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Pneumococcal protein PavA is important for nasopharyngeal carriage and development of sepsis

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Keywords: colonization; host–pathogen interactions; nasopharyngeal carriage; pneumococcus; sepsis; virulence
Accepted 18 November 2009

SUMMARY

The pneumococcal cell surface protein PavA is a virulence factor associated with adherence and invasion *in vitro*. In this study we show *in vivo* that PavA is necessary for *Streptococcus pneumoniae* D39 colonization of the murine upper respiratory tract in a long-term carriage model, with PavA-deficient pneumococci being quickly cleared from nasopharyngeal tissue. In a pneumonia model, *pavA* mutants were not cleared from the lungs of infected mice and persisted to cause chronic infection, whereas wild-type pneumococci caused systemic infection. Hence, under the experimental conditions, PavA-deficient pneumococci appeared to be unable to seed from lung tissue into blood, although they survived in blood when administered intravenously. In a meningitis model of infection, levels of PavA-deficient pneumococci in blood and brain following intercisternal injection were significantly lower than wild type. Taken collectively these results suggest that PavA is involved in successful colonization of mucosal surfaces and in translocation of pneumococci across host barriers. Pneumococcal sepsis is a major cause of mortality worldwide so identification of factors such as PavA that are necessary for carriage and for translocation from tissue to blood is of clinical and therapeutic importance.

INTRODUCTION

Streptococcus pneumoniae is responsible for a high burden of human disease and death. It is a leading cause of pneumonia, sepsis, meningitis and otitis media in children, the elderly and in immunocompromised individuals (Kadioglu *et al.*, 2008). The pneumococcus colonizes mucosal surfaces of the human upper respiratory tract and it is the establishment of colonization (or the carrier state) that is thought to be the basis of all pneumococcal diseases (Rosenow *et al.*, 1997; Bogaert *et al.*, 2004; Regev-Yochay *et al.*, 2004). An important prerequisite for host upper respiratory tract colonization and subsequent invasion and dissemination is the expression and interaction of particular pneumococcal virulence factors with host tissue receptors. It is well established that pneumococci adhere to and invade both epithelial cells and endothelium using cell-specific mechanisms for internalization and invasion (van der Flier *et al.*, 1995; Adamou *et al.*,1998; Zhang *et al.*, 2000; Elm *et al.*,

2004; Hammerschmidt *et al.*, 2005; Hammerschmidt, 2006; Talbot *et al.*, 1996).

Pneumococcal adhesins such as phosphorylcholine (ChoP) and PspC (also referred to as CbpA) have been directly implicated in adherence to epithelial cells (Cundell *et al.*, 1995; Rosenow *et al.*, 1997; Weiser, 2005). Other virulence factors such as surface-associated exoglycosidases including neuraminidase A (NanA), β-galactosidase (BgaA) and β-*N*-acetyl-glucosaminidase (StrH) are known to be involved in adhesive interactions with host tissue glycoconjugates (King *et al.*, 2006). Others, such as hyaluronidase and α-enolase, have been implicated in interactions with extracellular matrix components (Bergmann *et al.*, 2001; Jedrzejas *et al.*, 2002). The pneumococcal factors that enable host epithelial and tissue barriers to be breached are not well understood, but interactions of pneumococci with polymeric immunoglobulin receptor (Zhang *et al.*, 2000) and with platelet activating factor receptor (Cundell *et al.*, 1995) are potential mechanisms. Identification of the factors promoting transition from colonization to invasive infection is of primary scientific and clinical interest.

Pneumococci are known to bind to the immobilized form of fibronectin in a trypsin-sensitive manner (van der Flier *et al.*, 1995). This interaction involves, at least in part, pneumococcal protein PavA, which interacts with fibronectin and is associated with virulence in mouse models (Holmes *et al.*, 2001). As in other gram-positive cocci such as *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus gordonii*, recognition of fibronectin has been shown to be important for adhesion to epithelial cells and for subsequent invasion (Molinari *et al.*, 1997, 2000; Okada *et al.*, 1997, 1998; Dziewanowska *et al.*, 1999; Nobbs *et al.*, 2007). The PavA protein shows about 85% identity to *S. pyogenes* and *S. gordonii* fibronectin-binding proteins Fbp54 and FbpA (Christie *et al.*, 2002) respectively, and is localized to the pneumococcal cell outer surface. This occurs despite the absence of a signal sequence required for protein export via the general secretory pathway, and lack of a typical LPXTG cell wall anchorage motif (Holmes *et al.*, 2001; Pracht *et al.*, 2005). Other proteins of streptococci that also lack these motifs and that are nevertheless associated with the outer surface include Fbp54 (Courtney *et al.*, 1994), streptococcal surface dehydrogenase (Pancholi & Fischetti, 1992), surface enolase of *S. pyogenes* (Pancholi & Fischetti, 1998) and pneumococcal α-enolase (Bergmann *et al.*, 2001). These proteins, therefore, constitute a novel class of surface proteins of gram-positive bacteria (Chhatwal, 2002).

It has previously been shown that pneumococcal mutants deficient in PavA were significantly reduced in their abilities to adhere to or invade human epithelial and endothelial cells *in vitro* (Holmes *et al.*, 2001; Pracht *et al.*, 2005). In a peritonitis model with BALB/c mice, we demonstrated that mice intraperitoneally infected with isogenic PavA-deficient mutants survived significantly longer than those mice inoculated with wild-type strain, and that no PavA-deficient pneumococci could be detected in blood or spleen from infected mice (Holmes *et al.*, 2001). Results obtained from signature-tagged mutagenesis experiments have independently identified PavA as a virulence factor in pneumococcal infection using CD-1 mice (Lau *et al.*, 2001). In addition, in a meningitis model with C57BL/6 mice, it was shown that mortality was substantially reduced in mice infected with PavA-deficient pneumococci compared with those infected with wild-type pneumococci (Pracht *et al.*, 2005). These results may be associated with evidence that PavA is essential for pneumococci to escape phagocytosis, and that *pavA* mutants cause less severe immune responses in the host (Noske *et al.*, 2009).

There is therefore strong evidence that, in experimental models of meningitis and peritonitis, PavA is an important virulence factor, and that its absence leads to significant attenuation of pneumococcal virulence. However, there has been no detailed study of the role of PavA during acute pulmonary infection accompanied by bacteraemia, nor of its activities in acute upper respiratory tract infection or in long-term asymptomatic nasopharyngeal carriage. As colonization of the nasopharynx is a prerequisite for development of pneumococcal disease, we have investigated the function of PavA in this environmental niche using a model of long-term nasopharyngeal carriage, and in a model of acute pulmonary infection.

In this article we show that PavA is necessary for long-term carriage within the murine nasopharynx and for translocation of pneumococci from colonized niches into blood. This is consistent with *in vitro* data suggesting that PavA is involved in adherence to epithelial cells and for invasion of endothelial cells by

pneumococci (Pracht *et al.*, 2005). Because pneumococcal bacteraemia and sepsis are a major cause of mortality worldwide, identification of pneumococcal translocation factors would be useful in the development of new anti-infection strategies.

METHODS

Bacterial strains and growth media

Wild-type *S. pneumoniae* serotype 2, strain D39, was obtained from the National Collection of Type Cultures, London, UK (NCTC 7466). Its isogenic *pavA* mutant was generated as previously described (Holmes *et al.*, 2001) by allelic exchange of a 1073-base-pair cassette containing a chloramphenicol acetyltransferase gene (Claverys *et al.*, 1995) inserted into the *Apa*I site present within the N-terminal coding region of the *pavA* gene. Cell protein extracts from *pavA* mutants analysed by Western immunoblot were devoid of PavA polypeptide (Holmes *et al.*, 2001). Bioluminescent derivates of wild-type D39 and D39Δ*pavA* were generated by transformation of these strains with genomic DNA from the bioluminescent strain Xen10 (A66.1 serotype 3), which was purchased from Xenogen (Xenogen Corporation/Caliper Life Sciences, Alameda, CA). *S. pneumonaie*-Xen10 possesses a stable integration of the pAUL-A Tn*4001 luxABCDE* Km^r at a single integration site in the chromosome and colonies show a bright bioluminescence. Transformation of strain D39 or *pavA* mutant with the Xen10 genomic DNA resulted in stable bioluminescent derivatives bearing the pAUL-A Tn*4001 luxABCDE* Km^r at the same integration sites as in Xen10. The bioluminescent derivatives of D39 and D39Δ*pavA*, respectively had identical growth and phenotypic properties to the parental strains.

Pneumococci were grown in brain–heart infusion broth (Oxoid, Basingstoke, UK), in Todd–Hewitt broth (Difco, Franklin Lakes, NJ) containing 0.5% yeast extract (THY), or on blood agar base (Oxoid) supplemented with 5% [volume/volume (v/v)] defibrinated horse blood. Chloramphenicol (10 μg/ml) was added to the medium when appropriate. Bacteria were identified as pneumococci before experiments by Gram stain, catalase test, α-haemolysis on blood agar plates, and by optochin sensitivity. The capsular polysaccharide serotypes were confirmed by the Quellung reaction and no differences in capsule formation between the D39 wild-type or isogenic *pavA* mutant were evident; nor were there any differences in growth rate or pneumolysin production (Holmes *et al.*, 2001). Bacterial suspensions were standardized for inoculation and stored at −80°C. When required, suspensions were thawed at room temperature; bacteria were then harvested by centrifugation and suspended in sterile phosphate-buffered saline (PBS).

Mouse models of infection and carriage

Female outbred MF1 mice were used. All mice were 8–10 weeks old when infected and weighed 30–35 g (Harlan, Bicester, UK). These mice did not have detectable levels of anti-type 2 antibodies present in their sera (data not shown). Mice were lightly anaesthetized with 2.5% (v/v) fluothane (AstraZeneca, Macclesfield, UK) over oxygen (1.5–2 l/min) (Kadioglu *et al.*, 2002) and, for the acute pneumonia infection model (and survival experiment), 50 μl PBS containing 10^6 colony-forming units (CFU) *S. pneumoniae* were administered into the nostrils of anaesthetized mice (Kadioglu *et al.*, 2000). For the long-term asymptomatic nasopharyngeal carriage model, 10 μl containing 10^5 CFU *S. pneumoniae* was administered into the nostrils of the mice. Intravenous infections were administered as 10^5 CFU *S. pneumoniae* via the dorsal tail vein. *Streptococcus* CFU present in inocula were confirmed by viable plate counts on blood agar.

At pre-chosen time intervals following infection, groups of mice were deeply anaesthetized with 5% (v/v) fluothane and blood was collected by cardiac puncture. Immediately afterwards, the mice were sacrificed by cervical dislocation, and the lungs and nasopharyngeal tissue were removed separately into 10 ml sterile PBS, weighed, and then homogenized via an Ultra-Turrax T8 homogenizer (IKA, Staufen, Germany). Viable counts in homogenates and blood were determined by serial dilution in PBS and plating onto blood agar. For intravenous infections, mouse tail blood samples were taken from the same mice 3, 6, 24 and 48 h postinfection, and viable counts were determined as described above.

Animal model of pneumococcal meningitis

Meningitis was introduced by transcutaneous injection of 15 μl bacterial suspension containing 10^7 CFU/ml of *S. pneumoniae* D39 ($n = 5$), D39Δ*pavA* ($n = 4$) or

sterile PBS (uninfected controls, $n = 4$) into the cisterna magna under short-term anaesthesia with halothane (Manco *et al.*, 2006). Then, animals were allowed to wake up and food and water were supplied *ad libitum*. After 24 h, animals were scored clinically then deeply anaesthetized with ketamine; the brain was removed and the cerebellum was homogenized in PBS and CFU were determined (Pracht *et al.*, 2005). These animal experiments were approved by the government of Upper Bavaria, Germany.

Bioluminescent image analysis of pneumococcal infections

Dissemination of pneumococci after intranasal infection of female outbred CD1 mice (Charles River Laboratories, Wilmington, MA) was observed in real time using the IVIS® Lumina Imaging System (Xenogen Corporation). Pneumococci were grown in THY to and optical density at 600 nm of 0.35 (approximately 4×10^8 CFU/ml), collected by centrifugation, washed once with PBS, suspended in one-tenth of the original volume in PBS, and diluted to final concentrations of 10^7, 10^8 or 10^9 CFU. Six mice per group were lightly anaesthetized by intraperitoneal injection of ketamine and xylazine (Rompun®) and pneumococci were administered intranasally. Mice were imaged for 1 min at a number of time-points postinfection to monitor dissemination of pneumococci into the lungs. In addition, bioluminescent intensity was determined by quantification of the total photon emission using the LivingImage IgorPro 4.0 software package (Xenogen).

Statistical analyses

Data were analysed using GraphPad Prism software version 4 for Macintosh computers and are shown as mean ± SEM. Two tailed Student's *t*-test analyses were performed on data sets and differences were considered significant when $P < 0.05$.

RESULTS

Impact of *pavA* mutation on survival in mouse model of pneumococcal pneumonia

Groups of 10 MF1 mice were intranasally infected with either wild-type D39 pneumococci or its isogenic PavA-deficient mutant. In mice infected with PavA-deficient pneumococci, mortality was significantly lower than in wild-type-infected mice ($P < 0.01$). Only two out of 10 D39Δ*pavA* infected mice succumbed to infection during the 120-h period of the survival experiment. The remaining eight mice showed no signs or symptoms of illness during this period. In contrast, all 10 wild-type infected mice succumbed to their infections between 44 and 52 h postinfection (Fig. 1).

Role of PavA in upper and lower respiratory tract infection model

MF1 mice were challenged intranasally with either D39 wild-type or isogenic *pavA* mutant and samples were collected at 20 min, 3 h, 6 h, 24 h, 48 h and 72 h postinfection. Ten mice per time-point per pneumococcal strain were sacrificed and the nasopharynx and lungs of each mouse were removed to determine pneumococcal CFU. In wild-type-infected mice, pneumococcal CFU in the nasopharynx increased significantly ($P < 0.01$) over a 48-h period compared with mice infected with PavA-deficient pneumococci (Fig. 2). PavA-deficient pneumococci were just above the level of detection at 6 h postinfection and were completely cleared from the nasopharynx by 24 h postinfection (Fig. 2).

In wild-type-infected mice, pneumococcal CFU in lungs were significantly reduced ($P < 0.05$) during the

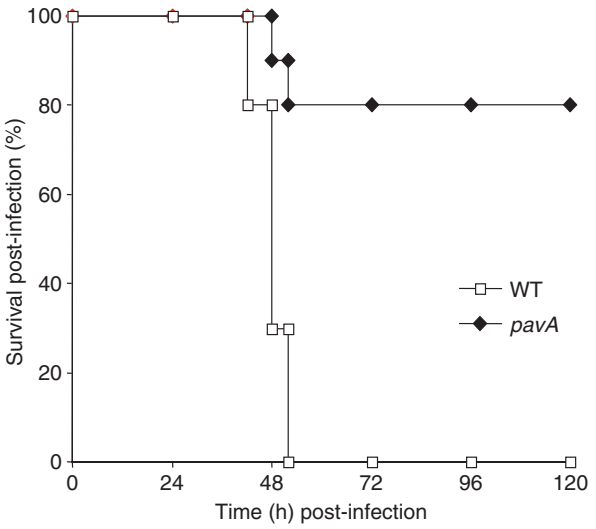

Figure 1 Mouse survival following intranasal challenge with wild-type D39 (WT) or *pavA* mutant pneumococci in an acute pneumonia model ($n = 10$ mice per bacterial strain).

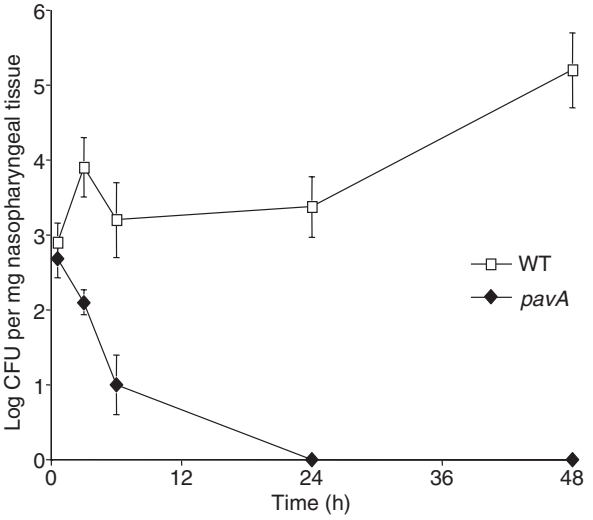

Figure 2 Acute pneumonia model. Wild-type D39 (WT) and *pavA* mutant colony-forming units (CFU) in total nasopharyngeal tissue following intranasal challenge in an acute pneumonia model (n = 10 mice, per time-point and bacterial strain, ± SEM).

first 6 h compared with PavA-deficient pneumococci (Fig. 3). Both wild-type and PavA-deficient pneumococci exhibited similar bacterial loads in the lungs by 24 h (Fig. 3). By 48 h, CFU of wild-type were significantly higher than those of the *pavA* mutants ($P < 0.01$). Numbers of PavA-deficient pneumococci were then diminished but continued to persist in lungs at 72 h postinfection, whereas wild-type-infected mice had to be sacrificed by 48–52 h because of ill health (Fig. 3).

PavA in septicaemia

Septicaemia, secondary to pulmonary pneumonia following intranasal challenge (Figs 2 and 3), was observed in wild-type-infected mice, but not in PavA-deficient-infected mice. Bacterial loads in blood were detected in wild-type-infected mice at 24 h and 48 h postinfection ($\log_{10}$ 5.2 ± 0.3 and $\log_{10}$ 6.8 ± 0.4, respectively). No CFU in blood were detected at any time-point for mice infected with the *pavA* mutant. To determine if this was the result of an inability of PavA-deficient pneumococcal cells to survive in blood, both strains were infected intravenously into groups of 10 mice each per strain and blood CFU was determined at 30 min, 3 h, 6 h, 24 h and 48 h postinfection. No significant differences were found in CFU, and wild-type-infected and *pavA*-mutant-infected mice exhibited similar bacterial loads in their blood throughout the 48 h period of the experiment (Fig. 4). Both groups of mice had to be sacrificed by 50–54 h postinfection because of ill health. There were no significant differences in survival times fol-

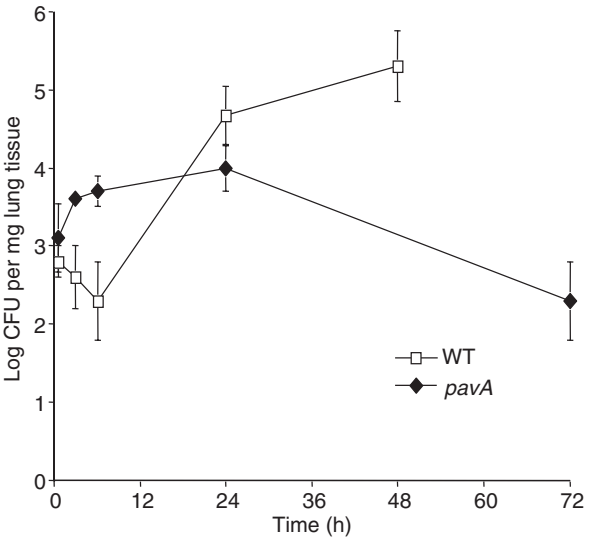

Figure 3 Wild-type D39 (WT) and *pavA* mutant pneumococci colony-forming units (CFU) in total lung tissue following intranasal challenge in an acute pneumonia model (experiment repeated twice, n = 10 mice, per time-point and bacterial strain, ± SEM).

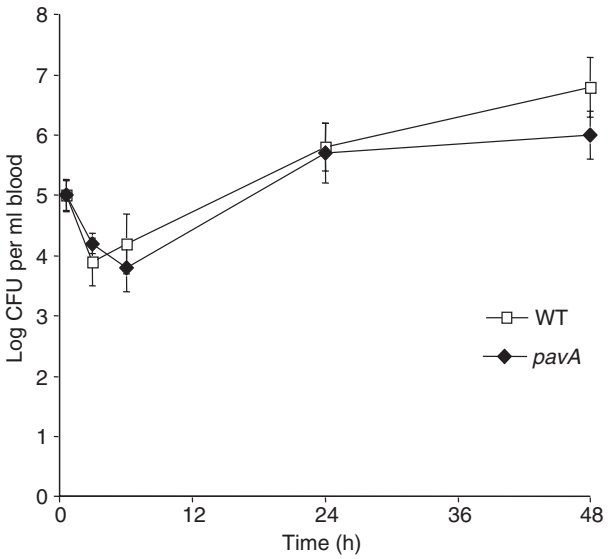

Figure 4 Sepsis model. Wild-type D39 (WT) and *pavA* mutant pneumococci colony-forming units (CFU) in blood following intravenous challenge in sepsis model (n = 10 mice, per time-point and bacterial strain, ± SEM).

lowing intravenous infection of wild-type or *pavA* mutants.

In vivo optical imaging of pneumococcal pneumonia

To image the *in vivo* spread of infection in real time following intranasal administration of pneumococci, bioluminescent optical imaging was used. Groups of six mice per strain and per dose were used. Progression of disease in mice infected with either bioluminescent wild-type D39 or its isogenic *pavA*-knockout was followed continuously in the same mice until they became moribund. Two additional dose ranges of 10-fold and 100-fold higher CFU (10^8 and 10^9) were used for bioluminescent PavA-deficient pneumococci-infected mice to determine the limit of attenuation with this mutant. Wild-type-infected mice developed severe lung infections encompassing the whole of the lung by 30–36 h postinfection. At this stage some mice also developed septicaemia, which rapidly spread the infection throughout the whole of the body and led to death by 42 h (Fig. 5).

All wild-type-infected mice died from their infections by 72 h. In contrast, no pneumococci were detected visually in *pavA*-mutant-infected mice at equivalent time-points (Fig. 5). Only when mice were infected with 10-fold or 100-fold increased CFU of PavA-deficient pneumococci (10^8 or 10^9, respectively) did they begin to show visible signs of bacterial infection and disease (Fig. 5). In mice infected with 10^8 CFU *pavA* mutant bacteria, whole lung infection was evident by 60 h postinoculation, but this was apparent much earlier (by 6 h postinfection) in mice infected with 10^9 CFU *pavA* mutant bacteria (Fig. 5).

Corresponding survival times (Fig. 6) of infected mice showed a median survival time of 58 h for wild-type D39-infected mice, while mice infected with the equivalent challenge dose of the *pavA* mutant strain all survived. With a 10-fold higher dose (10^8 CFU) of *pavA* mutant cells, infected mice also survived significantly longer than wild-type-infected mice (mean survival 168 h, $P < 0.05$; Fig. 6). There was no significant difference in survival compared to wild-type when a 100-fold higher dose (10^9 CFU) of the *pavA* mutant was used to infect mice ($P > 0.05$; Fig. 6). Hence, at the very highest infection dose (10^9 CFU) of PavA-deficient pneumococci, there was evidence that the systemic stage of an infection leading to whole body spread of pneumococci in mice could potentially be reached. However, in wild-type-infected mice, this stage was reached with a 100-fold lower dose.

PavA in meningitis

Intracisternal injection with strain D39 or *pavA* mutant pneumococci resulted in significant elevations of white blood cells in the cerebrospinal fluid 24 h after infection, documenting meningitis (data not shown). Compared with uninfected control animals, all infected animals also showed clinical signs of infection at 24 h. Bacteria could be isolated from the cerebellum (Fig. 7A) and from the bloodstream of infected mice (Fig. 7B). However, bacterial titres in the brain and the blood of animals that were infected with D39Δ*pavA* were significantly lower than in animals that were infected with the D39 wild-type strain (Fig. 7A,B).

Role of PavA in long-term nasopharyngeal carriage

Using our model of long-term nasopharyngeal carriage (in the absence of pulmonary infection or bacteraemia), MF1 mice were challenged intranasally with either D39 wild-type or isogenic *pavA* mutant and samples were collected at 20 min, and at 1, 2, 3, 7 and 14 days postchallenge. Ten mice per time-point per pneumococcal strain were culled and the nasopharynx of each mouse was removed for determining pneumococcal CFU. Wild-type D39 cells persisted in the nasopharynx of mice at approximately 10^3 CFU/mg throughout the 14-day period of this study (Fig. 8). No wild-type bacteria were detected in the lungs or in blood of mice at any of the time-points tested. At 20 min postchallenge there was no significant difference between PavA-deficient pneumococcal nasopharyngeal CFU compared with wild-type. From day 1 postchallenge onwards, CFU of the *pavA* mutant were significantly lower than wild-type CFU ($P < 0.05$; Fig. 8). By 7 and 14 days postchallenge, PavA-deficient mutant CFU in the nasopharynx were further significantly reduced ($P < 0.01$), and numbers were <10 CFU/mg tissue by 14 days postchallenge (Fig. 8).

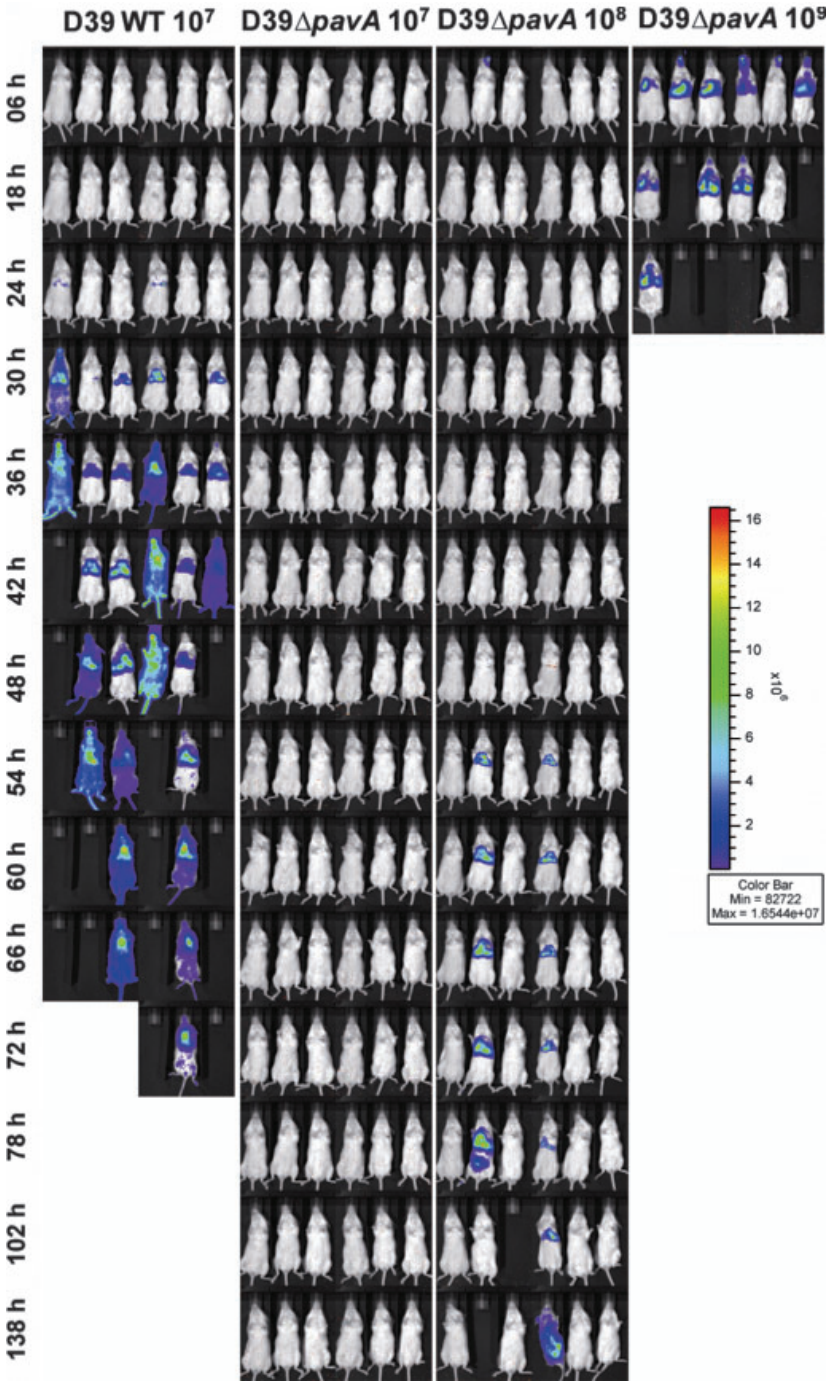

Figure 5 Bioluminescent image analysis of mice intranasally infected with wild-type D39 or *pavA* mutant pneumococci in an acute pneumonia model (n = 6 mice per bacterial strain).

DISCUSSION

Pneumococcal adherence to cells of the upper respiratory tract is an important precondition for successful colonization and subsequent invasion of host tissue. In this study we have shown that the pneumococcal outer cell surface protein PavA is strongly involved in colonization of the upper respiratory tract as PavA-deficient pneumococci are cleared from nasopharyngeal tissue. On the other hand, *pavA* mutants are not

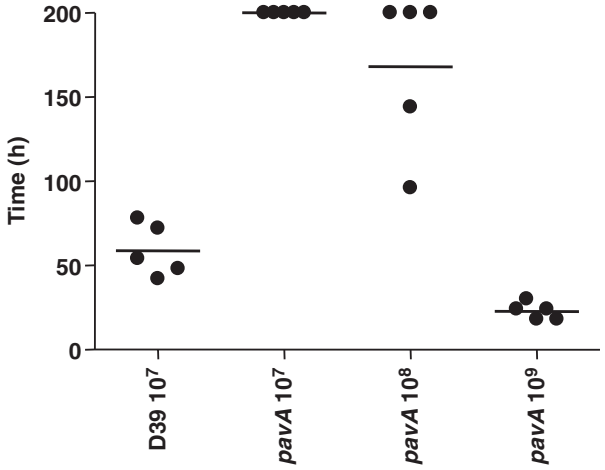

Figure 6 Effect of inoculum size on mouse survival following intranasal challenge with *pavA* mutant pneumococci in an acute pneumonia model (n = 5 mice per bacterial strain).

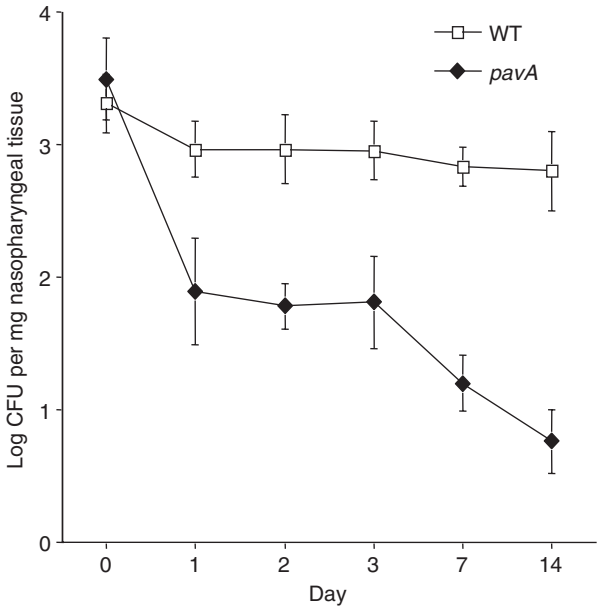

Figure 8 Nasopharyngeal carriage model. Wild-type D39 (WT) and *pavA* mutant pneumococci colony-forming units (CFU) in total nasopharynx following intranasal challenge in a long-term asymptomatic nasopharyngeal carriage model (n = 10 mice, per time-point and bacterial strain, ± SEM).

cleared from infection of the lower respiratory tract, and they are able to persist in lung tissue. Despite this persistence, PavA-deficient pneumococci appear to be unable to seed from lung tissue to blood, except at very high infection doses (100-fold higher than wild-type). We believe that this indicates a genuine inability, at normal infection doses, to successfully translocate from tissue to blood. This is not associated with significantly reduced numbers of PavA-deficient pneumococci early on in lung tissue infection. Indeed, during the first 6 h postinfection, PavA-deficient pneumococcal numbers were higher than wild-type. In addition, at the stage of development of bacteraemia in wild-type-infected mice (24 h), CFUs for both wild-type and mutant bacteria were similar. The *pavA* mutant is not impaired in ability to grow and survive in blood. When the wild-type or mutant were administered intravenously (directly) into blood, both behaved in the same manner and led to host death at equivalent times.

These results therefore appear to demonstrate genetic mutation in *S. pneumoniae* that retards seeding of bacteria from tissue to blood, hence stopping pneumonia from developing into a systemic infection. Mice infected with PavA-deficient pneumococci develop a longer-term chronic pneumonia, with no development of bacteraemia and no significant increase of morbidity (20% mortality over 120-h

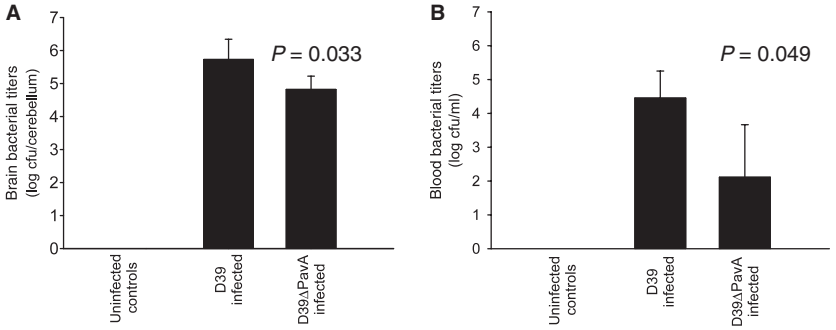

Figure 7 Intracisternal infection of mice with wild-type D39 (WT) or *pavA* mutant. The brain (A) and blood (B) colony-forming units (CFU) of *pavA*-mutant-infected animals were significantly lower statistically than those of wild-type-D39-infected animals.

period). Wild-type-infected mice on the other hand develop an acute pulmonary infection leading to bacteraemia and eventual death (100% mortality in 52 h). The development of lung infection without systemic spread by the *pavA* mutant is also demonstrated by the bioluminescent optical imaging. Even at 100-fold higher challenge doses, the foci of infection never progress beyond the confines of the lung in mice infected with PavA-deficient pneumococci. Yet in wild-type-infected mice (at 100-fold lower dose) it is clear that the infection progresses from pulmonary pneumonia to systemic septicaemia, as early as 30 h postinfection. Previous studies have shown that *pavA* mutants are attenuated in virulence following intraperitoneal inoculation (Holmes *et al.*, 2001). In the meningitis model of infection described in this paper, there were lower CFU in the brain for the *pavA* mutant compared with wild-type. This we suggest is because there is ongoing exchange of bacteria between blood and brain, and if dissemination of pneumococci is reduced then numbers in the brain are also lower. Significantly, PavA-deficient pneumococci are attenuated when administered to the tissues of the respiratory tract, but they are as virulent as wild-type when administered directly into blood. Taken collectively, these results strongly suggest that PavA plays an important role in bacterial translocation across tissue barriers into blood.

The mechanism by which PavA might regulate translocation across tissues is not clear at present. Recent evidence suggests that PavA is a key factor for live pneumococci to escape phagocytosis, and that less severe immune responses are generated to PavA-deficient pneumococci (Noske *et al.*, 2009). It might be that PavA is necessary for functional activities of other pneumococcal virulence determinants, perhaps by modulating immunogenic bacterial proteins (Pracht *et al.*, 2005). These various possibilities are currently under detailed investigation.

The data presented in this paper suggest that PavA is also involved in determining longer-term nasopharyngeal colonization by pneumococci and hence carriage. In our model, strain D39 pneumococci are carried at a constant level in the nasopharynx, in the absence of any lower respiratory tract infection or bacteraemia. Pneumococcal carriage is long term, beyond 28 days (Manco *et al.*, 2006), and is asymptomatic. Using this model, PavA-deficient pneumococci were unable to carry in the nasopharynx beyond 7 days, while wild-type pneumococci were carried for at least 14 days with no reduction in CFU. Clearly, pneumococcal factors other than PavA are also important in long-term carriage, as PavA-deficient pneumococci do not begin to be cleared from the nasopharynx until day 7. Other pneumococcal virulence factors such as pneumolysin and neuraminidase A (NanA) have also been shown to have important roles in nasopharyngeal colonization, although only in models of shorter-term acute infection accompanied by pneumonia and bacteraemia (Kadioglu *et al.*, 2002; Manco *et al.*, 2006). However, it is clear that PavA is involved in nasopharyngeal colonization both in the short-term acute infection model and in the longer-term asymptomatic carriage model.

PavA polypeptide is a fibronectin-binding protein (Holmes *et al.*, 2001), but is not the only fibronectin-interacting protein expressed by *S. pneumoniae* (van der Flier *et al.*, 1995; Pracht *et al.*, 2005). Mutants are reduced in adherence and invasion functions with respect to a range of host cells. It is not yet clearly established whether or not pneumococci adhere to and invade cells through a fibronectin-mediated interaction. Although it is possible that PavA may regulate the activities of virulence factors, there is no evidence to suggest that *pavA* mutants have lower levels of pneumolysin or of other known virulence determinants (Holmes *et al.*, 2001). The *Listeria monocytogenes* PavA orthologue FbpA, which mediates adherence to fibronectin, has been implicated to act as a chaperone that prevents degradation of specific virulence proteins such as listeriolysin (LLO) and internalin B (InlB) (Dramsi *et al.*, 2004) involved in invasion. The FbpA-like proteins show approximately 80% identities across the family (Christie *et al.*, 2002) so it is possible that they all exhibit functions in addition to fibronectin adherence.

NanA is a secreted protein anchored to cell wall peptidoglycan, and is thought to cleave terminal sialic acid residues from host glycoproteins. This has the potential to result in exposure of underlying receptors for adherence of bacteria and therefore promote colonization of the respiratory tract epithelium. It has previously been shown that NanA-deficient mutants were unable to colonize respiratory tissues of mice and seed into blood, despite being able to survive in blood when administered intravenously (Manco *et al.*, 2006). This phenotype is somewhat similar to the

pavA mutant phenotype, but *pavA* mutants are completely unaffected in neuraminidase activity (results not shown). It is conceivable therefore that NanA and PavA act in a concerted manner to successfully negotiate adherence. Subsequent invasion, and translocation of pneumococci across host tissues, may require both NanA and PavA, and might then lead to systemic disease.

ACKNOWLEDGEMENT

This study was funded by a grant from the Deutsche Forschungsgemeinschaft (DFG HA 3125/3-1) to S.H.

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