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Pneumococcal Surface Protein C Contributes to Sepsis Caused by *Streptococcus pneumoniae* in Mice

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The role of pneumococcal surface protein C (PspC; also called SpsA, CbpA, and Hic) in sepsis by *Streptococcus pneumoniae* was investigated in a murine infection model. The *pspC* gene was deleted in strains D39 (type 2) and A66 (type 3), and the mutants were tested by being injected intravenously into mice. The animals infected with the mutant strains showed a significant increase in survival, with the 50% lethal dose up to 250-fold higher than that for the wild type. Our findings indicate that PspC affords a decisive contribution to sepsis development.

Streptococcus pneumoniae is an important human pathogen causing both mild and severe diseases, including pneumonia, otitis media, sepsis, and meningitis. Several virulence factors participate in the pathogenesis of pneumococcal infection. In addition to the capsule, a number of proteins and enzymes are involved in pathogenesis and have also been proposed as vaccine candidates (16). Among these, pneumococcal surface protein C (PspC), also known as CbpA, SpsA, PbcA, and Hic, is considered a major virulence factor (5, 10–12, 14, 19). The *pspC* locus was characterized in a large number of clinical strains, and a high level of variability was found at both the *pspC* coding sequence and locus levels (4, 12). PspC proteins show a common organization, but two major groups differing in the anchor sequences may be distinguished. The N-terminal region of the PspC proteins is the result of the assembly of eight major sequence blocks that may be responsible for the different virulence phenotype associated with this surface protein (12). Several functions are attributed to PspC, including binding to the secretory component of human immunoglobulin A and to complement factors C3 and H (5, 7, 10, 11, 14). Binding to factor H (fH) is a defense strategy used by certain microorganisms for protection against complement attack and opsonophagocytosis. PspC also mediates adherence to lung cells and colonization of the nasopharynx and contributes to tissue invasion (3, 19). Immunization with purified PspC elicits protection against sepsis and carriage (1). It was recently shown that PspC-deficient mutants have a reduced ability to colonize the nasopharynx and infect the lungs (1). In the present work, the role of PspC in pneumococcal sepsis was analyzed in detail by constructing PspC-deficient mutants of type 2 and 3 pneumococci and by infecting mice intravenously.

Construction of *pspC* deletion mutants. Avery's classic strains D39 (type 2) and A66 (type 3) and derivatives thereof were used (Table 1). To improve the isolation of strains from infected mice, the streptomycin-resistant derivatives FP58 and HB565 were used for animal studies in our laboratory (Table 1). FP58 was obtained by the transformation of the D39 strain with a 410-bp PCR fragment containing the *str-41* allele from DP1004. Transforming DNA for the construction of mutants was obtained by the gene SOEing technique, as already described (13, 18). In the mutants, *pspC* was replaced with a chloramphenicol resistance cassette (*ami*/*cat*) (6). A 1,916-bp DNA fragment containing the *ami*/*cat* cassette (850 bp) flanked by regions upstream (540 bp) and downstream (526 bp) of *pspC* was generated (Fig. 1) and used to transform the FP58 and HB565 strains. The regions upstream and downstream of *pspC* were amplified from A66 by using the IF43-IF40 and IF41-IF30 primer pairs. Primer IF43 (5′-AATGAG AAACGAATCCTTAGCAATG-3′) is complementary to nucleotides (nt) 6368 through 6392 on section 190 of the TIGR4 genome (GenBank accession no. AE007507). In IF40 (5′-ATCCATTAAAAATCAAACAAATTTTCATGTTTATT TCCTTCTATATTTTTTCTTTA-3′), the underlined nt are complementary to IF38, while the last 31 nt correspond to nt 513 through 540 of *pspC*, (GenBank accession no. AF154012). In IF41 (5′-TCAGATAGGCCTAATGACTGGCTTTTATA AACCGAAGAAGTCATTGCCATCA-3′), the underlined nt are complementary to IF39 and the last 24 nt correspond to nt 1726 to 1749 of *pspC* (GenBank accession no. AF252857). Primer IF30 (5′-AAGATGAAGATCGCCTACGAACAC-3′) corresponds to nt 3347 through 3370 on section 190 of the TIGR4 genome (GenBank accession no. AE007507). The *ami*/*cat* cassette was amplified from plasmid pEVP3 (6) by using primers IF38 and IF39. Primer IF38 (5′-ATGAAAATTTGT TTGATTTTTAATGG-3′) corresponds to nt 175 through 200 of the *ami* promoter (GenBank accession no. X17337), while primer IF39 (5′-TTATAAAAGCCAGTCATTAGGCCTATC T-3′) is complementary to nt 1883 through 1910 of pC194 (GenBank accession no. V01277). Recombinants were selected for acquisition of chloramphenicol resistance by the multilayer plating procedure (13). Deletion of the *pspC* coding sequence in type 2 and type 3 chromosomes was confirmed by sequence analysis. *pspC* deletion mutants, designated FP30 (type 2) and FP20 (type 3) (Table 1), showed no difference

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TABLE 1. *S. pneumoniae* strains

Strain	Relevant properties[a]	Reference
D39	Type 2 Avery's strain	18
A66	Type 3 Avery's strain	18
DP1004	D39 unencapsulated derivative; *str-41*, Sm^r	18
FP58	Streptomycin-resistant derivative of D39; *str-41*, Sm^r	This study
HB565	Streptomycin-resistant derivative of A66; *str-1*, Sm^r	18
FP30	Derivative of FP58; Δ*pspC;* Sm^r, Cm^r	This study
FP20	Derivative of HB565; Δ*pspC;* Sm^r, Cm^r	This study

[a] *str-41* and *str-1* indicate point mutations conferring resistance to streptomycin (9, 20). Sm, streptomycin; Cm, chloramphenicol.

from the wild type in growth and competence development (data not shown).

Virulence of type 2 mutant. Mouse-passaged pneumococci, prepared as previously described (17), were used for inocula. Before being infected, the mice were kept under an infrared lamp (200 W) for 2 to 3 min and then given an intravenous (i.v.) injection into the tail vein. Bacteria were delivered in a total volume of 200 μl. The animals were monitored for 10 days. Differences in survival were analyzed by the Mann-Whitney-Wilcoxon test, considering the time point when mice died; for statistical purposes, animals still alive after 10 days were assigned a time to death of 240 h. Groups of 7-week-old female outbred MF1 mice ($n = 6$ to 8) (Harlan Nossan) were inoculated with a range of bacterial inocula (from 10^3 to 10^8 CFU) of either FP58 (type 2) or its isogenic *pspC* mutant, FP30. Differences in survival were detected only at the lowest doses. At a dose of 10^4 CFU per animal, infection by wild-type pneumococci was lethal in 87.5% of mice, while none of those inoculated with the mutant died ($P = 0.0016$). The 50% lethal dose (LD_{50}) was 2×10^3 CFU for the wild type and 3.7×10^4 CFU for the mutant, indicating a 19-fold attenuation in virulence (Fig. 2A).

Virulence of type 3 mutant. Experimental sepsis was repeated with type 3 pneumococci by infecting both MF1 (outbred) and CBA/Jico (inbred) mice with HB565 (wild type) and FP20 (*pspC* mutant) (Fig. 2B and C). CBA/Jico mice were chosen because of their sensitivity to pneumococcal infection (8). MF1 mice infected with the mutant showed an increase in survival for inoculum doses ranging from 10^5 to 10^7 CFU. Differences in survival (by Mann-Whitney-Wilcoxon test) were significant at the dose of 10^6 CFU ($P = 0.027$) (Fig. 2B). Survival of CBA/Jico mice was also significantly different both at 10^5 ($P = 0.0008$) and 10^6 ($P = 0.0019$) CFU, as all mice infected with the mutant survived and all control mice died (Fig. 2C). In MF1 outbred mice, the LD_{50} was 10^5 CFU for the wild type and 2.5×10^7 CFU for the mutant, while for CBA/Jico inbred mice, the LD_{50} was 2×10^4 CFU for the wild type and 3.2×10^6 CFU for the mutant (Fig. 2B and C). Depending on the mouse strain, PspC-negative mutants of type 3 pneumococci showed a 160- to 250-fold reduction of virulence in the i.v. sepsis model.

Previous studies using sepsis infection models were not able to show a convincing virulence attenuation of *pspC* mutants. When tested by being injected intraperitoneally, *pspC* mutants were not significantly reduced in virulence (2, 19), and only minor differences in time to death were found with an i.v. sepsis model (1). The present data show that PspC affords a decisive contribution to sepsis development, with mutants showing an attenuation of virulence of up to 250-fold. Our data were obtained with two different pneumococcal serotypes and both inbred and outbred mice. In our opinion, the i.v. route of inoculation and the use of a wide range of infecting doses were indeed instrumental in showing the important role of PspC in pneumococcal sepsis.

While PspC is required for nasopharyngeal colonization and lung infection in the mouse model (1, 19, 21), here we show that it is also very important for sepsis. PspC binds fH of the complement system in different pneumococcal serotypes, and its fH binding efficacy was demonstrated to vary among different strains (7, 14). Pneumococci can escape complement attack and opsonophagocytosis by recruiting fH with PspC in vitro

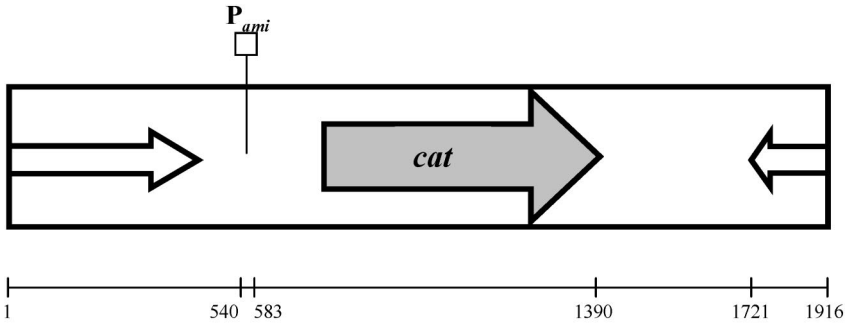

FIG. 1. Representation of the genetic construct for *pspC* deletion. The construct is constituted of a gene conferring resistance to chloramphenicol (*cat*) under the control of the *ami* promoter (6), flanked by the regions upstream and downstream of *pspC*. Upon transformation, *pspC* flanking sequences allow integration of the *ami*/*cat* cassette and deletion of *pspC*. The upstream region (540 bp) is complementary to nt 6392 to 5843 on section 190 of the TIGR4 genome (GenBank accession no. AE007507). The region between nt 541 and 582 corresponds to the pneumococcal *ami* promoter (nt 175 to 216, GenBank accession no. X17337), whereas the sequence spanning nt 583 to 1390 derives from the *Staphylococcus aureus* pC194 plasmid containing the *cat* gene (nt 1103 to 1910, GenBank accession no. V01277). The downstream flanking segment (526 bp) is composed as follows: nt 1391 to 1721 correspond to nt 2268 to 2598 of the A66 *pspC* locus (GenBank accession no. AF252857), whereas nt 1722 to 1916 are complementary to nt 3541 to 3347 on section 190 of the TIGR4 genome.

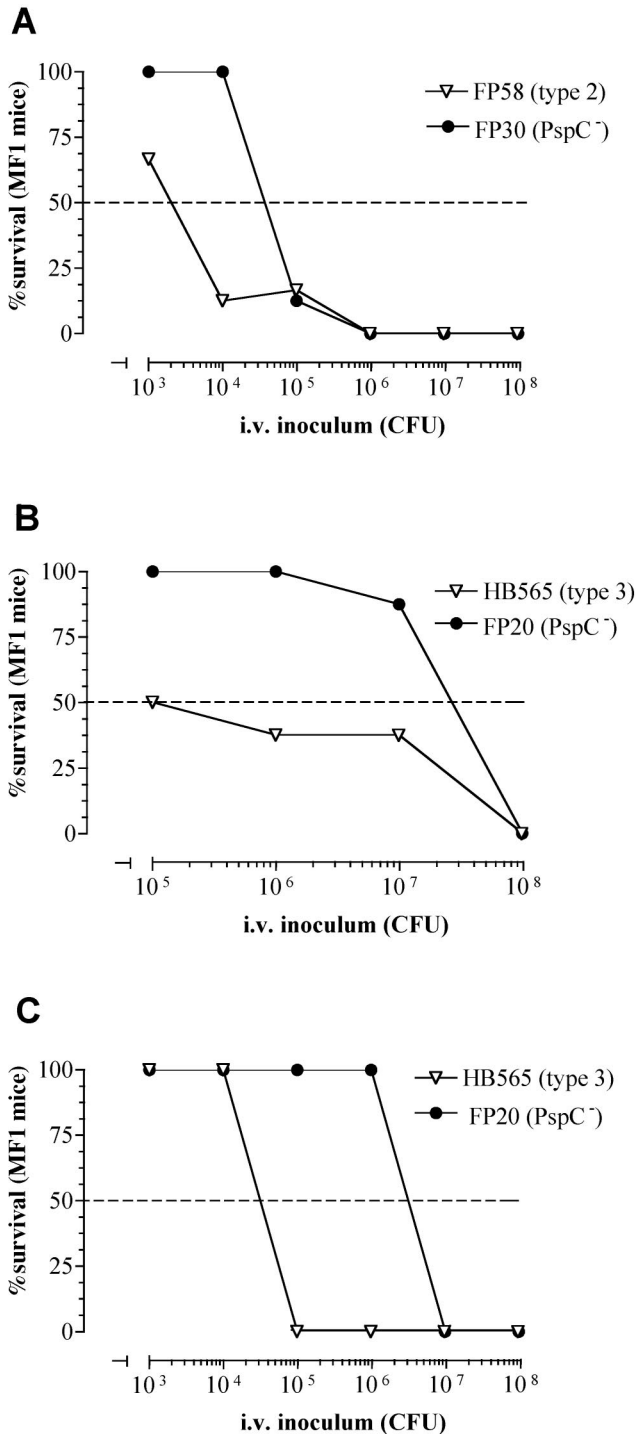

FIG. 2. Experimental murine model of pneumococcal sepsis. (A) Twelve groups of MF1 outbred mice ($n = 6$ to 8) were injected i.v. with different inocula (10^3 to 10^8 CFU) of either the type 2 wild type (FP58, open triangles) or the *pspC* mutant (FP30, solid circles). (B) Eight groups of MF1 mice ($n = 6$) were inoculated i.v. with increasing bacterial doses (10^5 to 10^8 CFU) of the type 3 HB565 (wild type, open triangles) or *pspC* mutant FP20 (solid circles). (C) Twelve groups of CBA/Jico inbred mice ($n = 6$ to 8) were infected i.v. with bacterial inocula (10^5 to 10^8 CFU) of either HB565 (open triangles) or FP20 (solid circles). Survival was recorded for 10 days. The percentage of mice surviving versus the dose of bacteria is shown. The LD_{50} are indicated by the dotted lines.

(15). Since the binding of *S. pneumoniae* to fH was shown to prevent complement-mediated phagocytosis, bacterial survival in the bloodstream should be compromised in PspC-deficient mutants. PspC-deficient mutants cannot bind fH (7, 14), and this inability is probably the key reason for the attenuation of virulence of these mutants in the sepsis model. This effect is more evident in type 3 than in type 2 *S. pneumoniae*, reflecting the higher binding affinity of fH observed for the type 3 strain.

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