AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

MOLECULAR PATHOGENESIS

Streptococcus pneumoniae Elaborates Persistent and Prolonged Competent State during Pneumonia-Derived Sepsis

Jingjun Lin,[a] Pyunghun Park,[a] Hua Li,[a] Myung Whan Oh,[a] Iwona T. Dobrucki,[b] Wawrzyniec Dobrucki,[b,c] Gee W. Lau[a]

[a]Department of Pathobiology, College of Veterinary Medicine, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA
[b]Molecular Imaging Laboratory, Beckman Institute For Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA
[c]Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA

ABSTRACT The competence regulon of pneumococcus regulates both genetic transformation and virulence. However, competence induction during host infection has not been examined. By using the serotype 2 strain D39, we transcriptionally fused the firefly luciferase (*luc*) to competence-specific genes and spatiotemporally monitored the competence development in a mouse model of pneumonia-derived sepsis. In contrast to the universally reported short transient burst of competent state *in vitro*, the naturally developed competent state was prolonged and persistent during pneumonia-derived sepsis. The competent state began at approximately 20 h postinfection (hpi) and facilitated systemic invasion and sepsis development and progressed in different manners. In some mice, acute pneumonia quickly led to sepsis and death, accompanied by increasing intensity of the competence signal. In the remaining mice, pneumonia lasted longer, with the competence signal decreasing at first but increasing as the infection became septic. The concentration of pneumococcal inoculum (1×10^6 to 1×10^8 CFU/mouse) and postinfection lung bacterial burden did not appreciably impact the kinetics of competence induction. Exogenously provided competence stimulating peptide 1 (CSP1) failed to modulate the onset kinetics of competence development *in vivo*. The competence shutoff regulator DprA was highly expressed during pneumonia-derived sepsis but failed to turn off the competent state in mice. Competent D39 bacteria propagated the competence signal through cell-to-cell contact rather than the classically described quorum-sensing mechanism. Finally, clinical pneumococcal strains of different serotypes were also able to develop natural competence during pneumonia-derived sepsis.

KEYWORDS *Streptococcus pneumoniae*, competence regulon, pneumonia-derived sepsis, SsbB, DprA

Streptococcus pneumoniae (pneumococcus) is an important human pathogen that causes otitis media, community-acquired pneumonia, bacteremia, pneumonia-derived sepsis, and meningitis, with significant morbidity and mortality, especially in children and elderly (1). Pneumococcus is widely known to colonize the nasopharyngeal tract (2). Capsule is one of the most important pneumococcal virulence factors (3). In 1928, Frederick Griffith discovered that coinoculation of a live avirulent noncapsulated (R, rough) serotype 2 pneumococcus bacteria with a heat-killed encapsulated (S, smooth) virulent serotype 2 strain bacteria would kill mice quickly, with a recovery of fully encapsulated serotype 2 pneumococci from blood (homogenous type R-S conversion) (4). Astonishingly, if either a heat-killed serotype 1 or 3 S strain was coinoculated with a live serotype 2 R strain, it killed some mice, with recovery of serotype 1 or 3 S colonies (heterogeneous type R-S conversion) from dead mice (4). The aforementioned study led to the discovery of DNA as the material responsible for genetic transformation (5).

Shortly after the first discovery of genetic transformation *in vivo*, competence induction of *S. pneumoniae* was successfully replicated *in vitro* and was found to require

Citation Lin J, Park P, Li H, Oh MW, Dobrucki IT, Dobrucki W, Lau GW. 2020. *Streptococcus pneumoniae* elaborates persistent and prolonged competent state during pneumonia-derived sepsis. Infect Immun 88:e00919-19. https://doi.org/10.1128/IAI.00919-19.

Editor Liise-anne Pirofski, Albert Einstein College of Medicine

Address correspondence to Gee W. Lau, geelau@illinois.edu.

Received 6 December 2019
Returned for modification 9 January 2020
Accepted 21 January 2020
Accepted manuscript posted online 27 January 2020
Published 23 March 2020

complicated culture medium and conditions, including supplementation of serum (6–8). Hypercompetent capsule-deficient pneumococcal strains derived from D39 were preferentially selected to study various mechanisms regulating competence development (9–11). The spontaneous competent state begins in early log phase and quickly spreads to the whole pneumococcal population, followed by rapid shutoff (9, 12). Competence can be induced by an "activator macromolecule" isolated from the pneumococcal cell surface, which was later identified as a 17-amino-acid peptide named the competence-stimulating peptide (CSP) (12, 13). Various mechanisms regulating the pneumococcal competence induction have been revealed. When the membrane-bound histidine-kinase ComD is activated by CSP, it phosphorylates the response regulator ComE, which in turn, induces the expression of 24 "early" competence genes (14). Two identical copies of "early" gene *comX1* and *comX2* encoding the alternative σ factor ComX, which induces more than 80 "late" competence genes, among which only 14 genes are required for DNA uptake and recombination. The transient competent state is rapidly turned off, and the cells become refractory to subsequent competence induction for an extended time (13, 15). The protein responsible for competence shut off was later identified to be the ComX-regulated DNA processing protein A (DprA) (16, 17).

Recently, debates have emerged regarding whether competence induction and signal propagation in pneumococcus are mediated through the cell-cell contact model versus the classically described quorum sensing-based diffusive CSP. For the D39 derivative R6 strain, it has been purported that CSP is tightly bound to the cell surface; however, for the Rx strain, which is derived from a different lineage of D39, CSP can be detected in the culture supernatant (13). The direct cell-cell contact sensing mechanism was examined in the pneumococcal strain R800 (a derivative of R6) and D39 (11). The authors of that study proposed that a subpopulation of pneumococcal cells overexpress and transmit CSP to other cells via cell-to-cell collision and that CSP peptides were bound to its cell surface receptor ComD (11). In contrast, another study concluded that competence induction was growth dependent and operated on the classical quorum-sensing principle where threshold accumulation of secreted diffusive CSP peptides was required (18).

The original Griffith experiment was performed by using a heat-inactivated S strain to facilitate the transformation of live R strains to a heterogeneous serotype S strain (for example, from serotype 2 R to serotype 3 S strain) during *in vivo* infection. However, attempts to convert live S strains to a heterogeneous serotype S strain (for example, from type 2 S to type 3 S strain) *in vivo* were unsuccessful (4, 19), suggesting that encapsulated S strains are more recalcitrant to genetic transformation. Interestingly, it has been reported that the encapsulated pneumococcal strains have an approximately 10,000,000-fold higher transformation rate during nasopharyngeal colonization than during lung infection (20).

Apart from genetic transformation, we and others have previously shown that the pneumococcal competence system is also important for virulence (21–25). Furthermore, by extensive deletion and genetic analysis, we identified the competent-state-specific "late" competence genes that are required for virulence but are independent of their DNA transformation (26–28). Therefore, there remains a great interest to further characterize the competence-regulated virulence. However, the spatiotemporal induction of competence during host infection and its importance remain unknown. Here, we constructed pneumococcal strains harboring competence-specific luciferase reporter to track the spatiotemporal dynamics of natural competence development in a mouse model of pneumonia-derived sepsis.

RESULTS

***S. pneumoniae* naturally develops a prolonged and persistent competent state during pneumonia-derived sepsis.** We used the serotype 2 strain D39 to construct the reporter strain AD2501 harboring a firefly luciferase transcriptionally fused to the *ssbB* gene (D39-*ssbB-luc*; Fig. 1A) (29). The *ssbB* gene encodes a single-stranded DNA-binding protein and is uniquely induced during competence induction and directly protects internalized single-stranded DNA (30). *ssbB* is one of the most highly induced "late" competence genes regulated by ComX (14) and therefore represents a good

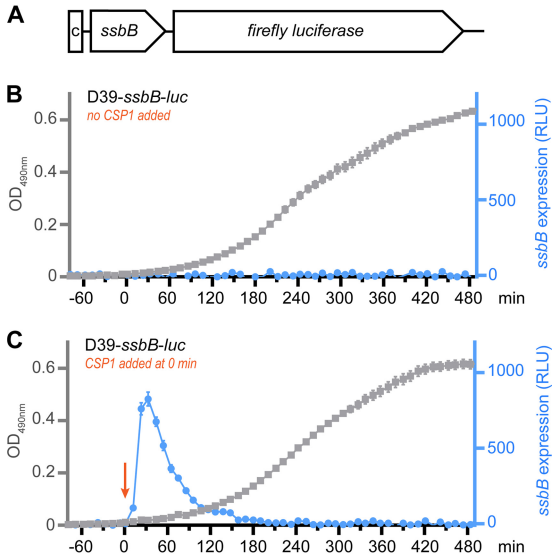

FIG 1 Provision of the CSP1 induces a transient competent state under *in vitro* culture conditions. (A) Schematic drawing of the firefly luciferase gene *luc* transcriptionally fused to the "late" competence gene *ssbB* of strain AD2501, which emits bioluminescence when pneumococcal cells enter the competent state. (B) The D39-*ssbB-luc* strain (AD2501) failed to naturally enter the competent state in the THY medium. (C) CSP1 (100 ng ml^{-1}) was added at time zero, and the bioluminescence was monitored for 480 min. The expression of *ssbB* peaked at 30 min and was abolished by 180 min. Results are shown as means ± the standard errors of the mean from 10 samples.

biomarker for monitoring competence induction (11, 16, 31). We tracked competence induction by measuring the luciferase activity in AD2501. The competent state did not naturally develop in strain AD2501 *in vitro* in the Todd-Hewitt broth supplemented with 5% yeast extract (THY) (Fig. 1B). After provision of 100 ng ml^{-1} CSP1, *ssbB-luc* expression in AD2501 began to increase at approximately 15 min and peaked at 30 min postexposure. The luciferase activities reduced to 10 and 2% of peak value at 2 and 3 h postexposure, respectively. The competent state failed to occur naturally for the remainder of the culturing period (Fig. 1C).

To track the competence induction *in vivo*, male adult CD-1 mice (8-week-old, 15 per cohort) were intranasally infected with 5×10^7 CFU of strain AD2501, and competence development was tracked spatiotemporally by using an IVIS SpectrumCT imaging system. Interestingly, pneumococcus developed natural competence during pneumonia-derived sepsis (Fig. 2; see Fig. S1 in the supplemental material). During the first 12 h, there was minimal to no detectable luciferase signal, suggesting either that the competent state was

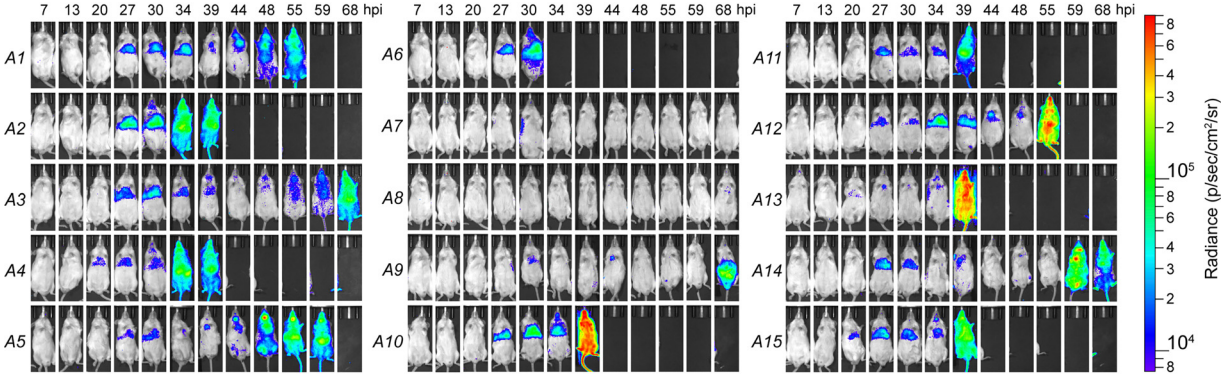

FIG 2 *S. pneumoniae* develops a prolonged competent state naturally during pneumonia-derived sepsis. Mice were intranasally infected with 5×10^7 CFU of the D39-*ssbB-luc* (AD2501) bacteria and tracked for competence induction for up to 68 hpi. Infected mice were imaged by using an IVIS SpectrumCT imaging system after subcutaneous injection of the D-luciferin potassium (100 mg kg^{-1}).

not initiated or that its induction was below the detection limit. After approximately 20 h, competence induction became detectable, and the kinetics of both the competent state and disease progression diverged into three outcomes. In the first group, mice (A2, A4, A6, A10, A11, A13, and A15) quickly progressed from pneumonia to bacteremia and sepsis, leading to death between 34 and 44 h postinfection (hpi). The induction of the competent state, as indicated by the luminescent signal output, increased persistently (Fig. 2). The second group of mice (A1, A3, A5, A12, and A14) died between 59 and 70 hpi. In these mice, the competence signal dropped or remained low before increasing substantially during the septic stage as they were approaching death (Fig. 2). The ebb and flow of competence signal in individually infected mice may reflect the struggle of pneumococcus to adapt and maintain stable lung infection. Finally, in the third group, two mice (A7 and A8) showed low to nondetectable competence induction and had much lower pneumococcus burdens in their lungs and spleens than the bacterial burdens of all moribund mice with competent pneumococci, reaching 10^8 CFU both in the lungs and spleens (see Fig. 4A and B, endpoint CFU). To rule out that *ssbB-luc* could be induced by noncompetence mechanisms, we deleted the *comCDE* genes in the strain AD2501 and found no *ssbB-luc* induction in any of the five mice infected by the strain AD2502 (Δ*comCDE-ssbB-luc*) through the entire time course of the infection despite recovery of pneumococcus found in both mouse lungs and spleens (Fig. S2). Thus, our results indicate that the temporal induction of pneumococcal natural competence during pneumonia-derived sepsis differs drastically from the *in vitro* competence induction. In contrast to the rapid on-and-off system *in vitro*, the *in vivo* competent state is prolonged and lasted for many hours.

Exogenously provided CSP1 has limited effect on the development of natural competence during pneumonia-derived sepsis. The significant difference between the pattern of competent state between *in vivo* versus *in vitro* conditions prompted us to investigate whether providing CSP1 exogenously could activate the competent state *in vivo*, and if so, whether the induction is transient (e.g., similar to *in vitro* pattern) or prolonged. CD-1 mice (cohorts of 10) were infected intranasally with 1×10^8 CFU of strain AD2501 (Fig. 3; Fig. S3). Group 1 mice (control cohort, Bx1 to Bx10) were left untreated and entered the competent state naturally at ~20 hpi (Fig. 3A). Group 2 mice (By1 to By10) were given CSP1 (100 ng per mouse) intranasally at 24 hpi (Fig. 3B). Although five mice in group 2 had entered competent state by 23 hpi, provision of CSP1 (at 24 hpi) did not induce the remaining 5 mice (By1, By2, By5, By6, and By7) to enter the competent state immediately (within 15 to 30 min) as observed under *in vitro* experimental conditions. Although mouse By7 showed a detectable competence induction 2.5 h after the CSP1 treatment (at 26.5 hpi), it could have been caused by either exogenously provided CSP1 or naturally developed competent. The remaining four mice (By1, By2, By5, and By6) entered the competent state between 35 and 40 hpi and eventually progressed to sepsis. These results suggest that exogenously provided CSP1 at 24 hpi failed to induce a competent state in pneumococcus. The provision of CSP1 did seem to increase the competence signal in mice By5 (nose) and By8. Furthermore, provision of CSP1 did not alter the prolonged competent state toward a transient competent state *in vitro* (e.g., By3 and By4). In the group 3 mice (Bz1 to Bz10), CSP1 was added immediately after bacterial inoculation (0 hpi), and no competence signal was detected within 1 hpi. However, competence signals were detected in three of five mice (Bz1 to Bz5) at 2 hpi. After a second dose of CSP1 instillation (2 hpi), all 10 mice showed competence induction (Fig. 3C). However, the competent state was almost turned off in all animals by 6 hpi, indicating that the prolonged competent state was not due to the extended stability of luciferase expressed in strain AD2501. Similar to the mouse cohorts in Fig. 3A and B, competence could be detected and sustained beginning around 20 hpi, with rapid (Bz1, Bz5, Bz6, Bz7, and Bz10) or slower (Bz2, Bz3, Bz4, Bz8, and Bz9) progression to pneumonia-derived sepsis and death. These results indicate that AD2501 bacteria in mouse lungs are not ready to enter competent state until approximately 20 hpi, suggesting that a time period of adaption to the host environment is crucial before pneumococcal bacteria can attain the naturally developed competent state.

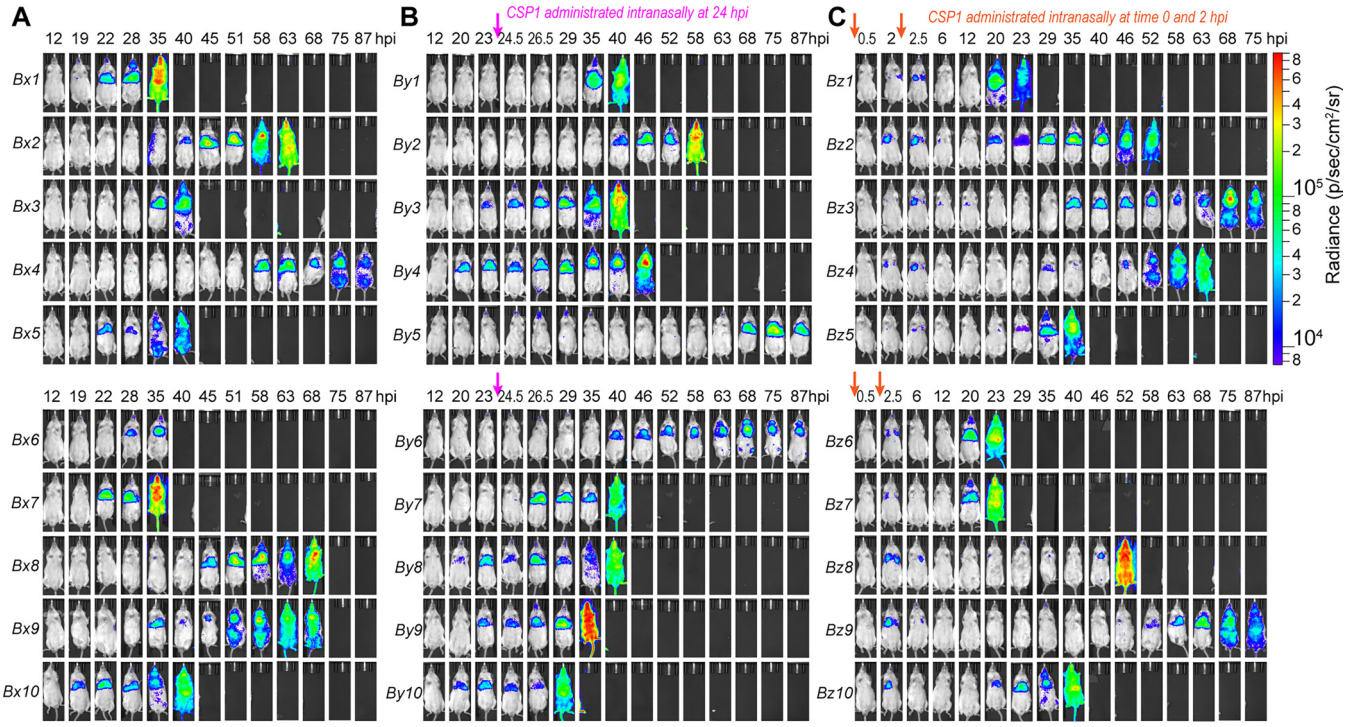

FIG 3 Exogenously provided CSP1 has limited effect on the development of natural competence during pneumonia-derived sepsis. Mice were intranasally inoculated with 10^8 CFU of D39-*ssbB-luc* (AD2501). (A) Naturally developed competent state in mice Bx1 to Bx10 in the absence of exogenously provided CSP1. (B) Mice By1 to By10 were intranasally instilled 100 ng of CSP1 at 24 hpi. (C) Mice Bz1 to Bz10 were intranasally instilled 100 ng of CSP1 (100 ng per mouse) at 0 and 2 hpi. Infected mice were imaged by using an IVIS SpectrumCT instrument after subcutaneous injection of the D-luciferin potassium (100 mg kg^{-1}).

Lung bacterial burden is not a key determinant in the development of natural competence during pneumonia-derived sepsis. Traditional model of quorum sensing is dependent on threshold accumulation of signaling molecules during growth (e.g., homoserine lactones at late exponential phase [CSP]) secreted by a bacterial population (15, 18, 32, 33). Because competence induction might be correlated with the bacterial density, we examined the number of pneumococci at the early stages of lung infection. In this experiment, 40 CD-1 mice were intranasally inoculated with 5×10^7 CFU of luciferase reporter strain AD2501. The first 10 mice were immediately sacrificed (within 10 min) and their lungs were homogenized. The inoculum in each mouse lung was $\sim 2 \times 10^7$ CFU, indicating efficient delivery of AD2501 bacteria into mouse lungs (Fig. 4A, 0.1 hpi). Therefore, the variation in the time of death of AD2501 infected mice (e.g., Fig. 3, from 30 h to >90 h) was likely a result of differences (e.g., genetics, immune capacity, and uneven distribution of inoculum in the lung) in individual mice.

At 12 hpi, another 10 mice were euthanized, and the pneumococcal burden in these lungs decreased slightly although the differences were not statistically significant compared to 0.1 hpi (Fig. 4A). At 24 hpi, 13/20 remaining mice had entered the competent state (Fig. 4C), with an average bacterial burden of 8×10^6 CFU. In contrast, in mouse lungs of which the AD2501 bacteria had not entered the competent state, the average pneumococcal burden was 1×10^6 CFU. However, the difference in pneumococcal burden between the two groups of animals was not statistically significant (Student *t* test, $P = 0.16$) (Fig. 4D), despite there being a positive correlation between luminescence output and lung CFU (Spearman $r = 0.6826$, $P = 0.0009$). It is worth noting that the mice that had not entered the competent state at the moment of euthanasia might have competence turned on shortly after, but we would not be able to know. Similarly, at 24 hpi, the difference in the bacterial burden of spleens between competent versus noncompetent mice was also statistically insignificant (Student *t* test, $P = 0.12$) (Fig. 4E), despite there being a positive correlation between luminescence output and spleen CFU (Spearman $r = 0.8047$, $P < 0.0001$).

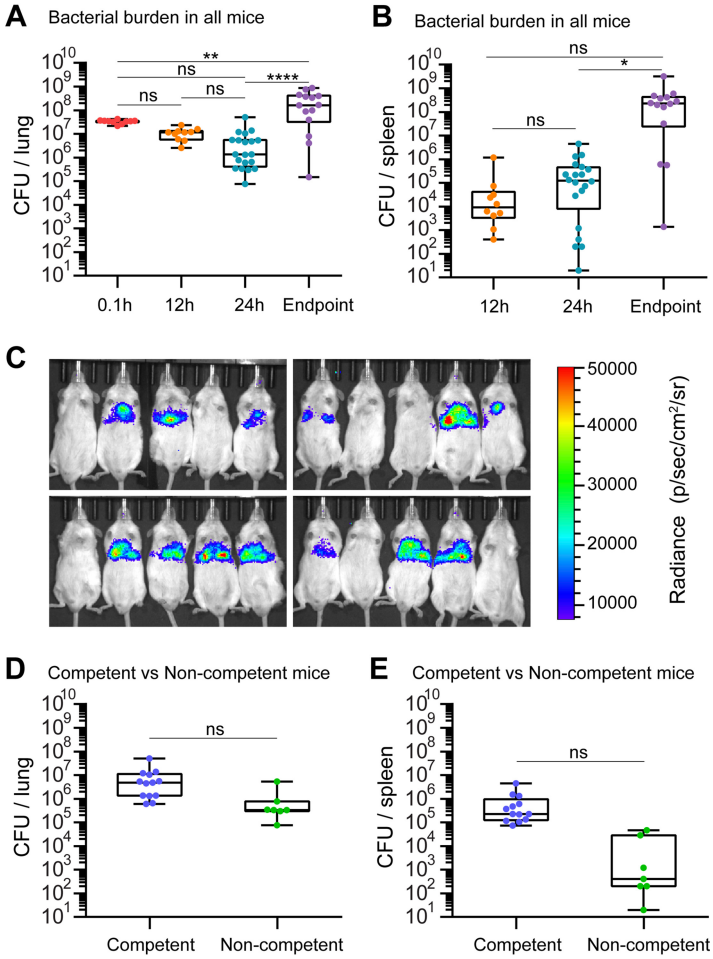

FIG 4 Pneumococcal burden does not affect the timing of naturally developed competence. Forty male CD-1 mice (7 weeks old) were inoculated with 5×10^7 CFU of D39-*ssbB-luc* (AD2501) intranasally. Infected mice ($n = 10$) were sacrificed immediately or at 12 hpi for determination of bacterial burden in lungs (A; 0.1 hpi, 12 hpi) and spleens (B; 12 hpi). (C) At 24 hpi, competence induction were determined by IVIS imaging in the remaining mice. After imaging, mice were euthanized and bacterial burdens in lungs (D) and spleens (E) from competent versus noncompetent mice were compared. The total bacterial burdens from lungs and spleens at 24 hpi were included in panels A and B, as well as the endpoint bacterial burden from lungs and spleens in mice derived from Fig. 2. Box-and-whisker plots are shown, with whiskers indicating the minimum and maximum values. One-way ANOVA, followed by Tukey's multiple-comparison test, was performed to compare statistical differences between four samples in panel A (ANOVA, $P < 0.0001$) and three samples in panel B (ANOVA, $P = 0.0178$). An unpaired Student *t* test was used to compare statistical differences between two samples in panels D (ns, $P = 0.1550$) and E (ns, $P = 0.1223$).

To compare the total bacterial burden in lungs and spleens more broadly, we also included the endpoint CFU of moribund mice from the Fig. 2 (data for mouse A6 were not recorded). The results revealed that both the lung burden and the systemic spread of pneumococcus (AD2501) started to increase at approximately 24 hpi, which temporally coincided with the competence induction. Eventually, both the lung and spleen reached a high pneumococcal burden of $>10^8$ CFU (Fig. 4A and B, endpoint). Collectively, these results suggest that the initial competence induction at approximately 20 hpi is not dependent on the number of pneumococcal cells, but rather, on adaptation to the lung environment.

Competence induction during pneumonia-derived sepsis is largely independent of the concentration of pneumococcal inoculum. To further examine whether bacterial load determines the timing of initial competence development, we challenged CD-1 mice with three different doses of luciferase reporter strain AD2501 (Fig. 5; Fig. S4). Group 1 mice (Cx1 to Cx5) received 5×10^7 CFU, group 2 mice (Cy1 to Cy5)

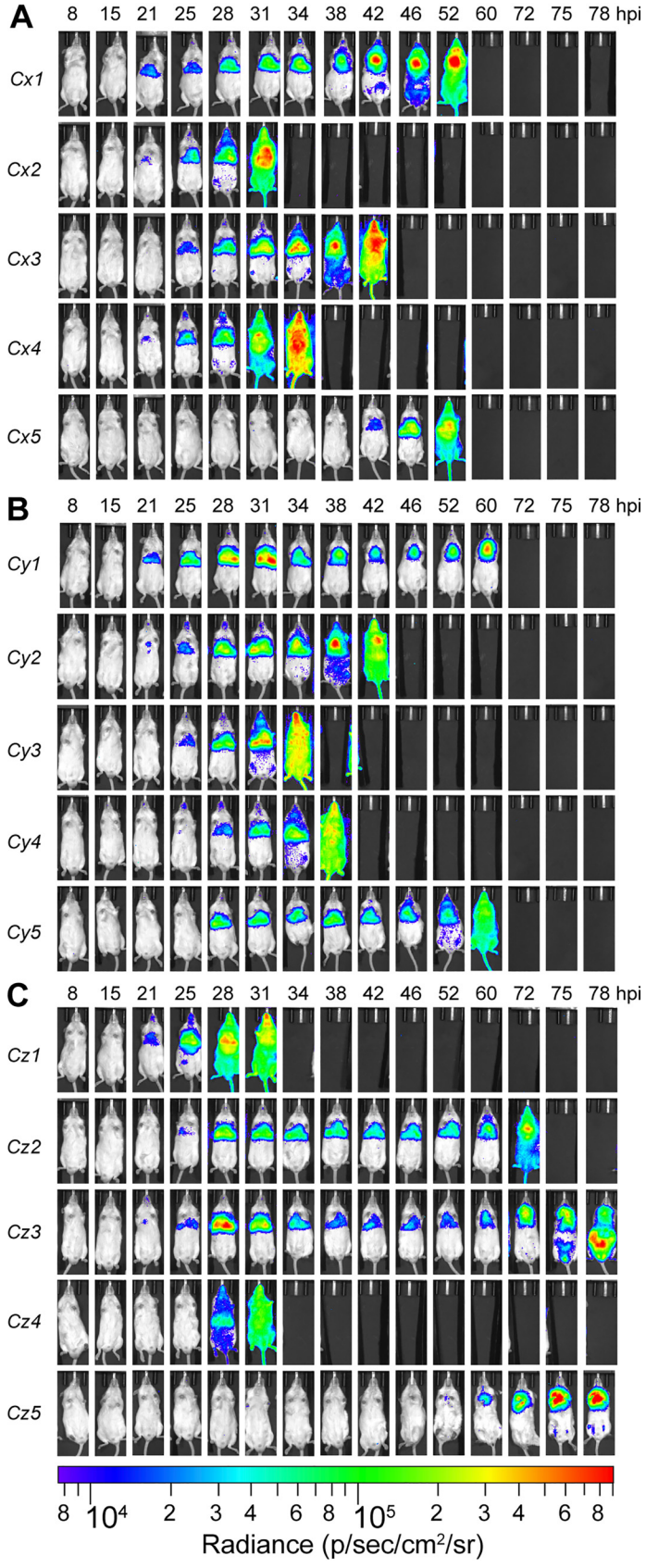

FIG 5 Pneumococcal inoculum levels do not significantly affect the timing of natural competence development. Three different doses of inoculum were intranasally administered into CD-1 mice (five

(Continued on next page)

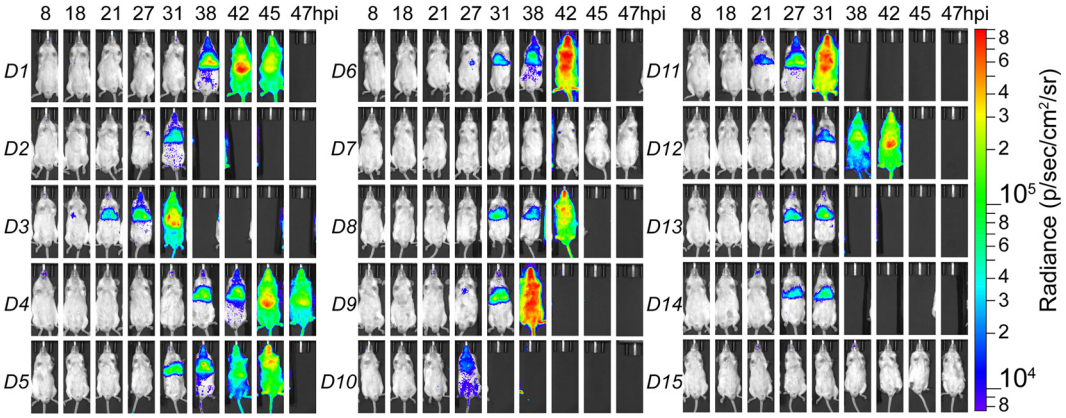

FIG 6 DprA is highly expressed but unable to shut off the competent state during pneumonia-derived sepsis. Fifteen mice were intranasally infected with 5×10^7 CFU of D39-*dprA-luc* (AD2503) in which the firefly luciferase gene (*luc*) was transcriptionally fused to the *dprA* gene. DprA expression was monitored by using the IVIS SpectrumCT imaging system after subcutaneous injection of the D-luciferin potassium (100 mg kg^{-1}).

received 5×10^6 CFU, and group 3 mice (Cz1 to Cz5) received 1×10^6 CFU. A major observable difference among the three groups of infected mice was that those infected with higher dose of AD2501 died slightly faster (Fig. 5A to C). However, despite the 50-fold change in the inoculum levels among the three different cohorts of mice, some of the mice from all three groups entered the competent state at approximately 21 to 25 hpi, and most of the mice showed detectable competence induction by 28 hpi. These results further suggest that bacterial number is not a key determinant affecting temporal competence induction. Collectively, data in Fig. 2 and 5 suggest that the pneumococcus needs to attain a physiological state with successful lung infection and full adaptation to the host environment before spontaneous competence induction.

DprA is highly expressed but unable to shut off the competent state during pneumonia-derived sepsis. The DprA protein has been reported to be critical for turning off the competent state *in vitro* (16, 17). Once induced, DprA dissociates the phosphorylated ComE dimer to interrupt the competence induction regulated by the ComCDE positive-feedback loop (16). Under *in vitro* conditions, it has been reported that DprA is highly expressed and stable (34), rendering competent pneumococcal cells refractory to subsequent induction by CSP (35). Deletion of the *dprA* gene causes elevated and prolonged expression of "late" competence genes (16, 17, 29, 34). Because of the prolonged and persistent competent state during pneumonia-derived sepsis, we examined whether the expression of DprA was compromised *in vivo*. Fifteen mice (D1 to D15) were intranasally infected with 5×10^7 CFU of the pneumococcal strain AD2503, which harbors a luciferase gene transcriptionally fused to the *dprA* gene in D39 (Fig. 6; see also Fig. S5A and B). The spatiotemporal expression of DprA in AD2503 was prolonged and persistent and mirrored that of D39-*ssbB-luc* (AD2501). Even toward the endpoint, DprA was still highly expressed but unable to shut off the competent state. Again, these results demonstrate that the spatiotemporal regulation of competence induction and maintenance of the competent state *in vivo* are not a simple replica of *in vitro* culture conditions.

A persistent competent state is conserved in other clinical strains of *S. pneumoniae* during pneumonia-derived sepsis. To examine whether the ability to develop competent state *in vivo* is conserved among other clinical serotypes of pneumococcus, we constructed the *ssbB-luc* reporter in the serotype 4 strain TIGR4 (AD2504)

FIG 5 Legend (Continued)
animals per cohort). (A) Group 1, 5×10^7 CFU D39-*ssbB-luc* (AD2501). (B) Group 2, 5×10^6 CFU strain AD2501. (C) Group 3, 1×10^6 CFU strain AD2501. Infected mice were imaged by using the IVIS SpectrumCT instrument after subcutaneous injection of the D-luciferin potassium (100 mg kg^{-1}).

and the serotype 3 strain 0100993 (AD2505) (21). CD-1 mice (10 per cohort) were intranasally infected with 2×10^7 CFU of strains AD2504 (Fig. 7A; see also Fig. S5C and D) and AD2505 (Fig. 7B; see also Fig. S5E and F), respectively. Importantly, both strains developed natural competence during lung infection. In the AD2504-infected cohort, 8/10 mice entered the competent state (Fig. 7A). In mouse Ex1 the competent state lasted for 17 h (35 to 52 hpi), whereas in mouse Ex3 the competent state lasted for at least 21 h (102 to 133 hpi) (Fig. 7A). A notable feature in the AD2504-infected mice was that natural competence seemed to develop near the endpoint and did not necessarily begin in the lung (Fig. 7A, mice Ex7 and Ex9). For AD2505, a naturally developed competent state was also detected during lung infection. For example, in mouse Ey2, the competent state lasted for 13 h (25 to 38 hpi), whereas in mouse Ey10 the competent state lasted for at least 30 h (63 to 93 hpi) (Fig. 7B). Collectively, these results suggest that, similar to D39, both TIGR4 and 0100993 develop natural competence that persists for a prolonged period of time during host infection.

Pneumococcus-free plasma isolated from septic-competence-positive mice failed to induce competent state *in vitro*. Recently, Prudhomme et al. reported that CSP was retained on the pneumococcal cell surface by ComD and activated competence by direct cell-to-cell contact (11), which is in agreement with earlier studies showing that CSP is tightly bound to the cell surface of the D39-derived strain R6 (12, 13). However, the proposed model was challenged by Moreno-Gamez et al., who reported that competence induction was dependent on a quorum-sensing mechanism relying on the threshold accumulation of the freely diffusible CSP (18). We sought to determine whether there were freely diffusible CSP peptides present in the blood of systemically infected competence-positive mice (e.g., Fig. 5, mouse Cx1 at 52 hpi), which may be the cause of the prolonged and high levels of competence during pneumonia-derived sepsis. We predicted that such high levels of CSP should be able to stimulate a noncompetent recipient reporter strain. We generated a *ssbB-luc* reporter strain in the Δ*dprA* genetic background (Δ*dprA-ssbB-luc*, AD2506), which has heightened sensitivity and response to competence induction due to its inability to shut off the competent state (29). The pneumococcal cells that secreted these CSPs were removed from the blood samples by filtration to eliminate possible competence transfer by cell-cell direct contact mechanism. Bacterium-free plasma samples prepared from such mouse blood, as well as whole blood from the same animal, were serially 2-fold diluted and added to the freshly grown recipient reporter strain AD2506. Because AD2501 was present in the whole blood recovered from the infected mice, the blood was also serially 2-fold diluted and mixed with THY medium only as a basal luminescence control (Fig. 8C). Interestingly, the pneumococcus-free plasma failed to induce AD2506 even with only a 2-fold dilution (Fig. 8A). In contrast, when the whole blood was mixed with the recipient reporter strain AD2506 (Fig. 8B), the bioluminescence signal was much stronger than in the control group that without AD2506 (Fig. 8C), suggesting that AD2506 was induced into the competent state (Fig. 8B and C). Similar results were obtained in nine additional mice, with data from one additional mouse shown (see Fig. S6 in the supplemental material). These results suggest the AD2501 bacteria in the blood of the infected mice have high ability to induce competence of AD2506. In contrast, the CSP in the bacterium-free plasma, if present, was inadequate to induce competence in AD2506. Finally, we also demonstrated that, when incubated at 37°C for 30 min, synthetic CSP1 was degraded in mouse blood or plasma but not in THY medium (Fig. S7). Collectively, our results suggest that competence induction in pneumococcal strain D39 during pneumonia-derived sepsis is likely mediated through a cell-cell contact mechanism rather than through threshold accumulation of freely diffusible CSPs that appear to be highly susceptible to degradation.

DISCUSSION

Encapsulated pneumococcal strains enter the competent state efficiently during pneumonia-derived sepsis. Capsule-deficient strains derived from D39, including CP1250 and R6 and its hypercompetent lineages R800 and Rx, are the most commonly

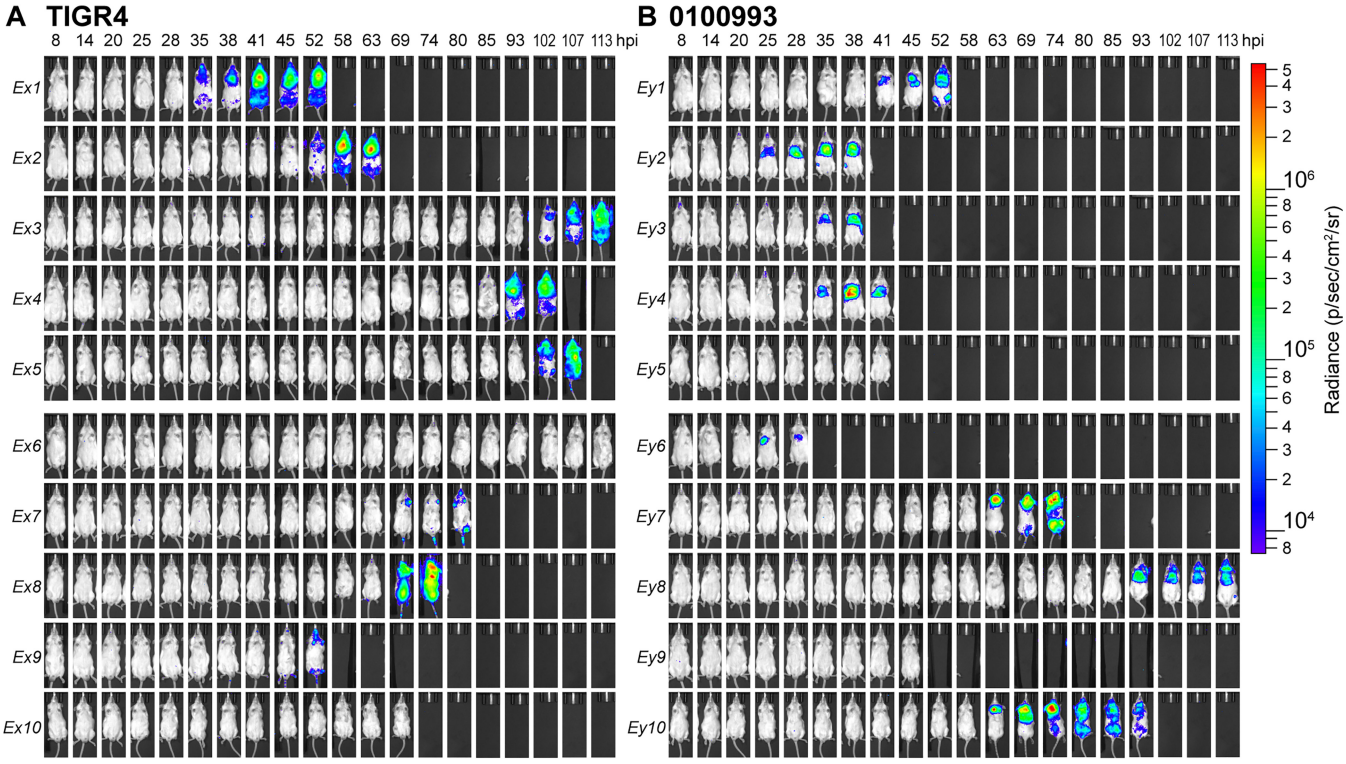

FIG 7 Additional clinical strains of *S. pneumoniae* with different serotypes develop natural competence during pneumonia-derived sepsis. CD-1 mice (10 per cohort) were intranasally infected with 2×10^7 CFU of the serotype 4 strain TIGR4-*ssbB-luc* (AD2504) (A) and the serotype 3 strain 0100993-*ssbB-luc* (AD2505) (B), respectively. Infected mice were imaged by using the IVIS SpectrumCT imaging system after subcutaneous injection of the D-luciferin potassium (100 mg kg^{-1}).

used to dissect the molecular mechanisms of pneumococcal competence development (16, 31, 36–38). To date, only a few competence studies have been performed in the encapsulated strains such as D39 (24, 26, 39–41). In addition to the strain selection, specially formulated complex liquid culture media optimized for transformation are required to allow spontaneous competence development (36). For example, we found that D39 was not able to enter the competent state spontaneously when cultured in the nutrient-rich THY medium most widely used to cultivate pneumococci (Fig. 1) (29). The competence-permissible C+Y medium, which contains defined amino acids, bovine serum albumin (BSA), and other nutrients, was formulated based on early findings that BSA or bodily fluids, including peritoneal washings and blood, provided better conditions for competence induction (6–8). One potential reason is that the pneumococcal cell surface protease HtrA (42), whose proteolytic activity can be detected in the culture supernatant, is capable of degrading CSP (42) but can be blocked by BSA (42). It is possible that the rich protein contents in bodily fluids (e.g., serum albumin) may act like BSA in the C+Y medium to allow CSP accumulation, enabling the onset of spontaneous competent state. Our study is the first to show that encapsulated pneumococcus from various serotypes can enter the competent state efficiently during pneumonia-derived sepsis.

Physiological adaptation to host environment is critical for competence initiation. At early stages of lung infection, the concentration of pneumococcal cells seems to play a negligible role in competence induction. For example, the pneumococcal burden was consistently higher (though not statistically significant) at 0.1 and 12 hpi than at 24 hpi, a time point when the infecting pneumococcal bacteria finally enter the competent state (Fig. 4A). These observations are further supported by the fact that provision of two doses of CSP at 0 and 2 hpi could only stimulate a transient, unsustainable competent state. These results suggest that pneumococcal strain D39 at

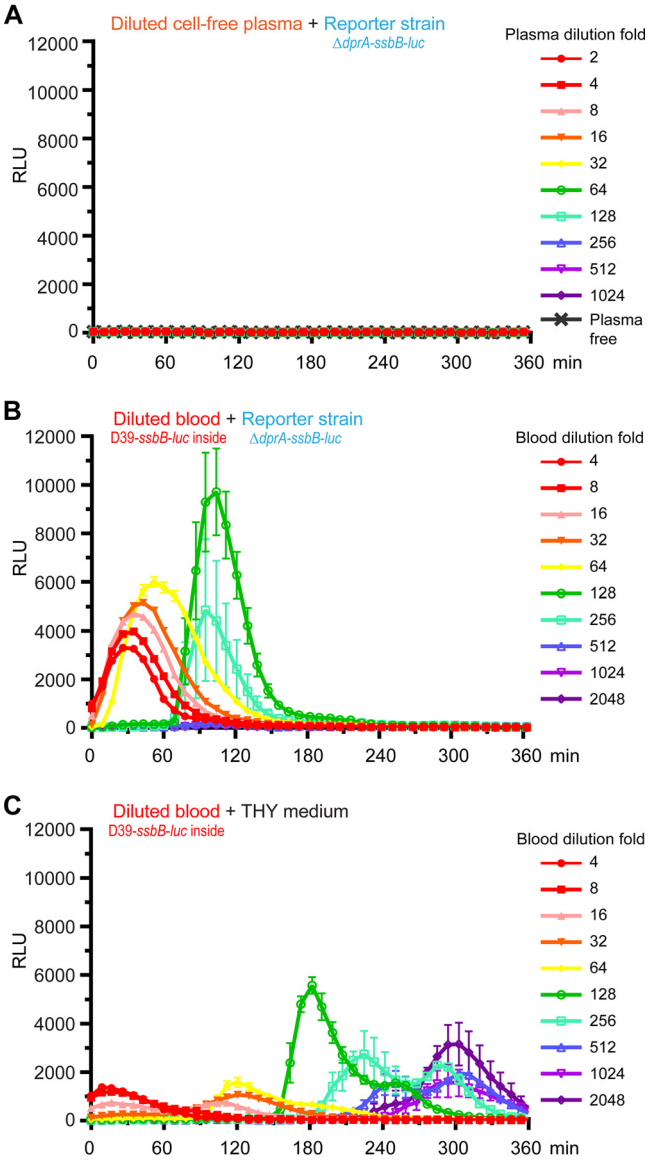

FIG 8 Whole blood containing the competent state pneumococcal bacteria induces competence development in noncompetent recipients. CD-1 mice ($n = 10$) were infected with 5×10^7 CFU of D39-*ssbB-luc* (AD2501). When the competent-state pneumococci had systemically spread to the entire circulatory system, as indicated by the IVIS SpectrumCT imaging system, whole blood was drawn from these mice. Cell-free plasma samples were acquired by centrifuging a portion of the whole blood to pellet the host cells, followed by passing the supernatant through a 0.22-μm filter to eliminate all pneumococcal bacteria. (A) This cell-free plasma was serially 2-fold diluted with THY medium and then mixed with the recipient reporter strain Δ*dprA-ssbB-luc* (AD2506) for competence induction. (B and C) Pneumococcus-containing whole blood was also serial 2-fold diluted and then mixed with THY with Δ*dprA-ssbB-luc* (AD2506) bacteria (B) or without AD2506 (C). The induction of competence was monitored by measuring the bioluminescence. Results are shown as means ± the standard errors of the mean from three replicates. Similar results were obtained from nine additional mice tested. One set of representative results is shown. It is worth noting that the dense red color of blood in low-dilution groups blocked the bioluminescence signal and therefore could not be used to directly compare results with the medium- or high-dilution groups.

the concentrations between 10^6 and 10^8 CFU need ~20 h to adapt and establish infection before the development of natural competence. Considering the fact that increasing the pneumococcal inoculum (e.g., from 1×10^6 CFU to 5×10^7 CFU) did not bring forth an earlier induction of the competent state, it is likely that physiological adaptation to the lung environment is at a higher hierarchical order that overrides the importance of higher local density of pneumococcal cells. Nevertheless, we cannot rule

out that more concentrated pneumococcal aggregates at individual lung microcompartments may independently enter the competent state. For example, we often observed competence signal reaching high levels locally rather than evenly distributed across the entire lung (Fig. 2, 3, and 5 are presented with adjusted scale bar, so the feature is not obvious, but such features can be seen in Fig. 4C). These results suggest that the freshly inoculated pneumococcus (AD2501) were not evenly dispersed, and only some pockets of the inoculated bacteria managed to adapt to the new host environment, avoided host killing, multiplied, and achieved the locally required density to enter the competent state, which eventually propagated through the entire lung.

Is there an additional competence inhibitor? We notice that contrary to efficient competence shutoff *in vitro*, highly expressed DprA was unable to turn off the competent state in pneumonia-derived sepsis, suggesting that competence termination during pneumonia-derived sepsis is a much more complex process. Interestingly, the existence of additional competence inhibitors in pneumococcal culture medium was suggested by Tomasz and Hotchkiss in the 1960s (12, 15). When added to the competent-state pneumococci, this uncharacterized inhibitor greatly decreased the rate of genetic transformation. It survived filtration, dialysis, and lyophilization (12). In addition, by diluting the culture medium continuously, competence could be maintained at high levels, likely due to the dilution of the inhibitor (15). Evidence supporting the existence of an additional competence inhibitor was observed by Weng et al. (17), who observed that, despite the extended presence of ComX in a protease-deficient pneumococcal mutant, its "late" competence gene expression still decreases (17). Weng et al. proposed the existence of a DprA-independent factor capable of shutting down the expression "late" competence gene even in the presence of ComX. This additional inhibitor may be linked to extracellular factors that can be efficiently diluted *in vivo* during pneumonia-derived sepsis, which is similar to the continuously diluted culture medium used by Tomasz (15).

Cell-cell direct contact or *bona fide* quorum sensing? Our *in vivo* pneumococcal surface-bound CSP cell-cell direct contact model of competence induction is in agreement with earlier (12, 13) and more recent (11) studies. The cell-free plasma experiment suggests that the freely diffusible CSP was only present in minimal amount in blood and was ineffective in driving the noncompetent pneumococcal cells into the competent state. On the contrary, competent-state pneumococcal cells in the blood were able to immediately induce competence in noncompetent Δ*dprA-ssbB-luc* recipient cells (AD2506), further supporting the cell-cell direct contact model. When bacterial numbers begin to increase after 24 hpi, newly replicated cells are likely to carry the competence signal, which may propagate the cell-cell contact signals and prolong the competent state. It is worth noting that we have not attempted to identify the presence of free CSP in the lung, since there is no good method to allow a timely recovery of the competent pneumococcus without exerting strong mechanical force and diluting the competent bacteria.

Low transformation rate in bacteremia/sepsis versus high transformation rate in nasopharyngeal colonization. Our study indicates that the competence system is readily activated at approximately 20 to 24 hpi. Interestingly, prior studies by us (41) and others (19, 20) have demonstrated that the frequency of genetic transformation in the encapsulated pneumococcus during acute pneumonia or bacteremia (through intraperitoneal injection) is very low. In contrast, the genetic transformation in the original studies with dead encapsulated strains and capsule-deficient rough strains occurred at a higher frequency (4, 6). Pneumococcus has a thick polysaccharide capsule layer covering the cell surface, which can be a physical barrier for DNA uptake (20, 44, 45). However, analysis of several recently sequenced pneumococcal genomes suggests that strains with capsules tend to have more frequent genetic exchanges within the genome (46, 47), and recombination events increase with larger capsule size or longer carriage duration (47), with capsules encoding the locus itself being an evolutionary hot spot (48). These results suggest that encapsulated pneumococcal strains are more capable of adapting and surviving longer during carriage, which allow for higher

incidence of genetic uptake and recombination events that increase genomic diversity. In the present study, the clinical serotype 3 strain 0100993, which is heavily encapsulated, showed detectable competence induction in nasal pharyngeal colonization (e.g., Fig. 7B, mice Ey2, Ey3, Ey4, and Ey7). Similarly, the encapsulated serotype 2 strain D39 also showed similar patterns (e.g., Fig. 5B, mice Cy2 and Cy4).

Additional contributing factors to genetic transformation may include the availability of donor DNA. In a previously published gene transfer study during nasopharyngeal colonization, a mixture of 5×10^6 CFU of two D39 derivatives each harboring a different antibiotic resistance marker was delivered intranasally. At 48 h postcolonization, a total of 1×10^5 CFU pneumococcal cells were recovered from each mouse; 8×10^3 CFU of these were recombinants (20). The reduced total recoverable bacteria might indicate the rich availability of genomic DNA released by dead pneumococcal cells within a more confined space. In contrast, other host compartments such as the lung, intraperitoneal cavity, and blood circulation system are larger in volume and therefore may reduce the availability of DNA. Finally, the ability of encapsulated pneumococcal strains to better survive the host assault will result in smaller amounts of donor DNA available for genetic transformation. Collectively, the aforementioned discussion could explain the discrepancy between a highly expressed competence system and an extremely low transformation rate in encapsulated strains (e.g., D39) during host infection.

Competence induction facilitates pneumonia-derived sepsis. How does the competent state confer an advantage to pneumococcus during pneumonia-derived sepsis? Competence induction is energy expensive with the upregulation of approximately 100 genes (14). There has been much debate about the true benefit of this costly multifaceted cellular process (49), among others, genomic fitness versus plasticity (50) and stress response (51, 52). Indeed, the competence-regulated genes are among the most highly expressed of all pneumococcal genes. However, among these, at least 70 genes are not required for transformation (14). Natural competence system has long been implicated to be involved in virulence (21, 53). Previous studies by our lab have shown that the expression of competence-dependent allolytic factors, including LytA, CbpD, and CibAB, benefits both acute pneumonia and bacteremia infections through the release of pneumolysin (26, 28). In addition, *in vitro* studies have shown that these allolytic factors contribute to fratricide, which increases DNA availability by killing a subpopulation of noncompetent pneumococcus (54–59). Fratricide was shown to be important for genetic transformation inside pneumococcal biofilms (60), which has been proposed to resemble nasopharyngeal colonization (20). However, our most recent studies have shown that allolysis, which is a prominent phenotype in hypercompetent capsule-deficient strains R800 and CP1250, is not significant in its parental strain D39 (29), suggesting that the increased expression of LytA, CbpD, and CibAB during the competent state contributes to virulence in functions other than fratricide. Instead, it is more likely that the release of pneumolysin and proinflammatory cell wall components mediated by LytA, CbpD, and CibAB during the prolonged competent state disrupt the alveolar-capillary barrier, leading to a systemic invasion into bloodstream, pneumonia-derived sepsis, multiorgan dysfunction, and eventual death in the mice (26, 61, 62).

In conclusion, we report here for the first time that during pneumonia-derived sepsis, the naturally developed competent state is prolonged and persistent and is recalcitrant to the shutoff mechanism mediated by DprA. The competent state facilitates the systemic invasion that leads to sepsis and death. Physiological adaptation of the pneumococcus to the host environment is critical for the successful induction of the competent state. Competent pneumococcal bacteria propagate the competence signal through cell-to-cell contact rather than the classically described quorum-sensing mechanism. Therapeutic strategies inhibiting the competence regulon, including the use of the dominant negative competence stimulating peptides we have previously developed (27, 41, 63), may offer potential adjunctive therapeutic strategies against pneumonia-derived sepsis caused by pneumococcus.

MATERIALS AND METHODS

Bacterial cultures. *S. pneumoniae* strains D39, TIGR4, and 00100993 were cultured in the THY medium (Becton Dickinson, Franklin Lakes, NJ). D39 cultured in the THY has tendency to form long chains, which leads to an underestimation of the actual number of bacteria inoculated into mice. To reduce inaccuracy, D39 was subcultured from an optical density at 600 nm (OD_{600}) of 0.2 THY culture previously started from a freshly picked single colony by spreading on the Columbia agar supplemented with 5% sheep blood (R01217; Thermo Scientific, Waltham, MA) for 5 h. Bacteria were gently washed off from the agar surface with saline and centrifuged at 3,000 × *g* for 4 min. The bacteria were then washed twice more with sterile saline and diluted to the desired concentration for mouse infection. Microscopic examination indicated that most bacteria were diplococci, which resembled a more natural state of pneumococcal morphology during host infection.

Construction of pneumococcal firefly luciferase reporter strains. Pneumococcal firefly luciferase reporter strains were generated as previously described (29). Briefly, a pEVP3-derived plasmid (64) containing firefly luciferase (pEVP3-*luc*) was inserted with an amplicon of *ssbB* gene through BamHI/KpnI digestion and ligation. The resultant plasmid, pEVP3-*luc-ssbB*, was transformed into the recipient pneumococcal strains D39, TIGR4, and 0100994 by selecting for chloramphenicol-resistant transformants (29).

Construction of D39-*dprA-luc* reporter strain AD2503. Plasmid pEVP3-*luc-dprA* was generated by using an amplicon of the *dprA* gene (forward primer, 5′-CGGGGTACCCCAATCAACAGATTTTGAAAGTTCT; reverse primer, 5′-CGCGGATCCGATTTTTTCTTAGAATTCTAGCTTAGGTC) through BamHI/KpnI digestion and ligation. The amplicon was transformed into pneumococcal strain D39 to create the D39-*dprA-luc* (AD2503) by selecting for chloramphenicol-resistant transformants (29).

Construction of Δ*comCDE-ssbB-luc* reporter strain AD2502. Genomic DNA of the Δ*comCDE* strain AD2064 (65) was extracted and used to transform the strain AD2501 by selecting for transformants that were doubly resistant to kanamycin and chloramphenicol. The intact *ssbB-luc* reporter in strain AD2501 was verified by PCR and DNA sequencing.

Ethics statement. Mouse studies were performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign (protocol 18135).

Mouse infection. Male CD-1 mice (7 to 8 weeks old) were anesthetized with isoflurane and intranasally administered 60 μl of pneumococcal inoculum. The inoculum levels were determined by serial dilution plating onto THY agar plates. At designated times or once moribund, the mice were euthanized, and their lungs or spleens were harvested, homogenized, and enumerated for pneumococcal burden.

***In vivo* imaging.** Infected mice were anesthetized with 3% isoflurane in an induction chamber and subcutaneously administered 100 mg kg^{-1} (body weight) of D-luciferin potassium (LUCK; GoldBio, St. Louis, MO) dissolved in DPBS (21031CV; Corning, Corning, NY) for 5 min before imaging. Anesthetized mice were imaged with the IVIS SpectrumCT imaging system (Perkin-Elmer, Waltham, MA). Luminescence images were acquired using the following settings: binning factor of 8, f number of 1, field of view at 25.4, and a luminescent exposure time of 60 s. Images were analyzed by Living Image software (Perkin-Elmer).

***In vitro* luciferase assays.** Pneumococcal strains harboring competence-regulated firefly luciferase (*luc*) reporters were cultured in 96-well plate at 37°C in a Wallac Victor 2 multilabel counter (Perkin-Elmer). D-Luciferin potassium (GoldBio) was added to the culture to a final concentration of 0.65 mM. The OD_{495} (to measure pneumococcal growth) and the luminescence output were both simultaneously measured. To induce competence, CSP1 was added to the culture medium to a final concentration of 100 ng ml^{-1}.

Competence induction in whole blood and pneumococcus-free plasma isolated from mice with pneumonia-derived sepsis. Mice infected with AD2501 were monitored for competence induction using an IVIS SpectrumCT instrument. When the acute lung infection had progressed into the septic stage where competent pneumococcal cells had systemically spread, whole blood was drawn from each mouse ($n = 10$) into a heparinized tube (Becton Dickinson) with shaking. Blood samples were immediately processed. A portion of whole blood was set aside for later dilution, while the remaining portion was first centrifuged at 1,000 × *g* for 5 min to remove eukaryotic cells, and then the upper-level plasma fraction was centrifuged at 12,000 × *g* for 5 min to further pellet pneumococcal bacteria. Pneumococcus-free plasma was obtained by centrifugation through a 0.22-μm filter (Costar 8160; Corning) at 12,000 × *g* for 5 min. Whole blood (containing pneumococcus) and the pneumococcus-free plasma were serial 2-fold diluted in the THY medium. Then, 100-μl portions of each sample (diluted or undiluted) were mixed with equal volumes of THY medium (containing 1.3 mM D-luciferin potassium) with or without the recipient reporter strain (Δ*dprA-ssbB-luc*; AD2506). Strain AD2506 was cultured in THY medium and used when the density reached an OD_{600} of 0.1 to 0.3, with no further dilution. The luminescence signal was recorded.

Statistical analyses. Quantitative data were expressed as means ± the standard deviations. Statistical significance values were compared using the GraphPad Prism statistical software package. Statistical differences among the group were determined by using the one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. Statistical differences when comparing between two samples within each group were determined by an unpaired Student *t* test. A significant difference was considered to be a *P* value of <0.05. Correlation analysis was performed using the nonparametric Spearman method.

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.9 MB.

ACKNOWLEDGMENTS

We thank David Briles (University of Alabama at Birmingham) for the pneumococcal strain D39 and Donald Morrison (University of Illinois at Chicago) for both the Janus Cassette and the pEVP3 plasmid.

This study was supported in part by the American Lung Association DeSouza Research Award (DS-192835-N), the National Institutes of Health (HL090699 and HL142626A1), and the University of Illinois Research Board award (RB16002) to G.W.L. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. The authors have no competing financial interests and are solely responsible for the experimental designs and data analysis.

J.L. and G.W.L. designed research. J.L., G.W.L., I.T.D., W.D., P.P, H.L., and M.W.O. performed research. J.L. and G.W.L. analyzed data. J.L. and G.W.L. wrote the paper.

REFERENCES

1. Musher DM, Thorner AR. 2014. Community-acquired pneumonia. N Engl J Med 371:1619–1628. https://doi.org/10.1056/NEJMra1312885.
2. Principi N, Marchisio P, Schito GC, Mannelli S, Ascanius Project Collaborative Group. 1999. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Pediatr Infect Dis J 18:517–523. https://doi.org/10.1097/00006454-199906000-00008.
3. Mac LC, Kraus MR. 1950. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed *in vitro*. J Exp Med 92:1–9.
4. Griffith F. 1928. The significance of pneumococcal types. J Hyg (Lond) 27:113–159. https://doi.org/10.1017/s0022172400031879.
5. Avery OT, Macleod CM, McCarty M. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J Exp Med 79:137–158. https://doi.org/10.1084/jem.79.2.137.
6. Dawson MH, Sia RH. 1931. *In vitro* transformation of pneumococcal types. I. A technique for inducing transformation of pneumococcal types *in vitro*. J Exp Med 54:681–699. https://doi.org/10.1084/jem.54.5.681.
7. Hotchkiss RD, Ephrussi-Taylor H. 1951. Use of serum albumin as source of serum factor in pneumococcal transformation. Fed Proc 10:200.
8. Austrian R. 1952. Bacterial transformation reactions. Bacteriol Rev 16:31–50.
9. Ottolenghi E, Hotchkiss RD. 1962. Release of genetic transforming agent from pneumococcal cultures during growth and disintegration. J Exp Med 116:491–519. https://doi.org/10.1084/jem.116.4.491.
10. Morrison DA, Lacks SA, Guild WR, Hageman JM. 1983. Isolation and characterization of three new classes of transformation-deficient mutants of *Streptococcus pneumoniae* that are defective in DNA transport and genetic recombination. J Bacteriol 156:281–290. https://doi.org/10.1128/JB.156.1.281-290.1983.
11. Prudhomme M, Berge M, Martin B, Polard P. 2016. Pneumococcal competence coordination relies on a cell-contact sensing mechanism. PLoS Genet 12:e1006113. https://doi.org/10.1371/journal.pgen.1006113.
12. Tomasz A, Hotchkiss RD. 1964. Regulation of the transformability of pneumococcal cultures by macromolecular cell products. Proc Natl Acad Sci U S A 51:480–487. https://doi.org/10.1073/pnas.51.3.480.
13. Havarstein LS, Coomaraswamy G, Morrison DA. 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. Proc Natl Acad Sci U S A 92:11140–11144. https://doi.org/10.1073/pnas.92.24.11140.
14. Peterson SN, Sung CK, Cline R, Desai BV, Snesrud EC, Luo P, Walling J, Li H, Mintz M, Tsegaye G, Burr PC, Do Y, Ahn S, Gilbert J, Fleischmann RD, Morrison DA. 2004. Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. Mol Microbiol 51:1051–1070. https://doi.org/10.1046/j.1365-2958.2003.03907.x.
15. Tomasz A. 1966. Model for the mechanism controlling the expression of competent state in *Pneumococcus* cultures. J Bacteriol 91:1050–1061. https://doi.org/10.1128/JB.91.3.1050-1061.1966.
16. Mirouze N, Berge MA, Soulet AL, Mortier-Barriere I, Quentin Y, Fichant G, Granadel C, Noirot-Gros MF, Noirot P, Polard P, Martin B, Claverys JP. 2013. Direct involvement of DprA, the transformation-dedicated RecA loader, in the shut-off of pneumococcal competence. Proc Natl Acad Sci U S A 110:E1035–E1034. https://doi.org/10.1073/pnas.1219868110.
17. Weng L, Piotrowski A, Morrison DA. 2013. Exit from competence for genetic transformation in *Streptococcus pneumoniae* is regulated at multiple levels. PLoS One 8:e64197. https://doi.org/10.1371/journal.pone.0064197.
18. Moreno-Gamez S, Sorg RA, Domenech A, Kjos M, Weissing FJ, van Doorn GS, Veening JW. 2017. Quorum sensing integrates environmental cues, cell density and cell history to control bacterial competence. Nat Commun 8:854. https://doi.org/10.1038/s41467-017-00903-y.
19. Dawson MH. 1930. The transformation of pneumococcal types. II. The interconvertibility of type-specific S pneumococci. J Exp Med 51:123–147. https://doi.org/10.1084/jem.51.1.123.
20. Marks LR, Reddinger RM, Hakansson AP. 2012. High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in *Streptococcus pneumoniae*. mBio 3:00200-12. https://doi.org/10.1128/mBio.00200-12.
21. Lau GW, Haataja S, Lonetto M, Kensit SE, Marra A, Bryant AP, McDevitt D, Morrison DA, Holden DW. 2001. A functional genomic analysis of type 3 *Streptococcus pneumoniae* virulence. Mol Microbiol 40:555–571. https://doi.org/10.1046/j.1365-2958.2001.02335.x.
22. Zhu L, Kuang Z, Wilson BA, Lau GW. 2013. Competence-independent activity of pneumococcal EndA [corrected] mediates degradation of extracellular DNA and nets and is important for virulence. PLoS One 8:e70363. https://doi.org/10.1371/journal.pone.0070363.
23. Oggioni MR, Trappetti C, Kadioglu A, Cassone M, Iannelli F, Ricci S, Andrew PW, Pozzi G. 2006. Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol Microbiol 61:1196–1210. https://doi.org/10.1111/j.1365-2958.2006.05310.x.
24. Kowalko JE, Sebert ME. 2008. The *Streptococcus pneumoniae* competence regulatory system influences respiratory tract colonization. Infect Immun 76:3131–3140. https://doi.org/10.1128/IAI.01696-07.
25. Ibrahim YM, Kerr AR, McCluskey J, Mitchell TJ. 2004. Control of virulence by the two-component system CiaR/H is mediated via HtrA, a major virulence factor of *Streptococcus pneumoniae*. J Bacteriol 186:5258–5266. https://doi.org/10.1128/JB.186.16.5258-5266.2004.
26. Zhu L, Lin J, Kuang Z, Vidal JE, Lau GW. 2015. Deletion analysis of *Streptococcus pneumoniae* late competence genes distinguishes virulence determinants that are dependent or independent of competence induction. Mol Microbiol 97:151–165. https://doi.org/10.1111/mmi.13016.
27. Zhu L, Lau GW. 2013. Therapeutic potential of the *Streptococcus pneumoniae* competence regulon. Expert Rev Anti Infect Ther 11:227–229. https://doi.org/10.1586/eri.13.10.
28. Lin J, Zhu L, Lau GW. 2016. Disentangling competence for genetic transformation and virulence in *Streptococcus pneumoniae*. Curr Genet 62:97–103. https://doi.org/10.1007/s00294-015-0520-z.

29. Lin J, Lau GW. 2019. DprA-dependent exit from the competent state regulates multifaceted *Streptococcus pneumoniae* virulence. Infect Immun 87:e00349-19. https://doi.org/10.1128/IAI.00349-19.
30. Attaiech L, Olivier A, Mortier-Barriere I, Soulet AL, Granadel C, Martin B, Polard P, Claverys JP. 2011. Role of the single-stranded DNA-binding protein SsbB in pneumococcal transformation: maintenance of a reservoir for genetic plasticity. PLoS Genet 7:e1002156. https://doi.org/10.1371/journal.pgen.1002156.
31. Guiral S, Henard V, Granadel C, Martin B, Claverys JP. 2006. Inhibition of competence development in *Streptococcus pneumoniae* by increased basal-level expression of the ComDE two-component regulatory system. Microbiology 152:323–331. https://doi.org/10.1099/mic.0.28425-0.
32. Fuqua WC, Winans SC, Greenberg EP. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol 176:269–275. https://doi.org/10.1128/jb.176.2.269-275.1994.
33. Mukherjee S, Bassler BL. 2019. Bacterial quorum sensing in complex and dynamically changing environments. Nat Rev Microbiol 17:371–382. https://doi.org/10.1038/s41579-019-0186-5.
34. Johnston C, Mortier-Barriere I, Khemici V, Polard P. 2018. Fine-tuning cellular levels of DprA ensures transformant fitness in the human pathogen *Streptococcus pneumoniae*. Mol Microbiol 109:663–675. https://doi.org/10.1111/mmi.14068.
35. Weyder M, Prudhomme M, Berge M, Polard P, Fichant G. 2018. Dynamic modeling of *Streptococcus pneumoniae* competence provides regulatory mechanistic insights into its tight temporal regulation. Front Microbiol 9:1637. https://doi.org/10.3389/fmicb.2018.01637.
36. Stevens KE, Chang D, Zwack EE, Sebert ME. 2011. Competence in *Streptococcus pneumoniae* is regulated by the rate of ribosomal decoding errors. mBio 2:e00071-11. https://doi.org/10.1128/mBio.00071-11.
37. Gagne AL, Stevens KE, Cassone M, Pujari A, Abiola OE, Chang DJ, Sebert ME. 2013. Competence in *Streptococcus pneumoniae* is a response to an increasing mutational burden. PLoS One 8:e72613. https://doi.org/10.1371/journal.pone.0072613.
38. Berge MJ, Mercy C, Mortier-Barriere I, VanNieuwenhze MS, Brun YV, Grangeasse C, Polard P, Campo N. 2017. A programmed cell division delay preserves genome integrity during natural genetic transformation in *Streptococcus pneumoniae*. Nat Commun 8:1621. https://doi.org/10.1038/s41467-017-01716-9.
39. Kjos M, Miller E, Slager J, Lake FB, Gericke O, Roberts IS, Rozen DE, Veening JW. 2016. Expression of *Streptococcus pneumoniae* bacteriocins is induced by antibiotics via regulatory interplay with the competence system. PLoS Pathog 12:e1005422. https://doi.org/10.1371/journal.ppat.1005422.
40. Domenech A, Slager J, Veening JW. 2018. Antibiotic-induced cell chaining triggers pneumococcal competence by reshaping quorum sensing to autocrine-like signaling. Cell Rep 25:2390–2400. https://doi.org/10.1016/j.celrep.2018.11.007.
41. Zhu L, Lau GW. 2011. Inhibition of competence development, horizontal gene transfer and virulence in *Streptococcus pneumoniae* by a modified competence stimulating peptide. PLoS Pathog 7:e1002241. https://doi.org/10.1371/journal.ppat.1002241.
42. Cassone M, Gagne AL, Spruce LA, Seeholzer SH, Sebert ME. 2012. The HtrA protease from *Streptococcus pneumoniae* digests both denatured proteins and the competence-stimulating peptide. J Biol Chem 287:38449–38459. https://doi.org/10.1074/jbc.M112.391482.
43. Reference deleted.
44. Yother J, McDaniel LS, Briles DE. 1986. Transformation of encapsulated *Streptococcus pneumoniae*. J Bacteriol 168:1463–1465. https://doi.org/10.1128/jb.168.3.1463-1465.1986.
45. Ravin AW. 1959. Reciprocal capsular transformations of pneumococci. J Bacteriol 77:296–309. https://doi.org/10.1128/JB.77.3.296-309.1959.
46. Rendueles O, de Sousa JAM, Bernheim A, Touchon M, Rocha E. 2018. Genetic exchanges are more frequent in bacteria encoding capsules. PLoS Genet 14:e1007862. https://doi.org/10.1371/journal.pgen.1007862.
47. Chaguza C, Andam CP, Harris SR, Cornick JE, Yang M, Bricio-Moreno L, Kamng'ona AW, Parkhill J, French N, Heyderman RS, Kadioglu A, Everett DB, Bentley SD, Hanage WP. 2016. Recombination in *Streptococcus pneumoniae* lineages increase with carriage duration and size of the polysaccharide capsule. mBio 7:e01053-16. https://doi.org/10.1128/mBio.01053-16.
48. Mostowy RJ, Croucher NJ, De Maio N, Chewapreecha C, Salter SJ, Turner P, Aanensen DM, Bentley SD, Didelot X, Fraser C. 2017. Pneumococcal capsule synthesis locus *cps* as evolutionary hotspot with potential to generate novel serotypes by recombination. Mol Biol Evol 34:2537–2554. https://doi.org/10.1093/molbev/msx173.
49. Johnston C, Martin B, Fichant G, Polard P, Claverys JP. 2014. Bacterial transformation: distribution, shared mechanisms and divergent control. Nat Rev Microbiol 12:181–196. https://doi.org/10.1038/nrmicro3199.
50. Engelmoer DJ, Donaldson I, Rozen DE. 2013. Conservative sex and the benefits of transformation in *Streptococcus pneumoniae*. PLoS Pathog 9:e1003758. https://doi.org/10.1371/journal.ppat.1003758.
51. Claverys JP, Prudhomme M, Martin B. 2006. Induction of competence regulons as a general response to stress in gram-positive bacteria. Annu Rev Microbiol 60:451–475. https://doi.org/10.1146/annurev.micro.60.080805.142139.
52. Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys JP. 2006. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science 313:89–92. https://doi.org/10.1126/science.1127912.
53. Bartilson M, Marra A, Christine J, Asundi JS, Schneider WP, Hromockyj AE. 2001. Differential fluorescence induction reveals *Streptococcus pneumoniae* loci regulated by competence stimulatory peptide. Mol Microbiol 39:126–135. https://doi.org/10.1046/j.1365-2958.2001.02218.x.
54. Steinmoen H, Knutsen E, Havarstein LS. 2002. Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population. Proc Natl Acad Sci U S A 99:7681–7686. https://doi.org/10.1073/pnas.112464599.
55. Havarstein LS, Martin B, Johnsborg O, Granadel C, Claverys JP. 2006. New insights into the pneumococcal fratricide: relationship to clumping and identification of a novel immunity factor. Mol Microbiol 59:1297–1307. https://doi.org/10.1111/j.1365-2958.2005.05021.x.
56. Claverys JP, Martin B, Havarstein LS. 2007. Competence-induced fratricide in streptococci. Mol Microbiol 64:1423–1433. https://doi.org/10.1111/j.1365-2958.2007.05757.x.
57. Eldholm V, Johnsborg O, Haugen K, Ohnstad HS, Havarstein LS. 2009. Fratricide in *Streptococcus pneumoniae*: contributions and role of the cell wall hydrolases CbpD, LytA, and LytC. Microbiology 155:2223–2234. https://doi.org/10.1099/mic.0.026328-0.
58. Eldholm V, Johnsborg O, Straume D, Ohnstad HS, Berg KH, Hermoso JA, Havarstein LS. 2010. Pneumococcal CbpD is a murein hydrolase that requires a dual cell envelope binding specificity to kill target cells during fratricide. Mol Microbiol 76:905–917. https://doi.org/10.1111/j.1365-2958.2010.07143.x.
59. Straume D, Stamsas GA, Salehian Z, Havarstein LS. 2017. Overexpression of the fratricide immunity protein ComM leads to growth inhibition and morphological abnormalities in *Streptococcus pneumoniae*. Microbiology 163:9–21. https://doi.org/10.1099/mic.0.000402.
60. Wei H, Havarstein LS. 2012. Fratricide is essential for efficient gene transfer between pneumococci in biofilms. Appl Environ Microbiol 78:5897–5905. https://doi.org/10.1128/AEM.01343-12.
61. Brown AO, Mann B, Gao G, Hankins JS, Humann J, Giardina J, Faverio P, Restrepo MI, Halade GV, Mortensen EM, Lindsey ML, Hanes M, Happel KI, Nelson S, Bagby GJ, Lorent JA, Cardinal P, Granados R, Esteban A, LeSaux CJ, Tuomanen EI, Orihuela CJ. 2014. *Streptococcus pneumoniae* translocates into the myocardium and forms unique microlesions that disrupt cardiac function. PLoS Pathog 10:e1004383. https://doi.org/10.1371/journal.ppat.1004383.
62. Alhamdi Y, Neill DR, Abrams ST, Malak HA, Yahya R, Barrett-Jolley R, Wang G, Kadioglu A, Toh CH. 2015. Circulating pneumolysin is a potent inducer of cardiac injury during pneumococcal infection. PLoS Pathog 11:e1004836. https://doi.org/10.1371/journal.ppat.1004836.
63. Koirala B, Lin J, Lau GW, Tal-Gan Y. 2018. Development of a dominant negative competence-stimulating peptide (dnCSP) that attenuates *Streptococcus pneumoniae* infectivity in a mouse model of acute pneumonia. Chembiochem 19:2380–2386. https://doi.org/10.1002/cbic.201800505.
64. Claverys JP, Dintilhac A, Pestova EV, Martin B, Morrison DA. 1995. Construction and evaluation of new drug-resistance cassettes for gene disruption mutagenesis in *Streptococcus pneumoniae*, using an ami test platform. Gene 164:123–128. https://doi.org/10.1016/0378-1119(95)00485-o.
65. Zhu L. 2013. The contribution of competence system to the virulence of *Streptococcus pneumoniae*. PhD dissertation. University of Illinois at Urbana-Champaign, Urbana, IL. http://hdl.handle.net/2142/46923.