Development of a non-invasive murine infection model for acute otitis media


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Otitis media (OM) is one of the most frequent diseases in childhood, and Streptococcus pneumoniae is among the main causative bacterial agents. Since current experimental models used to study the bacterial pathogenesis of OM have several limitations, such as the invasiveness of the experimental procedures, we developed a non-invasive murine OM model. In our model, adapted from a previously developed rat OM model, a pressure cabin is used in which a 40 kPa pressure increase is applied to translocate pneumococci from the nasopharyngeal cavity into both mouse middle ears. Wild-type pneumococci were found to persist in the middle ear cavity for 144 h after infection, with a maximum bacterial load at 96 h. Inflammation was confirmed at 96 and 144 h post-infection by IL-1β and TNF-α cytokine analysis and histopathology. Subsequently, we investigated the contribution of two surface-associated pneumococcal proteins, the streptococcal lipoprotein rotamase A (SlrA) and the putative proteinase maturation protein A (PpmA), to experimental OM in our model. Pneumococci lacking the slrA gene, but not those lacking the ppmA gene, were significantly reduced in virulence in the OM model. Importantly, pneumococci lacking both genes were significantly more attenuated than the ΔslrA single mutant. This additive effect suggests that SlrA and PpmA exert complementary functions during experimental OM. In conclusion, we have developed a highly reproducible and non-invasive murine infection model for pneumococcal OM using a pressure cabin, which is very suitable to study pneumococcal pathogenesis and virulence in vivo.

INTRODUCTION

In the first 3 years of life, otitis media (OM) affects 80% of the paediatric population (Giebink, 2001). Streptococcus pneumoniae, together with non-typhable Haemophilus influenzae and Moraxella catarrhalis, is considered to be one of the three major bacterial pathogens involved in OM (Lysenko et al., 2005; Ryan et al., 2006; Sabirov & Metzger, 2006, 2008). OM is the most common reason for children to visit a physician, to receive antibiotics or to undergo surgery, which, consequently, results in a high disease burden for both children and their parents. Treatment with antibiotics remains controversial and is complicated by the rising numbers of isolates resistant to antibiotics (Cripps et al., 2005; Ogguniyi et al., 2007). Despite the significant burden of OM on public health, the genetic basis of bacterial OM remains largely unclear (Chen et al., 2007).

Multiple animal models have been developed to study various aspects of the OM disease process, to increase our understanding of OM pathogenesis, and to design or improve preventive and treatment strategies (MacArthur & Trune, 2006). Different methods are used to provoke middle ear inflammation in various animal species: models using mice (Krekorian et al., 1990; MacArthur & Trune, 2006; Ryan et al., 2006; Sabirov & Metzger, 2008), rats (Fogle-Ansson et al., 2006; Melhus & Ryan, 2003; Tonnaer et al., 2003; van der Ven et al., 1999), gerbils, guinea pigs, chinchillas (Ehrlich et al., 2002; Fulghum & Marrow, 1996; Giebink et al., 1980) and monkeys (Alper et al., 2000) have previously been described in the literature. Among these, mice are increasingly becoming the model of choice in OM research. BALB/c mice are considered to be most susceptible to experimental acute otitis media (AOM).
K. Stol and others

(MacArthur et al., 2006; Melhus & Ryan, 2003; Ryan et al., 2006; Sandgren et al., 2005).

AOM can be evoked by the introduction of live or heat-killed bacteria, as well as by inflammatory substances (Ryan et al., 2006; Sabirov & Metzger, 2008). In most animal models, AOM is induced by direct inoculation of live bacteria into the middle ear cavity, via either a transtympanic or a transbullar route. In the transtympanic model, application of the inoculum into the middle ear cavity is achieved by direct injection through the tympanic membrane. The transbullar approach consists of a ventral midline incision in the neck under sedation. The bulla is exposed after blunt dissection and the middle ear is infected with a bacterial suspension using a thin needle through the bony wall (Ryan et al., 2006; Sabirov & Metzger, 2006). Another possibility is intranasal inoculation, where initial bacterial colonization occurs in the nasopharynx, which is followed by invasion of the middle ear cavity by the pathogen in about 50% of cases. This model resembles the natural route of infection in humans, and colonization of the nasopharynx is highly reproducible. However, development of OM after intranasal challenge is random and, consequently, reproducibility is low (Ryan et al., 2006; Sabirov & Metzger, 2008).

Reproducible induction of OM that maintains the physiological route of infection has been achieved in rats by Tonnaer et al. (2003). In their model, rats are inoculated intranasally, after which pneumococci are transferred to the middle ear cavity via the Eustachian tube by an increase of atmospheric pressure. In the current study, we used a similar model for mice, allowing us to study pneumococcal OM in a reproducible and consistent fashion.

Furthermore, we used our model to examine the contribution of two surface-associated pneumococcal proteins to OM: the streptococcal lipoprotein rotamase A (SlrA) and the putative proteinase maturation protein A (PpmA). SlrA and PpmA share homology with two distinct families of peptidyl-prolyl isomerases (PPIases). Both these surface-associated proteins are known to play a key role during pneumococcal infection (Hermans et al., 2006; Overweg et al., 2000). Previous research has shown a pivotal role of SlrA in adherence, colonization and immune evasion, whereas PpmA is involved in invasive disease and colonization of the nasopharyngeal cavity (Cron et al., 2009; Hermans et al., 2006; Overweg et al., 2000).

### METHODS

**Pneumococcal strains and growth conditions.** The pneumococcal strains used in this study are listed in Table 1, and were kindly provided by B. Henriques-Normark (Albiger et al., 2005). *S. pneumoniae* was routinely grown in Todd–Hewitt broth supplemented with 0.5% yeast extract or on Columbia blood agar (BA) plates at 37 °C and 5% CO₂. Prior to mouse infection experiments, bacteria were passaged in mice to maintain virulence as described previously (Alexander et al., 1994). Cultures of mouse-passaged *S. pneumoniae* strains were grown to an OD₆₀₀ of 0.2 (mid-exponential phase) and stored in aliquots at −80 °C in 15% glycerol. For in vivo challenges, aliquots were spun down and bacteria were resuspended in sterile PBS containing 1% methylcellulose, a viscous component, in order to minimize leakage of inoculum into the lungs (Tonnaer et al., 2003). When indicated, antibiotics were used at the following concentrations: streptomycin, 100 μg ml⁻¹; trimethoprim, 25 μg ml⁻¹; and erythromycin, 4 μg ml⁻¹.

**Construction of pneumococcal mutants.** To obtain the streptomycin-resistant SME215::*rpsL*, the *rpsL* gene encoding a streptomycin-resistant mutant of the ribosomal protein S12 was amplified from D39::*rpsL* (Hermans et al., 2006) with primer pair HB*rpsL*F (GTACAGGGACGTGCTGACAA) and HB*rpsLR* (CCCTTCTCC-ATGCTTTTGG). The *rpsL*-PCR product was introduced into SME215 by Csp-1-induced transformation and selection for streptomycin resistance. To delete the *slrA* gene from SME215, the *ΔslrA* region was amplified from D39A*slrA* (Hermans et al., 2006) with primer pair HB*slrAF* (TACACCAGGGAAATATGGA) and HB*slrAR* (TACACCAGGGCCATATGGA) and HB*slrAR* (TACACCAGGGCCATATGGA). The *ΔslrA*-PCR product was introduced into SME215 by Csp-1-induced transformation and selection for streptomycin resistance. To delete the *ppmA* gene from SME215, the *ΔppmA* region was amplified from D39A*ppmA* (Adrian et al., 2000) with primer pair HB*ppmA*F1 (ATGCTTTTGG). The **ppmA**-PCR product was introduced into SME215 by Csp-1-induced transformation and selection for streptomycin resistance. To obtain the SME215*ppmA* strain, a *ΔppmA*-PCR region was amplified from D39A*ΔppmA* (Adrian et al., 2000) with primer pair HB*ppmA*F1 (ATGCTTTTGG).

**Table 1. Pneumococcal strains and mutants used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<td><em>S. pneumoniae</em></td>
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<tr>
<td>D39</td>
<td>2</td>
<td>Tn4001 luxABCDE operon, Km’</td>
<td>NCTC 7466</td>
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<tr>
<td>TIGR4(lux)</td>
<td>4</td>
<td>Tn4001 luxABCDE operon, Km’</td>
<td>Albiger et al. (2007)</td>
</tr>
<tr>
<td>PJ1324(lux)</td>
<td>6B</td>
<td>Tn4001 luxABCDE operon, Km’</td>
<td>Sandgren et al. (2005)</td>
</tr>
<tr>
<td>SME215(lux)</td>
<td>19F</td>
<td>Tn4001 luxABCDE operon, Km’</td>
<td>Albiger et al. (2005), Sandgren et al. (2005)</td>
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<td>SME215(lux) strep</td>
<td>19F</td>
<td>SME215(lux) derivative, <em>rpsL</em>; Str’</td>
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<td>SME215(lux) ΔslrA</td>
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<td>SME215(lux) derivative, ΔppmA; <em>Trim</em></td>
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<td>This study</td>
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AACTGCTC) and HBppmAAR1 (GCAGCCTACAGCTAGCTTCC). The ΔppmA-PCR product was introduced into SME215 by CSP-1 induced transformation, and trimethoprim-resistant transformants lacking the ppmA gene were selected (Adrian et al., 2000; Hermans et al., 2006). The ΔslrAΔppmA double mutants were constructed by transformation of the ΔppmA-PCR product (amplified with primer pair HBppmA1 and HBppmAAR1) to each ΔslrA mutant and selecting for trimethoprim and erythromycin resistance. Deletion of slrA and ppmA in resulting transformants was confirmed by PCR and sequencing.

**Mouse strain and disease monitoring.** Experiments were conducted with 7-week-old, female, specific-pathogen-free BALB/c mice (Harlan). They conformed to Dutch animal protection legislation.

**Pressure cabin.** The pressure cabin consists of a perspex tube (30 cm, diameter 15 cm) with a fixed perspex wall. The perspex wall contains a manometer, an outlet valve, and a valve connected to a cylinder filled with compressed air. The removable front door is sealed with a rubber O-ring. Air pressure in the cabin can be increased stepwise (0–100 kPa) and monitored precisely (Tonnaer et al., 2003).

**Mouse OM model: single and co-infection.** Mice were lightly anaesthetized with 2.5 % (v/v) isoflurane over oxygen (1.5 l min⁻¹), and subsequently, a 10 μl suspension (containing 5 × 10⁶ c.f.u. bacteria or PBS/1 % methylcellulose as control) was applied onto both nostrils in alternating fashion (single infection and co-infection). Mice were placed in supine position with an initial pressure rise set at 10 kPa. When the mice regained consciousness and the first swallowing movements occurred, pressure was raised to 40 kPa, at 5 kPa per 15 s, in order to transfer the inoculum to the middle ear cavity. Thereafter, the pressure was lowered stepwise until atmospheric pressure was reached again. Pressure increase was evaluated using otomicroscopy and the tympanic membrane was scored for tympanic membrane perforation and for increased vascularization or bleeding.

For single infection with *S. pneumoniae* D39, TIGR4, SME215 or PJ1324, groups of 5–9 mice were sacrificed at 96 h post-inoculation. For single infection with *S. pneumoniae* SME215, groups of five mice were sacrificed at 1.5, 48, 96, and 144 h post-inoculation. Blood was collected using orbital puncture. The bulla enclosing the right middle cavity. Thereafter, the pressure was lowered stepwise until atmospheric pressure was reached again. Pressure increase was evaluated using otomicroscopy and the tympanic membrane was scored for tympanic membrane perforation and for increased vascularization or bleeding.

For single infection with *S. pneumoniae* D39, TIGR4, SME215 or PJ1324, groups of 5–9 mice were sacrificed at 96 h post-inoculation. For single infection with *S. pneumoniae* SME215, groups of five mice were sacrificed at 1.5, 48, 96, and 144 h post-inoculation. Blood was collected using orbital puncture. The bulla enclosing the right middle ear was dissected from the temporal bone and homogenized in 1 ml PBS-infected mice at the corresponding time points.

**RESULTS**

**Development of a non-invasive murine OM model**

The pathogenesis of pneumococcal OM involves bacterial translocation from the nasopharynx to the middle ear cavity through the Eustachian tube. In order to translocate bacteria in our model, we used a pressure cabin. First, we identified the appropriate pressure in our model with Evans blue dye. A pressure increase of 40 kPa was sufficient to transfer Evans blue dye from the nasopharynx into the middle ear cavity of BALB/c mice, without causing damage to the tympanic membrane. Otomicroscopy did not show rupture of the tympanic membrane, nor vascular dilatation of blood vessels present at the tympanic membrane (data not shown). Subsequently, the pressure cabin was used for bacterial infection with *S. pneumoniae* TIGR4 to test various doses and inoculum volumes. A dose of 5 × 10⁶ c.f.u. in a 10 μl volume resulted in reproducible infection, in line with the mouse colonization model used in our laboratory (Cron et al., 2009; Hendriksen et al., 2009). To examine strain-specific behaviour, mice were infected with the pneumococcal strains D39 (serotype 2), TIGR4 (serotype 4), PJ1324 (serotype 6B) and SME215 (serotype 19F). Ninety-six hours after infection mice were euthanized, whereupon ear and lung homogenates, nose lavage and blood were cultured. *S. pneumoniae* SME215 for experiments in which no mutant bacteria were recovered from a particular mouse, the number 20 (lower limit of detection) was used as the numerator. Mice with neither mutant nor wild-type bacteria in the middle ear were excluded from further analysis.

**Histology.** The skulls of all mice including both temporal bones, nose and maxilla were removed and processed for histological analysis. The specimens were formalin fixed in 4 % paraformaldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.4), decalcified using 10 % EDTA (Serva), and embedded in paraffin. Subsequently, all skulls were cut into 5 mm slices in an oblique direction. After deparaffinization through a series of xylene and graded ethanol baths, sections were stained with haematoxylin/eosin. Sections were photographed with a microscope (Carl Zeiss Axioskop 2 plus) coupled to a computer (FrogRes Capturepro 2.1) at an original magnification of 25 x and 200 x.

**Interleukin (IL)-1β and tumour necrosis factor (TNF)-α ELISA.** Commercially available kits were used to measure the IL-1β (Becton Dickinson) and TNF-α (U-cytech) concentrations in murine middle ear homogenate and nasopharyngeal lavage.

**Statistical analysis.** All data were analysed using SPSS 16.0 and GraphPad Prism version 4.0. For single infection a non-parametric test on log-transformed c.f.u. counts (mean, P<0.05) was used to calculate statistical significance. For co-infection the one-sample t-test on log-transformed CI scores (with an arbitrary mean of 0 and P<0.01) and comparative regression coefficients were used to calculate statistical significance.

Cytokine levels were calculated according to the manufacturer’s instructions. A non-parametric test (mean, SEM and P<0.05) was performed to calculate statistical significance between infected and PBS-infected mice at the corresponding time points.
and PJ1324 induced a median bacterial load in the middle ear of $1 \times 10^4$ c.f.u. and $5 \times 10^5$ c.f.u. respectively, at 96 h post-inoculation (Fig. 1). OM induced by S. pneumoniae TIGR4 was complicated by pneumonia and sepsis (as confirmed by clinical scores and bacterial culture) and consequently excluded from further analysis. Counts of S. pneumoniae D39 in the middle ear and nasopharynx were significantly lower compared to S. pneumoniae SME215 ($P = 0.003$) and PJ1324 ($P = 0.039$). Therefore, S. pneumoniae PJ1324 and SME215 were considered most suitable for our model. No bacteria or signs of inflammation were detected in middle ears of mice inoculated with PBS, indicating that transfer of local nasopharyngeal flora does not occur in our model.

Since the bacterial load of S. pneumoniae SME215 in the middle ear was significantly higher than that of S. pneumoniae PJ1324 ($P = 0.015$), SME215 was used to examine the bacterial kinetics in further detail at 1.5, 48, 96 and 144 h after infection (Fig. 2). Approximately $1 \times 10^5$ c.f.u. were detected in the middle ear cavity at 1.5 h post-inoculation; this number declined 50-fold at 48 h post-inoculation. Wild-type pneumococci persisted in the middle ear cavity up to 96 h post-infection (median $3 \times 10^5$ c.f.u.). The bacterial load in the nasopharynx reached $1 \times 10^6$ c.f.u. up to 96 h post-inoculation and $4 \times 10^5$ c.f.u. at 144 h. The bacterial load initially detected in the lungs was high, $1 \times 10^6$ c.f.u., but declined rapidly over time, with $5 \times 10^5$ c.f.u. at 48 h and $6 \times 10^5$ c.f.u. at 96 h post-inoculation (Fig. 2). No bacteria were recovered from blood (data not shown). Weight and temperature of the mice remained within the normal range and, importantly, mice did not show any signs of invasive disease; in particular no signs of pneumonia and sepsis were observed.

Immunology and histopathology

Both IL-1β and TNF-α levels in the middle ear were elevated throughout the course of infection. Importantly, levels in PBS-inoculated mice were below the detection limit at 1.5, 48 and 96 h post-inoculation and marginal at 144 h post-inoculation (TNF-α, 14.5 pg ml$^{-1}$; IL-1β, 24.4 pg ml$^{-1}$). The levels of TNF-α and IL-1β in the middle ear at 48 h and 96 h post-inoculation were significantly different from those in PBS-inoculated mice collected at the corresponding time points (Fig. 3A, B). The TNF-α cytokine levels detected in the nasopharynx were 10-fold lower than the levels in the middle ear, and significantly different at 48, 96 and 144 h post-inoculation when compared to PBS-inoculated mice (Fig. 3C). There was no significant difference between infected and PBS-infected mice in the IL-1β levels in the nasopharynx (Fig. 3D).

Histopathological examination showed an increased inflammation over time (Fig. 4). Neutrophils, lymphocytes and macrophages migrated into the middle ear cavity at 96 and 144 h post-inoculation (Fig. 4C, D). The mucous membrane, which is normally lined by a single layer of epithelium, by then was covered with a more irregular and multiple-layered epithelium (Fig. 4C, b and c; Fig. 4D, b and c). The loose connective tissue had expanded significantly, due to the presence of transient wandering immune cells that had migrated from local blood vessels to the site of infection (Fig. 4C, b and c; Fig. 4D, b and c).

![Fig. 1. Bacterial load in the middle ear (A), nasopharyngeal lavage (B), lungs (C) and blood (D) 96 h after inoculation with S. pneumoniae D39 (serotype 2), TIGR4 (serotype 4), PJ1324 (serotype 6B) and SME215 (serotype 19F). Each dot represents a single mouse. Mean values are represented by horizontal bars. The lower limit of detection, 20 c.f.u., is indicated by a dashed line.](image-url)
Usefulness of the murine OM model to study pneumococcal virulence

To assess the role of the pneumococcal proteins SlrA and PpmA in OM, we inactivated the \textit{slrA} and \textit{ppmA} genes in strain SME215 by insertion deletion mutagenesis, and tested the wild-type and \textit{ΔslrA}, \textit{ΔppmA} or \textit{ΔslrAΔppmA} mutants in a co-infection setup. Importantly, no differences in \textit{in vitro} growth between the wild-type strains and the mutants were detected, indicating that differences in virulence did not originate from altered growth rates.

In the middle ear, the bacterial load of the \textit{ΔslrA} mutant was significantly lower relative to the SME215 parent strain at 48 and 96 h post-infection (mean CI 0.14 and 0.07 respectively, a 10-fold reduction) (Fig. 5A). A significant difference between the wild-type and the \textit{ΔppmA} mutant was only found at 1.5 h after infection, not at later time points (mean CI 0.47, a fivefold reduction) (Fig. 5C). Deletion of both \textit{slrA} and \textit{ppmA} resulted in a significant decrease in middle ear bacterial load compared to wild-type at all time points: the CI score declined from 0.26 at 1.5 h post-inoculation to 0.003 at 96 h post-inoculation, representing a 3- to 300-fold reduction over time. In other words, the wild-type outcompeted the double knockout strain in a progressive and statistically significant fashion (Fig. 5E). Interestingly, we observed an additive effect upon

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**Fig. 2.** Bacterial load in the middle ear (A), nasopharynx (B) and lungs (C) at various times after inoculation with \textit{S. pneumoniae} SME215. Each dot represents one mouse. Mean values are indicated by horizontal bars. The lower limit of detection, 20 c.f.u., is indicated by a dashed line. No bacterial counts were detected in blood.

**Fig. 3.** IL-1β and TNF-α cytokine levels in the middle ear and nasopharynx. Each bar represents the mean value of five mice, error bars indicate SEM. (A) TNF-α in the middle ear; (B) IL-1β in the middle ear; (C) TNF-α in the nasopharyngeal lavage; (D) IL-1β in the nasopharyngeal lavage. Statistical significance was calculated between infected and mock-infected mice at the corresponding time point of collection. Significance (\(P<0.05\)) is depicted by *.
deletion of both genes, as the \( \Delta slrA\Delta ppmA \) mutant was significantly more attenuated in OM virulence than the \( \Delta slrA \) or \( \Delta ppmA \) single mutant (Fig. 5A, C, E).

Colonization of the nasopharynx was highly reproducible, yielding approximately \( 5 \times 10^7 \) c.f.u. at all time points. Throughout the course of the experiment, the \( \Delta slrA \), \( \Delta ppmA \) and \( \Delta slrA\Delta ppmA \) mutants were less able to colonize the nasopharynx in comparison to the parent strain (Fig. 5B, D, F). Deletion of \( slrA \) resulted in a significant difference in colonization of the nasopharynx from wild-type at all time points (mean CI varying from 0.06 to 0.14, a 7- to 17-fold reduction) (Fig. 5B). A significant difference between the wild-type and the \( \Delta ppmA \) mutant was seen at 12 h (mean CI 0.70), 48 h (mean CI 0.26) and 96 h (mean CI 0.15) h post-inoculation (Fig. 5D). In line with the results for the \( \Delta slrA\Delta ppmA \) mutant in the middle ear, outcompetition of the double mutant in the nasopharynx was progressive and statistically significant over time (CI 0.10–0.01, a 10- to 100-fold reduction) (Fig. 5F). Again, the \( \Delta slrA\Delta ppmA \) mutant was significantly more attenuated in virulence than the \( \Delta slrA \) or \( \Delta ppmA \) single mutant (Fig. 5B, D, F).

**DISCUSSION**

In the present study we developed a novel non-invasive murine OM model with the use of a pressure cabin. The mouse affords many advantages for *in vivo* research,
including ease of genetic manipulation, availability of inbred and transgenic strains and an extensively studied immune system. Moreover, experimental reagents for cellular and molecular studies are widely available (Krekorian et al., 1990; MacArthur & Trune, 2006; Ryan et al., 2006; Sabirov & Metzger, 2008). The model, which was adapted from a rat OM model described by Tonnaer et al. (2003), was used to investigate OM caused by S. pneumoniae and the contribution of the pneumococcal proteins SlrA and PpmA to the pathogenesis of OM.

For various reasons, the pressure cabin model is an improvement over the experimental AOM models currently used. First, pressure elevation facilitates the ascending infection of pneumococci from the nasopharynx to the middle ear cavity via the Eustachian tube, resembling the natural route of infection in humans. In comparison to AOM models, which rely on spontaneous OM development after nasopharyngeal colonization (Sabirov & Metzger, 2008), the percentage of successful middle ear infections is 100 % in our model. This is of major importance for future vaccination and challenge studies, since a 100 % successful infection rate is required to monitor the efficiency of a vaccine, i.e. reduction of bacterial load. Second, dermal pathogens like Staphylococcus epidermidis will not influence experimental results, whereas with the widely used transtympanic or transbullar approaches, a connection between the middle ear and its surroundings is established. Third, an inflammatory response due to local manipulation is avoided. Among others, MacArthur et al. (2006) described signs of inflammation due to direct instillation of PBS in the middle ear that were equivalent to those induced by lower doses of heat-killed bacteria. These inflammatory reactions were ascribed to the required tympanic membrane incision (Krekorian et al., 1990; MacArthur et al., 2006).

Of the pneumococcal strains tested, BALB/c mice were most susceptible to TIGR4 (serotype 4), followed by SME215 (serotype 19F), PJ1324 (serotype 6B) and D39 (serotype 2). These observations indicate pneumococcal strain-specific behaviour in vivo as described previously (Briles et al., 1992; Forbes et al., 2008). S. pneumoniae SME215 and PJ1324 persisted in the middle ear cavity for at least 96 h. These two strains were therefore considered suitable for experimental OM studies in vivo, and subsequent experiments were performed using S. pneumoniae SME215 only. On the day of inoculation with S. pneumoniae SME215, bacteria were detected in the lungs.
but they were cleared rapidly over time. We speculate that leakage to the lungs can be partially explained by the use of anaesthetics and pressure increase at the time of infection. Importantly, disease scores were low during this study, which implies absence of pneumonia or sepsis, and as a result – moribund state or early loss did not occur. This is in contrast to current chinchilla OM models, which often record a moribund disease state (Forbes et al., 2008). Since signs and symptoms of human OM mostly show a mild course of infection (Cripps et al., 2005), we consider our model to be a clinical representation of OM in humans. Moreover, infection with heat-inactivated bacteria to avoid systemic complications, as described in OM mouse models using direct tympanic membrane inoculation, was not necessary (MacArthur et al., 2006). Polymicrobial interactions between, for instance, S. pneumoniae, (non-typable) H. influenzae and M. catarrhalis, or combined bacterial and viral infections are of interest for future studies (Bakalez, 2004; Cripps et al., 2005; Lysenko et al., 2005; MacArthur et al., 2006).

The primary cytokines TNF-α and IL-1β were selected to monitor the initiation of the acute inflammatory response. TNF-α is produced mainly by macrophages or mast cells and is, among others, responsible for neutrophil migration (Maeda et al., 2004). Along with various other functions, IL-1β contributes to permanent pathological changes in the middle ear, mucosal damage, bone erosion, fibrosis and hearing loss. Higher concentrations of IL-1β were observed in purulent, acute, culture-positive effusions (Maeda et al., 2004; Skotnicka & Hassmann, 2008; Tong et al., 2008). Our results indicate a peak TNF-α and IL-1β response in the middle ear cavity at 96 h post-inoculation, whereas the maximum histopathological changes were shown at 144 h post-inoculation. These data confirm ongoing inflammation after onset of the inflammatory process by the primary cytokines: at 144 h, levels of both TNF-α and IL-1β were comparable to PSEP-treated mice, most likely since the number of white blood cells was sufficient to eradicate S. pneumoniae from the middle ear cavity. Using a rat OM model (Cripps & Kyd, 2007) reported a maximum TNF-α level of 1.3 × 10^3 pg ml^-1 at 24 h post-infection, while IL-1β was not detectable. They also reported cytokine levels in a mouse pneumonia model of pneumococcal infection: a maximum TNF-α level around 300 pg ml^-1 at 4 h post-infection and maximum IL-1β level near 6.5 × 10^2 pg ml^-1 at 24 h post-infection (Cripps & Kyd, 2007). However, as immune responses in in vivo experimental models are animal specific, bacterial-strain specific, and infection-site specific, it is difficult to directly compare the results. Unlike the strong inflammation in the middle ear (Tuomanen, 2000), there is a relatively mild host response to colonizing pneumococci in the nasopharynx (Chen et al., 2007), underscored by the marginal levels of IL-1β and TNF-α detected in the nasopharyngeal lavage in our study.

Bacterial surface-exposed proteins often play an important role in the interaction between pathogens and their host. Identification of novel surface-exposed proteins that play an important role in virulence should improve our understanding of OM pathogenesis and will facilitate new preventive strategies such as vaccine development. Virulence studies described in the literature have shown that most pneumococcal mutants display attenuated phenotypes in colonization, pneumonia, or sepsis mouse models, thus indicating niche-specific involvement in virulence of the respective genes (Chen et al., 2007; Cripps et al., 2005; Hava & Camilli, 2002; Hendriksen et al., 2007, 2008; Hermans et al., 2006; Ogunniyi et al., 2007; Tong et al., 2004; Tuomanen, 2000).

To our knowledge we report here the first OM virulence study performed in mice. Only very few studies have investigated the role of virulence factors in OM models (Chen et al., 2007; Tong et al., 2004, 2008). Chen et al. (2007) very nicely demonstrated the first extensive search for genetic requirements for pneumococcal OM by using signature-tagged mutagenesis in a chinchilla OM model and a murine colonization model. Since direct extrapolation from other models is problematic, given the animal-model specificity (Chen et al., 2007), we examined the contribution of two factors to OM and nasopharyngeal colonization directly. We showed that both single gene deletion of slrA and combined deletion of the slrA and ppmA genes significantly reduced the bacterial load during experimental AOM in both the nasopharynx and the middle ear compared to the wild-type strain. Pneumococci lacking the slrA gene, but not those lacking the ppmA gene, were significantly reduced in virulence in the OM model. Interestingly, the virulence of the pneumococci lacking both genes was significantly decreased compared to the ΔslrA or ΔppmA single mutant. This observation suggests complementary functions of StrA and PpmA in both experimental OM and nasopharyngeal colonization, resulting in an additive decrease in virulence when both genes are absent. Previous studies have shown a role for both StrA and PpmA in the early stage of infection, i.e. colonization (Cron et al., 2009; Hermans et al., 2006). Our results, even in a different mouse background and with a different pneumococcal strain, confirmed these findings. StrA is a functional PPlase involved in pneumococcal colonization, most likely by modulating the biological function of important virulence proteins, as described by Hermans et al. (2006). Although PpmA is a conserved surface protein with potential to elicit protective immune responses, the exact role of this protein is still unknown (Overweg et al., 2000).

In summary, we have developed an OM mouse model in which AOM can be established by various pneumococcal strains. This highly reproducible method is non-invasive, and infection is established in both ears. Consequently, multiple simultaneous applications are feasible, such as bacterial culture and histopathology. The model will be highly valuable for studying OM induced by S. pneumoniae, and OM-related pneumococcal virulence. It is also likely to be suitable for investigating the protective capacity of putative vaccine antigens against pneumococcal-induced
OM, since the route of infection does not bypass local immunity and the middle ear infection rate is 100%. Whether this model is also appropriate for other pathogens such as *H. influenzae* and *M. catarrhalis* is currently under investigation.

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