MAJOR ARTICLE

Emergence of Hypervirulent Mutants Resistant to Early Clearance During Systemic Serotype 1 Pneumococcal Infection in Mice and Humans

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(See the editorial commentary by Hakansson on pages 1–3.)

Background. *Streptococcus pneumoniae* serotype 1 has a high likelihood of causing invasive disease. Serotype 1 isolates belonging to CC228 are associated with low mortality, while CC217 isolates exhibit high mortality in patients.

Methods. Clinical pneumococcal isolates and mutants were evaluated in wild-type C57BL/6 mice, macrophage-depleted mice, neutrophil-depleted mice, and SIGN-R1 knockout mice. In vitro models included binding and phagocytosis by THP-1 cells, capsule measurements, hydrogen peroxide production, and viability assays.

Results. During early systemic infection in mice with serotype 1, large-colony variants appeared in blood. Similar large colonies were found in blood specimens from patients with invasive disease. Large morphotypes contained higher numbers of viable bacteria, grew faster, produced no or little hydrogen peroxide, and contained mutations in the *spxB* gene. *spxB* mutants were considerably more virulent in wild-type mice, less susceptible to early host clearance than wild-type strains after intravenous infection, but impaired in colonization. *spxB* mutants were less efficiently phagocytosed by macrophages than wild-type bacteria, which, in contrast to *spxB* mutants, caused more-severe disease when macrophages or SIGN-R1 were depleted.

Conclusions. Hypervirulent *spxB* mutants are selected in both mice and patients and are resistant to early macrophage-mediated clearance.

Keywords. Streptococcus pneumoniae; spxB; hydrogen peroxide; pneumococcal virulence; macrophages; SIGN-R1; phagocytosis; early clearance.

Streptococcus pneumoniae is a commensal pathogen adapted to its main ecological niche, the nasopharynx of preschool-aged children. Even though cases of healthy colonization vastly outnumber cases of invasive disease, *S. pneumoniae* is one of the most common causes of community-acquired pneumonia, sepsis, and meningitis worldwide. It is not known whether colonizing pneumococci appear in the bloodstream genetically unaltered or whether pathoadaptive mutations are selected for in the host that may enhance a given strain's capability of causing invasive disease.

Pneumococci comprise a large number of clonal types that may express one of at least 93 different capsular serotypes that differ in their odds ratio of causing invasive disease. Serotype 1 pneumococci have been found to exhibit a high invasive disease potential [1, 2] and cause infections among previously healthy individuals [3]. Serotype 1 isolates belong to the top 5 serotypes associated with invasive pneumococcal disease (IPD) in Europe, Asia, Africa, and Oceania [4]. Despite a high attack rate, disease severity has been reported by us and others to be relatively low, with low or no

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case-fatality rates [1, 3, 5]. However, epidemic outbreaks of pneumococcal meningitis caused by serotype 1 in Burkina Faso and Ghana have been reported to be associated with a high mortality (44%–74%). European serotype 1 isolates belong predominantly to CC228 (including ST227/228/306), whereas the Burkina Faso and Ghana isolates belonged to CC217 [6–8].

Pneumococci are unusual in that they produce high, potentially suicidal levels of hydrogen peroxide by converting pyruvate to acetyl phosphate and hydrogen peroxide via a pyruvate oxidase encoded by the *spxB* gene [9]. Hydrogen peroxide production causes bacterial death in the absence of exogenously added catalase. Therefore, *spxB* mutants tend to grow faster and form considerably larger colonies than wild-type bacteria on blood agar plates. It has been demonstrated that hydrogen peroxide–producing wild-type strains provide a competitive advantage over *spx*-mutant derivatives in animal colonization models [9–14]. Pneumococcal hydrogen peroxide production has also been shown to be important for outcompeting *Staphylococcus aureus* from the nose in mixed infections [15, 16]. Thus, during normal colonization the advantage of producing hydrogen peroxide outweighs the growth defect associated with its toxicity.

In this article, we demonstrate that *spxB* mutants emerge spontaneously as large colonies after culturing blood specimens from infected patients and from mice with invasive disease caused by serotype 1 pneumococci of CC228 and CC217. These hydrogen peroxide–deficient mutants are considerably more virulent in mice because of reduced early clearance but appear to be less successful in colonization. Also, we show that early resistance to eradication of *spxB* mutant bacteria in vivo is due to increased resistance to macrophage-mediated clearance.

MATERIAL AND METHODS

Bacterial Strains and Growth Conditions

Strains used in the study are described in Table 1. All strains were cultured at 37°C in Todd-Hewitt broth with 0.5% yeast extract (THY) with catalase to a final concentration of 2500 U/mL, in semisynthetic C + Y medium [17] or on blood agar plates at 37°C in 5% CO_2. Unless otherwise stated, strains were grown to mid-log phase (OD_{620}, 0.3–0.4) in static liquid culture. All deletion mutants were constructed using polymerase chain reaction (PCR) ligation mutagenesis (Supplementary Materials). Characterization was done with multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE; Supplementary Materials).

Colony Viability

Individual bacterial colonies from blood agar plates were resuspended in 1 mL of phosphate-buffered saline (PBS). Serial dilutions from each colony suspension were prepared in PBS, and the number of viable bacteria was determined.

Table 1. Characteristics of Clinical Isolates and In Vivo and In Vitro Mutants

Strain	Description	CFUs Per Colony, $\times 10^4$, Mean ± SD[a]	H_2O_2 Production per 5×10^7 Bacteria, mM, Mean ± SD[b]	Source
BHN32^{ST228}	Clinical isolate of serotype 1 with ST228 (MLST)	28 ± 7	0.41 ± 0.14	[11]
BHN123^{ST228}	Spontaneous mutant derived from BHN32 during in vivo experiment in mice; amino acid substitution in *spxB*, $Gly_{180}Asp$ *spxB* allele 1	534 ± 96	<0.025	This study
BHN685^{ST228}	BHN123 complemented with *spxB*	43 ± 12	0.36 ± 0.16	This study
BHN684^{ST228}	BHN32 knockout mutant in *spxB*	535 ± 83	<0.025	This study
BHN31^{ST306}	Clinical isolate of serotype 1 with ST306	41 ± 10	0.19 ± 0.05	[11]
BHN122^{ST306}	Spontaneous mutant derived from BHN31 during in vivo experiment in mice; amino acid substitutions in *spxB*, $Gly_{167}Cys$, $Asn_{445}His$, $Phe_{486}Val$ *spxB* allele 2	1100 ± 250	0.08 ± 0.03	This study
BHN124^{ST306}	Clinical isolate of serotype 1 with ST306	52 ± 16	0.21 ± 0.10	This study
BHN125^{ST306}	Spontaneous mutant derived from BHN124 in human; mutation, G→T, 79 bp 5′ of *spxB* start codon; transcription start site [24] *spxB* allele 3	1730 ± 530	<0.025	This study
BHN682^{ST217}	Clinical isolate of serotype 1 with ST217	14 ± 4	0.26 ± 0.04	This study
BHN683^{ST217}	Spontaneous mutant derived from BHN682 in human; amino acid substitution in *spxB*, $Asn_{119}Ile$ *spxB* allele4	206 ± 45	<0.025	This study
BHN734^{ST228}	BHN32*cps* knockout mutant	. . .	. . .	This study
BHN735^{ST228}	BHN123*cps* knockout mutant	. . .	. . .	This study
R6	Nonencapsulated laboratory strain	. . .	. . .	[12]
TIGR4 (T4)	Serotype 4	. . .	. . .	[13]
T4Δ*spxB*	TIGR4*spxB* knockout mutant	. . .	. . .	This study

Abbreviations: CFU, colony-forming unit; MLST, multilocus sequence type.

[a] Mean of 10 colonies.

[b] Mean of 4 assays.

Growth Rate Measurements

A description of the method by which the bacterial growth rate was measured is included in the Supplementary Materials.

Capsule Measurements

Capsular amount was measured using an indirect enzyme-linked immunosorbent assay (Supplementary Materials).

Hydrogen Peroxide Tolerance and Production Assays

Hydrogen peroxide tolerance and production was determined as described previously [18–20] (Supplementary Materials).

PCR and Sequencing

Genomic DNA was isolated using a tissue kit (Qiagen). PCR products were purified from solution by means of the GFX PCR and gel band purification kit (GE Healthcare) and were used in a sequence reaction involving the BigDye Terminator v.3.1 cycle sequencing reaction kit (Applied Biosystems). Sequences were read with an ABI 3130 ×l Genetic Analyzer

Binding and Phagocytosis Assay Using THP-1 Monocytic Cells

In brief, THP-1 cells were incubated with pneumococci for 1 hour at 37°C (ratio of bacteria to macrophages, approximately 20:1), washed with PBS, and lysed with saponin (Supplementary Materials). Total bound bacteria were calculated by subtracting colony-forming units (CFUs) internalized after gentamicin treatment from the total CFUs (bound and internalized). Binding and phagocytosis were expressed as a percentage of the number of bacterial CFUs used in the inoculum.

Animal Model

C57BL/6 wild-type age-matched male mice (5–10 weeks old) or C57BL/6 SIGN-R1$^{-/-}$ mutant mice were infected with 1×10^7 bacteria, intraperitoneally, intravenously in the tail vein, or intranasally. Mice inoculated intranasally were anesthetized by inhalation of isoflurane (Forene, Abbot). Clodronate liposomes (dichloromethylene diphosphonate) were purchased from ClodronateLiposomes.com and were prepared as described previously [21]. Mice were administered 200 μL (1 mg) of liposome-encapsulated clodronate or PBS intravenously into the tail vein 1 day before the pneumococcal challenge. A description of the method by which neutrophils were depleted is included in the Supplementary Materials.

Ethics Approval

The experiments were approved by the local ethical committee (Stockholms Norra Djurförsöksetiska Nämnd).

Statistical Analyses

Differences in survival were analyzed by the Mantel-Cox log-rank test. CFU counts in vivo were analyzed using the Mann–Whitney *U* test, and results with a *P* value of <.05 were considered statistically significant. CFU counts in vitro were analyzed using a 2-tailed *t* test. All analyses were performed in GraphPad Prism, version 5.04.

RESULTS

In Vivo Selection of Virulent Large Colony Variants After Systemic Infection With Serotype 1 Isolates in Mice and Humans

Two clinical isolates of serotype 1 belonging to CC228 (isolate BHN32^{ST228} of ST228 and BHN31^{ST306} of ST306) were used to infect C57BL/6 mice intraperitoneally. When recovering bacteria from blood specimens, both serotype 1 isolates yielded a mixture of large- and small-colony variants, the latter morphologically identical to the wild-type used as the inoculum. The normal small colonies showed a central indentation, whereas the large colonies were domed shaped (Figure 1*A*). The specific morphotypes were stable upon restreaking on blood agar plates and after frozen storage. The large morphotypes appeared between 24 and 48 hours after intraperitoneal challenge and occurred in about 70% of BHN31^{ST306}-infected mice at a ratio of 1 large colony to 10 wild-type colonies, whereas only about 20% of the BHN32^{ST228}-infected mice gave rise to the large phenotype and in a ratio of only 1 large colony to 100 wild-type colonies. An in vivo–selected large-colony variant of BHN31^{ST306} was tested using MLST and PFGE. It was found to be the same as the inoculum strain and was given the strain designation BHN122^{ST306}. The corresponding large-colony variant of BHN32^{ST228} was designated BHN123^{ST228}.

To investigate whether large-morphotype mutants are selected for during human infections we examined pneumococcal isolates from human blood specimens. Isolates from 2 patients gave rise to small and large morphotypes. PFGE and MLST revealed that the small- and large-colony variants were the same in each patient and belonged to ST306 and ST217, respectively. One small colony and 1 large colony from patient 1 were restreaked and designated BHN124^{ST306} and BHN125^{ST306}, respectively, and 1 each from patient 2 were streaked and called BHN682^{ST217} and BHN683^{ST217} (Table 1).

In Vivo–Selected Large Morphotype Mutants From Mice and Humans All Contain 1 or More Mutations in the Pyruvate Oxidase Gene *spxB*

Each of the in vivo–selected large morphotypes had completely lost or showed a lowered expression of hydrogen peroxide (Table 1). The large colony size correlated to a larger number of bacteria per colony as compared to wild-type colonies, and the growth was considerably better. Growth in wild-type strain was also enhanced by addition of catalase (Supplementary Figure 1*A* and 1*B*). Furthermore, the large-colony variants were more sensitive to exogenous hydrogen peroxide, compared with the parental strains (Supplementary Figure 1*C*). The

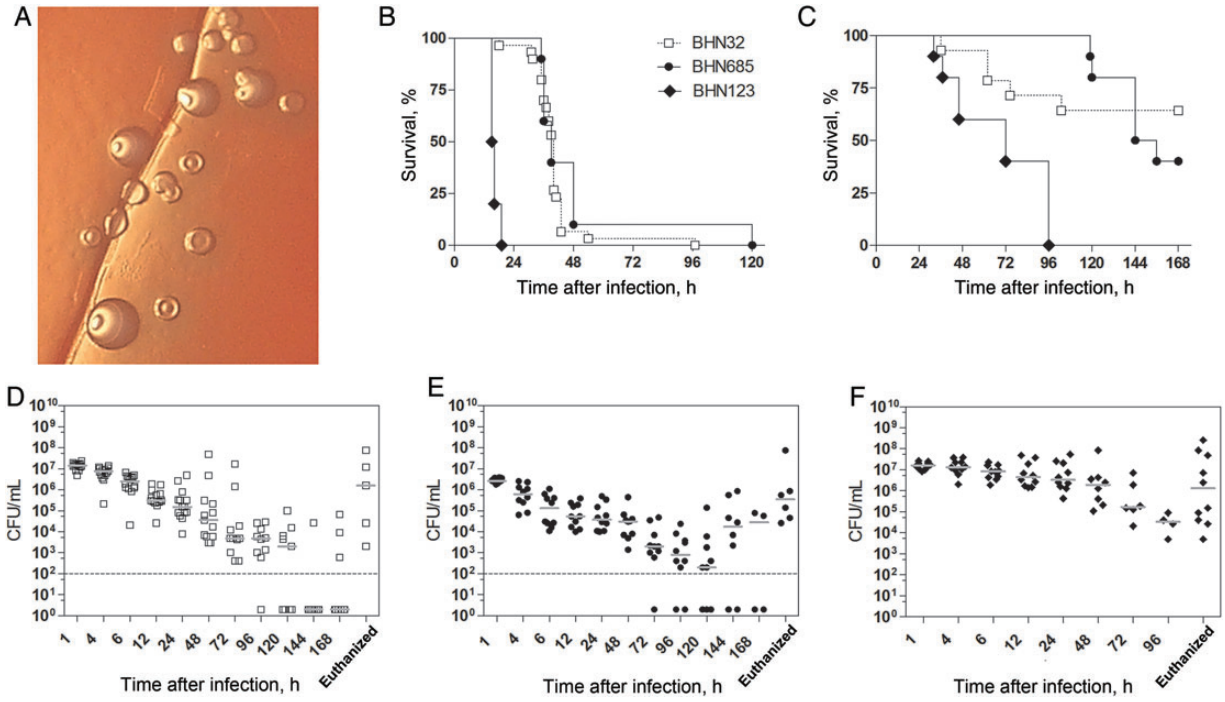

Figure 1. *spxB* mutant pneumococci of ST228 evoked in mice show large-colony morphotypes and are more virulent in murine intraperitoneal and intravenous infection models. Survival of C57BL/6 mice after infection with pneumococcal strains of serotype 1, ST228; wild-type BHN32^{ST228}, *spxB* mutant BHN123^{ST228}, and revertant BHN685^{ST228} (*spxB* complemented mutant). *A*, Colony morphology of *Streptococcus pneumoniae* serotype 1 clinical isolate wild-type BHN32^{ST228} (smaller colony) and spontaneous mutant BHN123^{ST228} *spxB* allele1 (larger colony). *B*, Survival time after intraperitoneal challenge. Mice infected with wild-type BHN32^{ST228} (n = 30) and revertant BHN685^{ST228} (n = 10) survived longer (40 hours and 39 hours, respectively) than those infected with the spontaneous *spxB* mutant, BHN123^{ST228} (n = 20), with a median survival time of 15.5 hours. Differences in survival times were statistically significant between mice infected with strains containing functional *spxB*, compared with the *spxB* mutants (BHN32^{ST228} vs BHN123^{ST228}, $P < .0001$; BHN32^{ST228} vs BHN685^{ST228}, $P = .2631$; BHN123^{ST228} vs BHN685^{ST228}, $P < .0001$). *C*, Survival after intravenous challenge. BHN32^{ST228} (n = 15) and BHN685^{ST228} (n = 10) showed a longer survival time than mutant BHN123^{ST228} (n = 10), with median survival times of 103 hours, 150 hours, and 72 hours respectively (BHN32^{ST228} vs BHN123^{ST228}, $P = .0017$; BHN32^{ST228} vs BHN685^{ST228}, $P = .5585$; BHN123^{ST228} vs BHN685^{ST228}, $P < .0001$). Levels of bacteremia are shown after intravenous challenge for wild-type BHN32^{ST228} (*D*) revertant BHN685^{ST228} (*E*), and mutant BHN123^{ST228} (*F*). Abbreviation: CFU, colony-forming unit.

above characteristics suggested mutations in the *spxB* gene, which was confirmed by sequencing (Table 1). Thus, the *spxB* gene in the large morphotype BHN123^{ST228} revealed a G to A substitution in base 539 resulting in a Gly180Asp amino acid substitution in SpxB relative to parental strain BHN32^{ST228}. BHN122^{ST306} contained as many as 5 nucleotide substitutions in *spxB*, compared with the parental strain BHN31^{ST306} (Table 1). We also sequenced the *spxB* gene from additional large-colony variants arising after intraperitoneal and intravenous infection with BHN32^{ST228} in different mice and found that they all contained different *spxB* mutations. Interestingly, when 3 large-colony variants were taken at different time points from the same mouse, they all turned out to carry different mutations in *spxB* (Supplementary Table 1).

Also, both large-colony variants BHN125^{ST306} and BHN683^{ST217} derived from the 2 human blood cultures were *spxB* mutants and deficient in hydrogen peroxide production in contrast to the respective small morphotypes (Table 1).

In Vivo–Selected *spxB* Mutants of Serotype 1 Are Hypervirulent in Mice Because of Decreased Early Clearance

Mice were challenged intraperitoneally with wild-type strain BHN32^{ST228}, *spxB* mutant strain BHN123^{ST228}, or the revertant strain BHN685^{ST228} (BHN123 complemented with the wild-type allele from BHN32). (Figure 1*B*). BHN685^{ST228} showed small colonies and produced similar levels of hydrogen peroxide as BHN32^{ST228} (Table 1). Mutant strain BHN123^{ST228} was considerably more virulent, with a median survival time of 15.5 hours, compared with 40 hours for BHN32^{ST228} and 39 hours for BHN685^{ST228} (Figure 1*B*). Thus, in vivo–selected *spxB* mutants obtained in mice challenged with a ST228 strain were hypervirulent after intraperitoneal challenge, and the increased virulence was mediated by an inactivated *spxB* locus.

Intravenous challenge of pneumococci allows a reproducible way to monitor bacterial numbers in blood over time. Intravenous infection using BHN32^{ST228}, BHN123^{ST228}, and BHN685^{ST228} revealed that mutant strain BHN123^{ST228} was

considerably more virulent than wild-type strain BHN32^{ST228} and revertant strain BHN685^{ST228} (Figure 1*C*). For both BHN32 and BHN685, there was a gradual decrease in bacterial numbers during the first 72 hours after intravenous challenge. Thus, the number of CFUs 48 hours after infection had decreased by 3 logs, from 10^7 one hour after infection to about 10^4. For surviving mice, blood counts remained at this low or even lower levels for up to 168 hours (Figure 1*D* and 1*E*). For BHN123^{ST228} the decline in the number of CFUs was less evident, and 48 hours after infection there was only 1 log decrease in the number of CFUs, compared with 1 hour after infection (Figure 1*F*). The data suggest that hypervirulence mediated by an inactivation of *spxB* is due to an increased resistance to early immune-mediated clearance.

One of the 2 patients with 2 morphotypes had a systemic infection caused by an isolate (BHN682^{ST217}) of ST217, a sequence type that has been associated with high mortality in humans. Upon intranasal challenge, the large-colony variant BHN683^{ST217} was more virulent than BHN682^{ST217}, with 45% and 10% of the infected mice, respectively, developing invasive disease (Figure 2*A*). Also, more bacteria were found in the blood at earlier time points for the large phenotype (Figure 2*B* and 2*C*). Even though all intranasally infected mice became colonized by both parent and mutant strains, as determined by culture of nasotracheal lavage, the average number of CFUs was slightly higher for BHN682^{ST217} (Figure 2*D*), suggesting that a functional *spxB* is of selective advantage during colonization in mice, as previously reported [11]. Intravenous challenge gave a dramatic difference between BHN683^{ST217} and BHN682^{ST217}, with no survival after 12 and 72 hours, respectively (Figure 2*E*). Even though the intravenous inoculum was similar for the 2 strains, the number of CFUs after 1 hour was already 1 log lower for the wild-type strain, compared with the *spxB* mutant (Figure 2*F* and 2*G*). For wild-type strain BHN682^{ST217}, the number of CFUs decreased for 6 hours, after which bacterial numbers increased and mice eventually succumbed of the infection (Figure 2*F*). In BHN683^{ST217}, there was no initial decrease in bacterial numbers. Instead, the number of CFUs increased during the first 6 hours of infection, until all mice succumbed (Figure 2*G*). When a mixed infection was performed with 99% wild-type strain BHN682^{ST217}and 1% *spxB* mutant strain BHN683^{ST217}, we found that the 2 bacterial populations behaved as different entities: the wild-type decreasing during the first 6 hours and then remaining stable until 12 hours after infection, whereas the *spxB* mutant rapidly increased to approximately half the bacterial population at 6 and 12 hours before increasing to >80% at the time mice were euthanized (Figure 2*H*). These data clearly demonstrate that the *spxB*

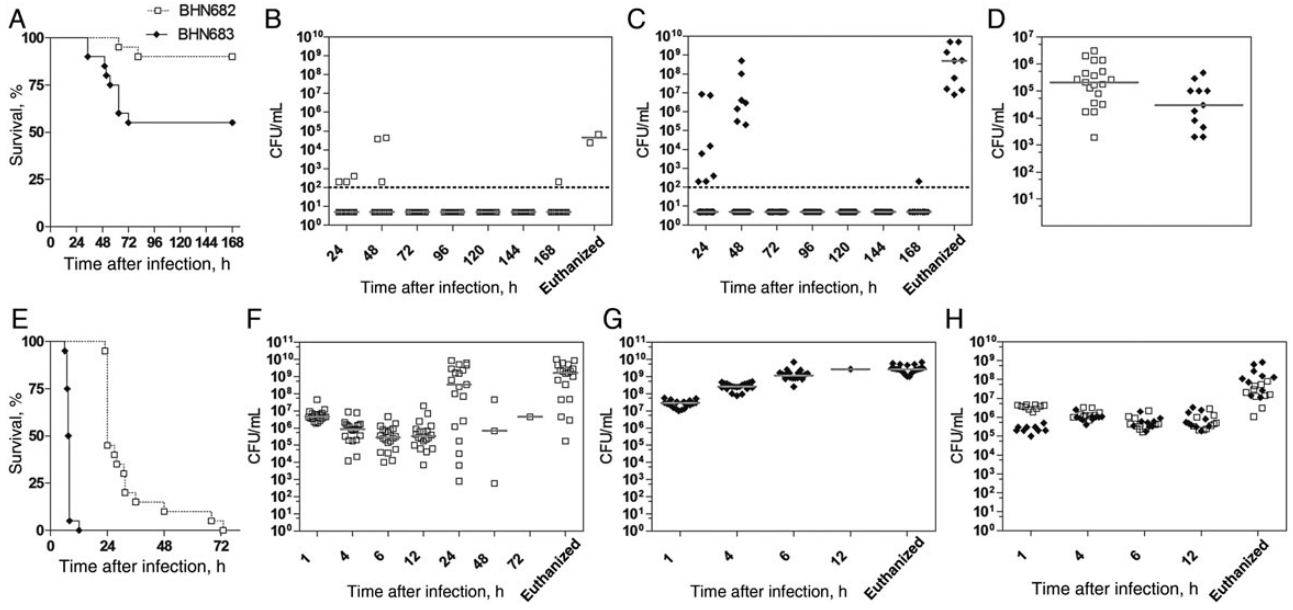

Figure 2. *spxB* mutant strains of ST217, evoked in patients with sepsis, are more virulent in mice after intranasal, and intravenous infection. *A*, Survival of C57BL/6 mice after intranasal challenge with wild-type BHN682^{ST217} (n = 20) and *spxB* mutant BHN683^{ST217} (n = 20). A significant difference was found in survival between the 2 strains ($P = .0099$). Levels of bacteremia are shown after intravenous challenge for BHN682^{ST217} (*B*) and for BHN683^{ST217} (*C*); dashed lines denote the lower limit of detection. *D*, A significant differences was found in bacterial load in the nasotracheal lavage fluid from surviving mice after intranasal challenge ($P = .0313$). *E*, Survival of mice after intravenous challenge with wild-type BHN682^{ST217} (n = 20) or *spxB* mutant BHN683^{ST217} (n = 20). The survival time of BHN683^{ST217} was shorter (median survival, 7.75 hours), compared with that for BHN682^{ST217} (24 hours; $P < .0001$). Level of bacteremia after intravenous challenge with wild-type BHN682^{ST217} (*F*) and mutant BHN683^{ST217} (*G*). *H*, Level of bacteremia after intravenous challenge with a mix of wild-type BHN682^{ST217} and mutant BHN683^{ST217} in ratio of 99 to 1. Abbreviation: CFU, colony-forming unit.

mutant BHN683^{ST217}, isolated from human blood, was more resistant to early host-mediated clearance, compared with wild-type strain BHN682^{ST217}.

To further study the role of *spxB* in other genetic backgrounds, we investigated the commonly used TIGR4 strain of serotype 4 (Supplementary Figure 2*A*–*C*). We observed a higher bacterial load in the blood for mice infected with the *spxB* mutant, compared with the wild-type strain, and a notable difference in the early clearance between the 2 strains, even though it was less pronounced than for type 1 strains.

SpxB Mutants Are Less Efficiently Phagocytosed by Macrophages In Vitro

*Spx*B mutants exhibit a faster growth rate in vitro in the absence of catalase but not in whole blood or serum (Supplementary Figure 2*D* and 2*E*). Thus, growth rate differences cannot explain the rapid emergence of *spxB* mutants in vivo. Instead our data suggest that the *spxB* mutants are resistant to a selective bottleneck occurring in the host during the initial phase of infection, to which the wild-type strain is sensitive. To see whether that bottleneck could represent macrophage-mediated clearance, the human derived monocytic-macrophage cell line THP-1 was used in binding and phagocytosis experiments. As shown in Figure 3*A* and 3*B*, the *spxB* mutants BHN123^{ST228} and BHN684^{ST228} (an in vitro–generated *spxB* knockout mutant of BHN32^{ST228}) adhered less well and were considerably less phagocytosed by THP-1 cells, compared with wild-type strain BHN32^{ST228} and the revertant strain BHN685^{ST228}. Also, for the ST217 pair, the in vivo–selected *spxB* mutant BHN683^{ST217} adhered less and was less phagocytosed, compared with wild-type strain BHN682^{ST217} (Figure 3*A* and 3*B*).

In Vivo Depletion of Macrophages Abolishes the Virulence Difference Between Wild-type and *spxB* Mutant Pneumococci

Clodronate liposomes were injected intravenously to deplete macrophages, as previously described [18]. The macrophage-depleted mice were challenged intravenously with the ST217 pair BHN682^{ST217} and BHN683^{ST217}. Macrophage-depleted mice were as susceptible to wild-type strain BHN682^{ST217} as to the *spxB* mutant strain BHN683^{ST217} and survived for <8 hours (Figure 4*A*). CFU counts in blood specimens revealed no initial bacterial clearance and that bacterial numbers were already increased for both wild-type and mutant bacteria 1 hour

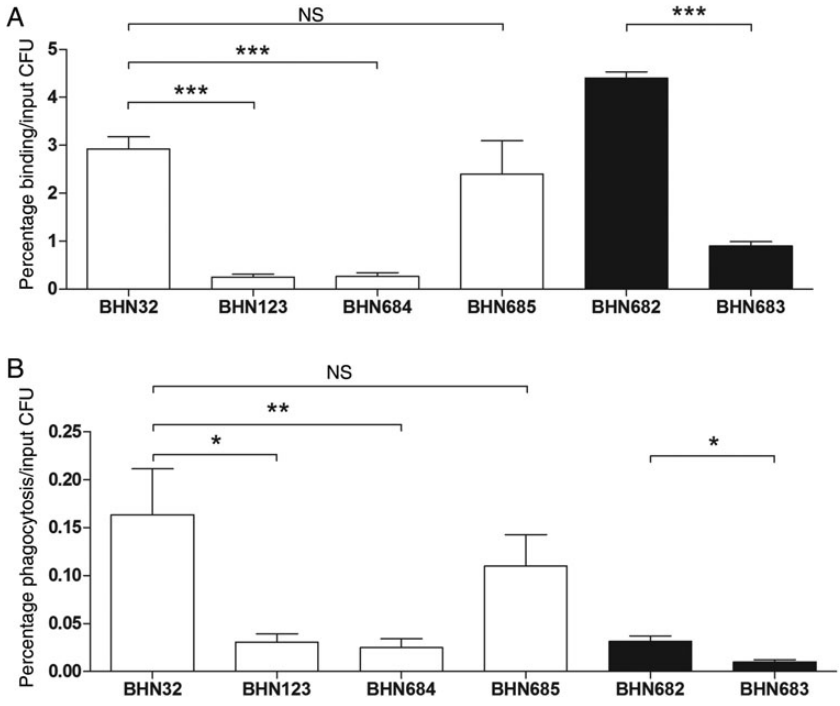

Figure 3. *spxB* affects the interaction between *Streptococcus pneumoniae* serotype 1 isolates and the human-derived macrophage cell line THP-1. Wild-type and mutant strains of serotype 1, ST228 (open bars), and ST217 (filled bars) were analyzed in binding and phagocytosis assays, using THP-1 cells. *A*, *spxB* mutations affect the binding of *S. pneumoniae* serotype 1 strains to macrophages. Binding is calculated by subtracting internalized bacteria from cell-associated bacteria, divided by initial inoculum. *B*, Spontaneous *spxB* mutant strains (BHN123^{ST228} and BHN683^{ST217}), as well as the in vitro–generated *spxB* knockout mutant strain (BHN684^{ST228}), were less phagocytosed by macrophages, compared with the corresponding wild-type strains and the revertant BHN685^{ST228}. The percentage phagocytosis represents the percentage of inoculated bacteria that were internalized. All data are presented as mean ± standard error of the mean. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001. Abbreviations: CFU, colony-forming unit; NS, not significant.

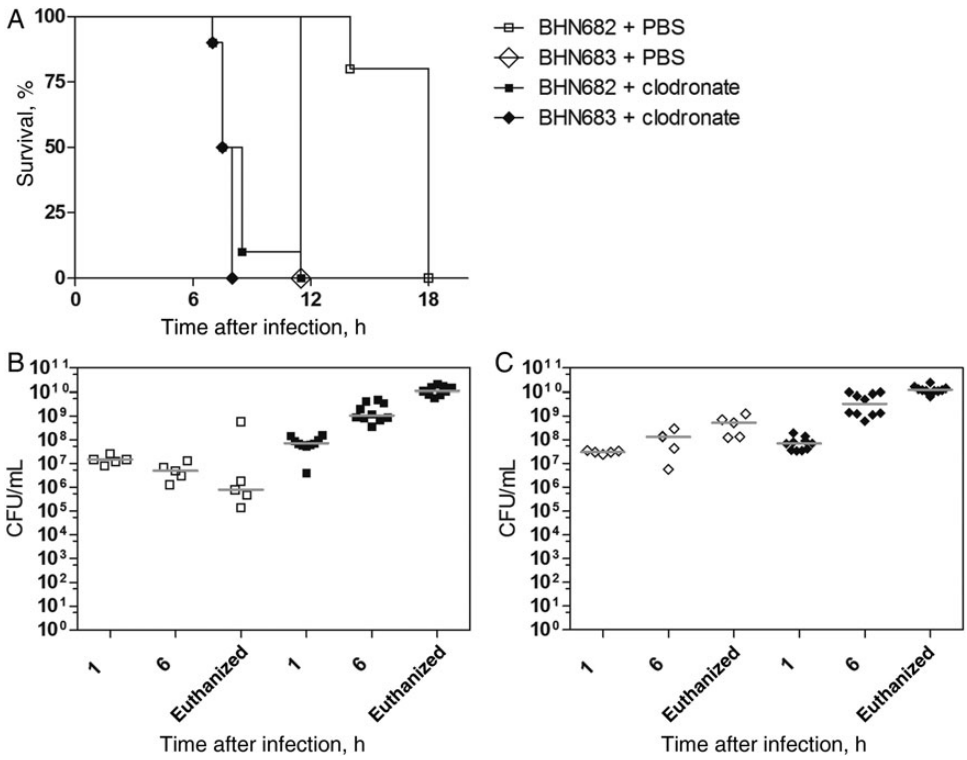

Figure 4. In vivo depletion of macrophages by using clodronate leads to increased resistance to early clearance of wild-type pneumococci. Survival of C57BL/6 mice treated with liposome-encapsulated clodronate or mice sham treated with phosphate-buffered saline (PBS) after intravenous challenge with wild-type BHN682^{ST217} or *spxB* mutant BHN683^{ST217}. *A*, Macrophage-depleted mice were more susceptible to *spxB*-proficient BHN682^{ST217}, compared with nondepleted mice (BHN682^{ST217} clodronate vs PBS, $P = .0005$). No significant differences were found in survival between macrophage-depleted mice challenged with wild-type BHN682^{ST217} or mutant BHN683^{ST217}. Bacteremia levels in mice depleted of macrophages with clodronate (*B*) or in controls treated with PBS (*C*) show that macrophage-depleted mice are more resistant to early clearance, compared with wild-type mice inoculated with wild-type *spxB*-proficient bacteria. Abbreviation: CFU, colony-forming unit.

after infection (Figure 4*B* and 4*C*). Next we investigated whether neutrophils are involved in resistance to early clearance (Figure 5A–C). Neutrophil depletion did not affect early clearance of wild-type strain or *spxB* mutants after systemic challenge, supporting our claim that it is primarily macrophages that clear wild-type bacteria more efficiently than *spxB*-mutant bacteria early after systemic challenge.

Effects of the C-Type Lectin SIGN-R1 on Early Pneumococcal Serotype 1 Clearance

It has been shown that mice lacking the C-type lectin SIGN-R1, which is preferentially expressed by marginal zone macrophages in the spleen, are significantly more susceptible to pneumococcal infection and fail to clear pneumococci from the circulation [22, 23]. We therefore infected SIGN-R1$^{-/-}$ mice intravenously with the ST217 pair BHN682^{ST217} and BHN683^{ST217} (Figure 6). There was no statistical difference between the in vivo–selected *spxB* mutant BHN683^{ST217} in wild-type and SIGN-R1$^{-/-}$ mice. However, wild-type BHN682^{ST217} was more virulent in SIGN-R1$^{-/-}$ mice, and there was no evidence for an initial clearance at early time points after BHN682^{ST217} infection of SIGN-R1$^{-/-}$, compared with wild-type mice. This suggests an important role for marginal zone macrophages expressing SIGN-R1 in the capacity to clear BHN682^{ST217} carrying the wild-type *spxB* allele but not in the clearing of its *spxB* mutant derivative strain BHN683^{ST217} (Figure 6).

DISCUSSION

Most if not all pneumococci have a functional *spxB* gene encoding a pyruvate oxidase, resulting in endogenous production of potentially suicidal levels of hydrogen peroxide. Here we demonstrate that in clinical isolates of serotype 1 belonging to 2 major CCs (CC228 and CC217), mutations in the *spxB* gene arise spontaneously during bacterial growth in the host, both in mice and in patients with sepsis. Mutations in *spxB* result in fitter, large-colony variants unable to produce endogenous hydrogen peroxide. Infection studies, particularly intravenous challenge, revealed that early clearance by the host was much less efficient for *spxB* mutants than for wild-type bacteria, resulting in a higher bacterial load and, as a consequence, an

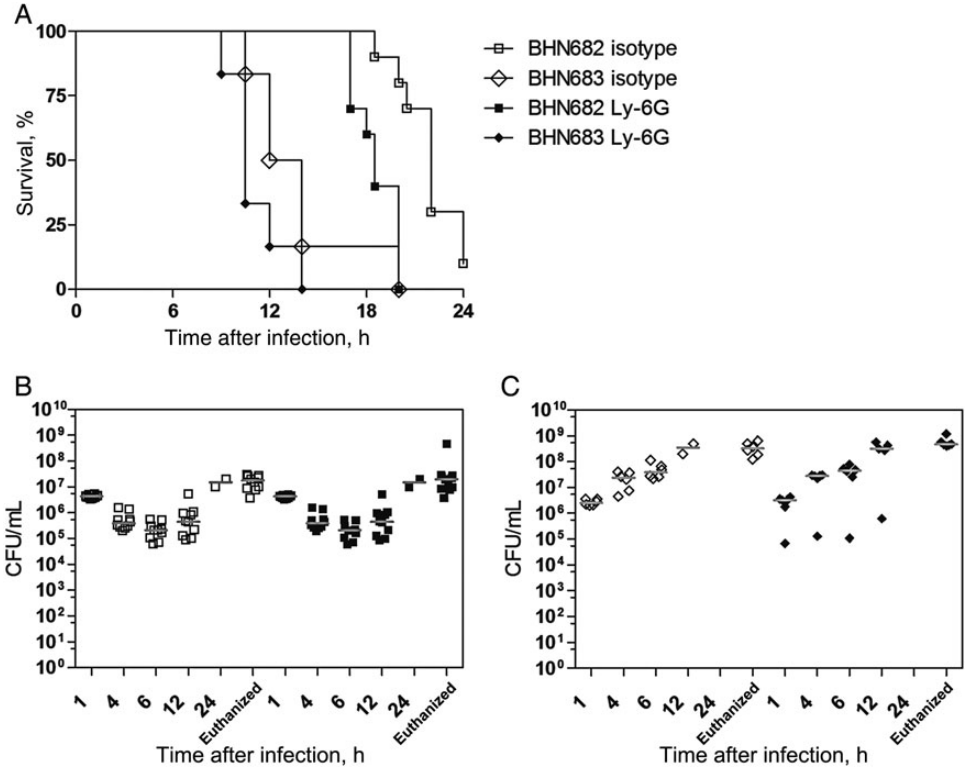

Figure 5. In vivo depletion of neutrophils by using antibody against the Ly-6G surface antigen does not affect early clearance but shortens survival time. Survival of C57BL/6 mice treated with antibody Ly-6G in phosphate-buffered saline (PBS) or mice sham treated with isotype antibody in PBS after intravenous challenge with wild-type BHN682^{ST217} or *spxB* mutant BHN683^{ST217}. *A*, Neutrophil-depleted mice were more susceptible to *spxB* proficient BHN682^{ST217}, compared with nondepleted mice (BHN682^{ST217} Ly-6G vs isotype antibody, $P = .0001$) No significant differences were found in survival between neutrophil-depleted mice and sham-treated mice challenged with *spxB* mutant BHN683^{ST217}. Bacteremia levels were the same in neutrophil-depleted and sham-treated mice after intravenous challenge with wild-type BHN682^{ST217} (*B*) or *spxB* mutant BHN683^{ST217} (*C*). Abbreviation: CFU, colony-forming unit.

earlier onset of severe disease. The phenotypes were reverted in a *spxB* revertant, arguing that selection of hypervirulence is due to *spxB* mutations.

In vivo selection of *spxB* mutant pneumococci in a host will depend on several parameters, such as the number of bacteria, the mutation frequency, the bottleneck created by the host immune system, and the fitness advantage for the mutant relative to the parental strain. In the present experiments, the challenge dose was 10^7 bacteria/mouse, and the calculated mutation frequency to rifampicin resistance was about 10^{-8} (data not shown). Since the *spxB* mutations represent a loss-of-function mutation, we expect the mutation frequency to be higher, possibly around 10^{-6}–10^{-7}, meaning that the inoculum might contain on average 1–10 *spxB* mutant bacteria. The in vivo–selected *spxB* mutants emerged early during infection but never outgrew the wild-type strain during the continued infection process. This finding, the fact that catalase is abundant in blood, and the similar growth behavior in serum and blood argue that it is resistance to early clearance, and not enhanced growth rate, that provides the selective force to enrich for *spxB* mutants during systemic infection. The mixing experiment shows that *spxB* bacteria do not affect early host clearance of wild-type bacteria, suggesting that mutant and wild-type bacteria behave as separate entities during infection.

Even though endogenous production of hydrogen peroxide may be detrimental to pneumococci, most clinical isolates carry an intact *spxB* gene. Expression of hydrogen peroxide or other functions associated with an intact pyruvate oxidase must therefore contribute to the fitness of pneumococci in their normal ecological niche, the human nasopharynx. This is supported by Regev-Yochay et al, who showed that a *spxB* mutant was outcompeted by its hydrogen peroxide–producing parental strain in an infant rat colonization model [14]. It has also been shown that production of hydrogen peroxide by pneumococci has a bactericidal effect on other organisms, such as *Staphylococcus aureus* and *Haemophilus influenzae*, that may confer a competitive advantage during nasopharyngeal colonization [15, 16]. In our intranasal infection, the in vivo–selected *spxB* mutants generated a lower number of colonizing bacteria, compared with the wild-type strain, suggesting that expression of *spxB* is of selective advantage to pneumococci during colonization. A reduction in colonization may also explain why a *spxB*

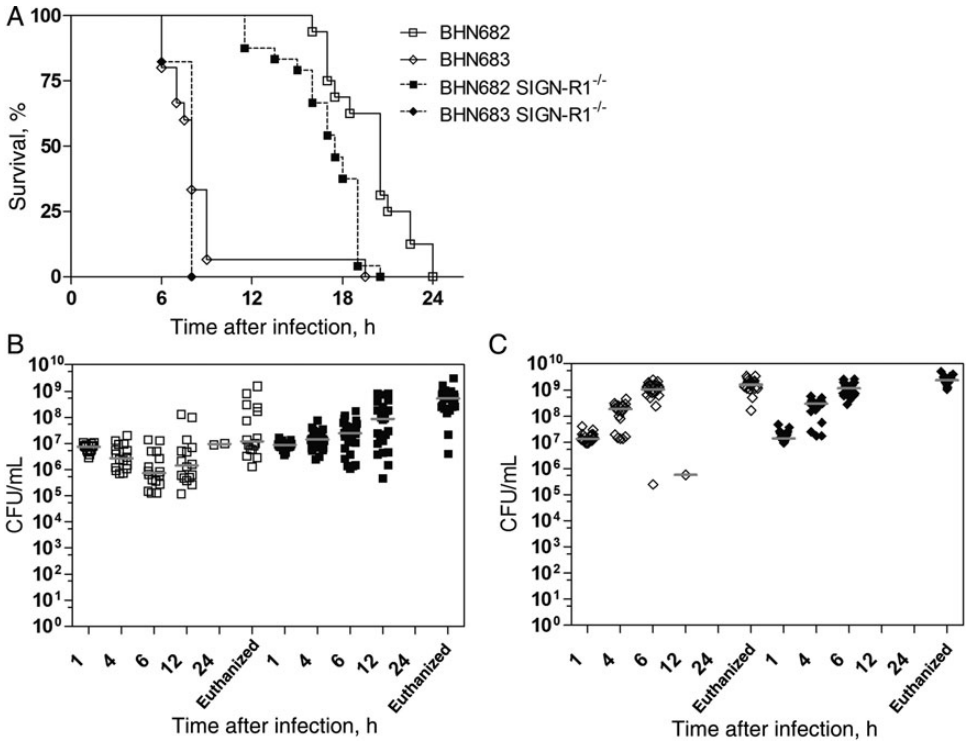

Figure 6. The C-type lectin SIGN-R1 affects early clearance of serotype 1 pneumococci of ST217 after intravenous infection. *A*, Survival of wild-type and SIGN-R1–deficient mice after intravenous challenge with wild-type BHN682^{ST217} or *spxB* mutant BHN683^{ST217}. SIGN-R1$^{-/-}$ mice infected with BHN682^{ST217} (n = 24) were more susceptible than wild-type mice infected with the same strain (n = 16), whereas wild-type mice infected with BHN683^{ST217} (n = 15) were as susceptible as SIGN-R1$^{-/-}$ mice infected with the same strain (n = 17). Bacterial counts (colony-forming units [CFUs]) in the blood in wild-type and SIGNRI$^{-/-}$ mice inoculated with strain BHN682^{ST217} (*B*) or BHN683^{ST217} (*C*). A higher bacterial load was found in SIGNR1$^{-/-}$ mice infected with BHN682^{ST217} (open circles), compared with wild-type mice inoculated with the same strain (open squares) at 4 hours (P = .0001), 6 hours (P = .0001), 12 hours (P = .003), and the time mice were euthanized (P = .013). Bacteremia levels were not significantly different between wild-type and SIGNR1$^{-/-}$ mice infected with BHN683^{ST217}.

knockout mutant of strain D39 (serotype 2) has been shown to be attenuated in an intranasal murine model [9, 24].

The data suggest that *spxB* mutants are selected for in vivo because they are more resistant to the initial clearance provided by macrophages. Thus, macrophage depletion, but not neutrophil depletion, resulted in equally susceptibility to wild-type bacteria and *spxB* mutant bacteria among mice. It has previously been shown that splenic marginal zone macrophages in mice are highly efficient in clearing pneumococci of serotypes 2 and 3 from the bloodstream, owing to their expression of the C-type lectin SIGN-R1 [22, 24]. Our data demonstrated that only the wild-type strain was affected by the absence of SIGN-R1, suggesting that SIGN-R1–expressing macrophages contribute to the clearance of wild-type bacteria, but not *spxB* mutant bacteria, from the bloodstream. Using the human derived monocytic cell line THP-1, we showed that the *spxB* mutants adhered less to and were phagocytosed in lower numbers by THP-1 cells, arguing that differences in early clearance depend on differences in the early eradication provided by macrophages.

Since the phenotypes observed could result from an altered or increased production of capsule, we measured capsule production during normal aerobic growth conditions but found no significant differences between wild-type and *spxB*-mutant bacteria (data not shown). However, in a recent study that used a serotype 2 strain, it was demonstrated that inactivation of *spxB* affects the sugar use pattern that results in the overproduction of capsular polysaccharide under semiaerobic conditions, a condition likely prevailing during systemic pneumococcal disease. This overproduction was partially mediated by an increased transcription of the capsule locus [25]. It is therefore possible that the in vivo selection of hypervirulent *spxB* mutants from systemic serotype 1 infection in mice and humans could be due to increased capsular-mediated resistance toward the early clearance performed by macrophages.

In the infectious process, as the bacteria move between different niches in the body, several bottlenecks will influence how mutants such as hypervirulent *spxB* mutants may be selected for in invasive pneumococcal disease. The selected mutants may differ from the parental strain in their ability to cause

disease, as was observed for the *spxB* mutants, and may thus have a clinical impact, even if they constitute only a fraction of the bacterial population in the bloodstream.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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References

1. Sandgren A, Sjostrom K, Olsson-Liljequist B, et al. Effect of clonal and serotype-specific properties on the invasive capacity of *Streptococcus pneumoniae*. J Infect Dis **2004**; 189:785–96.
2. Sleeman KL, Griffiths D, Shackley F, et al. Capsular serotype-specific attack rates and duration of carriage of *Streptococcus pneumoniae* in a population of children. J Infect Dis **2006**; 194:682–8.
3. Sjostrom K, Spindler C, Ortqvist A, et al. Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen. Clin Infect Dis **2006**; 42:451–9.
4. Hausdorff WP, Bryant J, Paradiso PR, Siber GR. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. Clin Infect Dis **2000**; 30:100–21.
5. Martens P, Worm SW, Lundgren B, Konradsen HB, Benfield T. Serotype-specific mortality from invasive *Streptococcus pneumoniae* disease revisited. BMC Infect Dis **2004**; 4:21.
6. Yaro S, Lourd M, Traore Y, et al. Epidemiological and molecular characteristics of a highly lethal pneumococcal meningitis epidemic in Burkina Faso. Clin Infect Dis **2006**; 43:693–700.
7. Leimkugel J, Adams Forgor A, Gagneux S, et al. An outbreak of serotype 1 *Streptococcus pneumoniae* meningitis in northern Ghana with features that are characteristic of Neisseria meningitidis meningitis epidemics. J Infect Dis **2005**; 192:192–9.
8. Brueggemann AB, Spratt BG. Geographic distribution and clonal diversity of *Streptococcus pneumoniae* serotype 1 isolates. J Clin Microbiol **2003**; 41:4966–70.
9. Spellerberg B, Cundell DR, Sandros J, et al. Pyruvate oxidase, as a determinant of virulence in *Streptococcus pneumoniae*. Mol Microbiol **1996**; 19:803–13.
10. Orihuela CJ, Gao G, Francis KP, Yu J, Tuomanen EI. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. J Infect Dis **2004**; 190:1661–9.
11. Sandgren A, Albiger B, Orihuela CJ, Tuomanen E, Normark S, Henriques-Normark B. Virulence in mice of pneumococcal clonal types with known invasive disease potential in humans. J Infect Dis **2005**; 192:791–800.
12. Hoskins J, Alborn WE Jr., Arnold J, et al. Genome of the bacterium Streptococcus pneumoniae strain R6. J Bacteriol **2001**; 183:5709–17.
13. Tettelin H, Nelson KE, Paulsen IT, et al. Complete genome sequence of a virulent isolate of Streptococcus pneumoniae. Science **2001**; 293:498–506.
14. Regev-Yochay G, Trzcinski K, Thompson CM, Lipsitch M, Malley R. SpxB is a suicide gene of *Streptococcus pneumoniae* and confers a selective advantage in an in vivo competitive colonization model. J Bacteriol **2007**; 189:6532–9.
15. Pericone CD, Overweg K, Hermans PW, Weiser JN. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. Infect Immun **2000**; 68:3990–7.
16. Regev-Yochay G, Trzcinski K, Thompson CM, Malley R, Lipsitch M. Interference between *Streptococcus pneumoniae* and *Staphylococcus aureus*: In vitro hydrogen peroxide-mediated killing by *Streptococcus pneumoniae*. J Bacteriol **2006**; 188:4996–5001.
17. Lacks S, Hotchkiss RD. A study of the genetic material determining an enzyme in Pneumococcus. Biochim Biophys Acta **1960**; 39:508–18.
18. Pericone CD, Park S, Imlay JA, Weiser JN. Factors contributing to hydrogen peroxide resistance in *Streptococcus pneumoniae* include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction. J Bacteriol **2003**; 185:6815–25.
19. Gibson CM, Mallett TC, Claiborne A, Caparon MG. Contribution of NADH oxidase to aerobic metabolism of Streptococcus pyogenes. J Bacteriol **2000**; 182:448–55.
20. Johnston JW, Myers LE, Ochs MM, Benjamin WH Jr, Briles DE, Hollingshead SK. Lipoprotein PsaA in virulence of *Streptococcus pneumoniae*: surface accessibility and role in protection from superoxide. Infect Immun **2004**; 72:5858–67.
21. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods **1994**; 174:83–93.
22. Kang YS, Kim JY, Bruening SA, et al. The C-type lectin SIGN-R1 mediates uptake of the capsular polysaccharide of *Streptococcus pneumoniae* in the marginal zone of mouse spleen. Proc Natl Acad Sci U S A **2004**; 101:215–20.
23. Lanoue A, Clatworthy MR, Smith P, et al. SIGN-R1 contributes to protection against lethal pneumococcal infection in mice. J Exp Med **2004**; 200:1383–93.
24. Ramos-Montanez S, Tsui HC, Wayne KJ, et al. Polymorphism and regulation of the spxB (pyruvate oxidase) virulence factor gene by a CBS-HotDog domain protein (SpxR) in serotype 2 *Streptococcus pneumoniae*. Mol Microbiol **2008**; 67:729–46.
25. Carvalho SM, Farshchi Andisi V, Gradstedt H, et al. Pyruvate oxidase influences the sugar utilization pattern and capsule production in *Streptococcus pneumoniae*. PLoS One **2013**; 8:e68277.