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Effect of Nonheme Iron-Containing Ferritin Dpr in the Stress Response and Virulence of Pneumococci

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***Streptococcus pneumoniae* (pneumococcus) produces hydrogen peroxide as a by-product of metabolism and provides a competitive advantage against cocolonizing bacteria. As pneumococci do not produce catalase or an inducible regulator of hydrogen peroxide, the mechanism of resistance to hydrogen peroxide is unclear. A gene responsible for resistance to hydrogen peroxide and iron in other streptococci is that encoding nonheme iron-containing ferritin, *dpr*, but previous attempts to study this gene in pneumococcus by generating a *dpr* mutant were unsuccessful. In the current study, we found that *dpr* is in an operon with the downstream genes *dhfr* and *clpX*. We generated a *dpr* deletion mutant which displayed normal early-log-phase and mid-log-phase growth in bacteriologic medium but survived less well at stationary phase; the addition of catalase partially rescued the growth defect. We showed that the *dpr* mutant is significantly more sensitive to pH, heat, iron concentration, and oxidative stress due to hydrogen peroxide. Using a mouse model of colonization, we also showed that the *dpr* mutant displays a reduced ability to colonize and is more rapidly cleared from the nasopharynx. Our results thus suggest that Dpr is important for pneumococcal resistance to stress and for nasopharyngeal colonization.**

Streptococcus pneumoniae (pneumococcus) resides primarily in the human nasopharynx, which is exposed to an oxygen-rich environment on the airway surface. During *in vitro* growth, pneumococci can generate hydrogen peroxide (H_2O_2) in the millimolar range. Despite this, *S. pneumoniae* lacks (H_2O_2-destroying) catalase, NADH peroxidase, and an H_2O_2-responsive regulator, such as PerR, all mechanisms that are used by other Gram-positive organisms for protection against H_2O_2 (1, 2). The mechanisms by which *S. pneumoniae* is able to survive and grow under oxygenated conditions without many of the defenses commonly found in aerobic and aerotolerant organisms are still unknown.

Hydrogen peroxide can interact with ferrous ions (Fe^{2+}) and form a highly reactive hydroxyl radical (·OH) through the Fenton reaction, causing DNA damage and increased toxicity to the cells (3). At the same time, iron is an essential nutrient for *S. pneumoniae*. Pneumococci can utilize ferric and ferrous ions, as well as hemoglobin and hemin, as iron sources, as demonstrated in studies with iron-deficient media (4–6). Two iron transporter operons, *piuBCDA* and *piaABCD*, appear to be important for acquisition of iron from the environment by pneumococci. Mutation of both operons led to severe attenuation in virulence (5, 7, 8). Therefore, intracellular free iron concentration in pneumococcus must be balanced to both support growth and minimize the damage caused by the Fenton reaction.

In *Escherichia coli*, a DNA-binding protein from starved cells (Dps) can capture free iron and protect DNA from oxidative damage (9). Dps is a ferritin-like protein and considered functionally similar to classical ferritins. Dps forms a spherical hollow assembly of 12 monomeric proteins and can hold up to 500 molecules of free iron per complex (10). A homolog of Dps, Dpr (Dps-like peroxide resistance), is conserved in Gram-positive bacteria, such as *Streptococcus mutans*, *Streptococcus pyogenes*, and *Streptococcus suis* (11–13). The structure of Dpr from *S. suis* is similar to that of Dps, and it can bind ferrous ions as well as other divalent cations, such as Cu^{2+}, Mn^{2+}, and Zn^{2+} (14, 15). Dpr is essential for aerobic survival of *S. mutans* and also prevents iron-dependent hydroxyl radical formation *in vitro* (16). Further studies showed that protection against H_2O_2 damage in *S. suis* by Dpr is dependent on its iron-binding capacity (17).

A *dpr* homolog is present in the pneumococcal genome. *dpr* expression is controlled by an orphan two-component regulator, RitR, which also controls several genes associated with oxidative stress (18). We hypothesized that Dpr is an important factor for resistance of oxidative stress caused by H_2O_2 in pneumococcus, by chelating free iron. To investigate the role of this gene in resistance to stress, a *dpr* mutant was generated by homologous recombination, and the phenotype of this mutant in response to stress and in animal models was assessed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* was grown on tryptic soy agar (TSA) with 5% sheep blood (BAP) or in Todd-Hewitt medium plus 0.5% yeast extract (THY) at 37°C in a 5% CO_2 incubator. Bovine liver catalase was purchased from Worthington Biochemical, Freehold, NJ. The hydrogen peroxide assay kit was purchased from Thermo Fisher Scientific (Rockland, IL). Other chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Iron binding assay. His-tagged Dpr was purified as described previously (19). Iron staining was performed as described previously (20, 21). Briefly, 1 mg/ml of Dpr and bovine serum albumin (BSA) were incubated in phosphate-buffered saline (PBS) with or without 1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ on ice for 30 min and then run on a 3 to 8%

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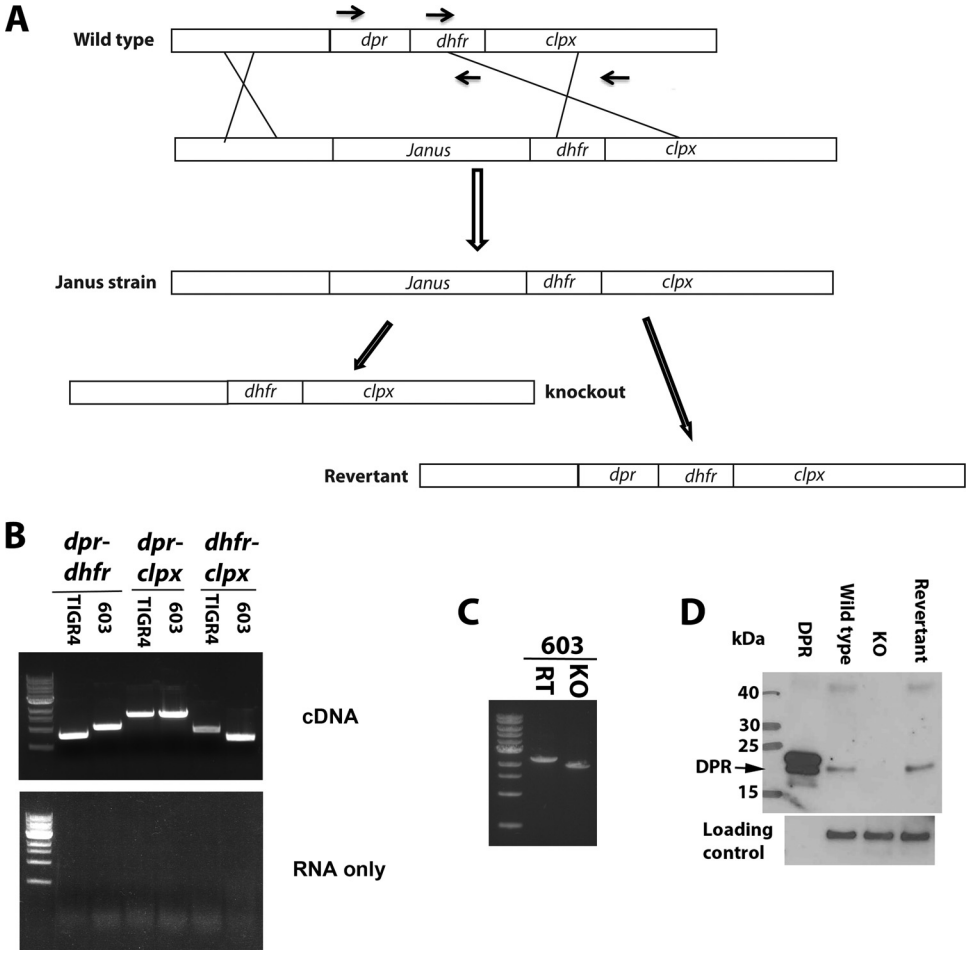


FIG 1 Generation of *dpr* mutants. (A) Schematic diagram of the generation of the *dpr* mutants and revertants using a bicistronic (Janus) cassette. Black arrows indicate the position of primers for reverse transcription-PCR in Fig. 1B. (B) *dpr*, *dhfr*, and *clpX* are in a single transcript. RNA was extracted from pneumococcal TIGR4 and 603 strains, and cDNA was generated by reverse transcription. PCR products using cDNA (top) or RNA (bottom) were amplified using primers listed in Table 1. (C) PCR amplification of genomic DNA from revertant and KO strains using two outside primers. (D) Western blotting was performed to confirm the phenotypes of the *dpr* KO and revertant strains in the 603 strain background. Pneumolysin was used as a loading control.

Tris-acetate gel under nondenaturing conditions. Iron-bound Dpr was stained with 1 mM 3-(2-pyridyl)-5,6-bis(2-[5-furyl sulfonic acid])-1,2,4-triazine (Ferene S) and 15 mM thioglycolic acid in 2% (vol/vol) acetic acid. Proteins were visualized with Coomassie brilliant blue staining.

Construction of the *dpr* mutants. The *dpr* mutants were constructed as illustrated in Fig. 1A. Primers are listed in Table 1. Genomic DNA was obtained using an extraction kit (Qiagen), following the manufacturer's protocol. Fragments of 800 bp upstream (using primers P1 and P2) and 800 bp downstream (using P5 and P6) of *dpr* and the Janus cassette (P3 and P4) were amplified from genomic DNA by PCR. An overlapping PCR was performed using these three PCR products as the template and primer sets P1 and P6. Bacteria were grown to an optical density at 600 nm (OD_{600}) of 0.05, and transformation was done as described previously (22). Transformants were identified on BAP supplemented with 600 μg kanamycin/ml and then sequenced to confirm that the Janus cassette was appropriately inserted. The Janus cassette was then replaced by recombination with either knockout (KO) or revertant constructs as illustrated in Fig. 1A. DNA for the KO strain was generated by overlapping PCR using the primer pairs P1 and P6 and products of P1 to P8 and P6 to P9 as a template. The DNA for the revertant strain was generated with primers P1 and P6 with wild-type genomic DNA as the template. Bacteria were selected on BAP with 1,000 μg streptomycin/ml and then sequenced using primers P0 and P7, both outside the cloning region. To generate a mutant in the TIGR4 background in which a chloramphenicol cassette was introduced to replace *dpr*, three DNA fragments were generated with P1 plus CM-P2, CM-P3 plus CM-P4, and CM-P5 plus P6 and then connected using overlapping PCR. Transformation was performed as described above. The full genome sequences of 603 and TIGR4 revertant (RT) and KO strains were obtained by Illumina HiSeq platform using a protocol described previously (23).

Operon detection. RNA was extracted from mid-log-phase bacteria using the RNeasy kit (Qiagen, Valencia, CA). cDNA was generated from RNA using reverse transcriptase and random primers. cDNA was then used as templates to amplify possible operons, and RNA was used as a control. Primers used for amplification are listed in Table 1.

Western blotting. Dpr antiserum was obtained by immunizing New Zealand White rabbits with purified Dpr protein (19). Mid-log-phase bacteria were pelleted and resuspended in SDS loading buffer. After being heated to 100°C for 10 min, samples were loaded onto 4 to 12% bis-Tris SDS gel and separated under denaturing conditions. Western blotting was performed as described before (24). Briefly, proteins were transferred to nitrocellulose membranes and blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBST) at room temperature for 1 h. The membrane was incubated with anti-Dpr (1:5,000 dilution) or anti-pneumolysin antisera (1:10,000) in 1% skim milk in PBST for 1 h at room temperature. The membrane was then washed three times and further

TABLE 1 Primers used in the current study

Primer[a]	Sequence
1572 P0 (−967)	GACAAGGAATTGGCGCATAAC
1572 P1 (−863)	CGCTAAACAGACCATTGAAC
1572 P2 (−19)	TTCCTCTGGAATAGGCATAGACATTATTTTTTGTAACCACTTC
1572 P3 (−19)	GAAGTGGTTACAAAAAATAATGTCTATGCCTATTCCAGAGGAA
1572–T4 P4 (534)	ATCTATGTTTCCTTATTTCTTACTAGGGCCCCTTTCCTTATGC
1572–T4 P5 (534)	GCATAAGGAAAGGGGCCCTAGTAAGAAATAAGGAAACATAGAT
1572–T4 P6 (1,359)	GACATGATTTCCTTCCATTC
1572–T4 P7 (1,491)	GAATGATTTCCTGAGCTAACTC
1572–T4 P8 (−19)	GAAGTGGTTACAAAAAATAATGTAAGAAATAAGGAAACATAG
1572–T4 P9 (−19)	CTATGTTTCCTTATTTCTTACATTATTTTTTGTAACCACTTC
1572-603 P4 (534)	CTATGTTTCCTTGTTTCTTACTAGGGCCCCTTTCCTTATGC
1572-603 P5 (534)	GCATAAGGAAAGGGGCCCTAGTAAGAAACAAGGAAACATAG
1572-603 P6 (1,364)	GGCATGATTTCCTTCCGTTC
1572-603 P7 (1,635)	GTGGTTATAAACCGCCACTG
1572-603 P8 (−19)	GAAGTGGTTACAAAAAATAATGTAAGAAACAAGGAAACATAG
1572-603 P9 (−19)	CTATGTTTCCTTGTTTCTTACATTATTTTTTGTAACCACTTC
1572–CM P2 (−16)	CATCAAGCTTATCGATACCGCATTATTTTTTGTAACCAC
1572–CM P3 (−16)	CGGTATCGATAAGCTTGATG
1572–CM P4 (534)	TGGAGCTGTAATATAAAAACCTTC
1572–CM P5 (534)	GGTTTTTATATTACAGCTCCATAAGAAATAAGGAAACATAG
1571RT-forward (727)	ATCGTCTGCCTTGGCATT
1571RT-reverse (807)	CCATCCCATCAAAGGTCAC
clpX RT T4-forward (1,656)	CGCATCAATTTCCACGATAC
clpX RT T4-reverse (1,760)	AGCTCTTAGCCAAGGTCTGG
clpX RT 603-forward (1,555)	AGAAGTTCCCAAACCAATCG
clpX RT 603-reverse (1,663)	GCGTATCGTGGAAATTGATG
1571-603RT-forward (958)	ATATTCTCGGTGGGAAGCAA
1571-603RT-reverse (1,056)	GACAAGTCAAACTCCTCAGGAAA
1572 P10 (248)	CAGCGAACGCTTGATTACAC
1572 P11 (350)	CACGTTCCAAGCTTTCTTCA
1572 P12 (−34)	GGTAGTAATCATTAAGAAGTGG

[a] Numbers in parentheses are the distance (bp) from the first base to the start codon of *dpr*.

incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG for 1 h at room temperature, and then it was developed using a chemiluminescent substrate. Purified Dpr was used as a positive control; pneumolysin was used as a loading control and probed with an antibody directed against pneumolysin.

Bacterial survival assays. Mid-log-phase bacteria ($OD_{600} = 0.5$) were used for bacterial survival assays. Bacteria were washed with PBS, subjected to different stress conditions as described below, and then plated on BAP: bacteria were incubated in THY with different concentrations of hydrogen peroxide (1 mM, 5 mM, or 10 mM for 30 min at room temperature) or at different pHs (pH 4 for 2 h, pH 7 for 2 h or pH 11 for 75 min) or subjected to elevated temperature (42°C for 2 h). An incubation time for each treatment was chosen such that about 99% of the KO bacteria would be killed. For iron sensitivity assays, mid-log-phase bacteria were diluted into ferrous chloride-containing THY medium and further incubated at 37°C.

Iron concentration measurements. RT and *dpr* mutant strains were grown in 1 liter of THY medium. Cells were collected when the OD_{600} reached 0.5 and then washed twice with ice-cold PBS. The cell pellets were then resuspended in 5 ml of PBS. Bacteria were lysed using a bead beater, following which the supernatant fraction was passed through a 30-kDa Amicon tube (Millipore). Retained fractions, the flowthrough, and whole bacteria were analyzed for iron content by inductively coupled plasma-mass spectrometry in the laboratory of J. Shine at the Harvard School of Public Health. The total iron concentration was normalized to total protein content of the bacterial lysates, which was measured by a micro-bicinchoninic acid (BCA) protein assay (Pierce).

Macrophage and neutrophil killing assays. Macrophage killing assays were performed as described previously with minor modifications (25, 26). Briefly, 5×10^5 immortalized wild-type murine macrophages (a gift from Douglas Golenbock, University of Massachusetts) per well were plated in 24-well cell culture plates for 16 h, and 2×10^6 CFU of either strain was added into each well. Cells were incubated at 37°C for 30 min and then washed with PBS three times. To quantify intracellular bacteria, cells were incubated in medium with 100 μg gentamicin/ml for 30 min at 37°C to kill all extracellular bacteria. Fresh medium was added to the culture, and the concentration of intracellular surviving bacteria at each time point was determined by plating cell lysates. Total CFU without addition of gentamicin at different time points were determined by plating dilutions on BAP after lysing the cells. Neutrophil surface killing assays were performed as described previously using HL-60 cell-derived neutrophils (27).

Nasal colonization model. To determine the ability of the *dpr* mutant to establish nasopharyngeal (NP) colonization in mice, mice were challenged with live encapsulated pneumococci as described previously (28) with 10^7 CFU of either RT or KO strains in 10 μl of PBS. Mice were euthanized, and nasal washes were collected at 6 h and 1, 2, 4, and 7 days after inoculation and plated on BAP supplemented with gentamicin for assessment of density of bacterial colonization. The competition between RT and KO strain *in vivo* was performed as described previously (29). Mice were challenged with 10^7 CFU of a 1:1 mixture of RT and KO strains in 10 μl of PBS at day 0, and samples from live animals were collected by applying 20 μl of ice-cold PBS to either nostril of a mouse and collecting droplets discharged by the animal at day 1, 3, and 7. Samples were cultured on blood agar plates (supplemented with 2 μg/ml of gentamicin) overnight, and the resulting colonies were harvested for genomic DNA extraction using a DNeasy blood and tissue kit (Qiagen, Valencia, CA). The absolute quantity of strain-specific genomic DNA in a sample was deter-

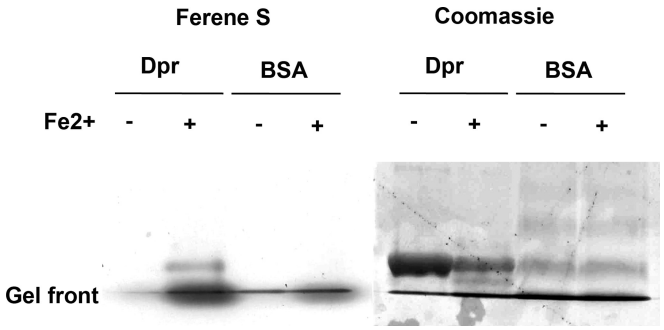


FIG 2 Binding of iron to Dpr. Purified Dpr and control BSA proteins were added to PBS with or without 1 mM Fe^{2+} and incubated on ice for 30 min. Proteins were separated with nondenaturing gel. Iron-bound proteins were visualized with Ferene S (left), and protein bands were visualized with Coomassie brilliant blue (right).

mined using strain-specific primer sets and then used to calculate the ratio of the two strains in the NP. RT and KO strain primers were designed around the *dpr* region. The upper and lower detection limits were set as total CFU and 1/total CFU, respectively.

Statistical analysis. PRISM (version 5.0, GraphPad Software, Inc.) was used for statistical calculations. Differences in cell killing assay were analyzed by two-way analysis of variance (ANOVA). NP colonization densities were analyzed by Mann-Whitney U tests. For all comparisons, a *P* value of <0.05 was considered significant.

RESULTS

Pneumococcal Dpr binds iron. The pneumococcal Dpr belongs to the ferritin-like protein family. A homolog of Dpr in *S. suis* binds iron and shares 62% identity and 80% similarity with the pneumococcal Dpr. To evaluate whether pneumococcal Dpr similarly binds iron, we purified Dpr from *E. coli*. As shown in Fig. 2 (left), Dpr can be stained by Ferene S (a colorimetric reagent for iron) in the presence of 1 mM Fe^{2+}, whereas no such band was seen when BSA was used as a control. The majority of Dpr that bound to iron remained in the same position as the non-iron-binding Dpr; interestingly, a small portion of Dpr shifted to a lower position, as revealed by Coomassie brilliant blue staining (Fig. 2, right), which suggests the possibility that Dpr may have two conformations when incubated with iron.

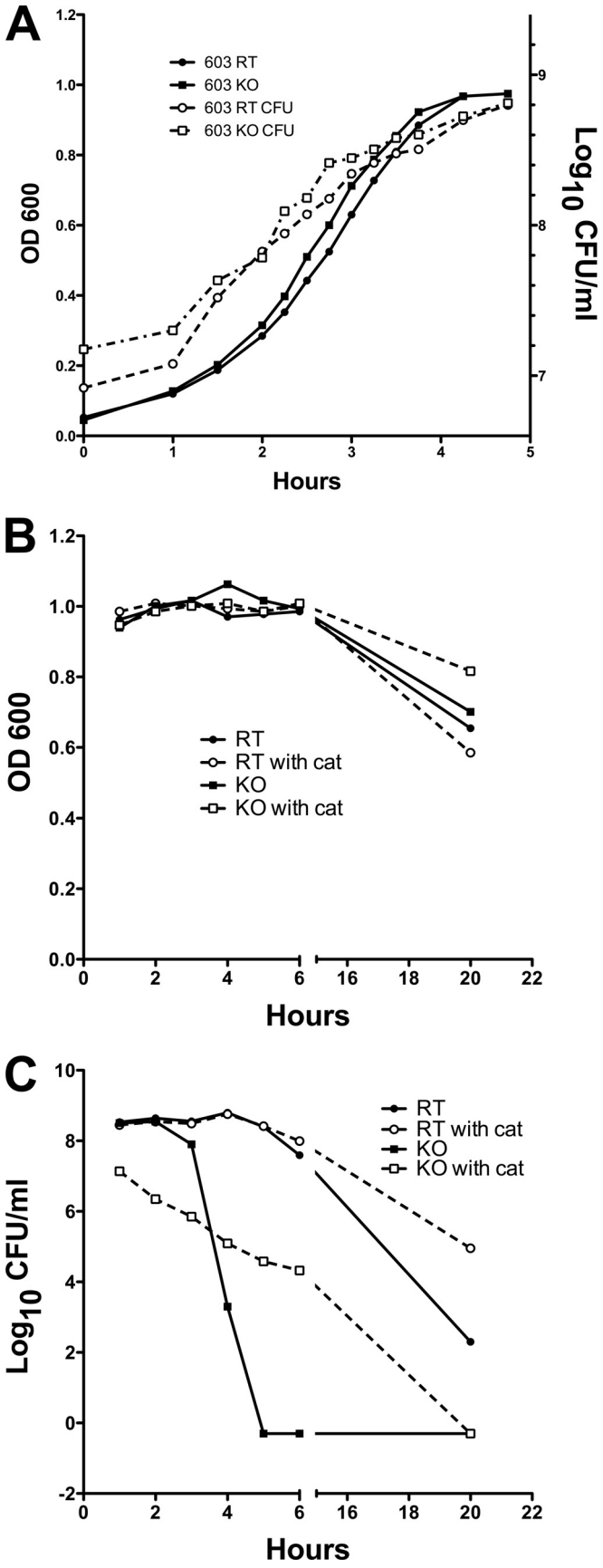
***dpr* is in an operon with *dhfr* and *clpX*.** As shown in Fig. 1A, two genes, *dhfr* (encoding dihydrofolate reductase) and *clpX*, are located downstream of *dpr* in the TIGR4 pneumococcal genome. These two genes have been previously shown to be essential in pneumococcus (30–32). In two published studies, attempts to generate a *dpr* mutant failed (1, 18). The details of previous attempts were not fully described, but we hypothesized that the inability to generate a *dpr* mutant may have been because of potential polar effects on the expression levels of downstream essential genes. To test the hypothesis that *dpr* is part of an operon, RNA was purified from pneumococcal strains TIGR4 and 603 (a serotype 6B clinical isolate) (28, 33). cDNA was generated using reverse transcriptase and used as the template to amplify PCR products covering the *dpr-dhfr*, *dhfr-clpX*, and *dpr-clpX* regions. All three amplifications resulted in readily detectable bands of PCR products for both strains (Fig. 1B, top), which were not seen in the absence of reverse transcriptase (Fig. 1B, bottom). These results clearly suggest that the genes *dpr*, *dhfr*, and *clpX* form part of an operon.

Generating *dpr* mutants. Having confirmed that these three genes are in an operon, we attempted to generate *dpr* mutants using a Janus bicistronic cassette (whose insertion in the genome confers kanamycin resistance and streptomycin susceptibility [34]) or with the simple insertion of a chloramphenicol (CM) resistance marker with terminator after the CM resistance cassette. Both approaches are based on homologous recombination, and the PCR products used for transformation share the same upstream and downstream sequences. We favored using the bicistronic Janus cassette for the following reasons: (i) in the absence of a terminator, the downstream genes are expressed under the promoter in the Janus cassette, and (ii) a clean deletion strain or a revertant with wild-type DNA can be generated, to control for any potential effect of transformation on bacterial growth and virulence, which allows a comparison of the phenotypes of deletion strains and a strain that has undergone similar transformation processes but still contains the gene of interest. TIGR4 and 603 strains were transformed using the same concentration of PCR products derived from the two strategies. Kanamycin-resistant colonies were obtained using the bicistronic Janus cassette strategy, but no CM-resistant colony was obtained when the CM cassette strategy was used. These results suggest that the inability to generate *dpr* mutant was likely due to a polar effect of the construct upon the downstream genes.

Subsequently, clean knockout (KO) and revertant (RT) strains were generated using homologous recombination, as illustrated in Fig. 1A. RT strains were used as comparators for the KO strains in all subsequent experiments. Candidate mutant strains were confirmed by using the primer pair P0 and P7 (which do not span the cloning region for DNA amplification) and sequencing the PCR product (Fig. 1C). Western blotting was performed by using cultures of 603 wild-type, KO, and revertant strains and probing with anti-Dpr rabbit antibody (Fig. 1D).

Growth phenotype of *dpr* mutant. The *dpr* mutant in the pneumococcal strain 603 background had no apparent growth defect on either blood agar plates or in THY medium, as shown in Fig. 3A. Both the OD_{600} and the number of CFU of the KO strain are similar to those of the RT strain. The growth curves of the *dpr* mutants in TIGR4 background, however, showed important growth defects in THY compared to TIGR4 RT or WT strains. Comparison of whole-genome sequences of the KO and the parent strain to identify potential additional mutations did not reveal any apparent explanation for this phenotype (data not shown). Due to the growth inhibition of the KO in the TIGR4 background, which could confound our examination of the role of *dpr*, we continued our studies using only the mutants generated in the 603 background.

Decreased survival of the *dpr* mutant in stationary phase. The production of H_2O_2 by *S. pneumoniae* has been shown to mediate pneumococcal cell death (35). We measured H_2O_2 production by RT and KO strains and found they were similar at every stage of growth (both strains produced over 100 μM at stationary phase). Based on the function of Dpr homologs in other bacteria, we hypothesized that the KO strain would have significantly decreased survival compared to the RT stain at stationary phase. As shown in Fig. 3A, while there were no differences in viability of the RT and KO strains at mid-log phase, important differences were noted at stationary phase: in contrast to the RT strain, the CFU counts in the KO strain decreased rapidly 3 h into stationary phase, while their OD_{600}s were not different from each other



(Fig. 3B and C). No viable KO bacteria could be detected after 5 h (Fig. 3C). The addition of catalase increased survival of the KO strain (Fig. 3C) but did not fully reverse the phenotype, suggesting that while H_2O_2 plays an important role, it may not be the only reason for the increased susceptibility of the KO strain at stationary phase.

Role of Dpr in resistance to stress. Dpr is thought to protect *S. pyogenes* under acid and alkaline stress conditions (13). We tested whether deletion of *dpr* would similarly affect the stress responses to pH changes in *S. pneumoniae*. Mid-log-phase bacteria were washed and resuspended in THY medium at pH 4, 7, or 11 at room temperature. No cell death was observed for either KO or RT at pH 7. As shown in Fig. 4, the survival rate of the KO strain at pH 4 or pH 11 for 120 and 75 min, respectively, was 3% that of the RT strain. This result thus confirmed the importance of Dpr in conferring resistance to extreme pH conditions. Our data also show that Dpr mediates resistance to lethal heat stress, as the KO strain incubated at 42°C for 2 h had a >2-log reduction in numbers of CFU compared to the RT strain.

To evaluate whether Dpr also plays a role in resistance to oxidative stress, RT and KO strains were tested for survival at a range of concentrations of H_2O_2. Whereas no killing was observed for the RT strain, viability of the KO strain was reduced in the presence of 5 mM or 10 mM H_2O_2 for 30 min, respectively (Fig. 4A; results are representative of at least three independent experiments). When incubated with 10 mM H_2O_2 for 2 h, viability was again reduced for the KO strain compared to the RT strain (Fig. 4B; results are representative of at least three independent experiments). These results strongly suggest that Dpr plays an important role in resistance to oxidative stress in pneumococcus, and the protection is likely due to its iron binding ability, as demonstrated in *S. suis* (17).

Sensitivity of *dpr* mutant to iron. Since Dpr mediates storage of iron inside cells in other bacteria, we tested the iron concentration in *dpr* mutant and RT strains. As shown in Table 2, the total iron content in the RT strain was 1,197.6 ng/mg protein, which is similar to what has been reported previously (1). In contrast, the total iron content in the KO strain was only 304.51 ng/mg protein, significantly lower than that of the RT strain ($P = 0.0079$ by the Mann-Whitney U test). We also examined whether the *dpr* mutation specifically lowered the iron content in the cytoplasmic fraction. Cell lysates were separated into fraction 1 (components larger than 30 kDa) and fraction 2 (components <30 kDa) using Amicon tubes (Millipore, MA). The iron content in fraction 1 from the RT strain was also much higher than that of the KO strain ($P = 0.0079$ by the Mann-Whitney U test), whereas the iron content of fraction 2 from both RT and KO strains was the same.

Dpr forms a dodecameric complex (15), resulting in a molecular mass of 240 kDa, and thus will not flow through a 30-kDa membrane. Therefore, the differences in iron content between the

FIG 3 Growth curve of *dpr* mutant and revertant strains. Bacteria were grown to an OD_{600} of 0.5 and diluted in THY medium to start the culture. (A) OD_{600} was monitored until growth of bacteria reached stationary phase, and the number of CFU at each time point was determined by serial dilution. (B) Optical density of the RT and KO strains in the presence (solid lines) or absence (dashed lines) of catalase (cat) after cells reached stationary phase. (C) Cell viability of RT and KO strains in the presence (solid lines) or absence (dashed lines) of catalase after cells reached stationary phase.

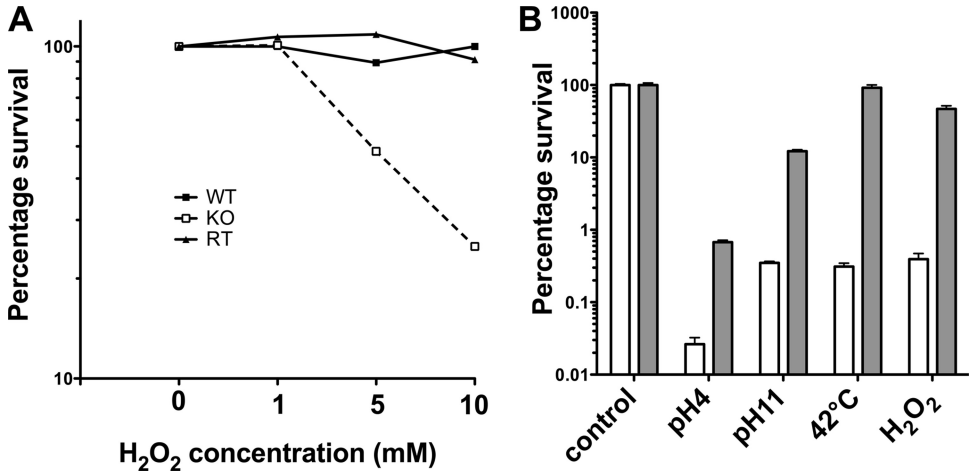


FIG 4 Sensitivity of the *dpr* deletion mutant to hydrogen peroxide, pH, and temperature stress conditions. (A) Bacteria were treated with different concentrations of H_2O_2 for 30 min. (B) Bacteria were treated at different pHs (pH 4 for 2 h or pH 11 for 75 min), subjected to elevated temperature (42°C for 2 h), or treated with 10 mM H_2O_2 for 2 h. Numbers of CFU were determined and normalized to the starting number of CFU. Open bar, KO strain; gray bar, RT strain. Results are from one experiment that is representative of at least three independent experiments.

RT and the KO strains in fraction 1 (>30 kDa) can most likely be ascribed to the lack of Dpr in the KO strain.

We then assessed whether excess iron in the culture medium would affect growth of the KO mutant strain. Although THY is not a chemically defined medium, the residual iron in THY does not cause growth inhibition in either strain, as shown in Fig. 3. Ferrous chloride was added to THY medium inoculated with mid-log-phase bacteria. As shown in Fig. 5, concentrations of ≥10 mM ferrous chloride were required to demonstrate inhibition of growth of the RT strain in THY. In contrast, the growth of KO strain was inhibited by the addition of as little as 0.5 mM in THY.

The *dpr* mutant is more susceptible to macrophage killing and defective in nasopharyngeal colonization. We examined whether the KO strain demonstrates increased susceptibility to phagocytic killing by macrophage and neutrophils. While we could not detect any differences in survival in a neutrophil surface killing assay (Fig. 6A), susceptibility to killing by macrophages was clearly increased in the KO strain. The adherence rate of KO strain to macrophages was similar to that of the RT strain (Fig. 6B); however, the KO strain was significantly more susceptible to killing by macrophages than the RT strain at 1, 2, and 3 h after gentamicin treatment to kill extracellular bacteria ($P < 0.0001$ by two-way ANOVA) (Fig. 6C).

Based on these results, we wished to examine whether the *dpr*

TABLE 2 Iron content in RT and *dpr* mutant strains

Fraction	Iron content (ng/mg of protein) in[a]:	
	RT strain	KO strain
Total bacteria	1,197.60 ± 38.17	304.51 ± 223.05
Lysate fraction 1[b]	666.11 ± 114.00	109.79 ± 25.82
Lysate fraction 2[b]	681.16 ± 360.24	649.81 ± 283.67

[a] Total iron content was determined in washed cells by inductively coupled plasma mass spectrometry. Values are the means ± standard deviations from five determinations. Results are representative of three independent experiments.
[b] Washed cells were lysed with a bead beater, and the soluble fraction was separated using a 30-kDa-cutoff concentrator. Fractions larger than 30 kDa are designated fraction 1, and fractions smaller than 30 kDa are designated fraction 2.

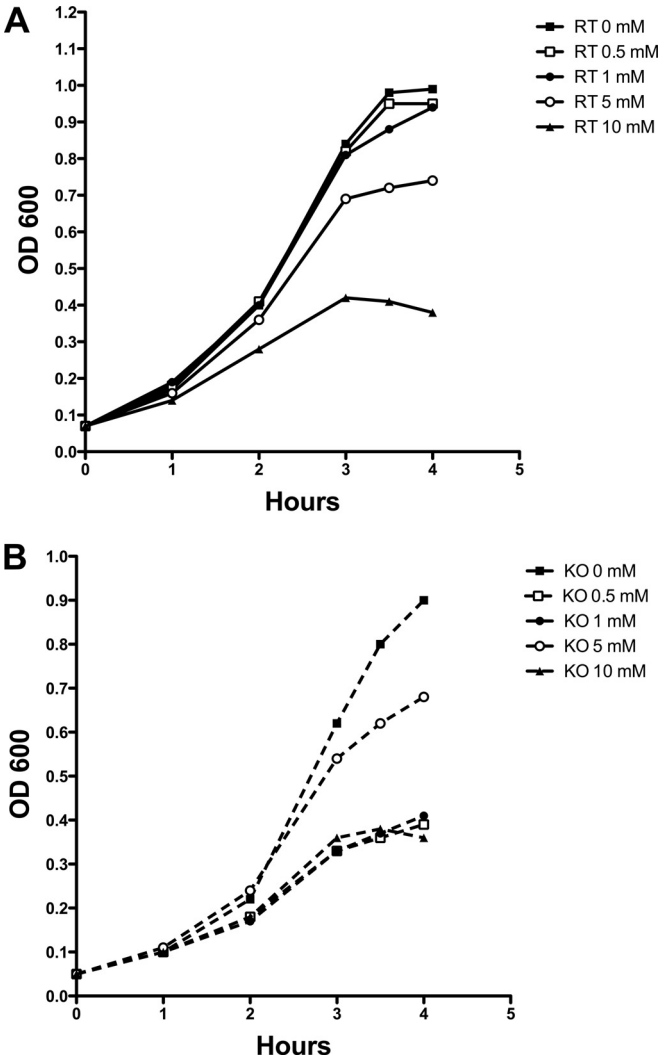


FIG 5 Growth of the RT (A) and KO (B) strains in the presence of ferrous chloride. Bacteria were grown to mid-log phase and diluted in THY containing different concentrations of ferrous chloride. Background absorbance caused by the addition of ferrous chloride was subtracted.

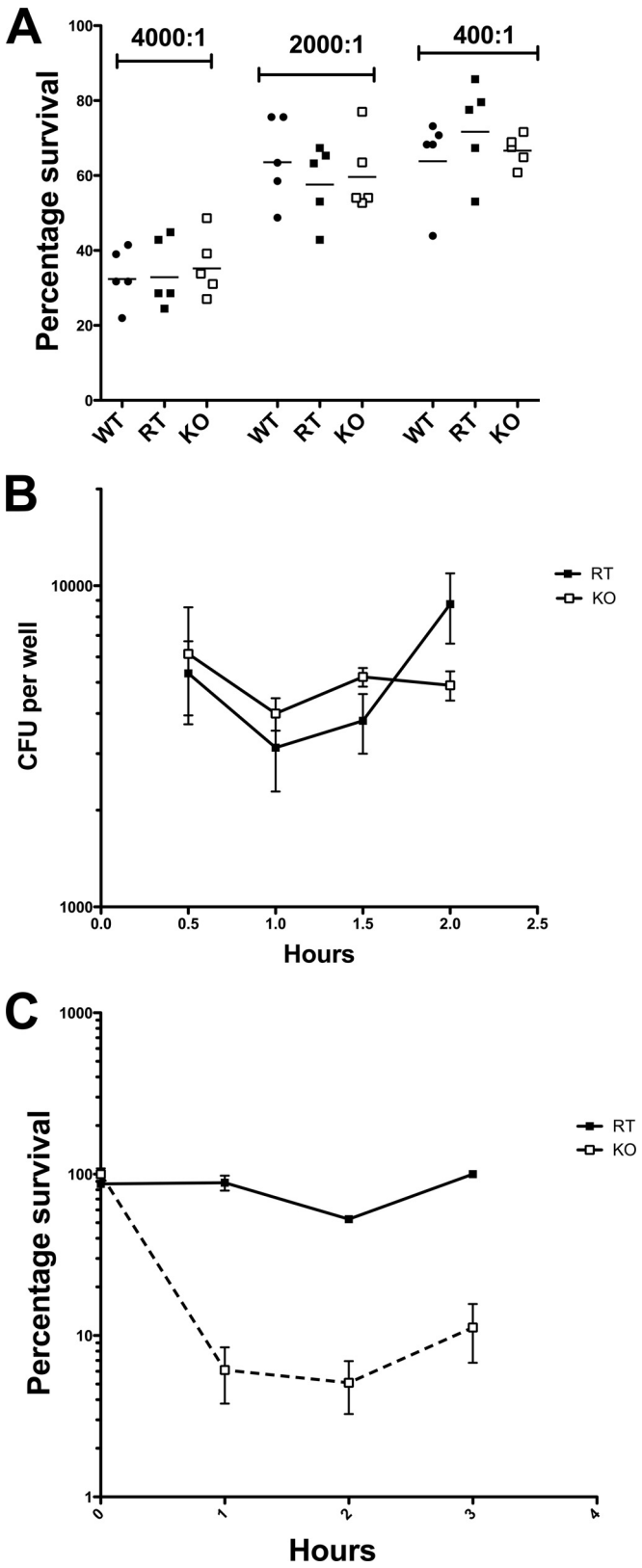


FIG 6 Compared to the RT strain, the *dpr* mutant is more susceptible to killing by macrophages but not neutrophils. (A) A neutrophil surface killing assay was performed using a range of cell-to-bacterium ratios. Percentages of surviving bacteria of the RT and KO strains are shown. (B) The RT and KO strains were incubated with murine macrophages at an MOI of 20 for 30 min. acrophages were washed with fresh medium, and numbers of total adherent plus intracellular bacteria were determined by plating cell lysates. (C) Surviving intracellular bacteria were counted after gentamicin treatment to kill extracellular bacteria, sequential washes, and plating of cell lysates.

mutant would be defective in colonization of mice, particularly since it had been shown that clearance of pneumococcal colonization is mediated by macrophages in mice that had not previously been exposed to pneumococcus (36). Thus, C57/BL6 mice were colonized with RT or KO strains and nasopharyngeal washes obtained at different time intervals. As shown in Fig. 7A, the density of colonization of the KO strain at 6 h was about 1 log lower than the density of colonization of the RT strain. The difference in densities of colonization between the two strains appears to persist for at least 2 more days, and by day 7, the KO strain was undetectable in nasal washes ($P < 0.0001$ compared to density of colonization by RT strain). To evaluate the early defect in colonization further, and to compare the two strains in the same animal, we performed mixed-infection competition studies, as described before (29). Ten mice were challenged with a 1:1 ratio of KO and RT strains and sampled at days 1, 3, and 7, and the ratio of KO to RT in nasal washes was determined using strain-specific primers and PCR. No KO strain was detected from nasal sampling at any of the time points (Fig. 7B), thus indicating that the KO strain was outcompeted by the RT strain as early as day 1.

Overall, these data indicate that the deletion of *dpr* greatly reduces the ability of the strain to colonize and renders the strain unable to compete with the RT strain in the NP.

DISCUSSION

S. pneumoniae lives in an oxygenated environment during colonization and encounters various oxidative stresses, derived presumably both from the host immune response and bacterial production of hydrogen peroxide. The precise mechanism by which pneumococcus neutralizes reactive oxygen species remains unclear, but several processes have been suggested, as reviewed in reference 37. Here we show that the *S. pneumoniae* Dpr binds iron and that the *dpr* mutant strain has significantly less total iron content. The *dpr* mutant strain has increased sensitivity to multiple stress conditions, including pH, temperature, iron, and oxidative stress. The *dpr* mutant is also significantly more sensitive to macrophage killing and highly attenuated in a nasal colonization model in mice.

dpr was first discovered by a library screening for a gene(s) responsible for resistance to peroxide or hydroperoxide toxicity in *S. mutans* (11). Dpr belongs to the Dps member family and has a structure similar to that of either ferritin or Dps (14, 15, 38–40). Studies have showed that *dpr* serves to protect Gram-positive bacteria against oxidative and other stresses (11–13). Unlike Dps, which protects DNA through direct association, Dpr does not bind to DNA (16). Instead, it has been suggested that its protection against oxidative stress is the result of chelation of intracellular free iron (17, 41). We show here that Dpr binds iron and that the *dpr* mutant has increased sensitivity to iron. These results are consistent with an iron-binding function to reduce the oxidative stress that results from the Fenton reaction. These results are also consistent with the findings of another study which showed that *dpr* expression increased in the presence of hemoglobin (42). Two iron-related regulators, RitR and IdtR, have been identified in

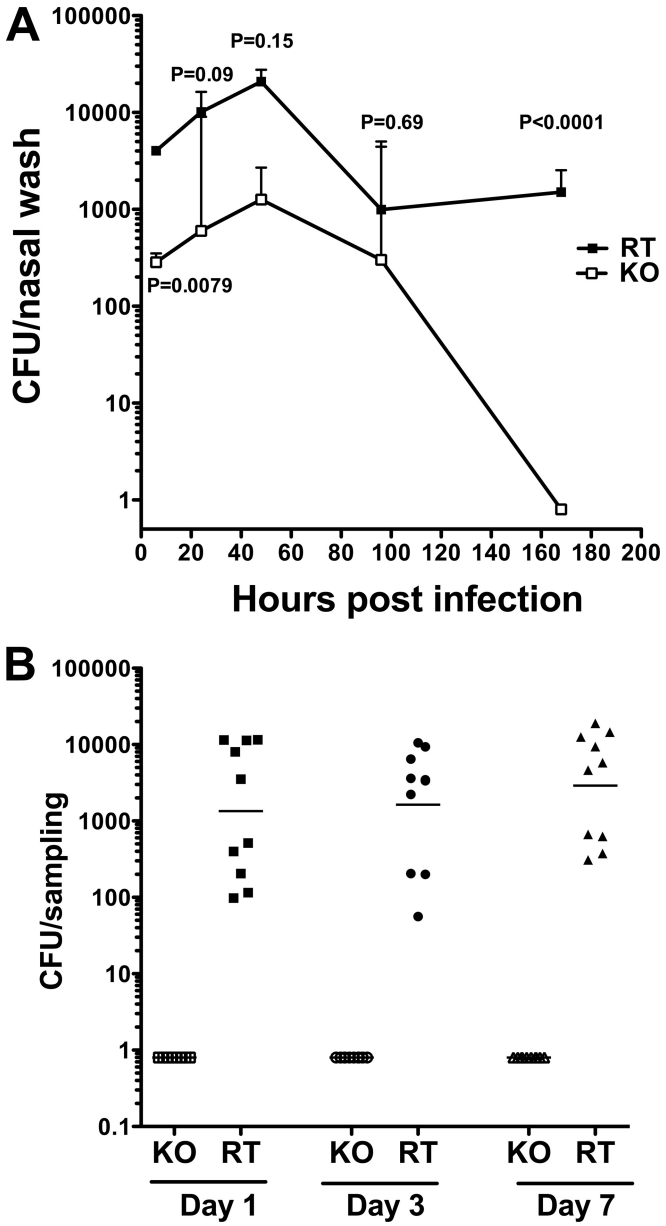


FIG 7 The ability of the *dpr* mutant to colonize the nasopharynx is severely impaired. (A) Groups of 5 or 10 mice were challenged intranasally with either the RT or KO strain, and their nasal colonization densities were determined at different time points. Colonization densities of the RT and KO strains at each time point were compared by the Mann-Whitney U test. (B) Competition between RT and KO strain in mice. Mice were intranasally challenged with 10^7 CFU of a 1:1 ratio of RT and KO strains, and samples were taken at days 1, 3, and 7. Total numbers of CFU were determined by growth on BAP. The absolute quantity of strain-specific genomic DNA in a sample was determined using strain-specific primer sets and then used to calculate the ratio of the two strains in the NP. No KO strain was detected at any time point.

pneumococcus (18, 43), and RitR was demonstrated to play a regulatory role in the expression of *dpr*, but whether IdtR is also involved in regulation of *dpr* expression still needs to be determined. Overall, our results are consistent with a role of *dpr* in conferring pneumococcal resistance to multiple stress conditions.

We also show here that *dpr* is in an operon with the downstream genes *dhfr* and *clpX*, suggesting that their transcriptional regulation and function may be related, as has been demonstrated in other bacterial species. In *E. coli*, ClpXP rapidly degrades Dps (the Dpr homolog) in stationary phase (44). However, whether Dpr concentration is similarly controlled by ClpX in pneumococcus in stationary phase is unknown.

The *dpr* mutant is significantly defective in resistance to macrophage killing and its ability to colonize mice. As an immunogen, Dpr has been tested in different pneumococcal animal models and shown to confer protection against pneumococcal colonization and invasive diseases (45–47). This study thus provides additional evidence for an important role of Dpr in pneumococcal pathogenesis.

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