Aquaporins (AQPs) are transmembrane proteins widely distributed in various organisms, and they facilitate bidirectional diffusion of water and uncharged solutes. The catalase-negative bacterium *Streptococcus oligofermentans* produces the highest H$_2$O$_2$ levels reported to date, which has to be exported to avoid oxidative stress. Here, we report that a *S. oligofermentans* aquaporin functions as a peroxiporin facilitating bidirectional transmembrane H$_2$O$_2$ transport. Knockout of this aquaporin homolog, So-AqpA, reduced H$_2$O$_2$ export by ~50% and increased endogenous H$_2$O$_2$ retention, as indicated by the cellular H$_2$O$_2$ reporter HyPer. Heterologous expression of So-aqpA accelerated exogenous H$_2$O$_2$ influx into *Saccharomyces cerevisiae* and *Escherichia coli* cells, indicating that So-AqpA acts as an H$_2$O$_2$-transferring aquaporin. Alanine substitution revealed Phe-40 as a key residue for So-AqpA-mediated H$_2$O$_2$ transport. Northern blotting, qPCR, and luciferase reporter assays disclosed that *So-aqpA* expression. Importantly, *So-aqpA* deletion decreased oxic growth and intraspecies competition and diminished the competitive advantages of *S. oligofermentans* over the caries pathogen *Streptococcus mutans*. Of note, *S. oligofermentans* orthologs with the *So-aqpA* promoter, indicating that MntR directly regulates H$_2$O$_2$-induced *So-aqpA* expression. Our work has uncovered an intrinsic, H$_2$O$_2$-inducible bacterial peroxiporin that has a key physiological role in H$_2$O$_2$ detoxification in *S. oligofermentans*.

Aquaporins (AQPs)\(^3\) belong to the major intrinsic protein (MIP) family and are widely distributed in all the cellular organelles. They form channels across biological membranes and facilitate bidirectional diffusion of water and uncharged solutes, such as glycerol and urea\(^1\), \(^2\). Since their first discovery in 1992\(^1\), numerous studies have demonstrated that human AQPs display important physiological functions and their dysfunctions cause many clinical disorders\(^3\), \(^4\). The plant AQPs are found to be involved in transpiration, root water uptake, seed desiccation, inhibition of self-pollination, and closure of leaf guard cells\(^5\), \(^6\). Phylogenetically, MIPs are clustered into two clades, the water-transporting AQPs and the glycerol-permeable aquaglyceroporins (GLPs)\(^7\). Both clades of the aquaporins possess an Asn-Pro-Ala (NPA) signature motif and an aromatic/arginine (ar/R) substrate selectivity filter; and ar/R consists of one conserved arginine and three other amino acids that are conserved within each subfamily\(^8\). Like many other membrane transporters, the activities of AQPs are subjected to regulation. The eukaryotic water-transporting aquaporins can be regulated by protein trafficking\(^9\), and phosphorylation, pH, or divergent cations mediated gating\(^10\), \(^11\). Recently, it was reported that pH regulates the permeability of an aquaglyceroporin, AQP7, by altering protonation of the key amino acid residues\(^12\).

Probably because of the similar electrochemical properties of H$_2$O and H$_2$O$_2$, some AQPs can transport H$_2$O$_2$ through the H$_2$O channel\(^6\), \(^13\). In recent years, a growing number of animal and plant aquaporin homologs have been verified to transport H$_2$O$_2$, which can promote development of some important physiological characteristics in eukaryotes\(^14\)–\(^16\). The human AQP3 and AQP8 facilitate H$_2$O$_2$ permeating across the cell membranes\(^17\), which allows mitochondria-generated H$_2$O$_2$, a key molecule in the redox signaling network, to permeate into other cellular compartments and regulate physiological processes\(^6\), \(^16\)–\(^19\). However, being an oxidant, the H$_2$O$_2$ level has to be strictly controlled to prevent cells from oxidative stress, which would subsequently cause disease and tumorigenesis\(^5\).

\(1\) To whom correspondence may be addressed. Tel.: 86-10-6480-7567; Fax: 86-10-6480-7429; E-mail: tonghuichun@im.ac.cn.

\(2\) To whom correspondence may be addressed. Tel.: 86-10-6480-7413; Fax: 86-10-6480-7429; E-mail: dongxz@im.ac.cn.

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This article contains Figs. S1–S3 and Table S1.

\(3\) The abbreviations used are: AQP, aquaporin; qPCR, quantitative PCR; MIP, major intrinsic protein; GLP, glycerol-permeable aquaglyceroporin; PALM, photoactivated localization microscopy; BHI, brain heart infusion; a.u., arbitrary units; ROI, region of interest; cfu, colony-forming unit; ANOVA, analysis of variance.
A Streptococcus aquaporin acting as peroxiporin

Because of the limited studies on prokaryotic AQP s (2, 20, 21), their functions are largely unknown, such as whether they also function to facilitate H$_2$O$_2$ excretion and whether this action has physiological significance. Based on the rate of H$_2$O$_2$ flux, Seaver and Imlay (22) found that a H$_2$O$_2$ transmembrane concentration gradient exists in Escherichia coli. This suggests that H$_2$O$_2$ permeability across the cellular membrane is limited, and the bacterial aquaporins may also function in facilitating H$_2$O$_2$ diffusion. Streptococci, a type of facultative anaerobic bacteria that lack catalase, are known to produce and accumulate high concentrations of H$_2$O$_2$ in cultures, indicating they possess effective H$_2$O$_2$ excretion pathways. Streptococci carry aquaglyceroporins in facilitating H$_2$O$_2$ efflux and the regulatory mechanisms. Through the integration of genetic, physiological, biochemical, and single-molecule imaging approaches, we identified a streptococcal peroxiporin, So-AqpA. By using an intracellular-specific H$_2$O$_2$ fluorescence reporter, HyPer, we demonstrated that So-AqpA, with Phe-40 as a key residue, facilitated the bidirectional permeation of H$_2$O$_2$ across the cellular membrane. Northern blotting and quantitative PCR, and photoactivated localization microscopy (PALM) super-resolution imaging determined that H$_2$O$_2$ induced the expression of So-aqpA gene at both transcriptional and translational levels. The two well-known redox transcriptional regulators PerR and MntR are involved in the H$_2$O$_2$-induced expression of So-aqpA. Deletion of the So-aqpA gene caused oxidative stress and reduced the intraspecies and interspecies competitive advantages of S. oligofermentans. This work reports for the first time the physiological roles of a bacterial peroxiporin, which could be a potential target for suppression of streptococci, especially the pathogenic species.

Results

The aquaporin homolog So-aqpA encodes an H$_2$O$_2$ facilitator

The S. oligofermentans genome carries three MIP family homologous genes: 1872_01445 encodes an aquaporin, so-aqpA; 1872_09070 and 1872_03455 encode glycerol uptake facilitator proteins and are designated as So-aqpB and So-aqpC, respectively, and the encoded proteins were designated as So-AqpB and So-AqpC. So-aqpC belongs to a three-gene operon for glycerol metabolism; thereby it was predicted to facilitate glycerol uptake. Phylogenetically, So-AqpA is related to the E. coli aquaporin Z (b0875), whereas the two aquaglyceroporins (So-AqpB and So-AqpC) are related to the E. coli aquaglyceroporin GlpF (h3927) and the human aquaporin AQP3 (360) (Fig. 1A). To determine whether these MIP family proteins function as H$_2$O$_2$ transporters in S. oligofermentans, So-aqpA and So-aqpB were deleted and the mutants were designated as ΔaqpA and ΔaqpB, respectively. Given that streptococci produce and accumulate endogenous H$_2$O$_2$ under oxic conditions, the two mutants and WT strain were cultured statically, and then the exponential phase cells were resuspended in fresh BHI medium. After incubated at 37 °C for 1 h, H$_2$O$_2$ concentrations in the cultures were measured as described in “Experimental procedures.” 113 ± 16 μM H$_2$O$_2$ was determined in the WT strain culture, whereas 64 ± 9 μM and 110 ± 13 μM H$_2$O$_2$ were determined in the ΔaqpA and ΔaqpB cultures, respectively. Next these three strains, and the So-aqpA complemented strain (aqpA-com) were cultured under higher oxygen supplies in 10 ml BHI in a 100-ml triangle flask for higher endogenous H$_2$O$_2$ production. Then H$_2$O$_2$ contents in the cultures were measured. We found that while about half amount of H$_2$O$_2$ was produced by the ΔaqpA, similar H$_2$O$_2$ yields were measured in the aqpA-com, the WT, and ΔaqpB strains (Fig. 1B). This suggests that So-AqpA is a H$_2$O$_2$ facilitator, but So-AqpB is not.

To further validate that So-AqpA acts as a H$_2$O$_2$ facilitator, its gene was heterogeneously expressed in Saccharomyces cerevisiae by the vector pYES2 and in E. coli by the vector pBl166 (24). As shown in Fig. 1C, good growth of the S. cerevisiae INVSc1 strain carrying a vacant vector occurred on the galactose agar plates that contained H$_2$O$_2$ up to 2.5 mM, whereas the So-aqpA-expressing strain grew poorly at 2 mM and no growth was shown at 2.5 mM H$_2$O$_2$. Accordingly, H$_2$O$_2$ minimal inhibitory concentration value was determined to be 3 mM for the So-aqpA-expressing strain compared with 6 mM for the empty vector-expressing S. cerevisiae. In addition, a green fluorescence protein (sgFP)-So-aqpA fusion was introduced into strain INVSc1, and the GFP fluorescence around the cytoplasmic membrane was observed under a confocal laser scanning microscope, confirming the expression of So-aqpA in S. cerevisiae (Fig. 1C). Subsequently, So-aqpA facilitated H$_2$O$_2$ permeation into E. coli cells was tested by monitoring the dynamics of exogenous H$_2$O$_2$ reduction based on the cytoplasmic catalase scavenging. The mid-exponential phase Luria broth (LB) cultures of E. coli DH5α expressing pBl166-aqpA and the vacant pBl166 were suspended in PBS, and by using 150 μM H$_2$O$_2$ as the initial concentration, the residual H$_2$O$_2$ amounts were measured over time. As shown in Fig. 1D, So-aqpA expression increased about 8% H$_2$O$_2$ uptake rate of E. coli until 4 min, and about 6% increase until 8 min. Using quantitative RT-PCR, the transcript copies of So-aqpA and 16S rRNA in E. coli were quantified as 369,564 ± 94,966/μg cDNA and 335,341 ± 81,442 × 10⁴/μg cDNA, respectively, thus, So-aqpA transcription in E. coli was determined as 0.11 ± 0.01 copies/1000 16S rRNAs. Taken together, these experiments demonstrated that So-AqpA is an H$_2$O$_2$ facilitator.

So-AqpA facilitates H$_2$O$_2$ efflux and influx

To test the direct involvement of So-AqpA in efflux of the S. oligofermentans endogenous H$_2$O$_2$, a HyPer fluorescent protein was used as an intracellular H$_2$O$_2$ reporter. The HyPer protein was constructed by Belousov et al. (25) by inserting the fluorescent protein cpYFP into the regulatory domain of the E. coli H$_2$O$_2$-sensing protein OxyR. When H$_2$O$_2$ oxidizes Cys-199 and Cys-208 to form a disulfide bond, HyPer emits green fluorescence. The S. oligofermentans lactate dehydrogenase promoter-HyPer gene fusion was inserted into the streptococci–
E. coli shuttle vector pDL278 (26) and introduced into E. coli DH5α cells. A similar peak of HyPer fluorescence, as reported previously (27), was observed in the E. coli–HyPer strain after 1-min treatment by 20 μM H₂O₂ (Fig. S1A). Following that, the pDL278-HyPer plasmid was introduced into the S. oligofermentans WT strain, ΔaqpA and the pyruvate oxidase deletion mutant (Δpox) (28) to generate cellular H₂O₂ real-time reporter strains WT-HyPer, ΔaqpA-HyPer, and Δpox-HyPer, respectively.

Next, So-AqpA-facilitated H₂O₂ export was examined in the three H₂O₂ real-time reporter strains that were cultured stat-
cally in BHI broth supplemented with 0 units, 300 units, and 1000 units catalase, respectively. Catalase was used to hydrolyze excreted H$_2$O$_2$ during growth so as to create an H$_2$O$_2$ gradient across cell membrane. The exponential phase cells were collected, and HyPer fluorescence was observed under a confocal laser scanning microscope. Fig. 2A showed significantly higher HyPer fluorescence intensity (~28 a.u. per region of interest (ROI)) in 1000 units catalase-treated ΔaqpA-HyPer cells compared with the WT-HyPer strain (~12 a.u. per ROI), whereas only slightly higher intensities were observed in 0 units and 300 units catalase-treated mutant cells (Fig. 2C). No HyPer fluorescence was observed at all in the Δpox-HyPer cells at the earlier
growth phase (Fig. S1B); this is consistent with the fact that the *pox* gene encoded pyruvate oxidase contributes to $H_2O_2$ production at the earlier growth phase of *S. oligofermentans* (28). This demonstrated that the *S. oligofermentans* *aqpA* gene encodes an $H_2O_2$ facilitator protein, and absence of the protein led to endogenous $H_2O_2$ retention. Although antibiotics can induce $H_2O_2$ production in bacteria, the observed HyPer fluorescence difference should be attributed to the studied genes, because all of the tested HyPer reporter strains were grown in BH1 broth plus spectinomycin to maintain the shuttle plasmid.

To explore the role of So-AqpA in facilitating $H_2O_2$ influx, $2 \text{ ml mid–explosive phase cells}$ of anaerobically grown WT-HyPer and $\Delta aqpA$-HyPer cells were collected and resuspended in PBS. Cells were first exposed to air for 15 min and then pulsed with $0.5 \text{ mm } H_2O_2$. Fluorescence of the WT-HyPer cells was observed at 5 min post pulsing under a confocal laser scanning microscope. However, only weak fluorescence was observed in the $\Delta aqpA$ mutant (Fig. 2B), which was 5-fold lower than that in the WT strain (Fig. 2D). These results demonstrate that So-AqpA functions as a $H_2O_2$ facilitator for its bidirectional diffusion across the cellular membrane.

**The key amino acid residues of So-AqpA for $H_2O_2$ permeation**

To probe the key amino acids of So-AqpA that are involved in $H_2O_2$ transport, we first performed an amino acid sequence alignment with the phylogenetic orthologs from eukaryotic and prokaryotic species. As shown in Fig. 3A, So-AqpA possesses the two characteristic NPA motifs and the four conserved amino acid residues for AQP substrate binding (29). Homology modeling of the So-AqpA protein was performed by automatic selection of the *Archaeoglobus fulgidus* aquaporin (identity 44%) as a template via the SWISS-MODEL web service. Fig. 3B shows that So-AqpA is a tetramer with each monomer forming a barrel shape, and the conserved Phe-40, Ile-165, Leu-174, and Arg-180 are situated at the substrate-binding sites, the two NPA motifs meet at the central part of the channel (Fig. 3C). Next, alanine substitution was performed for the four substrate-binding residues, and each of the residue-substituted So-AqpA mutants was introduced into the $\Delta aqpA$ strain by shuttle vector pB166. Using the same approach as described above, we determined that alanine substitution of Phe-40 (F40A) substantially and Arg-180 (R180A) moderately reduced $H_2O_2$ excretion, respectively (Fig. 1B). Mutation of the remaining two had no effect on excreted $H_2O_2$ content.

The effect of So-AqpA F40A mutation on $H_2O_2$ excretion was also tested using the HyPer fluorescence reporter. To accomplish this, F40A mutation was introduced into the chromosome at the So-aqpA locus to construct *aqpA*F40A strain, and then the HyPer fluorescence reporter was introduced into *aqpA*F40A strain to construct an *aqpA*F40A-HyPer strain. By comparing the HyPer fluorescence intensity with those of the WT-HyPer and $\Delta aqpA$-HyPer strains, failure of $H_2O_2$ efflux and influx was observed for the *aqpA*F40A-HyPer strain. As shown in Fig. 2, either by suspending the cells in 1000 units catalase-contained fresh BHI or by pulsing with $0.5 \text{ mm } H_2O_2$, similar HyPer fluorescence intensities were found for the *aqpA*F40A and $\Delta aqpA$ strain. Collectively, these experimental evidences demonstrate that Phe-40 is a key residue for So-AqpA to facilitate $H_2O_2$ transport.

**$H_2O_2$ induces transcription of the So-aqpA gene**

To investigate the link between So-AqpA’s function as a $H_2O_2$ facilitator and its role in detoxification for the bacterium, $H_2O_2$ induction of the *So-aqpA* expression was detected using Northern blotting and quantitative real-time PCR (qPCR) assays. A final concentration of $40 \mu M$ $H_2O_2$ was added into the mid–explosive phase cultures of *S. oligofermentans* that were grown anaerobically, and the same volumes of $H_2O_2$ were added to the non-$H_2O_2$ treatment controls. By using a DNA fragment of the *So-aqpA* gene as probe (Table S1), Northern blotting detected an RNA of about 0.7 kb only in $H_2O_2$-treated cells, indicating that $40 \mu M$ $H_2O_2$ significantly induced *So-aqpA* transcription (Fig. 4A); consistently, the qPCR assay also determined a 10.5-fold elevated abundance of the *So-aqpA* mRNA in response to $40 \mu M$ $H_2O_2$ (Fig. 4B). To test whether the endogenous $H_2O_2$ affected *So-aqpA* expression, its transcription was compared in the statically cultured WT strain and a double-gene deletion mutant of *pox* and *lox*, which encode the two primary $H_2O_2$ production proteins, pyruvate and lactate oxidase (28). As expected, qPCR determined a 4.3-fold reduced expression of the *So-aqpA* gene in the *pox/lox* double mutant (Fig. 4B).

A luciferase reporter was then used to test the *So-aqpA* expression profile in response to endogenous $H_2O_2$ during growth. The *So-aqpA* promoter was fused to the *luc* gene on pFW5-luc (30), and luciferase activities were determined during growth of the statically or anaerobically cultured *S. oligofermentans*. Fig. 4C shows that under static growth condition, relatively higher expression of the *So-aqpA* gene occurred at the earlier exponential phase, corresponding to the peak expression periods of the *pox* gene and $H_2O_2$ production (28). Luciferase reporter determined 2.7- to 26.7-fold higher activities in the statically grown cells than those in the anaerobically grown cells during the entire growth period, consistent with qPCR assayed up-regulation of *So-aqpA* in the static culture (Fig. 4B). This demonstrated that when cells accumulated relative high $H_2O_2$, expression level of the *So-aqpA* gene would be enhanced to fulfill its task in excreting endogenous $H_2O_2$ and attenuating oxidative stress in *Streptococcus*.

**Super-resolution PALM imaging reveals numerous So-AqpA protein molecules in $H_2O_2$-induced cell membrane**

Subsequently, we investigated how many So-AqpA protein molecules are present in one bacterial cell so as to meet the requirement for efficient endogenous $H_2O_2$ efflux. To accomplish this, the state-of-the-art super–resolution imaging method of PALM (31) was employed to obtain high-resolution cell images. First, a chromosomal AqpA-mMaple3 fusion was constructed in which the *So-aqpA* gene was tagged with the monomeric photoactivatable fluorescent protein mMaple3 (32). To visualize the So-AqpA protein expression in different growth phases and also in response to $H_2O_2$, the AqpA-mMaple3 strain was cultured statically and anaerobically, respectively. Cells of the static culture were collected at its earlier and mid-explosive growth phases and suspended in PBS.
buffer after washing. For the anaerobic culture, the mid-exponential phase cells were collected, and one aliquot was treated with 40 μM H₂O₂ for 20 min, whereas another was treated with the same volume of H₂O. Figure 5A shows the representative PALM images of the So-AqpA-mMaple3 fusion fluorescent proteins on cell membrane, each image included a cell chain consisting of three or four single cells. The mMaple3 signals indicated abundant So-AqpA protein molecules distributed on the cytoplasmic membrane. By using the Insight3 software, the protein numbers were counted on 18 cells for each sample (33), and the calculated So-AqpA protein molecules were 131 ± 22 and 122 ± 17 per cell in the earlier and mid–exponential phase statically grown culture, respectively. However, only an average of 41 ± 9 So-AqpA molecules per cell were counted in the anaerobic culture; and the protein number was increased by 1.6-fold upon H₂O₂ treatment (Fig. 5B). The super-resolution imaging not only reveals that S. oligofermentans possesses numerous transmembrane peroxiporin molecules, but also validates H₂O₂ induc-
0.1 nM biotin-labeled DNA was mixed with various concentrations of MntR promoter–luciferase reporter strain, P. caldolyticus WT strain and * and #, data are statistically significantly different in comparison between static cultures (details are described in “Experimental procedures”). Triplicate measurements were performed for three batches of cultures, and the averages ± S.D. are shown. * and #, data are statistically significantly different in comparison between statically grown WT strain and ΔperR (ΔperR and mntR ΔmntR), and statically grown (static) wild strain and p ox and lox double deletion mutants (Δpox/lox) (details are described in “Experimental procedures”). Triplicate measurements were performed for three batches of cultures, and the averages ± S.D. are shown. * and #, data are statistically significant compared with those of anaerobically grown WT strain as verified by Student’s t-test, p < 0.05. C, a luciferase reporter strain, PdpA-luc, in which the So-aqpA promoter was fused to the luciferase gene, was grown in BHI broth anaerobically or statically. At the indicated time points during growth, 100 μl cultures were collected in 1.5-ml Eppendorf tubes, and after 5 min exposure to air at room temperature, the luciferase activities (RLU, relative light units) were measured as described in “Experimental procedures.” OD_{600} was measured in parallel. Triplicate measurements were performed for three batches of cultures, and the averages ± S.D. are shown. *, data are statistically significant compared with those of anaerobically grown WT strain as verified by Student’s t-test, p < 0.05. D, a DNA fragment of the So-aqpA promoter was PCR amplified with 5’–end biotin-labeled primers (Table S1). 0.1 nmol biotin-labeled DNA was mixed with various concentrations of MntR protein in the EMSA-binding mixture and run in a native PAGE gel. Black arrow indicates the protein-DNA complex. Addition of increasing nonlabeled DNA (cold probe) decreased the protein-DNA complex.

MntR and PerR are involved in regulation of H2O2-induced expression of So-aqpA

To further explore the regulatory mechanisms that mediate H2O2 induction of the So-aqpA expression, we tested the possible involvement of two known redox regulatory repressors, the metalloregulator MntR (34) and the peroxide-responsive repressor PerR (35). Our unpublished transcriptomic data also showed that by deletion of either mntR or perR, H2O2-induced So-aqpA expression appeared to be attenuated. ΔmntR and ΔperR strains were then cultured anaerobically, and one aliquot of the mid–exponential phase cell was treated with 40 μM H2O2, while another was treated with the same volume of H2O2. After incubation at 37 °C for 20 min, the So-aqpA transcript copies were quantified by qPCR. As shown in Fig. 4B, comparing with the >10-fold induction of So-aqpA expression by H2O2, the induction was reduced to 4- and 1.8-fold in the mntR and perR deletion mutants, respectively. This confirmed a regulatory involvement of the two regulators in H2O2-induced So-aqpA transcription.

To determine whether the two regulatory repressors directly regulate the expression of So-aqpA, EMSA was performed to detect the associations of overexpressed MntR and PerR proteins with 5’–biotin–labeled So-aqpA promoter fragment. As shown in Fig. 4D, a protein-DNA complex appeared at a 1000:1 ratio of MntR to DNA, a comparable affinity to its regulated manganese transporting protein mntABC promoter in a parallel experiment (Fig. S2); this complex disappeared with addition of the competing nonlabeled cold probe. This indicates that MntR may directly regulate the expression of So-aqpA. Additionally, a putative MntR-binding sequence (TATTATAACCTA AAAATT, subscript letters indicate non-conserved bases) was
A Streptococcus aquaporin acting as peroxiporin

Figure 6. So-AqpA promotes the oxic growth and interspecies and interspecies competitive advantage of *S. oligofermentans*. A, growth profiles were determined for the WT strain, So-aqpA mutant (ΔaqpA), So-aqpA complemented (aqpAcom), and the Phe-40 substitution complemented (aqpAF40Acom) strains that were statically cultured in 10 ml or 40 ml BHI broth. Overnight BHI cultures of the tested strains were 1:30 inoculated into fresh BHI medium and incubated at 37 °C, and OD₆₀₀ was measured at the indicated time. The results are expressed as averages ± S.D. of three independent experiments. Suffix numbers after strain names indicate the culture volumes. B, exponential growing cells from the cultures above were collected, and HyPer fluorescence was observed and measured using the same procedure as described in Fig. 2. Representative images of three independent experiments are shown and fluorescence intensity a.u. per ROI is shown inside the parentheses in each image. Bar, 5 μm. C, competition between the wild strain and So-aqpA mutant was determined for three successive subcultured generations under high oxygen content. The same amounts of the two strains were co-inoculated or mono-inoculated into 10 ml BHI broth contained in a 100-ml flask, and the co-culture and mono-culture were subcultured for three successive generations. Colony-forming units (cfu) of each culture in each generation were counted on BHI agar plate; BHI plate containing 1 mg/ml kanamycin was used to count cfu of the ΔaqpA mutant in co-cultures. The results are averages ± S.D. of three independent experiments. *, significantly different from the So-aqpA deletion mutant in the first generation of co-culture as verified by one-way ANOVA analysis followed by Tukey’s post hoc test (*p < 0.05). D, growth suppression of *S. mutans* (*Sm*) by the *S. oligofermentans* WT strain and So-aqpA mutant (ΔaqpA) was tested on TPYG plate (0.5% tryptone, 0.5% peptone, 1% yeast, 1% glucose, 4% salt solution) (23). Overnight cultures of the tested strains were collected and adjusted to the same OD₆₀₀.1 0 μl of each strain were spotted side-by-side on the plates and incubated at 37 °C in the candle jar for 24 h. The experiments were repeated three times, and one representative experiment is shown.

To link growth inhibition with endogenous H₂O₂, cellular H₂O₂ levels were monitored using HyPer reporter. The mid–exponential phase cells of the WT-HyPer and ΔaqpA-HyPer strains were examined under a confocal microscope. As shown found at the So-aqpA promoter region, and this is consistent with a ~1.5-fold enhanced expression of So-aqpA detected in the mntR deletion mutant (Fig. 4B). We previously demonstrated that MntR is inactivated by H₂O₂ via cysteine oxidation (34), which may explain MntR-dependent H₂O₂ induction of the So-aqpA expression. However, direct association of PerR with So-aqpA promoter was not found (data not shown), and the regulatory mechanisms of PerR on So-aqpA expression need to be further explored.

**So-AqpA–based H₂O₂ export alleviates oxidative stress and provides *S. oligofermentans* with both intraspecies and interspecies competitive advantages**

Because the WT strain produced significantly higher H₂O₂ (912 ± 136 μM) in 10 ml than that (255 ± 108 μM) in 40 ml BHI broth, to investigate the physiological significance of So-AqpA, the So-aqpA mutants and WT strain were statically grown in 10 ml or 40 ml medium in a 100-ml flask to create relatively high and low oxygen contents, respectively.

To link growth inhibition with endogenous H₂O₂, cellular H₂O₂ levels were monitored using HyPer reporter. The mid–exponential phase cells of the WT-HyPer and ΔaqpA-HyPer strains were examined under a confocal microscope. As shown
in Fig. 6B, in the cells from either 10 ml or 40 ml culture, higher HyPer fluorescence intensities were always observed in ΔaqpA-HyPer compared with the WT-HyPer strain, whereas for each strain, higher HyPer fluorescence intensity was always detected in 10 ml compared with 40 ml cultures. The growth and cellular H₂O₂ measurements indicated that the aquaporin protein plays a significant role in protecting the bacterium from oxidative stress that is imposed by its own H₂O₂ production.

To examine the survivability of the ΔaqpA mutant among its parental WT strain, we co-cultured the two under higher and lower O₂ contents, respectively, and monocultures of the two strains were included as controls. By inoculating the same cell amounts of ΔaqpA and the WT strain into 40 ml and 10 ml BHI broth contained in a 100-ml flask, respectively, and until the stationary phase, the co-cultures and monocultures in 10-fold serial dilutions were plated on BHI agar plate with or without 1 mg/ml kanamycin. Significantly lower cell numbers were counted for the aqpA mutant (62 ± 3 × 10⁵ cells/ml) in the 10 ml co-culture compared with the WT strain (22 ± 8 × 10⁶ cells/ml). No significant difference was found between the ΔaqpA (60 ± 12 × 10⁵ cells/ml) and WT strain (65 ± 15 × 10⁶ cells/ml) in the 40 ml BHI co-culture. Furthermore, upon three consecutive subcultivations in 10 ml BHI, the percentage of the So-aqpA mutant gradually decreased from 21.7 to 1.2% in the first and third subculture of the co-culture (Fig. 6C), whereas cell numbers in the monospecies culture remained unchanged during subculturing. This further emphasizes the physiological significance of the aquaporin, which endows the bacterial cells with a selective advantage in intraspecies competition.

Previously, we found that S. oligofermentans overcompeted the caries-pathogen Streptococcus mutans using excreted H₂O₂, which is produced from the ample lactate generated by S. mutans so establishes a counterrattack strategy (23). To determine the contribution of So-AqpA to this interspecies competition, the WT strain and ΔaqpA mutant were respectively spotted adjacent to S. mutans on TPYG plates. Fig. 6D shows that the ΔaqpA mutant grew poorer and only slightly suppressed S. mutans compared with the WT strain. To further quantify the inhibition of WT and ΔaqpA strain against S. mutans, the two strains were co-incubated with S. mutans as a mix-species culture, by including S. mutans single-species culture as control. After 24 h incubation, the cfu of S. mutans was counted based on its different colony appearance from that of S. oligofermentans (23). Compared with the cell number (18 ± 2 × 10⁷ cells/ml) in monoculture, only 45% of live S. mutans (81 ± 11 × 10⁴ cells/ml) was detected in the co-culture with S. oligofermentans WT strain, whereas the live cells (145 ± 13 × 10⁵ cells/ml) were about 80% in the co-culture with ΔaqpA, thus the H₂O₂ excreted through So-AqpA can enhance about 35% inhibition effect of S. oligofermentans.

Discussion

In recent years, the physiological importance of aquaporin-facilitated transmembrane diffusion of H₂O₂, particularly in redox signaling, has been acknowledged in animals and plants (14–16). Yet, such information about the prokaryotic AQPs is almost missing. In this study, by using the ample H₂O₂-producing bacterium S. oligofermentans as a model, we demonstrated that a bacterial aquaporin, So-AqpA, functions to facilitate H₂O₂ transmembrane diffusion. The function not only detoxifies the endogenous H₂O₂ but also promotes the bacterium’s intraspecies and interspecies competitive abilities. Notably, H₂O₂ significantly induces So-aqpA expression at both the mRNA and protein levels, and two redox transcriptional regulators, PerR and MntR, are involved in the H₂O₂ induction. Therefore, So-AqpA is most likely an intrinsic peroxiporin as named by Henzler and Steudle (36) and is the first reported bacterial AQP with physiological importance.

H₂O₂, as a by-product, is generated in all metabolic pathways with the involvement of oxygen, but this oxidant molecule is scavenged rapidly by catalase in aerobic organisms (22, 37, 38). However, in the catalase-negative lactic acid bacteria like lactobacilli and streptococci, H₂O₂ efflux is an effective approach for detoxification. Although these bacteria carry genes encoding both AQPs and GLPs, so far only one in vitro study indicates that three GLPs from Lactobacillus plantarum promote H₂O₂ sensitivity when they are heterogeneously expressed in yeast (21). Whether these GLPs act as H₂O₂ facilitators and their physiological importance in bacteria remain unknown. The present study demonstrated a water-facilitator type of AQP in an ample H₂O₂-producing streptococcus acting as a H₂O₂ facilitator and contributing to H₂O₂ detoxification. However, inactivation of the gene did not improve streptococcus hypertonic growth (data not shown), thus suggesting that So-AqpA is not primarily a water facilitator.

Remarkably, H₂O₂ induces the expression of So-aqpA, so indicating that it is an intrinsic peroxiporin (Figs. 4 and 5). Two transcriptional regulators, PerR and MntR, which are specifically involved in regulation of the cellular redox state, are involved in the H₂O₂ induction, and MntR is determined to be a direct regulator. It is also found that H₂O₂ induces a human water facilitator AQP, aquaporin-4; however, the oxidant appears not to directly induce the AQP synthesis but through H₂O₂-promoted phosphorylation of Cav1, which can indirectly modulate AQP4 subcellular distribution (39). In contrast, H₂O₂ induction of the So-aqpA expression appears to be at the transcriptional level and is mediated by the global H₂O₂-responsive regulators. Thus, H₂O₂-induced So-AqpA synthesis could be a strategy used by the catalase-lacking streptococci for dealing with the endogenous H₂O₂; when at higher levels, H₂O₂ can be speedily exported for detoxification with the assistance of large amounts of So-AqpA. Noticeably, a relatively lower H₂O₂ induction was found on synthesis of So-AqpA protein than the transcription of the gene (Figs. 4 and 5). This discrepancy could be because in general the cellular proteins have longer life spans than mRNAs, so larger alteration could be detected for transcript abundance when bacteria encounter changed environments. In addition, posttranscriptional or posttranslational regulation could occur for bacterial AQPs gene expression, similar as reported for the eukaryotic AQPs (9, 10, 11).

The substrate selectivity of AQPs is based on the proper substrate size and formation of energetically favorable hydrogen bonds between substrates and AQP amino acid residues in the substrate path (8, 40). Because water and H₂O₂ molecules possess similar physicochemical properties (41), it is predicted that the water-permeable AQPs also facilitate H₂O₂ diffusion. This
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prediction is supported by some families of AQPs, such as the human AQP8 and AQP1, that are primarily water facilitators but also function in H$_2$O$_2$ permeation through cell membranes (42). In addition, the two molecules appear to pass through the same substrate channel, as transports of them are inhibited by the same amino acid mutations in eukaryotic AQPs (14). However, we cannot conclude that all AQPs transport H$_2$O$_2$, as the plant AQPs AtPIP2;3, AtPIP2;6, and AtPIP2;8 do not transport H$_2$O$_2$. Similarly, *E. coli* aqpZ (b0875) also does not transport H$_2$O$_2$ (20, 43, 44). So-AqpA may therefore represent a distinct type of AQP that acts primarily as a H$_2$O$_2$ facilitator, and its homologs are present in all the *Streptococcus* spp. and other catalase-negative bacteria like *Enterococcus* and *Lactococcus*. Such type of AQPs may also occur in plants, as expression of the *Solanaceae* XIP genes in yeast induce a high sensitivity to exogenous H$_2$O$_2$, even though they have no significant water-transport ability (45). Therefore, the distinct characteristics of the H$_2$O$_2$ facilitators need to be investigated but not limited to comparative analyses of the protein sequences.

Consistent with the majority of AQPs, So-AqpA is a six-transmembrane protein and possesses the conserved Asn-Pro-Ala motif which constitutes the substrate channel. Similar with most water-transporting AQPs, So-AqpA uses Phe-40 as one of the residues in the ar/R-selective filter, which was demonstrated to be the key residue for So-AqpA–mediated H$_2$O$_2$ transport. Differently, So-AqpA protein has no cysteine residue, which is present in almost all the eukaryotic AQPs (29, 46). This unique feature of the streptococcal aquaporins implies that it could be serve as a potential drug target for controlling infection of the pathogenic streptococci.

Collectively, this work reports a bacterial aquaporin that functions as a dedicated H$_2$O$_2$ facilitator (peroxiporin) and has important physiological roles for streptococci by detoxifying the endogenous H$_2$O$_2$ and endowing it intraspecies and interspecies competitive advantages.

**Experimental procedures**

**Bacterial strains and culture conditions**

*S. oligofermentans* AS 1.3089 (47) and its derivative strains (Table S1) were grown in brain heart infusion (BHI) broth (BD Difco, Franklin Lakes, NJ) statically or anaerobically under 100% N$_2$. Spectinomycin (1 mg ml$^{-1}$) or kanamycin (1 mg ml$^{-1}$) was used to select transformants.

**Construction of genetic strains**

All primers used in this study are listed in Table S1. So-aqpA and So-aqpB deletion strains were constructed using the PCR ligation method (48). The H$_2$O$_2$ reporter HyPer gene was amplified from the pHPer-N1 plasmid, which was kindly provided by Prof. Jiangyun Wang at the Institute of Biophysics, Chinese Academy of Sciences, and fused to the *S. oligofermentans* lactate dehydrogenase gene (*ldh*) promoter by overlapping PCR; meanwhile, the *So-aqpA* promoter and coding gene were PCR amplified. The purified PCR products were integrated into the compatible sites of pIB166 or pDL278 plasmids, and the correct recombinant plasmids pDL278-HyPer or pIB166-aqpA were transformed into the WT strain or *ΔaqpA* mutant to produce HyPer or *So-aqpA* ectopically expressed strains. So-AqpA F40A, I165A, L174A, or R180A mutations were introduced into the pHIB166-aqpA plasmid using a site-directed gene mutagenesis kit (Beyotime Biotechnology Co., Shanghai, China), and the correct constructs were transformed into the Δ*aqpA* mutant. The PCR-amplified monomeric gene sequence of the photoactivatable fluorescent protein mMAPle3, which was kindly provided by Xiaowei Zhuang (Harvard University), was 3’ fused to the *So-aqpA* gene by overlapping PCR. Meanwhile, the F40A mutated *So-aqpA* gene was PCR amplified from the pHIB166-aqpAF40A plasmid, and the purified PCR products were integrated into the *S. oligofermentans* WT genomic DNA via double crossover homologous recombination using kanamycin, amplified from plasmid pALH124 (49), as a selective marker to obtain aqpAF40A and *aqpA*-mMAPle3 strains. pDL278-HyPer was transformed into the aqpAF40A strain to obtain aqpAF40A-HyPer.

**Detection of intracellular hydrogen peroxide by HyPer imaging**

Mid-exponential phase HyPer reporter cells were pelleted, washed with PBS twice, and resuspended in 100 μl of PBS in a 1.5 ml Eppendorf tube. After exposure to air for 30 min in the dark at room temperature, 40 μl of cells were placed on a glass slide (25 × 75 mm, 1- to 1.2-mm thick), covered with a coverslip (14 × 14 mm, 0.17-mm thick), and then visualized under a confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems, Buffalo Grove, IL). Excitation was provided at 488 nm, with emission collected from a range of 500 to 600 nm. The gray values of 25 ROI by each containing five cells from each sample were measured using Leica Application Suite (LAS) Advanced Fluorescence software.

**Palm imaging**

The aqpA-mMAPle3 strain was cultured statically and anaerobically. Cells of the static culture were collected at the indicated time by centrifugation, washed twice, and resuspended in PBS. The anaerobic cultured cells were collected when OD$_{600}$ reached ~0.5. One aliquot was treated with 40 μM H$_2$O$_2$ for 20 min and another aliquot was treated with the same volume of H$_2$O and used as a control. After PBS washing twice, cells were resuspended in PBS. All cells were exposed to air for 30 min in the dark at room temperature, then the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed three times with PBS, and observed using PALM imaging. PALM imaging was performed using a Nikon TiE inverted microscope equipped with a 100 × oil-immersion objective (Nikon, PLAN APO, 1.49 NA) and an EMCCD camera (Andor-897). A 405-nm laser (Coherent, 100 milliwatt), 488-nm laser (Coherent, 100 milliwatt), and 561-nm laser (Coherent, 50 milliwatt) were used to either photoconvert or excite the fluorophores. AqpA-mMAPle3 was activated with a continuous 405-nm laser, which was slowly increased for optimal photoconversion rates and excited with a constant 561-nm laser (~2 kilowatts/cm$^2$). 100 nm Tetraspeck beads (Invitrogen) were used to calibrate for stage drift during data acquisition. Construction of super-resolution images was performed using Insight3 software, kindly provided by Dr. Bo Huang (University of California San Francisco). PALM data analysis such as drift
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correction and image rendering was carried out using custom-written MATLAB scripts.

**Determination of the excreted hydrogen peroxide in culture**

Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) in culture suspension was quantified as described previously (35). Briefly, 650 \(\mu\)l of culture supernatant was added to 600 \(\mu\)l of solution containing 2.5 mM 4-amino-antipyrine (4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one; Sigma) and 0.17 mM phenol. The reaction proceeded for 4 min at room temperature; horseradish peroxidase (Sigma) was then added to a final concentration of 50 milliunits/ml in 0.2 mM potassium phosphate buffer (pH 7.2). After 4 min incubation at room temperature, optical density at 510 nm was measured with a Unico 2100 visible spectrophotometer (Shanghai, China). The measurements were done for triplicate samples and repeated at least three times.

**Quantitative PCR**

Total RNA was extracted from the mid-log-phase (OD\textsubscript{600} ~0.4 to 0.5) cultures of tested strains using TRizol reagent (Invitrogen) as recommended by the suppliers. After quality confirmation on 1% agarose gel, RNA extracts were treated with RNase-free DNase (Promega). cDNAs were generated from 2 \(\mu\)g of total RNA with random primers using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) according to the supplier’s instructions and used for qPCR amplification with the corresponding primers (Table S1). Amplifications were performed with a Mastercycler ep realplex2 (Eppendorf AG, Hamburg, Germany). To estimate copy numbers of the So-aqpA mRNA, a standard curve of the So-aqpA 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 number of copies of So-aqpA transcript per 1000 16S rRNA copies is shown. All the measurements were done for triplicate samples and repeated at least three times.

**Northern blotting**

Total RNA was run on 5% Urea-PAGE for 100 min at 250 volts on ice. RNAs in the gel were transferred to positively charged nylon membranes (GE Healthcare) and cross-linked by using GS Gene Linker™ UV Chamber. The membranes were pre-hybridized in buffer (5 \(\times\) SSC, 5 \(\times\) Denhardt’s, 50% (v/v) deionized formamide, 0.5% (m/v) SDS, and 200 \(\mu\)g/ml salmon sperm DNA) for 4 h, and then hybridized with biotin-labeled NoraqpA (Table S1) at 42 °C overnight. The So-aqpA transcript was then detected using Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Scientific).

**Construction of So-aqpA luciferase reporter strain and assay of luciferase activity**

The So-aqpA luciferase reporter was constructed by inserting the promoter fragment of So-aqpA gene into the compatible sites on plasmid pFW5-luc (30) which carries luciferase reporter gene. The recombinant plasmid pFW5-PaqpA-luc was then transformed into the WT strain to produce PaqpA-luc strain. For luciferase activity assay, 100 \(\mu\)l of PaqpA-luc cells were collected into 1.5 ml-Eppendorf tubes, exposed to air for 5 min at room temperature, and 25 \(\mu\)l of 1 mM D-luciferin (Sigma-Aldrich) solution (in 1 mM citrate buffer, pH 6.0) was added, and then the assay was performed with a TD 20/20 luminometer (Turner Biosystems, Sunnyvale, CA). The optical density of the samples (OD\textsubscript{600}) was measured using a 2100 visible spectrophotometer (Unico, Shanghai, China) and used to normalize the luciferase activity. All the measurements were done on triplicate samples and repeated at least three times.

**EMSA**

The So-aqpA promoter fragment was PCR amplified using a biotin-labeled primer pair of aqpAEMSAS/AfpAEMSA (Table S1). EMSA was performed using Light Shift Chemiluminescent EMSA Kit (Pierce). Briefly, 0.1 nM biotin-labeled dsDNA probe was mixed with various amounts of MntR protein (0–200 nM) in the binding buffer (10 mM Tris-HCl, pH 8.0, 5% glycerol, 50 mM NaCl, 10 \(\mu\)g/ml BSA, 2 ng/\(\mu\)l poly (di-dC) and 0.1 mM MnCl\textsubscript{2}). The reaction mixtures stayed at 30 °C for 30 min, and then were electrophoresed on 8% polyacrylamide gel on ice. The DNA-protein complex was transferred onto a nylon membrane and detected by Chemiluminescent Nucleic Acid Detection Module kit (Thermo Scientific).

**Heterologous expression in Saccharomyces cerevisiae and observation of GFP fluorescence**

The *S. oligofermentans* So-aqpA gene was PCR amplified or fused to the green fluorescence protein (sgFPP) gene. Purified PCR products were double digested by HindIII and BamHI, and then integrated into the compatible sites of the pYES2 yeast expression vector (Thermo Fisher). Correct recombinant pYES2-So-aqpA, pYES2-So-aqpA-gfp, and pYES2 were transformed into *S. cerevisiae* INVSc1 using a yeast transformation kit (Labest Company, Beijing, China) and selected on SC-Ura medium (Coolaber Company, Beijing, China). Correct transformants were verified by plasmid extraction, PCR, and sequencing.

*S. cerevisiae* strains carrying pYES2-So-aqpA-gfp or pYES2 were grown in SC-Ura galactose medium to induce the So-aqpA-gfp gene expression. Then 500 \(\mu\)l cells were pelleted, washed with distilled water twice, and resuspended in 100 \(\mu\)l of distilled water in a 1.5 ml Eppendorf tube. After 30-min exposure to air in the dark at room temperature, 40 \(\mu\)l of cells were placed on a glass slide (25 \(\times\) 75 mm, 1- to 1.2-mm thick), covered with a coverslip (14 \(\times\) 14 mm, 0.17-mm thick), and then visualized under a confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems, Buffalo Grove, IL). Excitation was provided at 488 nm, and emission was collected from a range of 500 to 600 nm.

**Hydrogen peroxide sensitivity assay of S. cerevisiae**

Overnight cultures of *S. cerevisiae* INVSc1 strains carrying pYES2-So-aqpA or empty vector pYES2 grown in SC-Ura glucose were diluted into SC-Ura galactose medium to a final OD\textsubscript{600} of 0.4, and then induced at 30 °C for 6 h to allow So-aqpA gene expression. After induction, the two strains were diluted to OD\textsubscript{600} of 0.01, and 10 \(\mu\)l of the dilutions were spotted onto SC-Ura galactose agar plates containing various concentrations of H\textsubscript{2}O\textsubscript{2}. For minimal inhibitory concentration assay, 100-\(\mu\)l
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dilutions were added into a 96-well cell culture plate (Nunc), and 100 µl of 24 mM H2O2 were added to the first well and mixed, then 100 µl were taken out and added into the second well. Therefore, 2-fold serially diluted H2O2 concentration until 0.09 mM was generated. Growth was recorded after 6 days at 30 °C.

Assay of So-AqpA promoting Escherichia coli to uptake H2O2
Overnight cultures of E. coli carrying recombinant pIB166-aqpA or vacant vector pIB166 were 1:100 diluted into fresh LB and grown at 37 °C. After OD600 reached ~0.60, cells were collected by centrifugation at 5000 rpm for 10 min and washed twice with PBS. Cells were then diluted to OD600 0.1 with 10 ml of PBS, and H2O2 was added to a final concentration of 150 µM. 750 µl of cell suspension were centrifuged at 12,000 rpm for 2 min at indicated time points, and the residual H2O2 content in the supernatant was determined.

Statistical analysis
One-way ANOVA followed by Tukey's post hoc test or Student's t test was performed by using PASW Statistics 18 or Excel, respectively. The level of significance was determined at p < 0.05.

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