A Novel Aquaporin Subfamily Imports Oxygen and Contributes to Pneumococcal Virulence by Controlling the Production and Release of Virulence Factors

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ABSTRACT Aquaporins, integral membrane proteins widely distributed in organisms, facilitate the transport of water, glycerol, and other small uncharged solutes across cellular membranes and play important physiological roles in eukaryotes. However, characterizations and physiological functions of the prokaryotic aquaporins remain largely unknown. Here, we report that Streptococcus pneumoniae (pneumococcus) AqpC (Pn-AqpC), representing a new aquaporin subfamily possessing a distinct substrate-selective channel, functions as an oxygen porin by facilitating oxygen movement across the cell membrane and contributes significantly to pneumococcal virulence. The use of a phosphorescent oxygen probe showed that Pn-AqpC facilitates oxygen permeation into pneumococcal and Pn-AqpC-expressing yeast cells. Reconstituting Pn-AqpC into liposomes prepared with pneumococcal and Escherichia coli cellular membranes further verified that Pn-AqpC transports O2 but not water or glycerol. Alanine substitution showed that Pro232 in the substrate channel is key for Pn-AqpC in O2 transport. The deletion of Pn-aqpC significantly reduced H2O2 production and resistance to H2O2 and NO of pneumococci, whereas low-H2O2 treatment helped the ΔPn-aqpC mutant resist higher levels of H2O2 and even NO, indicating that Pn-AqpC-facilitated O2 permeation contributes to pneumococcal resistance to H2O2 and NO. Remarkably, the lack of Pn-aqpC alleviated cell autolysis, thus reducing pneumolysin (Ply) release and decreasing the hemolysis of pneumococci. Accordingly, the ΔPn-aqpC mutant markedly reduced survival in macrophages, decreased damage to macrophages, and significantly reduced lethality in mice. Therefore, the oxygen porin Pn-AqpC, through modulating H2O2 production and pneumolysin release, the two major pneumococcal virulence factors, controls the virulence of pneumococcus. Pn-AqpC orthologs are widely distributed in various pneumococcal serotypes, highlighting that the oxygen porin is important for pneumococcal pathogenicity.

IMPORTANCE Pneumococcus is the leading cause of community-acquired pneumonia, bacteremia, and meningitis. This work reports that a novel aquaporin subfamily represented by pneumococcal Pn-AqpC functions as an oxygen porin facilitating O2 influx into cells. Importantly, by mediating O2 influx, Pn-AqpC controls the production and release of H2O2 and Ply, the two major pneumococcal virulence factors. Moreover, by enhancing endogenous H2O2 production, Pn-AqpC significantly increases pneumococcal resistance to H2O2 and even NO, the major bactericidal chemical produced by macrophages. Consequently, the deletion of Pn-aqpC markedly decreased pneumococcal survival in macrophages and reduced damage to macrophages. Accordingly, the ΔPn-aqpC mutant significantly reduced pneumococcal virulence by controlling the production and release of virulence factors. Therefore, this work highlights that aquaporins, particularly Pn-AqpC, play important physiological roles in pneumococcal virulence.
**RESULTS**

**Pneumococcus possesses an atypical aquaglyceroporin, Pn-AqpC, representing a novel aquaporin subfamily.** Using the *S. oligofermentans* aquaporin So-AqpA (I872_01445) as a probe to query the genome of *S. pneumoniae* D39, an encapsulated serotype 2 strain, three genes (SPD_1320, SPD_1569, and SPD_2011) were hits at amino acid identities of 26%, 95%, and 31%, respectively. Phylogenetically, SPD_1569 clustered with So-AqpA and the water-facilitating aquaporin AqpZ of *E. coli* and thus was assigned as Pn-AqpA; SPD_2011 was clustered with the glycerol facilitator GlpF of *E. coli* and assigned as Pn-AqpB. However, SPD_1320 and some glycerol facilitators
from other lactic acid bacteria clustered to form a separate branch distantly related to *E. coli* GlpF (Fig. 1A) and thus assigned as Pn-AqpC. Aquaporins in the Pn-AqpC-affiliated branch were tentatively named atypical aquaglyceroporins and could represent a new aquaporin subfamily. These aquaporins congruously possess YVPR as the substrate-selective residues (Fig. 1B and C). Furthermore, the ar/R region diameter size in each Pn-AqpC monomer (Fig. S2A and B) was between those of the *E. coli* water (Fig. S2C)- and glycerol (Fig. S2D)-transporting aquaporins, implying a different substrate spectrum.

![Phylogenetic analysis identifies a new subfamily of aquaporins with unique substrate-selective residues.](image)

### FIG 1

Phylogenetic analysis identifies a new subfamily of aquaporins with unique substrate-selective residues. (A) A phylogenetic tree based on the amino acid sequences of the aquaporin orthologs was constructed using the maximum likelihood method with 1,000 replicates. The bar of 0.05 represents evolutionary distance. A dotted line frames the new subfamily of aquaporins (atypical). (B) The amino acid sequences of the aquaporins in panel A were aligned using ClustalW. The *E. coli* GlpF (b3927) secondary structure (top panel) and the amino acid positions of D39 Pn-AqpC (SPD_1320) (top row of the bottom panel) are shown. Asterisks specify the ar/R region residues, and black lines frame YVPR of the atypical aquaglyceroporins. (C) Distribution of the new subfamily of aquaporins among Lactobacillales. Phylogenetic analysis was implemented as described above for panel A on at most five protein sequences of each genus. Numbers inside parentheses are those containing the YVPR-type aquaglyceroporins; branches within the dark blue pie represent pneumococcal strains.
Therefore, we investigated the physiological functions of Pn-AqpC, a representative of the new aquaporin subfamily.

The absence of Pn-aqpC reduces H$_2$O$_2$ production but promotes the aerobic growth of pneumococcus. To probe the physiological functions of Pn-AqpC, Pn-aqpC was deleted in S. pneumoniae D39 and its nonencapsulated mutant R6. By reference to S. oligofermentans So-AqpA that facilitates H$_2$O$_2$ permeation, the function of Pn-AqpC in H$_2$O$_2$ transport was first evaluated in R6 and its ΔPn-aqpC mutant carrying a specific cellular H$_2$O$_2$ reporter HyPer gene (24). However, the HyPer reporter detected similar H$_2$O$_2$ influx into ΔPn-aqpC and wild-type (WT) cells when provided exogenous H$_2$O$_2$ (Fig. S3A, bottom), thus excluding a role of Pn-AqpC in H$_2$O$_2$ permeation, whereas significantly lower fluorescence was found in ΔPn-aqpC cells when no exogenous H$_2$O$_2$ was provided (Fig. S3A, top), indicating reduced H$_2$O$_2$ production when Pn-AqpC is absent.

Next, the H$_2$O$_2$ yields of the R6 and D39 wild-type strains and ΔPn-aqpC mutants were assayed in static cultures of 20 and 30 ml of brain heart infusion (BHI) broth in 100-ml flasks, respectively, which build gradient dissolved O$_2$ levels. Surprisingly, the two ΔPn-aqpC mutants both achieved better growth and lower H$_2$O$_2$ yields than the wild-type strains in the two culture volumes (Fig. 2A), while the Pn-aqpC-complemented strains (Pn-aqpC-com) recovered the wild-type phenotype (Fig. 2A). This suggested that Pn-AqpC might facilitate the transmembrane diffusion of O$_2$, a substrate for H$_2$O$_2$ formation. As similarly reduced cellular H$_2$O$_2$ and elevated aerobic growth were determined for the ΔPn-aqpC mutants of the nonencapsulated R6 and encapsulated D39 strains, the Pn-aqpC deletion-caused phenotype changes may not be related to the capsular polysaccharides; thus, strain R6 was investigated for the physiological functions per se of Pn-AqpC in the following experiments, except for animal studies.

Pneumococcal Pn-AqpC facilitates O$_2$ transport into cells. To verify the function of Pn-AqpC in transporting O$_2$, a dissolved oxygen microsensor Oxy meter (Unisense, Denmark) was used to measure the residual O$_2$ contents in stationary-phase cultures of pneumococci growing in 40 ml BHI broth in a 50-ml centrifuge tube, and 1.5- to 1.6-fold-lower O$_2$ consumption was determined for the R6 ΔPn-aqpC mutant than for the wild-type and Pn-aqpC-com strains (Fig. 2B). Similarly, using a phosphorescent oxygen probe, a 2.6-fold-lower O$_2$ uptake rate within 3 min was determined for the ΔPn-aqpC mutant than for the wild-type strain, while the Pn-aqpC-com strain recovered O$_2$ uptake levels of the wild type (Fig. 2C). Of note, the H$_2$O$_2$ yields in the wild type (122 ± 3 μM) and the ΔPn-aqpC mutant (18 ± 2 μM) were much lower than the theoretical stoichiometry (258 μM and 173 μM) calculated from the Oxy meter-measured oxygen consumption in the corresponding strains (258 ± 10 μM and 173 ± 21 μM). This indicates that other O$_2$ consumption pathways are present, such as NADH oxidase (Nox) catalyzing the oxidation of NADH to NAD$^+$ and H$_2$O by using O$_2$ as an electron acceptor (25). Therefore, we deleted nox in the wild type and the ΔPn-aqpC mutant, which reduced O$_2$ consumption by 30 ± 3.5 and 34 ± 5.8 μM, respectively. Nevertheless, there should have been other unknown pathways consuming the remaining 106 μM and 121 μM O$_2$ in the wild type and the ΔPn-aqpC mutant, respectively. Moreover, Pn-aqpC deletion did not alter the expression of spxB and lctO, which encode the two major H$_2$O$_2$ production enzymes pyruvate oxidase and lactate oxidase, respectively (Fig. S4). This indicates that the reduced H$_2$O$_2$ production in the ΔPn-aqpC mutant was due to decreased O$_2$ influx instead of reduced expression of the H$_2$O$_2$ production genes.

The O$_2$-facilitating function of Pn-AqpC was further verified by the coexpression of Pn-aqpC and the sperm whale myoglobin (Mb) gene in Saccharomyces cerevisiae INVSc1. Single-Mb-gene-expressing INVSc1-myo and INVSc1 strains were used as controls. In addition, an INVSc1-Pn-aqpC-gfp strain carrying a superfolder green fluorescent protein (GFP) gene (sfgfp) fusion to Pn-aqpC was constructed. The expressions of Pn-AqpC and Mb in S. cerevisiae were verified by Western blotting (Fig. SSA), and the cytoplasmic membrane localization of heterologously expressed Pn-AqpC in S. cerevisiae was confirmed via confocal microscopy examination (Fig. SSB). By measuring the characteristic oxymyoglobin (MbO$_2$) absorption at 541 nm in yeast protoplasts (26) (Fig. SSC) and purified MbO$_2$ (Fig. SSD), a significant MbO$_2$ increase (ΔA$_{max}$/optical

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FIG 2 Pneumococcal Pn-AqpC acts as an oxygen porin. (A) Growth of R6 (top) and D39 (bottom) wild-type (WT), ΔPn-aqpC, and Pn-aqpC-complemented (com) strains cultured statically in 20 and 30 ml of BHI broth in a 100-ml flask. H₂O₂ (millimolar) accumulations in the stationary-phase cultures are listed in the table at the bottom, with those of strains R6 and D39 in the top and bottom rows, respectively. (B) An oxygen microsensor Oxy meter was used to measure the residual dissolved O₂ in the stationary-phase cultures of the R6 wild type and its derivatives grown in 40 ml BHI broth in a 50-ml centrifuge tube. Oxygen consumption per OD₆₀₀ of cell mass was calculated by comparison to 283 μM O₂ in fresh medium. *, significantly different from other strains. (C) Mid-exponential-phase cultures of the R6 wild type and its derivatives were exposed to air, and the residual O₂ in the culture was measured using a phosphorescent oxygen probe. *, significantly different from the wild-type and Pn-aqpC-complemented strains. (D) Protoplasts of S. cerevisiae INVSc1 and INVSc1 carrying the myoglobin gene (INVSc1-myo) or coexpressed with Pn-aqpC (INVSc1-Pn-aqpC-myo) were exposed to air, and the A₅₄₁ increase per unit of biomass (ΔA₅₄₁/OD₆₀₀) was calculated. (E and I) The residual O₂ contents in the culture were determined using a phosphorescent oxygen probe. # and *, significantly different from INVSc1 and INVSc1-myo, respectively. (F and G) The recombinant GST–Pn-AqpC–His protein was purified (GST-tag), digested with 100 U thrombin to remove the GST tag to obtain Pn-aqpC–10×His (His-tag) (F), and reconstituted into pneumococcal (Spn) and E. coli liposomes (G). The Pn-AqpC proteins were examined on a 12% SDS-PAGE gel. The protein ladder is shown at the left. Black and gray arrows indicate the macromolecular aggregate and monomer of Pn-AqpC protein, respectively. (H and I) Pn-AqpC facilitating O₂ permeation across pneumococcal (Spn) and E. coli liposomes (I) was determined using a phosphorescent oxygen probe. *, the fluorescence intensity change at the respective time points was significantly different from that of the Pn-AqpC-devoid liposomes. (J) The R6 wild-type, ΔPn-aqpC, and Pn-aqpC-complemented (Pn-aqpC-com) strains were cultured (Continued on next page)
density at 600 nm (OD_{600}) was found in Pn-aqpC-expressing yeast compared to Pn-aqpC-devoid INVSc1 (Fig. 2D). Accordingly, the phosphorescent oxygen probe detected a more rapid decrease of the cultural O_{2} content of INVSc1-Pn-aqpC-myO than that of INVSc1-myO (Fig. 2E). These results collectively showed that Pn-AqpC facilitates oxygen permeation.

**Pn-AqpC facilitates oxygen transport across proteoliposomes.** To further confirm that Pn-AqpC facilitates the transport of O_{2} and other substrates, the recombinant glutathione S-transferase (GST)–Pn-AqpC–10×His protein was purified in the detergent octylglucoside (OG), and the GST tag was then removed (Fig. 2F). Two Pn-AqpC–10×His protein bands of ~32 kDa and ~68 kDa were identified as Pn-AqpC by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Fig. 2F and G; Table S1). Compared to the E. coli AqpZ tetramers that hardly dissociated with 1% SDS due to the strong hydrophobic characteristics (27), the 68-kDa protein was assumed to be a macromolecular aggregate, while the 32-kDa protein was assumed to be a monomer. The purified Pn-AqpC–10×His protein was reconstituted into the membrane lipid of pneumococci and E. coli (Fig. 2G). The phosphorescent oxygen probe was then wrapped within the proteoliposomes and Pn-AqpC-devoid liposomes to detect oxygen contents. This detected 3.2- and 1.5-fold-higher O_{2} influx in the first 40 s into the pneumococcal and E. coli proteoliposomes than the respective Pn-AqpC-devoid liposomes, respectively (Fig. 2H and I). Notably, 3-fold-higher O_{2} influx was determined for Pn-AqpC-devoid E. coli than for the pneumococcal liposomes within 80 s (Fig. 2H and I). These results demonstrated that Pn-AqpC sped up O_{2} permeation across the cellular membrane, and the pneumococcal membrane appears to have lower O_{2} permeability than that of E. coli.

Using a stopped-flow apparatus, Pn-AqpC facilitating water and glycerol permeation was measured based on osmosis-driven permeability. However, similar initial rates (k) were determined for proteoliposomes and Pn-AqpC-devoid liposomes in water and glycerol permeation (Fig. S3B to E). Therefore, Pn-AqpC specifically facilitates the permeation of O_{2} but not water or glycerol.

**Pn-AqpC acts as a prominent oxygen facilitator under lower oxygen levels.** Given that O_{2}, particularly at higher concentrations, permeates freely through the cytoplasmic membrane, the range of O_{2} levels wherein Pn-AqpC plays a role in facilitating O_{2} was tested by growing the R6 wild-type, ΔPn-aqpC mutant, and Pn-aqpC-com strains under gradient shaking speeds. Although the three strains exhibited similar growth rates under static and 200-rpm shaking conditions, the ΔPn-aqpC mutant grew slightly and markedly better under 60- and 120-rpm shaking conditions (Fig. 2J, left). Accordingly, significantly lower levels of H_{2}O_{2} were produced in the ΔPn-aqpC mutant cultured under static, 60-rpm, and 120-rpm conditions, but similar H_{2}O_{2} levels were generated in 200-rpm shaking cultures of wild-type, ΔPn-aqpC mutant, and Pn-aqpC-com strains (Fig. 2J, right). This shows that Pn-AqpC exerts an O_{2} facilitator role when the bacterium lives under lower O_{2} levels. Next, the role of Pn-AqpC in the H_{2}O_{2} production of pneumococci under 5% O_{2} was tested by mimicking the O_{2} concentrations in the human lower respiratory tract within the mucus layer or in close contact with pulmonary epithelial cells (11). The same amounts of the wild-type, ΔPn-aqpC mutant, and Pn-aqpC-com cultures were spotted onto a BHI agar plate and incubated in an O_{2} control in vitro glove box (Coy Laboratory Products). Compared with the wild-type and complemented strains, the ΔPn-aqpC mutant produced almost undetectable H_{2}O_{2} under 5% O_{2} (Fig. 2K); therefore, Pn-AqpC could play an important role in pneumococcal infection by facilitating O_{2} import for H_{2}O_{2} production, one of the pneumococcal virulence factors.

**FIG 2** Legend (Continued)

with shaking at different speeds. The optical density at 600 nm (left) and H_{2}O_{2} (millimolar) accumulating in stationary-phase cultures (right) were determined. # and * significantly different from the wild-type and Pn-aqpC-com strains and the respective static cultures, respectively. (K top and middle) Ten microliters of the mid-exponential-phase cultures in panel J was spotted onto a BHI agar plate and incubated in a 5% O_{2} environment for 10 h (top), and H_{2}O_{2} was then determined (middle) as described in Materials and Methods. (Bottom) Chemical H_{2}O_{2} with known concentrations was used as a reference. All experiments were conducted three times, and averages ± standard deviations (SD) (A, B, D, and J) or averages ± standard errors of the means (SEM) (C, E, H, and I) from one independent assay on triplicate samples are shown. For panels B to D and J, one-way ANOVA and Tukey’s test were performed; for panels E, H, and I, Student’s t test was performed (P < 0.05).
The substrate-selective residue Pro232 is essential for Pn-AqpC in facilitating O₂ permeation. To determine the key substrate-selective residues for Pn-AqpC in O₂ transport, alanine substitution was performed for each YVPR on the shuttle vector pDL278-Pn-aqpC and then transformed into the ΔPn-aqpC mutant to obtain the Tyr49A, Val223A, Pro232A, and Arg238A strains. The four mutants and the Pn-aqpC-com strain were grown in 10 ml BHI broth, and H₂O₂ yields in the stationary-phase cultures were used as a proxy for O₂ uptake. Threefold-reduced H₂O₂ yields were determined for the Pro232A mutant (0.48 ± 0.39 mM) compared with the Pn-aqpC-com strain (1.46 ± 0.21 mM), whereas no significant change was observed for the Tyr49A (1.38 ± 0.17 mM), Val223A (1.41 ± 0.18 mM), and Arg238A (1.27 ± 0.26 mM) strains.

Furthermore, using the oxygen microsensor Oxy meter, 1.4-fold-lower O₂ consumption for the Pro232A mutant was determined than for the wild-type and Pn-aqpC-com strains (Fig. 2B). The phosphorescent oxygen probe also measured 3.3- and 2.3-fold-lower O₂ consumption rates within 3 min for the Pro232A mutant than for the wild-type and Pn-aqpC-com strains, respectively (Fig. 2C). These results revealed that Pro232 is the key residue of Pn-AqpC in O₂ transport.

Elevated Pn-AqpC protein contents occur in aerobic cultures. Given the role of Pn-AqpC in O₂ transport, its synthesis in response to O₂ was determined. Using the phototactivated localization microscopy (PALM) superresolution imaging technique (28), numbers of Pn-AqpC proteins per cell were quantified in anaerobically, statically, and 120-rpm-shaking-grown cultures of the Pn-aqpC-mMaple3 strain, which carried an mMaple3 protein (29) fusion at the C terminus of Pn-aqpC. Figure 3A shows representative PALM images with mMaple3 fluorescence signals; each image included a 2- to 3-cell constituted cell chain, the typical morphology of pneumococcus. PALM data analysis indicated that the average numbers of Pn-AqpC protein molecules per cell were 23 ± 10 in anaerobic, 56 ± 6 in static, and 94 ± 9 in shaking cultures (Fig. 3A). Catalase treatment did not reduce the Pn-AqpC protein numbers (41 ± 21) in static culture; thus, O₂, but not H₂O₂, seems to induce Pn-AqpC expression.

Oxygen-induced Pn-AqpC expression was further verified by the GFP reporter strain Pn-aqpC-gfp. GFP fluorescence intensities showed a pattern of in anaerobic culture <60 rpm shaking culture <120 rpm shaking culture (Fig. 3B), whereas neither catalase treatment of the static culture nor H₂O₂ pulsing of the anaerobic culture changed the O₂-level-related Pn-AqpC abundances (Fig. 3C). Pn-AqpC was detected exclusively in the cellular membrane fraction (Fig. 3D), confirming its membrane protein identity as predicted by TMHMM (Fig. 51B). These findings indicated that O₂ induces the synthesis of Pn-AqpC.

The absence of Pn-AqpC reduces pneumococcal resistance to H₂O₂ and NO as well as Ply release. Given that endogenous H₂O₂ assists pneumococcus in resisting oxidative stress (7), the ΔPn-aqpC mutant reducing H₂O₂ resistance was presumed to be due to lower H₂O₂ production. As expected, a lower MIC of H₂O₂ was determined for the ΔPn-aqpC mutant (5 mM) than for the wild-type strain (8 mM). Consistently, the growth of ΔPn-aqpC in a BHI plate containing 10 mM H₂O₂ occurred only at a 10⁻⁵ dilution compared with the 10⁻⁶ dilutions of the wild-type and Pn-aqpC-com strains (Fig. 4A). Moreover, only 2.3% of ΔPn-aqpC mutant cells survived the challenge with 10 mM H₂O₂, compared with survival rates of >40% for the wild-type and Pn-aqpC-com strains (Fig. 4B). However, 40 μM H₂O₂ prepulsing significantly increased 10 mM H₂O₂ survival of the ΔPn-aqpC mutant (Fig. 4B), indicating that Pn-AqpC-promoted endogenous H₂O₂ production makes pneumococcus withstand exogenous H₂O₂ challenge.

Next, the role of Pn-AqpC in pneumococcal resistance to NO, another oxidant, was assayed as macrophages employ NO-dependent bactericidal mechanisms to clear infecting bacteria (8). Only 5% of the ΔPn-aqpC cells survived 5 mM NO, compared to about 20% survival of the wild-type and Pn-aqpC-com strains (Fig. 4C), suggesting the involvement of Pn-AqpC in NO resistance. Given that the proteins involved in H₂O₂ resistance also assist E. coli in resisting NO (30), we used 40 μM H₂O₂ to prepulse the three strains. H₂O₂ prepulsing increased the NO survival of the ΔPn-aqpC mutant by 6-fold but had no effect on the survival of the wild-type and Pn-aqpC-com strains...
This shows that lower H₂O₂ levels induce cross-protection of pneumococci from NO stress. Stationary-phase cells of pneumococci are usually autolyzed and thus release Ply (31), a major virulence factor. The deletion of Pn-aqpC appeared to significantly alleviate cell autolysis (Fig. 4D) and so may also reduce Ply release and the hemolytic activity of pneumococcus. To test this, 12-h-post-stationary-phase spent cultures of the wild-type, Pn-aqpC deletion, and complemented strains were 2-fold serially diluted, and horse red blood cells were added. Complete erythrocyte lysis was observed in ≤4-fold dilutions of the wild-type and Pn-aqpC-com cultures, but only partial hemolysis occurred in the 2-fold-diluted ΔPn-aqpC culture (Fig. 4E). Consistently, about 2.3-fold less Ply protein was detected in the 12-h-post-stationary-phase spent culture of the ΔPn-aqpC mutant (Fig. 4F). This shows that Pn-aqpC deletion reduces cell lysis as well as Ply release.

Deletion of Pn-aqpC reduces pneumococcal survival in macrophages and damage to macrophages. Macrophages, the first line of defense of the human immune system, utilize reactive oxygen and nitrogen species to kill invading microbes (8, 9). Given the reduced H₂O₂ production of the ΔPn-aqpC mutant, the effect of the Pn-aqpC deletion on pneumococcal survival in macrophages was investigated. First, 1 × 10⁵ macrophage RAW 264.7 cells were exposed to the nonencapsulated R6 wild-type cells.
type, Pn-aqpC deletion, and complemented strains at a multiplicity of infection (MOI) of 100:1. After 1 h of incubation, the bacterial cells attached to and internalized into macrophages were counted, and after additional 1-h and 1.5-h incubations, surviving pneumococcal cells within macrophages were counted. Although the numbers of viable bacterial cells of the three strains all significantly decreased, 4.5- and 3-fold-lower survival rates of the ∆Pn-aqpC mutant were determined after additional 1-h and 1.5-h incubations, respectively, than for the wild-type and Pn-aqpC-com strains (Fig. 5A). This validates the contribution of Pn-AqpC to pneumococcal survival in macrophages.

To query whether the reduced macrophage survival is caused by the increased NO sensitivity of the ∆Pn-aqpC mutant, diphenyleneiodonium chloride (DPI), an inhibitor of inducible nitric oxide synthase (iNOS) (32), was used to inhibit NO production by macrophages. The addition of DPI increased the number of living cells 4.9- and 5.6-fold for the wild-type and Pn-aqpC-com strains, respectively, whereas it enhanced the survival of the ∆Pn-aqpC mutant 8.4-fold in macrophages (Fig. 5A), indicating that Pn-aqpC deletion, and complemented strains at a multiplicity of infection (MOI) of 100:1.

An O2 Porin Contributes to Pneumococcal Virulence
AqpC-conferred pneumococcus oxidative stress resistance assists its survival in macrophages.

Given that H2O2 induces macrophage death (33), the role of the deletion of Pn-aqpC in pneumococcal damage to macrophages was examined. Upon bacterial

**FIG 5** Deletion of Pn-aqpC reduces pneumococcal survival and damage to macrophages and significantly attenuates virulence to mice. (A) Pneumococcal survival in macrophages was assayed by coincubation of 1 x 10^8 RAW 264.7 cells with the R6 wild type (WT) and derivatives at an MOI of 100:1. After 1 h of incubation, bacterial cells in the culture were removed, those attached to and internalized in macrophages were recorded, and this time point was set as 0 h. After additional 1- and 1.5-h (total, 2- and 2.5-h) incubations in fresh medium containing antibiotics, the pneumococcal cells that survived within macrophages were counted. The nitric oxide (NO) synthetase inhibitor DPI was added to reduce NO production by macrophages. (B to D) Using the same approach as the one described above for panel A, the contribution of Pn-AqpC to R6’s damage to macrophages was evaluated. (B) Live (green)/dead (red) cell staining of macrophages. The addition of 1 KU/ml catalase (cat) was used to assay the role of H2O2. (C) After 16 h of antibiotic treatment, macrophage death (percent) was calculated. (D) Bacterial damage to macrophages was also evaluated by leaked lactate dehydrogenase (LDH) activities. *, significantly different from the respective strain at 0 h (A); & significantly different from the wild-type and Pn-aqpC-com strains; #, significantly different from that without DPI treatment (A) or the respective strain without catalase addition (C and D) (P < 0.05 by one-way ANOVA and Tukey’s test). (E) BALB/c mice (n = 10; female) were intratracheally infected with 1 x 10^7 CFU of statically grown D39 wild-type and ΔPn-aqpC strains with or without 40 μM H2O2 pretreatment and the Pn-aqpC-com strain. PBS-administered mice were included as controls. Survival of the infected mice was monitored for up to 12 days, and representative survival curves from two independent experiments are shown. *, significantly different from the wild-type and Pn-aqpC-com strains (P < 0.05 by a log rank Mantel-Cox test). (F) Histopathological observation of the lung tissues of mice that survived PBS administration and ΔPn-aqpC infection and those that died from wild-type and Pn-aqpC-com infections.

Given that H2O2 induces macrophage death (33), the role of the deletion of Pn-aqpC in pneumococcal damage to macrophages was examined. Upon bacterial
challenging, only 30% of macrophage cells died from coinoculation with the ΔPn-aqpC mutant, compared to 72% and 65% cell death from coinoculation with the wild-type and Pn-aqpC-com strains, respectively (Fig. 5B and C), whereas the addition of 1 KU/ml catalase reduced macrophage damage from the wild type by 13% (59% with versus 72% without catalase) but did not alleviate the damage from the ΔPn-aqpC mutant (Fig. 5B and C), indicating that Pn-AqpC-promoted H2O2 production has some contributions to macrophage death. The roles of Pn-AqpC and H2O2 in damage to macrophages were also verified by the activities of lactate dehydrogenase in the cultures leaked from macrophages (Fig. 5D). However, the catalase-treated wild-type cells still caused significantly higher macrophage death (59%) than the ΔPn-aqpC mutant (30%), implying that Pn-AqpC itself or other Pn-AqpC-impacted factors, possibly the reduced release of Ply, contribute to macrophages death.

Pn-AqpC is required for pneumococcal virulence in a murine pulmonary infection model. Given that H2O2 and Ply are major virulence factors (1, 2), and the deletion of Pn-aqpC not only increased the H2O2 and NO susceptibility of but also reduced Ply release by pneumococci, the contributions of Pn-AqpC to pneumococcal virulence were evaluated in a murine pneumonia infection model. BALB/c mice were intratracheally infected with 1.0 × 10⁷ CFU of D39, its Pn-aqpC deletion mutant (pretreated with or without 40 μM H2O₂), and the Pn-aqpC-com strain. By monitoring mouse survival for 12 days postinfection, we found that the Pn-aqpC deletion significantly enhanced mouse survival to 78%, compared with 22% and 43% survival rates in the wild-type- and Pn-aqpC-com-infected groups, respectively (Fig. 5E), indicating that Pn-AqpC is involved in pneumococcal pathogenicity. Of note, the survival rate of mice infected with the 40 μM H2O₂-pretreated ΔPn-aqpC mutant was reduced to 60%, compared with 78% survival of those infected by the non-H2O₂-pretreated ΔPn-aqpC strain, suggesting that low-H2O₂-induced oxidative resistance enhances pneumococcal pathogenicity in addition to other virulence factors.

Neither was inflammatory cell immersion (Fig. 5F) observed nor were pneumococci recovered from the lung tissue of the surviving mice infected by the ΔPn-aqpC mutant, whereas 1.35 × 10⁸ ± 0.91 × 10⁸ and 1.54 × 10⁸ ± 0.72 × 10⁸ CFU/ml of pneumococci were recovered from the lungs of dead mice infected by the D39 wild-type and Pn-aqpC-com strains, respectively. These data confirmed the contribution of Pn-AqpC to pneumococcal pathogenicity.

DISCUSSION

To date, 13 and 120 aquaporin isoforms have been identified in humans and plants, respectively, and are delineated into three major subfamilies: the classical water-transporting aquaporins, glycerol-transporting aquaglyceroporins, and AQP supergene channel superaquaporins (14, 15, 34). They facilitate the transmembrane diffusion of water, glycerol, H2O2, CO2, and other small uncharged solutes (14–16, 26, 34, 35). Here, we report a new aquaporin subfamily represented by pneumococcal Pn-AqpC, which functions as an oxygen porin to facilitate oxygen uptake. Phylogenetically, the oxygen porins are distantly related to aquaglyceroporins and possess substrate-selective amino acid residues distinct from those of aquaporins and aquaglyceroporins. Importantly, the oxygen porin Pn-AqpC contributes significantly to the pathogenicity of S. pneumoniae. As depicted in Fig. 6, pneumococcal Pn-AqpC, which is increasingly synthesized under conditions of higher O2 contents, facilitates O2 influx into cells and thus promotes H2O2 production by pneumococcus. Endogenous H2O2 helps pneumococci adapt to higher exogenous H2O2 and NO levels; therefore, the deletion of Pn-aqpC reduced the H2O2 and NO resistance of pneumococci. Accordingly, the presence of Pn-AqpC promotes pneumococcal survival in macrophages and possibly other host immune cells. In addition, the absence of Pn-AqpC alleviates pneumococcal autolysis and, thus, Ply release and significantly reduces pneumococcal damage to macrophages. In support of this, the absence of Pn-AqpC significantly attenuated the virulence of pneumococcus in a murine pneumonia model. Thus, the new subfamily of prokaryotic aquaporins, represented by Pn-AqpC, might be virulence-related proteins.
To our knowledge, Pn-AqpC is the first reported oxygen porin with defined physiological functions. Although O2 freely diffuses across the cytoplasmic membrane (36), assays of both in vivo and heterogeneously expressed yeast and in vitro-reconstituted proteoliposomes all determined that Pn-AqpC increases O2 flux across the cellular membrane (Fig. 2), particularly when pneumococci are grown under lower O2 levels (Fig. 2J and K) similar to those in most host environments (11). In addition, the pneumococcal cellular membrane appears to have lower O2 permeability than that of E. coli, highlighting the role of Pn-AqpC in pneumococci, which could require controllable O2 influx for H2O2 synthesis. Enhanced Pn-AqpC contents were found in aerobically grown pneumococcus (Fig. 3), conforming to its oxygen facilitator mission; however, similar Pn-aqpC transcript levels were found in aerobic and anaerobic cultures (data not shown), implying the posttranscriptional regulation of Pn-AqpC expression. So far, an O2-facilitating function has been reported only for human AQP1 and Nicotiana tabacum PIP1;3 when ectopically expressed in yeast (26). They are affiliated with water-type aquaporins and distantly related to Pn-AqpC at very low protein identities (20% and 14%, respectively) and distinct selective filter residues; therefore, prokaryotic Pn-AqpC represents a novel subfamily of aquaporins. Analogous to other aquaporin orthologs, Pn-AqpC forms a tetramer, as indicated by structural homology modeling implemented in SWISS-MODEL (Fig. S2A) and a macromolecular aggregate formed by the purified Pn-AqpC–10×His protein (Fig. 2F and G). Although O2 is predicted to permeate through the central pore of the four monomers of human AQP1 (36), Pro232 in the Pn-AqpC ar/R region has been determined to be crucial for facilitating O2 transport (Fig. 2B and C). Thus, O2 could be transported through the oxygen porin substrate channel in addition to the tetramer central pore. Thus far, only O2, but neither H2O nor glycerol and H2O2, has been verified as the substrate of Pn-AqpC (Fig. 2; see also Fig. S3 in the supplemental material). Of note, distinct from most other reported aquaporins, Pn-AqpC does not contain cysteine residues and thus is not inactivated by mercury chloride (data not shown).

![Figure 6](mbio.asm.org) The newly identified oxygen-facilitating aquaporin Pn-AqpC modulates H2O2 production, ROS and RNS resistance, and pneumolysin (Ply) release and contributes significantly to the pathogenicity of pneumococcus. Pn-AqpC, an atypical aquaglyceroporin, functions as an oxygen porin to facilitate O2 influx and promotes pneumococcus to produce H2O2 via pyruvate oxidase (SpxB) and lactate oxidase (LctO). Endogenous H2O2 endows but deletion of Pn-aqpC reduces pneumococcus resistance to higher exogenous H2O2 and NO levels; therefore, the presence of Pn-AqpC enhances the survival of pneumococci in macrophages. Additionally, the presence of Pn-AqpC promotes pneumococcal cell lysis and, thus, Ply release. As the pneumococcal hemolysin Ply perforates eukaryotic cellular membranes and induces macrophage necrosis, the presence of Pn-AqpC enhances pneumococcal damage to macrophages. Consistently, the absence of Pn-AqpC significantly attenuates the virulence of S. pneumoniae in a murine pneumonia model.
Significantly, the oxygen porin Pn-AqpC contributes to pneumococcal pathogenicity, as the deletion of Pn-aqpC markedly attenuated lethality to mice (Fig. 5E). Through an exhaustive search, Pn-aqpC orthologs were found in all 8,183 pneumococcal genomes and contigs, which are attributed to 77 capsular serotypes and capsule-free strains. These orthologs exhibit 97% to 100% amino acid sequence identities with Pn-AqpC and 100% identity of YVPR in the ar/R region (Fig. 1C; Data Set S1). Additionally, Pn-AqpC orthologs are widely distributed among members of the genera Streptococcus and Lactococcus of the Streptococcaceae family, the genera Lactobacillus and Pediococcus of the Lactobacillaceae family, the genera Oenococcus and Weissella of the Leuconostocaceae family, and the genus Enterococcus of the Enterococcaceae family (Fig. 1C), so the members of this aquaporin subfamily appear to be restrictively present in facultative anaerobic bacteria, implying that they could contribute to the oxidative adaptation of these bacteria through O$_2$ influx-enabled endogenous H$_2$O$_2$ production. Of note, Pn-AqpC orthologs are particularly prominent in some pathogenic streptococcal species, such as Streptococcus pyogenes and S. mutans (Fig. 1A), implying their association with virulence. Although the non-H$_2$O$_2$-producing species S. mutans also possesses a Pn-AqpC ortholog (SMU_396), its O$_2$-facilitating role is not likely involved in H$_2$O$_2$ production through oxidases, whereas S. mutans encodes H$_2$O-forming NADH oxidase (Nox), which uses O$_2$ to oxidize NADH to NAD$^+$ to achieve cellular redox balance and energy production (37). Deletion of the nox gene reduced O$_2$ consumption by pneumococcus. A previous study also found that O$_2$ promotes O$_2$-tolerant S. mutans growth (38); thus, S. mutans AqpC could also have an important physiological role.

Based on the experimental evidence of pneumococcal survival in and damage to macrophages (Fig. 5A to D), we hypothesize that the mechanistic basis of Pn-AqpC in pneumococcal pathogenicity lies in its control of pneumococcal H$_2$O$_2$ production and Ply release. Compared with other pathogenic bacteria, streptococci are highly capable of resisting oxidative stress via endogenous H$_2$O$_2$-induced resistance to higher levels of exogenous H$_2$O$_2$ (6, 39), and this unique characteristic enables them to defend against the innate immune system of the infected host (9). H$_2$O$_2$ also contributes to pneumococcal virulence by damaging alveolar epithelial cell DNA and suppressing host innate immune systems (4, 5). Consistently, the major H$_2$O$_2$-producing enzyme pyruvate oxidase is crucial for the virulence of S. pneumoniae (40). This work identified that Pn-AqpC, by facilitating O$_2$ uptake, acts as a novel key component in controlling H$_2$O$_2$ production and oxidative stress resistance; thus, the absence of this membrane protein causes S. pneumoniae to be rapidly eliminated by macrophages and reduces damage to macrophages. Survival in macrophages could be important for pneumococcal invasion and is critical for pneumococcal bacteremia and persistence within hosts (10, 41). Remarkably, the presence of Pn-AqpC elevates pneumococcal autolysis and Ply release (Fig. 4E and F), probably due to endogenous H$_2$O$_2$ production, thus increasing the hemolytic activity of pneumococci. Ply, as the major virulence factor of pneumococci, has been known to mediate bacterial transmission, trigger inflammatory responses, and cause macrophage necrosis (3, 42, 43). Rapid autolysis and pneumolysin release were reported to increase the pathogenicity of pneumococcal serotype 1 (44). In addition, no correlations have been found between Pn-AqpC and other identified pneumococcal virulence factors, as the deletion of Pn-aqpC did not alter the transcription of lytA, psaA, pspC, spxB, and lctO (Fig. S4) or the capsule polysaccharide amounts (Fig. S6). Therefore, the virulence relevance of Pn-AqpC lies mainly in its oxygen-transporting function.

Collectively, Pn-AqpC, by facilitating O$_2$ uptake, modulates H$_2$O$_2$ production and Ply release, the two major virulence factors of pneumococci, and contributes remarkably to pneumococcal virulence. Pn-aqpC orthologs were found in all 8,183 pneumococcal genomes and contigs (Data Set S1). Therefore, the conserved membrane-integrated Pn-AqpC is exposed as a new potential target for fighting against pneumococcal disease.

**MATERIALS AND METHODS**

**Experimental strains and growth.** Experimental strains are listed in Table S2 in the supplemental material. Pneumococcus was grown in brain heart infusion (BHI) broth or agar plates (BD Difco) with 5%
sterile defibrinated sheep blood at 37°C with 5% CO₂. Pneumococcal strains were grown statically, with shaking, or anaerobically under 100% nitrogen. When required, kanamycin (1 mg/ml) and spectinomycin (300 μg/ml) were added.

**Construction of genetically modified strains.** All primers are listed in Table S2. The PCR ligation method (45) was used to construct the Pn-aqpC and nox deletion strains and His-, photoactivatable fluorescent protein mMaple3 (29), or superfolder green fluorescent protein (sfGFP)-tagged strains of *S. pneumoniae*. The spectinomycin and kanamycin resistance genes were derived from plasmids pDL278 (46) and pALH124 (47), respectively. The Pn-aqpC gene with its promoter was cloned into pDL278 for complemented strain construction. Alanine substitutions for Tyr49, Val223, Pro232, and Arg238 were implemented on pDL278-Pn-aqpC using a site-directed gene mutagenesis kit (Beyotime, China). Transformation was performed as described previously (48). Correct transformants were confirmed by PCR and DNA sequencing.

**Test of the transportable substrates of Pn-AqpC-constituted proteoliposomes.** The purified 10×His-tagged Pn-AqpC protein was reconstituted into liposomes made by the *S. pneumoniae* cellular membrane lipid (49, 50) and *E. coli* total lipid extract (Avanti) as previously described (16, 27). Detailed procedures are available in Text S1 in the supplemental material.

To examine the O₂ permeability of Pn-AqpC, cell membrane-impermeable and oxygen-quenchable phosphorescent oxygen probes (Cayman Chemical) were encapsulated into proteoliposomes and Pn-AqpC-devoid liposomes. The proteoliposomes and liposomes were vacuumed and N₂ gas flushed for 7 cycles, and 100 μl per well was then dispensed into a 96-well plate (Corning) under air. The fluorescence intensity of the phosphorescent oxygen probe was monitored (excitation at 380 nm and emission at 650 nm) for a recommended delay of 30 μs using a Synergy H4 hybrid multimode microplate reader (BioTek). Water and glycerol permeabilities were assayed using an SX20 stopped-flow spectrometer as previously described (27, 51). The experiments were repeated three times.

**PALM imaging.** Mid-exponential-phase Pn-aqpC-mMaple3 cells were exposed to air for 30 min in the dark and then observed using PALM imaging (28) as previously described (12). The superresolution images were constructed using Insight3 software (52), which was kindly provided by Bo Huang (University of California, San Francisco). PALM data analyses such as drift correction, protein abundance, and image rendering were carried out using custom-written Matlab scripts.

**Assay of pneumococcal survival in macrophages and damage to macrophages.** Mouse monocytic-macrophage RAW 264.7 cells (1 × 10⁵) were challenged for 1 h with pneumococcus at a multiplicity of infection of 100:1. After removing the bacteria, macrophages were incubated for another 1 and 1.5 h to count CFU of pneumococci within macrophages or for 16 h to determine macrophage death. Detailed procedures are available in Text S1 in the supplemental material.

**In vivo mouse infection experiment.** BALB/c mice (specific-pathogen-free [SPF] grade) were purchased from Vital River Company (Beijing, China). Animal experiments were approved by the Biomedical Research Ethics Committee of the Institute of Microbiology, Chinese Academy of Sciences. The protocol was approved by the Institutional Animal Care and Use Committee. *S. pneumoniae* D39 and derivative strains were intratracheally administered to 6- to 8-week-old female BALB/c mice at 1 × 10⁷ CFU in 20 μl phosphate-buffered saline (PBS), and PBS-administered BALB/c mice were included as controls. Mouse survival (10 per group) was monitored for 12 days. Mice were sacrificed under anesthesia, half-lungs were ground for enumerating pneumococcal CFU, and the other halves were used for histopathological observation.

**Statistical analysis.** One-way analysis of variance (ANOVA) followed by Tukey’s post hoc test and Student’s t test was performed using PASW Statistics 18 and Excel, respectively. A log rank Mantel-Cox test was performed using GraphPad Prism 8.0. The level of significance was determined at a P value of <0.05.

**Other procedures.** Detailed procedures are available in Text S1 in the supplemental material.

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**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**TEXT S1**, DOCX file, 0.04 MB.

**FIG S1**, TIF file, 1 MB.

**FIG S2**, TIF file, 2.3 MB.

**FIG S3**, TIF file, 1.8 MB.

**FIG S4**, TIF file, 0.1 MB.

**FIG S5**, TIF file, 1.3 MB.

**FIG S6**, TIF file, 0.7 MB.

**TABLE S1**, DOCX file, 0.02 MB.

**TABLE S2**, DOCX file, 0.03 MB.

**DATA SET S1**, XLSX file, 1.9 MB.

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An O$_2$ Porin Contributes to Pneumococcal Virulence

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