

Tissue-Specific Contributions of Pneumococcal Virulence Factors to Pathogenesis

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We assessed the ability of *Streptococcus pneumoniae* mutants deficient in either choline binding protein A (CbpA), pneumolysin (Pln), pyruvate oxidase (SpxB), autolysin (LytA), pneumococcal surface protein A, or neuraminidase A (NanA) to replicate in distinct anatomical sites and translocate from one site to the next. Intranasal, intratracheal, and intravenous models of disease were assessed in 4-week-old BALB/cJ mice by quantitation of bacterial titers in the relevant organs. Mice were also observed by use of real-time bioluminescent imaging (BLI). BLI allowed visualization of the bacteria in sites not tested by sampling. All mutants were created in D39 Xen7, a fully virulent derivative of capsular type 2 strain D39 that contains an optimized *luxABCDE* cassette. NanA, SpxB, and, to a lesser extent, CbpA contributed to prolonged nasopharyngeal colonization, whereas CbpA and NanA contributed to the transition to the lower respiratory tract. Once lung infection was established, Pln, SpxB, and LytA contributed to bacterial replication in the lungs and translocation to the bloodstream. In the bloodstream, only Pln and LytA were required for high-titer replication, whereas CbpA was required for invasion of the cerebrospinal fluid. We conclude that transitions between body sites require virulence determinants distinct from those involved in organ-specific replication.

Carriage of *Streptococcus pneumoniae* (the pneumococcus) in the nasopharynx is common and typically asymptomatic, yet, in infants, the elderly, and persons with underlying medical conditions, the pneumococcus is a major cause of community-acquired pneumonia, bacteremia, and meningitis [1, 2]. Although the pneumococcus has been studied for over a century, an understanding of the mechanisms that underlie the course of disease remains fragmented. In some individuals, the infection is compartmentalized, whereas, in others, it is not. Biopsies of lungs from individuals with pneumococcal pneumonia have demonstrated that the alveoli are filled with pneumococci, yet bacteremia is seen

in only a subset of these individuals. Similarly, of all the individuals with bacteremia, only a small subset develop meningitis [3]. The limitation of the spread of infection occurs despite ample opportunity for bacteria to cross into the bloodstream and, subsequently, cross the blood-brain barrier.

It is known that certain clones (multilocus sequence types) cause invasive disease more frequently than do others. Specific clones of serotypes 6 and 14 have been documented to be more invasive than carriage isolates within the same serotypes [4, 5]. Thus, although capsular polysaccharide is a requisite for pneumococcal virulence, the genotype or expression of other virulence determinants plays a significant role in determining the course of disease. In the present study, we sought to rigorously determine the roles of established pneumococcal virulence determinants, not only at different body sites but also in transitions from one site to the next. Mutants deficient in key virulence factors were tested independently in intranasal (inl), intratracheal (int), and intravenous (iv) challenge models of disease. In addition, we used real-time bioluminescent imaging (BLI) to follow the progression of disease in intact, live mice and, thus, to allow visualization of a mutant's capabilities and limitations at points of transition.

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Table 1. Bacterial strains used in the present study.

Strain	Description	Reference
<i>Streptococcus pneumoniae</i> D39X	Bioluminescent D39 bearing Tn4001 luxABCDE Km ^r	[8]
Virulence factor-deficient strains		
D39X cbpA ⁻	Bioluminescent CbpA-deficient derivative of D39X	[9]
D39X pln ⁻	Bioluminescent Pln-deficient derivative of D39X	[10]
D39X spxB ⁻	Bioluminescent SpxB-deficient derivative of D39X	[11]
D39X lytA ⁻	Bioluminescent LytA-deficient derivative of D39X	W. Haas, personal communication
D39X nanA ⁻	Bioluminescent NanA-deficient derivative of D39X	J. A. McCullers, personal communication
D39X pspA ⁻	Bioluminescent PspA-deficient derivative of D39X	[12]

NOTE. CbpA, choline binding protein A; LytA, autolysin; NanA, neuraminidase A; Pln, pneumolysin; PspA, pneumococcal surface protein A; SpxB, pyruvate oxidase.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* was grown on tryptic soy agar (Difco) plates supplemented with 3% defibrinated sheep blood or in defined semisynthetic casein liquid media [6] supplemented with 0.5% yeast extract. Erythromycin (1 µg/mL) and kanamycin (400 µg/mL) (both from Sigma) were added to the growth media as appropriate. *S. pneumoniae* cultures were inoculated from frozen stock and incubated at 37°C in 5% CO₂. *Escherichia coli* strains were grown in Luria-Bertani medium (Difco) at 37°C in an orbital shaker; erythromycin (1 mg/mL) was added to the *E. coli* cultures to maintain any plasmids.

Construction of mutants. D39 Xen7 (D39X), a stable, bioluminescent isolate of *S. pneumoniae* serotype 2 strain D39 [7], was created by transformation with the gram-positive *lux* transposon cassette Tn4001 luxABCDE Km^r, as described elsewhere [8]. Expression of the *lux* operon results in the production of luciferase and its aldehyde substrate, which leads to the generation of bioluminescence (visible light) from the bacteria, allowing them to be monitored and tracked in the live mice by use of a highly sensitive charge-coupled device camera. Bioluminescent derivatives of virulence-associated mutants were created by transformation of D39X with genomic DNA from preexisting mutants (*cbpA*⁻ [9], *pln*⁻ [10], *spxB*⁻ [11], *lytA*⁻ [W. Haas, personal communication], *nanA*⁻ [J. A. McCullers, personal communication], and *pspA*⁻ [12]) made by insertion-duplication mutagenesis. D39X has been used extensively in 2 studies and was not observed to have any properties distinct from its parent strain, D39 [8, 13]. To minimize the likelihood of changes introduced into D39X after transformation with genomic DNA, we purposely obtained genomic DNA from mutants created in D39 or its derivatives, the exception being the LytA mutant, which was in serotype 4 strain T4 [14]. Mutants were created by insertion-duplication mutagenesis with either pJDC9 or pVA891 [9–12]; therefore, the possibility for polar effects does exist. Transformation through natural competence was performed by use of competence stimulating pep-

tide-1 [15], and transformants were selected on blood agar plates containing erythromycin. Mutants were confirmed by polymerase chain reaction analysis and sequencing of the amplified fragments. Primers used to confirm each bioluminescent mutant were the same as those used to confirm the original mutant [9–12]. Table 1 lists all the bacterial strains used in the present study.

Mice. Female BALB/cJ mice (4–5 weeks old; The Jackson Laboratory) were maintained in biosafety level 2 facilities at the St. Jude Children's Research Hospital Animal Facility. All experimental procedures were done with mice anesthetized with either inhaled isoflurane (Baxter Healthcare) at 2.5% or MKX (1 mL of ketamine [Fort Dodge Laboratories] at 100 mg/mL, 5 mL of xylazine [Miles Laboratories] at 100 mg/mL, and 21 mL of PBS). MKX was administered by intraperitoneal (ip) injection at a dose of 0.05 mL/10 g of body weight.

Monitoring of bioluminescent pneumococcal infections. For all experiments and time points, a minimum of 2 replicates were performed, each with 6–10 mice, for a minimum of 12 mice sampled for each time point and experimental condition reported. The sole exception was cerebrospinal fluid (CSF) samples isolated from mice challenged with D39X *nanA*⁻ at 96 h, from which only 11 samples were collected. For int challenge, exponential cultures (OD₆₂₀ = 0.5) of D39X and its derivatives were centrifuged, and the bacteria were washed with and suspended in sterile PBS (BioWhittaker). Mice were anesthetized with isoflurane and challenged int [16] with 10⁷ cfu in 25 µL of PBS. Mice available at each time point were randomly selected 24, 48, 72, and 96 h after challenge and were imaged by use of the IVIS Imaging System 100 Series (Xenogen). After imaging, mice were killed, and bacterial titers in the nasal lavage, the blood, and the CSF were determined by serial dilution of the body fluids and plating on blood agar plates. The number of colony-forming units present in the lungs was assessed per gram of homogenized tissue. For int challenge [17], mice were anesthetized with isoflurane and infected int with 10⁵ cfu in 100 µL of PBS. Mice were imaged 2 days later, and bacterial

titors in the lungs and blood were determined. Mice infected iv were challenged by tail vein injection of 10^4 cfu in 100 μ L of PBS. Mice were imaged 24 h after infection, and blood and CSF were collected. For all cohorts and time points, a minimum of 2 experiments were performed with 6–10 mice/group and time point for each experiment. In all instances, the infectious dose administered was confirmed by serial dilution and plating of the bacterial suspension on blood agar plates.

Statistical analysis. Statistical analysis of bacterial titers in the nasal lavage, blood, lungs, and CSF was performed by use of a Mann-Whitney rank sum test.

RESULTS

Infection with the wild type (wt). Challenge of mice with D39X resulted in fulminate pneumonia, bacteremia, and meningitis regardless of the route of inoculation (figures 1–3). Bioluminescent images of mice infected with the *wt* showed a high-intensity signal emanating from the entire mouse by day 2. From previous experience, such lack of a discrete focal point

of luminescence indicates high-grade bacteremia [13]. Quantitative analysis of bacterial titers in mice infected inl revealed that the pneumococcus was present in all the sites tested and had spread from the nasopharynx to the central nervous system (CNS) within 2 days after challenge. Two days after inl challenge, mice infected with D39X had bacterial titers exceeding 10^7 cfu/g or mL in the lungs and the blood, respectively, and 10^3 cfu/mL in the CSF (table 1). Mice infected with D39X generally died of sepsis 2–3 days after inl or int challenge and 2 days after iv challenge.

Colonization of the nasopharynx. After inl challenge, 1 mutant, D39X *pspA*[−], showed an increased ability to colonize the nasopharynx, whereas mutants deficient in choline binding protein A (*CbpA*), pneumolysin (*Pln*), pyruvate oxidase (*SpxB*), autolysin (*LytA*), or neuraminidase A (*NanA*) showed decreased titers, compared with the *wt*, at day 1 (table 2). At day 2, only D39X *spxB*[−] and D39X *nanA*[−] had decreased titers in the nasal lavage (figure 1). The number of D39X *spxB*[−] in the nasopharynx decreased from almost 10^5 cfu/mL of nasal

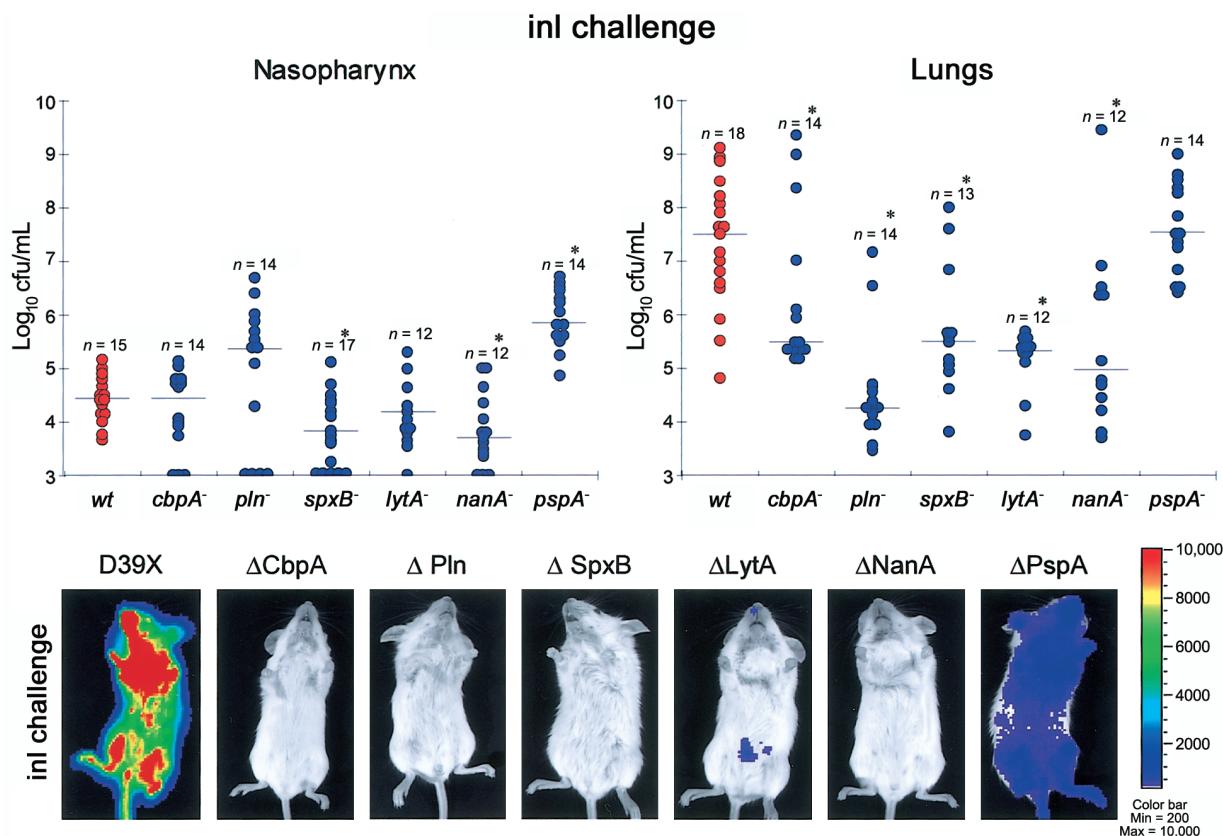


Figure 1. Bacterial titers in the nasal lavage and the lungs of mice infected intranasally (inl). Mice were infected inl with 10^7 cfu of D39X or its derivatives. Two days after challenge, mice were imaged by use of the IVIS Imaging System 100 Series (Xenogen), and, at the peak of disease, the number of bacteria in the nasopharynx and the lungs was determined by culture. Bacterial titers for each mouse from which samples were collected are plotted. Horizontal bars indicate the median bacterial titers. Asterisks indicate statistically significant differences, as determined by a Mann-Whitney rank sum test. *CbpA*, choline binding protein A; *LytA*, autolysin; *NanA*, neuraminidase A; *Pln*, pneumolysin; *PspA*, pneumococcal surface protein A; *SpxB*, pyruvate oxidase.

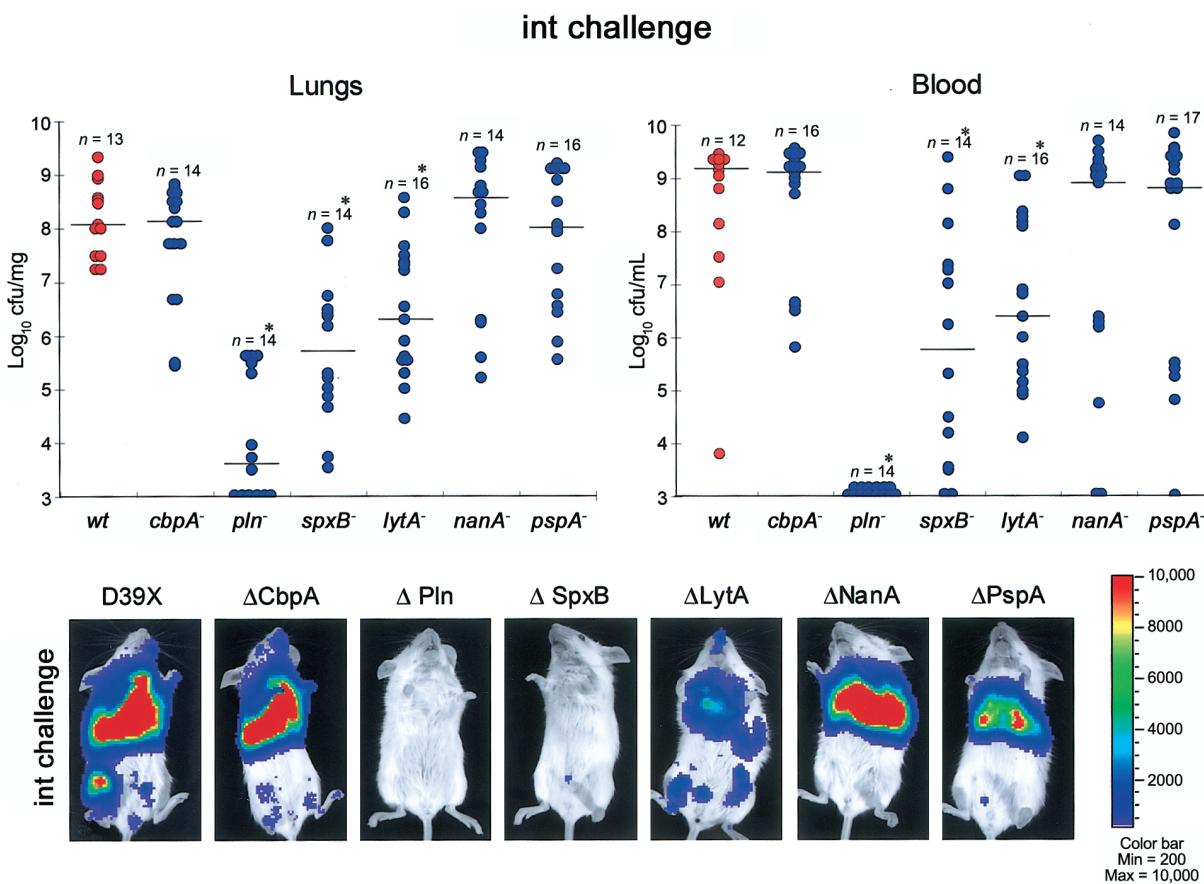


Figure 2. Bacterial titers in the lungs and the blood of mice infected intratracheally (int). Mice were infected int with 10^5 cfu of D39X or its derivatives. Two days after challenge, mice were imaged by use of the IVIS Imaging System 100 Series (Xenogen), and, at the peak of disease, the number of bacteria in the lungs and the blood was determined by culture. Data are reported as in figure 1. Asterisks indicate statistically significant differences, as determined by a Mann-Whitney rank sum test. CbpA, choline binding protein A; LytA, autolysin; NanA, neuraminidase A; Pln, pneumolysin; PspA, pneumococcal surface protein A; SpxB, pyruvate oxidase.

lavage at day 1 to almost undetectable levels at day 3 (table 2). Mice infected with D39X *nanA*⁻ also showed decreased nasal lavage titers at days 1 and 2 yet showed an increase at days 3 and 4. This increase corresponded to increased titers observed in the lungs during this time. Mice infected with D39X *cbpA*⁻ also had decreased titers, from 10^5 cfu/mL at day 1 to 10^3 cfu/mL at day 4 (table 2). Overall, these results suggest that CbpA, Pln, SpxB, LytA, and NanA all contribute to the initial ability to colonize a naive animal. Furthermore, SpxB and CbpA are required for sustained colonization of the nasopharynx.

Transition to pneumonia. int challenge of mice, with mutants deficient in CbpA, Pln, or LytA, resulted in less-fulminate pneumonia than that observed in mice infected with the *wt* (figure 1 and table 2). These mutants demonstrated a 100–1000-fold reduction in the number of bacteria present in the lungs 2 days after int challenge, despite having bacterial titers in the nasopharynx comparable with the *wt* at day 2 (figure 1 and table 2). These findings suggest that, although CbpA, Pln, and LytA do not contribute to colonization of the nasopharynx

at day 2, they are required for efficient transition of infection to the lower respiratory tract. Mutants deficient in SpxB or NanA were also reduced in the lungs at day 2. However, by use of an int model of infection, it was unclear whether the reduced titers observed in the lungs after challenge with these mutants were due to reduced titers in the nasopharynx (and subsequently fewer bacteria being aspirated into the lungs) or whether they reflected an inability of mutants deficient in SpxB or NanA to colonize and replicate in the lungs.

Development of pneumonia. To clarify whether the inability of the mutants to progress from the nasopharynx to the lungs stemmed from an inability to replicate in the lungs, we infected mice int, bypassing the nasopharynx. int challenge with the D39X mutants demonstrated that mutants deficient in Pln, SpxB, or LytA had a reduced capacity to replicate in the lungs (figure 2), whereas mutants deficient in CbpA or NanA replicated at levels comparable with those of the *wt*. Pln-deficient mutants exhibited a 5-log reduction in the number of pneumococci in the lungs, whereas mice infected with D39X *spxB*⁻

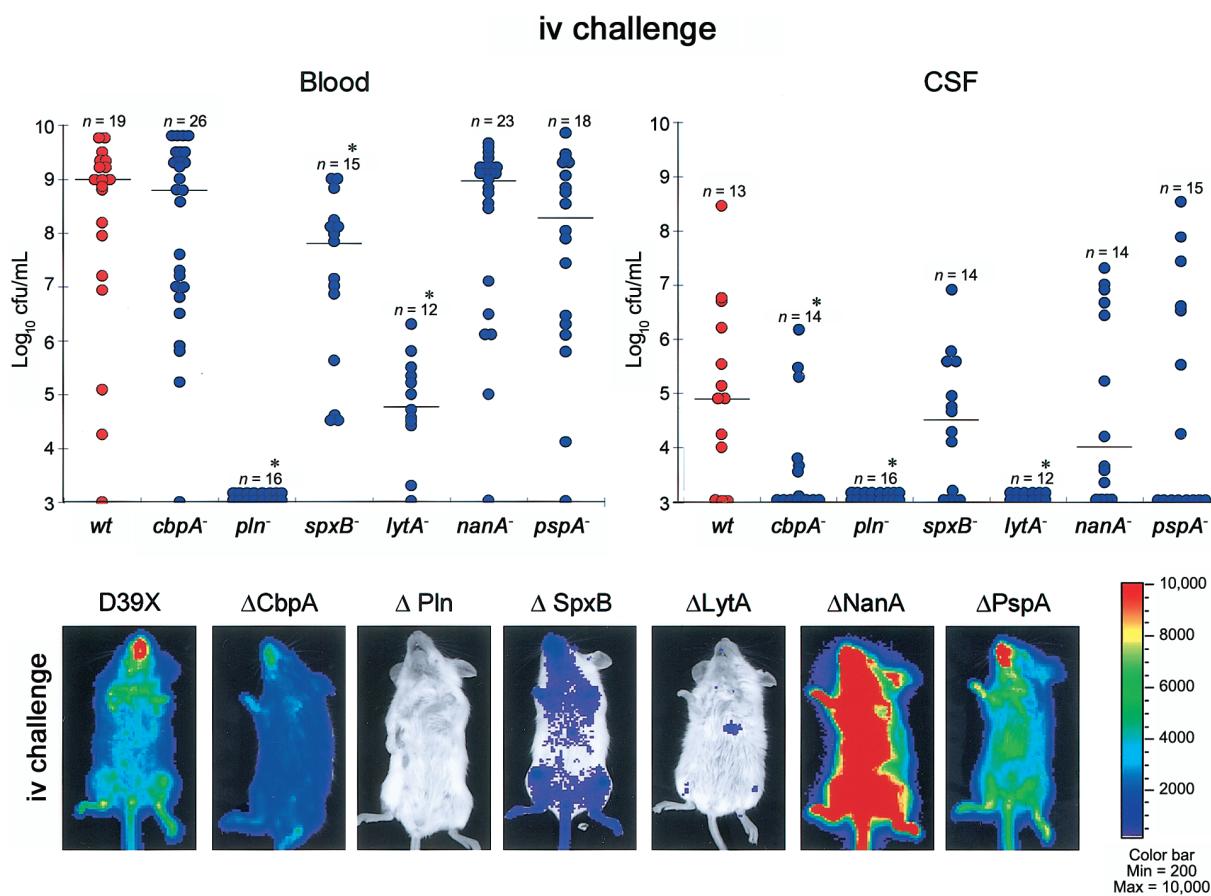


Figure 3. Bacterial titers in the blood and the cerebrospinal fluid (CSF) of mice infected intravenously (iv). Mice were infected iv with 10^4 cfu of D39X or its derivatives. One day after challenge, mice were imaged by use of the IVIS Imaging System 100 Series (Xenogen), and the blood and the CSF were cultured as described in figure 1. Data are reported as in figure 1. Asterisks indicate statistically significant differences, as determined by a Mann-Whitney rank sum test. CbpA, choline binding protein A; LytA, autolysin; NanA, neuraminidase A; Pln, pneumolysin; PspA, pneumococcal surface protein A; SpxB, pyruvate oxidase.

and D39X *lytA*⁻ exhibited a 1000-fold and a 10-fold reduction, respectively.

The inability of these mutants to cause severe pneumonia despite int challenge indicates that Pln, SpxB, and the major LytA are required for survival and replication in the lungs. Similarly, the observation that D39X *cbpA*⁻ and D39X *nanA*⁻ had *wt*-like titers in the lungs after int challenge, but not after int challenge, suggests that CbpA and NanA are required for progressing from the nasopharynx to the lungs but are not required after lower respiratory tract infection is established. That said, given that CbpA is an adhesin [18] and neuraminidase is involved in exposing receptors for adherence [19], these findings are consistent with the proposed roles of these proteins.

Bloodstream invasion. Mice infected int with mutants deficient in Pln, SpxB, or LytA had reduced titers in the bloodstream (figure 2). Because int challenge alone does not discern between the ability of a mutant to translocate to the blood from lungs or replicate once in the bloodstream, we challenged mice iv with 10^4 cfu with each mutant. By use of iv challenge with

the mutants, we found that Pln- or LytA-deficient mutants had a drastic decrease in the number of pneumococci in the blood, compared with the *wt*, whereas D39X *spxB*⁻ did not (figure 3). Mutants deficient in Pln were undetectable in the blood, indicating that Pln is required for survival in blood. Mice challenged with D39X *lytA*⁻ had a 5-log reduction in the blood, compared with those infected with the *wt*, again indicating that LytA is required for survival in the blood. In contrast, although D39X *spxB*⁻ also demonstrated a reduction in blood titers that reached statistical significance, the median bacterial titer exceeded 10^7 cfu/mL, a value associated with lethality. We conclude that the contribution of SpxB to survival in the blood is physiologically minimal but that SpxB is required for progression of the bacteria from the lungs to the blood (figure 2).

Development of meningitis. To cause meningitis, pneumococci in the blood must cross the cerebral endothelium. To model this capability, all strains were injected iv, and bacterial titers in the CSF were determined 24 h later. Quantitative analysis of bacterial titers in the CSF determined that only mutants

Table 2. Bacterial burden in mice after intranasal challenge with *Streptococcus pneumoniae*.

Strain	Median log number of cfu/g or mL															
	Nasopharynx				Lungs				Blood				CSF			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
D39X	5.60	4.40			5.48	7.55			5.37	7.72			—	3.80		
D39X <i>cbpA</i> ⁻	5.19	4.41	4.48	3.87	4.00	5.57	5.09	1.81	1.50	4.30	5.04	4.24	—	—	—	—
D39X <i>pln</i> ⁻	4.57	5.33	5.77	5.33	4.22	4.26	4.58	3.97	—	—	—	—	—	—	—	—
D39X <i>SpxB</i> ⁻	4.82	3.78	3.00	3.00	4.20	5.51	5.59	6.54	3.60	5.82	6.40	7.05	—	3.00	1.74	
D39X <i>lytA</i> ⁻	4.88	4.13	4.89	4.23	5.91	5.32	6.74	6.83	5.50	5.45	8.00	6.86	—	4.35	3.96	
D39X <i>nanA</i> ⁻	4.60	3.70	3.90	4.41	4.90	4.92	8.67	6.71	3.78	6.08	9.00	6.04	—	—	5.65	
D39X <i>pspA</i> ⁻	5.34	5.91	5.65		5.95	7.52	7.70		5.85	8.00	8.20		—	4.26	—	

NOTE. Median values were calculated from a minimum of 12 mice sampled at each time point for each experimental condition, the sole exception being cerebrospinal fluid (CSF) samples from mice infected with D39X *nanA*⁻ at 96 h, from which only 11 samples were collected. Bold type indicates a statistically significant ($P < .05$) difference in bacterial titers versus control (D39X), determined by use of a Mann-Whitney rank sum test. Dashes indicate that median titers of bacteria in the tissue sample are below detectable limits (1.0×10^3 cfu/g or mL). Empty boxes indicate that insufficient numbers of mice remained alive for accurate sampling.

deficient in CbpA, Pln, or LytA had diminished capacity to invade the CNS (figure 3). For D39X *pln*⁻ and D39X *lytA*⁻, this failure was most likely due to the consistently low titers present in the blood. Because Pln-deficient mutants were not detectable in the blood, it seems unlikely that they would move to the CSF. Mice challenged i.n. with D39X *lytA*⁻ had bacteria in the CSF 3 days after challenge, indicating that LytA-deficient mutants were capable of crossing into the CSF but required more time than did the *wt*.

Mutants deficient in CbpA had bacterial titers in the blood comparable with those in mice infected with the *wt* (10^8 cfu/mL of blood). Nonetheless, median bacterial titers in the CSF of mice infected i.v. with D39X *cbpA*⁻ were below detectable limits (figure 3). These findings suggest that CbpA is required for the translocation of bacteria from the bloodstream to the CSF but not for survival of the pneumococcus in the bloodstream.

DISCUSSION

Pneumococcal invasive disease is marked by a series of steps beginning with colonization of the nasopharynx and ending in meningitis. Since the discovery of *S. pneumoniae*, animal models have been used to assess the contribution of its virulence factors to disease. However, these studies have focused on organ-specific replication, and the majority of virulence factors have not been assessed in a manner that delineates their contribution to the transitions required to advance the course of disease. Furthermore, bacterial number has been typically ascertained at arbitrary time points, with no opportunity to fine-tune the timing of analysis to precise stages of disease. As such, although it is known that several virulence factors are required for invasive disease, our understanding of the exact contribution of these determinants to pathogenesis remains crude. In the present study, we have examined the contribution of the major pneumococcal virulence factors to bacterial success in distinct anatomical sites and have examined the ability of mutants de-

ficient in these virulence factors to spread from one anatomical compartment to the next in mice. BLI enabled near-continuous observation of the mice, whereas systematic titration of bacterial numbers in the nasopharynx, lungs, blood, and CSF generated confidence in sampling timing as reflecting discrete steps in disease. Although we confirmed some previously described properties, in several instances, we identified novel roles for known virulence factors. Figure 4 outlines the natural course of pneumococcal disease and summarizes the contribution of each virulence determinant as determined by the present study.

CbpA, which binds serum determinants, is an adhesin with 2 known human cell-surface receptors. It binds to the polymeric immunoglobulin receptor (pIgR) and mediates invasion across human nasopharyngeal epithelial cells [9] and participates with the platelet activating factor receptor (PAFr) in translocation across the cerebral endothelium [20]. Studies have shown that mutants deficient in CbpA have a 100-fold reduction in their capacity to colonize the nasopharynx of rat pups [18] and that translocation of the pneumococcus across endothelial cells *in vitro* requires CbpA [20]. In the present study, we have demonstrated that CbpA is required to sustain prolonged bacterial titers in the nasopharynx and that CbpA contributes to the ability of the bacteria to spread from the nasopharynx to the lower respiratory tract. CbpA was not required for survival of the pneumococcus in the lungs or the blood, nor was it required for translocation of the bacteria from the lungs to the blood. However, CbpA was required for translocation from the blood to the CSF. The present study is the first report confirming CbpA-dependent translocation across the blood-brain barrier in an infected animal.

Pln is a pore-forming toxin that is present in the cytosol and is released during exponential growth [21] or cell lysis [22]. Previous studies have shown that Pln-deficient pneumococci have a reduced mortality in mice and a reduced capacity to replicate in the blood [10, 23, 24]. Our results concur with the

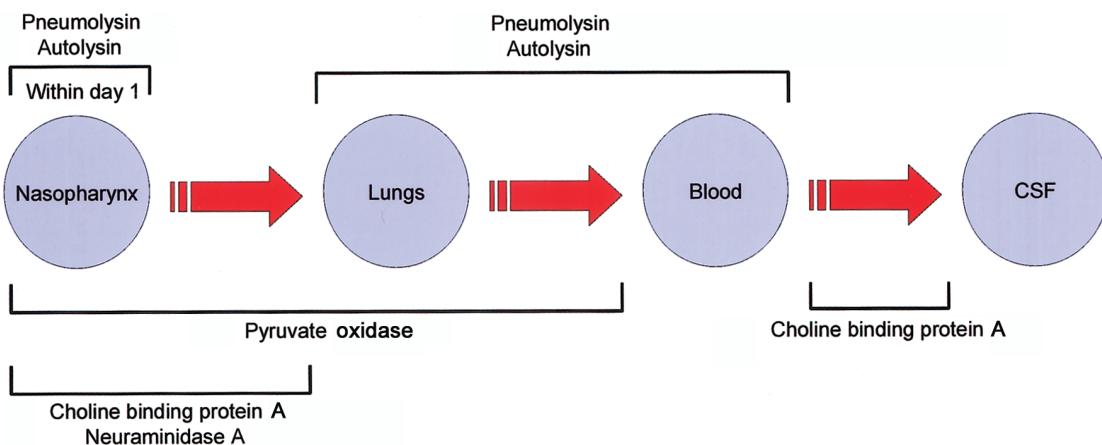


Figure 4. Schematic of the contribution of known virulence determinants to steps in pneumococcal invasive disease. CSF, cerebrospinal fluid.

results of these studies, clearly demonstrating that Pln is required for development of severe pneumonia and bacterial survival in the blood. Of all the mutants examined in the present study, Pln-deficient mutants were by far the most attenuated, indicating that, with the exception of capsular polysaccharide, Pln is the most broadly potent virulence factor. Several properties have been attributed to Pln that may explain its dramatic impact *in vivo*. Pln is cytotoxic [25], activates the classic complement pathway [17], inhibits the bactericidal activity of monocytes [26], and inhibits killing of the bacteria by neutrophils in a complement-dependent neutrophil killing assay [17]. Thus, the contribution of Pln to pathogenesis is multifactorial and correlates with prominent roles in bacteremia and acute lung injury.

Spellerberg et al. have shown that hydrogen peroxide produced during metabolism by SpxB is necessary for efficient *in vivo* colonization [11]. More recently, Percione et al. have demonstrated that hydrogen peroxide produced by the pneumococcus has inhibitory effects on other inhabitants of the upper respiratory tract [27]. Our results support both of these arguments, as the number of D39X *spxB*⁻ observed in the nasopharynx was reduced not only during early colonization (at day 1) but also declined steadily during the study.

Spellerberg et al. have also shown that SpxB contributes to bacterial titers in the lungs of rabbits and the blood of neonatal rats after *intratracheal* and *intraperitoneal* challenge [11]. The findings of the present study support the findings for the lungs, although we have found that SpxB plays a minimal role in the development of sepsis. Potentially high levels of catalase present in the blood may neutralize the effects of hydrogen peroxide.

LytA is a *N*-acetylmuramyl-L-alanine amidase [28] and is likely the major mechanism by which cell-wall components, such as peptidoglycan and teichoic acid, are released from the cell during infection [22]. It has been well established that LytA contributes to pneumococcal virulence, but the mechanism remains unclear. Peptidoglycan and teichoic acid are highly in-

flammatory—that is, both molecules activate the alternate complement pathway [29] and bind to lipopolysaccharide binding protein [30], CD14 [31], and Toll-like receptor 2 [32]. Pln is released during LytA-mediated lysis [22], although it is also secreted during the exponential phase [21]. In the present study and previous studies, mutants deficient in LytA demonstrated reduced titers in the lungs and the blood of infected mice. Thus, although the effect of LytA is apparently multifaceted, it appears that cell lysis mediated by LytA most likely contributes to the ability of the surviving bacteria to cause disease.

Pneumococci produce at least 2 distinct neuraminidases, NanA and NanB [33]. NanA is the major neuraminidase and removes terminal sialic acid from glycoconjugates found on the surfaces of host cells [34]. This is believed to expose cryptic receptors for the pneumococcus. Recently, studies have demonstrated that NanA contributes to the development of otitis media in a chinchilla model [35] and enhances bacterial adherence to chinchilla tracheas *ex vivo* [36]. The results of the present study are compatible with this hypothesis, demonstrating that NanA contributes to colonization of the nasopharynx and to the spread of the pneumococcus from the nasopharynx to the lungs. Unexpectedly, mutants deficient in NanA showed no defect in making transitions to other body sites. Because these steps also require attachment of the pneumococcus to host cells, these results suggest that alternative mechanisms of uptake, such as those involving PAFr, are responsible for translocation of bacteria from the lungs to the blood and from the blood to the CSF.

A surprising finding was the lack of attenuation of virulence of the D39X *pspA*⁻ mutant. Studies of serotype 4 and serotype 3 strains have demonstrated that pneumococcal surface protein A (PspA) is required for *in vivo* growth [37], affects the amount of complement deposited on the surface of the pneumococcus [38], and is a protective antigen [39]. McDaniel et al. have demonstrated that mutants deficient in PspA are cleared from the

blood within the first 2 h [40], yet our results suggest the contrary—that is, that PspA does not attenuate D39X. In support of our findings, findings by Berry et al. indicate that iv infection with the PspA deletion in D39, the serotype 2 parent strain of D39X, results in mortality identical to that due to the *wt* [23]. Likewise, Abejta et al. have shown that capsule type affects the accessibility of surface-bound complement [41]. Increased colonization by PspA⁻ mutants has also been observed elsewhere [42]. Thus, the contribution of PspA to virulence appears to depend on the serotype of the strain and the physical properties of the capsular polysaccharide.

Because the pneumococcus has no environmental niche and development of invasive disease does not contribute to spread of the bacteria to other hosts, one would speculate that the majority of virulence factors would contribute positively to nasopharyngeal colonization. In our mouse model, this was the case—all mutants tested, with the exception of PspA, were observed to have a reduced capacity to colonize the nasopharynx at day 1—yet this deficiency was observed to be short-term, as only SpxB and CbpA affected prolonged nasopharyngeal colonization (figure 1). However, one must consider that our challenge dose was designed to examine the transition of *S. pneumoniae* from the nasopharynx to the lower respiratory tract and was not aimed only at nasopharyngeal colonization. Because challenge dose plays a critical role in determining outcome and, thus, which genes are identified as required for virulence, a second study with a smaller challenge dose is warranted to more fully determine the virulence determinants required for nasopharyngeal colonization. Other virulence factors did not come into play until the bacteria entered the lower respiratory tract (figure 1). CbpA and NanA were apparently required for the initial colonization of the lower respiratory tract, whereas SpxB, Pln, and LytA were required for replication in the lungs. Unfortunately, we were not able to examine the contribution of CbpA-mediated pIgR-mediated invasion, because this occurs only in humans [9].

We conclude that, in a mouse model of disease, the ability of *S. pneumoniae* to cause bacteremia depends on the bacteria's ability to establish a lower respiratory tract infection and mediate cell damage in the lungs. Pln, cell-wall components (released by LytA), and hydrogen peroxide are the primary mediators of cell damage in the lungs and contribute to bacterial titers in the lungs and the bloodstream. It is interesting to speculate that the inflammation mediated by Pln, LytA, and hydrogen peroxide (in the lungs) is not an incidental consequence of pneumococcal infection but is instead a requirement for disease progression and pneumococcal replication during invasive disease. Edema and cell damage mediated by these virulence factors may be primary mechanisms to facilitate pneumococcal entry into the bloodstream. Likewise, complement depletion mediated by Pln and cell-wall products (LytA) may

be mechanisms by which the bacteria are able to replicate to high titers in the lungs and the blood, a conclusion consistent with the need for Pln in the development of meningitis. In contrast, actual entry into the CSF requires the specific ligand-receptor interaction between CbpA and its receptor and between choline on the cell wall and the PAFr.

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