Hydrogen peroxide-induced oxidative stress responses in *Desulfovibrio vulgaris* Hildenborough

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Summary

To understand how sulphate-reducing bacteria respond to oxidative stresses, the responses of *Desulfovibrio vulgaris* Hildenborough to H_2O_2-induced stresses were investigated with transcriptomic, proteomic and genetic approaches. H_2O_2 and induced chemical species (e.g. polysulfide, ROS) and redox potential shift increased the expressions of the genes involved in detoxification, thioredoxin-dependent reduction system, protein and DNA repair, and decreased those involved in sulfate reduction, lactate oxidation and protein synthesis. A gene coexpression network analysis revealed complicated network interactions among differentially expressed genes, and suggested possible importance of several hypothetical genes in H_2O_2 stress. Also, most of the genes in PerR and Fur regulons were highly induced, and the abundance of a Fur regulon protein increased. Mutant analysis suggested that PerR and Fur are functionally overlapped in response to stresses induced by H_2O_2 and reaction products, and the upregulation of thioredoxin-dependent reduction genes was independent of PerR or Fur. It appears that induction of those stress response genes could contribute to the increased resistance of deletion mutants to H_2O_2- induced stresses. In addition, a conceptual cellular model of *D. vulgaris* responses to H_2O_2 stress was constructed to illustrate that this bacterium may employ a complicated molecular mechanism to defend against the H_2O_2-induced stresses.

Introduction

Systems biology studies of the model sulfate-reducing bacterium (SRB) *Desulfovibrio vulgaris* Hildenborough have increased dramatically in the last few years. While traditionally classified as an obligate anaerobe, *D. vulgaris* has been found to be aero-tolerant (Dolla *et al*., 2006). Sulfate reducers are frequently found in habitats close to the oxic/anoxic zones (Cypionka, 2000) and *D. vulgaris* cells have been shown to swim towards a low concentration of oxygen (0.02–0.04%, v/v in anaerobic gas mixture) (Johnson *et al*., 1997). Furthermore, *Desulfovibrio desulfuricans* ATCC 27774 has been reported to grow in the presence of nearly atmospheric oxygen level (Lobo *et al*., 2007), although the growth of *Desulfovibrio* supported by oxygen respiration has not been reported. Therefore, it is expected that there is a protective mechanism in *D. vulgaris* cells to deal with the oxidative stress they may encounter in the environment.

Information from the genome sequences strongly suggests that *D. vulgaris* protection mechanisms against oxidative stresses are unique and complex. In addition to the well-known reactive oxygen species (ROS) detoxification system of microbes (e.g., Sod, KatA, AhpC), *D. vulgaris* utilizes a defence system with the

rubredoxin oxidoreductase (Rbo)/rubrerythrin (Rbr) enzymes. Rbo exhibits superoxide reductase activity and Rbr exhibits NADH peroxidase activity (Jenney *et al*., 1999; Lumppio *et al*., 2001; Fournier *et al*., 2003; Rodionov *et al*., 2004). Second, an orthologue of *Bacillus subtilis perR*, the hydrogen peroxide sensor and response regulator (Bsat *et al*., 1998; Fuangthong *et al*., 2002; Gaballa and Helmann, 2002; Mostertz *et al*., 2004), and two *perR* paralogues, *fur* and *zur*, are computationally identified in the *D. vulgaris* genome. However, no orthologues of the *Escherichia coli* H_2O_2 and O_2-response regulators OxyR and SoxR/SoxS (Pomposiello and Demple, 2001) have been identified. In addition, membrane-bound cytochrome *c* oxidase (*cox*, *DVU1811–1815*), a cytochrome *d* ubiquinol oxidase (*cydBA*, *DVU3270–3271*) and a cytoplasmic rubredoxin : oxygen oxidoreductase (*roo*, *DVU3185*) have been identified in the genome (Heidelberg *et al*., 2004) and may contribute to the removal of oxygen species.

Multiple studies have attempted to elucidate the mechanisms of the oxidative stress response in *D. vulgaris* and some genes were found to be involved in stress responses under different oxidative stress conditions. For example, PerR regulon genes comprised the few upregulated genes in a study with low O_2 (0.1%) exposure (Mukhopadhyay *et al*., 2007). Rubredoxin : oxygen oxidoreductase (Roo) enhanced the survival rate of *D. vulgaris* under microoxic conditions (1% air) (Wildschut *et al*., 2006). Sor (superoxide reductase) was shown to be a key player in oxygen defence under fully oxic condition when *D. vulgaris* cells were stirred continuously in air (Fournier *et al*., 2003). Thiol-peroxidase, BCP-like protein and putative glutaredoxin were more abundant in *D. vulgaris* cultures oxidized by continuous bubbling with pure oxygen (Fournier *et al*., 2006). Thioredoxin reductase gene, *trxB*, was found to be upregulated in response to air (Zhang *et al*., 2006) or pure oxygen flushing (Pereira *et al*., 2008). While these studies have improved our understanding of oxidative stress response in SRB, the genome-wide mechanistic picture of the *D. vulgaris* response to oxidative stress remains elusive.

In this study, a genome-wide analysis of the *D. vulgaris* response to H_2O_2, known to be a more reactive oxidant than superoxide (Miller and Britigan, 1997), was carried out to provide more insights into oxidative stress response mechanisms in *D. vulgaris*. Due to the accumulation of hydrogen sulfide in *D. vulgaris* cultures, oxidized compounds such as polysulfide could be produced, maintaining an elevated redox potential even after decomposition of H_2O_2. Together with metabolic activity assays, temporal transcriptional and translational profiling analyses provided a comprehensive picture of the direct and indirect effects of H_2O_2 on the oxidative stresses. Examination of the stress response of deletion mutants of *fur* and *perR* indicated that PerR and Fur may be coordinately involved in the regulation of oxidative stress response in *D. vulgaris.*

Results and discussion

Temporal changes of physiology and metabolic activities of D. vulgaris *cells in response to H_2O_2*

As the first step, different concentrations of H_2O_2 (0, 0.5, 1, 2, 4, 8 and 10 mM) were tested for their effects on the growth of mid-log-phase cells. About 3 h delay of growth was observed for low concentrations of H_2O_2 (0.5–2 mM) treatment, while 4 mM or higher concentrations of H_2O_2 arrested growth for proportionately longer times (data not shown). Therefore, 1 mM of H_2O_2 was used in this study.

The temporal changes of physiology and metabolic activities of *D. vulgaris* cells after the addition of H_2O_2 were examined. With the addition of H_2O_2, the colour of cell culture turned yellowish and slightly milky, suggesting the formation of polysulfide and sulfur, respectively, due to the chemical reaction between H_2O_2 and accumulated sulfide in the culture. Therefore, the effect of H_2O_2 treatment on cell growth was monitored as the recovery of sulfate reduction activity (Fig. 1A). The H_2O_2-dependent formation of polysulfide, which has been shown to be inhibitory to SRB (Kaster *et al*., 2007; Johnston *et al*., 2009), was monitored by determining the absorbance at 410 nm over time. Polysulfide was formed immediately after the addition of H_2O_2 and quickly diminished over time and became almost undetectable at 240 min after H_2O_2 treatment (Fig. 1B). It is presently unclear how much the relatively low concentration of Fe (II) (present as insoluble sulfide), which decreased from initially 40 μM to about 10 μM, contributed to the stress response. The redox potential of the cell culture shifted to a higher level following the addition of H_2O_2, and almost recovered at 240 min (data not shown). In addition, inhibitory effects of H_2O_2 and H_2O_2-derived chemical species on metabolism were demonstrated by decreased lactate oxidation and sulfate reduction (Fig. S1). These data suggest that H_2O_2-induced oxidative stresses include direct effects from H_2O_2 and indirect effects from derived chemical species such as polysulfide and ROS, along with the increase in the redox potential.

Overall gene expression patterns of D. vulgaris *responses to H_2O_2-induced stresses*

The temporal genome-wide transcriptional changes after addition of 1 mM H_2O_2 were examined by the *D. vulgaris* whole-genome microarray. In terms of gene number and fold change, the transcriptional response reached a peak

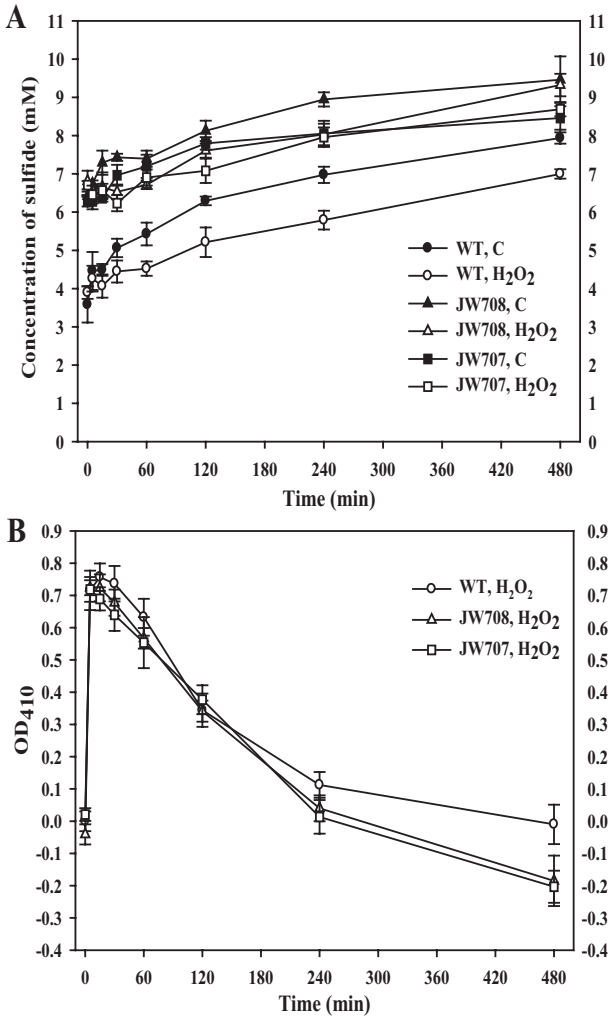

Fig. 1. Effects of H_2O_2 treatment on cell growth and production of derived chemical species in WT *D. vulgaris* and deletion mutants of *perR* (JW708) and *fur* (JW707).
A. Concentration of sulfide was monitored to indicate the effect of H_2O_2 treatment on cell growth.
B. Polysulfide was formed following the addition of H_2O_2 and eliminated over time.
The data shown are the averages of three biological replicates with standard deviation.

at 120 min with 485 genes upregulated and 527 genes downregulated (Fig. 2A), representing approximately 14% and 15% of the total open reading frames on the array respectively. The gene expression profiles of control (C30–C480) and treatment (T30–T480) samples were clearly separated by axis 1 (DC1), and the early (T30, T60 and T120) and late responses (T240 and T480) were well separated by axis 2 (DC2) in detrended correspondence analysis (DCA) of the microarray data (Fig. 2B).

In terms of functional categories of responsive genes, COG categories of O (post-translational modification, protein turnover, chaperones) and R (general function prediction) had the highest number of genes upregulated at 30 min (Fig. S2), suggesting the immediate damaging effect of H_2O_2-induced oxidative stress on cellular proteins. In addition to genes in these two categories, genes in COG functional categories T (signal transduction mechanisms), C (energy production and conversion), M (cell envelope biogenesis, outer membrane), E (amino acid transport and metabolism), N (cell motility and secretion) and L (DNA replication, recombination and repair) were significantly differentially transcribed at 60 min and

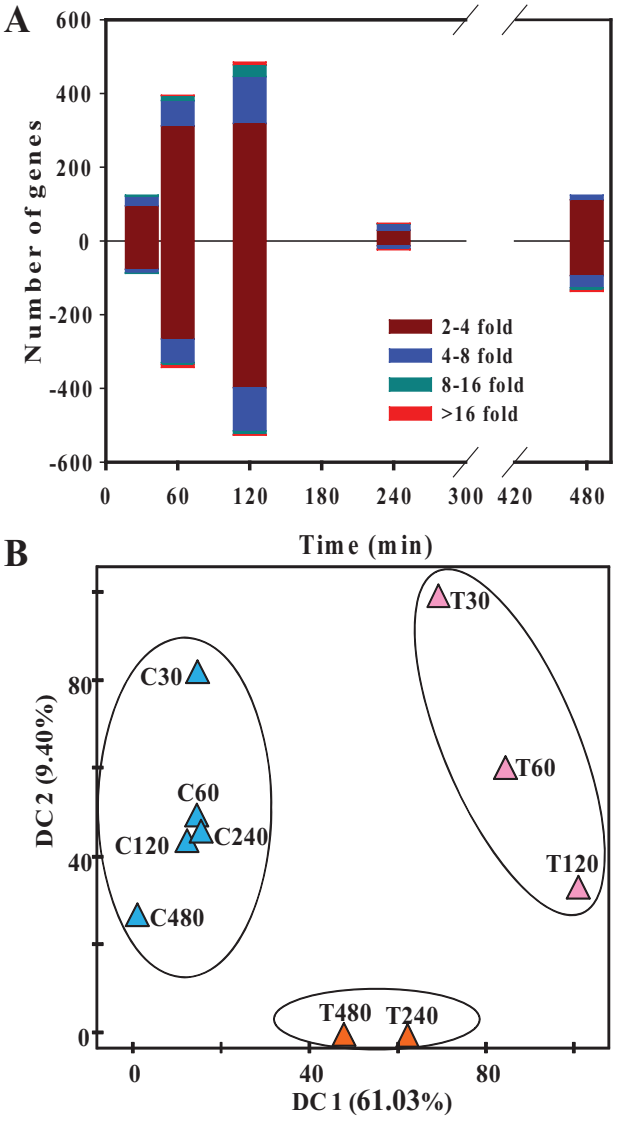

Fig. 2. Temporal profiling of the transcriptomic response.
A. Numbers of genes differentially transcribed following the addition of 1 mM H_2O_2 ($|\log_2 R$ (treatment/control)$| > 1$, $|Z| > 1.5$). Positive and negative numbers indicate number of genes with increased and decreased levels of transcription in the treatment cultures versus control respectively.
B. Detrended correspondence analysis (DCA) of the transcriptional changes. Overall similarity of the microarray gene expression profiles for H_2O_2-treated and control samples among the different time points was shown. C30–C480: control; T30–T480: treatment.

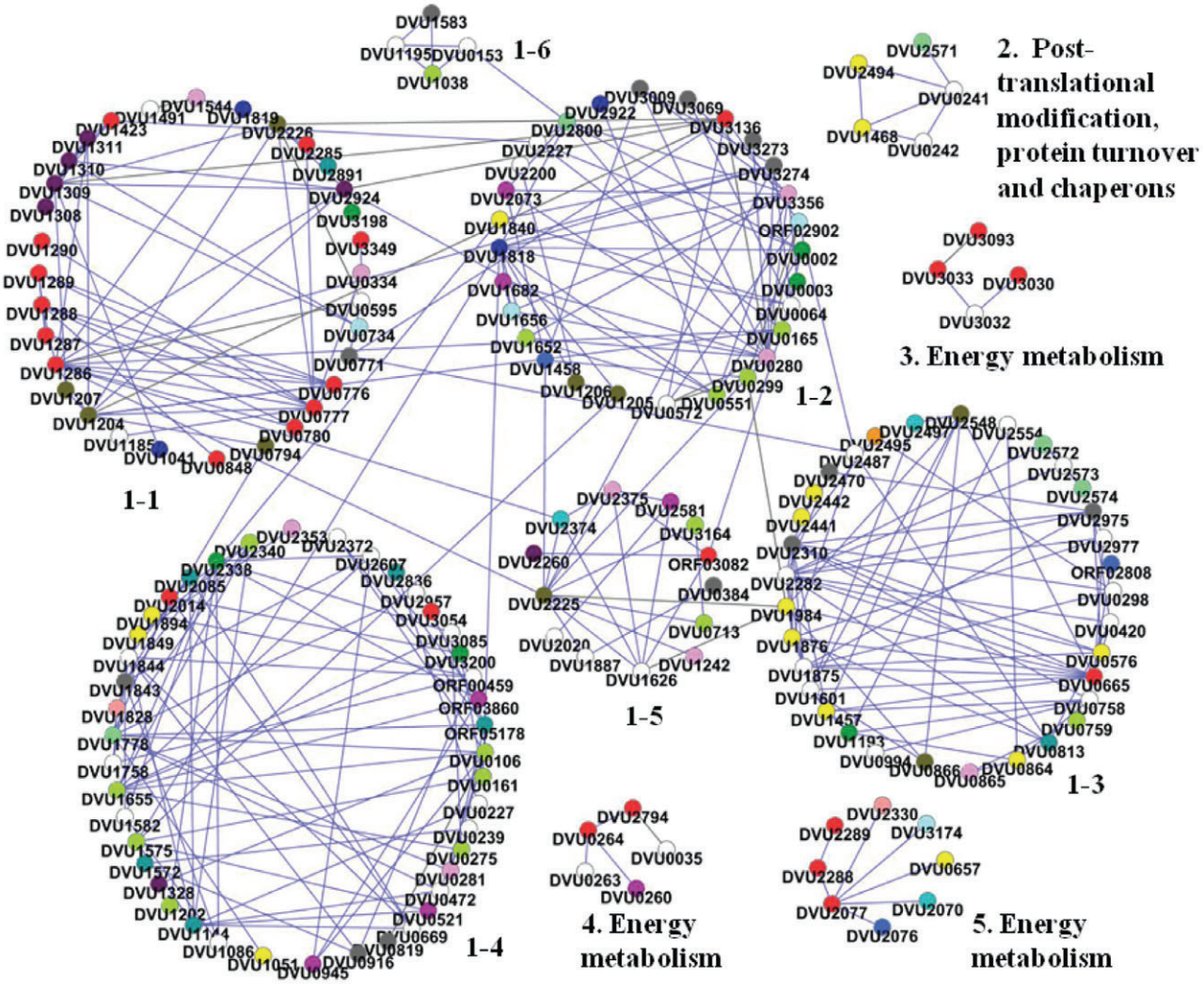

Fig. 3. Gene coexpression network from the H_2O_2 stress microarray profiles generated by the random matrix theory approach. Modules with more than four genes are shown. Annotations for genes identified by DVU numbers can be found at Microbes Online (http://www.microbesonline.org/). Each node represents a gene. Blue and grey lines indicate positive and negative correlation coefficients respectively. Colours were assigned to nodes according to their gene function categories: red, energy production and conversion; yellow, post-translational modification, protein turnover, chaperons; green, DNA replication, recombination and repair; purple, signal transduction mechanisms; brown, lipid transport and metabolism; green-yellow, carbohydrate, amino acid or nucleotide transport and metabolism; light green, inorganic ion transport and metabolism; magenta, translation, ribosomal structure and biogenesis; pink, cell envelope, biogenesis, outer membrane; dark cyan, transcription; orange, secondary metabolite biosynthesis, transport and catabolism; light cyan, coenzyme transport and metabolism; blue, intracellular trafficking, secretion and vesicular transport; light blue, cell motility/signal transduction mechanisms; salmon, cell cycle control, cell division and chromosome partitioning; cyan, defence mechanisms; dark grey, general function prediction; white, function unknown.

120 min (Fig. S2). In contrast, fewer genes with expression changes were detected at both 240 and 480 min, which is consistent with the changes of the derived chemical species such as polysulfide and metabolic activity.

In order to further understand the transcriptional responses to H_2O_2-induced oxidative stress, a gene coexpression network was constructed with the microarray data. The resulting network contained a total of 175 genes that were partitioned into five subnetworks (modules, with more than four genes) (Fig. 3). Module 1 was the largest module including 155 genes involved in different functional categories and further divided into submodules 1-1 to 1-6. As expected, genes from the same operon tend to link together in the subnetworks and all modules contain functionally coherent sets of genes.

Further insights into gene interactions in different functional categories were obtained by examining individual modules/submodules. In submodule 1-1, genes predicted to be involved in 'energy production and conversion', such as *atpGAF1* (*DVU0776–77*, *DVU0780*) and *dsrMKJOP* (*DVU1286–1290*), were tightly linked to genes (*DVU1308–1311*) involved in 'translation, ribosomal structure and bio-

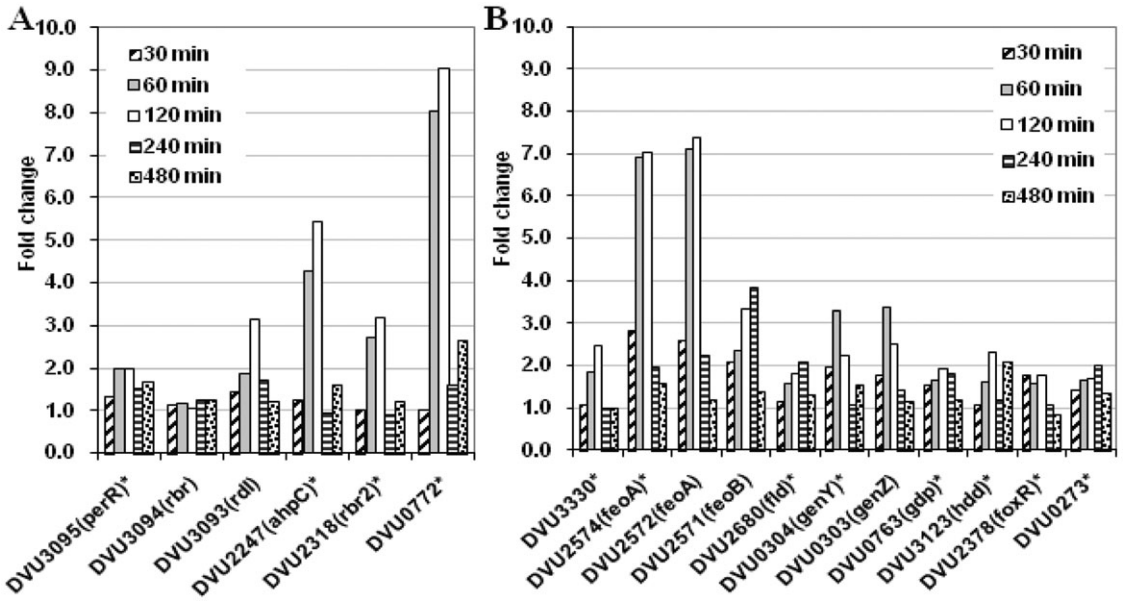

Fig. 4. Expression profiling of predicted PerR (A) and Fur (B) regulons across the time course. *: predicated regulator binding site found in the upstream of the gene.

genesis'. Iron transport gene *feoB* (*DVU2571*) was indirectly correlated to genes involved in 'post-translational modification, protein turnover, chaperons' in Module 2. A predicted oxidative stress response gene *DVU3093* (*rdl*, rubredoxin-like protein, upregulated, Table S2) was negatively correlated with downregulated genes *DVU3033* (encoding an iron–sulfur cluster-binding protein) and *DVU3030* (*ackA*, acetate kinase) in Module 3.

The gene coexpression network provides an advantage for functional prediction of hypothetical genes due to the fact that functionally related genes are connected to each other in the gene coexpression networks (Luo *et al*., 2007). Therefore, unknown function genes *DVU1875* (predicted to encode a DafA protein) and *DVU1601* (encoding a Clps domain-containing protein) in submodule 1-3 could be functionally involved in 'post-translational modification, protein turnover, chaperons'. Hypothetical genes *DVU3032* in Module 3 and *DVU0263* (predicted to encode a tetrahaem cytochrome *c*3 protein) in Module 4 could be involved in 'energy production and conversion'. In addition, the gene coexpression network shed light on the importance of genes based on the number of links for each gene. Genes involved in sulfate reduction (*DVU1286* and *DVU1288*), ATP production (*DVU0776–0777*), protein synthesis (*DVU1309–1311*), thioredoxin-dependent pathway [*DVU1457* (*trxB*)], transcriptional regulator (*DVU1144*), protein damage repair (*msrA*) and several genes encoding ribosomal proteins were examples of genes with the highest number of connections (Table S1). The putative nitroreductase gene *DVU3136* had six connections and was one of the most upregulated genes (Table S2), suggesting that *DVU3136* is actively involved in the stress response as is its homolog in *E. coli* (Liochev *et al*., 1999). These results suggested that the network analysis of gene expression could provide useful information for understanding gene function and interaction in the oxidative stress response.

Responses of key pathways/genes to H_2O_2-induced oxidative stresses

To gain more insights of the molecular mechanisms of the *D. vulgaris* oxidative stress response, the microarray data were further examined for representative functional groups/genes as follows.

Detoxification enzymes. The widespread ROS detoxification system genes including *sodB*, *katA* and *ahpC* as well as genes involved in rbo/*rbr* system in *D. vulgaris* may be used to protect the cell against oxidative stress. Among these genes, the expression of *ahpC* (*DVU2247*) was increased more than fourfold at 60 and 120 min (Fig. 4A), *rbr* was increased less than twofold, *katA* (*DVUA0091*) was significantly downregulated. The transcripts of *sodB* (*DVU2410*), *rub* (rubredoxin), *rbo* and *ngr* (nigerythrin, homologue of *rbr*) did not change significantly during the stress (data not shown), suggesting that the baseline concentrations of these enzymes may be sufficient for responding to the oxidative stress. In contrast, the gene expression of *rdl* (rubredoxin-like protein) and *rbr2* (putative rubrerythrin, homologue of *rbr*) increased more than threefold (Fig. 4A). Therefore, Rdl with Rbr2 rather than

Rbr or Ngr might play major roles in H_2O_2-induced stress response. Consistently, no obvious oxidative stress phenotype was found for the *D. vulgaris rbr* mutant when cells were exposed to H_2O_2 (Fournier *et al*., 2003).

Thioredoxin-dependent reduction systems. With H_2O_2 treatment, *trxB* (*DVU1457*, thioredoxin reductase) was significantly upregulated and transcripts of *trx* (thioredoxin) and *DVU0725* (thioredoxin domain-containing hypothetical protein) were increased as well. Thioredoxins function as hydrogen donors for the reduction of enzymes involved in DNA synthesis, protein repair and sulfur assimilation as well as the direct or indirect reduction of H_2O_2 (Zeller and Klug, 2006). In addition to the significant induction of *ahpC* as mentioned above, reductant-dependent protein repair system genes *msrAB* (*DVU1984/0576*) were significantly upregulated (Table S1). These data strongly suggested the involvement of the thioredoxin-dependent systems in the oxidative stress response.

DNA replication, recombination and repair. Different from the immediate upregulation of genes involved in 'post-translational modification, protein turnover, chaperons' (Fig. S2), *DVU2907* (*umuD*) was the only significantly increased gene involved in 'DNA replication, recombination and repair' at 30 min. At 60 min, besides *umuD*, *DVU0771* (encoding a putative molybdenum-protein-binding domain protein/site-specific recombinase, phage integrase), *DVU2003* (encoding a putative transposase) and *DVU1515* (*dcm*, encoding a putative type II DNA modification methyltransferase) were significantly upregulated. At 120 min, expression of more genes such as *DVU1193* (*radC*, encoding a putative DNA repair protein), *DVU1899* (encoding a putative DNA repair protein RecO) and *DVU1789* (*dnaG*, encoding DNA primase) increased (Fig. S3).

Signal transduction. Two-component signal transduction is a common mechanism that bacteria utilize to sense and respond to environmental changes. Genes *DVU3382* (encoding a histidine kinase containing a PAS sensory domain) and *DVU3381* (encoding a transcriptional regulatory protein) in one predicted operon were significantly upregulated (Fig. S3). The immediate and consistent upregulation of *DVU3382/3381* suggests that these genes may be involved in sensing the oxidative stress and conducting the stress response. However, additional experimental evidence is required to identify the biological roles of DVU3382 in sensing redox changes.

SRB signature genes. There were 46 SRB signature genes including genes involved in dissimilatory sulfate reduction pathways, oxidoreductase activities and oxidative stress responses (Chhabra *et al*., 2006). Microarray data from this study showed that sulfate reduction pathway genes including *dsrMKJOP*, *dsrABC* and *qmoABC* were downregulated (Fig. S3), which agreed with the slower growth under oxidative stress conditions.

Regulation of H_2O_2-induced oxidative stress response by PerR and Fur

PerR regulon has been predicted to be involved in oxidative stress responses (Rodionov *et al*., 2004). Fur, a paralogue of PerR and regulator of iron homeostasis, has been shown to be important for bacterial growth and stress responses (Touati *et al*., 1995; Hassett *et al*., 1996; Andrews *et al*., 2003). As shown in Fig. 4A, the transcripts of PerR regulon genes *ahpC*, *rdl*, *rbr2* and *DVU0772* increased more than threefold, while *perR* and *rbr* transcripts increased less than threefold. All of the predicted Fur regulon genes were upregulated with *feoA-feoAB* and *genYZ* showing the highest upregulation (Fig. 4B). Upregulation of all predicted PerR and Fur regulon genes in H_2O_2-induced oxidative stress response is distinct from other stress responses in this strain. De-repression of PerR regulon is observed when *D. vulgaris* cells were exposed to 0.1% O_2; however, only a few Fur regulon genes are differentially expressed (Mukhopadhyay *et al*., 2007). Although heat shock induces an increase in the expression of all PerR regulon genes, only *feoAB* and *gdp* are upregulated in the Fur regulon (Chhabra *et al*., 2006). In contrast, when exposed to nitrite, the transcription of most of the Fur regulon genes is increased whereas only the PerR-regulated *ahpC* is consistently upregulated at 30–90 min (He *et al*., 2006).

To further characterize the roles of PerR and Fur in H_2O_2-induced oxidative stress responses, transcriptional responses of Δ*perR* (JW708) and Δ*fur* (JW707) mutants following addition of 1 mM H_2O_2 were investigated. Under standard growth conditions, as expected, the de-repression of PerR regulon genes such as *ahpC*, *rbr2* and *DVU0772* was observed in Δ*perR* mutant (Table 1). De-repression of all Fur regulon genes except *DVU3123* was found in the Δ*fur* mutant. In addition, 12 genes (*DVU2379–DVU2390*) downstream of *foxR* (genes with less than threefold increases not shown) (Table 1) were de-repressed in Δ*fur* mutant, which is consistent with the gene transcription data reported by Bender and colleagues (2007). Interestingly, *ahpC* and *rbr2* were observed to be de-repressed in the mutant Δ*fur* as well. With H_2O_2 treatment, most of the de-repressed genes were not further responsive in the mutants. Genes that were de-repressed in the mutants but not responsive to oxidative stress could be considered as PerR- or Fur-

Table 1. Selected transcriptomics data in mutants under standard growth condition and H_2O_2 stress.

			De-repression of genes		Response to H_2O_2 in strain		
			No stress		1 mM H_2O_2 versus 0 mM at 120 min		
DVU No.	Name	Annotated function	Δ*fur*/WT	Δ*perR*/WT	Δ*fur*	Δ*perR*	WT
DVU0763[a]*	*gdp*	GGDEF domain protein	**4.5**	–0.5	–0.6	1.2	**1.7**
DVU2377		Hypothetical protein	**2.3**	0.3	–0.3	0.6	0.7
DVU2378[a]	*foxR*	Transcriptional regulator, AraC family	**3.3**	0.0	–0.1	0.1	0.8
DVU2379	*pqqL*	Peptidase, M16 family, putative	**2.3**	–0.6	–0.3	0.4	0.4
DVU2380	*atpX*	ABC transporter, ATP-binding protein	**2.7**	0.4	–1.4	–0.1	1.0
DVU2381		Conserved hypothetical protein	**4.6**	–0.2	–1.1	0.2	0.7
DVU2383		*tonB*-dependent receptor domain protein	**5.0**	–0.3	–1.2	–0.2	–0.1
DVU2384		ABC transporter, periplasmic substrate-binding protein	**1.8**	0.1	–0.4	0.3	1.1
DVU2388	*tolQ-1*	*tolQ* protein	**2.0**	0.1	–0.3	–0.3	0.5
DVU2389	*tolR*	Biopolymer transport protein, ExbD/TolR family	**1.7**	0.3	–0.6	0.6	0.8
DVU2390		*TonB* domain protein	**1.6**	–0.2	–0.6	0.5	0.5
DVU2456		Hypothetical protein	**1.8**	1.1	–0.2	–0.5	0.1
DVU2560		Conserved domain protein	**1.6**	1.3	0.1	–0.7	0.3
DVU2564***	*bioF*	8-Amino-7-oxononanoate synthase	**1.8**	–0.3	0.5	1.1	**2.1**
DVU2571***	*feoB*	Ferrous iron transport protein B	**4.0**	–0.5	–0.3	0.8	**2.2**
DVU2572*	*feoA*	Ferrous iron transport protein A	**4.6**	–0.4	0.2	**2.3**	**3.2**
DVU2573*		Hypothetical protein	**3.6**	–0.5	0.2	**1.7**	**3.4**
DVU2574[a]*	*feoA*	Ferrous iron transporter component *feoA*	**3.0**	–1.3	–0.2	**2.4**	**2.4**
DVU2680[a]*	*fld*	Flavodoxin, iron-repressed	**5.3**	–1.5	–1.0	1.1	**2.4**
DVU2681*		Hypothetical protein	**5.0**	–1.3	–1.0	**1.8**	**1.9**
DVU3122		Hypothetical protein	**4.4**	0.1	–0.8	0.4	–0.3
DVU3124		Hypothetical protein	**1.7**	–1.5	–0.2	1.0	–0.3
DVU3330[a]		Hypothetical iron-regulated P-type ATPase	1.4	–0.9	–0.1	0.9	0.3
DVU3331		Hypothetical protein	**2.2**	–0.2	0.0	0.9	0.6
DVU3332		Heavy metal translocating P-type ATPase	**1.9**	–0.8	–0.5	0.3	–0.2
DVU3333		Hypothetical protein	**2.3**	–0.1	–0.2	0.4	0.7
DVU0273[a]***		Conserved hypothetical protein	**4.4**	–1.4	–0.6	0.9	1.4
DVU0303*	*genZ*	Hypothetical protein	**4.6**	–0.8	–0.5	**1.8**	**3.2**
DVU0304[a]*	*genY*	Hypothetical protein	**4.5**	–1.0	–0.2	1.5	**3.3**
DVU0251		Membrane protein, putative	**2.1**	**2.5**	0.0	–1.1	0.8
DVU2247[b]***	*ahpC*	Alkyl hydroperoxide reductase C	**3.1**	**3.6**	0.4	–0.8	**3.5**
DVU2318[b]***	*rbr2*	Rubrerythrin, putative	**2.1**	**4.6**	0.9	–0.5	**2.9**
DVU0772[b]**		Hypothetical protein	0.9	**2.1**	**1.8**	1.5	**5.1**
DVU0712		Amino acid ABC transporter, periplasmic-binding protein	0.5	**1.9**	–1.1	**–1.6**	0.1
DVU0881	*fusA*	Translation elongation factor G, putative	1.4	**1.9**	–0.9	–0.8	0.9
DVU1131		Hypothetical protein	0.9	**1.8**	0.4	0.0	0.8
DVU1139		Bacteriophage DNA transposition B protein, putative	0.5	**1.7**	0.1	0.0	0.2
DVU1141		Hypothetical protein	0.9	**2.0**	–0.2	–0.3	0.1
DVU1142		Transcriptional regulator, putative	0.1	**1.9**	0.2	0.2	0.2
DVU0231		Hypothetical protein	0.7	**1.7**	–0.7	–0.7	–0.3
DVU2688		Bacteriophage transposase A protein	0.6	**1.8**	0.5	0.8	0.4
DVU2699	*slt*	Transglycosylase SLT domain protein	0.6	**1.7**	–0.6	–0.7	–1.1
DVU2793		Electron transport complex protein RnfD,	0.2	**1.6**	–0.1	–0.8	–0.2
DVU3270	*cydB*	Cytochrome *d* ubiquinol oxidase, subunit II	1.1	**1.7**	–1.1	**–3.5**	–0.4
DVU3271	*cydA*	Cytochrome *d* ubiquinol oxidase, subunit I	0.8	**1.9**	–0.9	**–2.3**	–0.4
DV00024**		Conserved hypothetical protein	0.4	**2.7**	1.0	–0.2	**2.3**
DVU0172	*phsB*	Thiosulfate reducatase (*phsB*)	1.0	**1.8**	0.7	0.2	0.7
DVU2347	*argD*	Acetylornithine aminotransferase	–0.3	**2.2**	–0.7	–1.5	–0.5
DVU2348	*dut*	Deoxyuridine 5-triphosphate nucleotidohydrolase	–0.2	**2.3**	–0.5	**–2.8**	–1.1
DVU0186		Conserved hypothetical protein	0.6	**2.0**	0.9	0.6	0.9

a. Containing predicted Fur binding sites.
b. Containing predicted PerR binding sites.
*: Fur-dependent; **: PerR-dependent; ***: PerR- and Fur-dependent.
Boldface indicates more than threefolds of gene expression change ($|\log_2 R| = 1.6$).

dependent oxidative response genes. As shown in Table 1, eight genes (Fur regulon genes *gdp*, *fld*, *genYZ*, *feoA-DVU2573-feoA* and Fur-de-repressed gene *DVU2681*) were Fur-dependent, two genes (PerR regulon gene *DVU0772* and PerR-de-repressed gene *DVU0024*) were PerR-dependent and five genes [Fur regulon genes *DVU0273*, *feoB*, Fur-de-repressed gene *DVU2564* (*bioF*) and PerR regulon genes *ahpC* and *rbr2*] were PerR- and

Fur-dependent. On the other hand, 33 genes were found to be upregulated in both wild type (WT) and mutants Δ*perR* and Δ*fur* when stressed with H_2O_2 (Table S3), but not de-repressed in unchallenged mutants Δ*perR* and Δ*fur* (Table 1), which suggested that these genes were not regulated by either PerR or Fur in oxidative stress response.

The results of physiological and metabolic changes in the deletion mutants, Δ*perR* and Δ*fur* supported the roles of these two genes. Compared with WT, the recovery of hydrogen sulfide production in H_2O_2-treated mutants Δ*perR* and Δ*fur* was quicker (Fig. 1A), suggesting that loss of function of PerR or Fur leads to increased resistance to H_2O_2 treatment. In addition, the elimination of polysulfide was faster in both mutants although a significant difference between mutants and WT was observed only at 480 min after H_2O_2 treatment (Fig. 1B); the recovery of decreased lactate oxidation/acetate accumulation and sulfate reduction in mutants was quicker (Fig. S1). Interestingly, with H_2O_2 treatment, the recovery of redox potential shift in Δ*fur* was much quicker than Δ*perR* and WT (results not shown). These data suggest a functional overlap as well as the difference between PerR and Fur, and further studies are needed to provide more insights into our understanding of the mechanism of PerR and Fur in oxidative stress responses.

Proteomic analysis of D. vulgaris *responses to H_2O_2-induced oxidative stresses*

The *D. vulgaris* response to oxidative stress at the protein level was assessed with iTRAQ proteomics strategy. A total of 379 proteins were detected with 9 significantly increased and 18 significantly decreased (Table 2) in 120 min (an observed response peak for gene transcription) samples. The abundance of DVU0273, a predicted Fur regulon protein, was significantly increased, implicating a role of Fur in oxidative stress response. An increase in protein level of DVU1078, a single-strand nucleic acid-binding R3H domain protein, implied the damaging effects on DNA molecules. In addition, the increase of CysK (DVU0663, cysteine synthase A) in protein content suggested that biosynthesis and/or repair of iron–sulfur cluster proteins were necessary under the oxidative stress. Sixteen out of the 18 proteins with significantly decreased levels were ribosomal proteins and decreased transcripts were found for five ribosomal protein encoding

Table 2. Proteomic and microarray data for proteins with the most significant changes in abundance.

			Microarray $\log_2 R$					iTRAQ $\log_2 R$
DVU No.	Name	Annotated function	30 min	60 min	120 min	240 min	480 min	120 min
DVU0799	NA	Conserved hypothetical protein	NA	NA	–1.5 (–0.0)	NA	NA	2.3 (4.2)
DVU1375	NA	Hypothetical protein	0.2 (0.4)	–0.3 (–0.5)	–0.9 (–1.4)	–0.3 (–0.5)	–0.3 (–0.6)	1.3 (2.4)
DVU3199	NA	Conserved hypothetical protein TIGR00103	NA	–0.6 (–1.1)	–1.0 (–1.9)	0.1 (0.1)	–0.0 (–0.1)	1.3 (2.4)
DVU0273	NA	Conserved hypothetical protein	0.5 (0.9)	0.7 (1.4)	0.8 (1.4)	1.0 (1.0)	0.4 (0.8)	1.3 (2.3)
DVU0797	NA	Conserved hypothetical protein	NA	NA	NA	NA	NA	1.1 (2.0)
DVU0508	*infB*	Translation initiation factor IF-2	–0.4 (–0.7)	–0.4 (–0.8)	–0.2 (–0.4)	0.2 (0.3)	0.4 (0.8)	1.1 (2.0)
DVU1265	NA	Hypothetical protein	1.0 (1.8)	1.2 (2.1)	**1.5 (2.0)**	–0.0 (–0.0)	0.4 (0.7)	**1.1 (2.0)**
DVU0663	*cysK*	Cysteine synthase A	0.1 (0.2)	0.2 (0.3)	0.5 (0.9)	0.5 (0.5)	–0.3 (–0.5)	1.1 (2.0)
DVU1078	NA	R3H domain protein	–0.6 (–0.0)	NA	–0.2 (–0.0)	–0.1 (–0.1)	0.8 (1.4)	1.2 (2.2)
DVU1326	*rpsM*	Ribosomal protein S13	–0.4 (–0.8)	–0.8 (–1.5)	**–1.0 (–2.0)**	0.0 (0.0)	0.2 (0.4)	**–1.3 (–2.1)**
ORFA00060	NA	Transcriptional regulator, AbrB family	NA	NA	NA	NA	NA	–1.5 (–2.4)
DVU1303	*rplC*	Ribosomal protein L3	–0.2 (–0.3)	–0.5 (–1.0)	**–1.0 (–1.8)**	0.0 (0.0)	0.1 (0.2)	**–1.3 (–2.2)**
DVU1304	*rplD*	Ribosomal protein L4	–0.0 (–0.1)	–0.2 (–0.3)	–0.6 (–1.0)	–0.2 (–0.4)	0.1 (0.1)	–1.3 (–2.2)
DVU1318	*rplF*	Ribosomal protein L6	–0.0 (–0.1)	–0.3 (–0.5)	**–1.0 (–1.6)**	–0.1 (–0.2)	0.3 (0.6)	**–1.3 (–2.2)**
DVU2518	*rplM*	Ribosomal protein L13	–0.3 (–0.5)	–0.6 (–1.1)	**–1.3 (–2.4)**	0.2 (0.3)	0.2 (0.4)	**–2.0 (–3.3)**
DVU1310	*rplP*	Ribosomal protein L16	–0.4 (–0.8)	–1.0 (–1.9)	**–1.6 (–2.9)**	–0.2 (–0.4)	–0.0 (–0.0)	**–1.4 (–2.3)**
DVU1330	*rplQ*	Ribosomal protein L17	–0.2 (–0.5)	–0.5 (–1.1)	–0.8 (–1.4)	0.2 (0.4)	0.2 (0.4)	–1.3 (–2.1)
DVU1319	*rplR*	Ribosomal protein L18	–0.1 (–0.2)	–0.2 (–0.4)	–0.6 (–1.0)	–0.2 (–0.3)	0.2 (0.3)	–2.0 (–3.3)
DVU0835	*rplS*	Ribosomal protein L19	0.6 (1.1)	0.2 (0.4)	0.2 (0.4)	0.4 (0.7)	0.4 (0.8)	–2.0 (–3.3)
DVU1314	*rplX*	Ribosomal protein L24	0.0 (0.1)	–0.6 (–1.0)	–0.5 (–0.8)	0.1 (0.2)	–0.1 (–0.1)	–1.8 (–3.1)
DVU1211	*rpmB*	Ribosomal protein L28	–0.3 (–0.0)	–0.7 (–1.2)	–0.2 (–0.3)	0.6 (1.1)	0.6 (1.1)	–2.6 (–4.4)
DVU2519	*rpsI*	Ribosomal protein S9	NA	0.2 (0.0)	0.1 (0.2)	0.0 (0.0)	0.4 (0.8)	–2.0 (–3.3)
DVU1327	*rpsK*	Ribosomal protein S11	–0.5 (–0.9)	–0.4 (–0.7)	–0.7 (–1.2)	–0.1 (–0.2)	0.3 (0.6)	–1.4 (–2.4)
DVU0504	*rpsO*	Ribosomal protein S15	0.0 (0.1)	0.3 (0.6)	0.5 (0.9)	1.2 (2.0)	0.8 (1.4)	–1.9 (–3.1)
DVU0839	*rpsP*	Ribosomal protein S16	–0.0 (–0.0)	–0.1 (–0.2)	–0.4 (–0.8)	0.1 (0.1)	0.7 (1.3)	–1.6 (–2.6)
DVU1298	*rpsL*	Ribosomal protein S12	NA	NA	–0.4 (–0.7)	0.2 (0.3)	NA	–1.9 (–3.1)
DVU2091	*thiE-1*	Thiamine-phosphate	–0.1 (–0.2)	0.1 (0.3)	0.3 (0.4)	0.2 (0.3)	–0.3 (–0.5)	–3.2 (–5.5)

R: treatment/control. Values in parentheses are *Z* scores.
Boldface indicates more than twofolds of change ($|\log_2 R| \geq 1$) in both transcript and protein level.

genes. Overall, proteomics and transcriptomics assays were in a good agreement, and both analyses indicated the damaging effect of oxidative stress on protein and DNA with a corresponding increase in the expression of damage repair genes and a decrease in the expression of metabolic genes.

Conceptual cellular model of D. vulgaris *responses to oxidative stresses*

Our experimental results suggested that the molecular mechanism of oxidative stress response in *D. vulgaris* appears to be quite different from that of *B. subtilis* and *E. coli.* First, PerR and Fur may functionally overlap in regulating H_2O_2-induced oxidative stress response in *D. vulgaris* (Table 1). In *E. coli*, H_2O_2 response regulator OxyR regulates the responses by a thiol switch (Tao *et al*., 1993; Zheng *et al*., 2001) and regulator PerR in *B. subtilis* senses H_2O_2 by metal-catalysed oxidation (MCO) of histidine (Lee and Helmann, 2006). In addition, the upregulated thioredoxin-dependent pathway is independent of PerR and Fur regulation in *D. vulgaris* (Table S3), which is similar to *B. subtilis* PerR that does not control genes involved in disulfide reduction (Helmann *et al*., 2003; Imlay, 2008). In contrast, the *E. coli* OxyR regulon includes genes involved in maintaining intracellular thiols (Tao *et al*., 1993; Zheng *et al*., 2001). Given that the protein sequence of *D. vulgaris* PerR and *B. subtilis* PerR are highly conserved especially the functionally crucial Zn^{2+} binding site and Fe^{2+} or Mn^{2+} binding site (Fig. S4), two questions remain: (i) Does PerR regulate the oxidative stress response like PerR in *B. subtilis* through metal-catalysed oxidation of histidine? (ii) Is 'thiol switch' the key event for the response regulation which is similar to that of *E. coli* OxyR? Further studies are required to address how PerR, Fur or additional regulators regulate the oxidative stress responses in *D. vulgaris* or other microorganisms.

Considering all of the experimental results and our general knowledge together, a conceptual cellular model of the *D. vulgaris* oxidative stress response was constructed (Fig. 5). A dramatic effect on gene transcription was observed when the mid-log phase *D. vulgaris* culture was challenged with H_2O_2. Genes involved in energy conservation and protein biosynthesis were downregulated, and genes involved in 'post-translational modification, protein turnover, chaperons' or 'DNA replication, recombination and repair' were sequentially stimulated for repairing the damage from H_2O_2 and derived chemical species. Two major detoxification pathways, including *rdl/rbr2* and thioredoxin-dependent pathways such as *ahpC*, were induced. PerR and Fur may functionally overlap by co-regulating most of the PerR or Fur regulon genes. In addition, the induction of thioredoxin-dependent reduction pathways could be independent of PerR or Fur.

In conclusion, derived chemical species such as polysulfide, sulfur, ROS and the resulting increase in the redox potential following the addition of H_2O_2 could trigger a complicated oxidative stress response in *D. vulgaris*, and the molecular mechanisms employed to defend against such a stress could differ substantially from that of other bacteria such as *E. coli* and *B. subtilis*.

Experimental procedures

Bacterial strains, growth conditions and biomass production

Desulfovibrio vulgaris Hildenborough and deletion mutants of *fur* (JW707) and *perR* (JW708) were investigated in this study. Mutants were constructed as described in Bender and colleagues (2007). Defined medium LS4D (Mukhopadhyay *et al*., 2006) with 60 mM lactate/50 mM sulfate was used as standard growth medium and the cell cultures were grown at 30°C anaerobically.

To produce biomass for the transcriptomics and proteomics assays, the mid-log phase pre-cultured *D. vulgaris* cells were subcultured into production vessels in triplicate with 10% (v/v) inocula. H_2O_2 [100 mM, prepared from 30% (v/v) H_2O_2 (Sigma, 9.8 M)] was added to mid-log phase (OD_{600} about 0.4) cultures to a final concentration of 1 mM. Same volume of anoxic water was added to the control cell cultures. Biomass was harvested at 0, 30, 60, 120, 240 and 480 min after H_2O_2 treatment. All sampling occurred in the anaerobic chamber. In the same way, the biomass of deletion mutants of *D. vulgaris fur* and *perR* was produced and harvested at two time points – 0 min and 120 min after 1 mM H_2O_2 treatment.

Analysis of chemical species

Lactate and acetate were quantified with HPLC organic acid analysis column (HPX-87H ion exclusion column, Cat: 125-0140, Bio-Rad). Sulfate concentrations were measured using ion chromatography as described previously (Elshahed *et al*., 2001). Aqueous sulfide concentration was determined colorimetrically as described previously (Trüper and Schlegel, 1964). At each time point (0, 5, 15, 30, 60, 120, 240 and 480 min), 1 ml of cell culture was injected into 1 ml of anoxic zinc acetate to trap the sulfide and the mixture was kept at 4°C until measurement. Polysulfide was monitored by determining the absorbance of the cell culture at 410 nm (Johnston *et al*., 2009).

Isolation of total RNA, genomic DNA and fluorescence labelling

Isolation, purification and fluorescence labelling of total cellular RNA and genomic DNA (gDNA) were carried out as described previously (Zhou *et al*., 1996; Chhabra *et al*., 2006). Cy5-labelled cDNA and Cy3-labelled gDNA were dried and stored at –20°C before hybridization.

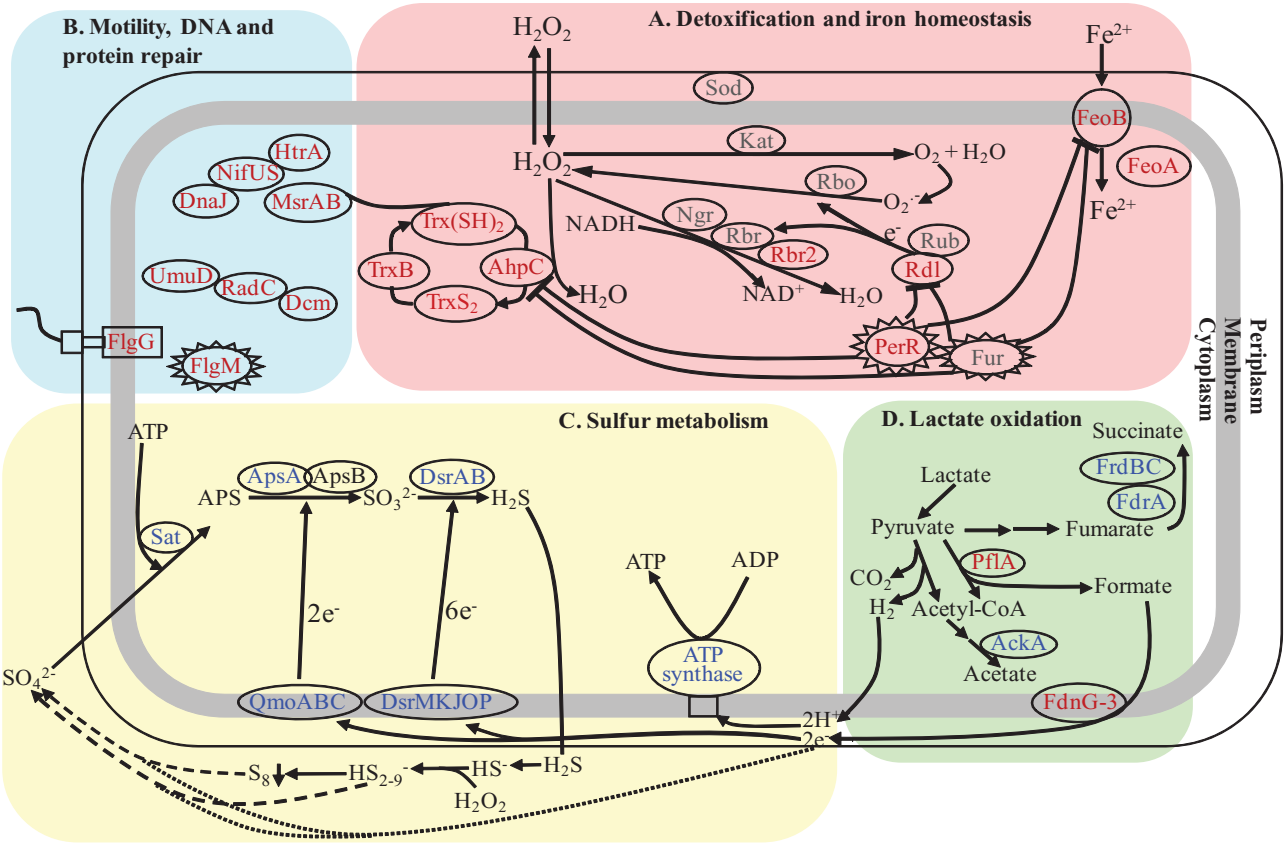

Fig. 5. A conceptual cellular model of *D. vulgaris* Hildenborough responses to H_2O_2. Dark red and dark blue font indicate increased or decreased gene expression, respectively; grey font represents genes without significant expression changes. The transcriptional regulators are marked with stars. The detoxification likely results from increased expression of the genes for Rdl/Rbr2 and the thioredoxin-dependent reduction pathway (A). Genes involved in iron influx (A), protein and DNA repair responses (B) were increased. PerR and Fur negatively co-regulated some of the PerR or Fur regulon genes (A). Genes for sulfate reduction (C) were decreased, while those genes encoding enzymes for the oxidation of lactate through pyruvate, acetyl-CoA and formate were increased (D).

Microarray hybridization and data analysis

The *D. vulgaris* whole-genome oligonucleotide (70mer) microarray covering 3482 of the 3531 protein-coding sequences of the *D. vulgaris* genome (He *et al.*, 2006) was used in this study. Array hybridizations and data analysis were performed as described previously (Chhabra *et al.*, 2006; Clark *et al.*, 2006; He *et al.*, 2006; 2010; Mukhopadhyay *et al.*, 2006). Briefly, the Cy3-labelled gDNA was used as control and co-hybridized with Cy5-labelled sample (TECAN HS4800, TECAN Group, Durham, NC). After 10 h of hybridization at 45°C with 50% (v/v) formamide in hybridization buffer, the microarray slides were dried and scanned for the fluorescent intensity (ScanArray Express microarray analysis system, Perkin Elmer, Boston, MA). The data processing was performed as described by Mukhopadhyay and colleagues (2006). The absolute Pearson correlation (uncentred) was used as the similarity metric and complete linkage hierarchical clustering was performed for cluster analysis. Microarray data for this study have been deposited in the NCBI GEO database under accession numbers GSE14345 and GSE14355.

Detrended correspondence analysis (DCA) was used to analyse the similarity of transcription profiling between different time points. Compared with the gene expression at time zero, the ORFs with more than twofold changes in gene expression ($|\log_2 R| > 1.0$, $|Z| > 1.5$) for at least one of the time points were kept for analysis. Five sets of data for control samples, C30–C480, and five data sets for treatment samples, T30–T480, were included in the analysis. The $\log_2 R$ value was transformed to fold change and value one was filled in for genes with no expression changes. DCA was run with PC-ORD (version 4, MjM Software Design).

Construction of gene coexpression network

The microarray data from all six time points were used for the construction of the gene coexpression network based on the random matrix theory approach (Luo *et al.*, 2007). First, all raw fluorescent intensities were normalized by the Cy3 signals generated from genomic DNA controls (Mukhopadhyay *et al.*, 2006). Second, for each spot, a ratio of Cy5/Cy3 was calculated and then logarithmic transformation of the ratio was performed. Third, a gene expression ratio of a treatment to a control was calculated by dividing a treatment Cy5/Cy3 ratio by a control Cy5/Cy3 ratio. All the data

sets at each time point were used for the gene coexpression network identification. The gene coexpression network presented here was generated with the cut-off of Pearson correlation coefficient of 0.95 between each pair of genes, which was determined by the network identification method (Luo *et al*., 2007). The submodule was separated by fast greedy modularity optimization (Clauset *et al*., 2004; Newman, 2006).

Proteomic analyses

Biomass harvested at 120 min after the addition of 1 mM H_2O_2 was used for proteomic analysis. Sample preparation, chromatography, mass spectrometry and data analysis for iTRAQ proteomics were performed as described previously (Redding *et al*., 2006; Mukhopadhyay *et al*., 2007). Protein $\log_2$ values with Z scores $\geq |2|$ were considered to be significantly changed. Each sample was run in duplicate to control the internal error. Reported protein ratios are an average of the internal and external technical replicates (four samples in total) with standard deviations.

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