Published in final edited form as:

Mol Microbiol. 2015 July ; 97(1): 151–165. doi:10.1111/mmi.13016.

Deletion analysis of *Streptococcus pneumoniae* late competence genes distinguishes virulence determinants that are dependent or independent of competence induction

Luchang Zhu^{1,#}, Jingjun Lin^{1,#}, Zhizhou Kuang¹, Jorge E. Vidal², and Gee W. Lau^{1,*}

¹Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, Illinois, United States of America

²Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, Georgia, United States of America

Summary

The competence regulon of *Streptococcus pneumoniae* (pneumococcus) is crucial for genetic transformation. During competence development, the alternative sigma factor ComX is activated, which in turn, initiates transcription of 80 “late” competence genes. Interestingly, only 16 late genes are essential for genetic transformation. We hypothesized that these late genes that are dispensable for competence are beneficial to pneumococcal fitness during infection. These late genes were systematically deleted, and the resulting mutants were examined for their fitness during mouse models of bacteremia and acute pneumonia. Among these, 14 late genes were important for fitness in mice. Significantly, deletion of some late genes attenuated pneumococcal fitness to the same level in both wild-type and ComX-null genetic backgrounds, suggesting that the constitutive baseline expression of these genes was important for bacterial fitness. In contrast, some mutants were attenuated only in the wild-type genetic background but not in the ComX-null background, suggesting that specific expression of these genes during competence state contributed to pneumococcal fitness. Increased virulence during competence state was partially caused by the induction of allolytic enzymes that enhanced pneumolysin release. These results distinguish the role of basal expression versus competence induction in virulence functions encoded by ComX-regulated late competence genes.

Graphical abstract—During genetic transformation of pneumococcus, the alternative sigma factor ComX regulates expression of 14 late competence genes important for virulence. The constitutive baseline expression of some of these genes is important for bacteremia and acute pneumonia infections. In contrast, elevated expression of DprA, CbpD, CibAB, and Cinbox are dependent on competence development, enhancing the release of pneumolysin. These results distinguish the role of basal expression versus competence induction in virulence determinants regulated by ComX.

*To whom correspondence should be addressed: geelau@illinois.edu; phone: 217-333-5077; fax: 217-244-7421.

#These authors contributed equally to the manuscript

The authors declare no conflict of interest.

The authors alone are responsible for the content and writing of the paper.

Keywords

Streptococcus pneumoniae; competence regulon; virulence factors; acute pneumonia; bacteremia

Introduction

Streptococcus pneumoniae (pneumococcus) is an important cause of human infections including pneumonia, otitis media, meningitis and sepsis. The effort to eradicate pneumococcal disease has been hampered by increasing prevalence of antibiotic resistance (Rivera and Boucher, 2011) and the limited protective spectrum of currently licensed vaccines (Mitchell *et al.*, 1997; Tomasz, 1999). Several large-scale insertion duplication and transposon insertion-mediated signature-tagged mutagenesis (STM) screens were conducted in animal models of acute pneumonia, bacteremia and otitis media to identify new virulence factors that might serve as novel drug or vaccine targets (Polissi *et al.*, 1998; Lau *et al.*, 2001; Hava and Camilli, 2002; Chen *et al.*, 2007). Intriguingly, these STM screens identified competence regulon genes including *comA*, *spd_0845*, *spd_1854* and *spd_2026* (Chen *et al.*, 2007); *comB* and *comD* (Lau *et al.*, 2001); *cbpD*, *coiA*, *cinA*, *dinF*, and *cglB* (Hava and Camilli, 2002); and *radA* (Polissi *et al.*, 1998; Hava and Camilli, 2002) to be important for host infection. Additional independent studies have shown that *lytA* (Berry *et al.*, 1989) and *cbpD* (Gosink *et al.*, 2000; Kausmally *et al.*, 2005) are important for virulence.

The development of competence for genetic transformation in pneumococcus resembles a quorum-sensing mechanism (Pestova *et al.*, 1996; Hakenbeck, 2000; Claverys *et al.*, 2006; Johnsborg and Havarstein, 2009). During growth, pneumococcus secretes and accumulates the competence stimulating peptide (CSP) pheromone in the environment. When a threshold concentration is exceeded, CSP interacts with and activates the histidine kinase ComD. Activated-ComD phosphorylates the response regulator ComE, which in turn, positively regulates the transcription of 24 “early” competence genes, including the gene that encodes the alternative sigma factor, ComX (Lee and Morrison, 1999; Luo and Morrison, 2003a; Luo and Morrison, 2003b; Piotrowski *et al.*, 2009). ComX binds to the “combox” in the promoter and associates with RNA polymerase, initiating the transcription of over 80 “late” competence genes (Peterson *et al.*, 2004). Among these, only 16 genes have been established as essential for genetic transformation (Peterson *et al.*, 2004). Even though some of the aforementioned genes have been shown to be important for virulence, with the exception of *comB*, *comD*, *lytA* and *cbpD*, the virulence role of other ComX-regulated late genes are not well characterized. Although some of these genes were identified as important for virulence in STM screens, potential attenuation as a result of polar effect due to insertion into an upstream gene within an operon raises concerns. In addition, attenuation due to competition with other STM mutant strains in a complex pool may not accurately reflect the extent of virulence of attenuation during 1:1 competition between wild-type and individual mutant, and during single infection. We hypothesized that the expression of the remaining ComX-regulated late competence genes might be beneficial for the overall fitness of pneumococcus during host infection. Especially, we want to resolve the intriguing question of whether the basal expression of these late genes is adequate for virulence, or a boost of

expression of these late genes during competence induction is important for host infection. In this study, we performed both systematic gene deletion and epistasis genetic analyses to examine the contribution of ComX-regulated late competence genes to the overall fitness of pneumococcus during mouse models of bacteremia and acute pneumonia infection.

Results and Discussion

ComX-deficient mutant is attenuated in mouse models of bacteremia and acute pneumonia

ComX, the alternative sigma factor specifically expressed in the competent pneumococcus cells, initiates the expression of many essential effectors for DNA uptake, processing, integration, as well as some genes of unknown functions (Lee and Morrison, 1999; Luo et al., 2003a). ComX is encoded by two identically redundant genes — *comX1* and *comX2* (Lee and Morrison, 1999). To determine if ComX played a role in survival and fitness during host infection, we analyzed *comX1*, *comX2* and *comX1 comX2* in direct competitive infection against parental wild-type D39 in mouse models of bacteremia and acute pneumonia. Deletion of either *comX1* or *comX2* gene alone did not alter the competitiveness of the mutants during blood and lung infection (Fig. 1A and B). However, *comX1 comX2* was only 20 and 23% as competitive as D39 during infection of blood and lungs, respectively. In addition, *comX1 comX2* was also attenuated in mouse models of single bacteremia and acute pneumonia by 1.34 and 1.29 logs, respectively (see Fig. 3A and B below).

ComX-regulated late competence genes that are important for survival and fitness during bacteremia and acute pneumonia

Attenuation of virulence in *comX1 comX2* prompted us to examine the contribution of ComX-regulated late competence genes to infection. Initially, we constructed 19 deletion mutants to allow full coverage of all the nonessential “late” gene operons that carry a combox and tested these mutants for competition against the wild-type D39 in a mouse model of bacteremia. Of these, 9 operons were identified to harbor virulence genes (Fig. 2). Each gene within these 9 operons was individually deleted in a nonpolar manner, and tested for attenuation during competitive infection against D39 in mouse models of bacteremia and acute pneumonia. As a result, a total of 14 genes were found to be important in bacteremia and/or acute pneumonia models infection (Fig. 2, Table 1).

Because competitive infection may not reveal the true levels of attenuation, we selected five mutants and examined their virulence in both the bacteremia and acute pneumonia models of single infections. All five mutants — *comX1 comX2*, *radC*, *recA*, *spd_0981*, and *spd_0031* — were attenuated in their ability to cause bacteremia (Fig. 3A). In addition, four out of the five mutants, with the exception of *radC*, were attenuated in their ability to cause acute pneumonia (Fig. 3B). These results suggest that *in vivo*, 1:1 competition assays between parental wild-type D39 and individual mutants are good indicators of virulence attenuation for single infections. Furthermore, ComX, RecA, SPD_0981 and SPD_0031 are important for infection in both lung and blood. In contrast, under our experimental conditions, RadC only plays a role during bacteremia but not acute pneumonia.

Interestingly, contrary to previously published insertional STM studies (Have and Camilli, 2002; Chen *et al.*, 2007), non-polar deletion mutants *coiA*, *dinF*, *cglB*, *spd_0845*, *spd_1854* and *spd_2026*, are not attenuated in virulence in current studies, as well as in a recently published study on the virulence of *cglABCDEFG* (Zhu *et al.*, 2013). There are several potential reasons for the discrepancy: (i) the virulence potential of pneumococcal strain D39 and derivatives are different than the TIGR4 strain used by Hava and Camilli (2002) and the otitis media strain ST556 by Chen *et al.* (2007); (ii) *Spd_0845*, *Spd_1854* and *Spd_2026* may only play a role in otitis media; and (iii) these genes play a minimal role in virulence in respective infection models, and their attenuation is only obvious in a complex STM mutant pool of up to 96 strains but not during 1:1 competition or single infection.

Functional annotation and analysis of ComX-regulated genes important for virulence

Various mutants are tentatively grouped based on their known or predicted functions (Table 1). Detailed characterization of some individual mutants are presented and briefly discussed below:

Stress responses

radA— *radA* was only 41% and 32% as competitive as to the parental wild-type D39 in the bacteremia and acute pneumonia models of infection, respectively (Table 1, Fig. 2). These results concurred with two previous STM screens that identified *radA* as important for survival within host (Polissi *et al.*, 1998; Have and Camilli, 2002). Protein sequence analysis indicate that RadA is a RecA-like NTPase that possesses an ATP binding site, a walker A motif and a walker B motif. RadA is important for genetic recombination and transformation in *Escherichia coli* and *Bacillus subtilis* (Beam *et al.*, 2002; Carrasco *et al.*, 2002). However, its role in the genetic transformation of pneumococcus remains controversial. Burghout *et al.* (2007) have shown that RadA is required for transformation. In contrast, Peterson *et al.* (2004) showed that it is dispensable for transformation. Nevertheless, *radA* is more susceptible to mutagen MMS than its parental wild-type (Burghout *et al.*, 2007), suggesting that RadA plays a role in DNA repair.

recA— *recA* was only 0.4% and 0.3% as competitive as D39 (Table 1) and was attenuated by 2.38 and 4.75 logs in bacteremia and acute pneumonia models of single infections, respectively (Fig. 3A and B). The pneumococcal *recA* gene (Fig. 4A) encodes a DNA recombination protein essential for genetic transformation (Martin *et al.*, 1992; Martin *et al.*, 1995). RecA protects the single stranded DNA (ssDNA) (Berge *et al.*, 2003) and plays an important role in searching for homologous sequences to allow DNA recombination (Kowalczykowski *et al.*, 1994; Steffen and Bryant, 2000)

recA grew slower than D39 (Fig. 4B). We found that *recA* was about 4-fold more sensitive to hydrogen peroxide (H₂O₂) than D39 (Fig. 4C). Pneumococcal pyruvate oxidase SpxB is responsible for the production of endogenous H₂O₂ (Spellerberg *et al.*, 1996; Regev-Yochay *et al.*, 2007). *spxB* produces 100-fold less H₂O₂ than D39 (Spellerberg *et al.*, 1996). Deletion of the *spxB* gene in the *recA* genetic background partially restored the growth defect of *recA* (Fig. 4B). These results indicate that RecA protects pneumococcus against endogenous H₂O₂-mediated DNA damage.

Interestingly, DNA sequence analysis reveals the presence of two promoters (*p1* and *p2*) regulating the expression of *recA* gene. *p1* is located at the upstream of the *cinA* gene. *p1* harbors a combox, which is also called the “cinbox”, and is only active during the competence stage (Pearce *et al.*, 1995). Activation of *p1* is required for the optimal recombination proficiency during genetic transformation (Mortier-Barriere *et al.*, 1998). The *p2* promoter is located immediately upstream of the *recA* gene. *p2* is constitutive and is required for the baseline expression of the *recA* gene (Pearce *et al.*, 1995). Deletion of *p1* neither influenced the *in vitro* growth nor altered the H₂O₂ sensitivity of the pneumococcus (Fig. 4C). In contrast, deletion of *p2* generated a mutant with a growth defect (Fig. 4B) and increased susceptibility to H₂O₂, phenotypes that were very similar to *recA* (Fig. 4C). Collectively, these results suggest that *p2* regulates the growth and stress response to H₂O₂, and that both processes are independent of the induction of competence for genetic transformation.

spd_1778— *spd_1778* was only 9.8% and 15% as competitive as D39 in mouse models of bacteremia and acute pneumonia infection, respectively (Table 1). Spd_1778 is a member of the bacterial RmuC family of DNA recombination proteins. RmuC of *E. coli* limits the rate of DNA inversions at short inverted repeats (Slupska *et al.*, 2000), suggesting a potential role in DNA recombination. Furthermore, RmuC is a component of the *E. coli* LexA regulon, which controls bacterial response to DNA damage (van Dyk *et al.*, 2001). van Opijnen and Camilli (2012) have reported that the RmuC (SP_1981 in pneumococcal strain TIGR4) is involved in the resistance against DNA gyrase inhibitor Norfloxacin and the DNA damaging agent methyl methanesulfonate. These reports further suggest a role for Spd_1778 in protecting pneumococcus against DNA damage.

spd_0031— *spd_0031* was only 8% and 5% as competitive as D39 in mouse models of bacteremia and acute pneumonia (Table 1). It was also 1.75 log and 1.64 log more attenuated during single bacteremia and acute pneumonia infections (Fig. 3). *spd_0031* encodes a protein that belongs to the PrsW family of peptidases. In *Clostridium difficile*, PrsW cleaves the anti-sigma factor RsiT to allow increasing expression of the sigma factors CsfT and CsfU. In turn, CsfT and CsfU positively regulate resistance to antimicrobial peptides that damage the bacterial membrane and to other agents that cause cell envelope stress (Ho and Ellermeier, 2011). The *prsW* mutant of *C. difficile* is 30-fold less virulent than wild-type (Ho and Ellermeier, 2011). Similarly, PrsW of *B. subtilis* degrades the anti-sigma factor RsiW, and senses antimicrobial peptides and cell envelope stress (Ellermeier and Losick; 2006).

radC— *radC* was 10% as competitive as D39 in bacteremia model of infection. No significance attenuation could be observed in acute pneumonia (Table 1). The CIs were reflected in the single infection, where *radC* is attenuated by 1.55 log in the bacteremia, but only by 0.75 log in the acute pneumonia (Fig. 3A and B). The *radC* gene was originally named after the *radC102* mutant of *E. coli* (Felzenszwalb *et al.*, 1984). This mutant has a moderate increase in sensitivity to radiation and X-ray, suggesting that RadC plays a role in DNA repair. However the relevant mutation was subsequently shown to be in the *recG* gene. Thus, the original *radC* mutation was in fact an allele of *recG* (Lombardo and Rosenberg,

2000). The function of pneumococcal RadC remains to be determined. Claverys and co-workers have shown that pneumococcal RadC plays no role in the repair of DNA lesions, recombination during genetic transformation, gene conversion nor in mismatch repair (Attaiech *et al.*, 2008). Protein sequence analysis suggests that RadC belongs to the JAB superfamily of metalloproteins that might function as a nuclease.

Transporter

spd_1828— *spd_1828* was only 14.8% as competitive as D39 in a mouse model of bacteremia infection, but nearly as competitive (66%) as the parental wild-type in the acute pneumonia infection (Table 1). *spd_1828* was first identified by Bartilson and colleagues (2001) to be the CSP-inducible gene *CPIP788*. Similar to our results, these authors found that *CPIP788* was significantly attenuated during blood infection but was as virulent as the wild-type in the lungs. Bioinformatic analysis indicates that Spd_1828 is a 43 aa protein harboring an ABC ATPase domain with similarity to the iron (III) dicitrate transport ATP-binding protein FecE in both *Neisseria polysaccharea* and *N. meningitidis*, which is the conserved domain described in Figure 5. However, Spd_1828 of D39 and other pneumococcal isolates are much shorter (43 aa – 64 aa) when compared to homologs from other streptococcal species and from other bacteria, including the *Neisseria* spp (217 aa – 278 aa). This suggests that there may have been a mis-annotation of Spd_1828 in D39 and other pneumococcal isolates (Fig. 5). Alternatively, Spd_1828 of pneumococcus has undergone a species-specific truncation during evolution.

Cellular metabolism

spd_0030— *spd_0030* was only 2.8% and 13% as competitive as D39 in the mouse models of bacteremia and acute pneumonia, respectively (Table 1). *spd_0030* encodes a pneumococcal homolog of carbonic anhydrase (PCA), an enzyme that catalyzes the reversible hydration of carbon dioxide ($\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$) (Hewett-Emmett and Tashian, 1996; Lindskog, 1997; Smith *et al.*, 1999; Smith and Ferry, 2000). There are three distinct families — α , β , and γ -PCAs — with no significant sequence identity or structural similarity (Hewett-Emmett and Tashian, 1996). DNA sequence analysis revealed that PCA belongs to the β -class and is highly conserved among various pneumococcal isolates (Smith and Ferry, 2000). CO_2 is important for the growth of pneumococcus (Kempner and Schlayer, 1942), likely because it needs CO_2 or HCO_3^- during the biosynthesis of nucleic acids, amino acids, and fatty acids (Aguilera *et al.*, 2005). Under CO_2 -rich conditions, pneumococcus may obtain CO_2 through diffusion. However, under CO_2 -poor conditions, fixation of the gas by PCA is required for pneumococcal growth (Burghout *et al.*, 2010). The growth defects of the PCA-deficient mutants under low CO_2 conditions could be restored by addition of unsaturated fatty acids, suggesting that PCA-mediated CO_2 fixation is at least associated with HCO_3^- -dependent de novo biosynthesis of unsaturated fatty acids (Burghout *et al.*, 2010). Similar to previous observations (Hewett-Emmett and Tashian, 1996), *spd_0030* was unable to grow at 37°C in the absence of 5% CO_2 (Fig. 6). Finally, consistent with our mouse infection results, PCA-mediated CO_2 fixation pathways have been shown to be important for invasion of the human brain microvascular endothelial cells and uptake by the murine J774 macrophage cells (Burghout *et al.*, 2010).

cinA— *cinA* was not attenuated in the mouse model of bacteremia, but slightly less competitive than D39 (CI 0.64) during lung infection (Table 1). A *cinA*-insertion mutant was less competitive during lung infection in a STM screen (Hava and Camilli, 2002). *cinA* is the first gene in the *cinA-recA-dinF-lytA* operon. Protein sequence analysis suggests that CinA has a molybdopterin binding domain with similarity to proteins involved in the biosynthesis of molybdopterin cofactor.

As described earlier, the *pI* promoter in the *cinA-recA-dinF-lytA* operon (Fig. 4A) contains a combox that is only active during the competence stage (Pearce *et al.*, 1995). Interestingly, *pI* was more attenuated than *cinA* in the acute pneumonia model of infection (Table 1). In addition, *pI* was less competitive than D39 in the bacteremia infection (Table 1), suggesting that increased expression of *cinA*, *recA*, *dinF* and *lytA* during competence might be important for virulence. Because *dinF* was not attenuated in both models of mouse infection (data not shown), it was likely that the ability to upregulate the expression of *cinA*, *recA* and *lytA* during competence contributed to pneumococcal fitness *in vivo*. Collectively, these results suggest the existence of competence-induced virulence versus basal expression-dependent virulence in pneumococcus (see Fig. 9 below).

spd_0981— *spd_0981* was only 15% and 24% as competitive as D39 in mouse models of bacteremia and acute pneumonia (Table 1). It was also attenuated by 2.18 log and 1.43 log during single infection of blood and lung (Fig. 3). *Spd_0981* encodes a protein with a CYTH-like superfamily domain. Enzymes belonging to this superfamily hydrolyze triphosphate-containing substrates, require metal cations as cofactors, and have a unique active site located at the center of an eight-stranded antiparallel beta-barrel tunnel (the triphosphate tunnel). The name CYTH originated from gene designation for the bacterial class IV adenylyl cyclases (CyaB) (Smith *et al.*, 2006) and from thiamine triphosphatase (Kohn *et al.*, 2012). This domain superfamily also contains RNA triphosphatases that catalyze the first step in mRNA capping (Gong *et al.*, 2006). Enzymes with this domain are believed to be involved in the nucleotide or organic phosphate metabolism (Iyer and Aravind, 2002).

Allolytic genes

Allolysis is a unique physiological phenomenon that occurs in the pneumococcal cells competent for genetic transformation. This process involves the lysis of a subpopulation of pneumococcal cells, resulting in the release of cell wall components, DNA, as well as virulence factors, including pneumolysin (Guiral *et al.*, 2005). During competence, ComX positively regulates the expression of four allolytic genes: *lytA*, *cbpD*, *cibA* and *cibB* (Guiral *et al.*, 2005). *LytA* is a major pneumococcal autolysin important for virulence (Berry *et al.*, 1989). *lytA* is the fourth gene in the *cinA* operon (Fig. 4A). The expression of *lytA* gene is driven by a constitutive promoter and a competence-specific promoter upstream of *cinA* gene (*pI*). The expression of *lytA* increases about 4-fold in pneumococcal cells competent for genetic transformation (Peterson *et al.*, 2004). *CbpD* is a cell wall hydrolase specifically expressed in the pneumococcal cells competent for genetic transformation (Peterson *et al.*, 2004; Kausmally *et al.*, 2005). It is required for partial cell lysis and DNA release during competence (Kausmally *et al.*, 2005). A *cbpD* mutant was identified in a STM screen in

mouse lungs (Hava and Camilli, 2002). *cibA* and *cibB* are two genes in the same operon that encode two double-glycine bacteriocins important for the competence-mediated autolysis (Fig. 2) (Guiral *et al.*, 2005). Our results showed that *cbpD*, *cibAB*, and *lytA* were all less competitive than D39 in mouse models of bacteremia and acute pneumonia (Table 1). Both *cbpD* and *cibAB* are moderately attenuated in single infection of bacteremia and acute pneumonia in mice (Fig. 7A and B). Similar to a previous report (Orihuela *et al.*, 2004), *lytA* was attenuated during single bacteremia and acute pneumonia. Moreover, our results indicated that the level of virulence attenuation in *lytA* was more severe than in *cbpD* and *cibAB* (Fig. 7A and B). Collectively, these results suggest that CbpD, LytA and CibAB are important for virulence during both acute pneumonia and bacteremia.

We performed genetic epistasis analyses to determine the hierarchical order of *cbpD*, *cibAB* and *lytA*, during single bacteremia and acute pneumonia infection. The results indicated that the *cbpD cibAB* mutant was not more attenuated than *cbpD* or *cibAB* alone (Fig. 7A and B). Although *cbpD lytA*, *cibAB lytA* and *cbpD cibAB lytA* were slightly more attenuated than *lytA*, the differences were not statistically significant. Moreover, *cbpD cibAB lytA* was not more attenuated than *cbpD lytA* or *cibAB lytA*. These results suggest that LytA is the most dominant autolytic enzyme during pneumococcal pathogenesis. Nevertheless, it is likely that LytA acts together with minor autolytic enzymes CbpD or CibAB to cause synergistic host infections, which is in line with their observed synergistic action *in vitro* during autolysis. One possible mechanism of further reduction in virulence of *cbpD lytA*, *cibAB lytA*, and *cbpD cibAB lytA* mutants could be related to decreasing levels of toxic effect of pneumolysin release in autolysis *in vivo*. Finally, CbpD and CibAB may have redundant function within the same pathway, as the loss-of-function in both proteins does not confer additional attenuation than individual protein alone.

Competence regulation

dprA— *dprA* was only 7% and 1% as competitive as D39 in bacteremia and acute pneumonia models of infection, respectively (Table 1). DprA is a dual functional competence protein essential for genetic transformation. During transformation, DprA binds to ssDNA and delivers it to RecA for recombination (Mortier-Barriere *et al.*, 2007). In addition, DprA is required for the protection of ssDNA during competence. In the absence of DprA, the incoming ssDNA is degraded rapidly and could not be processed for recombination (Berge *et al.*, 2003). Interestingly, two recent studies have reported that DprA is also required for shutting off the competence development (Mirouse *et al.*, 2013; Weng *et al.*, 2013). DprA binds to ComE and prevents the binding of the transcriptional activator to its promoter. *dprA* has difficulty exiting from the competence state and has a growth defect when stimulated with the CSP.

If the inability of *dprA* to exit the competence state contributed to its virulence attenuation, we hypothesized that a *dprA* mutant constructed in a pneumococcal strain deficient in competence induction would still be as virulent as its parental strain. We deleted the *dprA* gene in the *comB* genetic background. Because of its inability to export CSP, *comB* was not able to develop competence by itself. As shown in Fig. 8, *dprA comB* was as competitive as *comB* in mouse models of bacteremia and acute pneumonia infection.

Because neither *comB* nor *dprA comB* is able to develop spontaneous competence, these results suggest that the impact of DprA on bacterial competitiveness is dependent on competence induction. Moreover, because mutations in other genes for DNA uptake and integration, such as *ssbB* and *coiA*, did not reduce the competitiveness of the mutants (Fig. 8), it was likely that the DNA uptake and integration functions of DprA were not important for infection. Therefore, DprA contributes to bacterial fitness during infection by regulating physiological exit from the competence state rather than by processing DNA. Collectively, genetic analysis of the *dprA* locus suggests the existence of competence-induced virulence versus basal expression-dependent virulence in pneumococcus.

Competence dependent and independent virulence among the late genes within the competence regulon

Among the 14 deletion mutants, we had noticed that some strains were more attenuated than *comX1 comX2*. For example, both *spd_1778* and *spd_0030* were more attenuated than the *comX1 comX2* during competition with D39 (Table 1). In addition, deletion analysis of *dprA* locus in the *comB* genetic background (Fig. 8B), as well as the comparative study between *cinA* versus *p1* (with deletion of *combox*) (Table 1), suggest the existence of competence-related virulence versus basal expression-dependent virulence in pneumococcus. These results compelled us to examine additional analyses to differentiate the existence of competence-related virulence versus basal expression-dependent virulence in pneumococcus. We compared the competitiveness of 10 mutants derived from the D39 (*cbpD*, *cibAB*, *p1*, *lytA*, *radC*, *spd_0030*, *spd_0031*, and *spd_0981*, *spd_1778*, *spd_1828*) against the parental strain D39, versus the same 10 mutants derived from the *comX1 comX2* mutant background (e.g., *cbpD comX1 comX2*) against the parental strain *comX1 comX2*, using a mouse model of bacteremia. Among these mutants, *lytA*, *radC*, *spd_0030*, *spd_0031*, *spd_0981*, *spd_1778*, and *spd_1828* constructed in both D39 and *comX1 comX2* backgrounds were attenuated during bacteremia infection when competed against D39 and *comX1 comX2*, respectively (Fig. 9). These results suggest that the loss of virulence in these mutants is not dependent on the induction of competence by ComX. Rather, the baseline expression of these genes is important for bacterial fitness during mouse bacteremia infection. In contrast, *cbpD*, *cibAB*, and *p1* were attenuated in D39 background but not in the *comX1 comX2* background, suggesting that the attenuation of these three mutants was ComX-dependent (Fig. 9). Thus, increased expression of CbpD, CibAB and of the genes downstream of the *combox* in the *cinA-recA-dinF-lytA* operon during competence induction is important for bacterial fitness during infection. These results, together with those of the *dprA* (Fig. 8), indicate the existence of competence-induced virulence, which can be uncoupled from the basal expression-dependent virulence in pneumococcus.

Competence dependent virulence among the late genes is partially due to the release of pneumolysin

Pneumolysin is an important virulence factor of *S. pneumoniae* (reviewed in Mitchell and Dalziel, 2014), and is released during autolysis in pneumococcal cells competent for genetic transformation (Guiral *et al.*, 2005). We examined whether the competence dependent-virulence mediated by autolytic enzymes CbpD, CibAB and LytA regulated by *p1 combox*

was caused by increased release of pneumolysin during competence-induced allosolysis. A non-polar deletion mutant in the *ply* gene encoding pneumolysin (Shak *et al.*, 2013) was used as negative control. In addition, *dprA* was included to determine the effect of inability to turn off competence induction would have on the release of pneumolysin. As expected, Western blot analysis indicated that *ply* did not express pneumolysin (Fig. 10A, Fig. S1). After induction by CSP1, the level of pneumolysin in culture supernatants increased by 3-fold in the wild-type strain D39. Significantly, the changes of pneumolysin release were minimal in the *comX1 comX2*, *lytA* and *cbpD* during competence induction. In contrast, induction of competence by CSP1 induced a 2.3-fold increase in the release of pneumolysin in *cibAB*, *p1*, and *dprA*. Collectively, these results suggest that ComX regulates the induction of pneumolysin release by upregulating the allosolytic enzymes. *LytA* and *CbpD* but not *CibAB* are the main contributors to the release of pneumolysin. Importantly, *dprA* displayed higher basal levels of pneumolysin release when compared to D39, confirming that inability to turn off the competent state contributed to excessive allosolytic enzymes that increased pneumolysin release and potentially bacterial death and attenuation during host infection. Various release profiles of pneumolysin mirrored the extent of sheep blood hemolysis by various pneumococcus mutants (Fig. 10B), with the attenuation of blood hemolysis in the *comX1 comX2* was comparable to *ply* and *lytA*.

To determine whether the virulence of *LytA*, *CbpD*, *CibAB* were dependent on their ability to release pneumolysin, we compared the competitiveness of D39-derived *lytA*, *cbpD* and *cibAB* against the parental strain D39, versus the same mutants derived from the *ply* mutant background (e.g., *cbpD ply*) against the parental strain *ply*, using a mouse model of bacteremia. The *lytA* constructed in both D39 and *ply* backgrounds were attenuated during bacteremia infection when competed against D39 and *ply*, respectively (Fig. 10C). These results suggest that the loss of virulence in *lytA* was not exclusively dependent on the release of pneumolysin during competence induction. Rather, the baseline expression of *LytA* is important for bacterial fitness during mouse bacteremia infection. In contrast, *cbpD* and *cibAB* were attenuated in D39 background but not in the *ply* background, suggesting that the attenuation of these two mutants was due to their reduced ability to release pneumolysin (Fig. 10C). Collectively, these results suggest that increased virulence during competence induction is at least partially dependent on elevated release of pneumolysin mediated by allosolytic enzymes.

Conclusions

In this study, we conducted a systematic deletion analysis on ComX-regulated “late” competence genes. We demonstrated that ComX and 14 ComX-regulated genes contribute to bacterial fitness during acute pneumonia and/or bacteremia infections in mice. Most significantly, induction of *dprA* contributes to pneumococcal fitness by appropriately regulating exit from competence. Specific induction of *cbpD*, *cibAB* and *p1* promoter-regulated genes during competence contributes to bacterial virulence by enhancing the release of pneumolysin. In contrast, constitutive baseline expression of *lytA*, *radC*, *spd_0030*, *spd_0031*, *spd_0981*, *spd_1778*, and *spd_1828* is important for bacterial fitness during bacteremia infection. Collectively, these results suggest that the competence regulon

needs to be tightly regulated to optimize pneumococcal fitness and survival during host infection.

Experimental procedures

Bacterial strains and growth conditions

S. pneumoniae D39 (Avery *et al.*, 1979) was a generous gift from Dr. David Briles (University of Alabama-Birmingham). Bacteria were streaked from frozen stocks onto the Todd Hewitt Broth (THB) agar containing 5% defibrinated horse blood and incubated at 37°C with 5% CO₂. Fresh colonies were grown in THB to desired density.

Construction of gene replacement mutants

Pneumococcal genes were deleted using the Janus cassette according to the previous published methods (Sung *et al.*, 2001). Briefly, ~ 500 bp of DNA fragments flanking the gene targeted for deletion were amplified by PCR, and were ligated to the two ends of kanamycin and/or erythromycin resistance cassettes by overlapping PCR. Following gel purification, the PCR-amplified ligated products were transformed into appropriate recipient strains as we have previously described (Lau *et al.*, 2001). Transformants were selected on THB agar supplemented with 200 µg/ml of kanamycin or 0.3 µg/ml of erythromycin.

Ethics statement

The animal study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign (Protocol Number: 12230).

Mouse models of single acute pneumonia and bacteremia infection

CD1 mice (6-week old) (Charles River) were housed in positively-ventilated microisolator cages with automatic recirculating water, located in a room with laminar, high efficiency particle accumulation–filtered air. The animals received autoclaved food, water, and bedding. For the mouse model of acute pneumonia, animals were anesthetized with isoflurane and intranasally administered 10⁶ colony forming unit (CFU) pneumococcal cells. After 48 hr, lungs were removed from the euthanized animals, homogenized and enumerated for bacterial burden. For the bacteremia model, CD1 mice were inoculated intraperitoneally with 10⁴ CFU of pneumococcal cells. After 24 hr, pneumococcal burden in the spleens were enumerated. Moribund animals that displayed rough hair coat, hunched posture, distended abdomen, lethargy or inability to eat or drink were euthanized immediately.

In vivo and in vitro competitive assays

In vivo competition assays were performed as previously described (Mei *et al.*, 1997; Lau *et al.*, 2001). Briefly, CD 1 mice were inoculated with a pneumococcal suspension containing a 1:1 mixture of parental strain (e.g., D39) and its mutant derivative, using the bacterial concentration described above for single infections. At designated times, mouse lungs or spleens were harvested, homogenized and plated onto THB agar plates with and without

antibiotics. The competitive index (CI) was defined as the output ratio of mutant to wild-type bacteria divided by the input ratio of mutant to wild-type bacteria. A mutant with a CI of 0.7 or lower was tentatively defined as attenuated as previously described (Mei *et al.*, 1997; Lau *et al.*, 2001).

For *in vitro* growth competitions, both parental strain and each deletion mutant were allowed to grow in THB to OD_{600nm} of 0.1. One ml of both cultures were mixed thoroughly, and then diluted 100-fold in fresh THB. Five ml of the diluted mixture was then allowed to grow for 6 hr, serially diluted and plated onto THB agar with or without antibiotics for CFU determination.

Hemolysis assays

Hemolytic assays were performed as previously described [Paton *et al.* 1983], with some modifications. Pneumococcal strains were grown in THB to OD_{600nm} of 0.2 and then treated with CSP1 (10ng/ml) or PBS. After 20 min of incubation, supernatants were collected by pelleting cells at 3,000 × g for 15 min and filtering through 0.22 µm syringe filters (Millipore). Two-fold serial dilutions of the supernatants was performed with fresh THB before incubating with 10 mM dithiothreitol (DTT) at room temperature for 15 min. 500 µl of each sample was then mixed with 200 µl of 2% sheep red blood cells. After incubating at 37°C for 30 min, the mixture was centrifuged at 3,000 × g for 5 min. The absorbance of the cell-free supernatant was measured at OD_{541nm}. The hemolytic units (HU) were reported as the highest dilution that could lyse 50% of the red blood cells.

Western blot analysis of pneumolysin release

Two milliliters of supernatants prepared in the aforementioned hemolytic assays were concentrated to final volume of 40 µl (concentration factor 50x) using centrifugal filters (Amicon Ultra –2mL 30K Centrifugal Filter) following manufacture's instruction. Ten microliters of concentrated samples were subjected to Western blot analysis by probing with primary antibody against pneumolysin (Santa Cruz Biotechnology sc-80500, 1:1000 dilution). Pneumolysin expression was detected with a secondary goat anti-mouse IgG-HRP (Santa Cruz Biotechnology sc-2005, 1:10,000 dilution) by using the ECL substrate (Bio-Rad, 170-5060). Relative abundance of pneumolysin was quantified by densitometry using the ImageJ software.

Statistical analyses

Quantitative data were expressed as the mean ± standard error. Statistical significance of was compared using the GraphPad Prism statistical software package. A significant difference was considered to be $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. David Briles (University of Alabama at Birmingham) for the pneumococcal strain D39; Dr. Donald Morrison (University of Illinois at Chicago) for genomic DNA samples containing the *rpsL* and *pcomX-lacZ* constructs; and Dr. Malcolm Winkler (Indiana University) for his gift of the *spxB* mutant. Also, we want to thank Shawn Choe for critical editing of this manuscript. This work was supported by the University of Illinois Research Board Arnold O Beckman Award, the American Lung Association DeSouza Research Award (DS-192835-N) and the NIH (HL090699) to GW Lau.

References

- Aguilera J, van Dijken JP, De Winde JH, Pronk JT. Carbonic anhydrase (Nce103p): an essential biosynthetic enzyme for growth of *Saccharomyces cerevisiae* at atmospheric carbon dioxide pressure. *Biochem J.* 2005; 391:311–316. [PubMed: 15948716]
- Attaiech L, Granadel C, Claverys JP, Martin B. RadC, a misleading name? *J Bacteriol.* 2008; 190:5729–5732. [PubMed: 18556794]
- Avery OT, MacLeod CM, McCarty M. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Inductions of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J Exp Med.* 1979; 149:297–326. [PubMed: 33226]
- Bartilson M, Marra A, Christine J, Asundi JS, Schneider WP, Hromockyj AE. Differential fluorescence induction reveals *Streptococcus pneumoniae* loci regulated by competence stimulatory peptide. *Mol Microbiol.* 2001; 39:126–135. [PubMed: 11123694]
- Beam CE, Saveson CJ, Lovett ST. Role for radA/sms in recombination intermediate processing in *Escherichia coli*. *J Bacteriol.* 2002; 184:6836–6844. [PubMed: 12446634]
- Berge M, Mortier-Barriere I, Martin B, Claverys JP. Transformation of *Streptococcus pneumoniae* relies on DprA- and RecA-dependent protection of incoming DNA single strands. *Mol Microbiol.* 2003; 50:527–536. [PubMed: 14617176]
- Berry AM, Lock RA, Hansman D, Paton JC. Contribution of autolysin to virulence of *Streptococcus pneumoniae*. *Infect Immun.* 1989; 57:2324–2330. [PubMed: 2568343]
- Burghout P, Bootsma HJ, Kloosterman TG, Bijlsma JJ, de Jongh CE, Kuipers OP, Hermans PW. Search for genes essential for pneumococcal transformation: the RADA DNA repair protein plays a role in genomic recombination of donor DNA. *J Bacteriol.* 2007; 189:6540–6550. [PubMed: 17631629]
- Burghout P, Cron LE, Gradstedt H, Quintero B, Simonetti E, Bijlsma JJ, et al. Carbonic anhydrase is essential for *Streptococcus pneumoniae* growth in environmental ambient air. *J Bacteriol.* 2010; 192:4054–4062. [PubMed: 20525828]
- Carrasco B, Fernandez S, Asai K, Ogasawara N, Alonso JC. Effect of the recU suppressors sms and subA on DNA repair and homologous recombination in *Bacillus subtilis*. *Mol Genet Genomics.* 2002; 266:899–906. [PubMed: 11810266]
- Chen H, Ma Y, Yang J, O'Brien CJ, Lee SL, Mazurkiewicz JE, Haataja S, Yan JH, Gao GF, Zhang JR. Genetic requirement for pneumococcal ear infection. *PLoS One.* 2007; 3:e2950. [PubMed: 18670623]
- Claverys JP, Prudhomme M, Martin B. Induction of competence regulons as a general response to stress in gram-positive bacteria. *Annu Rev Microbiol.* 2006; 60:451–475. [PubMed: 16771651]
- van Dyk TK, DeRose EJ, Gonye GE. LuxArray, a high-density, genomewide transcription analysis of *Escherichia coli* using bioluminescent reporter strains. *J Bacteriol.* 2001; 183:5496–5505. [PubMed: 11544210]
- Ellermeier CD, Losick R. Evidence for a novel protease governing regulated intramembrane proteolysis and resistance to antimicrobial peptides in *Bacillus subtilis*. *Genes Dev.* 2006; 20:1911–1922. [PubMed: 16816000]
- Felzenszwalb I, Sargentini NJ, Smith KC. Characterization of a new radiation-sensitive mutant, *Escherichia coli* K-12 radC102. *Radiat Res.* 1984; 97:615–625. [PubMed: 6374741]
- Gong C, Smith P, Shuman S. Structure-function analysis of *Plasmodium* RNA triphosphatase and description of a triphosphate tunnel metalloenzyme superfamily that includes Cet1-like RNA triphosphatases and CYTH proteins. *RNA.* 2006; 12:1468–1474. [PubMed: 16809816]

- Gosink KK, Mann ER, Guglielmo C, Tuomanen EI, Masure HR. Role of novel choline binding proteins in virulence of *Streptococcus pneumoniae*. *Infect Immun*. 2000; 68:5690–5695. [PubMed: 10992472]
- Guiral S, Mitchell TJ, Martin B, Claverys JP. Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements. *Proc Natl Acad Sci U S A*. 2005; 102:8710–8715. [PubMed: 15928084]
- Hakenbeck R. Transformation in *Streptococcus pneumoniae*: mosaic genes and the regulation of competence. *Res Microbiol*. 2000; 151:453–456. [PubMed: 10961458]
- Hava DL, Camilli A. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. *Mol Microbiol*. 2002; 45:1389–1406. [PubMed: 12207705]
- Hewett-Emmett D, Tashian RE. Functional diversity, conservation, and convergence in the evolution of the alpha-, beta-, and gamma-carbonic anhydrase gene families. *Mol Phylogenet Evol*. 1996; 5:50–77. [PubMed: 8673298]
- Ho TD, Ellermeier CD. PrsW is required for colonization, resistance to antimicrobial peptides, and expression of extracytoplasmic function sigma factors in *Clostridium difficile*. *Infect Immun*. 2011; 79:3229–3238. [PubMed: 21628514]
- Hui FM, Morrison DA. Genetic transformation in *Streptococcus pneumoniae*: nucleotide sequence analysis shows comA, a gene required for competence induction, to be a member of the bacterial ATP-dependent transport protein family. *J Bacteriol*. 1991; 173:372–381. [PubMed: 1987129]
- Hui FM, Zhou L, Morrison DA. Competence for genetic transformation in *Streptococcus pneumoniae*: organization of a regulatory locus with homology to two lactococcal A secretion genes. *Gene*. 1995; 153:25–31. [PubMed: 7883181]
- Iyer LM, Aravind L. The catalytic domains of thiamine triphosphatase and CyaB-like adenylyl cyclase define a novel superfamily of domains that bind organic phosphates. *BMC Genomics*. 2002; 3:33. [PubMed: 12456267]
- Johnsborg O, Håvarstein LS. Regulation of natural genetic transformation and acquisition of transforming DNA in *Streptococcus pneumoniae*. *FEMS Microbiol Rev*. 2009; 33:627–642. [PubMed: 19396959]
- Kausmally L, Johnsborg O, Lunde M, Knutsen E, Havarstein LS. Choline-binding protein D (CbpD) in *Streptococcus pneumoniae* is essential for competence-induced cell lysis. *J Bacteriol*. 2005; 187:4338–4345. [PubMed: 15968042]
- Kempner W, Schlayer C. Effect of CO(2) on the Growth Rate of the Pneumococcus. *J Bacteriol*. 1942; 43:387–396. [PubMed: 16560508]
- Kohn G, Delvaux D, Lakaye B, Servais AC, Scholer G, Fillet M, et al. High inorganic triphosphatase activities in bacteria and mammalian cells: identification of the enzymes involved. *PLoS One*. 2012; 7:e43879. [PubMed: 22984449]
- Kowalczykowski SC, Eggleston AK. Homologous pairing and DNA strand-exchange proteins. *Annu Rev Biochem*. 1994; 63:991–1043. [PubMed: 7979259]
- Lau GW, Haataja S, Lonetto M, Kensit SE, Marra A, Bryant AP, et al. A functional genomic analysis of type 3 *Streptococcus pneumoniae* virulence. *Mol Microbiol*. 2001; 40:555–571. [PubMed: 11359563]
- Lee MS, Morrison DA. Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. *J Bacteriol*. 1999; 181:5004–5016. [PubMed: 10438773]
- Lindskog S. Structure and mechanism of carbonic anhydrase. *Pharmacol Ther*. 1997; 74:1–20. [PubMed: 9336012]
- Lombardo MJ, Rosenberg SM. radC102 of *Escherichia coli* is an allele of recG. *J Bacteriol*. 2000; 182:6287–6291. [PubMed: 11053371]
- Lucas R, Czikora I, Sridhar S, Zemskov E, Gorshkov B, Siddaramappa U, et al. Mini-review: novel therapeutic strategies to blunt actions of pneumolysin in the lungs. *Toxins (Basel)*. 2013; 5:1244–60. [PubMed: 23860351]
- Luo P, Li H, Morrison DA. ComX is a unique link between multiple quorum sensing outputs and competence in *Streptococcus pneumoniae*. *Mol Microbiol*. 2003a; 50:623–633. [PubMed: 14617184]

- Luo P, Morrison DA. Transient association of an alternative sigma factor, ComX, with RNA polymerase during the period of competence for genetic transformation in *Streptococcus pneumoniae*. *J Bacteriol.* 2003b; 185:349–358. [PubMed: 12486073]
- Martin B, Ruellan JM, Angulo JF, Devoret R, Claverys JP. Identification of the *recA* gene of *Streptococcus pneumoniae*. *Nucleic Acids Res.* 1992; 20:6412. [PubMed: 1475203]
- Martin B, Garcia P, Castanie MP, Claverys JP. The *recA* gene of *Streptococcus pneumoniae* is part of a competence-induced operon and controls lysogenic induction. *Mol Microbiol.* 1995; 15:367–379. [PubMed: 7538190]
- Mei JM, Nourbakhsh F, Ford CW, Holden DW. Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. *Mol Microbiol.* 1997; 26:399–407. [PubMed: 9383163]
- Mirouze N, Berge MA, Soulet AL, Mortier-Barriere I, Quentin Y, Fichant G, et al. Direct involvement of DprA, the transformation-dedicated RecA loader, in the shut-off of pneumococcal competence. *Proc Natl Acad Sci U S A.* 2013; 110:E1035–1044. [PubMed: 23440217]
- Mitchell TJ, Alexander JE, Morgan PJ, Andrew PW. Molecular analysis of virulence factors of *Streptococcus pneumoniae*. *Soc Appl Bacteriol Symp Ser.* 1997; 26:62S–71S. [PubMed: 9436318]
- Mitchell TJ, Dalziel CE. The biology of pneumolysin. *Subcell Biochem.* 2014; 80:145–60. [PubMed: 24798011]
- Mortier-Barriere I, de Saizieu A, Claverys JP, Martin B. Competence-specific induction of *recA* is required for full recombination proficiency during transformation in *Streptococcus pneumoniae*. *Mol Microbiol.* 1998; 27:159–170. [PubMed: 9466264]
- Mortier-Barriere I, Velten M, Dupaigne P, Mirouze N, Pietrement O, McGovern S, et al. A key presynaptic role in transformation for a widespread bacterial protein: DprA conveys incoming ssDNA to RecA. *Cell.* 2007; 130:824–836. [PubMed: 17803906]
- van Opijnen T, Camilli A. A fine scale phenotype-genotype virulence map of a bacterial pathogen. *Genome Res.* 2012; 22:2541–2551. [PubMed: 22826510]
- Orihuela CJ, Gao G, Francis KP, Yu J, Tuomanen EI. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. *J Infect Dis.* 2004; 190:1661–1669. [PubMed: 15478073]
- Paton JC, Lock RA, Hansman DJ. Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. *Infect Immun.* 1983; 40:548–552. [PubMed: 6840851]
- Pearce BJ, Naughton AM, Campbell EA, Masure HR. The *rec* locus, a competence-induced operon in *Streptococcus pneumoniae*. *J Bacteriol.* 1995; 177:86–93. [PubMed: 7798154]
- Pestova EV, Havarstein LS, Morrison DA. Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. *Mol Microbiol.* 1996; 21:853–862. [PubMed: 8878046]
- Peterson SN, Sung CK, Cline R, Desai BV, Snesrud EC, Luo P, et al. Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. *Mol Microbiol.* 2004; 51:1051–1070. [PubMed: 14763980]
- Piotrowski A, Luo P, Morrison DA. Competence for genetic transformation in *Streptococcus pneumoniae*: termination of activity of the alternative sigma factor ComX is independent of proteolysis of ComX and ComW. *J Bacteriol.* 2009; 191:3359–3366. [PubMed: 19286798]
- Polissi A, Pontiggia A, Feger G, Altieri M, Mottl H, Ferrari L, Simon D. Large-scale identification of virulence genes from *Streptococcus pneumoniae*. *Infect Immun.* 1998; 66:5620–5629. [PubMed: 9826334]
- Ramos-Montanez S, Tsui HC, Wayne KJ, Morris JL, Peters LE, Zhang F, 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; 67:729–746. [PubMed: 18179423]
- 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; 189:6532–6539. [PubMed: 17631628]

- Rivera AM, Boucher HW. Current concepts in antimicrobial therapy against select gram-positive organisms: methicillin-resistant *Staphylococcus aureus*, penicillin-resistant pneumococci, and vancomycin-resistant enterococci. *Mayo Clin Proc.* 2011; 86:1230–1243. [PubMed: 22134942]
- Shak JR, Ludewick HP, Howery KE, Sakai F, Yi H, Harvey RM, Paton JC, Klugman KP, Vidal JE. Novel role for the *Streptococcus pneumoniae* toxin pneumolysin in the assembly of biofilms. *MBio.* 2013; 4(5):e00655–13. [PubMed: 24023386]
- Slupska MM, Chiang JH, Luther WM, Stewart JL, Amii L, Conrad A, Miller JH. Genes involved in the determination of the rate of inversions at short inverted repeats. *Genes Cells.* 2000; 5:425–437. [PubMed: 10886369]
- Smith KS, Jakubzick C, Whittam TS, Ferry JG. Carbonic anhydrase is an ancient enzyme widespread in prokaryotes. *Proc Natl Acad Sci U S A.* 1999; 96:15184–15189. [PubMed: 10611359]
- Smith KS, Ferry JG. Prokaryotic carbonic anhydrases. *FEMS Microbiol Rev.* 2000; 24:335–366. [PubMed: 10978542]
- Smith N, Kim SK, Reddy PT, Gallagher DT. Crystallization of the class IV adenylyl cyclase from *Yersinia pestis*. *Acta Crystallogr Sect F Struct Biol Cryst Commun.* 2006; 62:200–204.
- Spellerberg B, Cundell DR, Sandros J, Pearce BJ, Idanpaan-Heikkila I, Rosenow C, Masure HR. Pyruvate oxidase, as a determinant of virulence in *Streptococcus pneumoniae*. *Mol Microbiol.* 1996; 19:803–813. [PubMed: 8820650]
- Steffen SE, Bryant FR. Purification and characterization of the RecA protein from *Streptococcus pneumoniae*. *Arch Biochem Biophys.* 2000; 382:303–309. [PubMed: 11068882]
- Steinmoen H, Knutsen E, Havarstein LS. 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.* 2002; 99:7681–7686. [PubMed: 12032343]
- Sung CK, Li H, Claverys JP, Morrison DA. An rpsL cassette, janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. *Appl Environ Microbiol.* 2001; 67:5190–5196. [PubMed: 11679344]
- Tomasz A. New faces of an old pathogen: emergence and spread of multidrug-resistant *Streptococcus pneumoniae*. *Am J Med.* 1999; 107:55S–62S. [PubMed: 10451010]
- Weng L, Piotrowski A, Morrison DA. Exit from competence for genetic transformation in *Streptococcus pneumoniae* Is regulated at multiple levels. *PLoS One.* 2013; 8:e64197. [PubMed: 23717566]
- Zhu L, Lau GW. Inhibition of competence development, horizontal gene transfer and virulence in *Streptococcus pneumoniae* by a modified competence stimulating peptide. *PLoS Pathog.* 2011; 7:e1002241. [PubMed: 21909280]
- Zhu L, Kuang Z, Wilson BA, Lau GW. Competence-independent activity of pneumococcal EndA mediates degradation of extracellular DNA and NETs and is important for virulence. *PLoS One.* 2013; 8:e70363. [PubMed: 23936195]

Fig. 1. ComX is important for bacteremia and acute pneumonia infections

(A) CD-1 mice (n=5) were intraperitoneally challenged with a suspension containing a 1:1 mixture of D39 and each mutant strain (1×10^4 CFU/strain). After 24 hr, spleens were harvested, homogenized, serially diluted and plated onto THB agar plates with or without kanamycin to determine the competitive indexes (CI). Each dot indicates the CI from one mouse. Bar shows the mean CI. * $p < 0.05$ when comparing the CI of $\Delta comX1 \Delta comX2$ against D39 using the GraphPad Prism statistical software.

(B) CD 1 mice (n = 5) were intranasally challenged with 1:1 mixture of D39 and each mutant strain (5×10^6 CFU/strain). After 48 hr, lungs were harvested for CI determination. * $p < 0.05$ when comparing the CI of $\Delta comX1 \Delta comX2$ against D39 using the GraphPad Prism statistical software.

Fig. 2.

ComX-regulated “late” competence gene loci important for host infection. Nineteen operons that contained the 54 late competence genes were non-polarly deleted by using the Janus cassette, and tested for their ability to compete against D39 in mouse model of bacteremia and acute pneumonia. Of these, deletion of 9 operons yielded mutants with reduced virulence. Subsequently, each gene within these 9 operons was deleted, and tested for virulence. Of these, 14 genes were identified to be important for infection in mouse models of bacteremia and/or acute pneumonia (See Table 1). Red boxes: Genes required for genetic transformation. Shaded boxes: Genes required for infections. “C” = combox in the promoter of each gene locus. Boxes with dotted lines: essential genes that were not included during the deletion process. *spd_0974* and *spd_0976* are transcribed as antisense RNA during competence.

Fig. 3. Virulence confirmation of five deletion mutants by single bacteremia and acute pneumonia infections

(A) CD-1 mice (n = 7) were intraperitoneally infected with 1×10^4 of D39, *comX1 comX2*, *recA*, *spd_0981*, *radC*, or *spd_0031*. Mouse spleens were harvested 24 hr post-infection for bacterial enumeration. * $p < 0.05$ when comparing the log₁₀ CFU of individual mutants against D39 by using the GraphPad Prism statistical software.

(B) CD-1 mice (n = 10) were intranasally infected with 5×10^6 of D39, *comX1 comX2*, *recA*, *spd_0981*, *radC*, or *spd_0031*. Mouse lungs were harvested 48 hr post-infection for bacterial enumeration. * $p < 0.05$ when comparing the log₁₀ CFU of individual mutants against D39 by using the GraphPad Prism statistical software.

Fig. 4. Contribution of *recA* and its promoters to growth and to resistance to H₂O₂ stress

(A) The organization of the *cinA-recA-dinF-lytA* operon. *p1* harbors a combox, which is active during competence. *p2* is a constitutive promoter of *recA*.

(B) The growth curves of D39 versus *recA*, *p1*, *p2* and *recA spxB*. The growth experiments were performed in the THB medium in triplicates for three times. The mean $\pm$ standard deviation from one typical experiment is shown.

(C) H₂O₂ sensitivity of D39 versus various mutants. 1×10^3 CFU of each strain were seeded into 1 ml of THB supplemented with indicated concentrations of H₂O₂. Pneumococcal strains were then incubated 8 hr at 37°C with 5% CO₂. “-” indicated no growth. “+” indicated positive growth. The experiments were repeated independently three times with similar results. The results from a representative experiment are shown.

Fig. 5.

Spd_1828 is truncated in pneumococcus. Protein alignment of Spd_1828 against various homologs from pneumococcal strains, and other *Streptococcus* and *Neisseria* species. DNA alignments were performed using a software program from DNA STAR. Boxed region indicate the ATP-binding domain.

Fig. 6.

spd_0030 has growth defect in the absence of added CO₂. *D39* and *spd_0030* were grown to OD₆₀₀ of 0.1, and then subjected to 10-fold serial dilutions. Each diluent (20 μ l) was spotted onto the THB agar. The plates were incubated at 37°C overnight with or without 5% CO₂ and photographed. The experiments were repeated independently three times with similar results. The results from one representative experiment are shown.

Fig. 7. Altolysis genes are important for bacteremia and acute pneumonia infections

(A) CD-1 mice ($n = 6$) were intraperitoneally infected with 1×10^4 of D39, *cbpD*, *cibAB*, *lytA*, *cbpD cibAB*, *cbpD lytA*, *cibAB lytA*, or *cbpD cibAB lytA*. Mouse lungs were harvested 24 hr post-infection for bacterial enumeration. * $p < 0.05$ when comparing the log₁₀ CFU of individual mutants against D39 by using the GraphPad Prism statistical software.

(B) CD-1 mice ($n = 7$) were intranasally infected with 5×10^6 of pneumococcal strains described in A. Mouse lungs were harvested 48 hr post-infection for bacterial enumeration. * $p < 0.05$ when comparing the log₁₀ CFU of individual mutants against D39 by using the GraphPad Prism statistical software.

Fig. 8. A factor that regulates exit from competence development is important for virulence. Analysis of *dprA* competitiveness against D39 or *comB*, as well as *coiA* and *ssbB* competitiveness against D39

(A) CD-1 mice ($n = 5$) were intranasally infected with 1:1 ratio (1×10^4 bacteria/strain) of D39 versus *dprA*, or *comB* versus *dprA comB*. After 24 hr, spleens were isolated for CI determination. Each dot indicates the CI from an individual mouse. Bar shows the mean CI. * $p < 0.05$ when comparing the CI of *dprA* against D39 by using the GraphPad Prism statistical software.

(B) CD-1 mice ($n = 5$) were intranasally infected with 1:1 ratio (2.5×10^6 bacteria/strain) of D39 versus *dprA*, or *comB* versus *dprA comB*. After 48 hr, lungs were harvested for CI determination. Each dot indicates the CI from an individual mouse. Bar shows the mean CI. * $p < 0.05$ when comparing the CI of *dprA* against D39 by using the GraphPad Prism statistical software.

Fig. 9.

In vivo competition assays differentiate the importance of basal versus competence-induced expression in genes required for bacteremia infection in CD-1 mice (n = 5). Competition assays of late competence gene mutants in D39 and *comX*-null genetic backgrounds. Columns 1–2: D39 versus *cbpD*, *comX1 comX2* vs. *cbpD comX1 comX2*; columns 3–4: D39 vs. *cibAB*, *comX1 comX2* vs. *cibAB comX1 comX2*; columns 5–6: D39 vs. *p1*, *comX1 comX2* vs. *p1 comX1 comX2*; columns 7–8: D39 vs. *lytA*, *comX1 comX2* vs. *lytA comX1 comX2*; columns 9–10: D39 vs. *radC*, *comX1 comX2* vs. *radC comX1 comX2*; columns 11–12: D39 vs. *spd_0030*, *comX1 comX2* vs. *spd_0030 comX1 comX2*; columns 13–14: D39 vs. *spd_0031*, *comX1 comX2* vs. *spd_0031 comX1 comX2*; columns 15–16: D39 vs. *spd_0981*, *comX1 comX2* vs. *spd_0981 comX1 comX2*; columns 17–18: D39 vs. *spd_1778*, *comX1 comX2* vs. *spd_1778 comX1 comX2*; columns 19–20: D39 vs. *spd_1828*, *comX1 comX2* vs. *spd_1828 comX1 comX2*. **p* < 0.05 when comparing the CI of each mutant derived from D39 against D39 versus the CI of each mutant derived from *comX1 comX2* against *comX1 comX2* by using the GraphPad Prism statistical software.

Fig. 10.

Competence-dependent virulence is partially mediated through the enhanced release of pneumolysin. (A) Hemolytic analysis of cell-free culture supernatants from the wild-type D39, *ply*, *comX1 comX2*, *dprA*, *lytA*, *cbpD*, *cibAB*, and *p1*. Hemolytic assays were independently performed six times in triplicates. Results from one typical experiment is shown. (B) Western blot analysis of concentrated culture supernatants from (A) by using an anti-pneumolysin antibody. Experiments were performed independently three times. Western blots from one typical experiment are shown. Densitometry analysis of pneumolysin release can be found in Fig. S1. (C) *In vivo* bacteremia infection in CD-1 mice ($n = 5 - 9$) between D39 against *lytA*, *cbpD*, and *cibAB*, and between *ply* against *ply lytA*, *ply cbpD*, and *ply cibAB*. * $p < 0.05$ when comparing the CI of *cbpD* and *cibAB* against D39 versus the CI of *cbpD ply* and *cibAB ply* against *ply* by using the GraphPad Prism statistical software.

Table 1

Attenuated late competence gene knockout mutants identified in this study

Strains	Deleted gene(s)	Function	Bacteremia CI	Pneumonia CI	Growth CI
REFERENCE STRAINS					
D39	/		1	1	1
<i>comX1 comX2</i>	<i>comX1, comX2</i>		0.2	0.23	0.97
STRESS RESPONSES					
<i>radA</i>	<i>radA</i>	DNA repair	0.41	0.32	1.13
<i>recA</i>	<i>recA</i>	DNA recombination and repair	0.004	0.003	0.2
<i>spd_1778</i>	<i>spd_1778</i>	RnuC family protein, DNA repair	0.098	0.15	0.96
<i>spd_0031</i>	<i>spd_0031</i>	PrsW protease family	0.08	0.05	0.97
<i>radC</i>	<i>radC</i>	Unknown	0.1	0.78	1.02
TRANSPORTER					
<i>spd_1828</i>	<i>spd_1828</i>	ABC transporter	0.148	0.66	0.96
CELLULAR METABOLISM					
<i>spd_0030</i>	<i>spd_0030</i>	Carbonic anhydrase	0.028	0.13	0.89
<i>cinA</i>	<i>cinA</i>	Biosynthesis of molybdopterin	1.11	0.64	1.02
<i>p1</i>	<i>combox</i>	Promoter of <i>cinA</i> operon	0.41	0.32	1.08
<i>spd_0981</i>	<i>spd_0981</i>	Hypothetical protein	0.15	0.24	0.98
ALLOLYSIS					
<i>lytA</i>	<i>lytA</i>	Autolysin	0.53	0.16	1.13
<i>chpD</i>	<i>chpD</i>	Amidase or endopeptidase	0.43	0.35	0.92
<i>cibA cibB</i>	<i>cibA-cibB</i>	Bacteriocins	0.5	0.37	1.18
COMPETENCE REGULATION AND DNA PROCESSING					
<i>dprA</i>	<i>dprA</i>	DNA processing protein	0.07	0.01	0.77