite normal. Even if we cannot exclude that a part of H_2O_2 was chemically reduced by the end-product of the sulfate reduction, our data suggest that the observed H_2O_2 consumption corresponds to a cell-mediated reaction. Most probably, H_2O_2 stresses include direct effects from H_2O_2 itself and indirect effects from H_2O_2-derived reactive chemical species together with increased redox potential.

The data show that addition of either 0.1 or 0.3 mM H_2O_2 to a mid-exponential culture results in a rapid consumption of the H_2O_2 in a cell-mediated reaction. However, exposure to 0.3 mM H_2O_2 appears to be much more toxic to the cells as all tested genes were strongly downregulated even when H_2O_2 was no longer detectable in the culture. This phenomenon provides evidence for the high stress state of the cells, which is not the case when they are exposed to a lower concentration of H_2O_2 (0.1 mM). In the presence of 0.3 mM H_2O_2, even if all genes encoding proteins related to peroxide scavenging (PerR regulon, *ngr*, *tpx*) were strongly downregulated, the remaining global peroxidase activity could account for the rapid H_2O_2 consumption. These results demonstrate a sharp contrast in the responses of *D. vulgaris* to low and high levels of H_2O_2, by analogy to data between 0.1% oxygen exposure and air stress (Fournier *et al.*, 2006; Mukhopadhyay *et al.*, 2007).

Our results show that the primary response of *D. vulgaris* Hildenborough to H_2O_2 stress is finely regulated. In addition to regulating genes directly involved in H_2O_2 detoxification such as the PerR regulon members, nigerythrin and thiol peroxidase-encoding genes, H_2O_2 also regulates the expression of *sod* and *sor* genes, involved in the elimination of superoxide anions. All these genes thus belong to the H_2O_2 stimulon and are directly involved in the defense mechanisms that allow cells to counterbalance the toxic effects of H_2O_2 and its derived chemical species in low concentrations. This mechanism thus allows cells to adapt successfully to temporary ROS presence and to survive in a variety of natural biotopes that undergo periodic exposure to oxidative conditions. It is noteworthy that the expression of all these genes is inversely regulated depending on the H_2O_2 concentration, suggesting subtle and complicated regulation mechanisms of oxidative stress responses in *D. vulgaris* that need further studies to be completely characterized.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Sequences of primers used in the study.

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