**Streptococcus pneumoniae** Serine Protease HtrA, but Not SFP or PrtA, Is a Major Virulence Factor in Pneumonia

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Abstract

*Streptococcus (S.) pneumoniae* is a common causative pathogen in pneumonia. Serine protease orthologs expressed by a variety of bacteria have been found of importance for virulence. Previous studies have identified two serine proteases in *S. pneumoniae*, HtrA (high-temperature requirement A) and PrtA (cell wall-associated serine protease A), that contributed to virulence in models of pneumonia and intraperitoneal infection respectively. We here sought to identify additional *S. pneumoniae* serine proteases and determine their role in virulence. The *S. pneumoniae* D39 genome contains five putative serine proteases, of which HtrA, Subtilase Family Protein (SFP) and PrtA were selected for insertional mutagenesis because they are predicted to be secreted and surface exposed. Mutant D39 strains lacking serine proteases were constructed by in-frame insertion deletion mutagenesis. Pneumonia was induced by intranasal infection of mice with wild-type or mutant D39. After high dose infection, only D39ΔhtrA showed reduced virulence, as reflected by strongly reduced bacterial loads, diminished dissemination and decreased lung inflammation. D39ΔprtA induced significantly less lung inflammation together with smaller infiltrated lung surface, but without influencing bacterial loads. After low dose infection, D39ΔhtrA again showed strongly reduced bacterial loads; notably, pneumococcal burdens were also modestly lower in lungs after infection with D39Δsfp. These data confirm the important role for HtrA in *S. pneumoniae* virulence. PrtA contributes to lung damage in high dose pneumonia; it does not however contribute to bacterial outgrowth in pneumococcal pneumonia. SFP may facilitate *S. pneumoniae* growth after low dose infection.

Introduction

The bacterium *Streptococcus (S.) pneumoniae* is a major global cause of human disease [1]. *S. pneumoniae* is the most frequent cause of community-acquired pneumonia and a common pathogen in sepsis, the incidence being greatest at the extremes of age and in immune compromised individuals [2]. Although the discovery of antibiotics and the development of vaccines have reduced the health burden associated with pneumococcal infections, *S. pneumoniae* still causes over 2 million deaths annually [1] and increased bacterial resistance against currently available antibiotics could make pneumococcal infections an even larger health threat in the future [3]. Consequently, additional knowledge about this bacterium and its virulence factors is of importance.

In 1991, Courtney [4] was the first to describe a role for serine proteases as virulence factors for *S. pneumoniae*. Since then, serine protease orthologs have been found in many bacteria and numerous roles in virulence and pathogenesis have been described [5–16]. One of these serine proteases is HtrA (high-temperature requirement A), which has been identified as a virulence factor in several bacterial species. In *S. pneumoniae*, HtrA is important for bacterial stress response and protein quality control, and has a role in competence [11,14,17]. As a posttranslational regulator, HtrA is involved in bacteriocin activity and cell division [8,15,16]. HtrA has been identified as an important virulence factor for *S. pneumoniae*, i.e. HtrA deficient pneumococci demonstrated a dramatically reduced virulence in models of pneumonia and bacteremia [11]. Another investigation identified PrtA (cell wall-associated serine protease A) as a pneumococcal serine protease important for virulence after intraperitoneal infection [5].

In this study, we searched for additional serine proteases as virulence factors of *S. pneumoniae*. By reannotating the *S. pneumoniae* D39 genome using a subsystems approach [18] and by screening all proteins for the presence of serine protease associated domains using Interproscan [19], we identified SFP (subtilase family protein) as an additional surface-exposed pneumococcal serine protease besides HtrA and PrtA, and generated directed gene
Materials and Methods

Serine protease search

All proteins encoded in the genome of S. pneumoniae D39 where screened for the presence of serine protease associated domains using Interproscan [19]. Domains IPR009003 and IPR001940 identified HtrA, domain IPR008357 identified SFP, while domains IPR000299 and IPR015500 identified both SFP and PrtA as predicted to encode serine proteases. An additional search for proteins predicted to have serine protease activity was performed by examining membership of GO category 0008236, resulting in the identification of SPD_1765 and SPD_1920. Blast analysis of the proteome of S. pneumoniae D39 with HtrA, SFP and PrtA with an E-value cut-off of 0.1 showed limited protein sequence similarity of SFP with PrtA, but no other putative serine proteases were identified. Furthermore the S. pneumoniae D39 genome was re-annotated using the +6RAST subsystems approach [18] and the resulting annotations where searched for protease encoding proteins. No other obvious serine proteases could be detected using these methods. Subcellular localization prediction of the putative serine proteases was performed with SignalP [20].

Construction of directed deletion mutants

Directed-deletion mutants of S. pneumoniae D39 lacking HtrA (D39Δhtra), SFP (D39Δsfp) or PrtA (D39ΔprtA) were generated by allelic exchange of the target gene with a spectinomycin resistance marker essentially as described previously [21]. Briefly, an extension PCR was performed to join 400-500 bp 5' and 3' flanking sequences of the target gene with the spectinomycin resistance cassette (obtained from pR412T7). The resulting PCR products were introduced by competent stimulating peptide (CSP-l)-induced transformation into D39. Transformants were selected on the basis of spectinomycin resistance and were checked by PCR for recombination at the desired location on the chromosome. Subsequently, the D39 WT strain was transformed with 1 μg chromosomal DNA isolated from the mutants to prevent the accumulation of inadvertent mutations elsewhere on the chromosome. At the same time, D39 was mock-transformed to obtain a coupled WT strain. All primers used in this study are shown in Table 1.

In vitro growth assay

Mid-log growing mutant or WT S. pneumoniae were diluted to an optical density (OD) of 0.1 (620 nm wavelength) in Todd Hewitt broth (Oxoid microbiology products, Hampshire, UK) with 0.5% Yeast extract (THY). Cultures were incubated at 37°C in a 5.0% CO2 incubator. OD was measured every hour for the next 5 hours.

Animals

Specific pathogen-free C57BL/6 male and female mice were purchased from Harlan Sprague-Dawley (Horst, the Netherlands). Experimental groups were age- and sex matched, and housed in the Animal Research Institute Amsterdam under standard care. All experiments were conducted with mice between 10 and 12 weeks of age.

S. pneumoniae Serine Protease Virulence Factors

S. pneumoniae in an compared several outcome parameters 48h after infection. Every hour for the next 5 hours.

Experimental study design

Pneumonia was induced by intranasal inoculation with S. pneumoniae D39, D39Δhtra, D39Δsfp or D39ΔprtA (serotype 2; 5×10^7 or 5×10^8 colony forming units (CFU) in 50 μL isotonic saline) using previously described methods [22–24]. Mice were euthanized 48 hours after induction of pneumonia (N = 8 mice per group). Blood was obtained from the inferior vena cava and diluted 4:1 with citrate. Bronchoalveolar lavage fluid (BALF), lung, spleen and liver were harvested as described [22–24] and organs were homogenised in five volumes of sterile isotonic saline. The left lung lobe was fixed in 10% buffered formalin and embedded in paraffin. Total cell numbers in BALF were determined by an automated cell counter (Coulter Counter, Coulter Electronics, Hialeah, FL, USA). Differential cell counts were performed on cytosin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Dudingen, Switzerland). For bacterial quantification blood, BALF, and organ homogenates were serially diluted by 10-fold in sterile isotonic saline and plated onto sheep-blood agar plates. Following 16 hours of incubation at 37°C CFU were counted. For further measurements, homogenates were diluted 1:1 with lysis buffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% (v/v) Triton X-100, pH 7.4) with protease inhibitor mix and incubated for 30 minutes on ice, followed by centrifugation at 600 g for 10 minutes. Supernatants were stored at –20°C until analysis.

Histopathology

Four-micrometer sections of the left lung lobe were stained with hematoxylin and eosin (H&E). Slides were coded and scored by a pathologist blinded for group identity for the following parameters: interstitial inflammation, endothelialitis, bronchiitis, oedema, pleuritis and presence of thrombi. All parameters were rated separately from 0 (condition absent) to 4 (most severe condition). The total histopathological score was expressed as the sum of the scores of the individual parameters, with a maximum of 24. In addition, pulmonary infiltrate was scored as a percentage of total lung surface occupied by confluent infiltrates.

Assays

Interleukin (IL)-6, tumor necrosis factor alpha (TNF-α), keratinocyte-derived cytokine (KC) and IL-1β were measured using commercially available ELISA kits (R&D Systems, Abingdon, UK). Myeloperoxidase (MPO; Hydult, Uden, the Netherlands) was measured by ELISA according to manufacturers’ instructions.

Statistical analysis

Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation, or as medians with interquartile ranges. Comparisons between groups were first performed using Kruskal-Wallis one-way analysis of variance test; in case of significant differences, differences between groups were tested using the Mann-Whitney U test. All analyses were done using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). P-values < 0.05 were considered statistically significant.
displays diminished growth and dissemination.

We infected mice with 5 × 10^5 CFU of either WT D39, D39ΔhtrA, D39Δsfp or D39ΔprtA via the airways and quantified bacterial loads at the primary site of infection (lungs) and distant body sites (blood, spleen and liver) 48 hours later (figure 2). WT S. pneumoniae D39, D39Δsfp and D39ΔprtA had multiplied in the lungs, accompanied by dissemination to spleen and liver; the counts of these three pneumococcal strains were similar in all body sites examined. In contrast, D39ΔhtrA counts were markedly lower in the lungs and in all distant body sites when compared with the other three strains (P < 0.0005). In addition, no bacteria could be detected in the blood of D39ΔhtrA infected animals (P < 0.005).

### Reduced lung inflammation during pneumonia caused by S. pneumoniae D39ΔhtrA or D39ΔprtA

This model of pneumococcal pneumonia is associated with histological features in the lung characteristic for lower respiratory tract infection, including interstitial inflammation, endothelialitis, edema, inflammatory infiltrates and pleuritis [25]. To determine the impact of the three S. pneumoniae serine proteases on the induction of these inflammatory alterations, we semi-quantitatively scored lung histology slides after pneumonia caused by WT D39, D39ΔhtrA or D39ΔprtA (figure 3). The lungs of D39ΔhtrA infected mice displayed significantly less inflammation and a smaller infiltrated lung surface (P < 0.01). Remarkably, in spite of similar bacterial loads, the lungs of D39ΔprtA infected mice also displayed lower histopathology scores when compared with lungs of WT D39 infected mice, together with a smaller infiltrated lung surface (P < 0.05).

Considering that neutrophils play a key role in the inflammatory response during respiratory tract infection by S. pneumoniae [1,26], we next determined total cell and neutrophil counts in BALF after infection with WT D39, D39ΔhtrA, D39Δsfp or D39ΔprtA (figure 4). We additionally measured MPO concentration in whole lung homogenates, as a marker of neutrophil content of lung tissue. Total cell counts were lower in BALF.
obtained from D39ΔhtrA infected mice (P < 0.05); a similar trend was observed for neutrophil influx. In accordance, whole lung MPO concentrations were lower after infection with D39ΔhtrA (P < 0.05). The deletion of either sfp or prtA did not influence cell recruitment. Cytokines and chemokines play an eminent role in the regulation of inflammation during pneumonia [1,26]. Therefore, we measured cytokines (TNFα, IL-1β, IL-6) and a chemokine (KC) in whole lung homogenates as an additional readout for pulmonary inflammation. Lung IL-1β, IL-6 and KC levels were lower in D39ΔhtrA when compared with the other three S. pneumoniae strains (table 2).

**Modest role for SFP after low dose infection**

The data presented above confirm that HtrA plays a significant role in S. pneumoniae virulence [11]. We argued that the...
contribution of PrtA and SFP to \textit{S. pneumoniae} virulence could be more subtle and as such not noticed in our model with a relatively high bacterial dose. Accordingly, we repeated our infection experiments with a 10-fold lower inoculum ($5 \times 10^4$ CFU). These studies again revealed the reduced virulence of D39\textit{ΔhtrA}, as reflected by lower bacterial loads in lungs ($P < 0.005$) and liver ($P < 0.005$) at 48 hours post infection (figure 5). Of interest, pneumococcal burdens were also modestly but significantly lower in lungs after infection with D39\textit{Δsfp} ($P < 0.05$); this difference with WT D39 was not observed in blood or distant organs. Cell influx in low dose pneumonia was modest (Figure 6), with less than half of the total cell counts in BALF compared to high dose infection. No difference in total cell numbers was observed between WT or mutant \textit{S. pneumoniae} infected mice (figure 6A). Neutrophil influx determined by cell differentiation on cytospin slides however was diminished in D39\textit{ΔhtrA} infected animals ($P < 0.05$).

\textbf{Figure 2.} \textit{S. pneumoniae} D39\textit{ΔhtrA}, but not D39\textit{Δsfp} or D39\textit{ΔprtA}, displays diminished growth and dissemination \textit{in vivo}. Mice were infected with WT or mutant \textit{S. pneumoniae} ($5 \times 10^5$ CFU) via the intranasal route and euthanized 48 hours later. Bacterial counts were determined in lung (A), blood (B), spleen (C) and liver (D). Data are expressed as box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 8 mice per group at each time point. *** $P < 0.005$ versus WT \textit{S. pneumoniae}.

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### Table 2. Lung cytokine and chemokine levels.

<table>
<thead>
<tr>
<th>\textit{S. pneumoniae}</th>
<th>D39</th>
<th>D39\textit{ΔhtrA}</th>
<th>D39\textit{Δsfp}</th>
<th>D39\textit{ΔprtA}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung homogenate, t = 48h (pg/mL)</td>
<td></td>
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</tr>
<tr>
<td>IL-6</td>
<td>1689 (257 – 2864)</td>
<td>174 (136 – 220) **</td>
<td>1076 (220 – 3045)</td>
<td>260 (174 – 3717)</td>
</tr>
<tr>
<td>KC</td>
<td>7164 (747 – 8697)</td>
<td>362 (322 – 444) **</td>
<td>3218 (659 – 7962)</td>
<td>482 (300 – 8355)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>724 (116 – 1117)</td>
<td>81 (73 – 97) **</td>
<td>363 (129 – 859)</td>
<td>106 (79 – 1047)</td>
</tr>
</tbody>
</table>

Mice were infected with the \textit{S. pneumoniae} strain indicated ($5 \times 10^5$ CFU) via the intranasal route and euthanized 48 hours later. Data are expressed as medians and interquartile range of 7 or 8 mice per group. ** $P < 0.01$ versus WT \textit{S. pneumoniae}.

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or mutant *S. pneumoniae*.

After high dose infection *D39* diminished systemic dissemination and decreased lung inflammation, as reflected by strongly reduced bacterial loads, diminished systemic dissemination and decreased lung inflammation. After high dose infection *D39ΔhtrA* induced significantly less lung inflammation without influencing bacterial loads. Pneumococcal burdens were also modestly but significantly lower in lungs after induction of pneumonia with *D39ΔprtA*. These data reveal two additional pneumococcal virulence factors that can modify the host response during pneumonia, albeit to a more modest extent than HtrA.

**Discussion**

Serine protease orthologs have been found in many bacteria, contributing to their virulence to a significant extent [4-14]. Previous research identified the serine protease HtrA as a major virulence factor in *S. pneumoniae* during experimentally induced pneumonia [11,14]. We here sought for additional *S. pneumoniae* serine proteases and determined their role in virulence during respiratory tract infection *in vivo*. Our main findings are that the *S. pneumoniae* D39 genome expresses three putative secreted, surface-exposed serine proteases: HtrA, SFP and PrtA. We confirmed reduced virulence after high and low dose infection of *D39* [*S. pneumoniae*], as reflected by strongly reduced bacterial loads, diminished systemic dissemination and decreased lung inflammation.

HtrA additionally has a distinct role in bacteriocin activity by reducing pneumococcal stress and osmotic stress [11]. Another study revealed an important role for HtrA during *S. pneumoniae* cell division [15]. HtrA has also been shown to provide a competitive advantage in *in vitro* competition [16]. Pneumococci mediate intracellular and interspecies competition *in vivo* and have been shown to provide a competitive advantage *in vivo* [16]. In the present study, we confirmed the strongly reduced virulence of *D39ΔhtrA* in pneumonia [11]. *D39ΔhtrA* infected animals displayed more than 3 logs lower bacterial counts in their lungs at 48 hours after infection; in addition, their lungs showed minimal signs of lower respiratory tract infection upon histopathological examination.

There was significantly less dissemination to distant organs in *D39ΔhtrA* infected mice. Notably, *D39ΔhtrA* could be detected in spleen and liver, although blood cultures were sterile in all experiments. Ibrahim et al [11] concluded that *D39ΔhtrA* did not disseminate from the lungs based on negative blood cultures; our data, however, indicate that *D39ΔhtrA* is able to spread to distant body sites.

It is known that apolactoferrin can kill many species of bacteria, including *Streptococcus pneumoniae*. Lactoferrin, an N-terminal peptide of apolactoferrin, and fragments of it are even more bactericidal than apolactoferrin. PrtA cleaves apolactoferrin, greatly enhancing the killing activity of apolactoferrin and its cleavage products [13]. Thus, in theory, PrtA deficiency might cause increased rather than decreased virulence due to a diminished capacity of apolactoferrin to kill *S. pneumoniae*. Nonetheless, the only *in vivo* study performed thus far with *S. pneumoniae* showed decreased virulence compared to WT *S. pneumoniae* when injected intraperitoneally in mice [5]. Our pneumonia model is more relevant to determine the role of PrtA in *S. pneumoniae* virulence, as pneumonia is the primary illness caused by pneumococci. In both high and low dose infection, bacterial outgrowth, pulmonary cell influx and cytokine release were similar after induction of pneumonia with *D39ΔprtA* or WT *S. pneumoniae*. Interestingly, histopathology scores of lungs and the percentage of infiltrated lung surface were significantly lower in *D39ΔprtA* infected animals than in mice inoculated with WT *S. pneumoniae*, suggesting that PrtA has a modest role in the induction of pulmonary inflammation during pneumococcal pneumonia without influencing cellular influx or cytokine release in the lungs, and bacterial multiplication and dissemination.

To our knowledge, the role of SFP in pneumococcal virulence has not been studied before. SFP has homology with *Streptococcus agalactiae* CspA, a serine protease capable of inactivating chemokines *in vivo* [7]. While after high dose infection the growth of *D39Δsfp* was...
indistinguishable from that of WT S. pneumoniae, this mutant strain demonstrated slightly reduced growth in the lungs in low dose pneumonia. This modest role of SFP mediated virulence should be confirmed in additional studies using different S. pneumoniae strains.

We identified two additional hypothetical proteins, SPD_1765 and SPD_1920, predicted to have serine protease activity. These were not examined further here as their serine protease domain was likely not exposed to the outer cell surface. It may be of interest to examine the roles of intracellular serine protease activity for SPD_1765 and SPD_1920 in S. pneumoniae virulence in future research.

The current investigation addressed the role of three serine proteases expressed by S. pneumoniae (HtrA, SFP and PrtA) in virulence in vivo by comparing bacterial growth and dissemination and the accompanying inflammatory response in the lung after 48 hours of airway infection by newly generated deletion mutants. This time point was selected since our main endpoint of interest was bacterial growth; the 48-hour time point reflects late stage pneumonia shortly before mice are expected to die. Our data confirm the previously reported important role for HtrA in S. pneumoniae virulence [11]. In this previous paper, reconstitution of HtrA into D39ΔhtrA reverted D39ΔhtrA to its full virulence [11]. We here created a new HtrA deletion mutant and our investigation is limited by the fact that we did not reconstitute HtrA in our independently generated D39ΔhtrA strain. Of note, however, our main objective was to identify new pneumococcal serine proteases as virulence factors in pneumonia. Since our results only reveal a significant role for the already established HtrA [11], we think that the absence of a HtrA reconstitution experiment does not jeopardize our main conclusion (i.e., that the other proteases identified do not significantly contribute to the virulence of S. pneumoniae). In contrast to its reported role in intraperitoneal infection [5] we here show that PrtA does not influence bacterial multiplication and dissemination in pneumococcal pneumonia, however, PrtA had a modest role in the induction of pulmonary inflammation. Finally, in the first studies reported to date, we provide evidence that SFP may facilitate S. pneumoniae growth after low dose infection of the lower respiratory tract, although clearly these data need to be confirmed in independent experiments involving more time points. Together these data firmly establish that virulence of S. pneumoniae is dominated by HtrA.

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Figure 5. **HtrA strongly contributes to virulence after low dose infection with a more modest role for SFP.** Mice were infected with a 10-fold lower inoculum of WT or mutant *S. pneumoniae* (relative to the infectious dose used in the experiments shown in figures 2–4; 5 × 10⁴ CFU) via the intranasal route and euthanized 48 hours later. Bacterial counts were determined in lung (A), blood (B), spleen (C) and liver (D). Data are expressed as box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 8 mice per group at each time point. * P < 0.05, ** P < 0.01, *** P < 0.005 versus WT *S. pneumoniae*.

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Figure 6. **Diminished cell and neutrophil influx during pneumonia caused by low dose *S. pneumoniae D39 ΔhtrA.** Mice were infected with a 10-fold lower inoculum of WT or mutant *S. pneumoniae* (relative to the infectious dose used in the experiments shown in figures 2–4; 5 × 10⁴ CFU) via the intranasal route and euthanized 48 hours later. Cell (A) and neutrophil (B) influx were determined on BALF cytospin preparations. Data are expressed as box- and whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. N = 8 mice per group at each time point. * P < 0.05 versus WT *S. pneumoniae*.

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Author Contributions
Conceived and designed the experiments: SFdS PWMH CvtV TvdP. Performed the experiments: SFdS HB. Analyzed the data: AZ JJTHR.

References

Contributed reagents/materials/analysis tools: HB AZ PWMH. Wrote the paper: SFdS TvdP.