Interplay between Manganese and Iron in Pneumococcal Pathogenesis: Role of the Orphan Response Regulator RitR


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Streptococcus pneumoniae (the pneumococcus) is a major human pathogen that is carried asymmetrically in the nasopharynx by up to 70% of the human population. Translocation of the bacteria into internal sites can cause a range of diseases, such as pneumonia, otitis media, meningitis, and bacteremia. This transition from nasopharynx to growth at systemic sites means that the pneumococcus needs to adjust to a variety of environmental conditions, including transition metal ion availability. Although it is an important nutrient, iron potentiates oxidative stress, and it is established that in S. pneumoniae, expression of iron transport systems and proteins that protect against oxidative stress are regulated by an orphan response regulator, RitR. In this study, we investigated the effect of iron and manganese ion availability on the growth of a ritR mutant. Deletion of ritR led to impaired growth of bacteria in high-iron medium, but this phenotype could be suppressed with the addition of manganese. Measurement of metal ion accumulation indicated that manganese prevents iron accumulation. Furthermore, the addition of manganese also led to a reduction in the amount of hydrogen peroxide produced by bacterial cells. Studies of virulence in a murine model of infection indicated that RitR was not essential for pneumococcal survival and suggested that derepression of iron uptake systems may enhance the survival of pneumococci in some niches.

S. pneumoniae (the pneumococcus) continues to be responsible for massive global morbidity and mortality, causing more deaths worldwide than any other single pathogen (1). It is carried asymmetrically in the nasopharynx of a high proportion of the human population, but in a subset of these individuals, it translocates to deeper sites, causing a broad spectrum of diseases, including pneumonia, meningitis, bacteremia, and otitis media (2). In developing countries, 1 to 2 million children under 5 years of age die each year from pneumonia, of which S. pneumoniae is the single most common cause, accounting for 20 to 25% of all deaths in this age group (3, 4). Even in developed countries, where effective antimicrobial therapy is readily accessible, morbidity and mortality are substantial. In these countries, deaths from pneumococcal disease occur primarily among those over 60 years of age, with case fatality rates of 10 to 20% for pneumonia and up to 60% for bacteremia (4).

Although it is recognized that the host niches occupied by S. pneumoniae differ greatly, there is still a lack of information about the effect of a changing microenvironment on the physiological properties of the bacterium and how this relates to pathogenesis. Over the last decade, the importance of transition metal ions in host-pathogen interactions has been recognized, with manganese emerging as a transition metal ion of particular importance to the cellular physiology of the pneumococcus (5, 6). This ion is a prosthetic group in superoxide dismutase (SodA) and also has been shown to have direct antioxidant properties (7, 8). Also, CpsB, a protein that is essential for the cycle of polysaccharide capsule assembly, export, and attachment to the cell wall, is a manganese-dependent tyrosine phosphatase (9). Manganese is present at low concentrations in host tissues (range, 0.4 to 1.1 μM in the blood, nasopharynx, and lungs of mice (10), and transport of manganese is dependent on the ABC-type permease PsaBCA (11). The correlation of hypersensitivity of psa mutants of S. pneumoniae to oxidative stress with loss of ability to transport manganese explains the avirulence of pneumococcal psa mutants in murine models (5, 8).

Even though S. pneumoniae lacks a respiratory chain, it can tolerate and consume oxygen. Two enzymes are of particular importance: NADH oxidase, which reduces molecular oxygen to water and consumes NADH produced during glycolysis (12–14), and pyruvate oxidase (SpxB), which converts pyruvate to acetyl-phosphate and hydrogen peroxide (H₂O₂) (15, 16). The actions of these enzymes provide the pneumococcus with an increased yield of ATP per mole of glucose. However, the H₂O₂ that is generated has the potential to cause oxidative damage to the bacterium. The pneumococcus lacks catalase, but it does possess a thiol-peroxidase (PsaD) (5, 8). Manganese plays a pivotal role in the defense of the cell against peroxide stress, although the biochemical basis for its action is still a matter of intensive study (17–19). An important factor in the protection of S. pneumoniae against peroxide stress is the relatively low requirement for iron; the pneumococcus has only a few enzymes containing iron-sulfur clusters, and it lacks a respiratory chain (20–22). Thus, the potential for production of damaging hydroxyl radicals from reaction of ferrous ions (Fe²⁺) and hydrogen peroxide (the Fenton reaction) is reduced. Nevertheless, S. pneumoniae does possess a Dpr protein (Dps-like per-
oxide resistance protein) that, like its Escherichia coli homologue (Dps), is able to protect the cell against iron and oxidative stress (23, 24). The response regulator RitR has previously been identified as being central to the response to iron and oxidative stress by uptake systems in S. pneumoniae (25).

In the present study, we investigated the effect of mutation of ritR on pneumococcal growth and physiology in media differing Fe/Mn ratios. Furthermore, we compared the pathogenesis properties of the virulent encapsulated strain D39 and an isogenic ritR mutant using a murine model of infection. These observations provide new insights into the interplay between transition metal ions in pneumococcal survival and virulence.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The virulent S. pneumoniae type 2 strain D39 (NCTC 7466) and derived strains were routinely grown in liquid culture at 37°C in chemically defined medium (CDM) (26). The strains used in this study are listed in Table 1. CDM was modified to include 4 mM l-lactate and 10 mM d-glucose as carbon sources, to exclude iron to derive Mn-CDM (final Mn concentration, 33 μM), and to exclude manganese to derive Fe-CDM (final Fe concentration, 20 μM). Cells were plated on blood agar base (Becton, Dickinson) supplemented with 5% defibrinated horse blood (blood agar plates) and grown at 37°C in the presence of 5% CO2. When antibiotic selection was required, media were supplemented with 0.2 μg/ml erthyromycin or 200 μg/ml spectinomycin. For mouse challenge experiments, strains were cultured in nutrient broth supplemented with 10% horse serum (serum broth).

**DNA manipulation and genetic techniques.** Chromosomal DNA was purified by using the GenElute bacterial genomic DNA kit (Sigma). PCR was performed by using Kod Hot Start DNA polymerase (Merck) (for amplification of the ritR deletion fragments) or Taq polymerase (New England BioLabs) (for screening assays) according to the manufacturers’ instructions. S. pneumoniae D39 ∆ritR was constructed by deletion-replacement using overlap extension PCR mutagenesis and direct transformation as previously described (27). The 1-kb region upstream of ritR was amplified using primers ritR1 and ritR2, while the 1-kb region downstream of ritR was amplified using primers ritR3 and ritR4. The erythromycin cassette was amplified using primers ery-F and ery-R. Three PCR fragments generated were joined together using primers ritR1 and ritR4 to form the deletion fragment. The ritR and sodA double deletion was constructed using a similar method. The 1-kb region upstream of sodA was amplified using primers sodA1 and sodA2, while the 1-kb region downstream of sodA was amplified using primers sodA3 and sodA4. The spectinomycin cassette was amplified using primers spec-F and spec-R. All deletion mutations were confirmed by Sanger sequencing. The primer sequences used in this study are listed in Table 2.

Complementation of D39 ∆ritR was performed by marker rescue. The entire region, including the 1-kbp regions upstream and downstream of ritR, was amplified using primers ritR1 and ritR4. The purified PCR fragment was transformed into D39 ∆ritR (27), and potential complemented strains were screened for the loss of erythromycin resistance. Erythromycin-sensitive colonies were screened by PCR, and the ritR complemented strain was confirmed via Sanger sequencing. All samples were submitted to the Australian Equine Genomic Research Centre (University of Queensland, Brisbane, Australia) for Sanger sequence analysis.

**Growth curve analysis.** Cells were grown overnight in CDM, washed three times in phosphate buffer, and diluted to an optical density at 600 nm (OD600) of 0.01 in fresh medium. The cells were statically grown in a microaerobic environment (25 ml medium in a 50-ml tube). The OD600 of the cells was measured every hour initially, increasing to every half hour during the exponential phase.

**Hydrogen peroxide concentration measurement.** Cells were grown microaerobically in CDM to mid-exponential phase (OD600 ~0.6). The cells were pelleted by centrifugation, and the supernatant was filtered through a 0.22-μm filter. Hydrogen peroxide was detected in the filtered cells by using the Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen). The final value was normalized to the number of cells present by measuring the total protein content in accordance with the QuantiPro BCA assay kit (Sigma) instructions.

**Quantitative gene expression studies.** RNA was isolated from 5 ml of cells harvested at the desired growth phase in accordance with the RNeasy Mini Kit (Qiagen). The isolated RNA was DNase treated using the RNeasy Free DNase set (Qiagen) and quantified using a Nanodrop instrument (Thermo Scientific). One microgram of RNA was converted to cDNA using the SuperScript III first-strand synthesis system for reverse transcriptase (RT)-PCR (Invitrogen). Real-time reverse transcriptase PCR was performed on the 1:5-diluted cDNA using the following primers: 16S-F, 16S-R, ritR-F, ritR-R, gnd-F, gnd-R, psaA-F, psaA-R, psaD-F, psaD-R, zwf-F, zwf-R, spxB-F, and spxB-R. The primer sequences used in this study are listed in Table 2. The PCR was performed using SYBR green Master Mix (Applied Biosystems) according to the manufacturer’s instructions. The reaction was performed using a 7900 HT real-time PCR machine (Applied Biosystems) under the following conditions: 95°C for

**Table 1:** Strains used in the study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>D39</td>
<td>S. pneumoniae virulent type 2 strain</td>
<td>22</td>
</tr>
<tr>
<td>D39 ∆ritR</td>
<td>S. pneumoniae D39 ritR::erm deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>D39 ∆sodA</td>
<td>S. pneumoniae D39 ritR::erm and sodA::aad double-deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>D39 ∆ritR::ritR</td>
<td>S. pneumoniae D39 ritR complemented strain</td>
<td>This study</td>
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**Table 2:** Primers used in the study

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<th>Primer</th>
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<td>ritR1</td>
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<td>ritR2</td>
<td>5’-TTGGTGTGATTAACCTTCTCC</td>
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<tr>
<td>ritR3</td>
<td>5’-GGGGAGGATATATTTGATACGG</td>
</tr>
<tr>
<td>ritR4</td>
<td>5’-GAATGATTGAGCGAGTTGG</td>
</tr>
<tr>
<td>ery-F</td>
<td>5’-CAATGATTTATCTTACGATGTAATACCTCTTTTTCTTTTTT</td>
</tr>
<tr>
<td>ery-R</td>
<td>5’-CTCATGAAATATTTCCCTCGG</td>
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<tr>
<td>sodA1</td>
<td>5’-GGATGTTGAGCGAGTTGG</td>
</tr>
<tr>
<td>sodA2</td>
<td>5’-CGATGTTGATTTATCTTACGATGTAATACCTCTTTTTCTTTTTT</td>
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<td>sodA3</td>
<td>5’-AACATGATTTATCTTACGATGTAATACCTCTTTTTCTTTTTT</td>
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<td>sodA4</td>
<td>5’-ACAGTCGACCTTGAGTTGCTA</td>
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<tr>
<td>spec-F</td>
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</tr>
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<td>spec-R</td>
<td>5’-AAATCCTGTTATTTATCAAGATTTATAGATTTATAGAT</td>
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</tr>
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<td>ritR-F</td>
<td>5’-TATCCCAACACCCGACACGTT</td>
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<td>ritR-R</td>
<td>5’-AAAGATCCGCAAGCCCTT</td>
</tr>
<tr>
<td>gnd-F</td>
<td>5’-GTCACGCAATTGAGC</td>
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<td>gnd-R</td>
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</tr>
<tr>
<td>spxB-R</td>
<td>5’-AGGGGATGACCTTTACCAAG</td>
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10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min; and a final dissociation cycle of 95°C for 2 min, 60°C for 15 s, and 95°C for 15 s. All data were analyzed using SDS 2.2.2 software (Applied Biosystems). Relative gene expression was calculated using the 2^(-ΔΔCt) method with 16S as the reference gene.

Murine model of infection. For mouse survival experiments, 10 5- to 6-week-old outbred female CD1 (Swiss) mice (per strain) were injected intraperitoneally with 5 × 10^5 CFU of either D39 wild-type (WT), D39 ΔritR, or complemented cells; the mice were observed over 8 days; and survival times were recorded. For the intranasal-challenge experiments, 20 mice (per strain) were first anesthetized by intraperitoneal injection with pentoobarbitone sodium (Nembutal; Rhone-Merieux) before 5 × 10^6 CFU of the D39 WT, D39 ΔritR, or ritR complemented strain was pipetted into the nares and involuntarily inhaled. Ten mice (per strain) were euthanized at 24 h and 48 h postchallenge, and the numbers of viable cells in the blood, lungs, nasal wash, and nasal tissue were determined as follows. Blood (1 ml) was collected from the heart, the nasopharynx was washed with 1 ml phosphate-buffered saline (PBS), and the lungs and the nasopharyngeal cavity were excised, washed briefly, placed in 1 ml of sterile PBS, and homogenized using a Precellys 24 homogenizer (Bertin Technologies). Samples were then serially diluted and plated on blood agar (supplemented with 0.2 μg/ml erythromycin where needed) to determine the numbers of viable D39 WT, D39 ΔritR, and ritR complemented cells. All animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals (National Health and Medical Research Council, Canberra, Australia) and were approved by the University of Adelaide Animal Ethics Committee.

Analysis of the intracellular metal concentration. Cells harvested from the desired growth phase were pelleted and washed with phosphate buffer, resuspended in 80% nitric acid, and incubated at 80°C for 24 h. The sample was then diluted to 2% nitric acid and submitted for inductively coupled plasma mass spectrometry (ICP-MS) analysis at the National Research Centre for Environmental Toxicology (University of Queensland, Brisbane, Australia). The final value was normalized to the number of cells present by measuring the total protein content in accordance with the QuantPro BCA assay kit (Sigma) instructions.

Statistical analysis. Differences in metal ion concentrations, hydrogen peroxide concentrations, relative gene expression, and numbers of CFU/ml in mouse studies were analyzed using a two-tailed t test (Minitab 15 statistical software).

RESULTS

Analysis of the growth of the ritR mutant in media containing altered Fe/Mn ratios. To determine whether the Fe/Mn ratio influenced the growth of *S. pneumoniae*, we developed two media based on a CDM (26). One of them had a high Fe/Mn ratio (Fe-CDM), while the other had a low Fe/Mn ratio (Mn-CDM) (see Materials and Methods). Figure 1A shows that the growth curves for wild-type D39 and the ritR mutant in Mn-CDM were essentially the same. However, in Fe-CDM, growth of the wild-type strain was unaffected, but the ritR mutant did not emerge from lag phase and did not grow in this high-Fe/Mn medium. Complementation of the ritR mutant restored its ability to grow in Fe-CDM (Fig. 1A), confirming that the phenotype of the mutant is attributable to the ritR mutation.

The above data confirmed previous work (25) showing that the ritR mutant is unable to tolerate high Fe levels. To determine whether Mn could influence this loss of Fe tolerance, we titrated Mn into Fe-CDM and tested growth as a function of the Fe/Mn ratio. Figure 1B shows that increasing Mn restored growth of the ritR mutant in Fe-CDM. The molecular basis of the protection afforded to bacteria by manganese is still not fully understood, although two interesting hypotheses have emerged that may explain its antioxidant activity (18, 28), in addition to its role as a component of Mn-dependent superoxide dismutases and catalases. *S. pneumoniae* possesses an Mn-dependent superoxide dismutase (SodA), but not an Mn-dependent catalase. To investigate whether SodA contributed to the rescue of the ritR mutant by Mn, we constructed a ritR sodA double mutant. Figure 1C shows that...
addition of Mn to the Fe-CDM still rescued this double mutant from Fe-induced growth inhibition, indicating that the removal of superoxide by SodA is not a factor in the Mn-dependent suppression of Fe-induced stress.

Effect of Mn on the intracellular Fe concentration. To further understand the biochemical basis of the Mn-dependent rescue of the ritR mutant from Fe-induced stress, we determined the cellular content of these two metal ions using ICP-MS. Cells were collected during mid-exponential phase and prepared for ICP-MS analysis as described in Materials and Methods. However, due to the inability of the ritR mutant to grow beyond an OD600 of 0.1 in Fe-CDM, a larger volume of cells was collected during mid-lag phase, and all the results were adjusted based on the amount of protein present per sample. Figure 2A shows that in the wild-type and ritR complemented strains, the concentration of Fe in cells grown in Fe-CDM was about 20-fold higher than that observed for cells grown in Mn-CDM. However, this was only a small increase compared to the situation for the ritR mutant, where it was observed that a higher concentration of Fe had accumulated in cells grown in Fe-CDM (>120-fold increase). Thus, the ritR mutant hyperaccumulates Fe, consistent with previous observations that this response regulator is a repressor of the expression of iron uptake systems in S. pneumoniae (25). We also investigated the effect of increasing Mn on the cellular concentrations of Fe and Mn in the ritR mutant grown in Fe-CDM. Figure 2B shows that addition of a minimum of 1 μM Mn resulted in a significant decrease in Fe accumulation (from 11 to 2 μmol/mg protein; P < 0.001). Furthermore, Fe accumulation decreased even further to 0.8 μmol/mg protein as added Mn was increased to 10 μM, although this did not reach statistical significance (P > 0.05). Subsequently, the internal Fe level remained low at around 0.6 to 0.7 μmol/mg protein when the added Mn was further increased to 100 μM (P > 0.05). Thus, it can be concluded that the Mn-dependent rescue of the ritR mutant from Fe-induced stress is correlated with a decrease in the cellular Fe content.

Effect of Mn on hydrogen peroxide production. Hydrogen peroxide is produced by the pneumococcus as a consequence of the action of pyruvate oxidase (SpxB) (15). In cells grown in Mn-CDM, the wild-type, ritR mutant, and complemented ritR mutant produced similar amounts of hydrogen peroxide (1.4, 1.1, and 1.3 μmol per mg protein, respectively; P > 0.05) (Fig. 3A). However, in Fe-CDM, hydrogen peroxide production was much higher in the ritR deletion mutant than in the wild type and the complemented ritR mutant (23.0 compared to 1.4 and 1.6 μmol per mg protein, respectively; P = 0.0001) (Fig. 3A). Addition of as little as 10 μM Mn to Fe-CDM resulted in a decrease in hydrogen peroxide production in the mutant (P < 0.05) (Fig. 3B), with further reduction achieved at higher Mn concentrations (P < 0.05) (Fig. 3B).

Effect of differing Fe/Mn ratios on the gene expression profile of S. pneumoniae. In order to begin to understand the physiological basis of the protective effects of manganese, samples were collected during the mid-exponential phase of growth in either Fe-CDM or Mn-CDM, with the exception of the ritR mutant in Fe-CDM, where a larger volume of sample was collected during the mid-lag phase of growth, and relative gene expression was measured. Expression of the following genes was tested: ritR, spxB, gnd, zwf, psaA, and psaD, which encode RitR, pyruvate oxidase, 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, pneumococcal surface adhesin A (manganese ABC transporter), and a putative thiol peroxidase, respectively. ritR was expressed at an approximately 2-fold-higher level when the cells were grown in a high-Fe environment compared to a high-Mn environment (P < 0.05) (Fig. 4A). This was consistent with previous studies indicating that ritR is upregulated by Fe (25).

Hydrogen peroxide is produced by the actions of two enzymes, lactate oxidase (LctO) and pyruvate oxidase (SpxB). As shown in Fig. 3A, it was observed that hydrogen peroxide levels were significantly higher in the ritR mutant when grown in Fe-CDM. The gene expression levels of lctO showed no significant difference in all strains when grown in either medium (data not shown). However, the gene expression of spxB was approximately 2-fold higher in the ritR mutant when grown in Fe-CDM than when grown in Mn-CDM (P < 0.05) (Fig. 4B). Additionally, the spxB gene expression in the ritR mutant when grown in Fe-CDM was 2-fold...
higher than expression in the wild-type and complemented strains when grown in both media ($P < 0.05$) (Fig. 4B).

A high-Fe environment can result in increased oxidative stress to bacteria (17, 29, 30). There are a number of ways bacteria combat this stress, most of which require NADPH as the ultimate source of reducing power to drive the detoxification of reactive oxygen species (31–33). In the pneumococcal genome, *gnd* is located directly upstream of *ritR* and encodes 6-phosphogluconate dehydrogenase, the second enzyme in the hexose monophosphate (HMP) shunt that diverts glucose 6-phosphate from glycolysis to bacteria (17, 29, 30). There are a number of ways bacteria combat this stress, most of which require NADPH as the ultimate source of reducing power to drive the detoxification of reactive oxygen species (31–33). In the pneumococcal genome, *gnd* is located directly upstream of *ritR* and encodes 6-phosphogluconate dehydrogenase, the second enzyme in the hexose monophosphate (HMP) shunt that diverts glucose 6-phosphate from glycolysis.
(the Embden-Meyerhof pathway) to the pentose phosphate pathway and generates NADPH in the process (20). The relative gene expression of \( gnd \) was higher in cells grown in Fe-CDM than in those grown in Mn-CDM, consistent with the hypothesis that a high-Fe environment increases oxidative stress on the bacteria. \( (P < 0.05) \) (Fig. 4C). Furthermore, the relative gene expression of \( gnd \) was approximately 3-fold higher in the \( ritR \) mutant when grown in Fe-CDM compared to growth in Mn-CDM and the wild-type and the complemented \( ritR \) mutant in both media \( (P < 0.05) \) (Fig. 4C).

The gene encoding the metal-binding component of the manganese ABC transporter, \( psaA \), was expressed at approximately 12-fold-higher levels in both the wild type and the complemented \( ritR \) mutant when the cells were grown in Fe-CDM than when they were grown in Mn-CDM \( (P < 0.05) \) (Fig. 4E). However, \( psaA \) was expressed at only 2-fold-higher levels in the \( ritR \) mutant when it was grown in Fe-CDM than when it was grown in Mn-CDM \( (P < 0.05) \) (Fig. 4E). In Fe-CDM, the \( ritR \) mutant expressed \( psaA \) at approximately 2-fold-lower levels than the wild-type and the complemented strain \( (P < 0.05) \) (Fig. 4E).

The putative thiol peroxidase, PsAD, has been thought to be involved in defense against oxidative stress (5). In general, the trend indicated slightly higher \( psaD \) gene expression in Mn-CDM than in Fe-CDM, with only the wild-type data reaching statistical significance (Fig. 4F). This could indicate that the enzyme is Mn regulated, as it is situated downstream of the \( psaBCA \) operon. Furthermore, studies have shown that transcription of \( psaD \) from the \( psaD \) promoter is weaker than that from the \( psaBCA \) promoter (5, 34).

**Virulence of a D39 \( \triangle ritR \) deletion mutant in a mouse model of infection.** Previous studies (35, 36) have suggested that RitR might have an important role in virulence. We used the \( ritR \) deletion mutant of \( S. \) pneumoniae D39 and compared its virulence with that of the otherwise isogenic wild-type and complemented strains. After intraperitoneal challenge, the average mouse survival time was slightly higher when inoculated with the \( ritR \) mutant than when inoculated with the wild type and the complemented \( ritR \) mutant \( (P < 0.05) \) (Fig. 5). This result was, however, not statistically significant, and all mice failed to survive past 2 days postinfection. Following an intraperitoneal infection, after 24 h, there was essentially no difference in the numbers of CFU of the pneumococcus recovered from blood (Fig. 6A), and this was also

**FIG 5** Survival time post-intraperitoneal challenge of female CD1 (Swiss) mice. The median mouse survival time postchallenge is represented by the horizontal line.

**FIG 6** Numbers of pneumococci recovered from blood 24 h post-intraperitoneal challenge (A), blood 24 h post-intranasal challenge (B), blood 48 h post-intranasal challenge (C), and lungs 48 h post-intranasal challenge (D). \( t \) tests were performed for D39 versus D39 \( \Delta ritR \) and D39 versus D39 \( \Delta ritR::ritR; \) * \( P < 0.05; ** \( P < 0.01. \)

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the case following an intranasal challenge (Fig. 6B). However, at 48 h, there was actually an increased recovery of the ritR mutant compared to the wild-type and complemented strains from both blood and lungs (Fig. 6C and D, respectively). Taken together, these results indicate that RitR is not essential for pneumococcal survival in a mouse model of infection.

**DISCUSSION**

Manganese has been established for some time as a key transition metal ion for the growth and virulence of pneumococci (5, 37–39). This view is largely derived from the observation that the manganese ABC transporter PsaBCA is critical for the survival of *S. pneumoniae* (5, 8, 11), but more specifically, it is known that SodA is also essential for virulence (7). These observations support the view that Mn plays an important role as an antioxidant, potentially via a variety of mechanisms (7, 28, 39–42). In contrast to Mn, Fe is a promoter of oxidative stress, especially in combination with hydrogen peroxide, since this leads to the production of hydroxyl radicals via the Fenton reaction (43). This is potentially devastating for *S. pneumoniae*, since it produces hydrogen peroxide as the product of pyruvate oxidation during growth in the presence of oxygen. *S. pneumoniae* has a limited demand for Fe; the bacterium lacks a respiratory chain and hence possesses no cytochromes (20), although it does require iron for the formation of enzymes containing FeS clusters, including anaerobic ribonucleotide reductase (20). In addition, mutation of the genes encoding the ABC transporters required for iron acquisition (*piaABCD* and *piuBCDA*) leads to attenuation of *S. pneumoniae* in a murine model of infection (44, 45).

It has been established that coordinate RitR-dependent repression of expression of Fe uptake systems (25, 44–46) and the derepression of genes (*dps*) involved in protection against Fe and hydrogen peroxide stress appear to contribute to protection of pneumococci against the toxic effects of the Fenton reaction. As expected, we observed that loss of control of iron homeostasis and defense against Fe and peroxide led to susceptibility of the ritR mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high. We expected that Mn might rescue the mutant under these conditions, and this mutant to Fe toxicity when the Fe/Mn ratio was high.
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REFERENCES