AEM
Journals.ASM.org

PerR-Regulated Manganese Ion Uptake Contributes to Oxidative Stress Defense in an Oral Streptococcus

Xinhui Wang,[a,b] Huichun Tong,[a] Xiuzhu Dong[a]

State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China[a]; University of Chinese Academy of Sciences, Beijing, China[b]

Metal homeostasis plays a critical role in antioxidative stress. *Streptococcus oligofermentans*, an oral commensal facultative anaerobe lacking catalase activity, produces and tolerates abundant H_2O_2, whereas Dpr (an Fe^{2+}-chelating protein)-dependent H_2O_2 protection does not confer such high tolerance. Here, we report that inactivation of *perR*, a peroxide-responsive repressor that regulates zinc and iron homeostasis in Gram-positive bacteria, increased the survival of H_2O_2-pulsed *S. oligofermentans* 32-fold and elevated cellular manganese 4.5-fold. *perR* complementation recovered the wild-type phenotype. When grown in 0.1 to 0.25 mM $MnCl_2$, *S. oligofermentans* increased survival after H_2O_2 stress 2.5- to 23-fold, and even greater survival was found for the *perR* mutant, indicating that PerR is involved in Mn^{2+}-mediated H_2O_2 resistance in *S. oligofermentans*. Mutation of *mntA* could not be obtained in brain heart infusion (BHI) broth (containing ~0.4 μM Mn^{2+}) unless it was supplemented with ≥2.5 μM $MnCl_2$ and caused 82 to 95% reduction of the cellular Mn^{2+} level, while *mntABC* overexpression increased cellular Mn^{2+} 2.1- to 4.5-fold. Thus, MntABC was identified as a high-affinity Mn^{2+} transporter in *S. oligofermentans*. *mntA* mutation reduced the survival of H_2O_2-pulsed *S. oligofermentans* 5.7-fold, while *mntABC* overexpression enhanced H_2O_2-challenged survival 12-fold, indicating that MntABC-mediated Mn^{2+} uptake is pivotal to antioxidative stress in *S. oligofermentans*. *perR* mutation or H_2O_2 pulsing upregulated *mntABC*, while H_2O_2-induced upregulation diminished in the *perR* mutant. This suggests that *perR* represses *mntABC* expression but H_2O_2 can release the suppression. In conclusion, this work demonstrates that PerR regulates manganese homeostasis in *S. oligofermentans*, which is critical to H_2O_2 stress defenses and may be distributed across all oral streptococci lacking catalase.

Oxidative stress is encountered by all organisms on earth (1, 2), as deleterious reactive oxygen species (ROS), including superoxide ion (O_2^-), hydroxyl radical (HO·), and hydrogen peroxide (H_2O_2), are generated when molecular oxygen participates in electron transfer reactions or is auto-oxidized by reduced enzyme cofactors (2–4). Therefore, organisms have developed various mechanisms to protect against ROS, such as detoxifying enzymes (5, 6). Aerobes generally employ the superoxide dismutase (SOD)-catalase cascade to eliminate cellular ROS (4–7). Anaerobes use the superoxide reductase (SOR)-dependent oxide decomposition pathway to reduce O_2^- to H_2O (8). Intact H_2O_2 is not particularly deleterious to cells, but it can react with cellular Fe^{2+} via Fenton chemistry to form highly toxic HO· (2). Ferritin and miniferritin (Dps family) proteins chelate Fe^{2+} to prevent the deleterious Fenton reaction (9). Moreover, not only is manganese ion (Mn^{2+}) a cofactor of SOD (7, 10), but it does not undergo Fenton chemistry (11). Rather, it acts as an inorganic enzyme to dismute O_2^- by complexing with small molecules in bacterial cells (11–13). Hence, ROS damage to cells is intimately related to metal ion homeostasis (6).

Expression of antioxidant genes is controlled by redox-sensing transcriptional factors. A Fur family regulator, peroxide-responsive regulator (PerR), is the prototype of the redox-sensing transcriptional repressor found mainly in Gram-positive bacteria (6, 14, 15). Upon sensing cellular H_2O_2, PerR::Fe derepresses the transcription of antioxidant genes, including catalase, alkylhydroperoxide reductase (AhpC/AhpF), heme biosynthesis enzyme, and ferrous and zinc ion homeostatic genes (6). For example, the PerR protein of *Bacillus subtilis* regulates oxidant-detoxifying enzymes and metal ion homeostatic genes, namely, the Fe^{2+} binding protein MrgA (a Dps family homolog) and the Zn^{2+} transporter ZosA (6, 16, 17). PerR is also involved in Fe^{2+} and Zn^{2+} homeostasis and the oxidative-stress response in *Streptococcus pyogenes* (18, 19).

Streptococci are facultative anaerobes performing fermentative metabolism. During aerobic growth, they not only convert O_2^- to H_2O_2 via SOD, but produce H_2O_2 catalyzed by various oxidases (20–24). However, they do not possess the main H_2O_2-degrading enzyme catalase, and the identified peroxidases, such as AhpC/AhpF and glutathione peroxidase, also contribute little to H_2O_2 resistance in streptococci (20, 25). Thus, streptococci must employ unique mechanisms for H_2O_2 stress defense. Recent studies demonstrate that metal ion homeostasis is pivotal for H_2O_2 defense in streptococci (6). A *dps*-like nonspecific DNA-binding protein (Dpr), which chelates Fe^{2+}, and the Zn^{2+} pump protein PmtA contribute to oxidative-stress resistance in streptococci (18, 26, 27). In addition, inactivation of Mn^{2+} transporter genes in some streptococci diminishes H_2O_2 and O_2^- resistance (28–30). Both *dpr* and *pmtA* are reported to be regulated by PerR (18, 26); however, how Mn^{2+} uptake is responsive to oxidative stress remains unclear.

Received 6 January 2014 **Accepted** 28 January 2014
Published ahead of print 31 January 2014
Editor: H. L. Drake

Address correspondence to Huichun Tong, tonghuichun@im.ac.cn.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00064-14.

doi:10.1128/AEM.00064-14

Oral streptococci inhabit dental plaque biofilms, where dynamic interspecies interaction occurs and the outcome of competition determines oral health. The oral cavity is an environment under fluctuating oxidative stress. The ability of oral streptococci to defend against oxidants determines whether they can win the interspecies competition and therefore determines oral health. *Streptococcus oligofermentans* is isolated from noncariogenic dental plaque in humans (31). It generates copious H_2O_2 via multiple pathways (22–24) and inhibits the growth of the dental caries pathogen *Streptococcus mutans*. Compared to other streptococci, *S. oligofermentans* has the greatest H_2O_2 tolerance (32), making it a model organism in studying bacterial anti-oxidative-stress mechanisms. Although the Dpr protein plays a role in H_2O_2 resistance in *S. oligofermentans* (32), the *dpr* mutant still retains partial H_2O_2 tolerance, indicating that other mechanisms may confer oxidant resistance. Here, by using physiological, biochemical, and genetic approaches, we demonstrate that manganese is important for *S. oligofermentans* H_2O_2 tolerance and that, in the presence of H_2O_2, PerR releases repression of the manganese transporter *mntABC* and the Fe^{2+}-chelating protein *dpr* genes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All *Streptococcus* strains were routinely incubated in brain heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C as a static culture under low oxygen levels or anaerobically under 100% N_2. BHI agar (1.5% [wt/vol]) plates were used to select mutant strains and count colonies. Antibiotics (1 mg ml^{-1} kanamycin and 1 mg ml^{-1} spectinomycin) were added to the BHI medium when necessary. The *Escherichia coli* strains (33) were grown in Luria-Bertani (LB) medium at 37°C with shaking and were used for plasmid amplification. When needed, kanamycin (50 μg ml^{-1}) and spectinomycin (250 μg ml^{-1}) were added for recombinant selection.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics and description[a]	Reference or source
E. coli		
DH5α	*supE44 lacU169* (φ80d *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 luxS*	33
S. oligofermentans		
Wild type	AS 1.3089; Kan^{s} Sp^{s}	31
Δ*perR*	AS 1.3089 *perR*::Kan; Kan^{r}; AS 1.3089 with *perR* deletion	This study
Δ*mntA*	AS 1.3089 *mntA*::Kan; Kan^{r}; AS 1.3089 with *mntA* deletion	This study
perR-com	AS 1.3089 *perR*::Kan pDL278-*perR*; Kan^{r} Sp^{r} Δ*perR* with *perR* complement	This study
mntABC-exp	AS 1.3089 pDL278-*mntABC*; Sp^{r}; AS 1.3089 with *mntABC* ectopic expression	This study
P*mntABC*::*luc*	AS 1.3089 pFW5-P*mntABC*-*luc*; Sp^{r}; AS 1.3089 with P*mntABC*::*luc* fusion	This study
Δ*perR* P*mntABC*::*luc*	AS 1.3089 *perR*::Kan pFW5-P*mntABC*-*luc*; Kan^{r} Sp^{r} Δ*perR* with P*mntABC*::*luc* fusion	This study
Plasmids		
pALH124	Kan^{r}	38
pDL278	Sp^{r}	40
pFW5-*luc*	Sp^{r}	41
pDL278-*perR*	Sp^{r}; pDL278 with AS 1.3089 *perR* gene under its inherent promoter	This study
pDL278-*mntABC*	Sp^{r}; pDL278 with AS 1.3089 *mntABC* gene under its inherent promoter	This study
pFW5-P*mntABC*-*luc*	Sp^{r}; pFW5-*luc* with AS 1.3089 *mntABC* promoter	This study

[a] Kan^{r}, kanamycin resistant; Sp^{r}, spectinomycin resistant.

DNA manipulation. Standard recombinant DNA techniques were used for plasmid construction and PCR product ligation. All restriction and ligation enzymes were purchased from New England BioLabs (Beverly, MA). *S. oligofermentans* genomic DNA was extracted and purified using the method of Marmur (34) with slight modifications (35). All primers (see Table S1 in the supplemental material) were designed according to the complete genome sequence of *S. oligofermentans* (36) and synthesized by Sangon Company (Shanghai, China). PCR amplifications were performed with KOD-Plus-Neo (Toyobo, Japan), and purification of PCR products was carried out with a Qiagen (Valencia, CA) QIAquick PCR Purification Kit. DNA extracted from agarose gels was purified with a Tiangen (Beijing, China) Tiangel Midi Purification Kit, and plasmids were extracted and purified with a Tiangen (Beijing, China) Tianprep Mini Plasmid Kit.

Construction of mutant strains. Peroxide-responsive repressor (*perR*) and metal ABC transporter substrate-binding lipoprotein (*mntA*) gene deletion mutants were constructed by the PCR ligation method (37). Briefly, two ~550-bp fragments of the upstream and downstream sequences of the *perR* and *mntA* genes, respectively, were amplified by PCR using *S. oligofermentans* genomic DNA as a template. The purified PCR products were digested with BamHI. The nonpolar kanamycin resistance gene cassette was cut from plasmid pALH124 (38) by digestion with BamHI. All three fragments were purified and mixed at a 1:1:1 molar ratio. A fused fragment was formed by T4 DNA ligase treatment and transformed into the *S. oligofermentans* wild-type strain using a published method (39). Transformants were selected on BHI (for the *perR* mutant) or BHI supplemented with 0.1 mM $MnCl_2$ (for the *mntA* mutant) agar plates containing 1 mg ml^{-1} kanamycin. The corresponding gene deletion was confirmed with PCR and sequencing.

perR-complemented and *mntABC* overexpression strains were constructed as described below. The entire gene fragments, including the coding region and the promoter sequence of *perR* and *mntABC*, were amplified from chromosomal DNA of *S. oligofermentans* by PCR with the primers listed in Table S1 in the supplemental material. Then, 762- and 2,660-bp PCR products were purified and double digested with EcoRI and SalI. After gel purification, the PCR products were inserted into *E. coli*-*Streptococcus* shuttle plasmid pDL278 (40), which was cut with the same enzymes. Positive transformants were selected on LB agar plates containing 250 μg ml^{-1} spectinomycin and identified by PCR and sequencing. The recombinant plasmids pDL278-*perR* and pDL278-*mntABC* were then transformed into the *S. oligofermentans perR* mutant and wild-type strains, respectively. Transformants were selected on BHI agar plates containing 1 mg ml^{-1} kanamycin and 1 mg ml^{-1} spectinomycin or 1 mg ml^{-1} spectinomycin and identified by PCR and sequencing.

Construction of luciferase reporter strains. For construction of the P*mntABC*-*luc* reporter strain, DNA fragments corresponding to a 430-bp sequence upstream of the start codon of the *mntABC* gene were amplified from chromosomal DNA using PCR with primers listed in Table S1 in the supplemental material. The purified PCR product was double digested with BamHI and NheI, gel purified, and ligated to plasmid pFW5-*luc* (41), which was digested with the same enzyme. The ligation mixtures were transformed into *E. coli* DH5α. Positive transformants were identified by

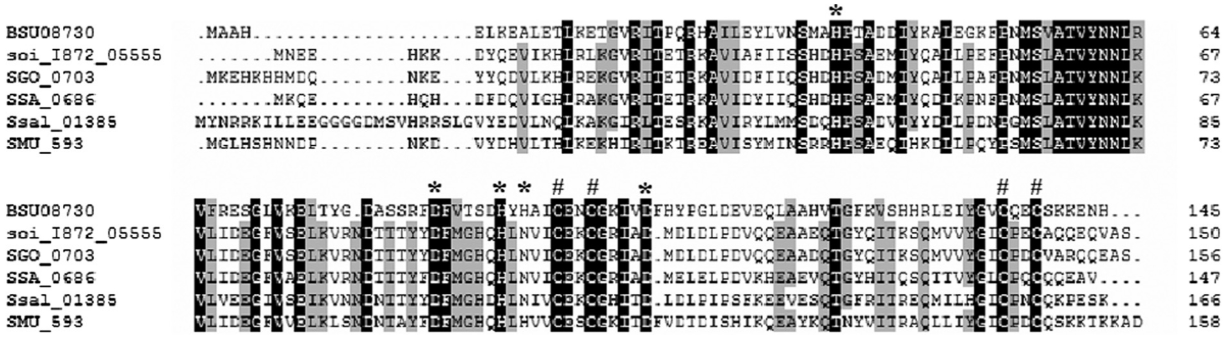

FIG 1 Sequence alignment of PerR proteins from *S. oligofermentans*, *B. subtilis*, and other representative species of oral streptococci. Amino acid sequences of PerR were retrieved from the protein database of NCBI. *, conserved amino acid residue essential for manganese or ferric ion binding in *B. subtilis*; #, conserved cysteine residue for zinc ion binding. BSU08730, *B. subtilis*; soi_I872_05555, *S. oligofermentans*; SGO_0703, *S. gordonii*; SSA_0686, *S. sanguinis*; Ssal_01385, *S. salivarius*; SMU_593, *S. mutans*. Black shading indicates the homology level is 100%; gray shading indicates the homology level is ≥75%.

PCR and sequencing. The recombinant plasmid pFW5-P*mntABC-luc* was then transformed into the *S. oligofermentans* wild-type strain and the *perR* mutant, and positive transformants were identified by PCR, sequencing, and luciferase activity.

Assay of hydrogen peroxide sensitivity. *S. oligofermentans* strains were grown in BHI broth under static or anaerobic conditions at 37°C overnight. The overnight cultures were diluted 1:100 into fresh BHI broth and incubated under static or anaerobic conditions. When the optical density at 600 nm (OD_{600}) reached 0.6 to 0.7, cells (1 ml) were removed and harvested by centrifugation. After two washings with phosphate-buffered saline (PBS), the cells were resuspended in 1 ml of fresh BHI broth. Cell aliquots (200 μl) were distributed into 1.5-ml Eppendorf tubes. One aliquot was challenged with 20 mM H_2O_2, and an aliquot without H_2O_2 treatment was used as a control. After incubation at 37°C for 10 min, the cells were collected, washed twice with PBS buffer, and resuspended in 200 μl BHI broth. Cell chains were separated by sonication for 30 s with a UP 200S sonicator (Germany). The samples were then serially 10-fold diluted. Appropriate dilutions were plated on BHI agar, and CFU were counted after 24 h of incubation in a candle jar at 37°C. Survival (percent) was calculated as the ratio of CFU in the H_2O_2-challenged sample to those in controls. Experiments were executed in triplicate, and each was repeated at least three times independently.

Metal content measurement. Concentrations of iron, manganese, and zinc in static and anaerobic cultures of various *S. oligofermentans* strains were measured using inductively coupled plasma mass spectrometry (ICP-MS). Overnight BHI cultures of tested strains were diluted 1:50 in fresh BHI broth and incubated at 37°C under static or anaerobic conditions. Mid-log-phase cells were harvested by centrifugation at 13,400 × *g* for 10 min. The cell pellets were washed twice in PBS with 1 mM EDTA and once in PBS without EDTA and then resuspended in 1 ml of PBS. One hundred microliters of suspension was used to measure the protein concentration with a bicinchoninic acid (BCA) protein analysis kit according to the manufacturer's recommendations. The remaining 900 μl of suspension was collected by centrifugation at 13,400 × *g* for 10 min. The pelleted bacterial cells were resuspended in 500 μl of nitric acid (ultrapure). After overnight incubation at room temperature, the cell suspension was brought to 1.5 ml with deionized distilled water. Then, metal ions were analyzed by ICP-MS (DRCII; PerkinElmer) at Beijing University Health Science Center. Beryllium, indium, and uranium standard solutions (PerkinElmer; National Institute of Standards and Technology [NIST] certified) were used to calibrate the ICP-MS. Experiments were conducted in triplicate, and each was repeated at least three times. The metal content was expressed in nmol per mg protein.

Luciferase activity measurement. Twenty-five microliters of 1 mM D-luciferin (Sigma-Aldrich, St. Louis, MO) solution (suspended in 1 mM citrate buffer, pH 6.0) was added to 100-μl samples, and luciferase activity assays were performed as previously described (39) using a TD 20/20 luminometer (Turner Biosystems, Sunnyvale, CA). The sample optical density (OD_{600}) was measured with a 2100 visible spectrophotometer (Unico, Shanghai, China) and used to normalize the luciferase activity. All measurements were performed in triplicate, and all experiments were repeated at least three times.

Hydrogen peroxide measurement. H_2O_2 in liquid culture was measured as described previously (24). Briefly, 650 μl of culture supernatant was added to 600 μl of solution containing 2.5 mM 4-amino-antipyrine (4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one; Sigma) and 0.17 M phenol. The reaction proceeded for 4 min at room temperature; horseradish peroxidase (Sigma) was then added to a final concentration of 50 mU/ml in 0.2 M potassium phosphate buffer (pH 7.2). After 4 min of incubation at room temperature, the OD_{510} was measured with a Unico (Shanghai, China) 2100 visible-light spectrophotometer. A standard curve was generated with known concentrations of chemical H_2O_2.

Quantitative PCR. Total RNA was extracted from mid-log-phase ($OD_{600} = 0.4$ to 0.5) cells using TRIzol reagent (Invitrogen, Carlsbad, CA) as recommended by the suppliers. After quality confirmation with a 1% agarose gel, the RNA was treated with RNase-free DNase (Promega, Madison, WI) and analyzed by PCR for possible chromosomal DNA contamination. cDNA was generated from 2 μg total RNA with random primers using Moloney murine leukemia virus reverse transcriptase (Promega) according to the supplier's instructions and used for quantitative-PCR (qPCR) amplification with the corresponding primers (see Table S1 in the supplemental material). Amplifications were performed with a Mastercycler ep realplex^2 (Eppendorf, Germany). To estimate copy numbers for a given mRNA, a standard curve of the tested gene was generated by quantitative PCR using 10-fold serially diluted PCR product as the template. The 16S rRNA gene was used as the biomass reference. The copy number of each gene was normalized to the number of 16S rRNA copies. The number of copies of the transcript of each gene per 1,000 16S rRNA copies is shown.

RESULTS

PerR functions as a peroxide-responsive repressor in *S. oligofermentans*. To identify *perR* homologs in *S. oligofermentans*, the *perR* gene from *B. subtilis* was used as a probe to query the complete genome. An open reading frame (I872_05555) annotated as "ferric transport regulator protein" with 38% amino acid identity was hit, and it was noted as PerR. Furthermore, PerR orthologs were found in almost all oral streptococci. PerR from *S. oligofermentans* had the highest identity (97%) with that of *Streptococcus cristatus* and had 58 to 87% identity with other oral streptococci. Amino acid sequence alignment of PerR proteins from representative oral streptococci and *B. subtilis* (Fig. 1) revealed that the essential amino acid residues for metal ion binding (H37, H91,

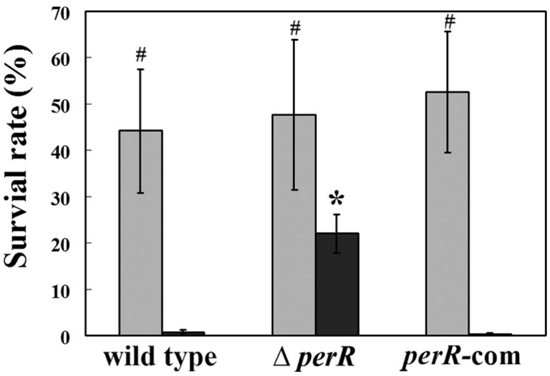

FIG 2 Survival of *S. oligofermentans* wild-type, *perR* mutant, and *perR*-complemented strains after H_2O_2 pulsing. The strains were incubated statically (gray bars) and anaerobically (black bars), and mid-log-phase cultures were collected and subjected to 20 mM H_2O_2 pulsing for 10 min. Viable cells were counted based on CFU on a BHI agar plate after serial dilution. Survival was calculated as the ratio of CFU in H_2O_2-pulsed samples to those without H_2O_2 treatment. The data are expressed as means ± standard deviations of three independent experiments. *, data are statistically significant in comparison to values of anaerobically cultured wild-type and *perR*-com strains as verified by Student's *t* test ($P < 0.01$); #, data are statistically significant compared to the values of the respective strains cultured anaerobically, as verified by Student's *t* test ($P < 0.05$).

D85, and D104 for manganese or ferric ions; C96, C99, C136, and C139 for zinc ions) in *B. subtilis* (15) were conserved in all oral streptococcal PerR proteins examined except H93, where an asparagine (N) is found in four streptococcal species, namely, *S. oligofermentans*, *Streptococcus gordonii*, *Streptococcus sanguinis*, and *Streptococcus salivarius*. Sequence conservation suggests that the oral streptococcal PerR proteins function similarly to that of *B. subtilis*.

To identify the function of PerR in *S. oligofermentans*, a *perR* deletion mutant was constructed and verified by PCR and sequencing. Both the *perR* mutant and the wild-type strain were cultured under anaerobic condition to exclude endogenous H_2O_2 production. Mid-log-phase cultures (OD_{600} = 0.6 to 0.7) were collected and subjected to H_2O_2 pulsing (20 mM) for 10 min. Then, the survival rate was quantified as described in Materials and Methods. As shown in Fig. 2, *perR* mutant survival (22.05%) was ~32-fold higher than that of the wild-type strain (0.67%). Furthermore, a *perR*-complemented strain, *perR*-com, showed significantly reduced survival (0.34%) compared with the *perR* mutant and similar to that of the wild-type strain. Thus, PerR has been demonstrated to be a peroxide-responsive repressor in *S. oligofermentans*.

***perR* deletion causes increased cellular manganese.** Because PerR regulates metal ion homeostasis in *B. subtilis* (6), its involvement in cellular metal ion balance in *S. oligofermentans* was examined. The wild-type, *perR* mutant, and *perR*-com strains were cultured anaerobically in BHI broth, and mid-log-phase cells (OD_{600} = 0.6 to 0.7) were collected to measure cellular metal by ICP-MS. Blank BHI broth was also included to determine the baseline metal content. As shown in Table 2, Mn^{2+} was 4.5-fold higher in the *perR* mutant, while its levels were similar in *perR*-com and the wild-type strain. However, iron and zinc were at similar levels in the three strains. This indicates that PerR specifically downregulates cellular manganese, likely by depressing the expression of Mn^{2+} transporters.

Mn^{2+} is important for H_2O_2 tolerance in *S. oligofermentans*. To elucidate the role of cellular Mn^{2+} in H_2O_2 tolerance, the *S. oligofermentans* wild-type strain was cultured anaerobically in BHI broth supplemented with $MnCl_2$ at different concentrations. Mid-log-phase cultures (OD_{600} = 0.6 to 0.7) were collected and subjected to H_2O_2 pulsing as described above. The survival of *S. oligofermentans* increased 2.5- and 23-fold upon addition of 0.1 and 0.25 mM $MnCl_2$, respectively (Fig. 3). In addition, 10.7-fold more manganese was detected in cells grown with 0.1 mM $MnCl_2$ than in cultures without $MnCl_2$ supplementation (Table 2). These data suggest that Mn^{2+} plays a significant role in the H_2O_2 tolerance of *S. oligofermentans*. Furthermore, addition of 0.25 mM $MnCl_2$ enhanced the shaking growth of *S. oligofermentans* by ~35%, similar to that with catalase addition (200 U/ml); this confirms that Mn^{2+} can be an H_2O_2 scavenger in *S. oligofermentans*.

***perR* inactivation increases Mn^{2+}-assisted H_2O_2 survival of *S. oligofermentans*.** To find the possible linkage between PerR and

TABLE 2 Cytoplasmic metal ion concentrations in various *S. oligofermentans* strains cultured statically or anaerobically

	Metal ion concn[a]					
	Static culture			Anaerobic culture		
Strain	Mn	Fe	Zn	Mn	Fe	Zn
Wild type	4.13 ± 0.96[d]	7.77 ± 1.42	2.37 ± 0.49	0.64 ± 0.15	6.33 ± 1.20	7.78 ± 1.50
perR mutant	4.59 ± 0.19	6.97 ± 0.56	2.12 ± 0.01	2.88 ± 0.29[d]	7.17 ± 1.70	6.21 ± 0.70
perR-com	ND	ND	ND	0.58 ± 0.06	6.96 ± 0.27	7.30 ± 1.40
mntA mutant[b]	0.44 ± 0.04[e]	ND	ND	0.33 ± 0.07[e]	ND	ND
mntABC-exp	8.82 ± 0.21[f]	ND	ND	2.86 ± 0.81[d]	ND	ND
Wild type-2.5Mn[b]	9.02 ± 0.34	ND	ND	1.86 ± 0.33	ND	ND
Wild type-Mn[c]	ND	ND	ND	6.85 ± 0.09[d]	ND	ND
perR mutant-Mn[c]	ND	ND	ND	9.80 ± 0.67[g]	ND	ND
mntABC-exp-Mn[c]	ND	ND	ND	15.46 ± 3.6[g]	ND	ND

[a] Data are the means ± standard deviations of three independent cultures; metal content is expressed as nmol/mg protein. ND, not determined.

[b] Strain grew in BHI broth with the addition of 2.5 μM $MnCl_2$.

[c] Strain grew in BHI broth with 0.1 mM $MnCl_2$ added.

[d] Value with significant difference from the anaerobically incubated wild-type strain ($P < 0.05$; Student's *t* test).

[e] Value with significant difference from the wild-type strain growing in 2.5 μM $MnCl_2$ (wild type-2.5Mn) ($P < 0.01$; Student's *t* test).

[f] Value with significant difference from the statically incubated wild-type strain ($P < 0.05$; Student's *t* test).

[g] Value with significant difference from the anaerobically incubated wild-type strain growing in 0.1 mM Mn^{2+} (wild type-Mn) ($P < 0.01$; Student's *t* test).

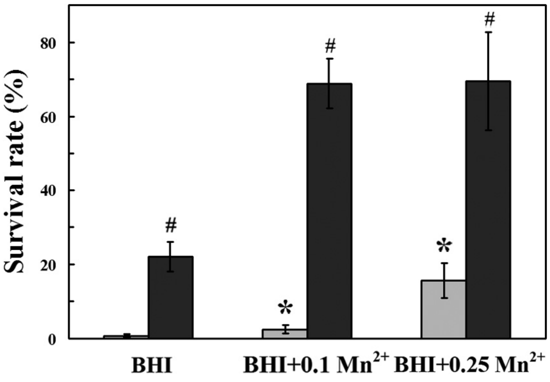

FIG 3 Impact of manganese ion on the H_2O_2-stressed survival of *S. oligofermentans* wild type and *perR* mutant. *S. oligofermentans* strains were anaerobically cultured in BHI broth with or without 0.1 and 0.25 mM $MnCl_2$. After H_2O_2 pulsing, viable cells were counted, and survival was quantified as described for Fig. 2. The data are expressed as means ± standard deviations of three independent experiments. Gray bars, wild-type strain; black bars, *perR* mutant. *, data are statistically significant in comparison to the values of the wild-type strain growing in BHI broth, as verified by Student's *t* test ($P < 0.05$); #, data are statistically significant compared to the values of the wild-type strain growing in the same medium, as verified by Student's *t* test ($P < 0.05$).

Mn^{2+} in H_2O_2 resistance, an *S. oligofermentans perR* mutant was cultured anaerobically in BHI broth supplemented with 0.1 and 0.25 mM $MnCl_2$ and then tested for H_2O_2 survival as described above. The results showed that *perR* inactivation increased H_2O_2 survival by 66% and 54% upon addition of 0.1 and 0.25 mM Mn^{2+}, respectively, which was more than the increase in H_2O_2 survival (21%) in BHI broth (Fig. 3). Accordingly, addition of 0.1 mM $MnCl_2$ increased cellular manganese more in the *perR* mutant than in the wild-type strain (Table 2). This indicates that PerR regulates manganese ion uptake, which contributes to H_2O_2 resistance of *S. oligofermentans*.

***mntABC* encodes a high-affinity Mn^{2+} transporter in *S. oligofermentans*.** To identify genes responsible for transporting Mn^{2+} in *S. oligofermentans*, two manganese transporter genes, *scaCBA* and *mntH*, which, respectively, belong to the manganese ABC transporter and the eukaryotic Nramp (*n*atural *r*esistance *a*ssociated *m*acrophage *p*rotein) families (42, 43), from *S. gordonii* were used as probes to query the complete genome of *S. oligofermentans*. Two targets were found: I872_09645-09655, a gene cluster encoding a putative manganese ABC transporter complex, designated *mntABC* (I872_09645, metal ABC transporter substrate-binding lipoprotein; I872_09650, ABC transporter membrane-spanning permease–manganese transport; I872_09655, ATP binding protein), and I872_08215, encoding a putative manganese transporter, Nramp, named *mntH*. The amino acid sequences of MntA, MntB, MntC, and MntH of *S. oligofermentans* shared 93%, 99%, 89%, and 89% identity with ScaA, ScaB, ScaC, and MntH from *S. gordonii*, respectively.

To measure the activity of the two manganese transport-related genes in *S. oligofermentans*, gene expression was measured in wild-type cells cultured statically or anaerobically in BHI broth. qPCR determined that in mid-log-phase cells (OD_{600} = 0.6 to 0.7), *mntA* expression was higher in all the cultures than *mntH* expression (Table 3), indicating that *mntABC* may be the main manganese ion transporter in *S. oligofermentans*. Both *mntA* and *mntH* increased (3.3- and 4.3-fold) their expression in the static culture, suggesting that the oxidative state induces the expression of the Mn^{2+} transporter genes *mntABC* and *mntH*.

Inactivation of *mntA* could be achieved in BHI culture of *S. oligofermentans* only when supplemented with an additional ≥2.5 μM Mn^{2+}. Since only 418 nM Mn^{2+} was detected in BHI broth, MntABC is predicted to be essential for *S. oligofermentans* in a low-Mn^{2+} environment. To identify the function of MntABC in the uptake of Mn^{2+}, the wild-type strain and the *mntA* mutant were grown in BHI broth supplemented with 2.5 μM Mn^{2+}, and mid-log-phase cells (OD_{600} = 0.6 to 0.7) were collected to measure cellular metal by ICP-MS. Compared to the wild-type strain, Mn^{2+} decreased by 95% and 82% in the statically and anaerobically cultured *mntA* mutant (Table 2), respectively, confirming that MntABC functions as a high-affinity Mn^{2+} transporter in *S. oligofermentans*.

Furthermore, 2.1- and 4.5-fold-greater manganese levels were found in a strain in which *mntABC* is ectopically expressed (*mntABC*-exp) when it was statically and anaerobically cultured, respectively (Table 2), verifying the metal-transporting function of MntABC in *S. oligofermentans*.

MntABC inactivation reduces the oxidative-stress tolerance of *S. oligofermentans*. To investigate the role of the Mn^{2+} transporter MntABC in H_2O_2 tolerance in *S. oligofermentans*, both the wild-type strain and the *mntA* mutant were cultured statically in BHI broth supplemented with 2.5 μM Mn^{2+}. Mid-log-phase cells (OD_{600} = 0.6 to 0.7) were collected and subjected to H_2O_2 pulsing as described above. Compared to the wild-type strain, *mntA* mu-

TABLE 3 Transcript levels of *mntA*, *mntH*, and *dpr* in the *S. oligofermentans* wild-type, *perR* mutant, and *perR*-complemented strains cultured statically or anaerobically

	Transcript level[a]					
	Wild type		*perR* mutant		*perR*-com	
Gene	Static	Anaerobic	Static	Anaerobic	Static	Anaerobic
mntA	14.86 ± 3.02	4.49 ± 1.37[b]	11.88 ± 1.69	25.86 ± 8.13	ND	4.27 ± 0.74[b]
mntH	0.26 ± 0.07	0.06 ± 0.01[c]	0.30 ± 0.02	0.13 ± 0.06	ND	ND
dpr	6.17 ± 1.49	0.58 ± 0.15[b]	15.90 ± 2.81	23.30 ± 8.81	ND	0.06 ± 0.01[b,d]

[a] Data are shown as means ± standard deviations of three independent experiments. Gene expression is shown as the means ± standard deviations of copy number/0.001 16S rRNA gene copy. ND, not determined.

[b] Data are statistically significant compared to the corresponding genes in a statically cultured wild-type strain and a statically or anaerobically cultured *perR* mutant; Student's *t* test ($P < 0.05$).

[c] Data are statistically significant compared to the corresponding genes in a statically cultured wild-type strain; Student's *t* test ($P < 0.05$).

[d] Data are statistically significant compared to the corresponding genes in an anaerobically cultured wild-type strain; Student's *t* test ($P < 0.05$).

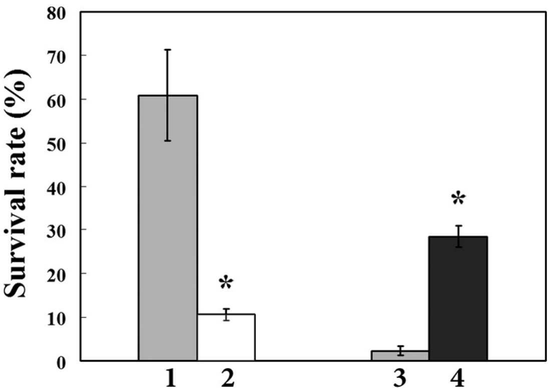

FIG 4 Role of MntABC-mediated manganese uptake in H_2O_2 stress tolerance in *S. oligofermentans*. The wild-type strain (1) and *mntA* mutant (2) were grown statically in BHI broth with addition of 2.5 μM $MnCl_2$, and the wild-type strain (3) and *mntABC*-exp (4) were grown anaerobically in BHI broth with supplementation of 0.1 mM $MnCl_2$. After H_2O_2 pulsing for 10 min, viable cells were counted, and survivors were quantified as described for Fig. 2. The data are expressed as means ± standard deviations of three independent experiments. *, data are statistically significant in comparison to the wild-type strain incubated under the same conditions, as verified by Student's *t* test ($P < 0.01$).

tation reduced H_2O_2 survival 5.7-fold (Fig. 4), indicating that MntABC-mediated Mn^{2+} uptake plays a role in protecting *S. oligofermentans* from H_2O_2 attack.

To further verify the role of MntABC in H_2O_2 resistance, the *mntABC*-exp strain was grown in BHI broth supplemented with 0.1 mM $MnCl_2$ and anaerobically incubated. Cells at mid-log phase were treated with H_2O_2 (20 mM) for 10 min. About 12-fold-higher survival was determined for the *mntABC*-exp strain than for the wild-type strain, and cellular manganese was significantly increased in the *mntABC*-exp strain, as well (Table 2).

Inactivation of *perR* upregulates *mntABC* and *dpr* expression. Increased Mn^{2+} was found in the *perR* mutant, so correlations among *perR*, *mntABC*, and *mntH* were tested in anaerobically cultured wild-type, *perR* mutant, and *perR*-com strains. Using qPCR, 5.8-fold upregulation of *mntA* was detected in the *perR* mutant, while similar expression levels were found in *perR*-com and the wild-type strain. Similarly, *dpr*, encoding an Fe^{2+}-chelating protein, was upregulated 40-fold in the *perR* mutant, whereas *perR* complementation greatly reduced *dpr* expression to as much as ~10-fold lower than that in the wild-type strain (Table 3). This shows that PerR represses expression of both *mntABC* and *dpr*. However, no significant change in *mntH* expression was detected in the *perR* mutant (Table 3), indicating that *mntH* is not subjected to PerR regulation.

Furthermore, a putative PerR-binding sequence "Per box" (AATTAGAAGCATTATAATT) was found in the promoter region of *dpr* but not in that of *mntABC*. Electrophoretic mobility shift assay (EMSA) detected PerR's binding only to the *dpr* promoter (see Fig. S1 in the supplemental material) but not to *mntABC* (data not shown). This suggests direct regulation of *dpr* by PerR but indirect regulation of *mntABC* in *S. oligofermentans*.

H_2O_2 releases PerR repression of *mntABC*. Since an effect of *perR* mutation on cellular manganese, H_2O_2 survival, and *mntABC* expression was not observed in statically cultured *S. oligofermentans* (Tables 2 and 3 and Fig. 2) and abundant H_2O_2 was produced only in statically cultured cells (see Fig. S2 in the supplemental material) while none was detected in anaerobic cultures under the detection limit of 1.90 μM H_2O_2, H_2O_2 may be a substance that interferes with PerR regulation of *mntABC*. To confirm this, an *mntABC* luciferase reporter strain was constructed by introducing the integrative plasmid pFW5-P*mntABC-luc* into the *S. oligofermentans* wild-type strain and the *perR* mutant. The constructed *S. oligofermentans* P*mntABC*::*luc* and Δ*perR* P*mntABC*::*luc* strains were cultured anaerobically, and 20 μM H_2O_2, a concentration similar to that produced in statically cultured cells (see Fig. S2 in the supplemental material), was added to the early log-phase cells (OD_{600}, ~0.2). After cultivation for 20, 40, 60, and 80 min, the luciferase activity and OD_{600} were measured. *mntABC* expression was significantly upregulated in H_2O_2-pulsed cultures

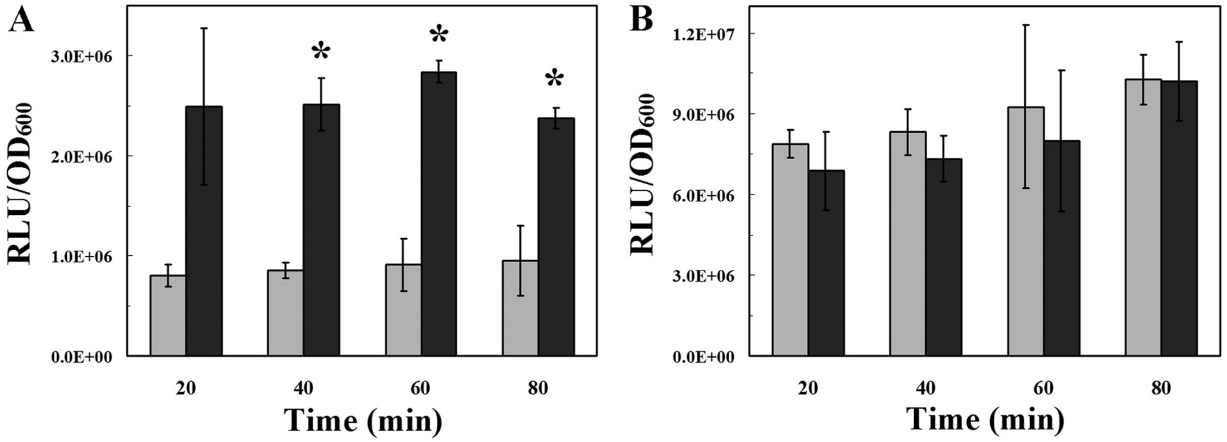

FIG 5 H_2O_2 induced *mntABC* expression in the wild-type strain (A) and *perR* mutant (B) of *S. oligofermentans*. Overnight BHI cultures of *S. oligofermentans*::P*mntABC-luc* and *S. oligofermentans* Δ*perR*::P*mntABC-luc* were diluted 1:30 in fresh BHI broth and incubated anaerobically. Then, 20 μM H_2O_2 was added to early-log-phase cells (OD_{600} = ~0.2). Cells were collected at 20, 40, 60, and 80 min. The OD_{600} and luciferase activity (relative light units [RLU]) were measured as described in Materials and Methods. *mntABC* expression is expressed as RLU/OD_{600} unit. Gray bars, no H_2O_2 addition; black bars, 20 μM H_2O_2 addition. The experiments were repeated 3 times, and the data are expressed as means ± standard deviations of three reads of each independent experiment. *, data are statistically significant in comparison to the values of the wild-type strain growing in BHI broth without H_2O_2 addition at the respective time points, as verified by Student's *t* test ($P < 0.01$).

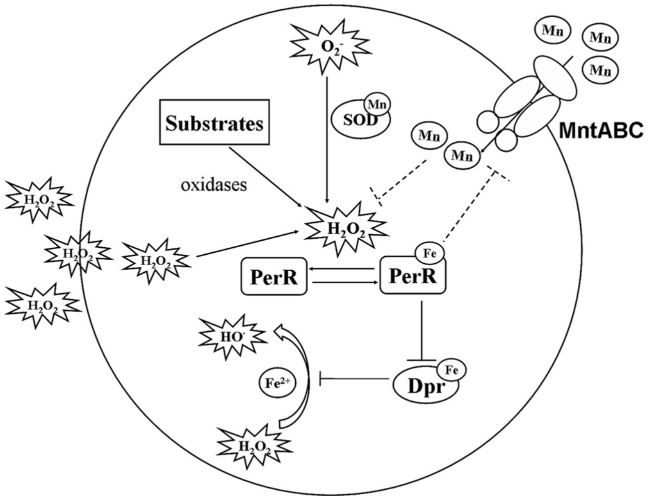

FIG 6 Diagram depicting the antioxidative mechanisms in *S. oligofermentans*. Endogenous H_2O_2 produced during aerobic growth or exogenous H_2O_2 releases PerR's inhibition of the Mn^{2+} transporter gene *mntABC* and the Fe^{2+}-chelating protein gene *dpr*. Mn^{2+} might protect *S. oligofermentans* from O_2^- by activating SOD and from H_2O_2 by unknown mechanisms. Dpr prevents Fenton reactions by chelating Fe^{2+}, avoiding the production of toxic HO·.

(Fig. 5A); however, no significant difference in *mntABC* expression was found between the H_2O_2-pulsed and nonpulsed *perR* mutant cells (Fig. 5B). This indicates that H_2O_2-induced *mntABC* expression depends on the presence of *perR*. Similar to the qPCR results (Table 3), 9- to 10-fold-elevated *mntABC* expression was detected in the non-H_2O_2-pulsed *perR* mutant compared to wild-type cells (Fig. 5), supporting the conclusion that PerR negatively regulates transcription of *mntABC*.

DISCUSSION

The oral commensal *S. oligofermentans* produces ample H_2O_2 (4.6 mM) via multiple pathways. In particular, lactate oxidase activity enables it to inhibit the caries pathogen *S. mutans* (22–24). *S. oligofermentans* is also resistant to higher H_2O_2 levels (5.5 mM) than other bacterial species, although it has no catalase (22). However, the activity of the ferric iron-chelating protein Dpr is insufficient for such higher H_2O_2 tolerance, as demonstrated by our previous work (32). Here, we report that *S. oligofermentans* contains extraordinarily high concentrations of manganese, which acts synergistically with Dpr to enable greater H_2O_2 tolerance than in other bacteria. Figure 6 depicts a model of anti-oxidative stress in *S. oligofermentans*. In the presence of endogenous or exogenous H_2O_2, PerR derepressed the expression of the manganese transporter *mntABC* and ferric iron-chelating protein *dpr* genes. MntABC facilitates Mn^{2+} internalization. Mn^{2+} can act as a cofactor of SOD or a catalase-like inorganic catalyzer; it can also substitute for Fe^{2+} in protein active sites to reduce protein oxidation. Dpr protein, by trapping Fenton reaction-stimulating Fe^{2+}, prevents inert H_2O_2 from converting to highly reactive HO·.

Fe and Mn are essential trace metal elements for bacteria, although the cellular Fe level is usually higher than that of Mn, e.g., the Mn/Fe ratio of 0.0072 (0.0197 nmol Mn and 2.72 nmol Fe/mg protein) found in *E. coli* (44). It has been reported that the poor Fenton reagent metal ions Mn^{2+} and Zn^{2+} prevent H_2O_2-derived HO· production (11), and a higher Mn/Fe ratio (0.24) contributes to oxidative-stress and radiation resistance in *Deinococcus radiodurans* (44). Moreover, the SOD-lacking *Lactobacillus plantarum* accumulates a high concentration of cellular Mn (20 to 35 mM) to defend against oxidative stress (10, 45). In this work, a high Mn/Fe ratio (0.48) was also detected in statically cultured *S. oligofermentans* (Table 2), but a high zinc level was not observed. Furthermore, a higher cellular Mn^{2+} level was found in *S. oligofermentans* (4.13 ± 0.96 nmol/mg protein) than in other streptococci (1.12 ± 0.23 and 1.57 ± 0.14 nmol/mg protein in *Streptococcus pneumoniae* and *S. mutans*) in the parallel measurements in this study. Supplementation experiments also confirmed the role of Mn^{2+} in the protection of *S. oligofermentans* against H_2O_2 (Fig. 3). How Mn^{2+} offers protection against H_2O_2 has been hypothesized. Stadtman et al. predicted a catalase-like activity of the Mn^{2+}-bicarbonate complex in scavenging H_2O_2 (46), a finding consistent with the observation in *S. oligofermentans* that, although high H_2O_2 concentrations are found in the culture media, cellular H_2O_2 is undetectable. The H_2O_2-scavenging activity of Mn^{2+} must be confirmed, as only barely detectable H_2O_2 degradation was found by mixed incubation of $MnCl_2$ with cell extracts of *S. oligofermentans* (data not shown).

Mn^{2+} is thought to reduce chemical oxidation of many proteins by substitution for the active Fenton-reactive Fe^{2+} at protein active sites (47). The growth of streptococci is reported to require iron (48), and high cellular iron levels (6 to 8 nmol/mg protein) are found in *S. oligofermentans*, as well, when cultured in BHI broth (Table 2). This suggests that Mn^{2+} substitution for Fe^{2+} can occur in the oral streptococcus under the current experimental conditions. Released Fe^{2+} might then be chelated by Dpr protein, avoiding Fenton chemistry.

Among the three types of manganese transporters, the manganese ABC transporter is key for bacterial uptake of Mn^{2+} (49). Manganese ABC transporter mutation affects biofilm formation and oxidative-stress resistance of *S. gordonii* and *S. mutans* (28, 49, 50), as well as the virulence of a number of bacterial pathogens, including *S. pneumoniae*, *S. pyogenes*, and *Yersinia pestis* (11, 29, 49). The *mntA* mutant can be obtained only in BHI medium (which contains trace amounts of Mn^{2+} [$\sim$0.4 μM]) supplemented with ≥2.5 μM Mn^{2+}. This suggests that MntABC acts as a main Mn^{2+} transporter at low Mn^{2+} levels in *S. oligofermentans*, while other manganese transporters, such as MntH, might function at relatively high extracellular Mn^{2+} levels. MntABC protects *S. oligofermentans*, not only from H_2O_2 pulsing (Fig. 4), but also from attack by paraquat, an O_2^- producer (data not shown), suggesting that MntABC-transported Mn^{2+} plays an important role in anti-oxidative stress in *S. oligofermentans*.

In response to H_2O_2 stress, *E. coli* employs OxyR, a transcriptional activator, to promote expression of the manganese transporter *mntH* and a Fur family regulator, which controls iron transporter genes (47, 51, 52). Gram-positive bacteria use PerR to repress zinc and iron transporters. In the absence of H_2O_2, PerR::Fe binds to the conservative DNA sequence (Per box) in the promoter regions, while H_2O_2 can oxidize and inactivate PerR::Fe protein, leading to derepression of zinc- and iron-transporting genes (6, 15, 53, 54). PerR in *S. oligofermentans* has been verified to be a peroxide-responsive repressor, as well (Fig. 2). However, unlike those of *B. subtilis* and *S. pyogenes* (6, 18), the PerR protein of *S. oligofermentans* represses the manganese transporter gene *mntABC*, in addition to *dpr*, so enhanced cytoplasmic Mn^{2+} was observed in the *perR* mutant (Tables 2 and 3 and Fig. 5), and this contributed to H_2O_2 resistance (Fig. 3). In addition, a physiological concentration of H_2O_2-induced *mntABC* transcription occurs only in the anaerobically cultured wild-type strain, but not in the *perR* mutant (Fig. 5). This indicates that the PerR protein of *S. oligofermentans* is involved in H_2O_2-induced *mntABC* gene expression. Collectively, PerR has been determined to play a pivotal role in sensing the cellular oxidative status and regulating Mn^{2+} and Fe^{2+} homeostasis in *S. oligofermentans*.

MntR, a member of the DtxR family, has been shown to regulate manganese transporter transcription in response to the cellular manganese concentration (55). This study also found that an MntR ortholog repressed the expression of *mntABC* in *S. oligofermentans*; however, *mntR* expression was not affected by *perR* mutation (data not shown). These data suggest that *mntABC* can independently respond to the cell redox status and manganese levels. Detailed regulation by PerR of *mntABC* and manganese homeostasis is under study in our laboratory through transcriptomics.

perR mutation does not significantly affect the antioxidant phenotype and *mntABC* expression when *S. oligofermentans* is statically cultured (Tables 2 and 3 and Fig. 2), suggesting that H_2O_2 produced under low oxygen levels inactivates *perR*. In accordance with this finding, PerR represses *mntABC* and *dpr* much more in anaerobic culture than in statically cultured cells (Table 3), and accordingly, more manganese is measured in the statically cultured wild-type strain (Table 2). More importantly, the survival of statically cultured *S. oligofermentans* is significantly higher than that in anaerobic culture after high-dose H_2O_2 pulsing (Fig. 2), indicating that antioxidant gene derepression promotes *S. oligofermentans* resistance to a high level of H_2O_2. Therefore, *S. oligofermentans* produces H_2O_2, not only as a chemical weapon in interspecies competition, but also as a strategy of “autoimmunity” to defend against more drastic oxidative stress. Orthologs of PerR and MntABC are found in almost all oral streptococci, suggesting that a PerR-regulated Mn^{2+}-based antioxidative mechanism can be generally used by these streptococci devoid of catalase. The oral commensals, such as *S. sanguinis* and *S. gordonii*, also suppress the growth of *S. mutans* by producing H_2O_2 (21), and this plays a role in oral health.

ACKNOWLEDGMENT

This study was supported by the National Natural Science Foundation of China, grant no. 31370098.

REFERENCES

1. **Lushchak VI.** 2011. Adaptive response to oxidative stress: bacteria, fungi, plants and animals. Comp. Biochem. Physiol. C Toxicol. Pharmacol. **153:** 175–190. http://dx.doi.org/10.1016/j.cbpc.2010.10.004.
2. **Imlay JA.** 2003. Pathways of oxidative damage. Annu. Rev. Microbiol. **57:** 395–418. http://dx.doi.org/10.1146/annurev.micro.57.030502.090938.
3. **Korshunov S, Imlay JA.** 2010. Two sources of endogenous hydrogen peroxide in *Escherichia coli*. Mol. Microbiol. **75:**1389–1401. http://dx.doi.org/10.1111/j.1365-2958.2010.07059.x.
4. **Imlay JA.** 2013. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat. Rev. Microbiol. **11:**443–454. http://dx.doi.org/10.1038/nrmicro3032.
5. **Imlay JA.** 2008. Cellular defenses against superoxide and hydrogen peroxide. Annu. Rev. Biochem. **77:**755–776. http://dx.doi.org/10.1146/annurev.biochem.77.061606.161055.
6. **Faulkner MJ, Helmann JD.** 2011. Peroxide stress elicits adaptive changes in bacterial metal ion homeostasis. Antioxid. Redox Signal. **15:**175–189. http://dx.doi.org/10.1089/ars.2010.3682.
7. **Yesilkaya H, Kadioglu A, Gingles N, Alexander JE, Mitchell TJ, Andrew PW.** 2000. Role of manganese-containing superoxide dismutase in oxidative stress and virulence of *Streptococcus pneumoniae*. Infect. Immun. **68:** 2819–2826. http://dx.doi.org/10.1128/IAI.68.5.2819-2826.2000.
8. **Jenney FE, Jr, Verhagen MF, Cui X, Adams MW.** 1999. Anaerobic microbes: oxygen detoxification without superoxide dismutase. Science **286:**306–309. http://dx.doi.org/10.1126/science.286.5438.306.
9. **Ilari A, Ceci P, Ferrari D, Rossi GL, Chiancone E.** 2002. Iron incorporation into *Escherichia coli* Dps gives rise to a ferritin-like microcrystalline core. J. Biol. Chem. **277:**37619–37623. http://dx.doi.org/10.1074/jbc.M206186200.
10. **Archibald FS, Fridovich I.** 1981. Manganese, superoxide dismutase, and oxygen tolerance in some lactic acid bacteria. J. Bacteriol. **146:**928–936.
11. **Culotta VC, Daly MJ.** 2013. Manganese complexes: diverse metabolic routes to oxidative stress resistance in prokaryotes and yeast. Antioxid. Redox Signal. **19:**933–944. http://dx.doi.org/10.1089/ars.2012.5093.
12. **Archibald FS, Fridovich I.** 1981. Manganese and defenses against oxygen toxicity in *Lactobacillus plantarum*. J. Bacteriol. **145:**442–451.
13. **Daly MJ.** 2009. A new perspective on radiation resistance based on *Deinococcus radiodurans*. Nat. Rev. Microbiol. **7:**237–245. http://dx.doi.org/10.1038/nrmicro2073.
14. **Bsat N, Herbig A, Casillas-Martinez L, Setlow P, Helmann JD.** 1998. *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. Mol. Microbiol. **29:**189–198. http://dx.doi.org/10.1046/j.1365-2958.1998.00921.x.
15. **Lee JW, Helmann JD.** 2006. The PerR transcription factor senses H_2O_2 by metal-catalysed histidine oxidation. Nature **440:**363–367. http://dx.doi.org/10.1038/nature04537.
16. **Helmann JD, Wu MFW, Gaballa A, Kobel PA, Morshedi MM, Fawcett P, Paddon C.** 2003. The global transcriptional response of *Bacillus subtilis* to peroxide stress is coordinated by three transcription factors. J. Bacteriol. **185:**243–253. http://dx.doi.org/10.1128/JB.185.1.243-253.2003.
17. **Ma Z, Lee JW, Helmann JD.** 2011. Identification of altered function alleles that affect *Bacillus subtilis* PerR metal ion selectivity. Nucleic Acids Res. **39:**5036–5044. http://dx.doi.org/10.1093/nar/gkr095.
18. **Brenot A, Weston BF, Caparon MG.** 2007. A PerR-regulated metal transporter (PmtA) is an interface between oxidative stress and metal homeostasis in *Streptococcus pyogenes*. Mol. Microbiol. **63:**1185–1196. http://dx.doi.org/10.1111/j.1365-2958.2006.05577.x.
19. **Grifantini R, Toukoki C, Colaprico A, Gryllos I.** 2011. Peroxide stimulon and role of PerR in group A *Streptococcus*. J. Bacteriol. **193:**6539–6551. http://dx.doi.org/10.1128/JB.05924-11.
20. **Yesilkaya H, Andisi VF, Andrew PW, Bijlsma JJ.** 2013. *Streptococcus pneumoniae* and reactive oxygen species: an unusual approach to living with radicals. Trends Microbiol. **21:**187–195. http://dx.doi.org/10.1016/j.tim.2013.01.004.
21. **Zhu L, Kreth J.** 2012. The role of hydrogen peroxide in environmental adaptation of oral microbial communities. Oxid. Med. Cell. Longev. **2012:** 717843. http://dx.doi.org/10.1155/2012/717843.
22. **Tong H, Chen W, Merritt J, Qi F, Shi W, Dong X.** 2007. *Streptococcus oligofermentans* inhibits *Streptococcus mutans* through conversion of lactic acid into inhibitory H_2O_2: a possible counteroffensive strategy for interspecies competition. Mol. Microbiol. **63:**872–880. http://dx.doi.org/10.1111/j.1365-2958.2006.05546.x.
23. **Tong H, Chen W, Shi W, Qi F, Dong X.** 2008. SO-LAAO, a novel L-amino acid oxidase that enables *Streptococcus oligofermentans* to outcompete *Streptococcus mutans* by generating H_2O_2 from peptone. J. Bacteriol. **190:**4716–4721. http://dx.doi.org/10.1128/JB.00363-08.
24. **Liu L, Tong H, Dong X.** 2012. Function of the pyruvate oxidase-lactate oxidase cascade in interspecies competition between *Streptococcus oligofermentans* and *Streptococcus mutans*. Appl. Environ. Microbiol. **78:**2120–2127. http://dx.doi.org/10.1128/AEM.07539-11.
25. **King KY, Horenstein JA, Caparon MG.** 2000. Aerotolerance and peroxide resistance in peroxidase and *perR* mutants of *Streptococcus pyogenes*. J. Bacteriol. **182:**5290–5299. http://dx.doi.org/10.1128/JB.182.19.5290-5299.2000.
26. **Fujishima K, Kawada-Matsuo M, Oogai Y, Tokuda M, Torii M, Komatsuzawa H.** 2013. *dpr* and *sod* in *Streptococcus mutans* are involved in coexistence with *S. sanguinis*, and PerR is associated with resistance to H_2O_2. Appl. Environ. Microbiol. **79:**1436–1443. http://dx.doi.org/10.1128/AEM.03306-12.
27. **Tsou CC, Chiang-Ni C, Lin YS, Chuang WJ, Lin MT, Liu CC, Wu JJ.** 2010. Oxidative stress and metal ions regulate a ferritin-like gene, *dpr*, in

Streptococcus pyogenes. Int. J. Med. Microbiol. **300:**259–264. http://dx.doi.org/10.1016/j.ijmm.2009.09.002.

28. **Jakubovics NS, Smith AW, Jenkinson HF.** 2002. Oxidative stress tolerance is manganese (Mn^{2+}) regulated in *Streptococcus gordonii*. Microbiology **148:**3255–3263.
29. **Janulczyk R, Ricci S, Bjorck L.** 2003. MtsABC is important for manganese and iron transport, oxidative stress resistance, and virulence of *Streptococcus pyogenes*. Infect. Immun. **71:**2656–2664. http://dx.doi.org/10.1128/IAI.71.5.2656-2664.2003.
30. **Tseng HJ, McEwan AG, Paton JC, Jennings MP.** 2002. Virulence of *Streptococcus pneumoniae*: PsaA mutants are hypersensitive to oxidative stress. Infect. Immun. **70:**1635–1639. http://dx.doi.org/10.1128/IAI.70.3.1635-1639.2002.
31. **Tong H, Gao X, Dong X.** 2003. *Streptococcus oligofermentans* sp. nov., a novel oral isolate from caries-free humans. Int. J. Syst. Evol. Microbiol. **53:**1101–1104. http://dx.doi.org/10.1099/ijs.0.02493-0.
32. **Zhu B, Tong H, Chen W, Dong X.** 2009. Role of Dpr in hydrogen peroxide tolerance of *Streptococcus oligofermentans*. Wei Sheng Wu Xue Bao **49:**1341–1346.
33. **Surette MG, Miller MB, Bassler BL.** 1999. Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. U. S. A. **96:**1639–1644. http://dx.doi.org/10.1073/pnas.96.4.1639.
34. **Marmur J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3:**208–218. http://dx.doi.org/10.1016/S0022-2836(61)80047-8.
35. **Dong X, Xin Y, Jian W, Liu X, Ling D.** 2000. *Bifidobacterium thermacidophilum* sp. nov., isolated from an anaerobic digester. Int. J. Syst. Evol. Microbiol. **1:**119–125. http://dx.doi.org/10.1099/00207713-50-1-119.
36. **Tong H, Shang N, Liu L, Wang X, Cai J, Dong X.** 2013. Complete genome sequence of an oral commensal, *Streptococcus oligofermentans* strain AS 1.3089. Genome Announc. 1:pii: e00353-13. http://dx.doi.org/10.1128/genomeA.00353-13.
37. **Lau PC, Sung CK, Lee JH, Morrison DA, Cvitkovitch DG.** 2002. PCR ligation mutagenesis in transformable streptococci: application and efficiency. J. Microbiol. Methods **49:**193–205. http://dx.doi.org/10.1016/S0167-7012(01)00369-4.
38. **Liu Y, Zeng L, Burne RA.** 2009. AguR is required for induction of the *Streptococcus mutans* agmatine deiminase system by low pH and agmatine. Appl. Environ. Microbiol. **75:**2629–2637. http://dx.doi.org/10.1128/AEM.02145-08.
39. **Tong H, Zhu B, Chen W, Qi F, Shi W, Dong X.** 2006. Establishing a genetic system for ecological studies of *Streptococcus oligofermentans*. FEMS Microbiol. Lett. **264:**213–219. http://dx.doi.org/10.1111/j.1574-6968.2006.00453.x.
40. **LeBlanc DJ, Lee LN, Abu-Al-Jaibat A.** 1992. Molecular, genetic, and functional analysis of the basic replicon of pVA380-1, a plasmid of oral streptococcal origin. Plasmid **28:**130–145. http://dx.doi.org/10.1016/0147-619X(92)90044-B.
41. **Podbielski A, Spellerberg B, Woischnik M, Pohl B, Lutticken R.** 1996. Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). Gene **177:**137–147. http://dx.doi.org/10.1016/0378-1119(96)84178-3.
42. **Kolenbrander PE, Andersen RN, Baker RA, Jenkinson HF.** 1998. The adhesion-associated sca operon in *Streptococcus gordonii* encodes an inducible high-affinity ABC transporter for Mn^{2+} uptake. J. Bacteriol. **180:**290–295.
43. **Kehres DG, Zaharik ML, Finlay BB, Maguire ME.** 2000. The NRAMP proteins of *Salmonella typhimurium* and *Escherichia coli* are selective manganese transporters involved in the response to reactive oxygen. Mol. Microbiol. **36:**1085–1100. http://dx.doi.org/10.1046/j.1365-2958.2000.01922.x.
44. **Daly MJ, Gaidamakova EK, Matrosova VY, Vasilenko A, Zhai M, Venkateswaran A, Hess M, Omelchenko MV, Kostandarithes HM, Makarova KS, Wackett LP, Fredrickson JK, Ghosal D.** 2004. Accumulation of Mn(II) in *Deinococcus radiodurans* facilitates gamma-radiation resistance. Science **306:**1025–1028. http://dx.doi.org/10.1126/science.1103185.
45. **Archibald F.** 1986. Manganese: its acquisition by and function in the lactic acid bacteria. Crit. Rev. Microbiol. **13:**63–109. http://dx.doi.org/10.3109/10408418609108735.
46. **Stadtman ER, Berlett BS, Chock PB.** 1990. Manganese-dependent disproportionation of hydrogen peroxide in bicarbonate buffer. Proc. Natl. Acad. Sci. U. S. A. **87:**384–388. http://dx.doi.org/10.1073/pnas.87.1.384.
47. **Anjem A, Varghese S, Imlay JA.** 2009. Manganese import is a key element of the OxyR response to hydrogen peroxide in *Escherichia coli*. Mol. Microbiol. **72:**844–858. http://dx.doi.org/10.1111/j.1365-2958.2009.06699.x.
48. **Brown JS, Holden DW.** 2002. Iron acquisition by Gram-positive bacterial pathogens. Microbes Infect. **4:**1149–1156. http://dx.doi.org/10.1016/S1286-4579(02)01640-4.
49. **Jakubovics NS, Jenkinson HF.** 2001. Out of the iron age: new insights into the critical role of manganese homeostasis in bacteria. Microbiology **147:**1709–1718.
50. **Arirachakaran P, Luengpailin S, Banas JA, Mazurkiewicz JE, Benjavongkulchai E.** 2007. Effects of manganese on *Streptococcus mutans* planktonic and biofilm growth. Caries Res. **41:**497–502. http://dx.doi.org/10.1159/000110882.
51. **Antelmann H, Helmann JD.** 2011. Thiol-based redox switches and gene regulation. Antioxid. Redox Signal. **14:**1049–1063. http://dx.doi.org/10.1089/ars.2010.3400.
52. **Zheng M, Storz G.** 2000. Redox sensing by prokaryotic transcription factors. Biochem. Pharmacol. **59:**1–6. http://dx.doi.org/10.1016/S0006-2952(99)00289-0.
53. **Mongkolsuk S, Helmann JD.** 2002. Regulation of inducible peroxide stress responses. Mol. Microbiol. **45:**9–15. http://dx.doi.org/10.1046/j.1365-2958.2002.03015.x.
54. **Herbig AF, Helmann JD.** 2001. Roles of metal ions and hydrogen peroxide in modulating the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA. Mol. Microbiol. **41:**849–859. http://dx.doi.org/10.1046/j.1365-2958.2001.02543.x.
55. **Que Q, Helmann JD.** 2000. Manganese homeostasis in *Bacillus subtilis* is regulated by MntR, a bifunctional regulator related to the diphtheria toxin repressor family of proteins. Mol. Microbiol. **35:**1454–1468. http://dx.doi.org/10.1046/j.1365-2958.2000.01811.x.