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The Contribution of PspC to Pneumococcal Virulence Varies between Strains and Is Accomplished by Both Complement Evasion and Complement-Independent Mechanisms

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Pneumococcal surface protein C (PspC) is a virulence factor of *Streptococcus pneumoniae* previously shown to play a role in bacterial adherence, invasion, and evasion of complement. We investigated the role of this protein in our murine models of pneumococcal pneumonia with different pneumococcal strains. The deletion of *pspC* in strains of serotypes 2, 3, and 19F did not significantly alter host survival times in the pneumonia model. In contrast, *pspC* deletion significantly reduced the virulence of the serotype 4 strain, TIGR4, in both the pneumonia and bacteremia models. Therefore, *pspC* is a systemic and pulmonary virulence determinant for *S. pneumoniae*, but its effects are influenced by the pneumococcal strain. Finally, pneumonia infection of complement-deficient ($C3^{-/-}$) mice enhanced *pspC* virulence, illustrating that PspC-mediated complement evasion contributes to virulence. However, other functions of PspC also contribute to virulence, as demonstrated by the finding that the *pspC*-deficient TIGR4 mutant was still attenuated relative to the wild-type parent, even in the absence of C3.

Streptococcus pneumoniae (the pneumococcus) is a human pathogen capable of causing diseases such as otitis media, meningitis, bacteremia, and pneumonia. Control of *S. pneumoniae* is impaired by increasing antibiotic resistance and suboptimal vaccines. As a result, there is a need for better characterization of pneumococcal virulence factors and potential vaccine antigens. One such virulence factor and vaccine candidate is pneumococcal surface protein C (PspC, also referred to as Sp2190, CbpA, SpsA, PbcA, and Hic). The *pspC* gene was found in all clinical strains analyzed, and a high level of variability was detected at both the *pspC* coding sequence and locus levels. PspC proteins show a common organization, but two major groups differing in anchor sequences may be distinguished. The first group of proteins is characterized by a typical choline binding domain while the second group shows a C-terminal LPXTG anchoring domain (13).

PspC has proven a promising vaccine candidate in mouse models (2, 5, 19). It has also been shown to contribute to virulence and nasopharyngeal colonization in mouse and rat models (2, 11, 12, 20, 27). PspC has been shown to be involved in ability to cause sepsis in an intravenous model of infection with strains of serotype 2 and 3 (12). Several functions are accredited to PspC that may contribute to its role in virulence. These include complement evasion through the binding of the complement regulator, factor H, and its recruitment onto the pneumococcal surface (8, 14, 15, 26). PspC can also bind complement component C3, human secretory immunoglobulin A (IgA), and the human polymeric Ig receptor and contributes to pneumococcal attachment and invasion (9, 10, 17, 27, 28). Recent findings indicate that the lack of PspC significantly enhances the susceptibility of pneumococci to both bactericidal activity and secretory response by the microglial cells, suggesting that this molecule may play an important role in the invasion of the central nervous system by pneumococcus (24).

To gain more insight into the role of *pspC* in pneumococcal virulence, we assessed *pspC*-deficient mutants in different strains in our murine pneumonia model. We show that the contribution of *pspC* to virulence in pneumonia differs between strains. In addition, the use of complement-deficient ($C3^{-/-}$) mice shows that *pspC* contributes to virulence both through its interaction with complement and through other, complement-independent functions.

MATERIALS AND METHODS

Mice. Female, 8- to 12-week-old, outbred MF1 mice (25 to 30 g) (Harlan Olac, Bicester, United Kingdom) and C57BL/6 mice (20 to 25 g) (Harlan Olac) gender matched with complement C3-deficient mice ($C3^{-/-}$ mice [30]; bred in house; 20 to 25 g) were used. All experiments were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

Bacteria. The pneumococcal strains used were serotype 2 (D39, NCTC 7466; Central Public Health Laboratory, London, United Kingdom), serotype 3 (HB565, a derivative of strain A66 [12]), serotype 4 (TIGR4, ATCC BAA-334), and serotype 19F (G54) (25). The PspC types of the strains (as defined in reference 13) are D39 (PspC3.1), TIGR4 (PspC3.4), G54 (PspC7.1), and HB565 (Hic). Pneumococci were cultured on blood agar base number 2 (Oxoid, Basingstoke, United Kingdom) plus 5% (vol/vol) horse blood (E&O Laboratories, Bonnybridge, United Kingdom) or brain heart infusion broth (Oxoid, Basingstoke, United Kingdom). Strains were validated via the Quellung reaction, sensitivity to optochin and multilocus sequence typing (performed at the Scottish Meningococcus and Pneumococcus Reference Laboratory, Stobhill Hospital, Glasgow, United Kingdom). The production of *pspC*-negative D39 and HB565 has been described elsewhere (12). In these strains, the *pspC* gene was deleted

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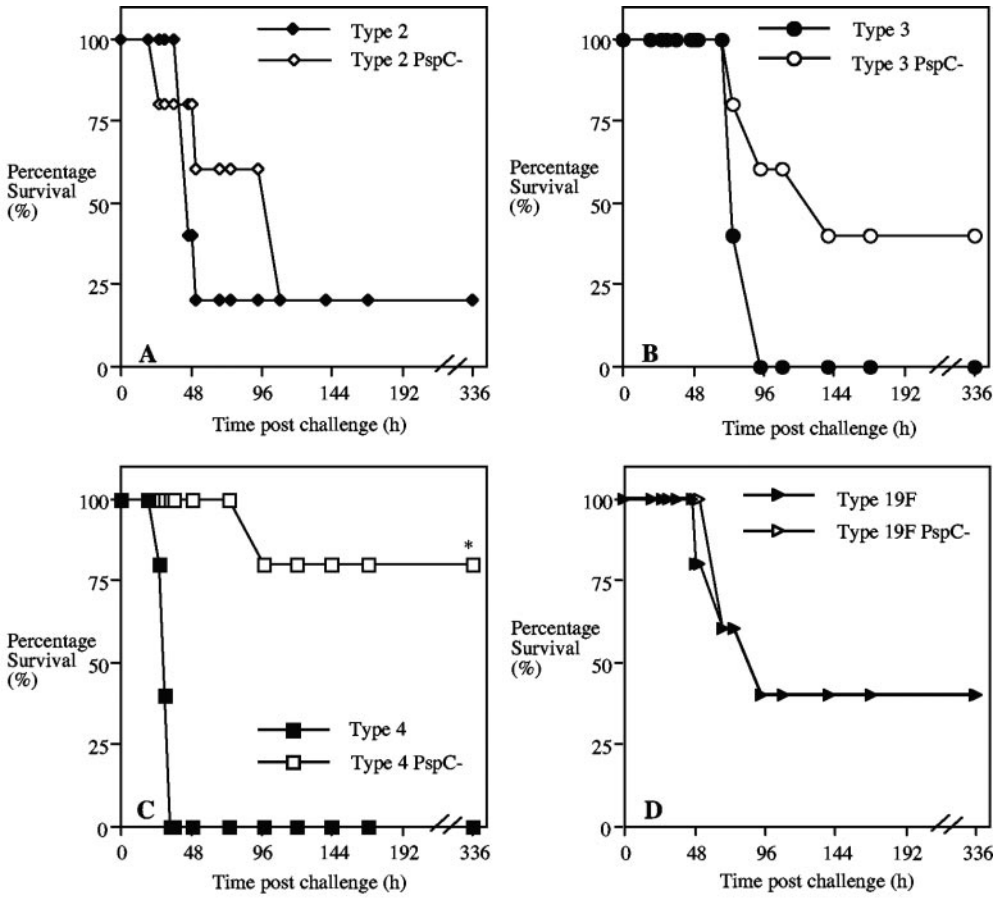

FIG. 1. Survival of MF1 mice ($n = 5$) following intranasal infection with wild-type or *pspC* mutant *S. pneumoniae* in strains D39 (A), HB565 (A66 derivative) (B), TIGR4 (C), and G54 (D). *, longer survival for mice infected with the *pspC* mutant bacteria than wild-type bacteria ($P < 0.01$).

with a chloramphenicol resistance cassette joined by PCR on both sides to sequences flanking the *pspC* locus. The same cassette was used to delete the *pspC* locus from strains TIGR4 and G54.

For in vivo studies, pneumococci were passaged intraperitoneally through mice and stored at −80°C as previously described (1). Bacteria for infections were thawed rapidly, harvested by centrifugation, and resuspended in sterile phosphate-buffered saline (PBS; Oxoid).

Infection of mice. To induce pneumonia, mice were anesthetized with halothane and the inoculum administered intranasally in a 50-μl volume. The infectious dose for each pneumococcal strain was predetermined (10^6 CFU for serotypes 2, 3, and 4 and 10^7 CFU for serotype 19F).

For the intravenous infection for the bacteremia model, 10^4 CFU of wild-type and *pspC*-deficient TIGR4 pneumococci in a 50-μl volume were injected via a tail vein.

For both infections, signs of disease were monitored frequently until mice were deemed to have irreversibly succumbed to the infection, at which time they were humanely sacrificed. Mice displaying no signs of illness for 336 h were considered to have survived the infection.

Sample collection and processing. Following cervical dislocation, cardiac blood was sampled. To lavage the nasopharynx, the trachea was clamped and 2 ml sterile PBS passed through the nasopharynx via a 16-gauge nonpyrogenic angiocatheter (F. Baker Scientific, Runcorn, United Kingdom) was used to lavage the lungs with 2 ml PBS to give bronchoalveolar lavage fluid. Lavaged lungs were then removed and homogenized in 5 ml PBS by using a glass handheld tissue homogenizer (Jencons, Leighton Buzzard, United Kingdom). Viable bacteria in samples were then counted by plating out serial 10-fold dilutions.

Statistical analysis. Bacteriology results are expressed as means ± 1 standard error of the mean. Samples containing fewer CFU per ml than the detection limit (log 1.92 CFU per ml for blood and log 0.92 CFU per ml for nasopharyngeal, airway, and lung samples) were ascribed a value just below the detection limit (log 1.91 or 0.91 CFU per ml). Comparisons of bacterial loads were performed using unpaired Student's *t* tests. Survival times were analyzed using nonparametric Mann-Whitney U analysis.

Statistical analyses were carried out using StatView 4.1 (Abacus Concepts, Berkeley, Calif.) with *P* values of <0.05 considered statistically significant for all analyses.

RESULTS

The influence of *pspC* on murine survival during pneumococcal pneumonia varies between strains. We investigated the role of *pspC* in pneumococcal virulence in four different strains, each of a different serotype. Mice infected intranasally with D39 (serotype 2) or G54 (serotype 19F) and deficient in *pspC* had survival times and survival rates similar to those of mice infected with the wild-type bacteria (Fig. 1A and D). Infection of mice with *pspC*-deficient HB565 (serotype 3) pneumococci resulted in survival times that were not significantly different from those of wild-type bacteria, although there was a trend towards attenuation of the mutant (Fig. 1B). Mice infected with *pspC*-deficient HB565 had a median survival time of 140 h, while those infected with wild type had a median survival time of 75 h. It has already been shown that

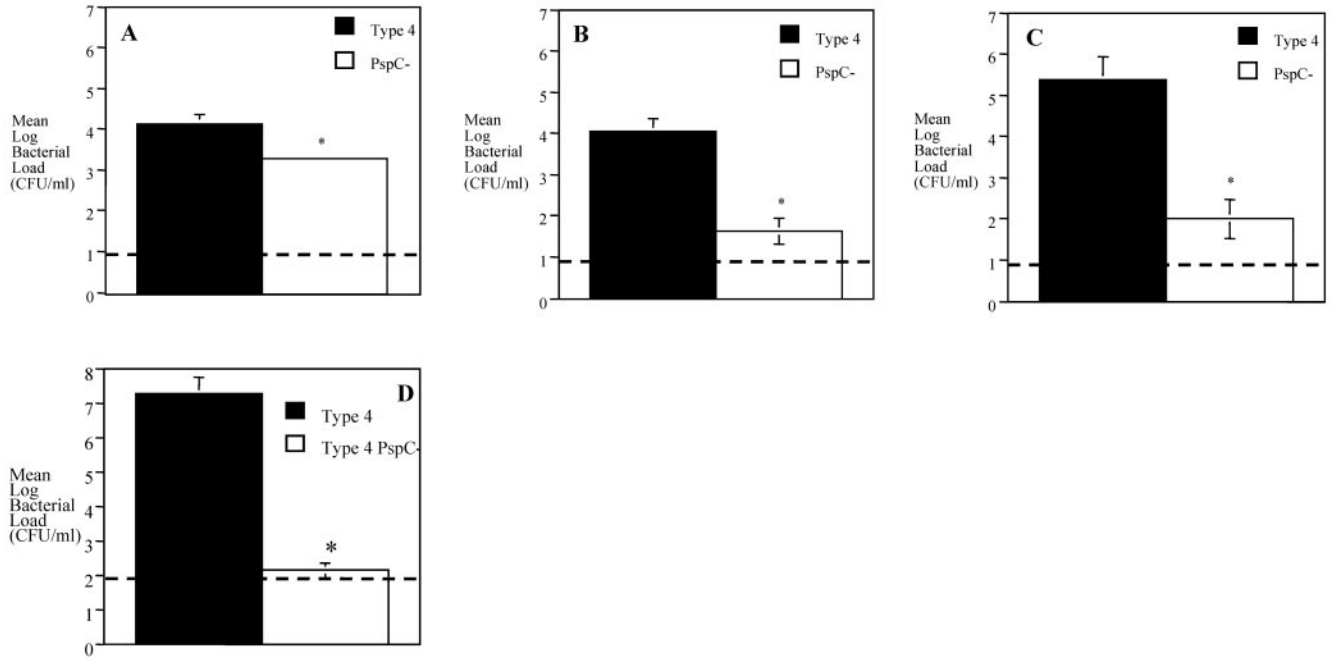

FIG. 2. Bacterial loads within the nasopharynx (A), lung airways (B), lung tissue (C), and blood (D) of MF1 mice ($n = 5$) 24 h following intranasal infection with wild-type or *pspC* mutant TIGR4. The dashed line represents the detection limit of the viable count assay. *, higher counts for the wild type than the mutant ($P < 0.01$).

D39 and HB565 strains deficient in *pspC* are deficient in their ability to cause sepsis following intravenous infection (12).

In contrast to the case for the strains described above, there was a significant attenuation following intranasal challenge when *pspC* was deleted from the serotype 4 strain TIGR4. Mice intranasally infected with TIGR4 deficient in *pspC* survived significantly longer than those infected with wild-type bacteria ($P < 0.01$) (Fig. 1C). Four of five mice infected with TIGR4 *pspC* survived the experiment, with one dying at 98 h (median, >336 h). In contrast, all mice infected with wild-type TIGR4 succumbed by 33 h postinfection (median, 31.5 h).

To further characterize the attenuation seen with the *pspC*-deficient TIGR4 mutant, bacterial viable counts in the lung tissues and blood were assessed following intranasal infection. When examined 24 h following infection, the levels of *pspC*-deficient TIGR4 were significantly reduced within the nasopharynx, lung airways, lung tissue, and blood compared to wild-type TIGR4 ($P < 0.01$ for all sites) (Fig. 2).

TIGR4 deficient in *pspC* is also attenuated following intravenous infection. Following intravenous infection, mice challenged with the *pspC*-deficient TIGR4 mutant survived longer than mice infected with wild-type TIGR4 ($P < 0.01$) (Fig. 3). Four of five mice in the *pspC*-deficient TIGR4 group survived (median, >336 h), while all animals infected with TIGR4 died (median, 32 h). When bacterial counts were assessed 24 h postinfection, wild-type TIGR4 had reached significantly higher levels of bacteremia than the *pspC* mutant ($P < 0.01$). Mice infected with wild-type TIGR4 had $\sim 10^6$ CFU/ml, while *pspC*-deficient TIGR4 counts were only $\sim 10^2$ CFU/ml. All five mice infected intravenously with wild-type bacteria were bacteremic at 24 h, but only 2/5 mice infected with the *pspC* mutant had detectable pneumococci within their bloodstream.

***pspC* contributes to virulence through complement-dependent and -independent functions.** It is believed that a major role of PspC in virulence is evasion of the complement pathway via the recruitment of factor H onto the bacterial surface (14, 15, 26). We investigated this hypothesis by infecting transgenic mice deficient in complement component C3 with wild-type and *pspC*-deficient TIGR4. Interestingly, the deletion of C3 enhances the bacterial levels of the *pspC* mutant to levels similar to those seen with wild-type TIGR4 in wild-type mice in all tissues (Fig. 4). Hence, the absence of complement appears to enhance the virulence of the *pspC* mutant. In addition, the *pspC* mutant counts are significantly higher in the blood of $C3^{-/-}$ mice versus the *pspC*-deficient mutant counts in wild-type mice (Fig. 4D). There was also a trend for higher counts in the lung tissue (Fig. 4C) but not in the airway or nasopharynx (Fig. 4A and B). This indicates that evasion of complement is a major activity in which *pspC* is involved but that the importance of this interaction varies with body site, being most important in the blood but of minor importance in the airways and nasopharynx. However, in the absence of complement, the *pspC* mutant is still attenuated relative to wild-type TIGR4 with regards to bacterial counts in the airways, lung tissue, and blood. This indicates that *pspC* also contributes to virulence independently of its interaction with complement.

In addition, comparisons of the levels of bacteria in wild-type and $C3^{-/-}$ mice show the increased susceptibility of the gene knockout mice to both wild-type and mutant pneumococci (Fig. 4). Finally, in wild-type C57BL/6 mice, the *pspC* mutant is attenuated relative to wild-type TIGR4, showing that this phenotype is not restricted to a single mouse strain, as the earlier data were generated with MF1 mice.

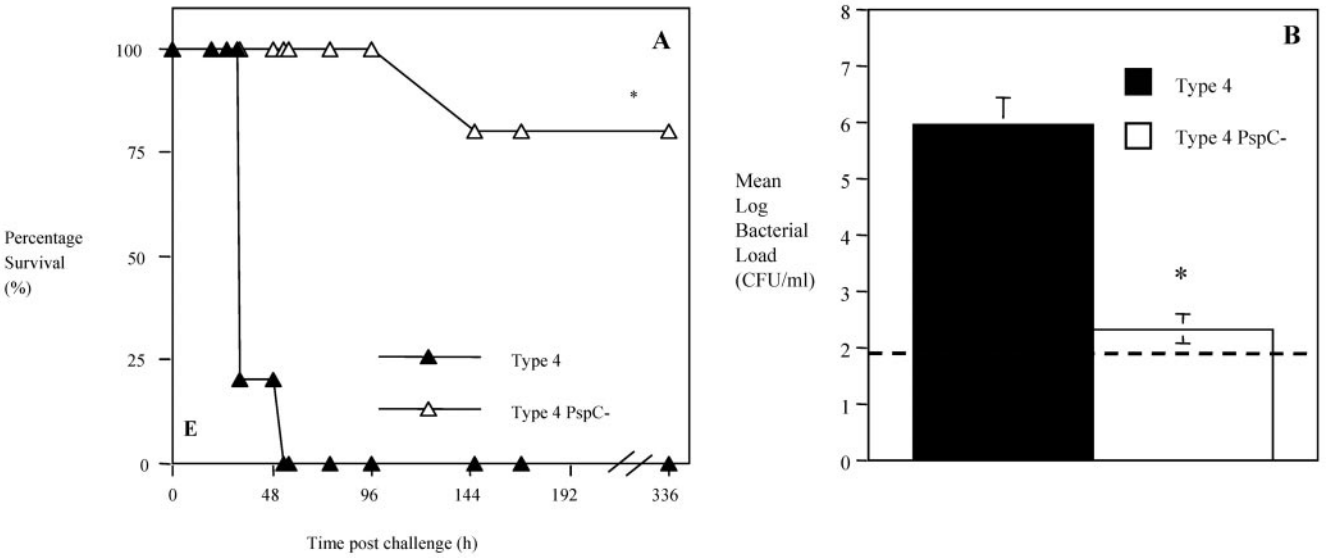

FIG. 3. Survival curve (A) and bacterial load (B) in the blood 24 h following intravenous infections of MF1 mice ($n = 5$) with wild-type or *pspC*-deficient TIGR4. The dashed line represents the detection limit of the viable count assay. *, higher counts for the wild type than the mutant ($P < 0.01$).

DISCUSSION

PspC is a known pneumococcal virulence factor with a variety of functions that likely contribute to the pathogenesis of disease. Previously, it has been shown to contribute to nasopharyngeal colonization, pneumonia, and bacteremia (2, 11, 12, 20, 27). To expand on previous data on the role of *pspC* during pneumococcal infections, we have compared the effect of *pspC* deletion in a four different strains. Furthermore, with the use of $C3^{-/-}$ mice, we have addressed the importance of PspC-complement interactions to virulence.

As determined by survival rates and survival times, the deletion of *pspC* caused significant attenuation only in the serotype 4 strain TIGR4 and not in the other three strains tested. While this does not rule out a role for *pspC* in the virulence of these other strains—indeed, there was a strong trend towards attenuation of the mutant in strain HB565—there was nonetheless a differential effect of deleting *pspC* between strains. Therefore, the influence of *pspC* in virulence varies between strains. Moreover, it is known that the deletion of *pspC* in strains D39 and HB565 causes attenuation when using a sepsis model (12). Thus, PspC seems to play a site-specific role (i.e., these strains are not attenuated in a pneumonia model but are in a sepsis model). Strain-to-strain variation in the influence of different pneumococcal virulence factors has been reported previously by our group and others (3, 7, 18, 21, 23). PspC is highly polymorphic, and this may contribute to the strain-specific effects we noted (9, 13). Interestingly, the ability to bind factor H is independent of capsule serotype. In this study, the attenuation of *pspC*-deficient TIGR4 was accompanied by significantly reduced bacterial counts in the lungs and blood of infected mice and was confirmed in a second mouse strain. Furthermore, the *pspC*-deficient TIGR4 mutant was also shown to be attenuated in a model of bacteremia following intravenous infection. Therefore, in TIGR4, *pspC* contributes to virulence in both pneumonia and bacteremia infections. Our data agree with and expand upon the identification of *pspC* in the TIGR4 signature-tagged mutagenesis screen of pneumonia virulence factors (11). Likewise, *pspC* has previously been shown to contribute to pneumococcal bacteremia, with strains A66 and D39 (2, 12), and to pneumonia, with strain EF3030 (2). In addition, in D39, it appears that *pspC* is important for translocation from the nasopharynx to the lungs and in crossing the brain-blood barrier (20). A role in colonization has also been suggested (2, 27).

The interaction of PspC with factor H recruits this complement regulator to the pneumococcal surface and acts to protect the bacterium from complement-mediated clearance (14, 15). This PspC-mediated recruitment of factor H onto the pneumococcus has been shown to occur in vivo (26). The central role of complement in immunity to the pneumococcus (22) suggests that this interaction may be important to virulence. However, this has never been assessed directly in vivo although it has been shown that preincubation with factor H enhances survival of bacteria following intravenous infection (26). To address the issue of the importance of PspC-factor H interactions during infection, we used gene knockout mice lacking C3, a central component of the complement regardless of the activation pathway. If complement evasion contributes to the function of PspC during infection, the removal of complement should restore/enhance the virulence of the mutant. Indeed, the removal of complement enhanced *pspC* mutant bacterial counts to levels similar to those with wild-type TIGR4 in wild-type mice. Furthermore, removal of complement increased the counts of the *pspC* mutant in the blood, lung tissue, and airways compared to mutant counts in the presence of complement, albeit that the difference between the lung tissue and airways was not statistically significant. Thus, *pspC*-complement interactions do appear to contribute to virulence. This effect was not seen in the nasopharynx and was only minimal in the airways, and so the importance of this interaction seems

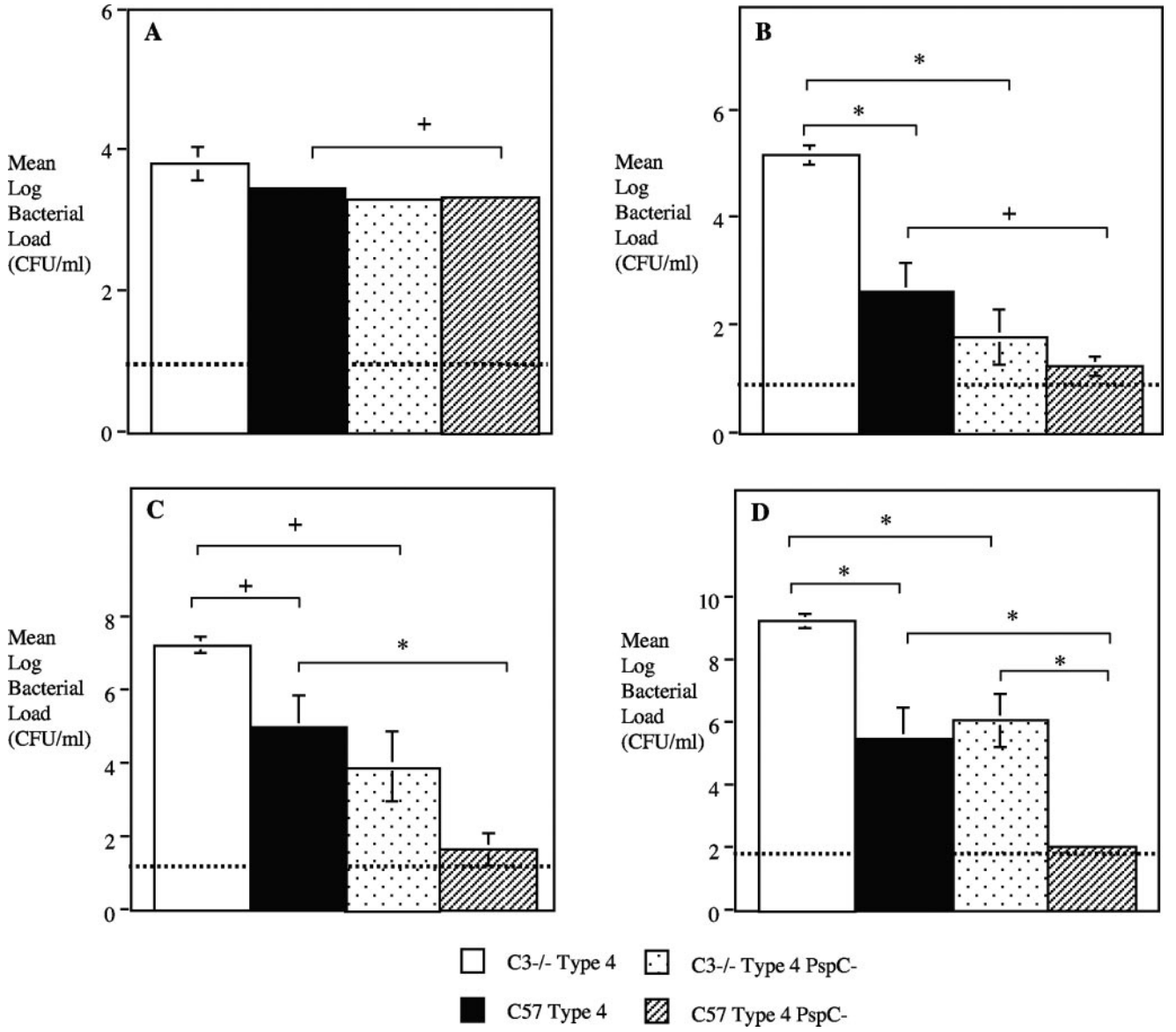

FIG. 4. Bacterial loads within the nasopharynx (A), lung airways (B), lung tissue (C) and bloodstream (D) of $C3^{-/-}$ or C57BL/6 mice ($n = 5$) 24 h following intranasal challenge with wild-type or *pspC* mutant TIGR4. The dashed line represents the detection limit of the viable count assay. + and * denote levels of significance (P) of <0.05 and <0.01, respectively.

dependent on anatomical location, being of most importance in the blood. This is the first demonstration of the in vivo significance of *pspC*-mediated complement evasion. However, in the airways, lung tissue, and blood, there is still attenuation of the *pspC* mutant bacteria compared to wild-type bacteria in the $C3^{-/-}$ mice. This suggests that PspC-complement interactions are not the only contribution of PspC in this infection and that other functions, such as adherence and invasion, must also contribute. This is in contrast to previous work with a *pspA* mutant (29). Infection of $C3^{-/-}$ mice fully restored the virulence of this mutant, showing that inhibition of complement was likely to be the major role in virulence for PspA (29). Therefore, PspC contributes to pneumococcal virulence via complement-dependent and -independent mechanisms. The results of our studies and those reported previously suggest that interaction of PspC with factor H is not a human-specific interaction (11), whereas the interaction with the polymeric Ig receptor is a human-specific trait (11). The complement-independent mechanisms observed in the mouse therefore do not involve the interaction with the polymeric Ig receptor. Finally, our data showing increased susceptibility of $C3^{-/-}$ mice to pneumococcal infection agree with our previous work and that from others in highlighting the importance of C3 in innate immunity to the pneumococcus (4, 6, 16).

Overall, in line with the polymorphic nature of the gene, the contribution of *pspC* to pneumococcal virulence varies between strains. This contribution, at least in TIGR4, appears to be via complement-dependent (evasion) and other, complement-independent functions likely to involve the role of PspC in adherence and invasion.

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