Molecular mechanisms of pneumococcal fratricide

Molekylære mekanismar bak fratricide i *Streptococcus pneumoniae*

Philosophiae Doctor (PhD) Thesis

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List of papers

Paper I

Eldholm V, Johnsborg O, Haugen K, Ohnstad HS & Håvarstein LS (2009)
Fratricide in *Streptococcus pneumoniae*: contributions and role of the cell wall hydrolases CbpD, LytA and LytC. Microbiology. 155; 2223-2234.

Paper II

Eldholm V, Gutt B, Johnsborg O, Brückner R, Maurer P, Hakenbeck R, Mascher T & Håvarstein LS (2010) The pneumococcal cell envelope stress-sensing system LiaFSR is activated by murein hydrolases and lipid II-interacting antibiotics. J. Bacteriol **192**: 1761-1773.

Paper III

Eldholm V, Johnsborg O, Straume D, Ohnstad HS, Berg KH, Hermoso JA & Håvarstein LS
Pneumococcal CbpD is a murein hydrolase that requires a dual cell-envelope binding-specificity to kill target cells during fratricide. Mol. Microbiol. (in press)

Other relevant papers by the author:

Johnsborg O, Eldholm V, Bjørnstad ML & Håvarstein LS (2008)
A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species. Mol. Microbiol. **69**: 245-253.

Johnsborg O, Eldholm V & Håvarstein LS (2007)
Natural genetic transformation: prevalence, mechanisms and function. Res. Microbiol. **158**: 767-778.

Abstract

The bacterium *Streptococcus pneumoniae* is endowed with the ability to take up naked DNA from the surroundings and recombine this DNA into the chromosome. This property, termed competence for natural genetic transformation, plays a fundamental role in the biology and evolution of this pathogen. Induction of the competent state is controlled by a quorum sensing-like system: binding of the secreted peptide pheromone CSP to its membrane-embedded receptor ComD activates its cognate cytoplasmic response regulator ComE. Phosphorylated ComE triggers expression of its target genes by binding to a conserved promoter element. Among the ComE regulated genes is *comM* encoding an immunity protein, and *comX* which encodes the alternative sigma factor σ^x. Among the σ^x regulated genes are genes encoding proteins involved in so-called fratricide (lat: killing of brothers), including the autolysin LytA and the putative murein hydrolase CbpD. LytC encoded by a σ^{70} dependent gene has also been shown to be important for pneumococcal fratricide. Recent research indicates that fratricide mediates release of DNA from the predated target cells. This liberated DNA can subsequently be taken up and incorporated into the genomes of the attacking cells. Competent pneumococci are able to kill and lyse both non-competent pneumococci and other closely related species. This phenomenon might thus be a central component underlying gene exchange between streptococci, a phenomenon that for example can lead to the spread of antibiotic resistance and vaccine escape. Fratricide might also play a role in the release of virulence factors during host invasion.

There is in other words mounting evidence that fratricide is an important phenomenon both from an evolutionary and a public health perspective. The main aim of the work presented here has been to gain a better understanding of pneumococcal fratricide with a focus on the molecular mechanisms underlying the phenomenon.

In paper I we show that lysis of target cells is more efficient when LytA and LytC are provided by the target cells than when they are provided by the attacking cells. Whereas LytC was found in copious amounts in the growth medium of both competent and non-competent cells, LytA could only be detected in whole-cell extracts. Elution of choline-binding proteins from the cell envelopes of competent cells did not result in release of detectable levels of LytA. A close correlation between cell death and LytA mediated autolysis was also established. Together, these results suggest that LytA might be an intracellular protein that is released upon cell death. CbpD could be detected in the supernatant, but elution with choline showed that most of the protein was attached to the cell envelope. CbpD is normally dependent on LytA and LytC for efficient lysis of target cells, but we found that in the presence of 1.0 mM EDTA, this dependency was lost.

In paper II it was established that upon competence-induction in the absence of ComM, the three-component system LiaFSR (Spr0342-HK-RR03) is induced by the concerted action of CbpD, LytA and LytC. The system was also found to protect competent cells against these lysins. Deletion of the response regulator LiaR led to a doubling of lysis upon competence-induction regardless of the presence of ComM, but in cultures harboring a functional ComM, lysis remained very low. In accordance with results from other Firmicutes, the LiaFSR system was found to be induced by the lipid II interacting antimicrobials bacitracin and nisin. Whole-genome microarray analysis of gene expression upon treatment with sub-lethal doses of bacitracin revealed a total of 18 genes, organized in six transcriptional units, to be up-regulated more than 2-fold in the wildtype strain compared to the corresponding *liaR* mutant. Deletion of two of these genes, *spr*0810 and *pcpC*, alone and in combination revealed that these two genes were responsible for most, but not all the protective effect of LiaFSR upon competence induction.

In the final part of the study, reported in paper III, zymogram analyses of protein extracts established that CbpD is a murein hydrolase.

CbpD consists of an N-terminal CHAP domain followed by two SH3b domains and a C-terminal choline-binding domain. A CbpD fusion protein with the CHAP domain exchanged with green fluorescent protein was found to target the division zone of pneumococci. Deletion studies revealed that this targeting was conferred on the protein by the choline-binding domain. The SH3b domains of CbpD were shown to bind peptidoglycan. Further studies established that both binding-specificities are essential for CbpD to lyse target cells. Finally, transmission electron microscopy revealed that CbpD causes ruptures in the division zone of cells under attack.

Samandrag

Streptococcus pneumoniae kan ta opp DNA frå omgjevnadane og inkorporere dette arvematerialet i sitt eige genom. Denne evna spelar ei fundamental rolle i evolusjonen til denne bakterien. Kompetanse for genetisk transformasjon er kontrollert av eit "quorum sensing" liknande system: Binding av peptidferomonet CSP til reseptoren ComD aktiverer responsregulatoren ComE. ComE aktiverer transkripsjon av ei fleire gener, blant anna immunitetsproteinet ComM og den alternative sigmafaktoren σ^x. Blant dei σ^x kontrollerte genene finn ein gener som kodar for protein involvert i såkalla "fratricide" (broderdrap), mellom anna CbpD og autolysinet LytA. LytC som er koda av eit σ^{70}-kontrollert gen er óg viktig for fratricide. Nyare forskning indikerer at fratricide medfører frigjering av DNA frå dei predaterte målcellene. Frigjort DNA kan i neste omgang takast opp og integrerast i genoma til angriparcellene. Kompetente pneumokokkar kan drepe og lysere både ikkje-kompetente pneumokokkar og andre nært beslekta artar. Dette fenomenet kan difor vere ein sentral komponent i genutveksling mellom streptokokkar, eit fenomen som mellom anna kan medføre spreiing av antibiotikaresistens. Det er óg mogeleg at fratricide bidreg til frigjering av virulensfaktorar ved invasjon av verten.

Det er altså mykje som tyder på at fratricide er eit viktig fenomen både i eit evolusjonært perspektiv og eit folkehelseperspektiv. Hovudmålet med arbeidet har vore å auke kunnskapen om dette fenomenet med eit fokus på dei molekylære mekanismane som ligg bak.

I artikkel I syner vi at lysis av målceller er meir effektiv når LytA og LytC vert produsert av målcellene sjølv enn om dei vert produsert av angriparane. LytC vart funne i store mengder i vekstmediet til både kompetente og ikkje-kompetente celler. LytA vart berre detektert i celle-ekstrakt, i hovudsak i kompetente celler. Eluering av cholin-bindande protein frå celleoverflata medførte ikkje frigjering av målbare mengder LytA. Vidare såg vi at det er ein nær korrelasjon mellom celledaud og LytA-indusert autolyse. Saman tyder desse resultata på at LytA kan vere eit intracellulært protein som vert frigjort og aktivert ved celledaud. CbpD vart funne i vekstmediet under kompetanse, men eluering med cholin indikerte at det meste av proteinet var bunde til celleoverflata. CbpD er normalt avhengig av LytA og LytC for effektiv lysis av målceller, men vi fann at tilsats av 1,0 mM EDTA gjorde CbpD i stand til effektivt å lysere målceller aleine.

I artikkel II vart det vist at tre-komponent systemet LiaFSR vert aktivert av CbpD, LytA og LytC ved kompetanse-induksjon i fråvér av ComM. Systemet viste seg óg å verne cellene mot desse lysina. Delesjon av responsregulatoren LiaR medførte ei dobling av lysis ved kompetanse-induksjon uavhengig av om ComM var til stades, men i kulturar med eit funksjonelt ComM heldt andelen lyserte celler seg låg. Som i andre Firmicute bakteriar, vart LiaFSR aktivert av dei lipid II interagerande antimikrobielle midla bacitracin og nisin. Microarray analysar av endringar i genekspresjon ved behandling med sub-letale dosar av bacitracin resulterte i deteksjon av 18 LiaR regulerte gener organisert i seks operon. Delesjon av to av desse gena, *spr0810* og *pcpC*, aleine og i kombinasjon, avslørte at desse to genene er ansvarlige for det meste, men ikkje all den beskyttande effekten av LiaFSR ved kompetanse-induksjon.

I den siste delen av studia, rapportert i artikkel III, viste vi ved hjelp av zymogram analysar av protein ekstrakt at CbpD er ein murein hydrolase. CbpD er sett saman av eit N-terminalt CHAP domene etterfulgt av to SH3b domener og eit C-terminalt cholin-bindande domene. Vi fann at eit fusjonsprotein av CbpD med CHAP domenet byta ut med grønt fluoroscerande

protein (GFP) i hovudsak bind celledelingssona i pneumokokkar. Delesjosstudier avdekka at det cholin-bindande domenet er ansvarleg for denne bindingsspesifisiteten. SH3b domena viste seg å binde peptidoglykan. Vidare studier viste at begge desse bindingsspesifisitetane er naudsynte for at CbpD skal lysere målceller. Til slutt avdekka transmisjons-elektronmikroskopi at CbpD påfører rifter i celledelingssona på angripne celler.

Introduction

Horizontal gene transfer between bacteria

Bacteria reproduce by clonal propagation rather than by sexual reproduction. Yet, the genetic structure of many bacterial species indicate panmixia within populations (86). The reason for this apparent contradiction is the occurrence of horizontal gene transfer (HGT). HGT can occur via three different mechanisms: conjugation (self-propagation of plasmids), transduction (DNA transfer via bacteriophage infection) or natural genetic transformation (NGT, active acquisition of DNA by the bacterium). The latter mechanism is the only one initiated and controlled by the bacterium itself, and is arguably the closest bacteria come to sex. There are ways to estimate the portion of the genes in a given genome that has been acquired by HGT. Such estimates often relies on discrepancies in GC content, codon usage and amino acid composition (20). In a study of 88 prokaryotic genomes, foreign genes were estimated to make up 0-22% of bacterial genomes. Most genomes were found to harbor a significant portion of transferred genes, whereas bacterial genomes harboring little or no foreign genes typically belong to endosymbionts undergoing genome reduction. A problem with such estimates is that they will always under-estimate the frequency of HGT, as gene transfer between closely related strains necessarily fail to generate a signal detectable in large scale studies. Indeed, as recombination efficiency positively correlates with the length and degree of homology of the donor DNA (98), it is reasonably to assume that HGT mainly occurs between closely related bacteria. Moreover, the host-specificity of bacteriophages and the requirement for physical contact in the case of conjugation further acts to restrict the flow of genetic material between distantly related bacteria (conjugation can occur between species, but conspecific bacteria are presumably overrepresented in the immediate vicinity of a given donor / recipient).

In addition to its role in the spread of antibiotic resistance, HGT is an important player in the general evolution of bacteria and has probably played a significant role in ensuring the near universality of the genetic code (45). This work focuses on NGT, for which *Streptococcus pneumoniae* has become a model organism.

The streptococci

Most streptococci can be found associated with human or animal hosts. Areas of colonization include the mucosa of the upper respiratory tract, oral cavity, dental surfaces and the intestines. Most streptococcal species are commensal members of the resident microbial population, but the genus also includes important pathogens. One such species is *Streptococcus mutans*, which inhabits dental surfaces and is the major etiological agent of dental caries. Other examples include the species *S. pneumoniae* and *Streptococcus pyogenes*, which are versatile human pathogens inflicting a devastating toll on human populations, especially in developing countries. *S. pyogenes* is responsible for a range of diseases including common pharyngitis and skin infections, life-threatening conditions such as pneumonia, bacteraemia, necrotizing fasciitis, streptococcal toxic shock syndrome and secondary sequelae including acute rheumatic fever and glomerulonephritis. According to current estimates, about half a million people are killed by *S. pyogenes* infections each year (102). *S. pneumoniae* is a leading cause of pneumonia, bacteraemia and meningitis in children worldwide. Pneumococcal infections cause about 826 000 deaths per year of children < 5 years old, amounting to ~ 11% of all such fatalities worldwide (71). In addition to causing disease in humans, *S. pneumonia* has been found to infect and probably kill wild chimpanzees (9). *S. suis* is a major pathogen of pigs and has the potential to infect humans in close contact with infected animals. *Streptococcus suis* cause meningitis, septicaemia, endocarditis, arthritis and septic shock in both pigs and

humans. *S. suis* appears to be an emerging human pathogen and infections in humans are associated with high mortality, but the incidence rate is still low (57).
Streptococcus sanguinis can colonize the heart valves and cause infective endocarditis following surgery, but is also a constituent of the protective resident microbiota of healthy individuals. *S. sanguinis*, *Streptococcus mitis* and *Streptococcus oralis* colonization protects against acute otitis media caused by *S. pneumoniae* and *Haemophilus influenzae*. Whether the protection is due to bacteriocin production or niche competition is not known. *S. salivarius* has also been speculated to protect against pharyngitis due to the production of bacteriocins active against *S. pyogenes* (91).

Streptococci are Gram-positive cocci and grow in pairs or chains. The cells are non-motile and generally spherical with sizes of 0.5-1.0 µM x 1.0-2.0 µM. (Figure 1). Belonging to the group lactic acid bacteria, they ferment sugars and sugar alcohols to lactic acid. ATP synthesis is based on substrate level phosphorylation only.

Species belonging to the streptococci have historically been classified at the crudest level according to their properties when grown on blood agar (73). Alpha haemolytic streptococci (e.g. *S. pneumoniae*) oxidize the iron in haemoglobin resulting in a green zone on blood agar plates. Beta haemolytic bacteria cause complete rupture of erythrocytes resulting in a clear zone on blood agar. The rest are labelled as gamma haemolytic, meaning that no lysis or oxidation takes place.

Beta-haemolytic streptococci are further characterised by Lancefield serotyping which is based on detection of specific carbohydrates in the bacterial cell wall (51). Based on this nomenclature *S. pyogenes* is often referred to as Group A Streptococci (GAS)

Standard methods of classification and phylogenetic analyses based on 16S ribosomal RNA sequences often fail to discriminate between members of the mitis group that includes *S. pneumoniae* and commensal species such as *S. mitis, S. oralis* and *S. infantis*. Members of these species are naturally competent for genetic transformation and horizontal transfer of DNA is thus thought to play a significant role in shaping their genomes. Using a polyphasic approach including genome hybridization, sequencing of four house-keeping genes, analysis of cell wall carbohydrates and more, Kilian *et al*. recently demonstrated that *S. mitis* strains are no more related to each other than to *S. pneumoniae* (figure 2) (50). The authors also found virulence genes typically associated with pneumococci to be randomly distributed among *S. mitis* strains (figure 2). Based on these findings, the authors convincingly argue that *S. mitis* lineages have evolved through genome reduction from a *S. pneumoniae*-like pathogen to their current largely commensal lifestyle. These findings are not only fascinating, but demand a re-evaluation of the species boundaries in mitis group streptococci, and maybe beyond (50).

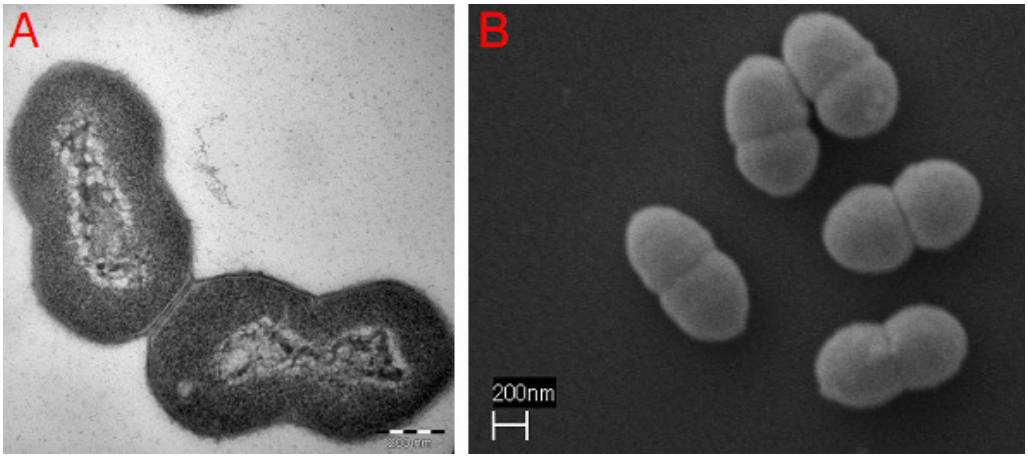

Figure 1. Transmission (A) and scanning (B) electron micrographs of *S. pneumoniae* cells.

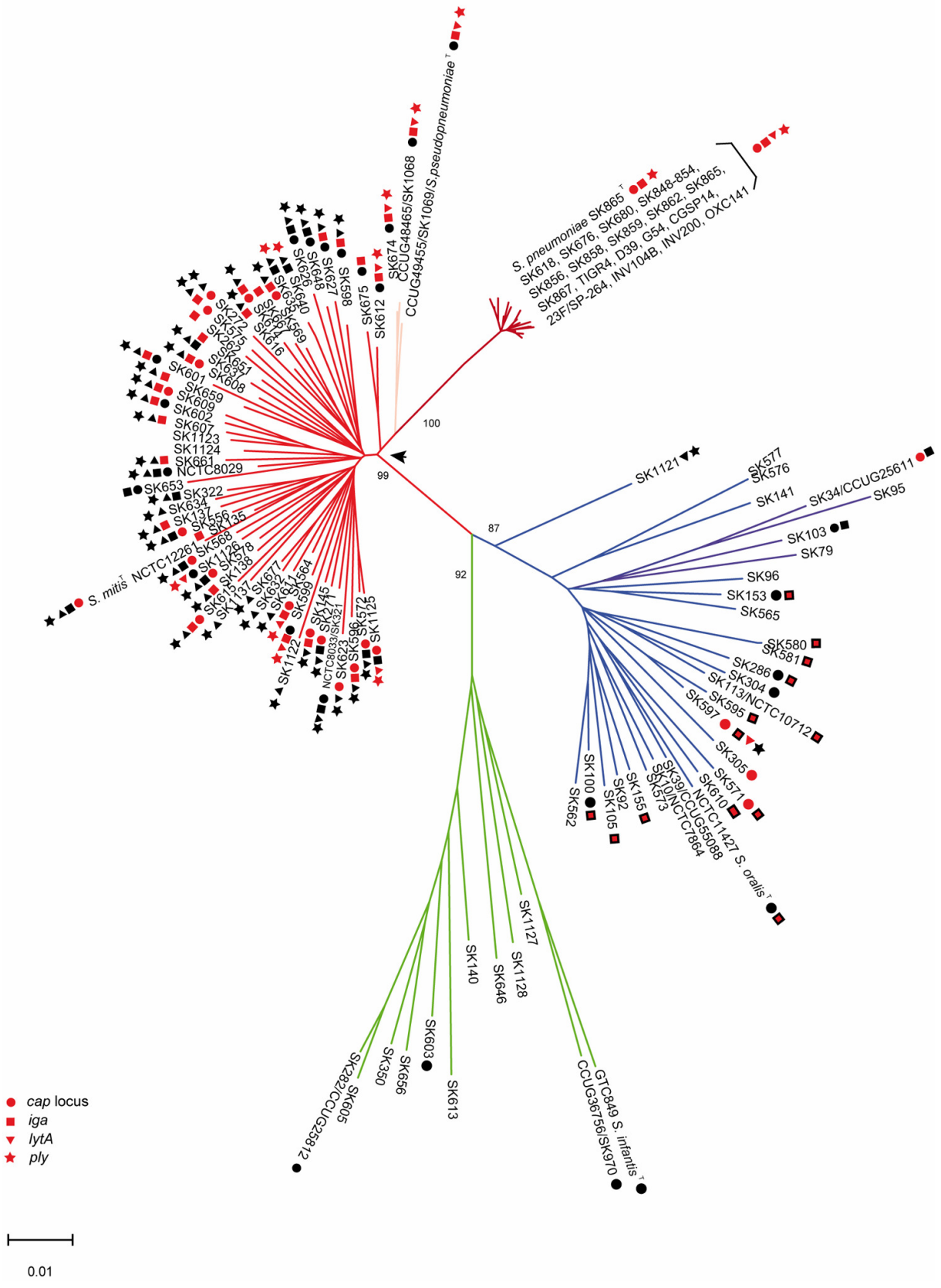

Figure 2. Mitis group phylogeny. Pneumoniae-mitis-pseudopneumoniae cluster (red lines), the Oralis cluster (blue lines) and the Infantis cluster (green lines). Presence of homologues of virulence factors usually associated with *S. pneumoniae* (*cap* locus, capsule synthesis operon; *iga*, IgA1 protease gene; *lytA*, autolysin gene; *ply*, pneumolysin gene) in the Mitis lineages is illustrated (present: red, absent: black). Black squares with a red center indicate IgA1 protease activity but an amplicon size not supporting the presence of an *iga* gene in the context found in *S. pneumoniae*. Scale bar indicates genetic distance. From Kilian *et al*. (50).

Streptococcus pneumoniae

As stated above, *S. pneumoniae* is a major global cause of death in humans. Important virulence factors harbored by pneumococci include the polysaccharide capsule, IgA protease, LytA and pneumolysin. One characteristic of *S. pneumoniae* is that all the genes encoding these virulence factors are found in all isolated clinical strains. Among the rest of mitis group streptococci, these virulence factors are distributed randomly, with many strains harboring none (50).

Teichoic acids (TAs) linked to peptidoglycan and lipids constitute an essential component of the Gram positive cell wall. TAs are polymers of glycerol or ribitol joined by phosphate groups. Amino acids or sugars are attached to the ribitol/glycerol groups. Most Gram-positives have one type of wall teichoic acid (WTA) and one membrane embedded lipoteichoic acid (LTA), but some have a more diverse arsenal of glycopolymers. One example is *Bacillus subtilis* that in addition to LTA has two distinct WTAs plus teichuronic acid that is produced under phosphate-limiting conditions (6). TAs from Gram-positive bacteria exhibit varying complexity and differ in net charge, sugar content and decoration of the repeating units (100). LTAs are generally less diverse than WTA with a repeating unit often consisting of glycerol phosphate (e.g. *Staphylococcus aureus*). Pneumococci, however, have LTA repeating units that are identical to WTA (19).
In *S. pneumoniae*, the teichoic acid pentamer repeating unit consists of ribitol phosphate, two molecules of N-acetylgalactosamine (GalNac), 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (AATGal) and glucose (42). The main genetic determinants of choline metabolism in *S. pneumoniae* are located in the *lic1* and *lic2* operons. Choline is transported into the cytoplasm and activated to cytidine 5-diphosphocholine (CDP-choline) by the products of *licABC* (*lic1* operon). Among the members of the *lic2* operon, LicD1 and LicD2 most probably attach the activated CDP-choline to membrane anchored teichoic acid residues, whereas the "flippase" TacF transport the choline-loaded teichoic acid precursors across the cell membrane (29).
The presence of choline covalently attached to the TA phosphoryl group is a characteristic property of pneumococci. Cell wall choline residues are central to many aspects of the pneumococcal physiology including docking of bacteriophages (55), interactions with human host proteins (5) and binding of surface proteins (104). Choline-binding surface proteins play important roles in *S. pneumoniae* and closely related mitis group streptococci such as *S. mitis* and *S. oralis*. Sequenced strains of *S. pneumoniae* harbor varying numbers of choline-binding proteins (>10), several of which are cell wall hydrolytic enzymes. Choline-binding proteins bind non-covalently to teichoic acid choline residues via choline-binding domains (CBD) consisting of a varying number of choline-binding repeat units 20-21 amino acids long. Choline-binding sites are located at the interface of two consecutive repeat units, where three structurally conserved aromatic residues form a cavity in which the choline moiety is stabilized.

S. pneumoniae is not in possession of an electron transport chain and does not carry out aerobic respiration. Pneumococci also lack a catalase that catalyzes the decomposition of hydrogen peroxide to oxygen and water and several other proteins involved in protection against oxidative stress in bacteria, such as NADH peroxidase and the regulators OxyR and PerR (34). Yet, pneumococci are able to grow in the presence of oxygen. When growing in oxygenic environments, the oxidation of pyruvate to acetyl-phosphate by the pyruvate oxidase SpxB plays an important role in pneumococcal metabolism, as acetyl-P can be used to generate ATP. A by-product of this reaction is the production of H_2O_2 (see reaction below).

$$\text{pyruvate} + P_i + O_2 \xrightarrow{\text{pyruvate oxidase(SpxB)}} \text{acetyl-P} + CO_2 + H_2O_2$$

S. pneumoniae produces H_2O_2 at levels sufficient to inhibit or kill *Haemophilus influenzae* and *Neisseria meningitides* in co-cultures (75) and exhibit cytotoxic effects on mammalian epithelial cells in vitro (17). How the pneumococcus escapes the toxic effects of

H_2O_2 production remains enigmatic, but might involve avoidance of the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow 3\ Fe^{3+} + OH^* + OH^-$). Extremely reactive hydroxyl radicals produced by this reaction is responsible for most of the H_2O_2 induced damage in *E. coli*, but does not play a significant role in *S. pneumoniae*. It has been speculated that the toxic effects of the Fenton reaction is avoided in one or more of the following ways: 1. Sequestration of Fe^{2+} away from DNA; 2. The absence of many enzymes containing $[4Fe\text{-}4S]^{2+}$ clusters known to leak iron cations upon oxidative stress; 3. Replacement of $[4Fe\text{-}4S]^{2+}$ clusters with $[2Fe\text{-}2S]^{2+}$ clusters more resistant to oxidation (76).

Various strains of *S. pneumoniae* exhibit high diversity in gene content, a property that is probably linked to their natural transformability. A comparative genomic analysis of 17 sequenced strains revealed a core genome containing 1454 of the total of 3170 genes identified (46%). 36% of the genes were distributed among some but not all strains, whereas 18% of the genes were unique to one strain only (33). Thus, any given strain harbors a portion of the pool of genes constituting the "supragenome". It was suggested that pneumococci have evolved various adaptations to escape the immune system and to cope with variations among hosts and the commensal microbiota (33). Shuffling of the genes responsible for these adaptations makes the pneumococcus a versatile colonizer and pathogen, without imposing on it the cost of maintaining the full supragenome in any given cell.

Coping with cell envelope stress

The cell envelope includes the cytoplasmic membrane, the murein layer and teichoic acids anchored in or covalently attached to the membrane or murein respectively. This envelope is essential to the bacterium, mainly because it is responsible for the integrity (or existence) of the cell but it also constitutes the interface between the cell and the environment. That is, sensing of what takes place outside the cell must happen at the cell envelope. Signals are subsequently transmitted from the outside to the inside of the cell and an appropriate response is mounted.

Two types of regulatory systems constitute the core of the Gram positive cell envelope stress response: extracytoplasmic function σ factors (σ^E) and two-component systems (TCS). As cell envelop stress is extracytoplasmic, systems to sense and respond to such stress needs a component that spans the cytoplasmic membrane. Both types of systems have a membrane-anchored sensor and a cytoplasmic transcriptional regulator (47). In the case of σ^E, a membrane-spanning anti-σ factor tightly binds its cognate σ^E under non-inducing conditions. Under inducing conditions, the σ^E is released and associates with the RNA polymerase in order to regulate transcription (32). The standard two-component system on the other hand, has a membrane-spanning sensor histidine-kinase (HK) which upon sensing a signal transfers a phosphate from its donor histidine to a receptor aspartate in the response-regulator (RR). In most cases, the phosphorylated (activated) RR directly activates or represses transcription of specific genes by binding recognition sequences in the genome.
The bacitracin-response in *B. subtilis* serves as a good example of a complex regulatory network responsible for coping with cell envelope stress. Upon encountering sub-lethal doses of bacitracin, two σ^E and three TCSs are induced. Among the up regulated TCSs are BceSR, which regulates the ABC transporter BceAB that probably function as a bacitracin resistance-determinant, and the LiaRS (previously yvqCE) system (43)

Two-component systems

The *S. pneumoniae* genome encodes 13 two-component systems (52). The two best described systems are ComDE and CiaRH.

ComDE is responsible for competence for genetic transformation in *S. pneumoniae*. The HK ComD is the receptor for the competence

pheromone CSP (36). Upon binding CSP, the sensor ComD activates ComE. By analogy to similar systems, the activation probably occurs by transfer of a phosphoryl group from a conserved histidine in ComD to a conserved aspartate in ComE. ComE binds a conserved direct repeat motif thereby directly activating transcription of the so-called early competence genes (77, 99). The *comCDE* operon is itself part of the ComE regulon and thus constitutes an autocatalytic loop that is activated when the threshold concentration of CSP is reached. The early competence genes also include *comX*, encoding an alternative σ factor (54). ComX activates transcription of the late competence genes. The late competence-inducible genes encode proteins involved in DNA uptake, recombination, competence-induced cell lysis (fratricide) and proteins of unknown function.

CiaRH is an enigmatic TCS involved in competence and beta-lactam resistance. The system has been studied quite extensively since it was first reported that a constitutive ON-mutation of the TCS resulted in increased β-lactam resistance and complete competence and transformation deficiency (26). CiaRH loss-of-function mutations, on the other hand, leads to a phenotype perfectly able to develop competence, even at sub-optimal pH values (13). CiaRH has been implicated in controlling early competence-development, as screening for *comCDE* over-expression phenotypes using mariner-mutagenesis yielded various *ciaR* mutants (58). CiaRH is itself indirectly controlled by ComDE, as it is among the late-competence genes/operons up regulated by ComX during competence (77) and has been proposed to ensure a safe exit from the stress involved in competence development (13). Remarkably, the five strongest promoters of the CiaR regulon direct transcription of small non-coding RNAs, two of which have been found to affect stationary-phase autolysis (30). The molecular basis of the signal responsible for CiaRH activation is still unknown. In addition to its induction by ComE, the CiaRH system has been reported to be induced by calcium deprivation (22), vancomycin (28) and penicillin (83).

Based on homology to corresponding systems in *Lactococcus* and *Bacillus*, HK03-RR03 is a member of a different kind of system where the HK lacks an extra-cytoplasmic domain. Instead, these systems contain a third component, a membrane embedded protein termed *liaF* in *B. subtilis* (*spr*0342 in *S. pneumoniae* R6), which is transcribed from the same promoter as the genes encoding the two-component regulatory system (48). This three-component system is termed LiaFSR in the eponymous system of *Bacillus subtilis*. The membrane-anchored protein LiaF acts as a negative modulator of the system through direct interaction with LiaS both in *B. subtilis* and *S. mutans* (48).
When LiaS and/or LiaF sense the appropriate stress-signal(s), LiaF-mediated inhibition of the LiaSR signal-transduction system is abolished, resulting in activation of LiaR-dependent promoters. The nature of the signal responsible for the release of LiaF from its interaction with LiaS remains unknown.
With the exception of the genera *Lactobacillus* and *Clostridium*, the LiaFSR system is conserved in Firmicutes. Based on regulon size and genomic context of the system, two groups can be distinguished (48): Group 1 includes the genera *Bacillus* and *Listeria*. In *Bacillus* the LiaR regulon primarily constitutes the *liaIH-(G)FSR* locus. The LiaR regulon of *Listeria* is similar, but the genes are split in two different loci, *liaIH* and *liaFSR*. In Group II, including *Staphylococcus* and *Lactococcus*, only the *liaFSR* operon is conserved. In species belonging to Group II, the LiaR regulon is much larger and LiaFSR seems to constitute the main cell-envelope stress response system (48). The HK/RR-03 has not yet been properly characterized in *S. pneumoniae*, but the characterized systems in other Firmicutes are induced by certain cell wall active antibiotics, especially those that interfere with the lipid II cycle (e.g. bacitracin, ramoplanin, vancomycin and nisin) (47).

Communication and quorum sensing

Intercellular communication in bacteria, so-

called quorum sensing (QS) is a means to coordinate behaviour that is unproductive for single cells to exhibit alone. The production of virulence factors, bioluminescence, biofilm formation, sporulation, mating, competence for genetic transformation and fratricide are all examples of processes governed by QS. The main types of QS systems can be summarized as follows (3, 97): The classical Gram positive QS relies on a short peptide pheromone (often post-translationally modified) that is secreted by a dedicated ABC transporter and sensed by a membrane-embedded HK. Upon binding the pheromone, the HK autophosphorylates and transfers the phosphoryl group to its cognate RR which then activates transcription of its target genes by binding specific DNA recognition motifs. The classical Gram negative QS relies on secretion of acylated homoserine lactones (AHLs) produced by a LuxI-type synthase. The AHLs diffuse freely across the cell envelope and binds a cytoplasmatic LuxR-type receptor. The activated receptor regulates transcription by binding recognition sequences in the promoter region of target genes. Gram negative species often produce several AHLs differing only in their acyl side chain and also harbor individual LuxR proteins that only respond to their specific AHL. Thus, several QS systems might interact to form complex regulatory systems. Of particular interest is the autoinducer AI-2 (a furanosyl borat diester) that is produced by both Gram positive and Gram negative species and might constitute some kind of *lingua franca* (2).

In their natural habitat, bacterial species often co-exist in ordered communities requiring communication for proper functioning. Indeed, research carried out in recent years has revealed a wealth of complex interactions taking place within and between bacterial species. For example, the *agr* autoinducing peptide (AIP) of *S. aureus* is synthesized in four different versions by four different pherogroups (25, 41). The various AIPs are modified peptides encompassing 8-9 variable amino acids containing a conserved five-membered thiolactone ring (61). The peptide pheromones not only activate their cognate AgrC receptor but also inhibit the AgrC receptors of competing pherogroups (25) . During host invasion, the pherogroup first reaching a quorum initiates a virulence cascade and out- competes cells belonging to other pherogroups (70). Another example illustrating the importance of QS has been found in the Gram positive family *Bacillus*. Although Gram positives do not produce AHLs themselves, a *Bacillus* strain isolated from soil was found to produce an enzyme deactivating AHLs produced by Gram negatives (16). Presumably, this is part of a strategy of gaining an advantage by interfering with the communication between competing strains.

QS was first discovered in pneumococci. In the 1960s, it was reported that competence development is a coordinated event taking place at a particular cell-density during exponential growth (72, 92, 94). Induction of the competence phenotype was demonstrated to rely on a proteinaceous compound, but the exact nature of this compound remained elusive until the mid 1990s when Håvarstein *et al*. finally reported the molecule to be a 17 amino acid peptide termed Competence Stimulating Peptide (CSP) (36).

Bacterial natural genetic transformation

Bacteria capable of taking up naked DNA from the immediate environment and incorporate it into their genomes are termed naturally transformable. A number of naturally transformable species are found in the low GC Gram positives (the Firmicutes) including *S. pneumoniae* and *B. subtilis*. In both species, the genes involved in competence development can be divided into early and late genes (11). The high GC Gram positives (the Actinobacteria) are less well studied with regard to NGT, but *Mycobacterium smegmatis* has been reported to be naturally transformable. Due to the presence of homologues of the competence genes *comEA* and *comEC* that are involved in translocation of DNA across the cytoplasmic membrane in a number of Actinobacteria, it has been speculated that NGT is a widespread property of these bacteria (45). Naturally

transformable species are also found across the alpha, beta, gamma and epsilson subdivisions of the Gram negative phylum Proteobacteria, including the important pathogens *Neisseria meningitides, Neisseria gonorrhoeae, Haemophilus influenzae* and *Helicobacter pylori*. (8, 60, 69) This indicates that NGT is an ancestral property of this lineage. Naturally competent bacteria have also been reported in the Gram negative phyla Deinococcus-Thermus, Cyanobacteria and Chlorobi.

NGT is usually a tightly regulated process occurring at certain phases of growth or in response to specific environmental stimuli. The exceptions are *N. gonorrhoeae* and *N. meningitides* that are competent throughout their lifecycles. The transforming DNA can in theory be supplied from any source, but there is mounting evidence that bacteria have evolved mechanisms to obtain more homologous DNA: Members of the Neisseriaceae and Pasteurellaceae strongly prefer to take up DNA containing their own specific DNA uptake signal sequence and most gonococci even harbor a horizontally acquired genetic island encoding machinery for active donation of DNA (31, 81).
Pneumococci seem to have evolved a more violent approach to obtain homologous DNA, as it was recently demonstrated that they can kill other mitis group streptococci and use the liberated DNA as a source of transforming DNA (44, 88).

Pneumococcal competence for genetic transformation

S. pneumoniae holds a unique place in the history of research in microbiology and genetics. In 1928, Frederick Griffith discovered "the transforming principle" working with pneumococci. He found that by a to him unknown mechanism, phenotypes could be transferred from dead to live pneumococci (24). Then, in 1944, Oswald Avery and co-workers reported their ground-breaking finding that the transforming agent was in fact DNA. This was the first proof that DNA is the genetic material of life on earth (1). Since then, natural genetic transformation has been shown to be a property of a wide range of Gram positive and Gram negative bacteria (45).

In pneumococci, competence for genetic transformation is a transient phenotype. Cultures grown in competence-permissive medium spontaneously develop competence at an $OD_{550\ nm}$ of 0.15 - 0.2. The ability to take up DNA is abruptly lost after about 30-45 minutes (93). CSP is encoded by *comC* and is synthesized with an N-terminal leader peptide containing a Gly-Gly motif (36). CSP is secreted by the ATP-binding cassette transporter ComA and the accessory protein ComB encoded by the *comAB* operon. Concomittant with CSP secretion, ComA cleaves off the prepeptide including the double glycine, resulting in maturation of the pheromone (37, 40).
As mentioned above, binding of the peptide pheromone CSP to the HK ComD induces its autophosphorylation, presumably followed by transfer of the phosphoryl group to the cognate RR ComE (38). Activated ComE binds a conserved direct repeat DNA sequence motif thereby directly activating transcription of the so-called early genes (77, 99). The core competence regulating operon *comCDE* is itself part of the ComE regulon and thus constitutes an autocatalytic loop. Microarray analyses revealed that ComE activates transcription of about 20 genes (77). Among the early genes are two copies of *comX* encoding an alternative σ factor (σ^X) (54, 77). ComX directs the transcription of about 80 late competence genes, some of which are involved in DNA uptake and recombination. Late competence genes involved in DNA uptake include the *comGA-comGD* operon encoding the building blocks that make up the transformation pseudopilus and the genes encoding the DNA receptor ComEA, the DNA import channel ComEC and the ATP-binding protein ComFA. Together with the endonuclease EndA, which is not induced during competence, these proteins form the DNA uptake machinery of *S. pneumoniae* (10). One strand of the double stranded DNA is degraded outside the cell, presumably by EndA (10) and single stranded

DNA (ssDNA) is imported in a 3´→ 5´ direction (62). Late genes involved in processing the incoming DNA include the genes encoding CoiA, DprA, RadA, RecA and SsbB (13, 77). As ssDNA enters the cell, SsbB protects the DNA from degradation by forming a nucleoprotein complex termed the eclipse comples (63). DprA competes with SsbB for DNA binding and conveys the ssDNA to RecA . DprA then promotes the homology-dependent interaction between the RecA-ssDNA filament and super-coiled DNA (65). The exact roles of CoiA and RadA remains unknown, but both have been implicated in recombining the imported DNA with the chromosome (14)

In addition to the early and late genes, the competence regulon includes two other classes of genes with different transcription profiles. One class comprises the delayed genes, for which mRNA keeps accumulating after the expression peaks of the early and late genes. The delayed genes are mainly stress related (77). The last class includes repressed genes, many of which are involved in protein synthesis (77).

Certain aspects of competence development and regulation remain incompletely understood. For example, induction of the competent state is not governed by simple quorum sensing, as it was recently shown that competence could be induced in response to the DNA-damaging agent mitomycin C and certain antibiotics (79). It is not understood how these cues are translated into induction of the competence regulon, but various regulatory proteins apart from ComCDE and ComX, such as the CiaRH system and the protein kinase StkP have been shown to play a role in competence development (13, 58, 85). Another example is the termination of competence. The ComE dependent ComW is necessary to activate ComX and protect the protein from ClpE-ClpP mediated degradation. (90). ComW was thought to regulate the shut-down of competence, but this termination was recently found to be independent of the proteolytic degradation of ComW and ComX, and remains to be elucidated (78). A schematic view of competence regulation is presented in figure 3.

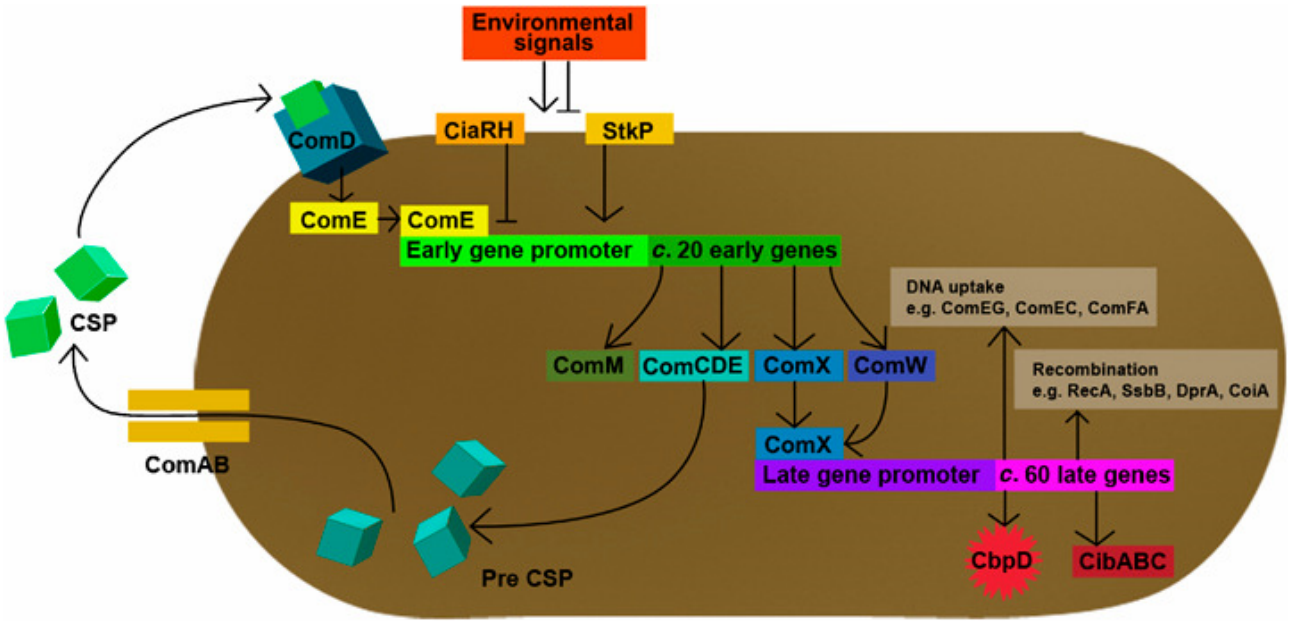

Figure 3. Schematic representation of competence regulation in *Streptococcus pneumoniae* (46). See text for details.

Pneumococcal fratricide

Among the ~120 genes regulated by competence induction, only ~25% are involved in transformation (77). Some of the remaining genes are involved in a phenomenon termed pneumococcal fratricide. In 2002, Steinmoen *et al.* (88) discovered that an intracellular β-galactosidase reporter and chromosomal DNA was released to the growth medium upon competence induction of the *S. pneumoniae* strain CP1415. Subsequent experiments with mixed cultures of competence proficient and competence deficient (*ΔcomE*) cells and similar experiments carried out on blood agar plates revealed that non-competent cells were actually lysed by their competent sister cells (27, 89). The efficiency of competence induction varies substantially between different strains and growth media, and in fact the discovery of fratricide in 2002 was a quite serendipitous one, as suboptimal induction of the growing *S. pneumoniae* culture resulted in a mix of competent and non-competent cells.

In liquid culture, fratricide is dependent on the choline-binding proteins LytA, LytC and CbpD (27, 49, 89). These proteins lyse non-competent cells whereas competent cells are protected. This immunity was recently found to be conferred by the putatively membrane-embedded early competence gene ComM (39).
LytA, the major pneumococcal autolysin, is an N-acetylmuramyl-L-alanine amidase responsible for the stationary phase lysis of pneumococci (35, 84). The protein is constitutively expressed but up-regulated during competence (64, 77). LytA consists of an N-terminal amidase domain and a C-terminal choline-binding domain (CBD) consisting of six choline-binding repeat units. The protein exists in its active form as a homodimer (18). The lysozyme LytC has an N-terminal CBD made up of 11 choline-binding repeat units. However, low sequence conservation of the two C-terminal repeats suggest that they might not function as choline-binding repeats, but constitute a linker between the CBD and the C-terminal lysozyme domain. Expression of LytC is unaffected by competence as it is expressed from a house-keeping promoter (21). The putative murein hydrolase CbpD is expressed during competence exclusively and is absolutely required for fratricide in liquid cultures (49). Yet, the fact that deletion of LytA and LytC together abolishes fratricide (66) means that the key effector CbpD depends on LytA or LytC in order to exert its full activity. CbpD is a modular enzyme consisting of an N-terminal cysteine, histidine-dependent amidohydrolase / peptidase (CHAP) domain followed by two Src homology 3b (SH3b) domains and a C-terminal choline-binding domain (CBD) consisting of four choline-binding repeat units (figure 4). That the CHAP domain actually functions as a cysteine protease is exceedingly likely, as a point-mutation in the putative active-site cysteine abolished its activity (49). The enzymatic specificity of the CHAP domain has not been solved, but it is highly homologous to domains found in a number of phage lysins and bacterial murein hydrolases whose characterized members either act as endopeptidases that cleave within murein stem peptides, or as amidases that cleave the N-acetylmuramyl-L-Ala bond (4, 53, 82). In eukaryotes, SH3 domains are involved in protein interaction by binding proline-rich regions (103). The existence of bacterial SH3 domains was predicted based on weak sequence homology and similar predicted folding a few years later (101). The function of bacterial SH3 domains is largely unknown, but in case of the lysostaphin homologue ALE-1 appears to target the protein to the peptidoglycan of target cells (56). Choline-binding domains are 20-21 amino acids in length and occur in a variable number of repeats in streptococcal choline-binding proteins. Proteins containing these domains bind phosphorylcholine moieties decorating wall- and lipoteichoic acids.

Guiral *et al.* (27) found that non-competent cells lyse and release intracellular pneumolysin (Ply) when they are co-cultivated with competent cells on agar plates. The authors also demonstrated that LytA and LytC can be provided either by the competent

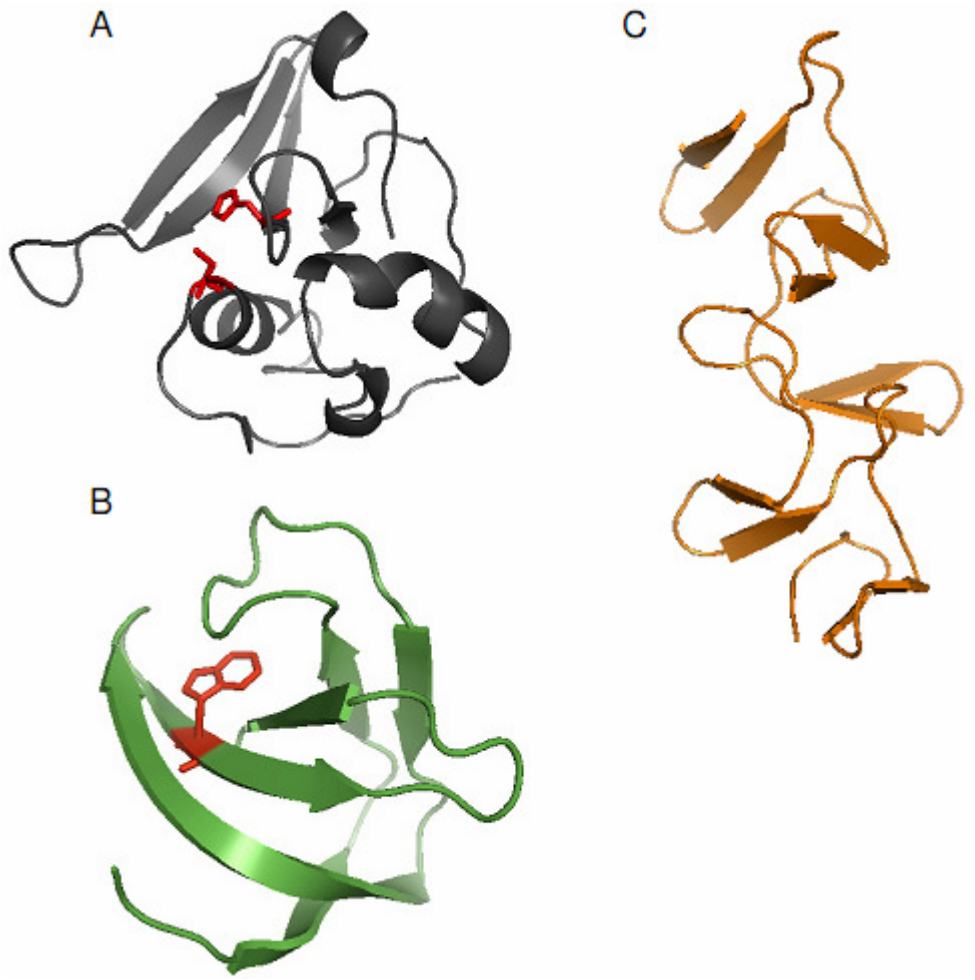

Figure 4. Individual 3D-models of the CHAP, SH3b and CBD domains of CbpD. The structures were obtained by threading using HHpred (87). (A) The CHAP domain. The model is based on the NMR solution structure of the *Staphylococcus saprophyticus* CHAP domain protein Ssp0609 (pdb: 2k3a). The active-site cysteine and histidine residues are highlighted in red and shown in stick configuration. (B). The SH3b domain. The model is based on the SH3b domain of the *Staphylococcus capitis* glycylglycine endopeptidase ALE-1 (pdb: 1r77). A conserved tryptophan residue is highlighted in red and rendered in stick configuration. (C) The CBD. The model is based on the CBD of the pneumococcal autolysis regulator CbpF (pdb: 2v05).

cells or the non-competent target cells (27). In contrast to what was found in liquid culture, deletion of CbpD alone did not significantly reduce lysis on agar plates. This led to the discovery of the bacteriocins CibA and CibB. CibA and CibB are part of a two-peptide bacteriocin system whereas a third protein CibC encoded by the same CibABC operon confers immunity to the bacteriocins (27).

Fratricide has been referred to as competence-induced cell lysis (88), heterolysis (89), allolysis (27) fratricide (killing of siblings) (23) and sobrinicide (killing of cousins) (11). As this predation can target both isogenic, conspecific and heterospecific bacteria and in all cases rely on the same molecular mechanism, fratricide is used in this work as it has become a generally accepted and widely used term.

The co-regulation of competence and fratricide indicates that these phenomena are physiologically linked. Indeed, recent research suggests that fratricide might have evolved as a mechanism for active acquisition of DNA. Johnsborg *et al.* recently demonstrated that transfer of a novobicin resistance gene from non-competent target cells to competent attackers during co-cultivation was virtually abolished upon deletion of *cbpD* in the competent cells (44). This illustrates that acquisition of DNA is directly linked to fratricide *in vitro* and there is no reason to believe that this is not the case also *in situ*.

Pneumococci were also found to be able to lyse the closely related species *S. mitis* and *S. oralis* in a CbpD-dependent manner and incorporate the liberated DNA into their genomes. Furthermore, this ability is not restricted to pneumococci, as *S. mitis* is able to "steal" DNA in the same CbpD-dependent manner from non-competent sibling cells as well as from non-competent *S. pneumoniae* and *S. oralis* cells (44).

Pneumococci and other members of the mitis group streptococci produce CSPs with highly variable peptide sequences (43). The CSP receptor ComD was recently found to exhibit a degree of promiscuousity in its ability to be activated by non-cognate CSPs, that is, both the cognate and various foreign CSPs are able to trigger the competence cascade in *S. pneumoniae* (44). This seems to imply that there exists a selective pressure for diversification of the pheromone, and at the same time to produce a ComD receptor that is able to sense as many different CSPs as possible. This would render any given strain able to maximize its killing range and minimize the possibility of being "blind folded" as competing strains mount their attack (Figure 5).

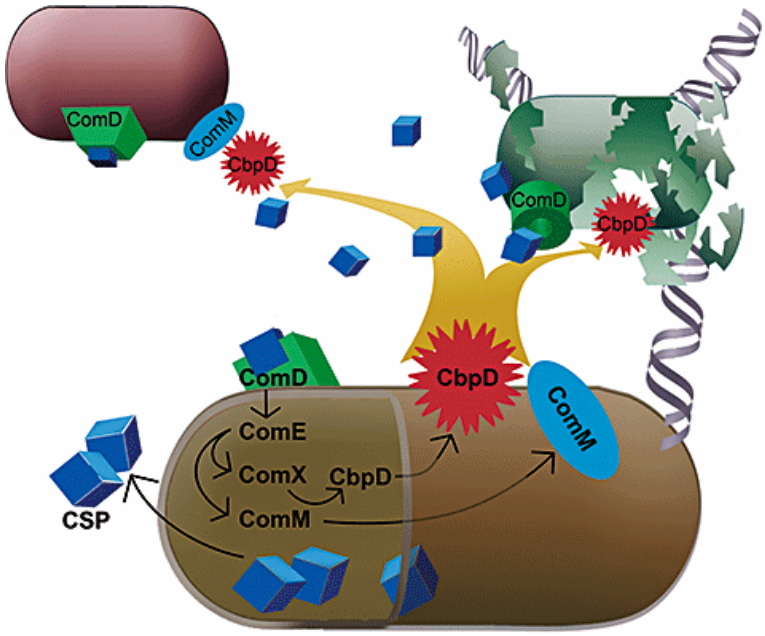

Figure 5. Model depicting CbpD -mediated cell lysis and DNA transfer within and between the species *S. pneumoniae*, *S. mitis* and *S. oralis* in their natural habitat. Competent streptococci (represented by brown cell in front) attack and lyse neighbouring non-competent streptococci belonging to different pherogroups (represented by green cell). DNA released by the lysed cells is subsequently taken up by the competent attackers. A wire-tapping cell belonging to a different pherogroup (dark red) responds to this non-cognate CSP, detects the oncoming attack and is able to neutralize the effect of CbpD by expressing the ComM immunity protein. ComD, CSP histidine kinase receptor; ComE, the cognate response regulator of ComD; ComX alternative sigma factor controlling expression of the late genes; CbpD, putative murein hydrolase; ComM, immunity protein providing protection against CbpD. Figure from Johnsborg et al. (44).

Aim of the study

Pneumococcal fratricide is likely to play an important role in gene exchange between different strains of pneumococci and their closest relatives (44). The phenomenon could thus be important for the spread of antibiotic resistance and vaccine escape by capsule switching. Pneumococcal fratricide has also been suggested to play a role in the release of virulence factors during host invasion (27). A detailed understanding of how pneumococci kill each other might also lead to new ideas on how to combat infections caused by these bacteria. The current study was initiated to gain a better understanding of the molecular mechanisms involved in pneumococcal predation, with key focus on sorting out the roles of CbpD, LytA and LytC, the main effectors of fratricide.

Main results

Paper I

LytA and LytC can be provided by competent attackers (*trans*) or non-competent target cells (*cis*), but are more efficient in *cis*.

CbpD produced by competent cells is essential for fratricide in liquid cultures (49). CbpD triggers the activity of the lysins LytA and LytC, that must be provided either in cis or trans for efficient lysis of target cells (27). We decided to study the contributions of LytA and LytC in attacker (Δ*comA*, competence inducible; Δ*ebg*, no β-galactosidase production) and target cells (Δ*comE*, non-competent; *hirL::lacZ*, constitutive LacZ producers) in more detail for a better understanding of fratricide. In order to achieve this, single and double Δ*lytA* Δ*lytC* mutants were constructed both in attacker and target backgrounds. The mutants were then assayed by measuring β-galactosidase leakage from target cells compared to total β-galactosidase levels after 30 minutes co-culture with competent attacker cells. We found that both LytA and LytC were ~3x more efficient when supplied in *cis* compared to *trans*. When Δ*lytA* Δ*lytC* double mutants were employed as both attackers and targets, only < 0.5 % of the target-cells lysed, revealing that CbpD is very inefficient on its own under the conditions used.

There is significant lytic activity in the supernatant of competent pneumococci

It is not known whether fratricide occurs mainly via cell-cell contact or whether the killing factors (CbpD, LytA and LytC) are released into the environment to exert their action. To test for lytic activity in the supernatant, attacker cells were induced to competence and supernatants harvested by sterile filtration 20 minutes post competence induction. We found significant lytic activity in the supernatant when this was mixed 1:1 with non-competent target cell, illustrating that cell-cell contact is not absolutely required for fratricide. Next, western analyses of mutants carrying His_6-tagged versions of LytA, LytC and CbpD were performed. CbpD was readily detected in concentrated supernatants of competent cultures, but elution with 2% choline indicated that most of the CbpD is attached to cell-envelope teichoic acids. LytC was found in copious amounts in the supernatant regardless of competence-induction and elution with choline. LytA on the other hand could not be detected in supernatants. In whole-cell extracts, LytA was detectable in non-competent cells, but was present in larger amounts in competent cells.

There is a close correlation between cell death and activation of LytA

The cellular localization of LytA remains undetermined, but our results (above) together with previous reports (7, 104) indicate that LytA is an intracellular protein. Treating early exponential phase Wt and ΔlytA cells with various concentrations of the detergent deoxycholate (DOC, 0.025-1 %) revealed a close correlation between cell death and activation of LytA. Upon treatment with 0.05% DOC, the Δ*lytA* mutant stopped growing, whereas half the cells in the Wt culture lysed and the rest stopped growing. LIVE/DEAD staining of both cultures revealed that all the cells were dead. Upon treatment with 0.025% DOC, both cultures kept growing, and nearly all the cells were viable. These results indicate that death comes first, lysis second. LytA mediated autolysis can be triggered by a wide range of treatments including CbpD activity, detergents, freeze-thawing, hydrogen peroxide and penicillin antibiotics, the common denominator seems to be cell death.

The activity of CbpD increases dramatically in the presence of EDTA

We serendipitously found that addition of 1mM EDTA dramatically increased the lytic

activity of supernatants prepared from competent cultures. The increase was dependent on the presence of CbpD, but was not a result of protection against proteolytic degradation of the protein. These results prompted us to investigate whether CbpD alone could lyse cells upon competence induction in the presence of EDTA. A few year ago the fratricide-immunity protein ComM was described for the first time (39), and it was demonstrated that only a subfraction of cells lyse upon competence induction of a monoculture of *comM* mutants. We assayed cultures of Δ*comM hirl::lacZ* mutants with or without additional deletions of *lytA* and *lytC* or *lytA, lytC* and *CbpD* for lysis upon competence induction with or without the addition of 1mM EDTA. Lysis was quantified by measuring β-galactosidase release as described above. It was found that ~20 % of the *comM* cells lysed in the absence of EDTA and > 50% in the presence of EDTA. Interestingly, the Δ*lytA,* Δ*lytC* Δ*comM* mutant exhibited very limited lysis (~ 2 %) in the absence of EDTA, but lysed efficiently in the presence of EDTA (~30%) The corresponding mutant with the additional deletion of *cbpD* on the other hand, did not lyse at all, even when supplemented with EDTA. Whether the divalent cation chelator EDTA acts on CbpD itself or interact with the target cells to make them more lysis-prone, remains unknown. Nevertheless, these results indicate that CbpD is endowed with stand-alone murein hydrolase activity.

Paper II

The three-component system LiaFSR regulon

The fact that only about 20 % of a culture of *comM* mutants lyse upon competence induction led us to speculate that there might exist additional back-up systems to restrict cell envelope stress-induced lysis in the absence of the fratricide-specific immunity conferred by ComM. Reports that the cell envelope stress sensing two-component system CesSR in *Lactococcus lactis* is activated by the bacteriocin Lcn972, the lipid II interacting antibiotics bacitracin and vancomycin (59), and in one report, lysozyme (96), prompted us to investigate the homologous system in pneumococci: spr0342-HK03-RR03 (from now designated LiaFSR).

By measuring luciferase production in a *liaR::luc* mutant, the lipid II-interacting antibiotics bacitracin, nisin and to some degree tunicamycin were found to induce the Lia system. As bacitracin was the strongest inducer, we performed a DNA microarray analysis comparing the global gene expression profile of the R6 wildtype and the isogenic *liaR* mutant in the presence of a sub-lethal concentration of bacitracin. The LiaFSR regulon was found to include genes encoding choline-binding protein PcpC (part of the Lia transcriptional unit), the heat inducible transcription repressor HrcA, Heat-shock protein GrpE and the PspC-like gene *spr*0810.

The LiaFSR system is activated by CbpD, LytA and LytC

Employing a luciferase reporter gene immediately donwstream of the LiaR regulated gene *spr*0810, we found that the Lia system is activated during competence in the absence of ComM. This activation was found to depend on the concerted action of CbpD, LytC and LytA. This establishes that peptidoglycan degrading enzymes can activate the LiaFSR cell envelope stress response in pneumococci. LytC is a lysozyme that cleaves the 1-4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine, whereas LytA cleaves the *N*-acetylmuramyl-L-Ala bond between the murein glycan chain and the stem peptides. The CHAP domain of CbpD is highly homologous to similar domains in phage lysins and bacterial murein hydrolases. Characterized CHAP protein family members are either endopeptidases or *N*-acetylmuramyl-L-Ala amidases. Our findings thus demonstrate that both glycan strand-cleaving enzymes and enzymes that cleave amide/peptide bonds can trigger the LiaFSR stress response system in *S. pneumoniae*. Activation of the Lia system by a

peptidoglycan degrading enzyme has so far only been observed in *L. lactis*. Studies of the CesSR system yielded conflicting results regarding the ability of lysozyme to activate the system. Veiga *et al.* found that lysozyme treatment increased expression of the *ces* operon (96), whereas Martínez *et al.* working with the same strain did not observe induction of a *lacZ* reporter gene under control of a CesR-responsive promoter upon lysozyme treatment (59). The results presented here clearly demonstrate that peptidoglycan degrading enzymes can activate the LiaFSR system of *S. pneumoniae*.

LiaFSR constitutes a second layer of defence during competence

In order to investigate whether the Lia system actually confers resistance to the murein hydrolase induced stress during fratricide, we deleted *liaR* in a "Wt" and Δ*comM* background. In a Wt background, cell lysis during the 30 minutes following competence induction increased from 1.2 % to 2.4 % of the total cell population upon deletion of *liaR*. Deletion of *liaR* in a Δ*comM* background increased lysis from ~ 20 % to 40 % of the cells. In both cases, deletion of *liaR* leads to a doubling of lysis, indicating that the LiaFSR system protects against competence-induced stress. It should be noted from the results above though, that ComM is sufficient to protect the vast majority of cells against lysis even in the absence of *liaR*.

Two members of the LiaFSR regulon, *spr0810* and *pcpC*, contributes to the LiaFSR-coordinated protection.

PcpC is a paralogue of the LytC regulator CbpF whereas *spr*0810 is a member of the phage-shock protein C superfamily (pfam04024), and we hypothesized that these proteins could be responsible for the murein hydrolase-protective phenotype conferred by LiaR. Indeed, deletion of these genes in a *ΔcomM* background revealed that these two genes together were responsible for most, but not all the protective properties of the LiaFSR during competence.

Paper III

CbpD is a murein hydrolase

CbpD harbors an N-terminal CHAP domain. The enzymatic specificity of the CHAP domain has not been solved, but it is highly homologous to corresponding domains found in a number of phage lysins and bacterial murein hydrolases. Zymogram analysis of total protein extracts prepared from a *wt* strain and a Δ*cbpD* mutant resulted in a distinct zone of clarification attributable to CbpD. This demonstrated for the first time that CbpD indeed is a murein hydrolase. Comparison of the clearing zone associated with CbpD in SDS-gels incorporating *wt* cells and *ΔlytC* cells revealed that the enzymatic activity of CbpD activates LytC, as the clearing zone appeared more rapidly and was significantly more pronounced when LytC was present than when it was absent.

Cell envelope binding properties of CbpD domains

To assay the cell envelope binding properties of CbpD, a fluorescent fusion protein consisting of CbpD with the CHAP domain exchanged with GFP was expressed in *E. coli*. The purified protein was found to primarily target the division zone of pneumococci. By assaying additional fusion proteins containing the SH3b domains or the choline-binding domain (CBD), the septum targeting was found to be conferred by the CBD. We then assayed the binding of the fusion proteins to purified peptidoglycan sacculi. This revealed that the SH3b domains of CbpD bind peptidoglycan.

The dual binding activities of the SH3b domains and the CBD is necessary for lysis of target cells

The effect of various alterations of CbpD was assayed by investigating the ability of competent cells to lyse target cells. Alterations included point-mutations in one or both SH3b domains, deletion of the SH3b

domains altogether and deletion of the CBD. Fratricide assays were carried out following a previously describe method (88). The experiments revealed that that both the SH3b domains and the CBD are essential for lysis of target cells.

CbpD causes ruptures in the septum of target cells.

Finally we analyzed CSP induced monocultures by transmission electron microscopy (TEM). Two strains were compared: both lacked the immunity protein ComM and the autolysins LytA and LytC, but one of the strains carried the additional deletion of *cbpD*. The TEM images revealed that CbpD causes ruptures in the division zone.

Discussion

The three papers constituting the foundation of the current thesis all shed light on various aspects of pneumococcal fratricide. The work, which concerns the molecular mechanisms underlying fratricide, has led to new insights that have significantly improved our understanding of the phenomenon. Yet, important questions remain unanswered.

As it is a key player in pneumococcal fratricide, CbpD was the main focus of this work. We have shown that CbpD is a murein hydrolase that mainly targets the division zone of target cells (paper III). The enzymatic specificity of CbpD has not been solved yet, but as it is a CHAP protein, it can be inferred that CbpD is a peptidoglycan degrading endopeptidase or amidase. In paper I, It was demonstrated that the reducing agent DTT stabilizes CbpD activity in cell-free supernatant from competent cultures, probably by protecting the active-site cysteine from oxidation. CbpD is dependent on LytA and LytC for full activity, but 1mM EDTA was found to empower CbpD with the ability to kill non-immune Δ*comM* cells efficiently without the aid of LytA and LytC. These observations might reflect important aspects of the regulation of CbpD activity *in situ,* but this remains to be investigated.

Our results also shed light on the cascade of events triggered by the protein, namely the activation of the amidase LytA and the lysozyme LytC. In paper III we demonstrated directly that it is the enzymatic activity of CbpD that leads to activation of LytC. Pérez-Dorado and co-workers have recently reported findings that support our data (74). A curious modular arrangement in the LytC structure results in a steric hindrance that obstructs the enzymatic domain from carrying out its lysozyme activity on cross-linked glycan chains (figure 6). In other words, some other enzyme must make specific cuts in the stem peptides in order to unleash LytC activity. This finding, as noted by Peréz-Dorado *et al.* (74), fits nicely with our finding that CbpD triggers LytC activity, as we reported in paper I and later confirmed in paper III.

Results presented in paper I suggest that LytA is an intracellular protein: 1. Localization studies of His-tagged proteins failed to detect any LytA associated with cell wall teichoic acids or in the culture medium; 2. The concentration of DOC sufficient to kill pneumococcal cells is slightly lower than the concentration necessary to cause total lysis of

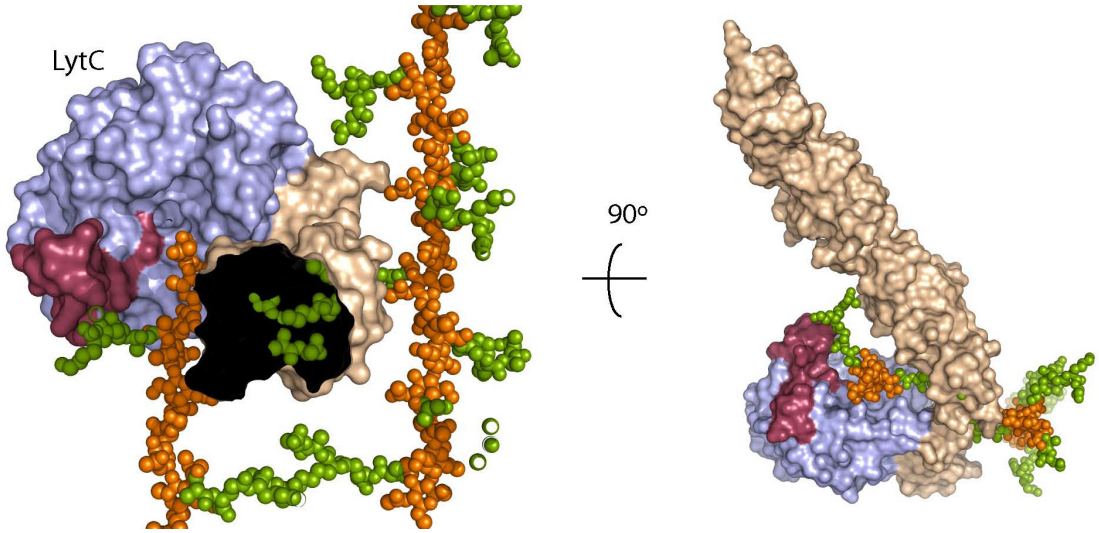

Figure 6. Modular arrangement in LytC with implications for interactions with peptidoglycan. Superimposition of LytC (catalytic module coloured in light blue and the CBD in light brown) onto peptidoglycan (glycan chains as orange spheres and peptide-stems as green spheres). Left, CBD of LytC is not fully depicted (the black area) to appreciate the steric hindrance of peptide stems of peptidoglycan that would exist in a crosslinked arrangement. The presence of the crosslinked peptide would abrogate binding. From Pérez-Dorado *et al.* (74).

the culture. The latter finding is in concordance with a view that leakage of intracellular LytA and not cell death alone is necessary to trigger LytA mediated autolysis. Previous studies demonstrated that LytA activity is inhibited when added to cultures of live pneumococci (15, 95), but how this suppression occurs remains to be investigated. In paper II, LytA was found to contribute to LiaSR activation upon competence induction of a Δ*comM* mutant. On the face of it, this finding does not correspond well with the notion that LytA is an intracellular protein that is only able to lyse dead cells, as dead cells obviously cannot increase the expression of genes. A possible explanation is that LytA leaks from dead cells and is able to take part in the attack on live cells. If this is the case, the peptidoglycan degrading activities of CbpD and LytC must somehow make live cells susceptible to LytA degradation of the cell wall of live cells.

Together, these findings now enable us to propose a model of how CbpD triggers fratricide: First, CbpD produced by competent cells bind the septa of non-competent target cells. Here, the protein makes specific cuts, either in the stem peptide or between the N-acetylmuramyl of the glycan chain and the first L-alanine of the stem peptide. This triggers the lysozyme activity of extracytoplasmic LytC. Together, CbpD and LytC damage the cell wall to such a degree that its integrity and the integrity of the cytoplasmic membrane are partially lost. This allows LytA leakage from the cytoplasm. LytA hydrolyzes the N-acetylmuramyl-L-alanine bonds of the cross-linked murein, possibly triggering even more LytC activity. The result of this cascade is lysis of the target cell.

The only phenotype described for a Δ*lytC* mutant outside a fratricide setting is diminished stationary phase autolysis at 30°C (21). Deletion of *lytC* has no visible effect on growth, yet copious amounts of LytC are present in or on the surface of the cell and in the growth medium during exponential growth. Whether LytC has other more prominent roles in pneumococcal physiology is an open question. Despite the lack of growth effects upon deletion of *lytC*, it is for example possible that the protein plays a role in recycling of murein sugars, but that the effect is masked by other protein(s) with overlapping functions Alternatively, a rich growth medium could mask the phenotypic effects of deleting *lytC*. Our results together with those obtained by Pérez-Dorado *et al.* (74), suggest that the cleavage of peptide cross-links by LytA or CbpD activates LytC . It is also known that endopeptidase and amidase domains are widespread in phage proteins. Interestingly, a phage lysin containing a CHAP and a lysozyme domain was shown to be present in the tail tip of *Staphylococcus aureus* phage φMR11 suggesting a role of the lysin in phage invasion (80). A search of published phage genomes reveals that proteins harboring a CHAP domain, usually in conjunction with a lysozyme domain is common in putative tail proteins of other Firmicutes phages as well. This prompts us to speculate whether LytC might be a constituent of an apoptosis mechanism that is activated upon phage entry. The specific cuts in the murein layer introduced by the phage to gain entry to the cell might activate LytC, triggering autolysis and thus prevent phage propagation. If this is the case, CbpD relies on a system that is in place for totally different purposes to trigger lysis of target cells. At this point of time, this is purely a matter of speculation. Hopefully, continued research will result in a better understanding of the role of LytC.

We have shown that the LiaFSR three-component system plays a role in protecting cells against competence-induced stress. However, the system does not seem to play a major role in protection of non-competent target cells, but rather seems to protect the competent cells themselves against cell envelope stress. The reason why only a subfraction of non-competent target cells lyse and die in fratricide assays has not been answered yet. There are at least two possible explanations for this observation: Pneumococcal predation could be fine-tuned not for a total wipe-out of target cells, but for limited lysis. This could be brought about *e.g.*

by quick deactivation of CbpD as observed in paper I. Alternatively, there could be as yet undetected systems that are set in motion in cells subjected to attack by murein hydrolases, rendering these cells immune to lysis. This could be brought about *e.g.* by changes in acetylation patterns in the peptidoglycan as observed in *Lactococcus lactis* (96).
In paper II we speculate that LiaFSR has diverged from a classical cell envelope stress response system into a second layer of defence against competence-induced cell envelope stress. Although ComM is sufficient to protect the vast majority of competent cells against their own lysins, the fraction of cells that lyse upon competence induction doubles from 1.3% to 2.6% when *liaR* is deleted. This does not look very impressive, but in an evolutionary time scale, this extra level of protection could very well be selected for. Upon competence induction, the early gene *comM* is expressed ~5 minutes before the late genes, including *cbpD* (77). This observation together with the imperfect protection conferred by ComM might provide clues to its mechanism of action. It seems likely that ComM does not directly inactivate or scavenge CbpD, but rather protects the cells in a more indirect manner, *e.g.* by bringing about changes in the structure of cell envelope peptidoglycan or teichoic acids. In a population of cells undergoing competence development, it is possible that some cells are unable to carry out these hypothesized changes in time, hence the need for a second layer of defence.

Unravelling the function(s) of fratricide remains a complicated task. That gene exchange is important seems exceedingly likely, but the evolutionary effect of this exchange is complicated to assess. A better understanding of this will have to be based on both laboratory work and bioinformatic analyses. If fratricide-mediated gene exchange is restricted to only closely related bacteria, the result will be homogenization of the taxon. If the process is very promiscuous, random shuffling of genes will not be enough for homogenization. The effect of gene exchange will of course also differ from the level of individual cells to higher taxonomic levels. A group of neighbouring cells can be said to diversify by incorporating different pieces of "foreign" DNA, even if the same process leads to homogenization at higher taxonomic levels. Previous studies at our laboratory suggests that predation-mediated gene exchange involving pneumococci is restricted to closely related mitis group streptococci (44). Whether this restricted killing is restricted enough to have a homogenizing effect on these bacteria remains a matter of speculation. The existence of different pherogroups complicates the matter further. If two pherogroups colonize the same space this should in theory favour gene exchange between the two groups: Members of the group first developing competence would be immune to the lysins thus produced, whereas members of the second pherogroup would succumb to these lysins resulting in liberation of DNA that can be taken up and incorporated into the genomes of the members of the first pherogroup. Future research will hopefully further our understanding of the importance of predation-mediated gene exchange. A recent study found strains of *S. mitis* to be no more related to each other than to pneumococci, thus questioning the species delineation of the mitis group streptococci (50). Possibly, the mitis group streptococci might come to be regarded as one species or a few species belonging to different "killing groups". A true species delineation of mitis group streptococci might never be agreed upon. The large variation in gene content in pneumococci is probably similar in other mitis group streptococci and DNA transfer within and between these species is blurring the boundaries between them. These species might even be said to share a mitis group supragenome.

In addition to gene exchange, a putative function of pneumococcal fratricide is killing of niche competitors. The pneumococcus is a transient colonizer, thus, upon entering a new host it has to establish a presence in an already established community. Predation could be a means of killing niche-competitors and creating a space for colonization by brute

force. Extracellular DNA has been shown to be important for the formation of *S. pneumoniae* biofilms (67), and DNA released by fratricide could possibly play a role in the formation of biofilms. It should be noted that a study found CbpD to be dispensable for biofilm formation (67), but this does not rule out a role of CbpD in biofilm formation in other settings *e.g.* including mixed cultures.

Finally, pneumococcal fratricide might be important for the release of virulence factors, such as pneumolysin (Ply). Competence-induction has been shown to cause Ply-mediated β-haemolytic growth on blood-agar plates (27). The authors also found that the Ply was released mainly from non-competent cells succumbing to lysins produced by competent cells. Whether this *in vitro* finding translates into *in vivo* relevance remain to be seen.

When discussing pneumococcal predation, a relevant question to ask is why this phenomenon is kept separate from bacteriocin-mediated killing. Like CbpD, many bacteriocins are able to kill non-immune isogenic bacteria although they are classically considered as killers of competitors. Pneumococci produce the immunity protein ComM to protect themselves against CbpD, just like bacteriocin producers produce specific immunity proteins for self protection. If one considers the proposed bacteriocin classification scheme by Cotter *et al.* (12), CbpD could be classified as a bacteriolysin together with, among others, lysostaphin. Lysostaphin produced by *Staphylococcus simulans* and the lysostaphin homologue ALE-1 produced by *Staphylococcus capitis* preferentially target *S. aureus* cells. As pneumococci are able to kill closely related bacteria belonging to other species, CbpD fits nicely in this proposed bacteriolysin category of bacteriocins. In summary, the main difference between bacteriocin mediated killing and CbpD-induced fratricide is the link to gene exchange in the latter.

Future perspectives and concluding remarks

Regarding the molecular details of fratricide, future research should focus on the enzymatic activity of CbpD and how this activity is controlled. In addition, continued research should aim at elucidating the ComM mediated immunity mechanism. In liquid culture, CbpD is the key player of fratricide, whereas the two-peptide bacteriocin CibAB plays a prominent role in fratricide on blood-agar plates. Unravelling the distinct roles of these proteins in more biologically relevant settings such as biofilms would be very interesting.

Determining the ultimate biological role(s) of fratricide will be an even more profound task. The phylogenetic boundaries of cross-species killing in mitis group streptococci and beyond, and the effect of these boundaries on shaping the evolution of these bacteria remain to be investigated. Bacteriocin-mediated killing of conspecific and heterospecific bacteria is widespread in the bacterial world. At present, only mitis group streptococci are known to couple such killing with acquisition of transforming DNA. It is possible that these phenomena could be co-regulated in other more distantly related naturally transformable bacteria as well. If this is the case, fratricide might be important for shaping the evolution of many bacterial species across this domain of life.

In vivo studies are needed in order to clarify whether fratricide contributes to virulence, for instance by releasing intracellular virulence factors. A possible role of fratricide during establishment of streptococcal communities upon invasion of an already established resident bacterial population could be elucidated by experiments with mixed culture biofilms.

Bacteria do not reproduce sexually. In higher organisms, sex is restricted to members of the same species. Sexual reproduction is essential to avoid accumulation of deleterious mutations in higher organisms (68), and as sex is restricted to members of the same species, conservation of the genome is ensured. In bacteria, transformation might be important for DNA repair and to grant bacteria access to a larger supragenome, and bacteria too have mechanisms to prevent the uptake of DNA from distantly related organisms. Naturally competent members of the families Neisseriaceae and Pasteurellaceae strongly prefer to take up DNA containing their own specific DNA uptake sequences (60). The Pasteurellaceae family is further divided into two subclades with different uptake sequences (81). This ensures that only DNA from closely related species is taken up and recombined with the genome. Johnsborg *et al*. demonstrated that competent pneumococci can lyse closely related mitis group streptococci but not the more distant relative *S. gordonii*. Subsequently, liberated DNA is taken up and incorporated into the genomes of the attacking pneumococci (44). Hence, fratricide seems to constitute a mechanism for active acquisition of homologous DNA. In paper III we show that CbpD is dependent on the cell envelope binding specificity of both the SH3b domains and the CBD in order to lyse target cells. The CBD is likely to restrict the killing range to target cells harboring choline containing teichoic acids, and further discrimination might be conferred by the SH3b domains. Incorporation of exogenous DNA into the genome is also limited by lack of homology and restriction modification systems that act to diminish incorporation of DNA from different strains and species. A narrow killing range of CbpD might thus seem redundant, but if acquisition of homologous DNA is an integral part of fratricide, it makes sense not to “waste” CbpD activity on unrelated bacteria.

References

1. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Inductions of transformation by a deoxyribonucleic acid fraction isolated from pneumococcus type III. J. Exp. Med. **79:**137-158.
2. **Bassler, B., E. Greenberg, and A. Stevens.** 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J. Bacteriol. **179:**4043-4045.
3. **Bassler, B. L.** 2002. Small Talk: Cell-to-Cell Communication in Bacteria. Cell **109:**421-424.
4. **Bateman, A., and N. D. Rawlings.** 2003. The CHAP domain: a large family of amidases including GSP amidase and peptidoglycan hydrolases. Trends Biochem. Sci. **28:**234-237.
5. **Bergmann, S., and S. Hammerschmidt.** 2006. Versatility of pneumococcal surface proteins. Microbiology **152:**295-303.
6. **Bhavsar, A. P., and E. D. Brown.** 2006. Cell wall assembly in *Bacillus subtilis*: how spirals and spaces challenge paradigms. Mol. Microbiol. **60:**1077-1090.
7. **Briese, T., and R. Hakenbeck.** 1985. Interaction of the pneumococcal amidase with lipoteichoic acid and choline. European Journal of Biochemistry **146:**417-427.
8. **Catlin, B. W.** 1960. Transformation of *Neisseria menigitides* by deoxyribonucleates from cells and from culture slime. J. Bacteriol. **79:**579-590.
9. **Chi, F., M. Leider, F. Leendertz, C. Bergmann, C. Boesch, S. Schenk, G. Pauli, H. Ellerbrok, and R. Hakenbeck.** 2007. New *Streptococcus pneumoniae* Clones in Deceased Wild Chimpanzees. J. Bacteriol. **189:**6085-6088.
10. **Claverys, J.-P., B. Martin, and P. Polard.** 2009. The genetic transformation machinery: composition, localization, and mechanism. FEMS Microbiology Reviews **33:**643-656.
11. **Claverys, J.-P., M. Prudhomme, and B. Martin.** 2006. Induction of Competence Regulons as a General Response to Stress in Gram-Positive Bacteria. Annu. Rev. Microbiol. **60:**451-475.
12. **Cotter, P. D., C. Hill, and R. P. Ross.** 2005. Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. **3:**777-788.
13. **Dagkessamanskaia, A., M. Moscoso, V. Henard, S. Guiral, K. Overweg, M. Reuter, B. Martin, J. Wells, and J.-P. Claverys.** 2004. Interconnection of competence, stress and CiaR regulons in *Streptococcus pneumoniae*: competence triggers stationary phase autolysis of ciaR mutant cells. Mol. Microbiol. **51:**1071-1086.
14. **Desai, B. V., and D. A. Morrison.** 2007. Transformation in *Streptococcus pneumoniae*: formation of eclipse complex in a *coiA* mutant implicates CoiA in genetic recombination. Molecular Microbiology **63:**1107-1117.
15. **Díaz, E., R. López, and J. L. García.** 1990. Chimeric phage-bacterial enzymes: a clue to the modular evolution of genes. Proc. Natl. Acad. Sci. **87:**8125-8129.
16. **Dong, Y.-H., J.-L. Xu, X.-Z. Li, and L.-H. Zhang.** 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc. Natl. Acad. Sci. U S A **97:**3526-3531.
17. **Duane, P. G., J. B. Rubins, H. R. Weisel, and E. N. Janoff.** 1993. Identification of hydrogen peroxide as a *Streptococcus pneumoniae* toxin for rat alveolar epithelial cells. Infect. Immun. **61:**4392-4397.

18. **Fernandez-Tornero, C., E. Garcia, R. Lopez, G. Gimenez-Gallego, and A. Romero.** 2002. Two New Crystal Forms of the Choline-binding Domain of the Major Pneumococcal Autolysin: Insights into the Dynamics of the Active Homodimer. J. Mol. Biol. **321:**163-173.
19. **Fischer, W., T. Behr, R. Hartmann, J. Peter-Katalinic, and H. Egge.** 1993. Teichoic acid and lipoteichoic acid of *Streptococcus pneumoniae* possess identical chain structures. Eur. J. Biochem. **215:**851-857.
20. **Garcia-Vallve, S., E. Guzman, M. A. Montero, and A. Romeu.** 2003. HGT-DB: a database of putative horizontally transferred genes in prokaryotic complete genomes. Nucl. Acids Res. **31:**187-189.
21. **Garcia, P., M. P. Gonzalez, E. Garcia, J. L. Garcia, and R. Lopez.** 1999. The molecular characterization of the first autolytic lysozyme of *Streptococcus pneumoniae* reveals evolutionary mobile domains. Mol. Microbiol. **33:**128-138.
22. **Giammarinaro, P., M. Sicard, and A.-M. Gasc.** 1999. Genetic and physiological studies of the CiaH-CiaR two-component signal-transducing system involved in cefotaxime resistance and competence of *Streptococcus pneumoniae*. Microbiology **145:**1859-1869.
23. **Gilmore, M. S., and W. Haas.** 2005. The selective advantage of microbial fratricide. Proc. Natl. Acad. Sci. U S A **102:**8401-8402.
24. **Griffith, F.** 1928. The significance of pneumococcal types. Journal of Hygiene **27:**113-159.
25. **Guangyong, J., R. Beavis, and R. P. Novick.** 1997. Bacterial Interference Caused by Autoinducing Peptide Variants. Science **276:**2027-2030.
26. **Guenzi, E., A.-M. Gasc, M. A. Sicard, and R. Hakenbeck.** 1994. A two-component signal-transducing system is involved in competence and penicillin susceptibility in laboratory mutants of *Streptococcus pneumoniae*. Mol. Microbiol. **12:**505-515.
27. **Guiral, S., T. J. Mitchell, B. Martin, and J.-P. Claverys.** 2005. From The Cover: Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: Genetic requirements. Proc. Natl. Acad. Sci. U S A **102:**8710-8715.
28. **Haas, W., D. Kaushal, J. Sublett, C. Obert, and E. I. Tuomanen.** 2005. Vancomycin Stress Response in a Sensitive and a Tolerant Strain of *Streptococcus pneumoniae*. J. Bacteriol. **187:**8205-8210.
29. **Hakenbeck, R., A. Madhour, D. Denapaite, and R. Brückner.** 2009. Versatility of choline metabolism and choline-binding proteins in *Streptococcus pneumoniae* and commensal streptococci. FEMS Microbiol. Rev. **33:**572-586.
30. **Halfmann, A., R. Hakenbeck, and R. Brückner.** 2007. A new integrative reporter plasmid for *Streptococcus pneumoniae*. FEMS Microbiol. Lett. **268:**217-224.
31. **Hamilton, H. L., N. M. Dominguez, K. J. Schwartz, K. T. Hackett, and J. P. Dillard.** 2005. *Neisseria gonorrhoeae* secretes chromosomal DNA via a novel type IV secretion system. Mol. Microbiol. **55:**1704-1721.
32. **Helmann, J. D.** 2002. The extracytoplasmic function (ECF) sigma factors. Adv. Microb. Physiol. **46:**47-110.
33. **Hiller, N. L., B. Janto, J. S. Hogg, R. Boissy, S. Yu, E. Powell, R. Keefe, N. E. Ehrlich, K. Shen, J. Hayes, K. Barbadora, W. Klimke, D. Dernovoy, T. Tatusova, J. Parkhill, S. D. Bentley, J. C. Post, G. D. Ehrlich, and F. Z. Hu.** 2007. Comparative Genomic Analyses of Seventeen *Streptococcus pneumoniae* Strains: Insights into the Pneumococcal Supragenome. J. Bacteriol. **189:**8186-8195.
34. **Hoskins, J., W. E. Alborn, Jr., J. Arnold, L. C. Blaszczak, S. Burgett,**

B. S. DeHoff, S. T. Estrem, L. Fritz, D.-J. Fu, W. Fuller, C. Geringer, R. Gilmour, J. S. Glass, H. Khoja, A. R. Kraft, R. E. Lagace, D. J. LeBlanc, L. N. Lee, E. J. Lefkowitz, J. Lu, P. Matsushima, S. M. McAhren, M. McHenney, K. McLeaster, C. W. Mundy, T. I. Nicas, F. H. Norris, M. O'Gara, R. B. Peery, G. T. Robertson, P. Rockey, P.-M. Sun, M. E. Winkler, Y. Yang, M. Young-Bellido, G. Zhao, C. A. Zook, R. H. Baltz, S. R. Jaskunas, P. R. Rosteck, Jr., P. L. Skatrud, and J. I. Glass. 2001. Genome of the Bacterium *Streptococcus pneumoniae* Strain R6. J. Bacteriol. **183:**5709-5717.

35. **Howard, L. V., and H. Gooder.** 1974. Specificity of the Autolysin of *Streptococcus* (*Diplococcus*) *pneumoniae*. J. Bacteriol. **117:**796-804.

36. **Håvarstein, L. S., G. Coomaraswamy, and D. A. Morrison.** 1995. An Unmodified Heptadecapeptide Pheromone Induces Competence for Genetic Transformation in *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. U S A **92:**11140-11144.

37. **Håvarstein, L. S., D. B. Diep, and I. F. Nes.** 1995. A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. Mol. Microbiol. **16:**229-240.

38. **Håvarstein, L. S., P. Gaustad, I. F. Nes, and D. A. Morrison.** 1996. Identification of the streptococcal competence-pheromone receptor. Mol. Microbiol. **21:**863-869.

39. **Håvarstein, L. S., B. Martin, O. Johnsborg, C. Granadel, and J.-P. Claverys.** 2006. New insights into the pneumococcal fratricide: relationship to clumping and identification of a novel immunity factor. Mol. Microbiol. **59:**1297-1037.

40. **Ishii, S., and T. H. Yano, Hideyuki.** 2006. Expression and Characterization of the Peptidase Domain of Streptococcus pneumoniae ComA, a Bifunctional ATP-binding Cassette Transporter Involved in Quorum Sensing Pathway. J. Biol. Chem. **281:**4726-4731.

41. **Jarraud, S., G. J. Lyon, A. M. S. Figueiredo, L. Gerard, F. Vandenesch, J. Etienne, T. W. Muir, and R. P. Novick.** 2000. Exfoliatin-Producing Strains Define a Fourth agr Specificity Group in *Staphylococcus aureus*. J. Bacteriol. **182:**6517-6522.

42. **Jennings, H. J., C. Lugowski, and N. M. Young.** 1980. Structure of the complex polysaccharide C-substance from *Streptococcus pneumoniae* type. Biochemistry **19:**4712-4719.

43. **Johnsborg, O., T. Blomqvist, M. Kilian, and L. S. Håvarstein.** 2007. Biologically active peptides in streptococci, p. 25-59. *In* R. Hakenbeck and S. Chatwal (ed.), Molecular Biology of Streptococci. Horizon Scientific Press, Norfolk.

44. **Johnsborg, O., V. Eldholm, M. L. Bjørnstad, and L. S. Håvarstein.** 2008. A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species. Mol. Microbiol. **69:**245-253.

45. **Johnsborg, O., V. Eldholm, and L. S. Håvarstein.** 2007. Natural genetic transformation: prevalence, mechanisms and function. Res. Microbiol. **158:**767-778.

46. **Johnsborg, O., and L. S. Håvarstein.** 2009. Regulation of natural genetic transformation and acquisition of transforming DNA in *Streptococcus pneumoniae*. FEMS Microbiol. Rev. **33:**627-642.

47. **Jordan, S., M. I. Hutchings, and T. Mascher.** 2008. Cell envelope stress response in Gram-positive bacteria. FEMS Microbiol. Rev. **32:**107-146.

48. **Jordan, S., A. Junker, J. D. Helmann, and T. Mascher.** 2006. Regulation of LiaRS-Dependent Gene Expression in *Bacillus subtilis*: Identification of Inhibitor Proteins, Regulator Binding Sites, and Target

Genes of a Conserved Cell Envelope Stress-Sensing Two-Component System. J. Bacteriol. **188:**5153-5166.

49. **Kausmally, L., O. Johnsborg, M. Lunde, E. Knutsen, and L. S. Havarstein.** 2005. Choline-Binding Protein D (CbpD) in *Streptococcus pneumoniae* Is Essential for Competence-Induced Cell Lysis. J. Bacteriol. **187:**4338-4345.
50. **Kilian, M., K. Poulsen, T. Blomqvist, L. S. Håvarstein, M. Bek-Thomsen, H. Tettelin, and U. B. S. Sørensen.** 2008. Evolution of *Streptococcus pneumoniae* and Its Close Commensal Relatives. PLoS ONE **3:**e2683.
51. **Lancefield, R. C.** 1933. A serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med. **57:**571-595.
52. **Lange, R., C. Wagner, A. de Saizieu, N. Flint, J. Molnos, M. Stieger, P. Caspers, M. Kamber, W. Keck, and K. E. Amrein.** 1999. Domain organization and molecular characterization of 13 two-component systems identified by genome sequencing of *Streptococcus pneumoniae*. Gene **237:**223-234.
53. **Layec, S., J. Gérard, V. Legué, M.-P. Chapot-Chartier, P. Courtin, F. Borges, B. Decaris, and N. Leblond-Bourget.** 2009. The CHAP domain of Cse functions as an endopeptidase that acts at mature septa to promote *Streptococcus thermophilus* cell separation. Mol. Microbiol. **71:**1205-1217.
54. **Lee, M. S., and D. A. Morrison.** 1999. Identification of a New Regulator in *Streptococcus pneumoniae* Linking Quorum Sensing to Competence for Genetic Transformation. J. Bacteriol. **181:**5004-5016.
55. **Lopez, R., E. Garcia, P. Garcia, C. Ronda, and A. Tomasz.** 1982. Choline-containing bacteriophage receptors in *Streptococcus pneumoniae*. J. Bacteriol. **151:**1581-1590.
56. **Lu, J. Z., T. Fujiwara, H. Komatsuzawa, M. Sugai, and J. Sakon.** 2006. Cell Wall-targeting Domain of Glycylglycine Endopeptidase Distinguishes among Peptidoglycan Cross-bridges. J. Biol. Chem. **281:**549-558.
57. **Lun, Z.-R., Q.-P. Wang, X.-G. Chen, A.-X. Li, and X.-Q. Zhu.** 2007. *Streptococcus suis*: an emerging zoonotic pathogen. Lancet Infect. Dis. **7:**201-209.
58. **Martin, B., M. Prudhomme, G. Alloing, C. Granadel, and J.-P. Claverys.** 2000. Cross-regulation of competence pheromone production and export in the early control of transformation in *Streptococcus pneumoniae*. Mol. Microbiol. **38:**867-878.
59. **Martínez, B., A. L. Zomer, A. Rodríguez, J. Kok, and O. P. Kuipers.** 2007. Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the lactococcal two-component system CesSR. Mol. Microbiol. **64:**473-486.
60. **Mathis, L. S., and J. J. Scocca.** 1982. *Haemophilus influenzae* and *Neisseria gonorrhoeae* recognize different specificity determinants in the DNA uptake step of genetic transformation. J. Gen. Microbiol. **128:**1159-1161.
61. **Mayville, P., G. Ji, R. Beavis, H. Yang, M. Goger, R. P. Novick, and T. W. Muir.** 1999. Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proc. Natl. Acad. Sci. U S A **96:**1218-1223.
62. **Méjean, V., and J.-P. Claverys.** 1988. Polarity of DNA entry in transformation of Streptococcus pneumoniae. Molecular and General Genetics MGG **213:**444-448.
63. **Morrison, D. A., I. Mortier-Barriere, L. Attaiech, and J.-P. Claverys.** 2007. Identification of the Major Protein Component of the Pneumococcal Eclipse Complex. J. Bacteriol. **189:**6497-6500.

64. **Mortier-Barrière, I., A. d. Saizieu, J.-P. Claverys, and B. Martin.** 1998. Competence-specific induction of *recA* is required for full recombination proficiency during transformation in *Streptococcus pneumoniae*. Mol. Microbiol. **27:**159-170.
65. **Mortier-Barrière, I., M. Velten, P. Dupaigne, N. Mirouze, O. Piétrement, S. McGovern, G. Fichant, B. Martin, P. Noirot, E. Le Cam, P. Polard, and J.-P. Claverys.** 2007. A Key Presynaptic Role in Transformation for a Widespread Bacterial Protein: DprA Conveys Incoming ssDNA to RecA. Cell **130:**824-836.
66. **Moscoso, M., and J.-P. Claverys.** 2004. Release of DNA into the medium by competent Streptococcus pneumoniae: kinetics, mechanism and stability of the liberated DNA. Mol. Microbiol. **54:**783-794.
67. **Moscoso, M., E. Garcia, and R. Lopez.** 2006. Biofilm Formation by *Streptococcus pneumoniae*: Role of Choline, Extracellular DNA, and Capsular Polysaccharide in Microbial Accretion. J. Bacteriol. **188:**7785-7795.
68. **Muller, H. J.** 1964. The relation of recombination to mutational advance. Mutat Res. **106:**2-9.
69. **Nedenskov-Sorensen, P., G. Bukholm, and K. Bovre.** 1990. Natural competence for genetic transformation in *Campylobacter pylori.* J. Infect. Dis. **161:**365-366.
70. **Novick, R. P., and E. Geisinger.** 2008. Quorum Sensing in *Staphylococci*. Annual Review of Genetics **42:**541-564.
71. **O'Brien, K. L., L. J. Wolfson, J. P. Watt, E. Henkle, M. Deloria-Knoll, N. McCall, E. Lee, K. Mulholland, O. S. Levine, and T. Cherian.** 2009. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet **374:**893-902.
72. **Pakula, R., and W. Walczak.** 1963. On the Nature of Competence of Transformable Streptococci. J Gen Microbiol **31:**125-133.
73. **Patterson, M. J.** 1996. Streptococcus. *In* S. Baron (ed.), Baron's Medical Microbiology, 4th ed. University of Texas Medical Branch.
74. **Pérez-Dorado, I., A. González, M. Morales, R. Sanles, W. Striker, W. Vollmer, S. Mobashery, J. L. García, M. Martínez-Ripoll, P. García, and J. A. Hermoso.** Insights into pneumococcal fratricide from crystal structure of the modular killing factor LytC. (in press) Nat. Struct. Mol. Biol.
75. **Pericone, C. D., K. Overweg, P. W. M. Hermans, and J. N. Weiser.** 2000. Inhibitory and Bactericidal Effects of Hydrogen Peroxide Production by *Streptococcus pneumoniae* on Other Inhabitants of the Upper Respiratory Tract. Infect. Immun. **68:**3990-3997.
76. **Pericone, C. D., S. Park, J. A. Imlay, and J. N. Weiser.** 2003. Factors Contributing to Hydrogen Peroxide Resistance in *Streptococcus pneumoniae* Include Pyruvate Oxidase (SpxB) and Avoidance of the Toxic Effects of the Fenton Reaction. J. Bacteriol. **185:**6815-6825.
77. **Peterson, S. N., C. K. Sung, R. Cline, B. V. Desai, E. C. Snesrud, P. Luo, J. Walling, H. Li, M. Mintz, G. Tsegaye, P. C. Burr, Y. Do, S. Ahn, J. Gilbert, R. D. Fleischmann, and D. A. Morrison.** 2004. Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. Mol. Microbiol. **51:**1051-1070.
78. **Piotrowski, A., P. Luo, and D. A. Morrison.** 2009. 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. **191:**3359-3366.
79. **Prudhomme, M., L. Attaiech, G. Sanchez, B. Martin, and J.-P. Claverys.** 2006. Antibiotic Stress

Induces Genetic Transformability in the Human Pathogen *Streptococcus pneumoniae*. Science **313:**89-92.

80. **Rashel, M., J. Uchiyama, I. Takemura, H. Hoshiba, T. Ujihara, H. Takatsuji, K. Honke, and S. Matsuzaki.** 2008. Tail-associated structural protein gp61 of *Staphylococcus aureus* phage φMR11 has bifunctional lytic activity. FEMS Microbiol. Lett. **284:**9-16.
81. **Redfield, R., W. Findlay, J. Bosse, J. S. Kroll, A. Cameron, and J. Nash.** 2006. Evolution of competence and DNA uptake specificity in the Pasteurellaceae. BMC Evolutionary Biology **6:**82.
82. **Rigden, D. J., M. J. Jedrzejas, and M. Y. Galperin.** 2003. Amidase domains from bacterial and phage autolysins define a family of γ-$_{D,L}$ -glutamate-specific amidohydrolases. Trends Biochem. Sci. **28:**230-234.
83. **Rogers, P. D., T. T. Liu, K. S. Barker, G. M. Hilliard, B. K. English, J. Thornton, E. Swiatlo, and L. S. McDaniel.** 2007. Gene expression profiling of the response of *Streptococcus pneumoniae* to penicillin. J. Antimicrob. Chemother. **59:**616-626.
84. **Sanchez-Puelles, J. M., C. Ronda, J. L. Garcia, P. Garcia, R. Lopez, and E. Garcia.** 1986. Searching for autolysin functions. Eur. J. Biochem. **158:**289-293.
85. **Saskova, L., L. Novakova, M. Basler, and P. Branny.** 2007. A eukaryotic-type serine/threonine protein kinase StkP is a global regulator of gene expression in *Streptococcus pneumoniae*. J. Bacteriol.**:**1606-1616.
86. **Smith, J. M., N. H. Smith, M. O'Rourke, and B. G. Spratt.** 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. **90:**4384-4388.
87. **Soding, J., A. Biegert, and A. N. Lupas.** 2005. The HHpred interactive server for protein homology detection and structure prediction. Nucl. Acids Res. **33:**W244-248.
88. **Steinmoen, H., E. Knutsen, and L. S. Havarstein.** 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.
89. **Steinmoen, H., A. Teigen, and L. S. Havarstein.** 2003. Competence-Induced Cells of *Streptococcus pneumoniae* Lyse Competence-Deficient Cells of the Same Strain during Cocultivation. J. Bacteriol. **185:**7176-7183.
90. **Sung, C. K., and D. A. Morrison.** 2005. Two Distinct Functions of ComW in Stabilization and Activation of the Alternative Sigma Factor ComX in *Streptococcus pneumoniae*. J. Bacteriol. **187:**3052-3061.
91. **Tagg, J. R., and K. P. Dierksen.** 2003. Bacterial replacement therapy: adapting 'germ warfare' to infection prevention. Trends Biotech. **21:**217-223.
92. **Tomasz, A.** 1965. Control of the competent state in Pneumococcus by a hormone-like cell product: an example for a new type of regulatory mechanism in bacteria. Nature **208:**155-159.
93. **Tomasz, A.** 1966. Model for the Mechanism Controlling the Expression of Competent State in Pneumococcus Cultures. J. Bacteriol. **91:**1050-1061.
94. **Tomasz, A., and R. D. Hotchkiss.** 1964. Regulation of the transformability of pneumococcal cultures by macromolecular cell products. Proc. Natl. Acad. Sci. U S A **51:**480-487.
95. **Tomasz, A., and S. Waks.** 1975. Enzyme replacement in a bacterium: Phenotypic correction by the experimental introduction of the wild type enzyme into a live enzyme defective mutant pneumococcus. Biochemical and Biophysical Research Communications **65:**1311-1319.
96. **Veiga, P., C. Bulbarela-Sampieri, S.**

Furlan, A. Maisons, M.-P. Chapot-Chartier, M. Erkelenz, P. Mervelet, P. Noirot, D. Frees, O. P. Kuipers, J. Kok, A. Gruss, G. Buist, and S. Kulakauskas. 2007. SpxB Regulates O-Acetylation-dependent Resistance of *Lactococcus lactis* Peptidoglycan to Hydrolysis. J. Biol. Chem. **282:**19342-19354.

97. **Waters, C. M., and B. L. Bassler.** 2005. QUORUM SENSING: Cell-to-Cell Communication in Bacteria. Annu. Rev. Cell Dev. Bi. **21:**319-346.
98. **Watt, V. M., C. J. Ingles, M. S. Urdea, and W. J. Rutter.** 1985. Homology requirements for recombination in *Escherichia coli*. Proc. Natl. Acad. Sci. **82:**4768-4772.
99. **Ween, O., P. Gaustad, and L. S. Håvarstein.** 1999. Identification of DNA binding sites for ComE, a key regulator of natural competence in *Streptococcus pneumoniae*. Mol. Microbiol. **33:**817-827.
100. **Weidenmaier, C., and A. Peschel.** 2008. Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. Nat. Rev. Microbiol. **6:**276-287.
101. **Whisstock, J. C., and A. M. Lesk.** 1999. SH3 domains in prokaryotes. Trends Biochem. Sci. **24:**132-133.
102. **WHO.** 2009. Fact sheet Group A Streptococcus.
103. **Wu, H., A. B. Reynolds, S. B. Kanner, R. R. Vines, and J. T. Parsons.** 1991. Identification and characterization of a novel cytoskeleton-associated pp60src substrate. Mol. Cell. Biol. **11:**5113-5124.
104. **Yother, J., and J. M. White.** 1994. Novel surface attachment mechanism of the *Streptococcus pneumoniae* protein PspA. J. Bacteriol. **176:**2976-2985.

Paper I

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Fratricide in *Streptococcus pneumoniae*: contributions and role of the cell wall hydrolases CbpD, LytA and LytC

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Pneumococci that have developed the competent state kill and lyse non-competent sister cells and members of closely related species during co-cultivation *in vitro*. The key component in this process, called fratricide, is the product of the late competence gene *cbpD.* In addition, the peptidoglycan hydrolases LytA and LytC are required for efficient lysis of target cells. Here, we have investigated the relative contribution and possible role of each of the proteins mentioned above. Previous studies have shown that CbpD is produced exclusively by competent cells, whereas LytA and LytC can be provided by the competent attackers as well as the non-competent target cells. By using an improved assay to compare the effect of *cis*- versus *trans*-acting LytA and LytC, we were able to show that target cells are lysed much more efficiently when LytA and LytC are provided *in cis*, i.e. by the target cells themselves. Western analysis demonstrated that considerable amounts of LytC are present in the growth medium. In contrast, we were not able to detect any extracellular LytA. This finding indicates that LytA- and LytC-mediated fratricide represent different processes. In the absence of LytA and LytC, only a tiny fraction of the target cells were lysed, demonstrating that CbpD does not function efficiently on its own. However, in the presence of 1 mM EDTA, the fraction of target cells lysed directly by CbpD increased dramatically, indicating that divalent cations are involved in the regulation of fratricide under natural conditions.

INTRODUCTION

Streptococcus pneumoniae is an important human pathogen with remarkable adaptation capabilities. It is the most common single cause of community-acquired infections, including bacterial pneumonia, bacteraemia, meningitis and otitis media. The pneumococcus causes 1–2 million deaths per year, most of which occur in developing countries, where this bacterial species is probably the most important pathogen of early infancy (Obaro & Adegbola, 2002).

Bacteria that have the ability to take up naked DNA from the environment and incorporate this DNA into their genomes by homologous recombination are said to be competent for natural genetic transformation. So far, about 70 different species of bacteria, many of which are important pathogens, have been shown to possess this property (Johnsborg *et al.*, 2007). In *S. pneumoniae* and related commensal streptococci, natural genetic transformation mediates intraspecies as well as interspecies gene transfer. It has for instance been firmly established that gene exchange by this mechanism has contributed significantly to the increasing incidence of penicillin-resistant *S. pneumoniae* worldwide (Hakenbeck, 1995; Chi *et al.*, 2007), a development that constitutes a major threat to public health (Jenkins *et al.*, 2008). Gene exchange by natural transformation gives the pneumococcus access to a large gene pool, which it shares with other pneumococcal strains and closely related commensal streptococci (Hiller *et al.*, 2007). Consequently, genes present in the common gene pool that give a selective advantage under certain types of stress (e.g. vaccination or treatment with antibiotics) will spread rapidly among these bacteria.

Recently, we reported that a competence-controlled predatory mechanism dramatically increases the efficiency of lateral gene transfer in *S. pneumoniae* and related commensal species (Johnsborg *et al.*, 2008). When developing the competent state, streptococci such as *S. pneumoniae*, *Streptococcus mitis* and *Streptococcus oralis*

Abbreviations: CSP, competence-stimulating peptide; LTA, lipoteichoic acid; TA, teichoic acid.

Details of the construction of *S. pneumoniae* mutants, and a table of primers, are available as supplementary material with the online version of this paper.

express a putative murein hydrolase, CbpD, which mediates lysis of non-competent target cells present in the same environment. As the DNA released from the lysed cells can be taken up by the competent attacker cells, the rate of gene transfer is greatly increased. Induction of the competent state in *S. pneumoniae* is controlled by the ComABCDE quorum-sensing system (Håvarstein *et al.*, 1995; Pestova *et al.*, 1996). Although the closely related ComABCDE systems of *S. mitis* and *S. oralis* have not been studied in the same detail as their pneumococcal counterpart, there is little doubt that these proteins have the same function in all three species. The ComABCDE quorum-sensing system consists of the *comC*-encoded competence-stimulating peptide (CSP) (Håvarstein *et al.*, 1995), its secretion apparatus (ComAB) (Hui & Morrison, 1991), and a two-component signal transduction pathway (ComDE) that senses the concentration of extracellular CSP (Håvarstein *et al.*, 1996). When the CSP pheromone reaches a critical concentration, transcription of the early and late competence genes is initiated, and the cells enter the competent state. The response regulator ComE, which upon phosphorylation binds to a conserved direct-repeat motif in the promoter region of target genes (Ween *et al.*, 1999), directly controls the expression of about 20 early competence genes. Among these are the gene(s) encoding ComX, an alternative sigma factor required for expression of approximately 80 late competence genes (Lee & Morrison, 1999). Curiously, only a fraction of the CSP-responsive genes, 7 early and 14 late gene products, are required for the transformation process (Dagkessamanskaia *et al.*, 2004; Peterson *et al.*, 2004). In addition, a few CSP-responsive genes are involved in the predatory lysis mechanism described above (for a review, see Claverys & Håvarstein, 2007). These include the early competence gene *comM* (Håvarstein *et al.*, 2006), and the two late competence genes *cbpD* and *lytA* (Steinmoen *et al.*, 2003; Guiral *et al.*, 2005; Kausmally *et al.*, 2005). The *comM* gene encodes an immunity protein that protects the competent cells against their own lysins (Håvarstein *et al.*, 2006). The mechanism behind this protection is not known. Similarly, the exact mode of action of the key component of the lysis mechanism, CbpD, has not been determined yet. However, since its CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) domain is homologous to the corresponding domain of a number of cell wall hydrolases that cleave peptide bonds within the stem peptides of bacterial peptidoglycan, it is reasonable to assume that CbpD performs the same function (Bateman & Rawlings, 2003; Rigden *et al.*, 2003). The major pneumococcal autolysin LytA is expressed in non-competent cells, but its expression increases during competence (Mortier-Barrière *et al.*, 1998; Dagkessamanskaia *et al.*, 2004; Peterson *et al.*, 2004). An additional constitutively expressed cell wall hydrolase, LytC, which is not part of the competence regulon, has also been shown to contribute to the lysis mechanism (Guiral *et al.*, 2005).

These proteins, CbpD, LytA and LytC, appear to be the principal players mediating lysis of non-competent target cells during fratricide in liquid cultures. However, when attacker and target cells are grown together within blood agar plates, two additional components, the bacteriocins CibAB, are involved as well (Guiral *et al.*, 2005). For reasons unknown, these bacteriocins do not contribute to lysis of target cells in liquid cultures (Håvarstein *et al.*, 2006, unpublished results).

In the current study, we have investigated the role and relative contributions of CbpD, LytA and LytC to fratricide in planktonic cultures of *S. pneumoniae*. Our results show that CbpD alone can kill and lyse target cells. However, the effect of CbpD is strongly amplified by the action of LytC and LytA, whose activities are directly or indirectly triggered by CbpD. Remarkably, in the presence of 1 mM EDTA, the dependency of CbpD on LytA and LytC decreases considerably, and CbpD becomes able to kill and lyse target cells efficiently on its own.

METHODS

Bacterial strains and growth conditions. Pneumococcal strains and plasmids used in this study are described in Table 1. The bacteria were grown in C medium (Lacks & Hotchkiss, 1960). Samples analysed on Western blots were collected from cultures grown in C medium without added BSA.

Construction of *S. pneumoniae* mutants. Construction of the DNA fragments and plasmids used in mutagenesis of the various streptococcal strains is described in the supplementary online material. The sequences of all primers used are given in Supplementary Table S1. DNA was introduced into *S. pneumoniae* by natural transformation. Pneumococcal cultures grown at 37 °C to an OD_{550} of around 0.1 were induced to competence using 100–250 ng ml^{-1} of CSP (N-EMRLSKFFRDFILQRKK-C). After phenotypic expression in liquid medium for 90–120 min at 37 °C, *S. pneumoniae* transformants were selected by plating on Todd–Hewitt agar supplemented with chloramphenicol (4.5 μg ml^{-1}), kanamycin (400 μg ml^{-1}), streptomycin (200 μg ml^{-1}), spectinomycin (200 μg ml^{-1}) or tetracycline (0.2 μg ml^{-1}).

Quantification of β-galactosidase release from target cells. To determine the relative contributions of *cbpD, lytA* and *lytC* to competence-induced lysis, the amount of β-galactosidase released from lysed target cells during co-cultivation with competent attackers was measured. All target cells contained a *lacZ* gene from *Escherichia coli* fused to a highly expessed gene termed *hirL* (SPD_1984). In addition, the endogenous β-galactosidase gene (*ebg*) was disrupted in both target and attacker cells. The procedure used was essentially as described by Steinmoen *et al.* (2002). Briefly, cultures of competence-inducible and non-inducible target cells were grown in parallel at 37 °C in a water bath. At OD_{550} ~0.3, the cultures were mixed in equal volumes, induced with 250 ng CSP ml^{-1}, and transferred to a water bath at 30 °C. Shifting the temperature from 37 °C to 30 °C has previously been shown to increase the efficiency of competence-induced lysis (Steinmoen *et al.*, 2003). Uninduced mixed cultures, receiving no peptide pheromone, were run in parallel as negative controls. After 30 min, samples were removed from the water bath and placed on ice. To measure the amount of β-galactosidase released from lysed target cells, cultures were filtered (0.2 μm) to obtain cell-free supernatants. β-Galactosidase assays of supernatants were carried out in Eppendorf tubes at 30 °C and contained 240 μl 5× Z buffer (5 mM $MgCl_2$, 250 mM β-mercaptoethanol, 50 mM KCl, 0.3 M

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype/relevant feature	Reference/source
Strains		
R484	R800, but *trt1 comE*::*kan102*, Kan^R	Håvarstein *et al.* (2006)
R704	R6 derivative, *comA*::*ermAM*, Ery^R	J. P. Claverys*
RH1	R704 but *ebg*::*spc*, Ery^R Spc^R	Johnsborg *et al.* (2008)
RH2	RH1, but *hirL*::*pLhirL*, Cm^R Ery^R Spc^R	Johnsborg *et al.* (2008)
RH3	RH2, but Δ*comE*::*kan*, Cm^R Ery^R Spc^R Kan^R	Johnsborg *et al.* (2008)
RH4	RH2, but Sm^R by transformation with CP1200 chromosomal DNA, Cm^R Ery^R Spc^R Sm^R	This study
RH5	RH4, but Δ*lytA*::*Janus*, Cm^R Ery^R Spc^R Kan^R	This study
RH6	RH5, but Δ*lytA* by transformation with PCR fragment targeting the Janus insertion, Cm^R Ery^R Spc^R Sm^R	This study
RH7	RH6, but Δ*comE*::*kan*, Cm^R Ery^R Spc^R Sm^R Kan^R	This study
RH8	RH2, but Δ*lytC*::*tet*, Cm^R Ery^R Spc^R Tc^R	This study
RH9	RH8, but Δ*comE*::*kan*, Cm^R Ery^R Spc^R Tc^R Kan^R	This study
RH10	RH6, but Δ*lytC*::*tet*, Cm^R Ery^R Spc^R Sm^R Tc^R	This study
RH11	RH10, but Δ*comE*::*kan*, Cm^R Ery^R Spc^R Sm^R Tc^R Kan^R	This study
RH12	RH2, but Δ*comM*::*Janus*, Cm^R Ery^R Spe^R Kan^R	This study
RH13	RH10, but Δ*comM*::*Janus,* Cm^R Ery^R Spc^R Tc^R Kan^R	This study
RH14	RH1, but Δ*lytA*::*kan*, Ery^R Spc^R Kan^R	This study
RH15	RH1, but Δ*lytC*::*tet*, Ery^R Spc^R Tc^R	This study
RH16	RH14, but Δ*lytC*::*tet*, Ery^R Spc^R Kan^R Tc^R	This study
RH17	RH1, but Δ*cbpD*::*kan*, Ery^R Spc^R Kan^R	Johnsborg *et al.* (2008)
RH201	RH421, but Δ*lytC*::*Janus*, Ery^R Spc^R Kan^R	This study
RH202	RH201, but replacement of Janus cassette with *lytC* gene encoding His-tag, Ery^R Spc^R Sm^R	This study
RH205	RH423, but *cbpD*::*N-terminal his tag* from pLCH, Ery^R Spc^R Str^R Cm^R	This study
RH207	RH421, but Δ*lytA*::*Janus*, Ery^R Spc^R Kan^R	This study
RH218	RH207, but *lytA*::*N-terminal his tag* by transformation with PCR construct, Ery^R Spc^R Sm^R	This study
RH220	RH10, but Δ*cbpD*::*Janus*, Cm^R Ery^R Spc^R Tc^R Kan^R	This study
RH221	RH220, but Δ*cbpD* by transformation with PCR fragment targeting the Janus insertion	This study
RH222	RH221, but Δ*comM*::*Janus*	This study
RH227	RH218, but Δ*lytC*::*Janus*, Ery^R Spc^R Kan^R	This study
RH228	RH202, but Δ*lytA*::*Janus*, Ery^R Spc^R Kan^R	This study
RH420	RH4, but Δ*comM*::*Janus*, Cm^R Ery^R Spc^R Kan^R	This study
RH421	RH1, but Sm resistant by transformation with CP1200 chromosomal DNA, Ery^R Spc^R Sm^R	This study
RH422	RH421, but Δ*cbpD*::*Janus*, Ery^R Spc^R Kan^R	This study
RH423	RH422, but Δ*cbpD* by transformation with PCR fragment targeting the Janus insertion, Ery^R Spc^R Sm^R	This study
CP1200	Rx derivative, *hex mal rpsL1*, Sm^R	Shoemaker & Guild (1974)
H4	Ek4166 but *lytA*::pFW13 by transformation with plasmid DNA	Steinmoen *et al.* (2003)
Plasmids		
pEVP3	Vector with *cat* cassette and promoterless *lacZ*; does not replicate in *S. pneumoniae*	Claverys *et al.* (1995)
pLhirL	pEVP3 derivative carrying an *hirL* targeting fragment; insertion into the *S. pneumoniae* genome generates a *hirL*::*lacZ* fusion	Steinmoen *et al.* (2002)
pLCH	pEVP3 derivative carrying a *cbpD* targeting fragment encoding a His_6-tag close to the 5′ end of the gene	This study
pLS590	*tet cat*	Lacks *et al.* (2000)

*Gift from Professor Jean-Pierre Claverys, CNRS, LMGM-UMR, Toulouse, France.

$Na_2HPO_4.7H_2O$, 0.2 M $NaH_2PO_4.H_2O$, 4 mg ONPG ml^{-1}, pH 7.0), 500 µl supernatant and 460 µl C medium. After terminating the reactions by addition of 0.5 ml of a 1 M Na_2CO_3 solution, hydrolysis of ONPG was recorded in a spectrophotometer at 420 nm. Enzyme activity was calculated according to Miller (1972).

Quantification of β-galactosidase release from monocultures of RH420, RH13 and RH222 was carried out essentially as described above. The strains were grown at 37 °C until they reached OD_{550} 0.3. Then, when applicable, 250 ng CSP ml^{-1} and/or 1 mM EDTA was added, and the cultures were transferred to a water bath at 30 °C.

After 30 min, samples were collected, sterile filtered, and assayed for β-galactosidase activity.

Stability of lytic activity in supernatants. RH1 cultures were grown to OD_{550} ~0.3 at 37 °C, transferred to a 30 °C water bath and induced to competence by addition of 250 ng CSP ml^{-1}. Twenty minutes post-induction, culture samples were sterile filtered and the resulting supernatants were immediately mixed with equal volumes of RH3 cultures (50 %, v/v) grown to OD_{550} ~0.35. After further incubation at 30 °C for 30 min, β-galactosidase released from lysed RH3 target cells was quantified as described above. To measure the total β-galactosidase activity present in target cells used to test the activity of supernatants from competent RH1 cultures, the cells were completely lysed by incubation at 30 °C for 10 min in the presence of 0.1 % (v/v) Triton X-100. After lysis, all samples were stored on ice until assayed. β-Galactosidase activity was assayed as described above except that 100 µl cell lysate and 960 µl C medium was used in the reactions. In order to assay the stability of the lytic activity of the filtrate, it was either supplemented with 5 mM DTT or left untreated at room temperature. Samples to be tested were withdrawn every 10 min for half an hour, and mixed with equal volumes of RH3 cultures at OD_{550} ~0.35. To investigate whether the stimulatory effect of EDTA was due to metalloproteases released during fratricide that degraded CbpD, sterile-filtered supernatants were prepared as described above and supplemented with 5 mM DTT. The supernatant was then split in two, and 1 mM EDTA was added to one of the parallels. After 1 h at room temperature, the supernatant without EDTA was divided again into two equal parts, one of which received 1 mM EDTA. Immediately afterwards, supernatants were added to RH3 target cultures (50 %, v/v) and incubated at 30 °C for 30 min. β-Galactosidase release from the target cells was quantified as described above.

Western blotting. Concentrated culture supernatants from competent pneumococci expressing His-LytA, His-LytC or His-CbpD were prepared as follows. At OD_{550} 0.3, cultures expressing each of the His-tagged lysins were divided into two parallel aliquots. One parallel received CSP (250 ng ml^{-1}) plus 1 % choline chloride, whereas the other received only CSP. After 30 min the cultures were sterile filtered and concentrated 70-fold using Amicon Ultra-15 centrifugal filter devices with a 10 kDa molecular mass cutoff. The concentrated supernatants were mixed with Laemmli sample buffer and stored frozen at −80 °C until analysed. Unconcentrated culture supernatants from competent and non-competent RH202 cells were prepared in the same manner, except for the step involving the Amicon Ultra-15 centrifugal filter devices.

To prepare whole-cell extracts, cultures were grown to OD_{550} 0.3 and induced as above. Uninduced cultures were run in parallel. Thirty minutes after addition of CSP, 1 ml samples of induced and uninduced cultures were transferred into Eppendorf tubes and centrifuged at 14 500 ***g*** for 1 min. The resulting pellets were dissolved in 40 µl sample buffer and stored as described above. After heating to ~95 °C for 5 min, samples (35 µl) were loaded onto 12.5 % SDS-PAGE precast gels (Bio-Rad), electrophoresed, and transferred to PVDF membranes. His-tagged LytA, LytC and CbpD proteins on the membranes were detected using the Pierce SuperSignal West Pico HisProbe kit according to the manufacturer's instructions.

Immunofluorescence microscopy. Cultures growing at 37 °C were transferred to 30 °C and induced to competence at OD_{550} 0.3. After 20 min, the cells were sedimented and stained with the LIVE/DEAD *Bac*Light bacterial viability kit (Invitrogen) according to the protocol. The cells were visualized using a Leica TCS SP5 AOBS confocal microscope.

Measurement of autolysis in RH1 and RH14 cultures subjected to varying concentrations of deoxycholate. Samples (3 ml) of vigorously growing cultures of pneumococcal strains RH1 and RH14 (Δ*lytA*) were pipetted into glass cuvettes and immediately transferred to a DU 800 spectrophotometer (Beckman Coulter) equipped with a temperature-controlled, six-cell block, cuvette holder preheated to 37 °C. The spectrophotometer was preprogrammed to measure OD_{550} automatically every 2 min for 52 min. After growing the RH1 and RH14 strains in the spectrophotometer for about 13 min, deoxycholate was added to a final concentration of 0.1, 0.05 and 0.025 % (w/v).

RESULTS

The relative contributions of LytA, LytC and CbpD to fratricide in liquid culture

It has been reported previously that LytA and LytC can be provided by either the competent attacker or the non-competent target cells during pneumococcal fratricide (Guiral *et al.*, 2005). This is an interesting observation that might help elucidate the molecular mechanism behind the process. We therefore decided to extend the study of Guiral *et al.* (2005) by performing a more comprehensive and quantitative analysis of the contributions of LytA and LytC to competence-induced cell lysis. By using non-competent target cells that produce cytoplasmic β-galactosidase constitutively, the level of cell lysis was determined by measuring the amount of β-galactosidase released into the growth medium during co-cultivation with competent attacker cells. Our results show that only about 0.4 % of the target cells are lysed when the *lytA* and *lytC* genes have been deleted in both attacker and target cells, demonstrating that CbpD is not very effective on its own under the conditions used (Table 2). Nevertheless, CbpD is an essential component of the lysis mechanism, as attacker cells lacking this protein are totally unable to kill and lyse their non-competent siblings (Kausmally *et al.*, 2005; Table 2). When CbpD is present, LytA and LytC both play an important role in the lysis process. LytC contributes more than LytA, however, as twice the number of target cells are lysed during co-cultivation of the strains RH14 (Δ*lytA*) and RH7 (Δ*lytA*) than in a corresponding experiment with the strains RH15 (Δ*lytC*) and RH9 (Δ*lytC*) (Table 2). In agreement with previously published results, our data show that LytA and LytC can be supplied by either attacker or target cells. However, by using a more quantitative assay than Guiral *et al.* (2005) we were able to compare the level of lysis obtained with *cis*-acting versus *trans*-acting LytA and LytC. Our results show that the origin of these cell wall hydrolases influences the efficiency of the fratricidal process. Almost four times as many target cells were lysed when LytA and LytC were supplied *in cis* (see co-cultivation of RH16 and RH3, Table 2) compared to the situation where these enzymes were supplied *in trans* (see co-cultivation of RH1 and RH11, Table 2). However, regardless of whether LytA and LytC are supplied *in cis* or *in trans*, they must somehow be activated by CbpD in order to attack and lyse non-competent target cells.

Table 2. Fraction of target cells lysed during co-cultivation of various *lytA*, *lytC* and *cbpD* mutants of competent (attacker) and non-competent (target) cells

	RH3 (WT)*	RH7 (Δ*lytA*)	RH9 (Δ*lytC*)	RH11 (Δ*lytA* Δ*lytC*)
RH1 (WT)*	33 ± 1.4 %†	20.4 ± 0.6 %	13.5 ± 0.7 %	5.6 ± 0.1 %
RH14 (Δ*lytA*)	27.2 ± 1.5 %	19.7 ± 1.1 %	12.0 ± 0.9 %	1.3 ± 0.6 %
RH15 (Δ*lytC*)	26.2 ± 0.5 %	10.1 ± 1.8 %	9.2 ± 0.3 %	0.6 ± 0.2 %
RH16 (Δ*lytA* Δ*lytC*)	21.0 ± 2.0 %	4.7 ± 1.2 %	3.2 ± 0.5 %	0.4 ± 0.1 %
RH17 (Δ*cbpD*)	0.03 ± 0.02 %	ND	ND	ND

*The RH1 and RH3 strains are designated wild-type (WT) because they have intact *cbpD*, *lytA* and *lytC* genes.
†The β-galactosidase release obtained with competent wild-type attackers (RH1) co-cultivated with non-competent wild-type target cells (RH3) was 171 Miller units (mean ± SEM). We found that this corresponds to 33 % of the total β-galactosidase content of intact plus lysed RH3 target cells. Thus, on average, 33 % of the target cells were lysed in this co-cultivation experiment. In all other cases, in which the experiments were carried out exactly as for RH1 and RH3, β-galactosidase release to the growth medium was measured in Miller units and then converted to percentage lysis of target cells, by dividing by 171 and multiplying by 33. ND, Not determined.

Internal and external levels of CbpD, LytA, and LytC in cultures of competent and non-competent pneumococcal cells

CbpD, LytA and LytC all contain choline-binding domains, whose function is to attach these proteins to cell wall teichoic acid (TA) and lipoteichoic acid (LTA) (López & García, 2004). It has not been clear, however, whether CbpD and LytC, which are secreted via the Sec pathway, primarily attach to TA and LTA of the cells producing them or if they diffuse into the medium. In the case of LytA, which lacks a signal peptide, its mode of release from the cytoplasm is still a subject of dispute (López & García, 2004). Our results show that when target cells lack both LytA and LytC, their absence can to a certain extent be compensated for by LytA and LytC originating from competent attacker cells (Table 2). Consequently, these enzymes must reach the target cells either by diffusing through the medium or via cell–cell contact. The same type of reasoning applies also for CbpD, which is produced by competent attacker cells but act at the surface of target cells. To gain further insight into these matters we added histidine tags (His_6-tags) to the N-termini of CbpD, LytA, and LytC for detection and tracking purposes. As CbpD and LytC both contain N-terminal signal peptides, the His-tag was introduced immediately downstream of the predicted signal peptidase cleavage site. The wild-type genes were replaced with the corresponding mutant genes by double-crossover recombination using the counter-selectable Janus cassette as described by Sung *et al.* (2001). The activities of the CbpD-His, LytA-His and LytC-His proteins were found to be the same or somewhat reduced compared to their non-modified counterparts. The kinetics of LytA-dependent stationary-phase autolysis was essentially identical for the mutant strain carrying the LytA-His construct (RH218) and the wild-type strain used as control. The strain carrying the CbpD-His construct (RH205) was, however, only about 40 % as efficient as the wild-type strain in provoking lysis of strain RH3 when used as an attacker in co-cultivation experiments with this target strain. Co-cultivation experiments, comparing the properties of the LytC-His-carrying strain RH228 (Δ*comA* Δ*lytA*) with its wild-type equivalent RH14 (Δ*comA* Δ*lytA*), revealed that RH228 was about 70 % as efficient as RH14 in provoking lysis of the RH11 (Δ*comE* Δ*lytA* Δ*lytC*) target strain. Together, these data demonstrate that although the activities of LytC-His and CbpD-His are moderately reduced compared to the wild-type versions of these proteins, the three His-tagged lysins have retained their functionality.

Cultures of strains RH202, RH205, and RH218, producing LytC-His, CbpD-His and LytA-His, respectively, were grown at 37 °C until they reached OD_{550} 0.3. They were then induced to competence by addition of CSP (250 ng ml^{-1}), and transferred to a water bath at 30 °C. After 30 min at 30 °C, samples of pelleted cells and sterile-filtered supernatants were harvested for Western analysis. Two experiments were run in parallel for each of the mutant strains. In one of the parallels, 1 % choline chloride was added to the culture in order to prevent binding of the secreted His-tagged lysin to TA and LTA in the pneumococcal cell wall. Western blots of SDS-PAGE gels loaded with equal amounts of pelleted bacterial cells revealed that LytC is produced in similar amounts in competent and non-competent pneumococci. In contrast, CbpD was only detected in competent cells (Fig. 1a). As expected, we found that the production of LytA increases during competence. A very weak band corresponding to LytA was detected in the lane loaded with non-competent RH218 cells (not visible in Fig. 1a), whereas a considerably stronger band appeared in the lane loaded with equal amounts of competent RH218 cells. These results are in accordance with previously published data based on Northern analysis, microarray analysis and the use of reporter assays (Mortier-Barrière *et al.*, 1998; Dagkessamanskaia *et al.*, 2004; Peterson *et al.*, 2004; Kausmally *et al.*, 2005). In the present study our primary interest was to trace the lysins after they had been translocated across the cytoplasmic membrane. Cell-free

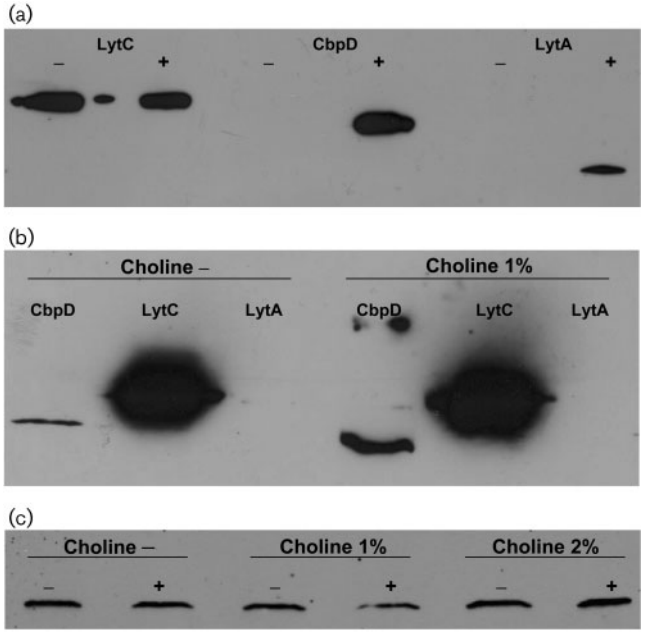

Fig. 1. Detection of His-tagged CbpD, LytA and LytC by Western analysis. (a) Equal amounts of competent (+) and non-competent (–) cells of the RH202, RH205 and RH218 strains were harvested and analysed by Western blotting to compare the amounts of CbpD, LytA and LytC present in whole-cell extracts. (b) Analysis of sterile-filtered supernatants from competence-induced cultures of the RH202, RH205 and RH218 strains. When the cultures reached OD_{550} 0.3, 1 % (w/v) choline chloride and 250 ng CSP ml^{-1} was added to one set of parallels, whereas the other (choline –) received only CSP. Thirty minutes after addition of CSP, the supernatants were collected, concentrated approximately 70-fold using Amicon Ultra-15 centrifugal filter units, and analysed by Western blotting. (c) Detection of LytC in unconcentrated culture supernatants from competent (+) and non-competent (–) RH202 cultures in the absence (choline –) or presence of 1 % and 2 % choline.

supernatants, with or without choline chloride added, were concentrated 70-fold using Amicon Ultra-15 centrifugal filter devices (molecular mass cutoff 10 kDa), and analysed by SDS-PAGE followed by Western blotting. We were unable to detect LytA-His in the concentrated supernatants, demonstrating that this autolysin remains within the cytoplasm of the producer cell, or that the amount of LytA-His released to the environment is below the sensitivity level of our assay (Fig. 1b). LytC-His was found to be present in the supernatant of competent as well as non-competent cells in similar amounts, and could also readily be detected directly in unconcentrated supernatants (Fig. 1c). Unexpectedly, concentrated supernatants from RH202 cultures supplemented with 1 or 2 % choline chloride did not contain more LytC-His than corresponding supernatants without choline chloride (Fig. 1c). In contrast, when strain RH205 was induced to competence in the presence of 1 % choline chloride, the amount of CbpD-His detected in the supernatant was much higher than in corresponding cultures where choline chloride had been omitted (Fig. 1b). This result implies that most of the secreted CbpD protein stays attached to TA and LTA in the pneumococcal cell wall, whereas only a subfraction is released to the growth medium.

Activation of LytA-dependent autolysis by deoxycholate requires a lethal concentration of this detergent

Our results show that CbpD somehow activates LytA during fratricide. Since a number of seemingly unrelated treatments of pneumococcal cells, such as exposure to CbpD, detergents, freezing and thawing, hydrogen peroxide, penicillin and other cell wall inhibitors (Dubos, 1937; Tomasz & Waks, 1975a; Mascher *et al.*, 2006; Regev-Yochay *et al.*, 2007), lead to LytA-dependent autolysis, we speculated that cell death is the common denominator that triggers the activity of LytA. To test this hypothesis we added various amounts of deoxycholate to pneumococcal strains RH1 and RH14 (Δ*lytA*) while they were in the early exponential phase of growth, and measured the effect of this treatment with respect to autolysis and cell viability. We found that at concentrations of deoxycholate of 0.1 % (w/v) and above, RH1 cells lysed completely while the mutant cells (RH14) lacking the *lytA* gene stopped growing (Fig. 2a). When a culture of the RH1 strain was treated with 0.05 % (w/v) deoxycholate its OD_{550} dropped rapidly from 0.15 to about 0.08 and then continued to decline at a slow rate (Fig. 2a). Even though 0.05 % (w/v) deoxycholate was not sufficient to trigger immediate autolysis of all the cells in an RH1 culture, RH14 cells subjected to the same concentration of the detergent stopped growing. Live/dead staining performed on the RH1 and RH14 cultures treated with 0.05 (w/v) deoxycholate (Fig. 2a) revealed that all cells in these cultures were dead (Fig. 2b). Interestingly, in RH1 cultures subjected to 0.025 % (w/v) deoxycholate only a minor fraction of the cells underwent autolysis, while the surviving majority continued to grow. The LytA-deficient RH14 cells also tolerated this concentration and kept multiplying (Fig. 2a). In this case, live/dead staining of RH1 and RH14 cells showed that nearly all cells in these cultures were alive (Fig. 2b). It can be concluded from these results that there is a close correlation between cell death and activation of LytA-dependent autolysis in *S. pneumoniae*.

Lytic activity in cell-free supernatants harvested from cultures of competent pneumococci

To find out if CbpD released into the growth medium by competent pneumococci is active, sterile filtered supernatants collected from competent RH1 cultures were assayed for lytic activity against RH3 target cells. The results showed that the level of cell lysis obtained with supernatant from RH1 cultures reached approximately 50 % of that obtained in standard co-cultivation experi-

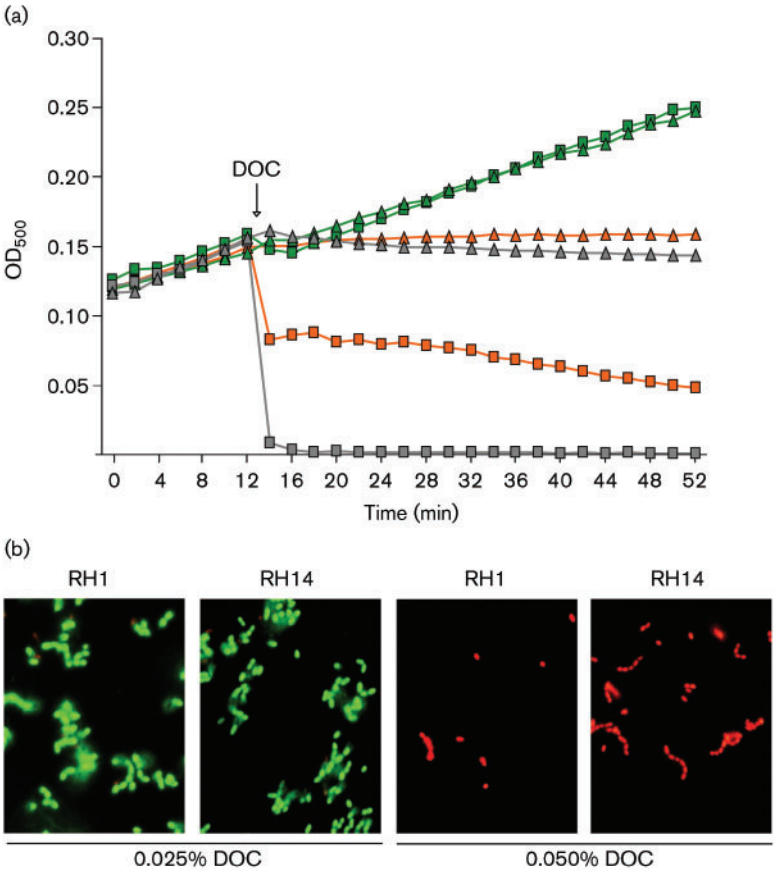

Fig. 2. Determination of the concentration of deoxycholate (DOC) required to trigger autolysis in RH1 cells and death of RH14 (Δ*lytA*) cells. RH14 cells will not undergo autolysis when treated with DOC, as they lack the autolysin LytA. (a) Cultures of RH1 (squares) and RH14 cells (triangles) were grown in a spectrophotometer equipped with a Peltier temperature-controlled cuvette holder heated to 37 °C. At the time indicated by the arrow, cultures were subjected to a final concentration of DOC corresponding to 0.1 % (grey symbols), 0.05 % (orange symbols) or 0.025 % (w/v) (green symbols). Growth and autolysis were monitored by measuring the OD_{550}. (b) RH1 and RH14 cultures treated with 0.05 and 0.025 % (w/v) deoxycholate were stained with the BacLight Bacterial Viability kit (Invitrogen) according to the producer's recommendations. Live cells fluoresce green, whereas dead cells fluoresce red. The data presented are representative of the results from three independent experiments.

ments between competent RH1 and non-competent RH3 cells. The lytic activity of the supernatants was completely lost upon addition of 1 mM iodoacetamide, a general inhibitor of cysteine proteases. As the CHAP domain of CbpD shows strong homology to known cysteine proteases, this finding suggests that the lytic activity detected in sterile-filtered supernatants depends on the presence of CbpD. In accordance with this, supernatants harvested from competent cultures of the RH17 (Δ*cbpD*) strain displayed no lytic activity at all.

When stored at room temperature, supernatants collected from competent RH1 cultures gradually lost their ability to lyse RH3 target cells (Fig. 3). The lytic activity dropped by two-thirds in 30 min, suggesting that CbpD is somehow inactivated and/or degraded by proteases. Interestingly, addition of 5 mM DTT to the RH1 supernatants slowed the decay rate considerably (Fig. 3). In an attempt to elucidate the mechanism behind this stabilizing effect we added 5 mM DTT to supernatants collected from competence-induced RH17 (Δ*cbpD*) cultures, i.e. supernatants lacking CbpD. When these supernatants were mixed with RH3 target cultures (50 %, v/v) no β-galactosidase release was detected (result not shown). We therefore concluded that DTT acts directly on CbpD, and that the reducing agent probably stabilizes the activity of CbpD by protecting its active-site cysteine from oxidation.

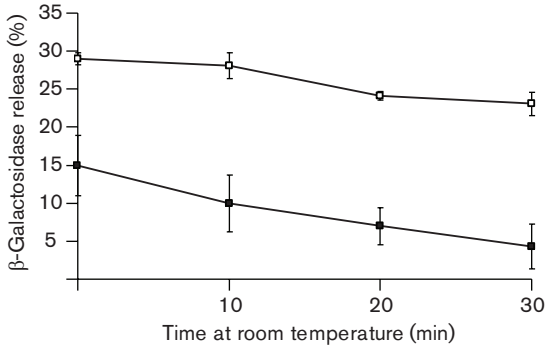

Fig. 3. Decay rate of the lytic activity present in the growth medium of competent pneumococcal cultures in the presence (□) and absence (■) of DTT. Sterile-filtered RH1 supernatants were left at room temperature for various periods of time and mixed (50 %, v/v) with non-competent RH3 target cells (OD_{550} 0.35) producing intracellular β-galactosidase. Release of β-galactosidase from the target cells (as indicated on the *y*-axis) represents the percentage of β-galactosidase activity measured in the growth medium relative to the total amount of this enzyme present in the RH3 cultures (extracellular + intracellular β-galactosidase). Results presented are the means ± SEM of three independent experiments.

To determine whether proteolytic degradation also contributed to the declining CbpD activity observed in Fig. 3, various protease inhibitors were employed. We found that addition of 1 mM EDTA strongly increased the lytic activity of supernatants harvested from competent RH1 cells. When supernatants collected from these cells were supplemented with 5 mM DTT, placed at room temperature for 1 h, and subsequently mixed with cultures of RH3 target cells (50 % v/v) for 30 min, approximately 20 % of the target cells were lysed. If, however, supernatants from competent RH1 cells were supplemented with 1 mM EDTA in addition to 5 mM DTT, the fraction of target cells lysed increased to around 69 %. This finding suggested that a CbpD-degrading metalloprotease, whose activity is inhibited by EDTA, is present in the supernatants. To establish whether this could be the case we performed experiments in which EDTA was added to RH1 supernatants after they had been rested at room temperature for 1 h, i.e. immediately before they were mixed with cultures of RH3 target cells. Following this procedure, 65 % of the target cells were lysed. The fact that EDTA has the same stimulatory effect, regardless of whether it is added to RH1 supernatants before or after they were incubated at room temperature for 1 h, argues strongly against the idea that EDTA protects CbpD from proteolytic degradation. Further control experiments showed that the chelating agent alone was not responsible for the stimulatory effect, as no lysis took place when supernatants from competent RH17 (Δ*cbpD*) cultures supplemented with 1 mM EDTA were mixed with RH3 target cells. Neither did the presence of EDTA affect the content of cytoplasmic *β*-galactosidase in RH3 cells treated with this compound for 30 min. Although EDTA does not appear to protect against proteolytic degradation, our results clearly show that its stimulatory effect depends on the presence of CbpD. In other words, removal of divalent cations by chelation somehow enhances the efficiency of the fratricide process when it takes place in liquid C medium under the conditions employed in this study.

The activity of CbpD increases dramatically in the presence of EDTA

To further elucidate the effect of EDTA, a series of experiments were carried out with strains in which the immunity gene *comM* had been deleted. When a monoculture of strain RH420 (Δ*comM hirL*::*lacZ*) is induced to competence at OD_{550} 0.3, approximately 20 % of the cells are lysed. In contrast, very little lysis (1–2 %) is observed in an RH2 (*hirL*::*lacZ*) culture, which expresses the ComM immunity protein during competence. The reason why a monoculture lacking the ComM immunity protein is not completely lysed is not understood. As shown in Fig. 4(a), the level of competence-induced cell lysis increased almost threefold in RH420 (Δ*comM hirL*::*lacZ*) cultures treated with 1 mM EDTA compared to untreated cultures. On the other hand, competence-induced cultures of strain RH222, lacking ComM, CbpD, LytA and LytC, did not lyse at all. Intriguingly, competent cultures of RH13 lysed efficiently in the presence of 1 mM EDTA, but very poorly without this metal chelator (Fig. 4a). The only difference between RH222 and RH13 is that the latter strain harbours a functional *cbpD* gene. Live/dead staining of competent and non-competent cultures of the RH421, RH222 and RH13 strains confirmed that in the presence of EDTA, the ability of CbpD to kill target cells on its own increases dramatically (Fig. 4b).

DISCUSSION

Our results show that when competent pneumococci were co-cultivated with their non-competent siblings, efficient lysis of the non-competent target cells depended on CbpD and the autolysins LytA and/or LytC. CbpD produced by the competent attacker cells was absolutely required, but was not very active on its own under the conditions employed. In the presence of LytA and/or LytC, however, a significant fraction of the target cells was lysed. An important step towards understanding the mechanism behind the lethal activity of CbpD will be to identify the cleavage site of its CHAP domain. Most characterized CHAP domains are cysteine proteases that function as peptidoglycan hydrolases. It is therefore reasonable to assume that the CHAP domain of CbpD cleaves within the peptidoglycan stem peptides or the seryl-alanine/alanyl-alanine dipetide bridges linking these stem peptides. Removal of the dipeptide bridges in a pneumococcal Δ*comE* strain by insertion of a Janus cassette in the *murM* gene did not affect the strain's susceptibility to CbpD (Fiser *et al.*, 2003; data not shown). This strongly indicates that the CHAP domain of CbpD, which has been demonstrated to be essential for its function (Håvarstein *et al.*, 2006), cleaves within the murein stem-peptide rather than in the dipeptide bridges.

The data presented in Table 2 show that LytC as well as LytA contributes significantly to fratricide. Both autolysins can be provided *in trans* by the attacker cells, but lysis of target cells is considerably more efficient when they are provided *in cis*. As depicted in Fig. 1, LytC is released to the growth medium in relatively high amounts by cells possessing a functional *lytC* gene. This result readily explains how the protein becomes available to LytC-deficient target cells during co-cultivation with LytC-proficient attackers. We do not know, however, whether the pool of LytC detected in the growth medium mainly represents free enzyme or enzyme attached to TA in shed cell wall material. In contrast to the abundance of extracellular LytC present in pneumococcal cultures, extracellular LytA is either absent or present in only very minute amounts. Even in the presence of 1 % choline, which should elute any LytA attached to TA and LTA in the pneumococcal cell wall, no band corresponding to LytA could be identified on the Western blots. This negative result is in accordance with data published by Yother & White (1994), who concluded that LytA is probably an

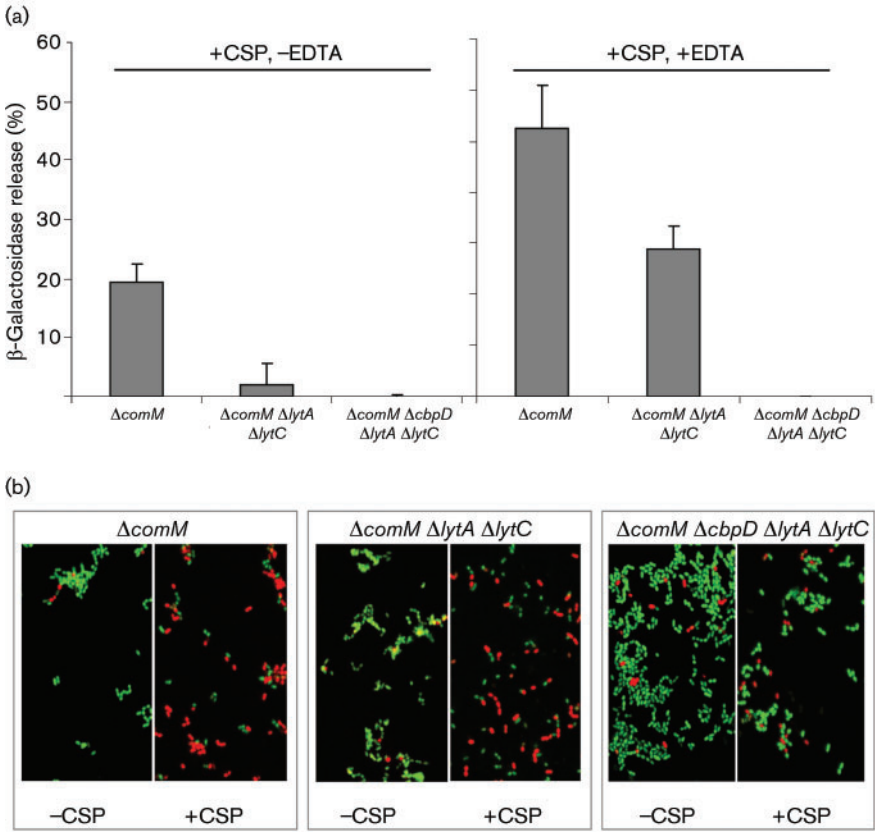

Fig. 4. Effect of EDTA on the efficiency of competence-induced self-lysis. All mutant strains used were deficient in ComM, an immunity protein that protects competent cells from succumbing to the activity of their own lysins. When monocultures of *comM* mutants are induced to competence a fraction of the cells will lyse. (a) β-Galactosidase release from CSP-induced cultures of RH420 (Δ*comM*), RH13 (Δ*lytA* Δ*lytC* Δ*comM*) and RH222 (Δ*cbpD* Δ*lytA* Δ*lytC* Δ*comM*). The cultures were either untreated (left panel) or supplemented with 1 mM EDTA (right panel) at the time of competence induction (the addition of CSP). After 30 min incubation at 30 °C the percentage β-galactosidase release, i.e. extracellular relative to total β-galactosidase activity, was determined using the same procedure as explained in the footnote to Table 2. Results presented are the means ± SEM of three independent experiments. (b) Live/dead staining of EDTA-treated (1 mM) non-competent (left panel) and competent (right panel) cultures of each of the mutant strains RH420 (Δ*comM*), RH13 (Δ*lytA*, Δ*lytC*, Δ*comM*) and RH222 (Δ*cbpD*, Δ*lytA*, Δ*lytC*, Δ*comM*). The cultures were harvested 20 min after CSP induction and stained with the BacLight Bacterial Viability kit (Invitrogen) according to the producer's recommendations. Live cells fluoresce green, whereas dead cells fluoresce red.

intracellular protein. As we applied a very sensitive detection system for His-tagged proteins, and concentrated our supernatant samples 70-fold, our assay must be considered significantly more sensitive than the one employed by Yother & White (1994). Taking this into account, it is highly likely that LytA is stored intracellularly, presumably associated with the inside of the cytoplasmic membrane, and that it is released only when the integrity of this membrane is lost. The results of an earlier study by Briese & Hakenbeck (1985), examining the distribution of LytA in different cellular fractions, lend support to this view. Our finding that a lethal concentration of deoxycholate is required to trigger LytA-dependent autolysis points in the same direction. Upon adding 0.05 % (w/v) deoxycholate to an RH1 culture we observed that all the cells died, whereas only about half of the cells underwent autolysis. This result indicates that pneumococcal cells are killed at a slightly lower concentration of deoxycholate than is required to initiate LytA-dependent autolysis, suggesting that release of LytA from an intracellular membrane-associated depot and not cell death per se triggers the autolytic mechanism. However, previous studies have shown that addition of extracellular purified LytA to exponentially growing LytA-deficient pneumococci does not trigger autolysis before the cells have reached the stationary phase (Díaz *et al.*, 1990). Similarly, Tomasz & Waks (1975) demonstrated that extracellular LytA is active when added to cell wall material or mechanically disrupted unfractionated bacteria, but is immediately inhibited when adsorbed to living cells. Evidently some mechanism exists that protects metabolically active pneumococci against their own autolysin. Based on previously published reports,

some of which have been referred to above, and the results presented in the current study, we propose the following mechanism of action of LytA: (i) in living cells LytA is not normally present in the cell wall, but is probably stored on the inside of the cytoplasmic membrane, (ii) various treatments that kill pneumococcal cells bring about loss of integrity of the cytoplasmic membrane, leading to release of LytA followed by autolysis, (iii) when LytA is released the enzyme must bind to phosphorylcholine residues present on TA and LTA in the pneumococcal cell wall in order to convert to its active form (Tomasz & Westphal, 1971; Giudicelli & Tomasz, 1984), and (iv) if the enzyme is added exogenously to living cells its activity is somehow suppressed.

Considering the differences between LytA and LytC with respect to localization, how do they contribute to fratricide and how are they activated by CbpD? Evidently, the relatively high concentration of LytC detected in the medium of non-competent pneumococcal cultures in the exponential phase of growth is not harmful to the cells, demonstrating that LytC is inactive under these circumstances. Thus, our results strongly indicate that CbpD activates LytC through direct interaction, through a mediator, or indirectly by, for example, introducing specific cuts in the peptidoglycan of target cells. According to this hypothesis, the combined or successive action of CbpD and LytC lyses the target cells and releases the β-galactosidase reporter enzyme. LytA-mediated lysis, on the other hand, probably represents an autolytic process triggered by CbpD. As mentioned above, it is well documented that exogenously added LytA is not able to degrade the cell wall of metabolically active cells (Lacks, 1970; Tomasz & Waks, 1975b; Díaz *et al.*, 1990). Therefore, activation of LytA by CbpD probably requires that CbpD alone, or together with LytC, kills the target cells first through limited hydrolysis of their cell walls. Presumably the damage introduced by CbpD or CbpD/LytC will cause disruption of the cytoplasmic membrane followed by release of intracellular LytA. As a consequence extensive LytA-mediated autolysis takes place, allowing efficient release of the β-galactosidase reporter enzyme. Although our results show that CbpD is able to kill and lyse a minor fraction of the target cells on its own (see Table 2), there is reason to believe that the β-galactosidase release assay employed underestimates the fraction of target cells killed directly by CbpD. Since the β-galactosidase enzyme used as a reporter is a tetramer with a molecular mass of about 420 kDa, the presence of relatively large pores or total disintegration of the cell wall is required for efficient release of the enzyme. For this reason, cells that are killed by CbpD via limited cell wall hydrolysis that leaves their peptidoglycan sacculus largely intact might escape detection. The ability of LytA to act *in trans* is probably due to the fact that 1–2 % of the attacker cells are lysed by other attackers during co-cultivation with non-competent target cells. The reason for this is probably that a small fraction of attacker cells does not respond to CSP under the conditions used, and therefore do not develop immunity. LytA leaking from these cells will presumably contribute to increased lysis of target cells that have been killed, but not completely disrupted by CbpD or CbpD/LytC.

The Western analyses presented in Fig. 1 show that 30 min after competence induction most secreted CbpD resides in the cell walls of the producer cells. This finding is somewhat counterintuitive, as it would be expected that CbpD must be excreted into the environment in order to reach the target cells. A possible explanation for this result is that fratricide under natural conditions involves cell–cell contact between attacker and target cells. As CbpD is probably attached to the TA and LTA protruding from the surface of competent cells, it is conceivable that direct contact between the cell walls of competent and non-competent cells could result in lysis of the latter. Undoubtedly, close contact between donor and recipient cells would greatly increase the chance that DNA released from the lysed target cells is captured and taken up by competent attackers. The fact that CbpD-mediated gene transfer between different strains of pneumococcus, and between pneumococci and their commensal relatives, most likely takes place within biofilms in the nasopharynx, supports the idea that close contact between attacking and attacked cells is the natural situation at which fratricide takes place.

Interestingly, addition of 1 mM EDTA strongly increases the efficacy of the fratricide mechanism, and enables CbpD to kill and lyse non-competent target cells efficiently on its own (see Fig. 4). This finding indicates that divalent cations are involved in the regulation of CbpD activity. Alternatively, the presence of the metal chelator may interfere with the physiology of the target cells in a way that makes them more susceptible to CbpD. However, as pneumococcal cultures treated with 1 mM EDTA grow at the same rate as untreated cultures for up to 1 h after addition of the chelator (results not shown), this alternative does not seem plausible. It is therefore likely that the ability of EDTA to stimulate fratricide is due to a specific mechanism rather than a general toxic effect on the target cells. CbpD may, for example, contain binding site(s) for divalent cations that upon removal by EDTA cause a conformational change that enhances the activity of this putative peptidoglycan hydrolase. It is also possible that divalent cations bound to the phosphate groups of TA and LTA or other anionic sites in the cell wall interfere with binding of CbpD to its target sites in the cell wall, and that removal of these ions makes the substrate more accessible for the enzyme (Neuhaus & Baddiley, 2003). These questions will be the subject of future investigations. Whatever the nature of the EDTA effect, our findings suggest that divalent cations play an important role in the regulation of fratricide under natural conditions.

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REFERENCES

Bateman, A. & Rawlings, N. D. (2003). The CHAP domain: a large family of amidases including GSP amidase and peptidoglycan hydrolases. *Trends Biochem Sci* **28**, 234–237.

Briese, T. & Hakenbeck, R. (1985). Interaction of the pneumococcal amidase with lipoteichoic acid and choline. *Eur J Biochem* **146**, 417–427.

Chi, F., Nolte, O., Bergmann, C., Ip, M. & Hakenbeck, R. (2007). Crossing the barrier: evolution and spread of a major class of mosaic *pbp2x* in *Streptococcus pneumoniae*, *S. mitis*, and *S. oralis*. *Int J Med Microbiol* **297**, 503–512.

Claverys, J. P. & Håvarstein, L. S. (2007). Cannibalism and fratricide: mechanisms and raison d'être. *Nat Rev Microbiol* **5**, 219–229.

Claverys, J. P., Dintilhac, A., Pestova, E. V., Martin, B. & Morrison, D. A. (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.

Dagkessamanskaia, A., Moscoso, M., Hénard, V., Guiral, S., Overweg, K., Reuter, M., Martin, B., Wells, J. & Claverys, J. P. (2004). Interconnection of competence, stress and CiaR regulons in *Streptococcus pneumoniae*: competence triggers stationary phase autolysis of *ciaR* mutant cells. *Mol Microbiol* **51**, 1071–1086.

Díaz, E., López, R. & García, J. L. (1990). Chimeric phage-bacterial enzymes: a clue to the modular evolution of genes. *Proc Natl Acad Sci U S A* **87**, 8125–8129.

Dubos, R. J. (1937). Mechanism of the lysis of pneumococci by freezing and thawing, bile, and other agents. *J Exp Med* **66**, 101–112.

Fiser, A., Sergio, R. F. & Tomasz, A. (2003). Cell wall branches, penicillin resistance and the secrets of the MurM protein. *Trends Microbiol* **11**, 547–553.

Giudicelli, S. & Tomasz, A. (1984). Attachment of pneumococcal autolysin to wall teichoic acids, an essential step in enzymatic wall degradation. *J Bacteriol* **158**, 1188–1190.

Guiral, S., Mitchell, T. J., Martin, B. & Claverys, J. P. (2005). Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements. *Proc Natl Acad Sci U S A* **102**, 8710–8715.

Hakenbeck, R. (1995). Target mediated resistance to β-lactam antibiotics. *Biochem Pharmacol* **50**, 1121–1127.

Håvarstein, L. S., Coomaraswami, G. & Morrison, D. A. (1995). An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proc Natl Acad Sci U S A* **92**, 11140–11144.

Håvarstein, L. S., Gaustad, P., Nes, I. F. & Morrison, D. A. (1996). Identification of the streptococcal competence-pheromone receptor. *Mol Microbiol* **21**, 863–869.

Håvarstein, L. S., Martin, B., Johnsborg, O., Granadel, C. & Claverys, J. P. (2006). New insights into the pneumococcal fratricide: relationship to clumping and identification of a novel immunity factor. *Mol Microbiol* **59**, 1297–1307.

Hiller, N. L., Janto, B., Hogg, J. S., Boissy, R., Yu, S., Powell, E., Keefe, R., Ehrlich, N. E., Shen, K. & other authors (2007). Comparative genomic analyses of seventeen *Streptococcus pneumoniae* strains: insights into the pneumococcal supragenome. *J Bacteriol* **189**, 8186–8195.

Hui, F. M. & Morrison, D. A. (1991). 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* **173**, 372–381.

Jenkins, S. G., Brown, S. D. & Farrell, D. J. (2008). Trends in antibacterial resistance among *Streptococcus pneumoniae* isolated in the USA: update from PROTEKT US Years 1–4. *Ann Clin Microbiol Antimicrob* **7**, 1–11.

Johnsborg, O., Eldholm, V. & Håvarstein, L. S. (2007). Natural genetic transformation: prevalence, mechanisms and function. *Res Microbiol* **158**, 767–778.

Johnsborg, O., Eldholm, V., Bjørnstad, M. L. & Håvarstein, L. S. (2008). A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species. *Mol Microbiol* **69**, 245–253.

Kausmally, L., Johnsborg, O., Lunde, M., Knutsen, E. & Håvarstein, L. S. (2005). Choline-binding protein D (CbpD) in *Streptococcus pneumoniae* is essential for competence-induced cell lysis. *J Bacteriol* **187**, 4338–4345.

Lacks, S. A. (1970). Mutants of *Diplococcus pneumoniae* that lack deoxy-ribonucleases and other activities possibly pertinent to genetic transformation. *J Bacteriol* **101**, 373–383.

Lacks, S. A. & Hotchkiss, R. D. (1960). A study of the genetic material determining an enzyme activity in pneumococcus. *Biochim Biophys Acta* **39**, 508–517.

Lacks, S. A., Ayalew, S., de la Campa, A. G. & Greenberg, B. (2000). Regulation of competence for genetic transformation in *Streptococcus pneumoniae*: expression of *dpnA*, a late competence gene encoding a DNA methyltransferase of the *Dpn*II restriction system. *Mol Microbiol* **35**, 1089–1098.

Lee, M. S. & Morrison, D. A. (1999). Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. *J Bacteriol* **181**, 5004–5016.

López, R. & García, E. (2004). Recent trends in the molecular biology of pneumococcal capsules, lytic enzymes, and bacteriophage. *FEMS Microbiol Rev* **28**, 553–580.

Mascher, T., Heintz, M., Zähner, D., Merai, M. & Hakenbeck, R. (2006). The CiaRH system of *Streptococcus pneumoniae* prevents lysis during stress induced by treatment with cell wall inhibitors and by mutations in *pbp2x* involved in β-lactam resistance. *J Bacteriol* **188**, 1959–1968.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Mortier-Barrière, I., de Saizieu, A., Claverys, J. P. & Martin, B. (1998). Competence-specific induction of *recA* is required for full recombination proficiency during transformation in *Streptococcus pneumoniae*. *Mol Microbiol* **27**, 159–170.

Neuhaus, F. C. & Baddiley, J. (2003). A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. *Microbiol Mol Biol Rev* **67**, 686–723.

Obaro, S. & Adegbola, R. (2002). The pneumococcus: carriage, disease, and conjugate vaccines. *J Med Microbiol* **51**, 98–104.

Pestova, E. V., Håvarstein, L. S. & Morrison, D. A. (1996). Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. *Mol Microbiol* **21**, 853–862.

Peterson, S. N., Sung, C. K., Cline, R., Desai, B. V., Snesrud, E. C., Luo, P., Walling, J., Li, H., Mintz, M. & other authors (2004). Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. *Mol Microbiol* **51**, 1051–1070.

Regev-Yochay, G., Trzcinski, K., Thompson, C. M., Lipsitch, M. & Malley, R. (2007). SpxB is a suicide gene of *Streptococcus pneumoniae* and confers a selective advantage in an *in vivo* competitive colonization model. *J Bacteriol* **189**, 6532–6539.

Rigden, D. J., Jedrzejas, M. J. & Galperin, M. Y. (2003). Amidase domains from bacterial and phage autolysins define a family of γ-D,L-glutamate-specific amidohydrolases. *Trends Biochem Sci* **28**, 230–234.

Shoemaker, N. B. & Guild, W. R. (1974). Destruction of low efficiency markers is a slow process occuring at a heteroduplex stage of transformation. *Mol Gen Genet* **128**, 283–290.

Steinmoen, H., Knutsen, E. & Håvarstein, L. S. (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.

Steinmoen, H., Teigen, A. & Håvarstein, L. S. (2003). Competence induced cells of *Streptococcus pneumoniae* lyse competence-deficient cells of the same strain during co-cultivation. *J Bacteriol* **185**, 7176–7183.

Sung, C. K., Li, H., Claverys, J. P. & Morrison, D. A. (2001). An *rpsL* cassette, Janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. *Appl Environ Microbiol* **67**, 5190–5196.

Tomasz, A. & Waks, S. (1975a). Mechanism of action of penicillin: triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis. *Proc Natl Acad Sci U S A* **72**, 4162–4166.

Tomasz, A. & Waks, S. (1975b). Enzyme replacement in a bacterium: phenotypic correction by the experimental introduction of the wild type enzyme into a live enzyme defective mutant pneumococcus. *Biochem Biophys Res Commun* **65**, 1311–1319.

Tomasz, A. & Westphal, M. (1971). Abnormal autolytic enzyme in a pneumococcus with altered teichoic acid composition. *Proc Natl Acad Sci U S A* **68**, 2627–2630.

Ween, O., Gaustad, P. & Håvarstein, L. S. (1999). Identification of DNA binding sites for ComE, a key regulator of natural competence in *Streptococcus pneumoniae*. *Mol Microbiol* **33**, 817–827.

Yother, J. & White, J. M. (1994). Novel surface attachment mechanism of the *Streptococcus pneumoniae* protein PspA. *J Bacteriol* **176**, 2976–2985.

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Fratricide in *Streptococcus pneumoniae*: contributions and role of the cell wall hydrolases CbpD, LytA and LytC

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SUPPLEMENTARY MATERIAL

Construction of *S. pneumoniae* mutants

Primers used in this work are listed in Supplementary Table S1. Transformtion of *S. pneumoniae* RH2 (*rpsL*$^+$) with genomic DNA from *S. pneumoniae* CP1200 (*rpsL1*, Sm^R) followed by selection on agar plates containing 200 μg streptomycin ml^{-1} gave rise to the RH4 (*rpsL1*, Sm^R) strain.

RH5 was constructed by transforming RH4 with a Janus cassette (Sung *et al.*, 2001) targeting *lytA*. Janus is a *kan-rpsL*$^+$ DNA cassette that confers resistance to kanamycin and dominant sensitivity to streptomycin in a streptomycin-resistant background. First, a ~1000 bp region upstream of *lytA* in strain R704 was amplified using the primers LytAF and LytARjan(KAN)-N. This PCR product was subsequently fused to the 5′ end of the *kan*::*rpsL*$^+$ cassette using the primers LytAF and RpsL41R. Next, a ~1000 bp DNA fragment corresponding to the downstream region of *lytA* in strain R704 was amplified using the primers LytA.1Fjan(RPSL) and LytAR. This PCR product was fused to the 3′ end of the *kan*::*rpsL* cassette. The final fragment, containing the *kan*::*rpsL*$^+$ flanked by the DNA sequences immediately upstream and downstream of *lytA* was used to transform the RH4 strain. Following selection on Todd–Hewitt agar containing 400 μg kanamycin ml^{-1}, a kanamycin-resistant colony, RH5, was picked and shown to be streptomycin-sensitive, verifying the insertion of the Janus cassette into the RH4 genome.

RH6 was derived from RH5 by replacing its Janus cassette by double-crossover homologous recombination using a PCR fragment targeting the chromosomal site containing the Janus insertion. PCR products corresponding to the upstream and downstream regions of the *lytA* locus were amplified using the primer pairs LytAF/LytDR, and LytAR/LytD.1F, respectively. The two DNA fragments were subsequently joined by overlap-extension PCR using the primers LytAF and LytAR. The resulting fragment was transformed into RH5, and following selection on Todd–Hewitt agar containing 200 μg streptomycin ml^{-1}, a colony was isolated. By means of PCR, this strain (RH6) was verified to contain a complete deletion of the Janus cassette as well as the *lytA* gene. The *comE* gene of RH6 was disrupted by transformation with genomic DNA from R484 (Δ*comE*::*kan*), resulting in the competence-deficient RH7 strain.

The *lytC* deletion mutant RH8 was constructed by transforming RH2 with a *lytC*::*tet* PCR construct. First, the DNA regions flanking *lytC* were amplified using the primer pairs LytCF/LytCtetR(tet) and

LytC1tetF(tet)/LytCR. The primers TetR and TetF were then used to amplify the tetracycline resistance gene from the plasmid pLS590. This PCR product was mixed with the PCR product corresponding to the upstream region of the *lytC* gene, and the two fragments were subsequently fused in a PCR using the primers LytCF and TetR. The resulting fragment was finally joined to the PCR fragment corresponding to the downstream region of *lytC* in an overlap-extension PCR with the primers LytCF and LytCR.

RH8 was transformed with genomic DNA from R484 to give rise to the competence-deficient (Δ*comE*::*kan*) mutant RH9. RH10 was constructed by transforming RH6 with the *lytC*::*tet* cassette described above. Subsequently, a competence-deficient mutant of RH10 was constructed by transforming this strain with genomic DNA from R484, resulting in strain RH11 (Δ*comE*::*kan*). RH12 was derived from RH2 by transforming this strain with a *comM* Janus cassette as described by Johnsborg *et al.* (2008). Next, genomic DNA from RH12 was used to transform RH10, giving rise to RH13 (Δ*comM*::*Janus)*. Genomic DNA from *S. pneumoniae* H4 was used to transform RH1, giving rise to RH14 (Δ*lytA*::*kan)*, whereas RH1 transformed with the *lytC*::*tet* cassette described above gave rise to RH15. Finally, this *lytC*::*tet* cassette was used to transform RH14, giving rise to the Δ*lytA*::*kan* Δ*lytC*::*tet* mutant RH16.

RH220 was constructed by transforming RH10 with a *cbpD*::*Janus* construct described in Johnsborg *et al.* (2008). The Janus insertion was subsequently removed using the protocol described previously (Johnsborg *et al.*, 2008), giving rise to the strain RH221. Finally, a *ΔcomM*::*Janus* fragment was amplified from RH12 using primers ComMF and ComM1R and transformed into RH221, resulting in RH222.

A strain expressing a LytC protein engineered to contain a His-tag close to its N terminus was constructed as follows: First, strain RH1 was transformed with genomic DNA from strain CP1200 giving rise to the streptomycin-resistant RH421 strain carrying the *rpsL1* allele. RH421 was next transformed with a Janus-cassette targeting *lytC*. The primers LytCF and LytCRKan were used to amplify a ~1000 bp DNA fragment corresponding to the 5′ region flanking *lytC*, whereas the primers LytC1Fjan and lytCR were used to amplify a ~1000 bp fragment corresponding to the 3′ region flanking *lytC*. The two fragments were then fused to the 5′ and 3′ ends of the *kan*::*rpsL*$^{+}$ Janus cassette in two successive overlap-extension PCRs using the primers LytCF and RpsL41R in the first reaction, and LytCF and LytCR in the second reaction. The resulting fragment was transformed into RH421, giving rise to RH201. To construct the *lytC* gene encoding an N-terminally His-tagged LytC protein, the primers LytCF and LytCHisR were first used to amplify a ~1000 bp DNA fragment flanking the 5′ end of *lytC*. This fragment included the part of *lytC* encoding its signal peptide sequence as predicted by SignalP 3.0 (Emanuelsson *et al.*, 2007). The primers LytCHisF and LytCR were used to amplify a DNA sequence encoding the mature LytC protein plus the N-terminal His-tag. Using the primers LytCF and LytCR, the two PCR fragments

were subsequently fused in an overlap-extension PCR in such a way that the His-tag was inserted between amino acids 14 and 15 in the secreted mature LytC protein. This PCR fragment was then used to transform RH201, giving rise to RH202. Deletion of *lytA* in this strain, by transformation with a *lytA*-targeting Janus cassette amplified from strain RH207 using the primers LytAF and LytAR, resulted in strain RH228.

The RH205 strain, expressing a CbpD protein engineered to contain a His-tag between amino acids 4 and 5 in the secreted mature protein, was constructed as follows. RH421 was transformed with a Janus cassette targeting *cbpD*, giving rise to RH422. The Janus insertion was subsequently removed by transformation with a PCR construct containing the flanking regions of *cbpD* only, giving rise to RH423. Next, a DNA fragment encoding His-CbpD was constructed. The primers CbpDF.1 and N-hisCbpDR were used to amplify a ~1000 bp DNA fragment flanking the 5′ end of *cbpD*. This fragment included the DNA sequence encoding the CbpD signal peptide as predicted by SignalP 3.0 (Emanuelsson *et al.*, 2007). The primers CbpDR.1 and N-his CbpDF were used to amplify the DNA sequence encoding the mature CbpD protein plus the N-terminal His-tag. The two PCR fragments were fused in an overlap-extension PCR using the primers CbpDF.1 and CbpDR.1. The resulting DNA fragment was subsequently digested with *Xho*I and *Dra*III, and ligated into the corresponding restriction sites of the non-replicative pEVP3 vector (Claverys *et al.*, 1995), resulting in the plasmid pLCH. Finally, the RH205 strain was generated by transformation with the pLCH plasmid, followed selection on chloramphenicol plates.

Strain RH218, expressing a modified version of LytA with a His-tag inserted between amino acids 1 and 2 at the N-terminal end, was constructed as follows: The *lytA*::*Janus* fragment described above was used to transform RH421, giving rise to RH207. Then a *lytA* gene encoding the N-terminal His-tag was synthesized by overlap-extension PCR. The primers LytAF and LytAHisR were used to amplify a ~1000 bp DNA fragment flanking the 5′ end of *lytA*, whereas the primers lytAR and lytAHisF were used to amplify the *lytA* gene and ~1000 bp flanking the 3′ end of the *lytA* gene. The products were subsequently fused in a PCR using the primers LytAF and LytAR. Transformation of strain RH207 with the resulting fragment generated strain RH218. Deletion of *lytC* in this strain with a *lytC*::*Janus* fragment amplified from strain RH201 with primers LytCF and LytCR resulted in strain RH227.

Supplementary Table S1. Oligonucleotide primer sequences

Primer	Sequence (5′–3′)	Reference
LytCF	TGTGGCTCAAATTGAGGCCA	This study
LytCHisR	CTACTTGAATGATGATGATGATGAGCTGTCGTTGTATCCTGCG	This study
LytCHisF	CATCATCATCATCATCATTCAAGTAGTTCAGAGCAAAATC	This study
LytCR	CCAAGTTCGGCTGCAAGCA	This study
LytCRKan	CACATTATCCATTAAAAATCAAACAGCTGTCGTTGTATCCTGCG	This study
LytC1Fjan	CTAAACGTCCAAAAGCATAAGGAAAGGGGCGCGTAGATGTCAGC	This study
LytCKOR	ATCACATCCCTCTTTCAAATCATCGCTGAATGCTGTTCCACCTAG	This study
LytCKOF	CGATGATTTGAAAGAGGGATG	This study
LytCtetR(tet)	TAATTACACTTCATAATTAATTCCTCCCCACCTAGCTTTTGCTACTTA	This study
LytC1tetF(tet)	AGGGATTTCTAAATCGTTAAGGGATCGGGCGCGTAGATGTCAGC	This study
TetF	GGAGGAATTAATTATGAAGTGTA	This study
TetR	GATCCCTTAACGATTTAGAAATCCCT	This study
ComMF	CTGCTCGCCTATTAGATGAC	This study
ComM1R	CCCCACGCTCTTGGCTAC	This study
CbpDF.1	ATGCACTCGAGGCTGCAGAAGGCTTGGTGGAGTCACATTCTATCCGTACA	This study
CbpDR.1	CTCTCTCACAGCGTGGGCATTACCCAGATCAGGTGCGGT	This study
N-hisCbpDF	CATCATCACCATCACCATAATGGATCGATTGCGCGTGGGGATGATTATCCTGCT	This study
N-hisCbpDR	CATTATGGTGATGGTGATGATGTCCTCTGGAATAGGCATAGACACTATC	This study
LytAR	TCAACCATCCTATACAGTGAA	This study
LytAF	TGTATCTATCGGCAGTGTGAT	This study
LytARjan (KAN)-N	AGCTTAAGATCTAGAGCTCGAGGATCCGCCGACTTGAGGCAAATC	This study
LytA.1Fjan	CTAAACGTCCAAAAGCATAAGGAAAGGGCCAGAATTCACAGTAGAG	This study

(RPSL)		
LytDR	CCGCTTCATTCTGTACGGTT	This study
LytD.1F	GCATTCAACCGTACAGAATGAAGCGGCCACGTTGACCCTTATCCAT	This study
LytAHisR	TTAATTTCATGATGGTGGTGATGATGCATATTCTACTCCTTATCAATTAA	This study
LytAHisF	CATCATCACCACCATCATGAAATTAATGTGAGTAAATTAAGAA	This study
RpsL41R	CTTTCCTTATGCTTTTGGAC	Johnsborg *et al.* (2008)

REFERENCES

Claverys, J. P., Dintilhac, A., Pestova, E. V., Martin, B. & Morrison, D. A. (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.

Emanuelsson, O., Brunak, S., von Heijne, G. & Nielsen, H. (2007). Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* **2**, 953–971.

Johnsborg, O., Eldholm, V., Bjørnstad, M. L. & Håvarstein, L. S. (2008). A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species. *Mol Microbiol* **69**, 245–253.

Sung, C. K., Li, H., Claverys, J. P. & Morrison, D. A. (2001). An rpsL cassette, Janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. *Appl Environ Microbiol* **67**, 5190–5196.

Paper II

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The Pneumococcal Cell Envelope Stress-Sensing System LiaFSR Is Activated by Murein Hydrolases and Lipid II-Interacting Antibiotics▽§

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In the *Firmicutes*, two-component regulatory systems of the LiaSR type sense and orchestrate the response to various agents that perturb cell envelope functions, in particular lipid II cycle inhibitors. In the current study, we found that the corresponding system in *Streptococcus pneumoniae* displays similar properties but, in addition, responds to cell envelope stress elicited by murein hydrolases. During competence for genetic transformation, pneumococci attack and lyse noncompetent siblings present in the same environment. This phenomenon, termed fratricide, increases the efficiency of horizontal gene transfer *in vitro* and is believed to stimulate gene exchange also under natural conditions. Lysis of noncompetent target cells is mediated by the putative murein hydrolase CbpD, the key effector of the fratricide mechanism, and the autolysins LytA and LytC. To avoid succumbing to their own lysins, competent attacker cells must possess a protective mechanism rendering them immune. The most important component of this mechanism is ComM, an integral membrane protein of unknown function that is expressed only in competent cells. Here, we show that a second layer of self-protection is provided by the pneumococcal LiaFSR system, which senses the damage inflicted to the cell wall by CbpD, LytA, and LytC. Two members of the LiaFSR regulon, *spr*0810 and PcpC (*spr*0351), were shown to contribute to the LiaFSR-coordinated protection against fratricide-induced self-lysis.

During a transient state termed competence for natural transformation, *Streptococcus pneumoniae* has the ability to take up naked DNA from the environment and incorporate this DNA into its genome by homologous recombination. A quorum sensing-like system consisting of the secreted competence-stimulating peptide (CSP), a membrane-embedded histidine kinase (ComD), and its cognate cytoplasmic response regular (ComE) controls competence development in this species (15, 16, 37). Upon entering the competent state, pneumococci start transcribing a number of competence-specific genes termed the early and late competence genes (26). A few of these genes are involved in a phenomenon termed fratricide. Naturally competent pneumococci use this mechanism to kill and lyse noncompetent sister cells present in the same environment, presumably to "steal" their DNA (19, 41). The key effector of fratricide in liquid medium is a putative murein hydrolase, CbpD, that together with the autolysins LytA and LytC degrades the cell wall of the noncompetent target cells (7, 12, 22). To avoid succumbing to their own toxins, competent pneumococci protect themselves by producing an immunity protein termed ComM (17). Although the mechanism by which ComM provides protection is not known, we speculated that the murein hydrolases involved in the fratricide mechanism might cause envelope stress in competent pneumococci.

In Gram-positive bacteria, the cell envelope consists of a thick murein cell wall, interspersed with (lipo)teichoic acids and the cytoplasmic membrane. The cell envelope is a crucial structure of the bacterial cell that fulfills a number of important functions. It protects the cell and gives it its shape but also functions as a molecular sieve and communication interface with the environment. Ensuring its integrity is therefore crucial for survival. Accordingly, bacteria have evolved numerous regulatory devices that monitor the integrity of and initiate protective responses in case of damage to the cell envelope. These systems are collectively termed cell envelope stress responses (20).

Most Gram-positive bacteria belonging to the phylum *Firmicutes* possess homologs of a cell envelope stress sensor system from *Bacillus subtilis*, designated LiaSR (30). In *Lactococcus lactis* and *Staphylococcus aureus* the corresponding two-component regulatory systems are called CesSR and VraSR, respectively (24, 29). In addition to their membrane-spanning histidine kinase sensors (LiaS/VraS/CesS) and their cognate response regulators (LiaR/VraR/CesR), these systems contain a third protein found to be essential for signal sensing. The gene encoding this protein, termed *liaF* in *B. subtilis*, is transcribed from the same promoter as the genes encoding the two-component regulatory system. Evidence indicates that LiaF, which is a membrane-anchored protein, acts as a negative modulator of the system through direct interaction with LiaS (21). When LiaS and/or LiaF sense the appropriate stress signal(s), LiaF-mediated inhibition of the LiaSR signal transduction system is

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relieved, resulting in activation of LiaR-dependent promoters. The exact signal(s) detected by LiaSR, VraSR, and CesSR are not known. In general, they are induced by cell wall-active antibiotics, especially those that interfere with the lipid II cycle (e.g., bacitracin, ramoplanin, vancomycin, and nisin) (20). In addition, direct cell wall damage, for example treatment with lysozyme, may in some cases also activate these systems (45). We therefore decided to investigate whether the pneumococcal two-component system HK03/RR03, which corresponds to LiaSR, VraSR, and CesSR, is activated during competence in response to the activity of CbpD, LytA, and LytC. In what follows, we will refer to the HK03/RR03 system as LiaSR. In accordance with the cell envelope stress-sensing systems from *B. subtilis*, *S. aureus*, and *L. lactis*, the pneumococcal system also contains a third component (*spr*0342) corresponding to LiaF.

Our results show that CbpD, together with LytA and LytC, activates the pneumococcal LiaFSR system in a *comM* deletion mutant, providing evidence that both glycan strand cleavage and hydrolysis of the stem peptide-*N*-acetylmuramyl bond can trigger LiaFSR activation. In addition, we found that the LiaFSR system contributes significantly to protect competent pneumococci against the potentially lethal effects of the fratricide mechanism. The LiaFSR is also induced by cell wall antibiotics that interfere with the lipid II cycle of cell wall biosynthesis. Using a combination of global transcriptional profiling, regulon mining, and comparative genomics, we defined the LiaR regulon in *S. pneumoniae*. We demonstrate that two regulon members, Spr0810 and PcpC (Spr0351), are important to counteract cellular lysis. Taken together, our data indicate that the LiaFSR system of *S. pneumoniae* has adapted its function to orchestrate a second, ComM-independent layer of protection against fratricide-induced self-lysis.

MATERIALS AND METHODS

Bacterial growth conditions and storage. Cultures from which RNA was extracted were grown in Todd-Hewitt broth (THB). All other experiments were carried out in C medium (25). Precultures of the bacteria were grown at 37°C to an optical density at 550 nm (OD_{550}) of 0.3 to 0.35, mixed with 0.5 vol of 50% glycerol, and stored at −80°C.

Construction of mutants and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1, while the sequences of all primers employed are given in Table S1 in the supplemental material. DNA was introduced into the streptococcal strains by means of natural transformation using 250 ng/ml of CSP to induce the competent state in the DNA recipient. Cultures of *S. pneumoniae* were grown at 37°C until they reached an OD_{550} of 0.15 to 0.2. Then, CSP and transforming DNA were added, and the cultures were further incubated for 90 to 120 min at 37°C before plating on selective medium to identify transformants. *S. pneumoniae* transformants were selected by plating on Todd-Hewitt agar supplemented with chloramphenicol (4.5 μg/ml), kanamycin (400 μg/ml), streptomycin (200 μg/ml), spectinomycin (200 μg/ml), or tetracycline (2.5 μg/ml).

RH237, which carries a deletion of the *comM* locus, was derived from strain RH420 (7), which contains a Janus cassette replacing *comM*. To construct RH237, the upstream and downstream regions (~1,000 bp) of *comM* were amplified with the primer pairs ComMF/ComMRdel and ComMFdel/ComMR, respectively. The fragments were subsequently fused in a PCR using the primers ComMF and ComMR. The resulting DNA fragment was transformed into RH420, resulting in removal of the Janus cassette.

Initially, an allelic replacement mutant of *liaR* was constructed by long-flanking homology (LFH) PCR, essentially as described previously (31, 46). In brief, a spectinomycin resistance cassette was amplified from plasmid pDG1726 (11). Two primer pairs (designated up-reverse/up-forward and do-reverse/do-forward) were designed to amplify ~1,000-bp DNA fragments flanking the region to be deleted (*liaR*) at its 5′ and 3′ ends. The resulting fragments are here called the "*liaR*-up" and "*liaR*-do" fragments, respectively. The 3′ end of the *liaR*-up fragment as well as the 5′ end of the *liaR*-do fragment extended into the gene to be deleted in a way that all expression signals of genes up- and downstream of the targeted gene remained intact. Extensions of ~25 nucleotides were added to the 5′ ends of the up-reverse and do-forward primers that were complementary (opposite strand and inverted sequence) to the 5′ and 3′ ends, respectively, of the amplified spectinomycin resistance cassette. Portions (100 ng) of the *liaR*-up and *liaR*-do fragments and a 3-fold molar excess of the spectinomycin resistance cassette were used together with the specific up-forward and do-reverse primers at standard concentrations in a second PCR. The PCR product was directly used to transform *S. pneumoniae*, resulting in strain TMSP008.

RH253 was constructed by transforming RH237 with a Janus cassette targeting *liaR*. The upstream region of *liaR* (~1,000 bp) was amplified with the primers Rr03F and Rr03Rkan and fused together with a Janus cassette in a subsequent PCR using the primers Rr03F and RpsL41R. The downstream region of *liaR* was amplified using the primers Rr03rpsl and Rr03R and fused together with the upstream region and the Janus cassette in a final PCR employing the primers Rr03F and Rr03R. Both *liaR* mutants behaved similarly with regard to phenotypes and target gene expression (data not shown).

To construct a *spr*0810 deletion mutant, 1,000 bp upstream and downstream of *spr*0810 was amplified using the primer pairs spr0810F/spr0810kanR and spr0810rpslF/spr0810R, respectively, and fused to the 5′ and 3′ends of a Janus cassette. Subsequent transformation of this DNA fragment into RH237 resulted in RH269. Similarly, a *pcpC*-targeting Janus cassette was amplified using the primer pairs pcpCF/pcpCkanR and pcpCrpslF/pcpCR and transformed into RH237, resulting in SPH-4. In order to construct the strain RH259, carrying a luciferase reporter gene immediately downstream of *liaR*, the plasmid pR424 (4) was digested with BamHI and HindIII. Next, ~400 bp of the 3′ region of *liaR* was amplified with the primers Rr03luc1 and Rr03luc2. The PCR fragment was subsequently cloned into the linearized vector using the In-Fusion Dry-Down PCR cloning kit (Clontech) according to the manufacturer's protocol, giving rise to pVEL5. This plasmid was transformed into RH421 (7), resulting in RH259.

To construct pVEL6 we utilized a method described by Geiser et al. (10). The primers spr0810pR424F and spr0810pR424R, carrying at their 5′ ends sequences homologous to a region immediately upstream of the *luc* reporter gene of the pR424 plasmid, were used to amplify a 487-bp region starting 184 bp upstream of *spr*0810 and ending at its stop codon. In order to incorporate the 487-bp sequence upstream of the *luc* reporter gene in pR424, the PCR product was purified and utilized as a primer (200 ng) for a second PCR with pR424 (100 ng) as a template. To get rid of methylated template plasmid, 20 U DpnI (New England BioLabs) was added to the reaction at the end of the thermal cycling. The new plasmid pVEL6, harboring the gene encoding *spr*0810 under the control of its own promoter upstream of the *luc* gene, was incorporated into the genome of the RH421 strain by insertion-duplication mutagenesis, giving rise to the RH270 strain. RH271 was constructed by transforming RH270 with a *comM*-targeting Janus cassette amplified from RH420 with the primers ComMF and ComMR. The cassette was subsequently removed by transforming RH271 with a fragment targeting the Janus cassette, amplified from RH237 with the same primers, resulting in RH272.

To delete *liaR* in this background, a Janus cassette targeting *liaR* was first amplified from RH253 using the primers Rr03F and Rr03R. This PCR product was transformed into RH272, resulting in RH273. Similarly, this Janus cassette was transformed into RH4, resulting in RH276.

In order to replace *cbpD* with a point-mutated (C75→A) version, *cbpD* was first deleted in RH272 by transforming the strain with a *cbpD*-targeting Janus cassette. This cassette was amplified from RH422 (7) using the primers CbpD-1098F and CbpDR. The resulting strain, RH283, was subsequently transformed with a PCR cassette that replaced the Janus insertion with a cbpD allele encoding the C75àA mutation. First, a fragment starting ~1,100 bp upstream of *cbpD* and ending just downstream of the active-site cysteine was amplified using the primers CbpD-1098F and $CbpD_{C75A}R$. The rest of *cbpD*, including ~1,000 bp of the *cbpD* downstream region, was amplified using the primers $CbpD_{C75A}F$ and CbpDR. The two fragments were then fused in a separate PCR using the primers CbpD-1098F and CbpDR. This fragment was transformed into RH283, resulting in RH284.

To construct RH291, RH421 (7) was first transformed with a PCR cassette replacing *lytC* with a Tet^r resistance gene, resulting in RH287. The Tet^r cassette was amplified from RH16 (7) using the primers LytCF and LytCR. RH287 was next transformed with the *comM*-targeting Janus cassette described above, giving rise to RH288. Subsequent removal of the Janus cassette by transformation with the PCR fragment described for RH237 resulted in RH289. This mutant was subsequently transformed with pVEL6 in order to introduce the luciferase reporter gene fusion of *spr*0810, resulting in RH290. Finally, a *lytA*-targeting Janus

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype/relevant feature[a]	Reference/source
Strains		
RH4	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc hirL*::*lacZ* (Cm^r) Sm^r by transformation with CP1200 chromosomal DNA; Ery^r Spc^r Cm^r Sm^r	7
RH229	Δ*comA*::*ermAM* (Ery^r) *spr1332*::*luc*; Ery^r Cm^r	This study
RH237	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc hirL*::*lacZ* (Cm^r) Δ*comM*; Ery^r Spc^r Cm^r Sm^r	This study
RH253	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc hirL*::*lacZ* (Cm^r) Δ*comM liaR*::*Janus*; Ery^r Spc^r Cm^r Kan^r	This study
RH259	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc liaR*::*luc* by transformation with pVEL5; Ery^r Spc^r Sm^r Cm^r	This study
RH269	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc hirL*::*lacZ* (Cm^r) Δ*comM*, Δ*spr*0810 Ery^r Spc^r Cm^r Kan^r	This study
RH270	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc spr*0810::*luc* by transformation with pVEL6; Ery^r Spc^r Sm^r Cm^r	This study
RH271	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc spr*0810::*luc* Δ*comM*::*Janus*; Ery^r Spc^r Cm^r Kan^r	This study
RH272	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc spr*0810::*luc* Δ*comM*; Ery^r Spc^r Cm^r Sm^r	This study
RH273	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc spr*0810::*luc* Δ*comM* Δ*liaR*::*Janus*; Ery^r Spc^r Cm^r Kan^r	This study
RH276	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc hirL*::*lacZ* (Cm^r) Δ*liaR*::*Janus*; Ery^r Spc^r Cm^r Kan^r	This study
RH283	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc spr*0810::*luc* Δ*comM* Δ*cbpD*::*Janus*; Ery^r Spc^r Cm^r Kan^r	This study
RH284	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc spr*0810::*luc* Δ*comM cbpD*$_{C75A}$; Ery^r Spc^r Cm^r Sm^r	This study
RH287	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc* Δ*lytC*::*tet*; Ery^r Spc^r Sm^r Tet^r	This study
RH288	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc* Δ*lytC*::*tet;* Δ*comM*::*Janus*; Ery^r Spc^r Tet^r Kan^r	This study
RH289	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc* Δ*lytC*::*tet*; Δ*comM*; Ery^r Spc^r Tet^r Sm^r	This study
RH290	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc* Δ*lytC*::*tet* Δ*comM spr*0810::*luc*; Ery^r Spc^r Tet^r Sm^r Cm^r	This study
RH291	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc* Δ*lytC*::*tet* Δ*comM spr*0810::*luc* Δ*lytA*::*Janus*; Ery^r Spc^r Tet^r Kan^r Cm^r	This study
RH294	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc spr1332*::*luc* Δ*comM*::*Janus*; Ery^r Cm^r Kan^r	This study
TMSP008	*liaR*::Spc^r	This study
TMSP011	*bgaA*::pBG0202	This study
TMSP013	*bgaA*::pBG0204	This study
TMSP014	*bgaA*::pBG0205	This study
TMSP015	*bgaA*::pBG0206	This study
SPH-4	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc hirL*::*lacZ* (Cm^r) Δ*comM* Δ*pcpC*::*Janus*; Ery^r Spc^r Cm^r Kan^r	This study
SPH-5	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc hirL*::*lacZ* (Cm^r) Δ*comM* Δ*spr*0810 Ery^r Spc^r Cm^r Sm^r	This study
SPH-6	Δ*comA*::*ermAM* (Ery^r) *ebg*::*spc hirL*::*lacZ* (Cm^r) Δ*comM* Δ*spr*0810 Δ*pcpC*::*Janus*; Ery^r Spc^r Cm^r Kan^r	This study
Plasmids		
pR424	ColE1(pEVP3) derivate, Cm^r, carries an *ssbB* targeting fragment and *luc* gene; insertion-duplication in *S. pneumoniae* generates a *ssbB*::*luc* (ssbB$^+$) fusion	4
pPPP2	ColE1 derivate, Amp^r Tet^r *'lacZ*	14
pVEL5	pR424 derivative, Cm^r; carries *liaR* targeting fragment and *luc* gene; insertion-duplication in *S. pneumoniae* generates a *liaR*::*luc* (rr03$^+$) fusion	This study
pVEL6	pR424 derivative, Cm^r; carries *spr*0810 targeting fragment and *luc* gene; insertion-duplication in *S. pneumoniae* generates a *spr*0810::*luc* (*spr*0810$^+$) fusion	This study
pR1332	pR424 derivative, Cm^r; carries *spr*1332 targeting fragment and *luc* gene; insertion-duplication in *S. pneumoniae* generates a *spr1332*::*luc* (*spr*1332) fusion	This study
pBG0202	pPPP2 derivative, P$_{liaF}$(−137–+57)::*lacZ*	This study
pBG0204	pPPP2 derivative, P$_{liaF}$(−61–+57)::*lacZ*	This study
pBG0205	pPPP2 derivative, P$_{liaF}$(−17–+57)::*lacZ*	This study
pBG0206	pPPP2 derivative, P$_{liaF}$(−64–+57)::*lacZ*	This study
pDG1726	pUC19 derivative with Spc^r cassette from pJL74	11

[a] All *Streptococcus pneumoniae* strains are R6 derivates. Cm^r, chloramphenicol resistance; Ery^r, erythromycin resistance; Spc^r, spectinomycin resistance; Sm^r, streptomycin resistance; Kan^r, kanamycin resistance, Amp^r, ampicillin resistance; Tet^r, tetracycline resistance.

cassette was amplified from RH11 (7) using the primers LytAF and LytAR and transformed into RH290, resulting in RH291.

RH294 was constructed as follows. First, an ~400-bp internal portion of *spr*1332 was amplified using the primers spr1332lucF and spr1332lucR. The fragment was cloned into pR424 linearized with BamHI and HindIII using an In-Fusion kit (Clontech) following the protocol supplied by the manufacturer. The resulting vector, pR1332, was transformed into R704 (17), resulting in RH229. This strain was transformed with the *comM*-targeting Janus cassette described above, giving rise to RH294. To construct SPH-5, the Janus cassette in RH269 was removed from the *spr*0810 locus. The upstream and downstream regions (~1,000 bp) of *spr*0810 were amplified with the primer pairs spr0810F/spr0810delR and spr0810delF/spr0810R, respectively. The fragments were subsequently fused in a PCR using the primers spr0810F and spr0810R. The resulting DNA fragment was transformed into RH269, resulting in removal of the Janus cassette. Subsequently, the *pcpC* gene was replaced with the Janus cassette described above in order to obtain a *spr*0810 *pcpC comM* mutant (SPH-6).

Ectopic integration of P$_{liaF}$-*lacZ* fusions were constructed based on the vector pPPP2. This vector carries a tetracycline resistance cassette for selection in *S. pneumoniae* and integrates into the *bgaA* locus. Promoter fragments were amplified using the primers TM1044, TM1276, TM1278, TM1279, and TM1339, thereby introducing EcoRI and BamHI sites. Standard cloning techniques were applied (39). The resulting plasmids pBG0202, pBG0204, pBG0205, and pBG0206 were used to transform *S. pneumoniae* R6 with tetracycline selection (3 μg/ml), resulting in strains TMSP011, TMSP013, TMSP014, and TMSP015.

Luciferase reporter assays. Mutants harboring the *luc* gene fused to *spr*0810 or *liaR* were grown in 280 μl C medium mixed with 20 μl of a 10 mM solution of d-luciferin from *Photinus pyralis* in 96-well Corning NBS plates. The cultures were incubated at 37°C in a FluoStar Optima plate-reader (BMG Labtech), and induced to competence by the addition of 250 ng/ml CSP at an OD_{492} of ~0.200. Uninduced cultures were run in parallel as controls. Growth and luminescence were measured at 3-min intervals.

β-Galactosidase assays. Quantification of β-galactosidase release from competence-induced cells and promoter induction assays were performed as described previously (7, 32, 41) based on the protocol of Miller (34).

Preparation of total RNA for DNA microarray analysis and Northern blotting. TMSP008 was grown anaerobically at 37°C in THB medium to mid-log phase. The culture was split and induced with bacitracin (10 μg/ml final concentration), with one sample remaining as the uninduced control. After 10 min of induction, 30 ml of each sample was mixed with 15 ml cold killing buffer (20 mM Tris-HCl,

pH 7.0, 5 mM $MgCl_2$, 20 mM NaN_3), harvested by centrifugation, and frozen in liquid nitrogen. For cell disruption, the pellet was resuspended in 200 μl killing buffer, immediately dropped into the Teflon vessel (filled and precooled with liquid nitrogen), and then disrupted with a Mikro-Dismembrator U (Sartorius). The resulting cell powder was resuspended in 3 ml of lysis solution (4 M guanidine-thiocyanate, 0.025 M Na-acetate, pH 5.2, 0.5% *N*-lauroylsarcosinate), and the RNA was extracted twice by phenol-chloroform/isoamylalcohol (25:24:1) followed by chloroform/isoamylalcohol (24:1) extraction and ethanol precipitation. RNA samples were DNase treated with the RNase-free DNase kit (Qiagen) according to the manufacturer's instructions and purified using RNeasy mini columns (Qiagen).

Probe preparation and Northern blot analysis. An internal fragment of *liaR* (~500-nucleotide length) was amplified by PCR using the primers TM1118 and TM1199 (see Table S1 in the supplemental material). The PCR fragments were purified by using the Qiagen PCR purification kit, and 1 μg of each fragment was labeled with digoxigenin (DIG) by *in vitro* transcription using the DIG RNA labeling mix (Roche) and the T7 RNA polymerase (Roche) according to manufacturer's protocol.

For Northern blot analysis, 5 μg or 10 μg of total RNA was denatured and loaded on a formaldehyde agarose gel. After electrophoresis, the RNA was transferred to a nylon membrane (Roche) in a downward transfer using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) as the transfer buffer. The RNA was cross-linked by exposing the damp membrane to UV light. The blot was prehybridized at 68°C for 1 h with prehybridization solution (0.2% sodium dodecyl sulfate [SDS], 0.1% *N*-lauroylsarcosinate, 5× SSC, 50% formamide, 2% blocking reagent), and labeled probe was added to the hybridization tube. Hybridization was performed overnight at 68°C. On the next day, the membrane was washed twice with low-stringency buffer (2× SSC, 0.1% SDS) at room temperature for 5 min, followed by two high-stringency washes (0.1× SSC, 0.1% SDS) at 68°C for 15 min. The DIG nucleic acid detection kit (Roche) was used for transcript detection. The blot was removed from the hybridization tube and placed in a box with 1× buffer 1 (10× buffer 1 is 1 M maleinic acid, 1.5 M NaCl, 0.3% Tween 20, pH 7.5) for 5 min at room temperature. The membrane was preincubated with buffer 2 (10% 10× buffer 1, 1% blocking reagent) for 30 min, treated with the antibody against DIG conjugated with alkaline phosphatase (AP) (Roche) for 30 min, and washed three times with 1× buffer 1 for 10 min. The blot was wrapped in plastic wrap, treated with the AP substrate CDP-Star (Roche) at a dilution of 1:200, and analyzed using a LumiImager (PeqLab).

The *S. pneumoniae* R6/TIGR4-Chip. The R6/TIGR4-Chip is a combined *S. pneumoniae* R6/TIGR4 oligoarray set, designed by the Department of Microbiology, University of Kaiserslautern, and produced in cooperation with the company OPERON (Huntsville, AL). The oligonucleotides are 70-mer sense oligonucleotides and have a 5′-amino-C6 linker. The oligoarray chip consists of 2,964 oligonucleotides in total: 2,347 oligonucleotides covering genes and unique intergenic regions which are longer than 200 bp of *S. pneumoniae* R6, 488 oligonucleotides covering genes and unique intergenic regions which are longer than 200 bp of TIGR4, 44 oligonucleotides covering additional sequences (repetitive elements, RNAs [tRNAs, rRNAs, RNaseP, small cytoplasmic RNA, small stable RNA]), and 1 oligonucleotide for the pbp2x variant of the penicillin-resistant isolate *S. pneumoniae* 2349, a representative of the clone Spain^{23F}-1 (2349.2x). The remaining 84 oligonucleotides represent controls: 12 randomized negative controls (HumV3con_1), 4 eukaryotic control genes, 10 positive controls, and 4 stringency controls (4 oligonucleotides with a 100%, a 90%, an 80%, and a 70% match to the corresponding gene), 32 tracking oligonucleotides (opHsV04 NC000001) representing randomly generated 30-mers (tracking oligonucleotides are randomly positioned in the 384-well [4 in each]), and 10 alien controls according to the Stratagene SpotReport alien oligoarray validation system (internal controls for the whole procedure [mRNA-cDNA hybridization]).

There were four types of oligonucleotides: (i) genes that were identical in R6 and TIGR4 (homology >95%); (ii) genes that occurred in only one of the two strains; (iii) variable genes, with one oligonucleotide covering the region conserved in both strains; and (iv) genes which varied over the whole length (on the DNA level approximately 18 to 30%): strain R6 was a reference strain. Altogether, 2,038 oligonucleotides represented R6/TIGR4 genes and 309 oligonucleotides were TIGR4 specific; 328 oligonucleotides represented intergenic regions of R6, and 160 represented those of TIGR4.

Microarray. Each oligonucleotide was spotted in duplicate onto a Schott Nexterion (Jena, Germany) slide E using the SpotArray TM24 Microarray Spotting System (PerkinElmer, Waltham, MA). Then, 30 μg of purified mRNA was synthesized from random hexamers into labeled cDNA by using the LabelStar array kit (Qiagen). The probes were then resuspended in 50 μl hybridization solution (Nexterion) plus 50 μl formamide and heated at 95°C for 5 min prior to hybridization.

Pretreatment, hybridization, and slide washing. Hybridizations were performed using a Tecan HS400 hybridization station. Prehybridization was carried out using the following steps: a 30-s wash with 0.5% SDS at 25°C; a 30-s rinse with H_2O at 25°C; and a 30-min incubation with prehybridization solution (4× SSC, 0.1% SDS, 0.1 mg/ml bovine serum albumin) at 42°C. The probe was then ready for injection. Hybridization was performed at 40°C with a medium agitation frequency for 16 h. Slide washing consisted of the following steps, all of which were performed at 30°C: 1 min with 2× SSC and 0.1% SDS, 1 min with 1× SSC, and 1 min with 0.1× SSC. The slides were finally dried under nitrogen (2.7×10^5 Pa) for 3 min.

Experimental data and replicates. For each strain to be analyzed, two independently grown cultures were used. Each mRNA preparation was divided into two parts, and each part was labeled with either Cy3 or Cy5 in order to eliminate any labeling bias during the following analyses. Since the oligonucleotides on the microarray were spotted in duplicate, a final number of four spots per experiment were analyzed. The chips were scanned with a resolution of 10 μm using the ScanArray4000 microarray analysis system. The hybridization spots were analyzed using the EasyScan method in the ScanArrayExpress software, version 4.0. A noise-to-background ratio of three was used as the cutoff. The microarray data were normalized using the LOWESS fit, and the resulting ratios were analyzed using Student's *t* test, available at the Nano+Bio-Center of the University of Kaiserslautern (http://nbc3.biologie.uni-kl.de/). Only genes that had reproducible changes in the transcript amount of greater than a 2-fold threshold were considered further. Original raw data can be found in the supplemental material or at http://microbial-stress.iab.kit.edu/87.php.

Comparative genomics and regulon predictions. To analyze the LiaR binding site in sequenced *S. pneumoniae* genomes, we used the Virtual Footprint algorithm, embedded in the Prodoric database at http://prodoric.tu-bs.de/vfp (36). To calibrate our LiaR-specific position weight matrix (PWM) and determine the parameter settings that enable us to discriminate false-positive hits from true target loci, we first screened the R6 genome. Based on the known positions of LiaR binding sites upstream of their target genes in three species, we restricted our search by applying the following cutoff criterion to filter out false-positive hits: repeats were considered potential LiaR binding sites only when they were located in noncoding regions upstream of genes with a maximum distance of 200 bp. Applying this criterion, we subsequently calibrated the sensitivity parameters, based on the known binding sites in *S. pneumoniae* R6, to a sensitivity of 0.93 (no threshold) and a core sensitivity of 0.90, with nonoccurrence penalty ON. The search window was limited to DNA regions 150 bp upstream of genes, and both "ignore match orientation" and "remove redundant" options were set ON (see http://prodoric.tu-bs.de/vfp/vfp_help.php#pwm for an explanation of these parameters).

RESULTS

Identification of the LiaR regulon by genome-wide transcriptional profiling. Bacitracin has been shown to be among the strongest inducers of LiaR-dependent gene expression in *B. subtilis*, *L. lactis*, and *S. aureus* (9, 29, 31). In all three cases, expression of the operon encoding the LiaRS two-component system is positively autoregulated. An initial test verified that the expression of the *liaFSR* operon of *S. pneumoniae* is also induced by bacitracin in a LiaR-dependent manner (data not shown). To identify LiaR-dependent genes (i.e., the LiaR regulon) in *S. pneumoniae*, we performed a DNA microarray analysis to compare the global gene expression profile of the R6 wild-type strain and the isogenic *liaR* mutant in the presence of sublethal amounts of bacitracin. For this purpose, both strains were grown to mid-logarithmic growth phase and induced with sublethal amounts of bacitracin (final concentration: 10 μg/ml) for 10 min. Cells were harvested and snap-frozen in liquid nitrogen. Subsequently, total RNA was prepared, reverse transcribed, and hybridized to a *S. pneumoniae* R6/TIGR4 biochip, based on the primer set designed by Eurofins MWG GmbH (Ebersbach, Germany; see Materials and Methods for details). A total of 18 genes, organized in six transcriptional units, were found to be upregulated more

TABLE 2. Identification of the LiaR regulon of *S. pneumoniae* by DNA microarray analysis

Locus tag	Gene	(Putative) function[a]	Fold changes[b]
*spr*0173	*arsC*	Arsenate reductase, glutaredoxin family; Spx-like	2.5
*spr*0174		Hypothetical protein	2.27
*spr*0342	*liaF*	Conserved membrane protein, putative regulator of LiaSR activity	2.7
*spr*0343	*liaS*	Histidine kinase, homolog of *B. subtilis* LiaS	2.86
*spr*0344	*liaR*	Response regulator, homolog of *B. subtilis* LiaR	11.1
*spr*0345	*alkD′*	DNA alkylation repair enzyme, truncated	7.7
*spr*0346	*′alkD′*	DNA alkylation repair enzyme, truncated	4.0
*spr*0347	*′alkD*	DNA alkylation repair enzyme, truncated	5.56
*spr*0348		Hypothetical protein	2.38
*spr*0349	*cpbG′*	Choline binding protein G, truncated	2.86
*spr*0350	*′cpbG*	Choline binding protein G, truncated	2.5
*spr*0351	*pcpC*	Choline binding protein	2.7
*spr*0453	*hrcA*	Heat-inducible transcription repressor	2.22
*spr*0454	*grpE*	Heat shock protein (activation of DnaK)	2.27
*spr*0810		Displays low level of homology to PspC from *E. coli*	6.0
*spr*1080		Hypothetical protein	2.22
*spr*1183	′ABC-NBD	Putative ABC transporter, ATP binding protein; possible multidrug efflux; truncated	2.04

[a] Gene annotations are taken from the MicrobesOnline database (http://www.microbesonline.org/).
[b] Fold changes express the average induction value of four replicates (two biological and two technical replicates) in the wild-type strain relative to an isogenic *liaR* mutant, both in the presence of bacitracin (final concentration, 10 μg/ml).

than 2-fold in the wild-type strain but not in the corresponding *liaR* mutant (Table 2). This set of genes includes the monocistronic *spr*0810 gene, which encodes a protein that belongs to the phage-shock protein C (PspC) superfamily. Another member of this superfamily (Llmg2163) was reported to be strongly upregulated by the corresponding two-component system (CesSR) in *L. lactis* (29).

Some target genes encode proteins with putative stress-related functions, such as the *hrcA-grpE* operon, which encodes a heat-shock regulator and a heat shock protein (18). Moreover, the *spr*0173/0174 operon encodes a putative transcriptional regulator with homology to Spx. Interestingly, Spx was also described as part of the CesSR regulon in *L. lactis* (45). Finally, spr1183 is a truncated monocistronic gene encoding the nucleotide-binding domain of a putative multidrug-efflux pump.

The largest and most strongly induced LiaR-dependent operon consists of 10 annotated genes (*spr*0342-*spr*0351; Fig. 1A) and includes the genes encoding the LiaFSR system (*spr*0343/44), thereby verifying the positive autoregulatory feedback loop observed for the homologous systems in *B. subtilis*, *L. lactis*, *S. aureus*, and *Streptococcus mutans* (21, 29, 43, 47). Induction of the *liaFSR* operon and its downstream genes by bacitracin has also recently been described for the *S. pneumoniae* strain D39 (28). The three truncated genes directly downstream of *liaSR* encode N-terminal, central, and C-terminal fragments of a potential DNA alkylation repair enzyme, homologous to AlkD. A comparative genomic analysis of this region, using the MicrobesOnline database (1), verified that these authentic frameshift mutations are present in all sequenced strains of *S. pneumoniae* (data not shown). The last three open reading frames of the operon encode the putative cell wall-anchored choline-binding proteins CbpG and PcpC (*spr*0351). The latter is a paralogue of CbpF. In strain R6, the *cpbG* gene is disrupted by an authentic frameshift mutation (*spr*0349/0350). Comparison with other pneumococcal strains reveals some heterogeneity, with some strains harboring an intact *cbpG* gene (i.e., CDC1873-00, SP11-BS70, SP14-BS69, ATCC 700669, and G54), while most other strains show various truncations (data not shown). The *pcpC* gene downstream of *cbpG* is found only in some pneumococcal strains, i.e., strains R6, D39, CDC1873-00, ATCC 700669, and Hungary19A-6.

Verification of the LiaR binding site and autoregulation of the *lia* operon by promoter deletion analysis and Northern blots. Because of its strong induction and known autoregulation, we next focused our attention on the *lia* operon (*spr*0342-0351). First, we analyzed the LiaR-dependent, bacitracin-inducible expression by Northern analysis. Northern analysis with a *liaR*-specific probe verified that the gene is expressed as part of a single ~5- to 6-kb transcript, corresponding well with the theoretical size of 5.3 kb (Fig. 1A). Its expression was induced by bacitracin in the wild-type strain but not in an isogenic *liaR* mutant (data not shown).

Binding sites for LiaR-like response regulators have been identified in all organisms studied in this respect to date, and the potential LiaR/CesR binding sites were predicted bioinformatically (21, 29). To verify the location of the postulated LiaR binding site, we performed a promoter deletion analysis. Promoter fragments upstream of *spr*0342 were transcriptionally fused to *lacZ*, using the promoter-probe vector pPPP2 (14) (see Table S1 in the supplemental material for primer sequences and Fig. 1B and C for details). The resulting plasmids (pBG0202-0206) were stably integrated in the chromosome of strain R6, resulting in strains TMSP011 and TMSP013-015 (Table 1). β-Galactosidase assays, performed in the presence and absence of bacitracin, demonstrated that the fragment of strain TMSP015, containing the complete inverted repeat representing the postulated LiaR binding site, was indeed still sufficient to mediate LiaR-dependent induction, whereas strain TMSP013, which lacks most of the 5′ half of the repeat, showed only a constitutive basal promoter activity. In strain TMSP014, where the predicted core promoter is missing, basal promoter activity was lost as well (Fig. 1D). Therefore, we conclude that the previously postulated inverted repeat Tcaa TCT—AGAcctA indeed represents the LiaR binding site of *S. pneumoniae.*

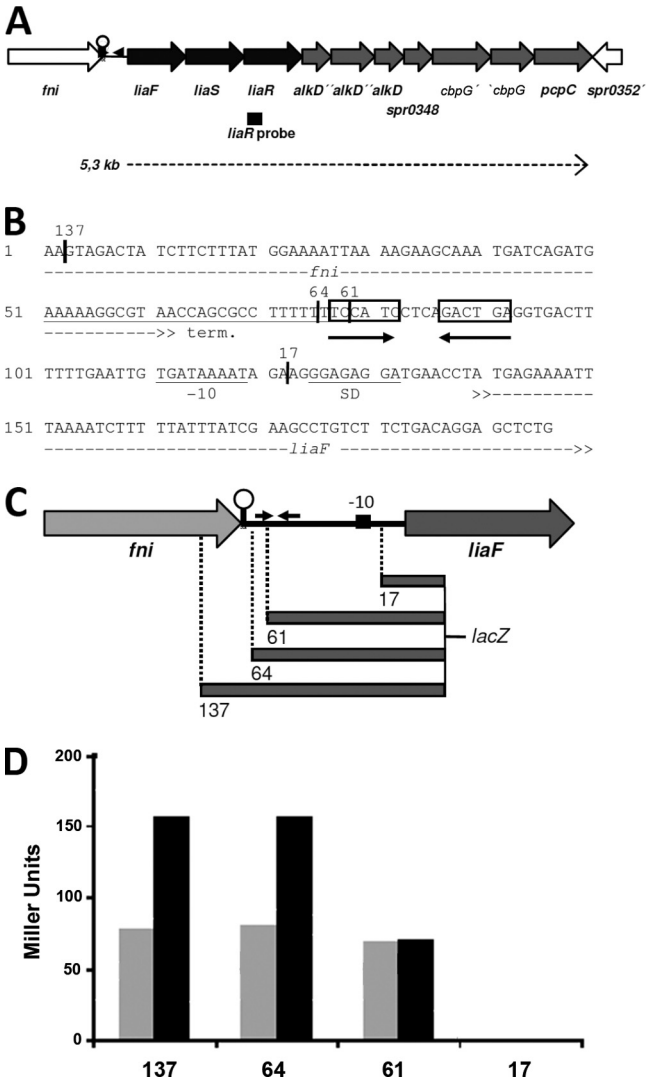

FIG. 1. Expression and promoter deletion analysis of the *liaFSR* operon. (A) Map of the *liaFSR* operon. The size of the *liaR*-specific transcript that was detected by Northern analysis is indicated by the arrow. (B) Sequence of the promoter region upstream of *liaF* (*spr*0342). (C) Schematic representation of the fragments cloned for the promoter deletion analysis of the *liaF* promoter. (D) β-Galactosidase activity of the fragments in the absence (light gray bars) and presence (black bars) of 10 μg/ml bacitracin. The numbers correspond to the fragments illustrated in panel C.

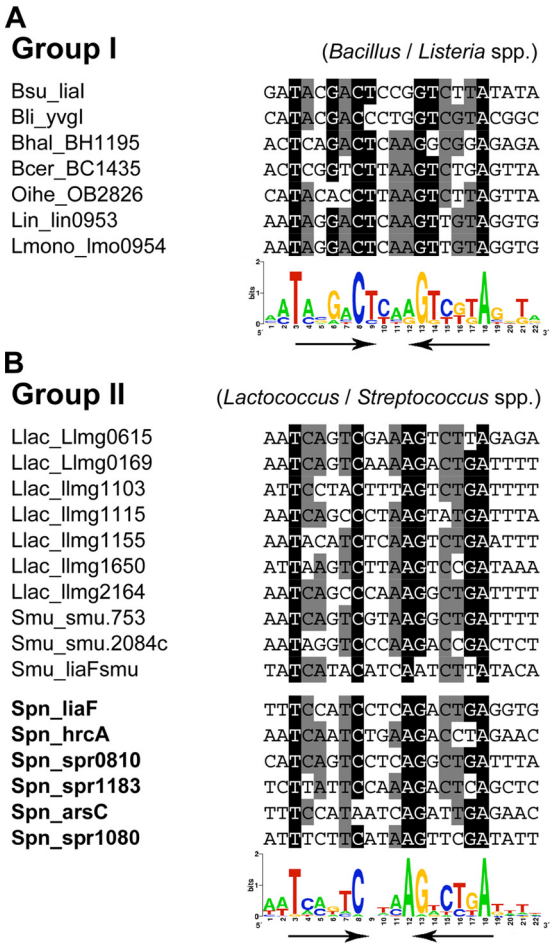

FIG. 2. LiaR binding sites and consensus sequence. (A) LiaR-like binding sites in *Bacillus* and *Listeria* species (group 1), as derived from the work of Jordan et al. (21). (B) LiaR/CesR-like binding sites in *Lactococcus* and *Streptococcus* species, based on the work of Martinez et al. (29) and this work. LiaR dependently expressed genes, as determined by DNA microarray analysis (Table 2), are highlighted in bold. Core residues (>80% conserved) and highly conserved residues (>60% conserved) are highlighted in black and gray, respectively. WebLogo (5) representations of the position weight matrices derived from these sequences are shown below. The inverted repeat is indicated by the two black arrows.

***In silico* identification of the LiaR binding site upstream of potential target genes.** Using this sequence motif but also the information from previous comparative genomics analyses of LiaR/CesR binding sites (21, 29, 43), we generated a LiaR/CesR consensus motif (Fig. 2). Two groups can be distinguished: the LiaR-like binding site upstream of *liaIH*-like operons in *Bacillus* and *Listeria* species (group 1; Fig. 2A) is a 7-2-7 inverted repeat, while the lactococcal/streptococcal binding motif represents a 6-4-6 motif (group 2; Fig. 2B). The core residues are identical in both groups, but the sequence conservation pattern shows a number of alterations.

Subsequently, we screened the promoter regions of potential LiaR target genes identified by our transcriptome analysis for the presence of LiaR binding motifs. This was done both by manual screening and by using the Virtual Footprint algorithm implemented in the Prodoric database (36), as described previously (21). We were able to identify a potential LiaR box in all of the six promoter regions (Fig. 2B). While the motifs upstream of *liaF*, *hrcA*, *spr*0810, and *spr*1183 were well conserved, the motifs of the two remaining loci (*arsC* and *spr*1080) showed some mismatches in core residues of the inverted repeat (Fig. 2B). The resulting motif is very similar for *L. lactis*, *S. mutans*, and *S. pneumoniae* but differs from the signature described for the genera *Bacillus* and *Listeria*, as mentioned above (Fig. 2). We therefore combined all identified LiaR boxes shown in Fig. 2B to develop an optimized position weight matrix (PWM) for streptococcal species. This PWM was subsequently used for genome-wide predictions of the LiaR regulon in other strains of *S. pneumoniae*.

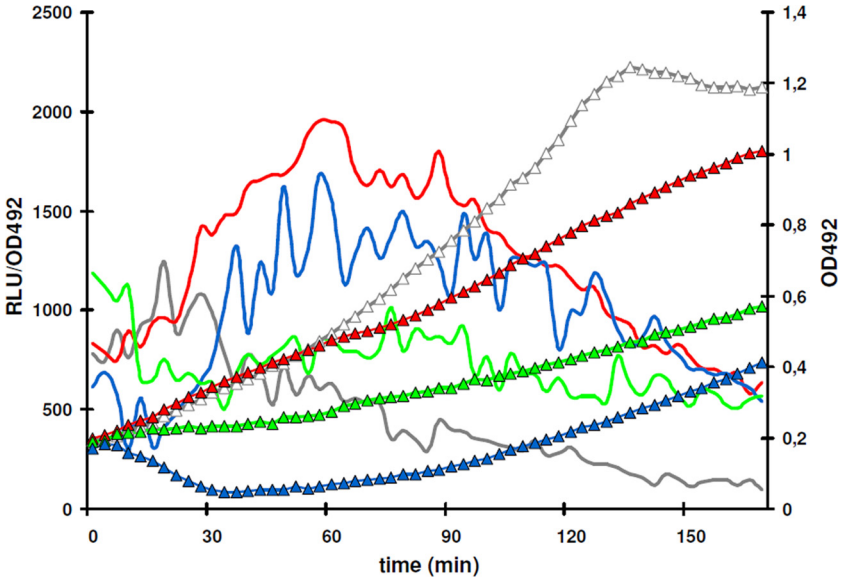

FIG. 3. Induction of the LiaSR system by antibiotics. A luciferase (*luc*) reporter gene was inserted immediately downstream of *liaR* (strain RH259). Bacterial growth curves are marked by triangles, whereas expression of *liaR*::*luc* transcriptional fusions is shown as unmarked curves. Luciferase activity is presented as relative luminescence units (RLU)/optical density (OD_{492}). Gray curves, untreated cultures; red curves, cultures grown in 5 μg/ml bacitracin; blue curves, cultures grown in 20 μg/ml nisin; green curves, cultures grown in 20 μg/ml tunicamycin. Antibiotics were added at time zero.

Prediction of the LiaR regulon in pathogenic strains of *S. pneumoniae* by comparative genomic profiling. The laboratory strain R6 has undergone extensive degenerative evolution, resulting in the loss of about 10% of the genome compared to pathogenic wild-type isolates (44). Both the number of pseudogenes found in the LiaR regulon and the lack of any antibiotic resistance phenotype linked to the Lia system made us wonder if the integrity of the Lia regulon has also been affected by this domestication. To get an idea of the potential degeneration of the LiaR regulon, we used the PWM of the streptococcal LiaR box described above (Fig. 2B) to screen all completely sequenced strains of *S. pneumoniae* that are embedded in the Prodoric database. Using the criteria and parameters described in Materials and Methods, we were able to retrieve nine potential motifs, including five of seven potentially LiaR binding sites of *S. pneumoniae* R6 (that we identified by the transcriptome analysis; see Table 2). The predicted LiaR binding site upstream of the remaining two loci shows significant mismatches in highly conserved residues of the binding site and could therefore not be retrieved (Fig. 2B).

In addition to the already known target loci, only four additional motifs were identified. In three cases (*spr*1303, *spr*1320, and *pgsA*), the potential target genes were located inside operons. For the fourth hit, *fucI* (*spr*1964), the motif was located 146 bp upstream of the start codon. Taken together, there is a good overall correlation between PWM-based binding-site predictions and the results from the genome-wide expression profiling (Table 2).

Subsequently, the same PWM and criteria were used to screen the four clinical *S. pneumoniae* strains implemented in the Prodoric genome database: D39 (NCTC 7466), TIGR4 (ATCC BAA-334), CGSP14, and Hungary19A-6. The results for all strains were consistent with the data retrieved from the scan of the R6 genome. Between seven and nine potential binding motifs were retrieved, including all target loci identified for strain R6 (data not shown). No additional potential LiaR target loci were identified. The data therefore indicate that no potential LiaR regulon members were lost in the course of domestication of the laboratory strain R6.

To further validate the quality of our PWM, we also screened the genome of *S. mutans* UA159, again using the same criteria and parameters as above. We identified five potential LiaR binding sites, including motifs upstream of the known target loci *spr*0810 and *liaFSR*.

Interestingly, the two target genes *hrcA* and *arsC*/*spx* are shared between *S. mutans* and *S. pneumoniae*. These results further strengthen our regulon prediction and the quality of the LiaR-specific PWM graphically illustrated in Fig. 2.

LiaSR in *S. pneumoniae* strain R6 is activated by lipid II-interfering antibiotics but does not confer resistance against them. The best-studied inducers of envelope stress-sensing systems of the LiaSR/VraSR/CesSR type are antibiotics interfering with the lipid II cycle, with bacitracin, ramoplanin, vancomycin, and nisin constituting the strongest inducers identified so far (20). We tested various antibiotics inhibiting different steps in the bacterial cell wall synthesis cycle for their ability to stimulate expression of the *liaSR* genes in *S. pneumoniae*. This time, we inserted the luciferase reporter gene immediately behind *liaR* itself, resulting in the strain RH259. Bacitracin, nisin, and tunicamycin were found to induce *luc* expression in this strain (Fig. 3), whereas a luminescence signal above background level could not be detected with cycloserine (10 to 500 μg/ml), ampicillin (0.0025 to 0.5 μg/ml), or vancomycin (0.1 to 2 μg/ml). The latter finding was unexpected, since vancomycin has previously been reported to upregulate *liaSR* expression in *S. pneumoniae* (13). In addition, vancomycin induces the

LiaFSR system of *B. subtilis* (32) and the *S. aureus* homolog VraSR (23).

For *B. subtilis* LiaSR, *S. aureus* VraSR, and *L. lactis* CesSR, it was shown that the two-component system mediates resistance to at least some of the inducing antibiotics. We therefore examined whether the same is true for *S. pneumoniae* LiaSR. We performed disk diffusion assays and killing curve experiments for all inducing antibiotics, basically as described previously (32), by comparing the sensitivity of the wild-type strain R6 and the isogenic *liaR* deletion mutant. No significant differences in the sensitivity toward any of the antibiotics tested were detected (data not shown). Interestingly, a weak increase of bacitracin susceptibility (2-fold) was recently observed for a *liaFSR* mutant of the ancestor strain D39, relative to the wild-type strain (28). The significance of this difference remains to be investigated.

LiaSR is activated during competence in the absence of the immunity gene *comM*. Because of the lack of an antibiotic resistance phenotype linked to LiaSR, we wondered if this system might be important in responding to other cell envelope perturbing conditions. In order to quantify LiaSR activity, the luciferase gene from *Photinus pyralis* (*luc*) was inserted immediately downstream of *spr*0810, one of the most strongly induced genes of the LiaR regulon, as demonstrated by our microarray analysis (Table 2). Expression of Luc in the resulting strain, RH270, was monitored by cultivating the bacteria in a 96-well plate in a Fluostar OPTIMA luminometer at 37°C as described previously (19). When competence was induced by the addition of 250 ng/ml of CSP to the culture (15), a slight increase in *luc* expression was observed (Fig. 4A). In order to determine whether ComM played a role in protecting against the observed cell envelope stress, *comM* was deleted using the Janus method (42). Interestingly, induction of competence in strain RH272 (Δ*comM spr*0810::*luc*) resulted in a strong upregulation of *spr*0810 expression (Fig. 4B). To verify that this upregulation was due to activation of the LiaSR system, *liaR* was deleted in the RH272 strain, giving rise to RH273. In strain RH273, the overall *spr*0810 expression was reduced, and the observed increase in luminescence following treatment with CSP was abolished (Fig. 4G). This result verifies that LiaSR is indeed responsible for regulation of *spr*0810 expression and shows that monitoring *spr*0810 expression can be used as a proxy for monitoring activation of the LiaSR system.

To rule out the possibility that the increased luminescence (expressed in relative luminescence units [RLU]/OD_{492}) observed for the *comM* mutant upon competence induction is an artifact resulting from the transient fratricide-induced drop in OD_{492}, we created a *comM* mutant carrying the *luc* gene fused to a aldo-keto reductase (*spr*1332) that has no role in competence or cell envelope maintenance. Competence induction in this strain resulted in only a brief and very modest increase in RLU/OD_{492} (Fig. 4H), demonstrating that the strong LiaSR activation observed during competence in cells lacking the ComM immunity protein is real.

Upregulation of LiaSR is attributable to the activity of CbpD, LytA, and LytC. To decide whether CbpD, the key player and trigger factor of the fratricide mechanism in *S. pneumoniae*, is responsible for activating LiaSR, the *cbpD* gene was deleted in strain RH272, giving rise to RH283. When RH283 was induced to competence, expression of the *luc* reporter gene returned to the level observed for strain RH270, demonstrating that CbpD activity is responsible for activating the LiaSR system in the absence of ComM (Fig. 4C). CbpD contains a proteolytic domain called CHAP (cysteine, histidine-dependent amidohydrolases/peptidases), which due to its strong homology to a number of phage lysins is believed to function as a murein hydrolase. In addition, CbpD contains two SH3b domains and four choline-binding repeats. While it has been firmly established that choline-binding repeats mediate noncovalent binding to cell wall teichoic (TA) and lipoteichoic acids (LTA) in *S. pneumoniae* (27), the exact function of the SH3b domains remains to be elucidated. The stress response elicited by CbpD is most probably generated by the enzymatic activity of the CHAP domain but could also be caused by the abrupt accumulation of this protein in the periplasm or the cell wall. To discriminate between these possibilities, we exchanged the wild-type *cbpD* gene with a mutated version in which the active site cysteine (C-75) had been replaced by an alanine residue. Induction of competence in this mutant strain, RH284 (Δ*comM spr*0810::*luc* $CbpD_{C75A}$), revealed that a functional CHAP domain is essential for the ability of CbpD to activate the LiaSR system (Fig. 4D).

It is well established that LytC is directly or indirectly activated by CbpD during fratricide and that this lysozyme is important for efficient lysis of target cells (7). Activation of the LiaSR system could therefore be attributable to LytC activity in addition to, or instead of, CbpD. We found that deletion of *lytC* (RH290) led to a change in the kinetics of the stress response. In the absence of LytC, the response reached approximately the same maximum level as in the RH272 strain, but expression of the Luc reporter was delayed and light production decayed more rapidly in the RH290 strain (Fig. 4E). The major autolysin in *S. pneumoniae*, LytA, has also been shown to be activated by CbpD and to contribute to the fratricide mechanism. It is therefore possible that the remaining stress response detected upon competence induction in strain RH290 was due to the *N*-acetylmuramoyl-*L*-alanine amidase (NAM-amidase) activity of LytA and only indirectly depended on the enzymatic activity of CbpD. To further elucidate these matters, the *lytA* gene was deleted in the RH290 strain, giving rise to RH291 (Δ*comM spr*0810::*luc* Δ*lytC* Δ*lytA*). The magnitude of the stress response in this strain was only slightly stronger than in the *cbpD* deletion mutant (Fig. 4F). Together, our results show that cell wall damage caused by the concerted action of the murein hydrolases CbpD, LytA, and LytC activates the LiaSR system in cells lacking the fratricide immunity protein ComM.

Deletion of *liaR* significantly increases lysis during competence. Considering that CbpD is able to elicit envelope stress that activates the LiaSR system, we wondered whether LiaSR in *S. pneumoniae* plays a part in protecting competent cells against their own lysins. To detect cell lysis caused by the fratricide mechanism, we took advantage of a *lacZ* reporter gene inserted downstream of the constitutive *hirL* promoter (41). The percent cell lysis was quantified by measuring β-galactosidase leakage to the supernatant relative to the total content of β-galactosidase in the culture (cells plus supernatant). First, *liaR* was deleted in the strain RH4 (*hirL*::*lacZ*), giving rise to RH276. The RH4 and RH276 strains were then induced to competence, and cell lysis was measured 30 min post CSP induction. Due to the presence of a functional *comM* gene, the RH4 strain (desig-

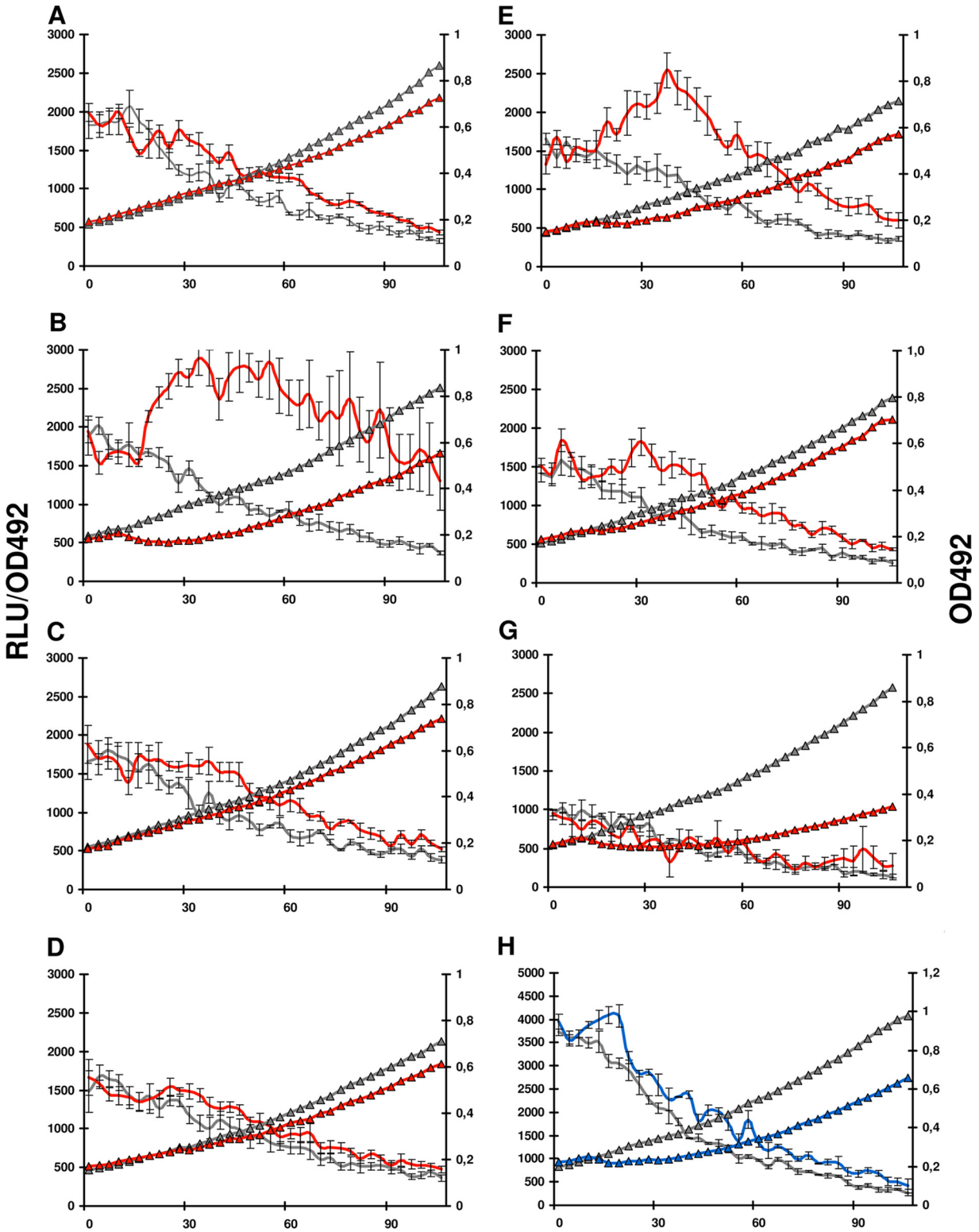

FIG. 4. Comparison of *liaSR* activation in competent and noncompetent pneumococci harboring various mutations in genes involved in the fratricide mechanism. The *luc* reporter was inserted immediately downstream of *spr*0810, a gene that is part of the LiaSR regulon. Bacterial growth curves are marked by triangles, whereas expression of *spr*0810::*luc* transcriptional fusions is shown as unmarked curves. Luciferase activity is presented as relative luminescence units (RLU)/optical density (OD_{492}). Gray curves represent noncompetent cultures, while colored curves represent CSP-induced competent cultures. CSP was added at time zero. (A) RH270 (*spr*0810::*luc*), (B) RH272 (*spr*0810::*luc comM*), (C) RH283 (*spr*0810::*luc comM cbpD*), (D) RH284 (*spr*0810::*luc comM* $cbpD_{C75A}$), (E) RH290 (*spr*0810::*luc comM lytC*), (F) RH291 (*spr*0810::*luc comM lytC lytA*), (G) RH273 (*spr*0810::*luc comM liaR*), (H) RH294 (*spr1332*::*luc comM*). Results presented in panels A to H are the means ± standard errors of results of three to five independent experiments.

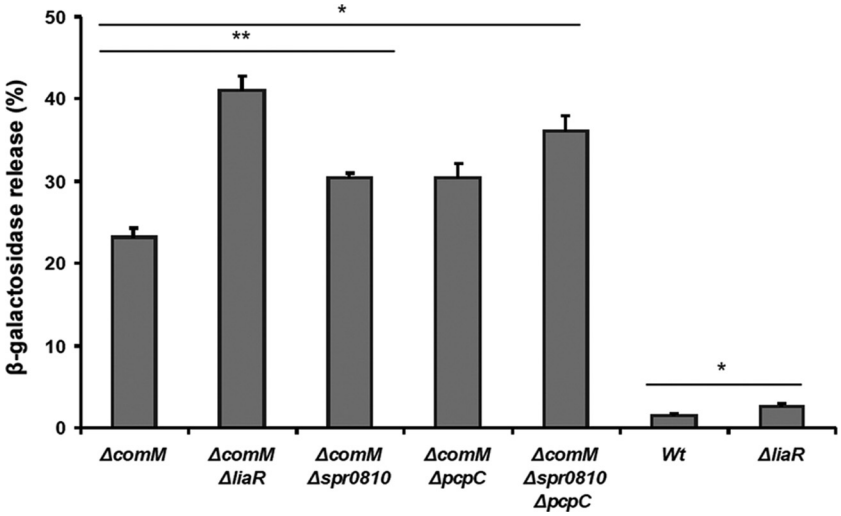

FIG. 5. Competence-induced cell lysis in various pneumococcal deletion mutants quantified by means of β-galactosidase release. All strains contain a *hirL*::*lacZ* fusion conferring constitutive production of an intracellular β-galactosidase reporter protein. The level of β-galactosidase activity (in Miller units) present in the cell-free supernatants is given as a percentage of the total activity present in supernatants plus intact cells. Strains used were as follows: RH237 (Δ*comM*), RH253 (Δ*comM* Δ*liaR*), SPH-5 (Δ*comM* Δ*spr*0810), SPH-4 (Δ*comM* Δ*pcpC*), SPH-6 (Δ*comM* Δ*spr*0810 Δ*pcpC*), RH4 (wild type), and RH276 (Δ*liaR*). Data represent the averages ± standard errors of results of at least three independent experiments. **, $P \leq 0.01$; *, $P \leq 0.05$.

nated WT in Fig. 5) underwent very limited lysis. Interestingly, the *liaR* mutant RH276 exhibited a doubling of lysis compared to the level for RH4, but the level of lysis remained low (Fig. 5). This indicates that ComM in itself is sufficient to prevent most cells from lysing, but that some cells succumb to competence-induced stress in the absence of a functional LiaSR system. Next, we deleted *liaR* in a strain (RH237) lacking ComM. The resulting mutant strain, RH253, turned out to be considerably more prone to lysis during competence than the parental strain. When the RH253 strain was assayed as outlined above, ~40% of the cell population lysed within 30 min. In contrast, only ~20% of the RH237 cells underwent lysis when they were assayed by the same procedure (Fig. 5). Together, these results show that without a functional LiaSR system, a doubling in cell lysis takes place in both ComM-proficient and -deficient cells.

We also investigated whether the LiaFSR system plays a role in protecting noncompetent target cells from competent attacker cells during cocultivation. These mixed-culture experiments, containing equal amounts of β-galactosidase-producing target cells and competent attacker cells, were carried out as described previously (41). Under the experimental conditions used, deletion of *liaR* in noncompetent target cells had no effect on their rate of survival (results not shown). This finding suggests that competent pneumococci experience an additional level or different kind of stress compared to noncompetent cells under attack and that a functional LiaSR system helps alleviate this stress.

Deletion of the *spr*0810 and *pcpC* genes. Based on the microarray analysis, we tried to identify candidate genes mediating the observed LiaR-dependent protection against CbpD, LytA, and LytC. Among the LiaR-dependent gene products upregulated in the microarray screen, we decided to further investigate *spr*0810 and PcpC. As mentioned previously, *spr*0810 encodes a protein predicted to belong to the phage shock protein C superfamily. The phage shock protein (Psp) system was first identified in *Escherichia coli* (3), and has since then been studied extensively in this species and *Yersinia enterocolitica* (6). The Psp systems of *E. coli* and *Y. enterocolitica* have six proteins in common (PspABCD and PspFG) that are also present in many other Gram-negative bacteria. In these bacteria, the Psp system responds to extracytoplasmic stress and appears to play a role in maintaining cytoplasmic membrane integrity and/or the proton motive force (33). All but the *spr*0810 gene product, which shows a rather low level of homology to *E. coli* PspC, appear to be absent from the pneumococcal genome. However, since both *E. coli* PspC and pneumococcal *spr*0810 are small membrane proteins possessing one centrally located transmembrane helix, they might perform comparable functions. The choline-binding protein PcpC (*spr*0351) is closely related to CbpF (*spr*0337), which has been shown to be a negative regulator of LytC activity in *S. pneumoniae* (35). As shown above, inactivation of the LiaFSR system increases competence-induced cell lysis (Fig. 5). As this effect is most prominent in the absence of ComM, we decided to delete the *spr*0810 and *pcpC* genes in a Δ*comM* background. We found that deletion of each of these genes led to a significant increase in competence-induced lysis, demonstrating that they contribute to alleviate stress caused by CbpD, LytA, and LytC. Damage caused by the fratricide mechanism was even less tolerated in a *spr*0810/*pcpC* double mutant. In this mutant, the level of cell lysis during competence reached ~36%, compared to about ~30% for each of the *spr*0810 and *pcpC* single mutants (Fig. 5). Taken together, our data clearly demonstrate an important role of LiaR-dependent gene expression as a second layer of defense, in addition to the primary immunity protein ComM, in counteracting fratricide-induced lysis.

DISCUSSION

All LiaFSR-like systems investigated so far are activated by agents or perturbations that affect the integrity of the bacterial

cell envelope. The strongest known inducers are lipid II cycle inhibitors such as of bacitracin, nisin, vancomycin, and ramoplanin. Other antibiotics that interfere with cell wall biosynthesis appear to be effective only in some species, suggesting some differences in the specificity of their LiaFSR systems. The eponymous LiaFSR system of *B. subtilis* is also weakly induced by more unspecific perturbations of the cell envelope, such as alkaline shock and secretion stress (20). For *S. aureus* VraSR, the antibiotics D-cycloserine and tunicamycin, which inhibit the enzyme (MraY) catalyzing the synthesis of lipid I, and even β-lactams and reduction in the level of *pbpB* transcription have been found to induce the system (9, 23). Furthermore, suboptimal transcription of *murF*, an enzyme that adds the D-alanyl-D-alanine dipeptide to the UDP-linked MurNAc-tripeptide, results in increased transcription of the VraSR system in *S. aureus* (40).

A different kind of cell envelope stress has been reported to induce the CesSR system in *L. lactis.* Veiga et al. (45) found that treatment of the MG1363 strain of this species with lysozyme increased expression of its *ces* operon. In contrast, Martínez et al. (29), working with the same strain of *L. lactis*, found that lysozyme at the concentrations tested was unable to induce expression of a *lacZ* reporter gene under the control of a CesR-responsive promoter. In the present study, we clearly show that enzymes that degrade peptidoglycan can activate the LiaFSR cell envelope stress-sensing systems of *S. pneumoniae*. LytC is a lysozyme that cleaves the β-1-4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the bacterial cell wall. Deletion of the gene encoding this lysozyme significantly altered the kinetics of the stress response in *S. pneumoniae* (Fig. 4). The response took longer to appear and decayed much faster than in the LytC-proficient parental strain used as a control. Further deletion of the gene encoding the NAM-amidase LytA reduced the LiaSR response to a level just above the detection limit of our system. LiaSR activation was totally dependent on the enzymatic activity of the CHAP domain of CbpD and its triggering of the glycan strand-cleaving activity of LytC or the NAM activity of LytA. The CHAP domain of CbpD is highly homologous to corresponding domains found in a number of phage lysins and bacterial murein hydrolases. Characterized members of the CHAP family has been found to act either as endopeptidases that cleave within murein stem peptides or as amidases that cleave the *N*-acetylmuramyl-L-Ala bond (2, 38). Thus, our findings indicate that both glycan strand-cleaving enzymes and enzymes that cleave amide/peptide bonds can elicit a stress response that activates the LiaSR system in *S. pneumoniae*.

Very little is known about the natural biological role of LytC. It is relatively highly expressed when pneumococci are grown in liquid culture under laboratory conditions, but mutants lacking a functional *lytC* gene display no growth defects or other strong phenotypes (8). However, Garcia et al. (8) showed that LytC mediates autolysis in the stationary phase when the pneumococcal cells are grown at 30°C, suggesting that LytC functions as an autolysin that becomes active at temperatures encountered by pneumococci colonizing the nasopharynx (8). Interestingly, it has also been found that LytC is activated by CbpD during fratricide (7). However, it has not been clear whether CbpD activates LytC through direct interaction or by an indirect mechanism. The fact that induction of the competent state in the $CbpD_{C75A}$ mutant did not trigger a LytC-provoked stress response demonstrates that a functional CHAP domain is required to activate LytC. Furthermore, since CbpD is able to trigger a small but significant stress response in the absence of LytC, the natural substrate of the CHAP protease is probably not LytC itself but the cell wall stem peptides. Based on these observations, our data strongly indicate that LytC is activated by cell wall damage inflicted by the enzymatic activity of CbpD.

Microarray analysis identified LiaR-controlled genes that might be involved in the observed LiaR-mediated protection against the murein hydrolases CbpD, LytA, and LytC. Three of the upregulated genes, *hrcA*, *grpE*, and *spr*0810, encode proteins that are involved in responses to various types of stress. HrcA regulates the expression of DnaK and GroEL by repressing the transcription of the operons encoding these molecular chaperones (18). DnaK and GroEL play a crucial role in protein folding, and is important for cell survival during heat shock and other types of environmental stress. The Psp system of Gram-negative bacteria responds to several types of stimuli, all of which have the potential to damage the cell envelope and dissipate the proton motive force. The only possible component of this system that has been identified in *S. pneumoniae* is the LiaR-controlled *spr*0810 gene. In *Y. enterocolitica* the product of the corresponding gene, PspC, functions both as a regulator of *psp* gene expression and as an effector that acts independently to support growth when the bacterium is subjected to extracytoplasmic stress (33). In contrast to the complex system regulated by HrcA, the Psp-like system in *S. pneumoniae* appears to consist of a single protein encoded on a monocistronic operon. We therefore decided to investigate whether deletion of the *spr*0810 gene in a *comM* mutant would further increase the fraction of cells undergoing CbpD-induced lysis during competence development. We found a modest but significant increase (~7%), indicating that *spr*0810 contributes to alleviate cell envelope stress elicited by the fratricide mechanism.

The *pcpC* gene is located at the 3′-end of the *liaFSR* operon, which encodes 10 predicted genes. PcpC is a paralog of CbpF, a choline binding protein that is one of the most abundant proteins in the pneumococcal cell wall. It was recently reported by Molina et al. (35) that external addition of 1.2 μM CbpF abolishes LytC-induced autolysis at 30°C *in vitro*. Since PcpC is highly homologous to CbpF, we speculated that this protein functions as a modulator of murein hydrolase activity as well. We therefore deleted the *pcpC* gene in a strain lacking ComM and measured the fraction of cells that lysed during competence. Similar to the results obtained with pneumococcal cells deficient in Spr0810, cell lysis increased by about 7% in the *pcpC* mutant compared to that in the parental strain. Together, our results show that both Spr0810 and PcpC contribute to stress relief during competence.

Fratricide-mediated self-lysis increased from about 23% in cells lacking a functional *comM* immunity gene (RH237) to 41% in a *comM*/*liaR* double mutant (RH253) (Fig. 5). To determine whether the LiaR-controlled gene products Spr0810 and PcpC can fully account for the protection provided by the LiaSR system, we constructed a *spr*0810/*pcpC* double mutant designated SPH-6 (Δ*spr*0810 Δ*pcpC* Δ*comM*). The level of self-lysis in a culture of this mutant reached 36%, approxi-

mately the sum of the increases measured for each of the *spr*0810 and *pcpC* single mutants relative to their parental strain RH237. As the combined effect of deleting *spr*0810 and *pcpC* does not fully amount to the observed difference between the RH237 and RH253 strains, it is likely that additional LiaR-controlled gene products are involved in counteracting CbpD-, LytC-, and LytA-induced stress. Possible candidates are HrcA and GrpE, which are involved in regulating the activity of the chaperone DnaK. In a wild-type strain the protective effect of the LiaSR system is mostly masked by ComM, which functions as a specific immunity mechanism against CbpD-induced fratricide. Still, even in a strain with a functional *comM* gene, fratricide-induced lysis doubles from 1.3% to 2.6% when LiaR is deleted (Fig. 5).

In *S. pneumoniae* the LiaFSR system does not protect the cells against the lipid II-interacting antibiotics that it responds to. Instead, it provides protection against self-lysis in competent cells. This finding makes it tempting to speculate that the LiaFSR system in *S. pneumoniae* has diverged from the classical cell envelope stress response to mediate a second layer of resistance against fratricide-induced lysis. It remains to be investigated whether other target genes of the LiaFSR system of *S. pneumoniae* have additional protective functions against other aspects of cell envelope stress.

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REFERENCES

1. **Alm, E. J., K. H. Huang, M. N. Price, R. P. Koche, K. Keller, I. L. Dubchak, and A. P. Arkin.** 2005. The MicrobesOnline web site for comparative genomics. Genome Res. **15:**1015–1022.
2. **Bateman, A., and N. D. Rawlings.** 2003. The CHAP domain: a large family of amidases including GSP amidase and peptidoglycan hydrolases. Trends Biochem. Sci. **28:**234–237.
3. **Brissette, J. L., M. Russel, L. Weiner, and P. Model.** 1990. Phage shock protein, a stress protein of *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **87:**862–866.
4. **Chastanet, A., M. Prudhomme, J.-P. Claverys, and T. Msadek.** 2001. Regulation of *Streptococcus pneumoniae* clp genes and their role in competence development and stress survival. J. Bacteriol. **183:**7295–7307.
5. **Crooks, G. E., G. Hon, J.-M. Chandonia, and S. E. Brenner.** 2004. WebLogo: a sequence logo generator. Genome Res. **14:**1188–1190.
6. **Darwin, A. J.** 2005. The phage-shock-protein response. Mol. Microbiol. **57:**621–628.
7. **Eldholm, V., O. Johnsborg, K. Haugen, H. S. Ohnstad, and L. S. Havarstein.** 2009. Fratricide in *Streptococcus pneumoniae*: contributions and role of the cell wall hydrolases CbpD, LytA and LytC. Microbiology **155:**2223–2234.
8. **Garcia, P., M. P. Gonzalez, E. Garcia, J. L. Garcia, and R. Lopez.** 1999. The molecular characterization of the first autolytic lysozyme of *Streptococcus pneumoniae* reveals evolutionary mobile domains. Mol. Microbiol. **33:**128–138.
9. **Gardete, S., S. W. Wu, S. Gill, and A. Tomasz.** 2006. Role of VraSR in antibiotic resistance and antibiotic-induced stress response in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **50:**3424–3434.
10. **Geiser, M., R. Cèbe, D. Drewello, and R. Schmitz.** 2000. Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. Biotechniques **31:**88–92.
11. **Guérout-Fleury, A.-M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene **167:**335–336.
12. **Guiral, S., T. J. Mitchell, B. Martin, and J.-P. Claverys.** 2005. Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements. Proc. Natl. Acad. Sci. U. S. A. **102:**8710–8715.
13. **Haas, W., D. Kaushal, J. Sublett, C. Obert, and E. I. Tuomanen.** 2005. Vancomycin stress response in a sensitive and a tolerant strain of *Streptococcus pneumoniae*. J. Bacteriol. **187:**8205–8210.
14. **Halfmann, A., R. Hakenbeck, and R. Brückner.** 2007. A new integrative reporter plasmid for *Streptococcus pneumoniae*. FEMS Microbiol. Lett. **268:**217–224.
15. **Håvarstein, L. S., G. Coomaraswamy, and D. A. Morrison.** 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. U. S. A. **92:**11140–11144.
16. **Håvarstein, L. S., P. Gaustad, I. F. Nes, and D. A. Morrison.** 1996. Identification of the streptococcal competence-pheromone receptor. Mol. Microbiol. **21:**863–869.
17. **Håvarstein, L. S., B. Martin, O. Johnsborg, C. Granadel, and J.-P. Claverys.** 2006. New insights into the pneumococcal fratricide: relationship to clumping and identification of a novel immunity factor. Mol. Microbiol. **59:**1297–1307.
18. **Jayaraman, G. C., J. E. Penders, and R. A. Burne.** 1997. Transcriptional analysis of the *Streptococcus mutans hrcA*, grpE and *dnaK* genes and regulation of expression in response to heat shock and environmental acidification. Mol. Microbiol. **25:**329–341.
19. **Johnsborg, O., V. Eldholm, M. L. Bjørnstad, and L. S. Håvarstein.** 2008. A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species. Mol. Microbiol. **69:**245–253.
20. **Jordan, S., M. I. Hutchings, and T. Mascher.** 2008. Cell envelope stress response in Gram-positive bacteria. FEMS Microbiol. Rev. **32:**107–146.
21. **Jordan, S., A. Junker, J. D. Helmann, and T. Mascher.** 2006. Regulation of LiaRS-dependent gene expression in *Bacillus subtilis*: identification of inhibitor proteins, regulator binding sites, and target genes of a conserved cell envelope stress-sensing two-component system. J. Bacteriol. **188:**5153–5166.
22. **Kausmally, L., O. Johnsborg, M. Lunde, E. Knutsen, and L. S. Havarstein.** 2005. Choline-binding protein D (CbpD) in *Streptococcus pneumoniae* is essential for competence-induced cell lysis. J. Bacteriol. **187:**4338–4345.
23. **Kuroda, M., H. Kuroda, T. Oshima, F. Takeuchi, H. Mori, and K. Hiramatsu.** 2003. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. Mol. Microbiol. **49:**807–821.
24. **Kuroda, M., K. Kuwahara-Arai, and K. Hiramatsu.** 2000. Identification of the up- and down-regulated genes in vancomycin-resistant *Staphylococcus aureus* strains Mu3 and Mu50 by cDNA differential hybridization method. Biochem. Biophys. Res. Commun. **269:**485–490.
25. **Lacks, S., and R. D. Hotchkiss.** 1960. A study of the genetic material determining an enzyme in Pneumococcus. Biochim. Biophys. Acta **39:**508–518.
26. **Lee, M. S., and D. A. Morrison.** 1999. Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. J. Bacteriol. **181:**5004–5016.
27. **Lopez, R., and E. Garcia.** 2004. Recent trends on the molecular biology of pneumococcal capsules, lytic enzymes, and bacteriophage. FEMS Microbiol. Rev. **28:**553–580.
28. **Majchrzykiewicz, J. A., O. P. Kuipers, and J. J. E. Bijlsma.** 2010. Generic and specific adaptive responses of *Streptococcus pneumoniae* to challenge with three distinct antimicrobial peptides, bacitracin, LL-37, and nisin. Antimicrob. Agents Chemother. **54:**440–451.
29. **Martínez, B., A. L. Zomer, A. Rodríguez, J. Kok, and O. P. Kuipers.** 2007. Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the lactococcal two-component system CesSR. Mol. Microbiol. **64:**473–486.
30. **Mascher, T.** 2006. Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in Firmicutes bacteria. FEMS Microbiol. Lett. **264:**133–144.
31. **Mascher, T., N. G. Margulis, T. Wang, R. W. Ye, and J. D. Helmann.** 2003. Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. Mol. Microbiol. **50:**1591–1604.
32. **Mascher, T., S. L. Zimmer, T.-A. Smith, and J. D. Helmann.** 2004. Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of *Bacillus subtilis*. Antimicrob. Agents Chemother. **48:**2888–2896.
33. **Maxson, M. E., and A. J. Darwin.** 2006. PspB and PspC of *Yersinia enterocolitica* are dual function proteins: regulators and effectors of the phage-shock-protein response. Mol. Microbiol. **59:**1610–1623.
34. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
35. **Molina, R., A. González, M. Stelter, I. Pérez-Dorado, R. Kahn, M. Morales, M. Moscoso, S. Campuzano, N. E. Campillo, S. Mobashery, J. L. García, P. García, and J. A. Hermoso.** 2009. Crystal structure of CbpF, a bifunctional

choline-binding protein and autolysis regulator from *Streptococcus pneumoniae*. EMBO Rep. **10:**246–251.

36. **Munch, R., K. Hiller, A. Grote, M. Scheer, J. Klein, M. Schobert, and D. Jahn.** 2005. Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. Mol. Microbiol. **21:**4187–4189.
37. **Pestova, E. V., L. S. Havarstein, and D. A. Morrison.** 1996. Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. Mol. Microbiol. **21:**853–862.
38. **Rigden, D. J., M. J. Jedrzejas, and M. Y. Galperin.** 2003. Amidase domains from bacterial and phage autolysins define a family of γ-$_{D,L}$-glutamate-specific amidohydrolases. Trends Biochem. Sci. **28:**230–234.
39. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
40. **Sobral, R. G., A. E. Jones, S. G. Des Etages, T. J. Dougherty, R. M. Peitzsch, T. Gaasterland, A. M. Ludovice, H. de Lencastre, and A. Tomasz.** 2007. Extensive and genome-wide changes in the transcription profile of *Staphylococcus aureus* induced by modulating the transcription of the cell wall synthesis gene *murF*. J. Bacteriol. **189:**2376–2391.
41. **Steinmoen, H., E. Knutsen, and L. S. Havarstein.** 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.
42. **Sung, C. K., H. Li, J. P. Claverys, and D. A. Morrison.** 2001. An *rpsL* cassette, Janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. Appl. Environ. Microbiol. **67:**5190–5196.
43. **Suntharalingam, P., M. D. Senadheera, R. W. Mair, C. M. Levesque, and D. G. Cvitkovitch.** 2009. The LiaFSR system regulates the cell envelope stress response in *Streptococcus mutans*. J. Bacteriol. **191:**2973–2984.
44. **Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J. Heidelberg, R. T. DeBoy, D. H. Haft, R. J. Dodson, A. S. Durkin, M. Gwinn, J. F. Kolonay, W. C. Nelson, J. D. Peterson, L. A. Umayam, O. White, S. L. Salzberg, M. R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A. M. Wolf, T. R. Utterback, C. L. Hansen, L. A. McDonald, T. V. Feldblyum, S. Angiuoli, T. Dickinson, E. K. Hickey, I. E. Holt, B. J. Loftus, F. Yang, H. O. Smith, J. C. Venter, B. A. Dougherty, D. A. Morrison, S. K. Hollingshead, and C. M. Fraser.** 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science **293:**498–506.
45. **Veiga, P., C. Bulbarela-Sampieri, S. Furlan, A. Maisons, M.-P. Chapot-Chartier, M. Erkelenz, P. Mervelet, P. Noirot, D. Frees, O. P. Kuipers, J. Kok, A. Gruss, G. Buist, and S. Kulakauskas.** 2007. SpxB regulates O-acetylation-dependent resistance of *Lactococcus lactis* peptidoglycan to hydrolysis. J. Biol. Chem. **282:**19342–19354.
46. **Wach, A.** 1996. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. Yeast **12:**259–265.
47. **Yin, S., R. S. Daum, and S. Boyle-Vavra.** 2006. VraSR two-component regulatory system and its role in induction of *pbp2* and *vraSR* expression by cell wall antimicrobials in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **50:**336–343.

SUPPLEMENTAL MATERIAL

Table S1. Oligonucleotide primer sequences

Primer	Oligonucleotide Sequence (5'→ 3')	Reference
CbpD-1098F	GTTGATTATCTTAGCAGCTCGT	This study
$CbpD_{C75A}F$	TCTCGTCAGGCTACTTCTTTTGTAGCCTTTCGT	This study
$CbpD_{C75A}R$	GCTACAAAAGAAGTAGCCTGACGAGAATACATGCGCCA	This study
CbpDR	CCAAGGGTTTGCTCGCAT	This study
ComMF	GACCGAACTTACCTTGAATGGA	This study
ComMFdel	AGAAAGCCTGTTTTTTATGGATGT	This study
ComMR	TGCCCCACGCTCTTGG	This study
ComMRdel	ATAACATCCATAAAAAACAGGCTTTCTCTTCTCTCTCCCTTCCTACCA	This study
LytAF	TGTATCTATCGGCAGTGTGAT	(1)
LytAR	TCAACCATCCTATACAGTGAA	(1)
LytCF	TGTGGCTCAAATTGAGGCCA	(1)
LytCR	CCAAGTTCGGCTGCAAGCA	(1)
pcpCF	CTGAGATTTCACCAACGTATGGT	This study
pcpCkanR	CACATTATCCATTAAAAATCAAACTTTATATATCCTCCAACATCAAATCCACT	This study
pcpCR	ACTGGCTAGCACAATACAGGA	This study
pcpCrpslF	CTAAACGTCCAAAAGCATAAGGAAAGTGAAGGCTAATTGTAAACTGTGAT	This study
RpsL41R	CTTTCCTTATGCTTTTGGAC	(2)
Rr03F	CTCCCACAGCTTGCTGGA	This study
Rr03Fdel	GAGTTTAGCAGATTTACTTGAGGA	This study
Rr03Frpsl	CTAAACGTCCAAAAGCATAAGGAAAGGAGTTTAGCAGATTTACTTGAGGA	This study
Rr03luc1	AACAAATTTTCATCAAGCTTCTTGATTGTGACCTCTTATT	This study
Rr03luc2	CCTTCAGCTAATTAGGATCTCTGCTAAACTCATCTAAAACTCCT	This study
Rr03R	AGATACTGTCGATAACTGGCTA	This study
Rr03Rdel	CTCCTCAAGTAAATCTGCTAAACTCCCTTATCTAACAGGGGAATACGG	This study
Rr03Rkan	CACATTATCCATTAAAAATCAAACCCTTATCTAACAGGGGAATACGG	This study
spr0810delF	TCTACAGAAGATTTGGAATAAGCA	This study
spr0810F	CACCCAGAGAATTACACTGCA	This study
spr0810kanR	CACATTATCCATTAAAAATCAAACAGTATATCACGGGGA	This study
spr0810pR424F	AATCAAACAAATTTTCATCAAGCTTCCATCCTTGCCCAA	This study
spr0810pR424R	GGATCCGGTGTAGACGTTAATCACCAAAACCAGCCTTCA	This study
spr0810R	CATCAAATGAAATGTAGCTTGCA	This study
spr0810rpslF	CTAAACGTCCAAAAGCATAAGGAAAGTCTACAGAAGATTTGGAATAAGCA	This study
spr1332lucF	AACAAATTTTCATCAAGCTTCGCAAGAAAAAATAGGTCTGTTAGATA	This study
TM1118	GCCTTGGAACTGC	This study
TM1199	CTAATACGACTCACTATAGGGAGAGGTGCTGAAAGGCATAGACAG	This study
TM1043	AGTCGAATTCGATCTACGCTTGATTATGTGTTCCC	This study
TM1044	ATGCGGATCCCAGAGCTCCTGTCAGAAGACAGGC	This study
TM1276	ACGGAATTCGTAGACTATCTTCTTTATGG	This study
TM1278	ACGGAATTCCATCCTCAGACTGAGGTGAC	This study
TM1279	ACGGAATTCAGGGAGAGGATGAACCTAGT	This study
TM1339	ACGGAATTCTTCCATCCTCAGACTGAGGTG	This study

1. **Eldholm, V., O. Johnsborg, K. Haugen, H. S. Ohnstad, and L. S. Havarstein.** 2009. Fratricide in *Streptococcus pneumoniae*: contributions and role of the cell wall hydrolases CbpD, LytA and LytC. Microbiology **155:**2223-2234.
2. **Johnsborg, O., V. Eldholm, M. L. Bjørnstad, and L. S. Håvarstein.** 2008. A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species. Mol. Microbiol. **69:**245-253.

Paper III

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Pneumococcal CbpD is a murein hydrolase that requires a dual cell envelope binding specificity to kill target cells during fratricide

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Summary

Pneumococci that are competent for natural genetic transformation express a number of proteins involved in binding, uptake, translocation and recombination of DNA. In addition, they attack and lyse non-competent sister cells present in the same environment. This phenomenon has been termed fratricide. The key effector of pneumococcal fratricide is CbpD, a secreted protein encompassing an N-terminal CHAP domain, two SH3b domains and a C-terminal choline-binding domain (CBD). CbpD is believed to degrade the cell wall of target cells, but experimental evidence supporting this hypothesis has been lacking. Here, we show that CbpD indeed has muralytic activity, and that this activity requires functional CBD and SH3b domains. To better understand the critical role played by the non-catalytic C-terminal region of CbpD, various translational fusions were constructed between the CBD and SH3b domains and green fluorescent protein (GFP). The results showed that the SH3b domains specifically recognize and bind peptidoglycan, while the CBD domain functions as a localization signal that directs CbpD to the septal region of the pneumococcal cell. Intriguingly, transmission electron microscopy analysis revealed that target cells attacked by CbpD ruptures at the septal region, in accordance with the binding specificity displayed by the CBD domain.

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Introduction

Pneumococci that are competent for natural genetic transformation kill and lyse non-competent sister cells or members of related species that are present in the same environment (for reviews, see Claverys and Håvarstein, 2007; Johnsborg and Håvarstein, 2009). This phenomenon has been termed fratricide. Proteins that are involved in the fratricide mechanism are encoded by early and late competence genes, and are consequently an integral part of the competence regulon. When pneumococci grow under conditions that are permissive for competence development they secrete a peptide pheromone (CSP) that regulates competence development by a quorum-sensing-like mechanism (Håvarstein *et al.*, 1995). By analogy with other two-component signal transduction systems, binding of extracellular CSP to its membrane-bound histidine kinase receptor, ComD, presumably results in transfer of a phosphoryl group to the cognate response regulator ComE (Håvarstein *et al.*, 1996; Pestova *et al.*, 1996). ComE then activates transcription of the early competence genes (Ween *et al.*, 1999), including the genes encoding the immunity protein ComM and the alternative σ-factor ComX (Lee and Morrison, 1999; Håvarstein *et al.*, 2006). ComM, which is predicted to be a polytopic integral membrane protein, protects competent cells against committing suicide by an unknown mechanism. ComX is required for transcriptional activation of the late competence genes, several of which are involved in pneumococcal fratricide. These are the genes encoding the key fratricide effector CbpD, two bacteriocins (CibAB) and the amidase LytA (Guiral *et al.*, 2005; Kausmally *et al.*, 2005). LytA is synthesized by non-competent cells, but its rate of expression increases during competence (Mortier-Barrière *et al.*, 1998). The only important component of the fratricide mechanism that is not part of the competence regulon is the lysozyme LytC, whose expression depends on σ^{70} (Guiral *et al.*, 2005). CbpD causes some lysis of target cells on its own, but its effect is multiplied manyfold by its activation of LytA and LytC (Eldholm *et al.*, 2009). The combined action of CbpD, LytA and LytC enables *Streptococcus pneumoniae* to lyse both non-competent pneumococci and related

species such as *Streptococcus mitis* and *Streptococcus oralis* (Johnsborg *et al*., 2008). As lysis coincides with competence, DNA released from target cells is taken up by competent attacker cells, resulting in increased efficiency of gene transfer (Steinmoen *et al*., 2002; Johnsborg *et al*., 2008).

CbpD, LytA, LytC and CibAB all contribute to fratricide when mixed cultures of attacker and target cells are grown on blood agar plates (Guiral *et al*., 2005). In liquid culture, however, CbpD together with LytA and LytC are the major players (Eldholm *et al*., 2009). CbpD is a modular enzyme consisting of an N-terminal CHAP domain followed by two bacterial SH3 (SH3b) domains and a C-terminal choline-binding domain (CBD) comprising four repeat units. The enzymatic specificity of the CHAP domain has not been determined, but it displays significant homology with corresponding domains found in a number of phage lysins and bacterial murein hydrolases. Characterized members of the CHAP family have been found to act either as endopeptidases that cleave within murein stem peptides, or as amidases that cleave the *N*-acetylmuramyl-L-Ala bond (Bateman and Rawlings, 2003; Rigden *et al*., 2003; Layec *et al*., 2009). In eukaryotes, SH3 domains confer upon their resident proteins the ability to interact with specific proline-rich sequences in various protein binding partners (Wu *et al*., 1991). Bacterial SH3 domains (Whisstock and Lesk, 1999) are poorly characterized compared with their eukaryotic counterparts, but available evidence suggests that they recognize bacterial peptidoglycan (Baba and Schneewind, 1996; Lu *et al*., 2006). Proteins harbouring CBDs bind non-covalently to choline moieties decorating the wall- and lipoteichoic acids of *S. pneumoniae* and related streptococci. CBDs consist of a variable number of tandem repeat units, each of which is 20–21 amino acids in length. Choline binding sites are located at the interface of two consecutive repeat units, where three structurally conserved aromatic residues form a cavity in which the choline quaternary ammonium moiety is primarily stabilized by cation-π interactions with the aromatic side chains. CbpD and LytA harbours four and six repeat units at their C-terminal ends, respectively, while the CBD of LytC consists of eleven N-terminally located repeat units (García *et al*., 1999; Varea *et al*., 2000).

In the present article we have shown experimentally that CbpD is a murein hydrolase, and that its ability to cleave stem peptides in the pneumococcal cell wall enhances LytC-mediated murein hydrolysis. Cellular localization studies using various green fluorescent protein (GFP) fusions demonstrated that the SH3b domains of CbpD bind specifically to peptidoglycan, while the choline-binding domain directs CbpD to the division zone. Furthermore, dual binding of the CbpD to teichoic acid and peptidoglycan via its CBD and SH3b domains, respectively, was shown to be required for efficient lysis of target cells. Finally, we used transmission electron microscopy (TEM) to show that target cells rupture at the division zone when attacked by CbpD, in accordance with the fact that CbpD predominantly accumulates in the septal region.

Results

CbpD is a murein hydrolase

CbpD is synthesized by pneumococci when they enter the competent state, and is secreted to the extracellular milieu via the Sec apparatus. Our previous studies revealed that a minor fraction of the secreted CbpD proteins is released to the growth medium, while the major fraction associates with the surface of the competent producer cells. This fraction could be eluted by 2% choline, demonstrating that cell-surface attachment of CbpD occurs mainly by non-covalent binding to teichoic acid via the choline-binding domain of the protein (Eldholm *et al*., 2009). CbpD is an essential component of the fratricide mechanism, but is not able to cause substantial lysis of target cells by itself in mixed cultures. Instead, CbpD activates the autolysins LytA and LytC, which in the presence of CbpD efficiently degrade the cell wall of non-immune cells. How CbpD activates these autolysins are not known. However, due to the presence of a CHAP domain at the N-terminus of CbpD, it has been assumed that it functions as an amidase or peptidase that catalyses the hydrolysis of peptide bonds in the cell wall cross-bridges. To verify this hypothesis we performed a zymogram analysis of total protein extracts prepared from various *S. pneumoniae* mutants to assay for murein hydrolase activity of CbpD. Proteins in the extracts were separated by SDS-PAGE on a gel containing heat-killed cells from a *S. pneumoniae* Δ*lytA*, Δ*lytC* double mutant (RH16). The zymogram analysis established for the first time that CbpD is indeed a murein hydrolase (Fig. 1A). The zymograms also showed that CbpD is present only in extracts from competent cells, whereas active LytA and LytC are present in the extracts regardless of competence induction.

The finding that CbpD functions as a murein hydrolase that by the nature of its CHAP domain must cleave amide or peptide bonds in the stem peptides of pneumococcal peptidoglycan rises the question of whether this process is responsible for triggering the activity of the amidase LytA and the lysozyme LytC. It has been demonstrated previously that exchanging the catalytic cysteine in the CHAP domain of CbpD with an alanine, or alkylating it with iodoacetamide, abolishes the enzyme's ability to activate LytA and LytC (Håvarstein *et al*., 2006; Eldholm *et al*., 2009), a result that together with the fact that CbpD

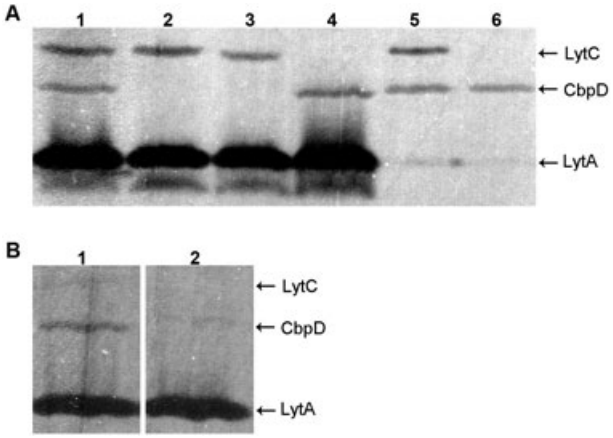

Fig. 1. Identification of pneumococcal murein hydrolases by zymography.
A. Zymogram analysis of total cell extracts prepared from various mutants of *S. pneumoniae*. Lytic activity was observed as bands of clear zones in the opaque gel. The lytic zones appear dark as the gels were photographed against a dark background. Competence-induced cells were harvested 15 min after addition of CSP (+). Uninduced cells were run in parallel without CSP (–). Lane 1: Wt + (strain RH1); lane 2: Wt – (strain RH1); lane 3: Δ*cbpD* + (strain RH17); lane 4: Δ*lytC* + (strain RH15); lane 5: Δ*lytA* + (strain RH14); lane 6: Δ*lytA*, Δ*lytC* + (strain RH16). Heat-inactivated cells of strain RH16 were incorporated in the SDS-PAGE gel. The zymograms were developed for 8 h.
B. Zymogram analysis of total cell extracts prepared from competence-induced RH1 cells harvested 15 min after addition of CSP. Heat-inactivated cells from strain RH14 (Δ*lytA*) were used in the zymogram depicted in lane 1, whereas cells from strain RH16 (Δ*lytA*, Δ*lytC*) were used in lane 2. The zymograms were developed for 1 h.

is a murein hydrolase proves that it is the amidase/endopeptidase function of CbpD that triggers the activity of the two autolysins. It follows from this that the combined action of CbpD and LytC should be more effective than CbpD alone in a zymogram assay like the one shown in Fig. 1A. To verify this, we compared the activity of CbpD in two different experimental set-ups. In one type of experiment, non-competent pneumococcal cells lacking both LytA and LytC were incorporated in the SDS-PAGE gel as a substrate for CbpD. In this case the clearing zone associated with CbpD resulted from the activity of this enzyme alone. In a parallel experiment the non-competent pneumococcal cells incorporated in the SDS-PAGE gel lacked only LytA. Consequently, the lysozyme LytC would be present in the cell wall of the cells attacked by CbpD, and should be activated by the action of this amidase/endopeptidase. The results of the zymogram analyses showed that the clearing zone caused by the combined action of CbpD and LytC appeared much faster and was more pronounced than the zone originating from the action of CbpD alone (Fig. 1B). It was not possible to perform a corresponding experiment with LytA proficient and deficient cells incorporated as substrate in the SDS-PAGE gels, because the presence of LytA caused complete lysis of the substrate making it impossible to detect the activity of CbpD and other murein hydrolases.

CbpD primarily targets the division zone

CbpD contains two SH3b domains and a CBD consisting of four repeat units. It is likely that both domain types are involved in attaching the protein to the cell envelope of target cells. CBDs specifically recognize and reversibly bind choline residues decorating teichoic and lipoteichoic acid in the cell wall of pneumococci (López *et al*., 2000; Hakenbeck *et al*., 2009). Bacterial SH3 domains are often part of proteins targeting the cell surface, suggesting that this type of domain recognizes the peptidoglycan, teichoic acid or some other part of the cell envelope. In the case of the lysostaphin homologue ALE-1, a murein hydrolase that specifically degrades *Staphylococcus aureus* cell walls, it has been reported that its C-terminal SH3b domain bind purified peptidoglycan in a manner that requires intact pentaglycine cross-bridges (Lu *et al*., 2006). To investigate whether CbpD targets a particular part of the pneumococcal cell envelope or binds evenly across the entire cell surface, we fused GFP to the C-terminal SH3b and choline-binding domains of CbpD. This was done by replacing the CHAP domain with GFP. The resulting fluorescent protein GFP–SH3b–SH3b–CBD was of similar size, and had the same number of domains as CbpD. In addition, an N-terminal His-tag was added to facilitate purification of the recombinant protein after overexpression in *Escherichia coli.* Our results show that the fusion protein mainly targets the division zone and poles of pneumococcal cells (Fig. 2A), but weak binding to the remaining part of the cell surface could be detected when longer exposure times were employed (data not shown).

The choline-binding module alone is sufficient to target CbpD to the division zone

The affinity of CbpD for the septal region could in principle be due to the SH3b domains, the choline-binding domain or both. To investigate these matters we expressed recombinant proteins engineered to contain a His-tagged GFP fused to different parts of CbpD. As cell wall teichoic acid (WTA) and lipoteichoic acid (LTA) are believed to be distributed evenly across the cell surface we considered it unlikely that the choline-binding domain is responsible for the observed binding pattern of CbpD (see Fig. 2A). We therefore initially focused on the SH3b domains. First, we deleted both SH3b domains and fused His-GFP directly to the choline-binding domain. Surprisingly, the resulting GFP–CBD product displayed the same binding pattern as the original GFP–SH3b–SH3b–CBD fusion protein (Fig. 2B). Even though each of the four domains of CbpD

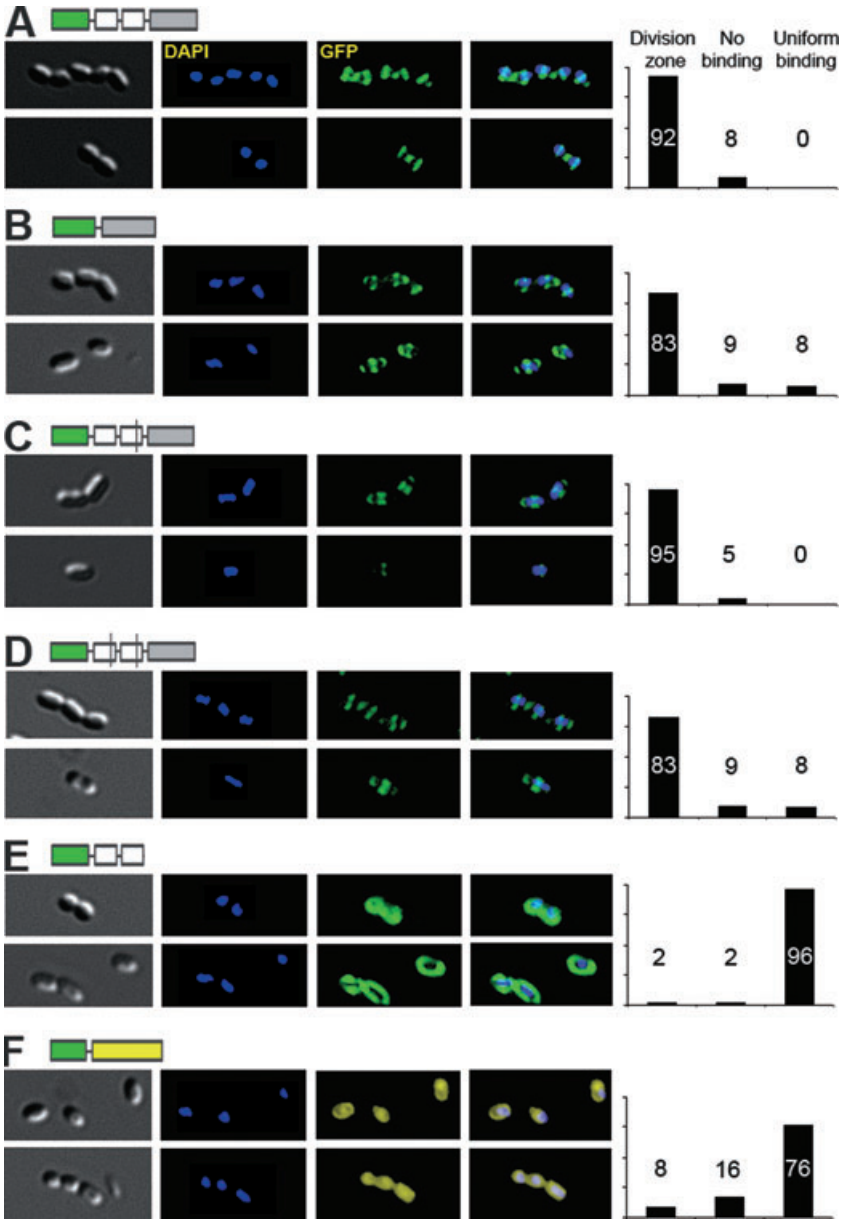

Fig. 2. Binding of various GFP fusion constructs to the surface of the *S. pneumoniae* RH14 strain. Green box represents GFP, open box represents a SH3b domain, grey box represents CBD from CbpD, yellow box represents CBD from CbpF. Vertical bars represent $W_{310}\rightarrow A$ and $W_{234}\rightarrow A$ mutations. Binding of the construct consisting of GFP fused to the CBD from CbpF is pseudocoloured yellow. Bars on the right-hand side show the distribution in per cent of the tree types of binding patterns observed with the different GFP fusion constructs.

is separated by a linker region, the possibility exists that deletion of the SH3b domains causes misfolding or affects the functionality of the downstream choline-binding domain. To inactivate the two SH3b domains without deleting them, highly conserved tryptophans at positions 234 and 310 were changed to alanines resulting in the constructs GFP–SH3b–SH3b$_{W310A}$–CBD and GFP–SH3b$_{W234A}$–SH3b$_{W310A}$–CBD (see Fig. 3B). Both of these mutated proteins targeted the septal and polar regions of the pneumococcal cell envelope, displaying a binding pattern indistinguishable from the parental construct (Fig. 2C and D). Deletion of the CBD on the other hand, leaving only the two SH3b domains fused to GFP, radically altered the cell surface localization of the protein. Without the CBD, the GFP–SH3b–SH3b protein bound evenly across the cell envelope (Fig. 2E).

To determine whether the binding pattern obtained with the choline-binding domain of CbpD (CBD/D) is typical for proteins carrying this type of domain, we fused GFP to the choline-binding domain of CbpF (CBD/F), a protein reported to be a regulator of autolysis (Molina *et al.*, 2009). Our results show that the GFP–CBD/F construct exhibited an even distribution on the cell surface, illustrating that septum localization is not a general property of choline-binding proteins (Fig. 2F). The GFP–CBD/D construct contains a linker (N-QLEGVTSSQNYQNQSGNISSYGS-NNSSTV-C) between the GFP and the CBD/D. To test whether this linker could be involved in division zone

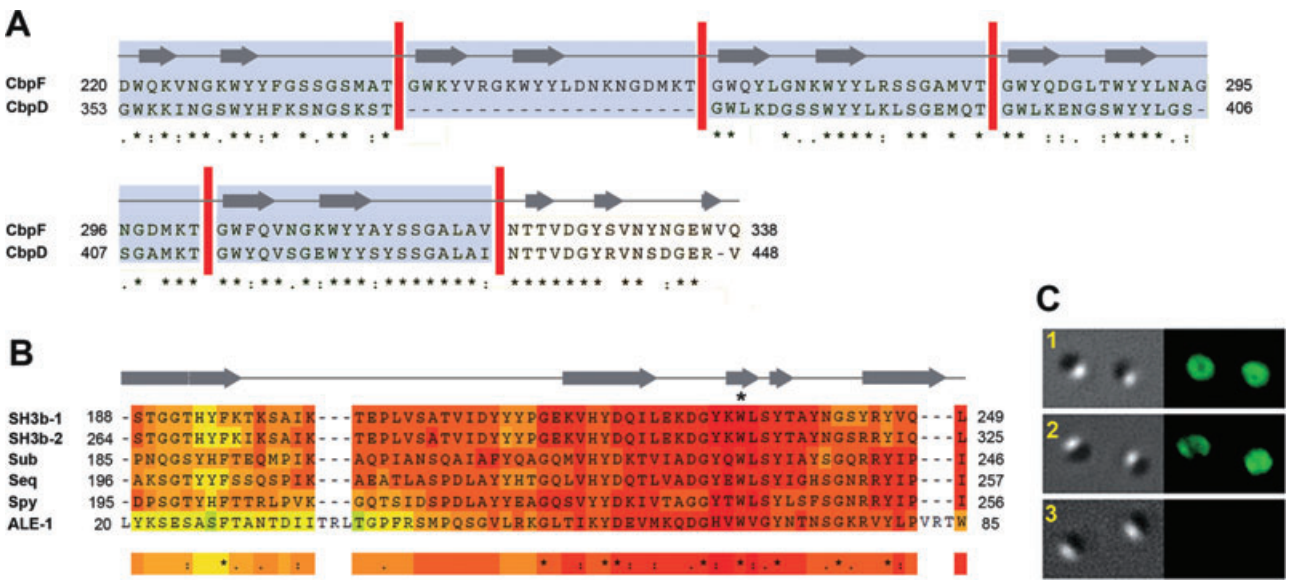

Fig. 3. Sequence analyses and binding studies of CBDs and SH3b domains.
A. Alignment of the CBDs (choline-binding repeats are shown in a shaded background and are separated by red bars) from CbpD and CbpF. The arrows above the alignment corresponds to β-strands in CbpF.
B. Alignment of the two SH3b domains from *Streptococcus pneumoniae* CbpD with SH3b domains from *Streptococcus pyogenes* CbpD, *Streptococcus uberis* CbpD, *Streptococcus suis* CbpD and *Staphylococcus aureus* ALE-1. The conserved tryptophan residue(s) chosen for site-directed mutagenesis is indicated by an asterisk. Colours indicate alignment score; from good (red) to poor (blue).
C. Binding of the GFP–SH3b–SH3b–CBD (1), GFP–SH3b–SH3b (2) and GFP–CBD (3) constructs to murein sacculi lacking teichoic acid. The sacculi were prepared from *S. pneumoniae* strain RH14.

targeting, it was inserted between GFP and CBD/F. In addition, another construct was made where the polar region (N-NYQNQSGNISSYGSNNSSTV-C) was removed from the GFP–CBD/D fusion protein. Neither of these changes affected the binding pattern of mutant proteins compared with their respective parental constructs (results not shown). In sum, these findings imply that CBD/D and CBD/F confer different binding specificities to the GFP fusion partner. Aligning the two CBDs using 3D-coffee (O'Sullivan *et al.*, 2004) that takes structural information into account [the structure of CbpF has been solved recently (Molina *et al.*, 2009)] reveals a high degree of conservation. The main difference is that CBD/D consists of four repeat units, whereas CBD/F has five (Fig. 3A).

The SH3b domains of CbpD bind peptidoglycan

SH3b domains are found in both Gram-positive and Gram-negative bacteria (Cabanes *et al.*, 2002). As mentioned above, the best-studied SH3b domains, with respect to their binding properties, are the targeting domains of the staphylococcal murein hydrolases lysostaphin and ALE-1 (Baba and Schneewind, 1996; Lu *et al.*, 2006). In these cases, the targeting domains recognize the pentaglycine interpeptide bridge characteristic of staphylococcal peptidoglycan. The pentaglycine bridge, however, is not recognized by SH3b itself, but by a separate binding motif consisting of nine amino acids situated at the N-terminal end of the SH3b domain (Lu *et al.*, 2006). A corresponding binding motif has not been found to be associated with SH3b-containing murein hydrolases from other bacterial species. The SH3b core domain is believed to bind peptidoglycan, but the specific portion of peptidoglycan recognized by SH3b has yet to be determined.

Having found that CbpD targets the cell envelope of *S. pneumoniae*, we decided to investigate whether the two SH3b domains are involved in peptidoglycan binding. We prepared cell wall sacculi from pneumococcal cells, and treated them with hydrofluoric acid to remove the teichoic acid component. The resulting sacculi, which consisted of pure peptidoglycan, bound the GFP–SH3b–SH3b–CBD fusion protein with an even distribution across the sacculi's surfaces (Fig. 3C). The GFP–SH3b–SH3b construct bound the sacculi in the same manner, whereas the GFP–CBD/D fusion protein was unable to bind. This finding demonstrates that the SH3b domains of CbpD specifically recognize the peptidoglycan component of the cell wall. It also shows that following hydrolysis of teichoic acid with hydrofluoric acid, the choline-binding domain is no longer able to mediate GFP-binding to pneumococcal sacculi.

Both the CBD and SH3b domains are required for efficient killing of non-competent cells during fratricide

After mapping the binding patterns of the fluorescent constructs described above, we wanted to investigate how

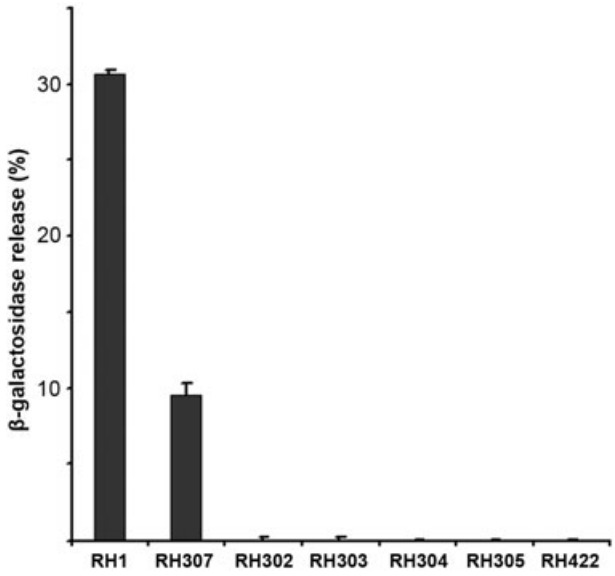

Fig. 4. Effect of specific mutations and domain deletions/alterations in CbpD on its ability to activate lysis of target cells. Lysis of target cells by attackers during co-cultivation experiments was determined by measuring the amount of intracellular β-galactosidase released to the growth medium relative to the total amounts of β-galactosidase present in the culture (target cells + medium). In all co-cultivation experiments RH3 (Δ*comE*, *hirL::lacZ*) was used as the target strain. The following strains were used as attackers: RH1 (produces wild-type CbpD), RH307 (produces CbpD chimera containing CBD from CbpF instead of its own CBD), RH302 (produces CbpD with deleted CBD), RH303 (produces CbpD lacking both SH3b domains), RH304 (produces CbpD with W310→A mutation in the C-terminal SH3b domain), RH305 (produces CbpD with W234→A and W310→A mutations in SH3b domains), RH422 (mutant with a deleted *cbpD* gene).

the corresponding alterations in the cell wall targeting domain of CbpD impacted on fratricide. During fratricide, CbpD produced by competent attacker cells triggers a chain of events that leads to lysis of non-competent target cells. All attacker strains used in this study were engineered to produce a CbpD variant with a functional CHAP domain, but carried the following mutations in other parts of the protein: deletion of CBD (RH302), deletion of both SH3b domains (RH303), W→A substitution in the C-terminal (RH304) or both (RH305) SH3b domains (see Fig. 3) or replacement of CBD/D with CBD/F from CbpF (RH307). These strains were compared with the wild-type attacker (RH1) in their ability to lyse non-competent target cells (RH3) upon competence induction (Fig. 4). RH3 cells constitutively express a β-galactosidase reporter protein that accumulates in the cytoplasm. Consequently, lysis of target cells by attackers during co-cultivation experiments can be monitored by measuring the amount of β-galactosidase released to the growth medium relative to the total amounts of β-galactosidase present in the culture (cells + medium) (Steinmoen *et al.*, 2002). Briefly, attacker strains and the target strain (RH3) were grown in C-medium to OD_{550} = 0.3. The attacker and target cultures were then mixed 1:1 concomitant with competence induction, and transferred to a 30°C water bath. After 30 min, β-galactosidase activity was measured in cell-free growth medium and total cell extracts. Based on these measurements the fraction of lysed target cells could be calculated.

Deletion of the two SH3b domains from CbpD completely abolished lysis of target cells during co-cultivation with competent attacker cells expressing this construct. The same result was obtained when the CBD/D domain was removed from the CbpD protein. Surprisingly, substituting a conserved tryptophan in only one of the two SH3b domains with an alanine (W_{310}→A) was enough to render the CbpD protein completely non-functional. Replacing the choline-binding domain of CbpD (CBD/D) with that of CbpF (CBD/F), on the other hand, resulted in a ~70% reduction in the lysis of target cells (Fig. 4). These results demonstrate that the dual binding of CbpD to teichoic acid and peptidoglycan via its choline-binding and SH3b domains, respectively, is essential for lysis of susceptible cells. The reduced lysis obtained upon substituting CBD/F for CBD/D indicates that CbpD functions more efficiently when it is directed to the septal region than when it binds to other parts of the pneumococcal cell wall.

CbpD causes ruptures in the division zone

Knowing that CbpD is a murein hydrolase, and that it localizes to the division zone, we used TEM to investigate whether CbpD mainly attacks this area of the cell wall. We took advantage of the fact that a subfraction (~20%) of cells lacking the immunity protein ComM lyse following competence induction (Håvarstein *et al.*, 2006). To avoid confusing the activity of CbpD with that of the autolysins LytA and LytC, both of which are activated by CbpD, we used a triple mutant (RH13) deficient in ComM, LytA and LytC for this experiment. The RH222 strain, containing an additional deletion of the *cbpD* gene, was used as a negative control. The strains were grown to OD_{550} = 0.3 and induced to competence. At time zero and 18 min after competence induction, 1 ml of samples of the cultures were withdrawn, mixed with 400 µl of ice-cold fixative and incubated on ice. EDTA has been shown to specifically enhance the effect of CbpD in the absence of LytA and LytC (Eldholm *et al.*, 2009). We therefore added 0.5 mM of this chelator to one parallel at the time of competence induction. Fixed and resin-embedded bacteria were thin-sectioned using ultramicrotomy, stained with uranyl acetate, and visualized using TEM. At time zero, there was no observable difference between the samples. Eighteen minutes after induction lysed cells could readily be seen in thin sections made from the RH13 cells, although the majority of the cells looked healthy (Fig. 5B). No lysed

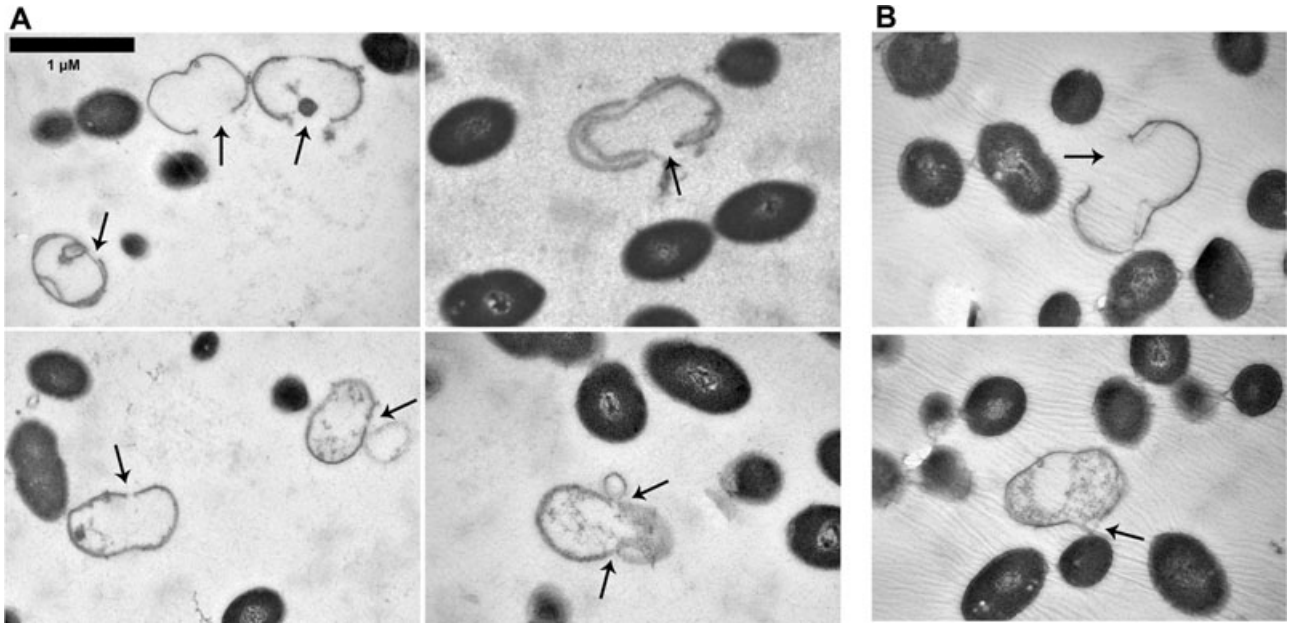

Fig. 5. Transmission electron micrographs of pneumococci 18 min after CSP induction. A Δ*lytA* Δ*lytC* double mutant (RH13) was used to specifically detect muralytic activity caused by CbpD. Lysis was most prominent in the presence of 0.5 mM EDTA (A), but lysed cells were also observed in the absence of EDTA (B). No lysed cells were found with the Δ*cbpD* Δ*lytA* Δ*lytC* triple mutant regardless of EDTA treatment (results not shown). Arrows indicate locations of cell rupture.

cells were seen in samples from CbpD-deficient RH222 cells (data not shown). In the presence of EDTA, lysis was more extensive in thin sections containing RH13 cells (Fig. 5A), whereas RH222 cells remained intact and seemingly healthy (data not shown). Intriguingly, in ruptured cells where the division zones are clearly visible, the cell wall was always split open in the septal region (Fig. 5). The fact that these ruptures only occur in the strain expressing CbpD demonstrates that this murein hydrolase is responsible for the observed cell wall damage. Together, our TEM data and results from the surface localization study outlined above strongly indicate that CbpD is a competence-specific murein hydrolase that predominantly attack the septal region of target cells.

Discussion

In the present work we have shown that the muralytic activity of CbpD depends on both its cell wall targeting domains. Furthermore, to cause efficient lysis CbpD must be directed towards the division zone of target cells, a function that is mediated by the enzyme's choline-binding domain.

Choline-decorated teichoic acid is found in *S. pneumoniae* and in a few closely related species; namely *Streptococcus pseudopneumoniae*, *S. mitis*, *S. oralis* and some strains of *Streptococcus infantis* (Kilian *et al.*, 2008). The implication of our findings is that the fractricide mechanism will be effective only when competent pneumococci encounter other strains or species containing choline-decorated teichoic acid in their cell walls. This is consistent with our previously published results demonstrating that *S. pneumoniae* can lyse members of the species *S. mitis* and *S. oralis*, but not *S. gordonii* (Johnsborg *et al.*, 2008). A consequence of the fratricide mechanism's limited target range is that it provides a means for competent cells to acquire homologous DNA. Interestingly, several streptococcal species lacking choline in their cell walls encode homologues of CbpD (Claverys *et al.*, 2007). However, in these cases the choline-binding domain has been exchanged with a completely unrelated type of domain with unknown properties. It is likely that this domain and CBD have equivalent functions, even though their binding specificities cannot be the same.

The SH3b domains of lysostaphin and its close homologue ALE-1 have both been shown to bind peptidoglycan from *S. aureus*. In the case of ALE-1, it was found that a nine-amino-acid motif adjacent to the classic SH3b domain recognizes the pentaglycine cross-bridge, explaining ALE-1's specificity for staphylococcal peptidoglycan. However, in the absence of this motif the SH3b domain of ALE-1 still retained some ability to bind purified peptidoglycan from *Lactobacillus plantarum*, a species that lacks interpeptide bridges between its stem peptides (Lu *et al.*, 2006). Interestingly, we found that our GFP–SH3b–SH3b construct binds to *L. plantarum* cells (result not shown). As the stem peptides of *L. plantarum* (L-Ala-D-iGlu-*meso*DAP-D-Ala-D-lactate) (Ferain *et al.*,

1996) are different from that of *S. pneumoniae* (L-Ala-D-iGln-L-Lys-D-Ala-D-Ala) (Garcia-Bustos *et al*., 1987), it is likely that the structure recognized by the SH3b domains of CbpD involves the conserved *N*-acetylglucosamine and/or *N*-acetylmuramic acid moieties. The sequence divergence between the SH3b domains of CbpD and the SH3b domain of ALE-1 is substantial (Fig. 3B), yet the fact that they all recognize peptidoglycan makes it tempting to speculate that this is a conserved role of bacterial SH3 domains. In addition to acting as targeting devices SH3b domains may also be crucial for substrate specificity by participating in the formation of the substrate-binding pocket together with the catalytic domain. This seems to be the case for AvPCP, a peptidoglycan cystein endopeptidase from *Anabaena variabilis* (Xu *et al*., 2009).

An important finding in the present study was that CbpD preferentially targets and ruptures the division zone. It is puzzling that CbpD should display such specificity considering that it is a 'weapon' produced by competent attacker cells to lyse and kill neighbouring target cells. Presumably, the fratricide mechanism would have been more efficient if CbpD was able to bind and hydrolyse any part of the cell wall instead of being directed towards a specific subsection of this megamolecule. One possible explanation is that the division zone represents the weakest part of the wall, and that CbpD as a result has evolved to target this region. In agreement with this, even distribution of the protein across the cell wall (by replacement of CBD/D with CBD/F) results in considerable reduction in lysis of target cells (Fig. 4). We speculate, however, that the restricted specificity of CbpD is an important part of the immunity mechanism that protects pneumococci against committing suicide during competence. It is likely that the immunity protein ComM, which in contrast to CbpD is encoded by an early competence gene, introduces a change in the peptidoglycan or teichoic acids of competent cells that blocks the activity of CbpD. Considering that ComM is expressed only a few minutes before CbpD, there might not be enough time to protect the whole cell wall. In addition, modification of the entire cell wall could be detrimental to the competent cell. If ComM only guards the cell division zone, however, protection can be achieved more quickly, and only a small part of the cell envelope will be transiently affected.

Very recently, Schlag *et al*. (2010) reported that WTA influences the cell surface localization of the staphylococcal Atl protein. This bifunctional autolysin is targeted to the cell-division zone via repeat domains that specifically recognizes and binds peptidoglycan. Interestingly, Schlag and co-workers' results suggest that the presence of WTA precludes or impairs binding of Atl to the peptidoglycan portion of the cell wall, and that Atl is able to bind to the septal region because this region does not contain WTA. Our results showed that the GFP–SH3b–SH3b–CBD construct targeted the septal region of sacculi even when they had been boiled in 4% SDS to remove LTA (data not shown), strongly indicating that WTA must be present in the septal region of pneumococcal cells. It follows from this that different mechanisms must be behind the observed septal positioning of CbpD and Atl. However, both mechanisms might depend on an uneven distribution of WTA and/or LTA across the bacterial cell surface.

Our results clearly show that it is the CBD and not the SH3b domains that targets CbpD to the septal region. To our knowledge, this is the first time that it has been demonstrated that a CBD alone is sufficient to target an entire/full-length protein to a specific location on the bacterial cell wall. Curiously, the targeting specificity displayed by CBD from CbpD is not common to all choline-binding proteins as the construct consisting of GFP fused to CBD from CbpF did not target the division zone, but displayed an approximately even distribution across the cell surface. This difference in location between the GFP–CBD/D and GFP–CBD/F constructs must be due to either subtle amino acid sequence differences or the number of choline-binding repeats. Interestingly, CbpD shows the lowest number of repeats among the members of the choline-binding family. Choline binding sites are formed at the interface of two consecutive repeats, where three aromatic residues non-covalently bind the choline moieties of teichoic acids. Considering the previously solved three-dimensional structure of CbpF (Molina *et al*., 2009) and the high sequence homology between CBDs of both proteins, it can be estimated that CBD/D could bind up to four choline moieties versus five for the CBD/F (Fig. S1), resulting in a lower choline affinity for CbpD than for CbpF. This dissimilarity together with subtle amino acid variations and/or modifications in the teichoic acid composition at the division zone could increase the choline affinity of CbpD at these specific loci. Alternatively, the higher choline-binding capability observed for CbpF could result in a less stringent binding requirement, allowing the protein to locate all across the cell surface.

Moreover, our experiments prove that the simultaneous presence of three separate cell wall targeting domains (the two SH3b domains plus the CBD) is required for CbpD activity. This contrasts with most pneumococcal choline-binding proteins, which only need a single targeting domain (a CBD) linked to the catalytic module to be fully active. As illustrated by the RH304 mutant strain, even a single-amino-acid substitution at one of the SH3b targeting domains completely abolishes the hydrolytic activity of CbpD. The unusual requirements observed for CbpD indicate that extremely precise recognition of the peptidoglycan is essential in order to obtain efficient and safe muralytic activity during fratricide.

In a previous study we found that lysis of target cells during fratricide only takes place in the presence of CbpD. However, fully efficient lysis required the presence of LytC

and LytA in addition to CbpD, strongly indicating that CbpD stimulates the muralytic activity of the two autolysins (Eldholm *et al*., 2009). In the present study we have obtained direct experimental evidence showing that CbpD and LytC together degrades the cell walls of pneumococci much more efficiently than CbpD alone (Fig. 1B). Very recently, Pérez-Dorado *et al*. (2010) proposed an explanation of this interesting phenomenon that is in full accordance with our findings. By determining for the first time the three-dimensional structure of LytC they were able to show that the modular configuration of LytC imposes limitations on its muralytic activity, and that prior cleavage of peptide stems by CbpD in theory could facilitate hydrolysis of non-cross-linked glycan strands by LytC. This means that during fratricide competent attacker cells lyse non-competent target cells by activating their autolysins. This amplification effect greatly enhances the impact of CbpD, and presumably allows the fratricide mechanism to function efficiently at a lower cost to the attacker cells.

In summary, we have reported here the function and localization of the multimodular murein hydrolase CbpD, which performs a highly regulated hydrolytic reaction on the cell wall peptidoglycan. Our experiments explain the co-ordinated role of the different modules of CbpD with respect to its lytic activity during pneumococcal fratricide. The particular composition of its choline-binding domain allows CbpD to target the division zone, while the presence of two in tandem SH3b domains attaches the protein to the peptidoglycan part of the cell wall. Most likely, the SH3b domains bind to the glycan strands and participate in the formation of the substrate-binding pocket together with the catalytic domain. Together our findings strongly indicate that the specific catalytic activity of CbpD set off a domino-like mechanism that activates LytC followed by LytA, resulting in rapid cell wall degradation and lysis of target cells.

Experimental procedures

Construction of S. pneumoniae *mutants*

Bacterial strains and plasmids used in this work are listed in Table 1, whereas the sequences of all primers used are given in Table S1. All *S. pneumoniae* transformations and experi-

Table 1. Strains and plasmids.

Strains/plasmids	Genotype/relevant feature	Reference/source
Strains		
RH1	*comA::ery, ebg::spc*, Ery^R, Spc^R	Johnsborg *et al*. (2008)
RH3	*comA::ery, ebg::spc, ΔcomE::kan, hirL*::pEVP3 (Cm^R), Ery^R, Spc^R, Kan^R, Cm^R	Johnsborg *et al*. (2008)
RH11	*comA::ery, ebg::spc, ΔcomE::kan, ΔlytA, ΔlytC::tc, hirL*::pEVP3 (Cm^R), Ery^R, Spc^R, Kan^R, Sm^R, Tc^R, Cm^R	Eldholm *et al*. (2009)
RH13	*comA::ery, ebg::spc, ΔcomM::Janus, ΔlytA, ΔlytC::tc, hirL*::pEVP3 (Cm^R), Ery^R, Spc^R, Kan^R, Tc^R, Cm^R	Eldholm *et al*. (2009)
RH14	*comA::ery, ebg::spc, ΔlytA::kan*, Ery^R, Spc^R, Kan^R	Eldholm *et al*. (2009)
RH15	*comA::ery, ebg::spc, ΔlytC::tc*, Ery^R, Spc^R, Tc^R	Eldholm *et al*. (2009)
RH16	*comA::ery, ebg::spc, ΔlytA::kan, ΔlytC::tc*, Ery^R, Spc^R, Kan^R, Tc^R	Eldholm *et al*. (2009)
RH17	*comA::ery, ebg::spc, ΔcbpD::kan*, Ery^R, Spc^R, Kan^R	Johnsborg *et al*. (2008)
RH222	*comA::ery, ebg::spc, ΔcomM::Janus, ΔlytA, ΔlytC::tc, ΔcbpD, hirL*::pEVP3 (Cm^R), Ery^R, Spc^R, Kan^R, Spc^R, Kan^R, Cm^R	Eldholm *et al*. (2009)
RH422	*comA::ery, ebg::spc, ΔcbpD::Janus*, Ery^R, Spc^R, Kan^R	Eldholm *et al*. (2009)
RH302	RH422 but *cbpDΔcbd*, Ery^R, Spc^R, Sm^R	This study
RH303	RH422 but *cbpDΔsh3-1 and Δsh3-2*, Ery^R, Spc^R, Sm^R	This study
RH304	RH422 but *cbpDW310→A*, Ery^R, Spc^R, Sm^R	This study
RH305	RH422 but *cbpDW234→A, W310→A*, Ery^R, Spc^R, Sm^R	This study
RH307	RH422 but *cbpD w/cbpF-CBD*, Ery^R, Spc^R, Sm^R	This study
E. coli BL21(DE3)pLysS	F^-, *ompT*, *hsd*S_B (r_B^- m_B^-), *gal, dcm* (DE3), pLysS (Cm^R), Cm^R	Invitrogen
L. plantarum WCFS1		Kleerebezem *et al*. (2003)
Plasmids		
pRSET/EmGFP	*gfp*, Amp^R	Invitrogen
pVEG1	pRSET derivate carrying a *gfp–cbpD* fusion excluding the CHAP domain, Amp^R	This study
pVEG2	pRSET derivate carrying *gfp* fused to the two SH3 domains of *cbpD*, Amp^R	This study
pVEG3	pRSET derivate carrying *gfp* fused to the CBD of *cbpD* (including the SH3–CBD linker), Amp^R	This study
pVEGx3	pRSET derivate carrying *gfp* fused to the CBD of *cbpD*, Amp^R	This study
pVEGx4	pRSET derivate carrying a *gfp–cbpDW310→A* fusion excluding the CHAP domain, Amp^R	This study
pVEGx5	pRSET derivate carrying a *gfp–cbpDW234→A*, *W310→A* fusion excluding the CHAP domain, Amp^R	This study
pVEGF	pRSET derivate carrying *gfp* fused to the CBD of *cbpF*, Amp^R	This study
pVEGxF	pRSET derivate carrying *gfp* fused to the CBD of *cbpF* via a polar linker derived from *cbpD*, Amp^R	This study

ments were carried out in C-medium (Lacks and Hotchkiss, 1960). Bacterial cultures were grown at 37°C and induced to competence using 250 ng ml^{-1} synthetic CSP-1 (Genosphere Biotechnologies). The cells were subsequently mixed with the appropriate DNA and incubated for 2 h before plating on Todd Hewitt agar (Difco). Selection of mutants was carried out using the appropriate antibiotics at the following concentrations: streptomycin (Sm) 200 μg ml^{-1}, kanamycin (Kan) 400 μg ml^{-1}, spectinomycin (Spc) 200 μg ml^{-1}, chloramphenicol (Cm) 3.5 μg ml^{-1}, tetracycline (Tc) 0.2 μg ml^{-1}, erythromycin (Ery) 2.5 μg ml^{-1}.

In order to construct a *S. pneumoniae* strain lacking the CBD of *cbpD*, the primers CbpD-1098 and SH3R-DStail were used to amplify a DNA fragment encompassing the *cbpD* CHAP-encoding domain together with ~1000 bp of the *cbpD* upstream region. Next, a DNA fragment comprising ~1000 bp of the *cbpD* downstream region was amplified using the primers CbpD-DSF and CbpDR. Finally, the two PCR products were fused in a PCR using primers CbpD-1098 and CbpDR. The resulting DNA product was transformed into RH422, resulting in RH302.

To construct a strain expressing a *cbpD* lacking the two SH3b domains, the primers CbpD-1098 and CbpDCHAPR were used to amplify a DNA fragment encompassing the *cbpD* CHAP-encoding domain together with ~1000 bp of the *cbpD* upstream region. Next, a DNA fragment comprising the CBD-encoding domain together with ~1000 bp of the *cbpD* downstream region was amplified using the primers CBD-CHAPoverlapF and CbpDR. Finally, the two PCR products were fused in a PCR using primers CbpD-1098 and CbpDR. The resulting DNA product was transformed into RH422, resulting in RH303.

cbpD variants carrying one or two point mutated SH3-encoding domains were constructed as follows: the primers CbpD-1098 and CbpDCHAPR were used to amplify a DNA fragment encompassing the *cbpD* CHAP-encoding domain together with ~1000 bp of the *cbpD* upstream region. Next, the remaining part of the *cbpD* was amplified with primers SH3F-CHAPtail and CbpD-StopR, using 50 ng of pVEGx4 (contains a fragment of *cbpD* encoding the SH3b domain mutation $W{\rightarrow}A_{310}$) or pVEGx5 (contains a fragment of *cbpD* encoding the SH3b domain mutations $W{\rightarrow}A_{234} + W{\rightarrow}A_{310}$) as template respectively. The two resulting PCR products were separately fused to the CHAP encoding DNA fragment described above using the primers CbpD-1098 and CbpD-StopR. Finally, a ~1000 bp DNA fragment comprising the region downstream of *cbpD* was amplified using the primers CbpDFstop and CbpDR, and fused to the two DNA products (carrying full-length *cbpD* with point mutations in one or both SH3b domains) using the primers CbpD-1098 and CbpDR. The two PCR products, carrying a *cbpD* gene with one ($W{\rightarrow}A_{310}$) or two ($W{\rightarrow}A_{234} + W{\rightarrow}A_{310}$) mutated SH3b-encoding domains were transformed into RH422, resulting in RH304 and RH305 respectively.

To construct RH307, the CBD-encoding part of *cbpD* was switched with that from *cbpF*. First, the primers CbpD-1098 and CbpDlinkerR were used to amplify a DNA fragment comprising the parts of *cbpD* encoding the two SH3b domains, the CHAP domain, and ~1000 bp of the *cbpD* upstream region. Next, the CBD-encoding domain of *cbpF* was amplified with the primers CbpFCBDF and CbpFstopR, and subsequently fused to the fragment described above using the primers CbpD-1098 and CbpFstopR. Finally, a ~1000 bp fragment comprising the *cbpD* downstream region was amplified with the primers CbpDDSCbpFlink and CbpDR, and fused to the CHAP–SH3b–CBD-encoding fragment using the primers CbpD-1098 and CbpDR. The resulting fragments was transformed into RH422 resulting in RH307.

Construction of plasmids and expression of recombinant proteins

The gene encoding EmGFP was amplified from pRSET/EmGFP (Invitrogen) with the primers GfpFNheI and GfpR. *cbpD* excluding the CHAP-encoding domain was amplified with the primers SH3F and CbpDRXhoI. The two fragments were subsequently fused with the primers GfpFNheI and CbpDRXhoI. The resulting PCR construct was digested with NheI and XhoI and ligated into the plasmid pRSET pre-cleaved with the same enzymes. The resulting plasmid pVEG1 encodes a protein carrying a His-tagged GFP–CbpD fusion in which the GFP replaces the CHAP domain.

Various domains were fused to the gene encoding GFP following the method described above. The portion of *cbpD* encoding the two SH3b domains was amplified with the primers SH3F and SH3RXhoI. This fragment was fused to *gfp* as described above and ligated into pRSET resulting in pVEG2. The *cbpD* CBD-encoding domain including the CBD–SH3b linker domain was amplified with the primers CBDF and CbpDRXhoI, fused to *gfp* and ligated into pRSET yielding pVEG3. Using 50 ng of pVEG3 as template, the CBD domain without the SH3b linker was amplified with primers LinkDelF and CbpDRXhoI whereas *gfp* followed by the nine amino acids (QLEGVTSSQ) was amplified with the primers GfpFNheI and LinkDelR. The two PCR fragments were fused together with primers GfpFNheI and CbpDRXhoI and ligated into pRSET as described above, yielding pVEGx3.

The CBD-encoding domain of *cbpF* was amplified with the primers CbpFCBDGF and CbpFCBDRXhoI, fused to *gfp* and ligated into pRSET resulting in pVEGF.

gfp and the polar SH3b linker in pVEG3 were amplified with primers GfpFNheI and CbpDlinkerR. The CBD-encoding part of *cbpF* was amplified with the primers CbpFCBDF and CbpFCBDRXhoI. The fragments were fused using the primer pair GfpFNheI + CbpFCBDRXhoI, and ligated into pRSET resulting in pVEGxF.

To introduce a point mutation ($W_{310}{\rightarrow}A$) in one SH3b domain (SH3b-2), 20 ng of pVEG1 was used as template in a QuickChange PCR reaction with primers SH3W310A-F and SH3W310A-R using the non-strand-displacing Pfu Turbo polymerase (Stratagene). The procedure was carried out as described by the manufacturer. Following thermal cycling, the amplification products were treated with dpnI for 1 h. Five microlitres of the reaction was used to transform One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen). PCR screening and sequencing of the resulting clones identified a clone carrying the desired plasmid, designated pVEGx4. Following the same procedure, 20 ng of pVEGx4 was used as template in a QuickChange PCR reaction with primers SH3W234AR and SH3W234AF, resulting in the plasmid, pVEGx5, which contains a W→A substitution in the conserved tryptophan of both SH3 domains.

All plasmids were transformed into BL21(DE3)pLysS *E. coli* cells (Invitrogen) following the standard protocol. Expression of proteins was carried out at 30°C for 2 h with shaking. The cells were then lysed by repeated freezing and thawing in liquid nitrogen and a 42°C water bath. Recombinant proteins were purified with the aid of Protino Ni-TED columns (Macherey-Nagel).

Zymogram analysis of murein hydrolase activity

Zymograms were carried out as described by Leclerc and Asselin (1989) with some minor modifications. In brief, 50 μg of total protein (estimated by Coomassie blue staining) from different *S. pneumoniae* strains was separated by SDS-PAGE as described by Laemmli (1970) using a 4% stacking gel and a 10% resolving gel. Prior to polymerization, the resolving gel solution was mixed with heat-treated (95°C for 10 min) cells from a 300 ml culture $OD_{550} = 0.2$ of strain RH16 (ΔlytA, ΔLytC) (Eldholm *et al*., 2009). Following electrophoresis at 2.5 V cm^{-2}, the gels were washed in de-ionized water for 2 × 30 min before adding the refolding buffer (50 mM NaCl, 20 mM $MgCl_2$, 0.5% Triton X-100 and 20 mM Tris-HCl, pH 7.4). The gels were incubated in refolding buffer until the lytic zones could be observed in the turbid gel.

Fluorescence microscopy

Bacterial cells were grown to $OD_{550} = 0.4$, placed on ice and paraformaldehyde and glutaraldehyde added to a final concentration of 2.5% and 0.006% respectively. After 1 h, the cells were washed in PBS (pH 7.4) and fixed on slides (Thermo Scientific)

Pneumococcal cell wall sacculi were prepared as described by Reusch (1982) with some modifications. Briefly, 50 ml of bacteria at $OD_{550} = 0.35$ were pelleted and dissolved in 3.3 ml of extraction fluid containing 1% SDS + 0.5% β-mercaptoethanol (SDS-ME). The cells were incubated at 85°C for 15 min and dH_2O added to a final volume of 5 ml. The resulting gel was sonicated for 15 s and harvested at 17 680 *g* for 30 min. The pellet was dissolved in 3.3 ml of SDS-ME and extracted with hot SDS-ME five times. The extracted cells were washed five times with ddH_2O, once with 2 M NaCl, once with ddH_2O, once with 2 M NaCl and four times with ddH_2O. The cells were treated with trypsin (1.5 μg ml^{-1} in PBS) overnight and pelleted at 16 000 *g* and dissolved in 1 ml of 4% SDS. The cells were then incubated at 85°C for 15 min, washed thrice with dH_2O and dissolved in 100 μl of PBS. In order to remove covalently attached teichoic acids, 60 μl of the sacculi were pelleted and dissolved in 250 μl of 48% hydrofluoric acid. After 48 h of incubation at 4°C, the sacculi were harvested by centrifugation at 16 000 *g* for 20 min, washed thrice with dH_2O and dissolved in 50 μl of PBS.

Slides with sacculi or fixed cells were incubated in room temperature for 8 min with ~15 μg ml^{-1} fluorescent fusion protein dissolved in PBS containing 0.05% Tween-20. When appropriate, 5 μg ml^{-1} DAPI was added to the protein solution. Following incubation, the slides were washed thrice for 1 min with in PBS containing 0.05% Tween-20.

Transmission electron microscopy

Pneumococci were grown at 37°C to $OD_{550} = 0.3$, induced to competence with CSP (250 ng ml^{-1}) and incubated at 30°C. In a parallel experiment, EDTA was added to a final concentration of 0.5 mM at the time of competence induction. At 0 and 18 min post induction, 1 ml of the cultures were withdrawn, added to 400 μl of ice-cold fix (7.5% paraformaldehyde + 0.018% glutaraldehyde) and incubated on ice. After 1 h of incubation, the cells were washed with PBS buffer, embedded in 0.5% low melting agarose, and further fixed with 2.5% paraformaldehyde and 0.006% glutaraldehyde overnight at 4°C. The cells were subsequently washed 3× with cacodylate buffer (0.1 M, pH 6.8), and post-fixed with 1% osmium in cacodylatbuffer for 1 h at 4°C. Subsequently, the cells were washed several times with cacodylate buffer and dehydrated with a graded series of ethanol (50%, 70%, 90%, 96% and 4× 100%), using 10 min incubations for each step. Finally, the cells were embedded in LRWhite resin. Ultrathin sections were cut with a diamond knife mounted on an ultramicrotome (LEICA EM UC 6) and sections were picked up with formvar- and carbon-coated slot copper grids. Counterstaining of the sections was performed with 4% aqueous uranyl acetate and 1% $KMNO_4$ for 10 min. The sections were examined with a FEI MORGAGNI 268 transmission electron microscope at an accelerating voltage of 80 kV, and photographs were recorded on VELETA camera.

Sequence alignments

Alignments were performed using 3D-coffee (O'Sullivan *et al*., 2004) as implemented in the phylogeny.fr package (Dereeper *et al*., 2008). The alignment of the CBD of *cbpD* and *cbpF* was curated by hand after aligning with 3D-coffe.

β-Galactosidase assay

β-Galactosidase assays to measure cell lysis was carried out as described previously (Steinmoen *et al*., 2002).

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References

Baba, T., and Schneewind, O. (1996) Target cell specificity of a bacteriocin molecule: a C-terminal signal directs lysostaphin to the cell wall of *Staphylococcus aureus*. *EMBO J* **15:** 4789–4797.

Bateman, A., and Rawlings, N.D. (2003) The CHAP domain: a large family of amidases including GSP amidase and peptidoglycan hydrolases. *Trends Biochem Sci* **28:** 234–237.

Cabanes, D., Dehoux, P., Dussurget, O., Frangeul, L., and Cossart, P. (2002) Surface proteins and the pathogenic potential of *Listeria monocytogenes*. *Trends Microbiol* **10:** 238–245.

Claverys, J.-P., and Håvarstein, L.S. (2007) Cannibalism and fratricide: mechanisms and raisons d'être. *Nat Rev Microbiol* **5:** 219–229.

Claverys, J.-P., Martin, B., and Håvarstein, L.S. (2007) Competence-induced fratricide in streptococci. *Mol Microbiol* **64:** 1423–1433.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., *et al*. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* **36:** W465–W469.

Eldholm, V., Johnsborg, O., Haugen, K., Ohnstad, H.S., and Håvarstein, L.S. (2009) Fratricide in *Streptococcus pneumoniae*: contributions and role of the cell wall hydrolases CbpD, LytA and LytC. *Microbiology* **155:** 2223–2234.

Ferain, T., Hobbs, J.N., Richardson, J., Bernard, N., Garmyn, D., Hols, P., *et al*. (1996) Knockout of the two *ldh* genes has a major impact on peptidoglycan precursor synthesis in *Lactobacillus plantarum*. *J Bacteriol* **178:** 5431–5437.

García, P., González, M.P., García, E., García, J.L., and López, R. (1999) The molecular characterization of the first autolytic lysozyme of *Streptococcus pneumoniae* reveals evolutionary mobile domains. *Mol Microbiol* **33:** 128–138.

Garcia-Bustos, J.F., Chait, B.T., and Tomasz, A. (1987) Structure of the peptide network of pneumococcal peptidoglycan. *J Biol Chem* **262:** 15400–15405.

Guiral, S., Mitchell, T.J., Martin, B., and Claverys, J.-P. (2005) Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements. *Proc Natl Acad Sci USA* **102:** 8710–8715.

Hakenbeck, R., Madhour, A., Denapaite, D., and Brückner, R. (2009) Versatility of choline metabolism and choline-binding proteins in *Streptococcus pneumoniae* and commensal streptococci. *FEMS Microbiol Rev* **33:** 572–586.

Håvarstein, L.S., Coomaraswamy, G., and Morrison, D.A. (1995) An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proc Natl Acad Sci USA* **92:** 11140–11144.

Håvarstein, L.S., Gaustad, P., Nes, I.F., and Morrison, D.A. (1996) Identification of the streptococcal competence-pheromone receptor. *Mol Microbiol* **21:** 863–869.

Håvarstein, L.S., Martin, B., Johnsborg, O., Granadel, C., and Claverys, J.-P. (2006) New insights into the pneumococcal fratricide: relationship to clumping and identification of a novel immunity factor. *Mol Microbiol* **59:** 1297–1307.

Johnsborg, O., and Håvarstein, L.S. (2009) Regulation of natural genetic transformation and acquisition of transforming DNA in *Streptococcus pneumoniae*. *FEMS Microbiol Rev* **33:** 627–642.

Johnsborg, O., Eldholm, V., Bjørnstad, M.L., and Håvarstein, L.S. (2008) A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species. *Mol Microbiol* **69:** 245–253.

Kausmally, L., Johnsborg, O., Lunde, M., Knutsen, E., and Håvarstein, L.S. (2005) Choline-binding protein D (CbpD) in *Streptococcus pneumoniae* is essential for competence-induced cell lysis. *J Bacteriol* **187:** 4338–4345.

Kilian, M., Poulsen, K., Blomqvist, T., Håvarstein, L.S., Bek-Thomsen, M., Tettelin, H., and Sørensen, U.B.S. (2008) Evolution of *Streptococcus pneumoniae* and its close commensal relatives. *PLoS ONE* **3:** e2683.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., *et al*. (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc Natl Acad Sci USA* **100:** 1990–1995.

Lacks, S., and Hotchkiss, R.D. (1960) A study of the genetic material determining an enzyme in pneumococcus. *Biochim Biophys Acta* **39:** 508–518.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Layec, S., Gérard, J., Legué, V., Chapot-Chartier, M.-P., Courtin, P., Borges, F., *et al*. (2009) The CHAP domain of Cse functions as an endopeptidase that acts at mature septa to promote *Streptococcus thermophilus* cell separation. *Mol Microbiol* **71:** 1205–1217.

Leclerc, D., and Asselin, A. (1989) Detection of bacterial cell wall hydrolases after denaturing polyacrylamide gel electrophoresis. *Can J Microbiol* **35:** 749–753.

Lee, M.S., and Morrison, D.A. (1999) Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. *J Bacteriol* **181:** 5004–5016.

López, R., González, M.P., García, E., García, J.L., and García, P. (2000) Biological roles of two murein hydrolases of *Streptococcus pneumoniae* representing examples of module shuffling. *Res Microbiol* **151:** 437–443.

Lu, J.Z., Fujiwara, T., Komatsuzawa, H., Sugai, M., and Sakon, J. (2006) Cell wall-targeting domain of glycylglycine endopeptidase distinguishes among peptidoglycan cross-bridges. *J Biol Chem* **281:** 549–558.

Molina, R., González, A., Stelter, M., Pérez-Dorado, I., Kahn, R., Morales, M., *et al*. (2009) Crystal structure of CbpF, a bifunctional choline-binding protein and autolysis regulator from *Streptococcus pneumoniae*. *EMBO Rep* **10:** 246–251.

Mortier-Barrière, I., de Saizieu, A., Claverys, J.-P., and Martin, B. (1998) Competence-specific induction of *recA* is required for full recombination proficiency during transformation in *Streptococcus pneumoniae*. *Mol Microbiol* **27:** 159–170.

O'Sullivan, O., Suhre, K., Abergel, C., Higgins, D.G., and Notredame, C. (2004) 3DCoffee: combining protein sequences and structures within multiple sequence alignments. *J Mol Biol* **340:** 385–395.

Pérez-Dorado, I., González, A., Morales, M., Sanles, R., Striker, W., Vollmer, W., *et al*. (2010) Insights into pneumococcal fratricide from crystal structure of the modular killing factor LytC. *Nat Struct Mol Biol* (in press).

Pestova, E.V., Håvarstein, L.S., and Morrison, D.A. (1996) Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. *Mol Microbiol* **21:** 853–862.

Reusch, V.M., Jr (1982) Isolation and analysis of sacculi from *Streptococcus sanguis*. *J Bacteriol* **151:** 1543–1552.

Rigden, D.J., Jedrzejas, M.J., and Galperin, M.Y. (2003) Amidase domains from bacterial and phage autolysins define a family of γ-D,L-glutamate-specific amidohydrolases. *Trends Biochem Sci* **28:** 230–234.

Schlag, M., Biswas, R., Krismer, B., Kohler, T., Zoll, S., Yu, W., *et al*. (2010) Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl. *Mol Microbiol* **75:** 864–873.

Steinmoen, H., Knutsen, E., and Håvarstein, L.S. (2002) Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population. *Proc Natl Acad Sci USA* **99:** 7681–7686.

Varea, J., Saiz, J.L., López-Zumel, C., Monterroso, B., Medrano, F.J., Arrondo, J.L.R., *et al*. (2000) Do sequence repeats play an equivalent role in the choline-binding module of pneumococcal LytA amidase? *J Biol Chem* **275:** 26842–26855.

Ween, O., Gaustad, P., and Håvarstein, L.S. (1999) Identification of DNA binding sites for ComE, a key regulator of natural competence in *Streptococcus pneumoniae*. *Mol Microbiol* **33:** 817–827.

Whisstock, J.C., and Lesk, A.M. (1999) SH3 domains in prokaryotes. *Trends Biochem Sci* **24:** 132–133.

Wu, H., Reynolds, A.B., Kanner, S.B., Vines, R.R., and Parsons, J.T. (1991) Identification and characterization of a novel cytoskeleton-associated pp60src substrate. *Mol Cell Biol* **11:** 5113–5124.

Xu, Q., Sudek, S., McMullan, D., Miller, M.D., Geierstanger, B., Jones, D.H., *et al*. (2009) Structural basis of murein peptide specificity of a γ-D-Glutamyl-L-Diamino acid endopeptidase. *Structure* **17:** 303–313.

Supporting information

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SUPPLEMENTARY MATERIAL

for

Pneumococcal CbpD is a murein hydrolase that requires a dual cell-envelope binding-specificity to kill target cells during fratricide.

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Methods

Molecular modeling of CBD/D from *S. pneumoniae*

Model of the CBD/D of *S. pneumoniae* was built on the basis of the crystal structure of the Choline-binding module of CbpF of *S. pneumoniae* (PDB-code: 2V05). Amino acid changes along the entire sequence were performed using the O program (Jones *et al.*, 1991), running a Silicon Graphics workstation. Side-chain rotamers were chosen from a database of more common conformers. The overall conformation of the model was energy minimized using the minimiser algorithm implemented in the CNS package (Brunger *et al.*, 1998). The stereo chemical quality of the model was checked with the program PROCHECK (Laskowski *et al.*, 1993).

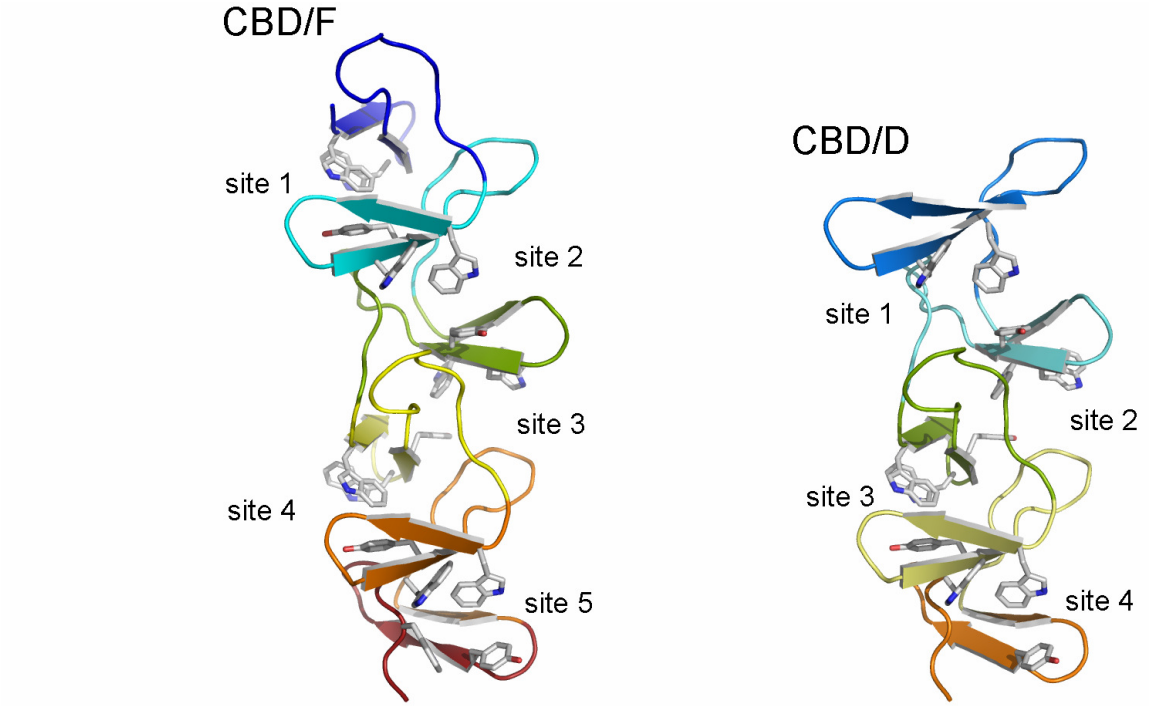

Figure S1. Structural comparison between CBD/F and CBD/D. Choline-binding repeats are colored differently and the aromatic residues involved in choline stabilization are highlighted in sticks. The choline-binding sites observed in both proteins are labeled. While CbpF presents five binding sites, CbpD should present only four.

Table S1

Oligonucleotides used in this study

CBD-CHAPoverlapF	CGATGACAGGATTTATTCATTTTAAAGATTTGCAGCTAGAGGGAGTGACTTCTTCACA	This study
CBDF	CGGCATGGACGAGCTGTACAAGCAGCTAGAGGGAGTGACTT	This study
CbpD-DSF	AAAATTGGAGTAGGAGAAATTTCCT	This study
CbpD-1098	GTTGATTATCTTAGCAGCTCGT	This study
CbpDCHAPR	CAAATCTTTAAAATGAATAAATCCTGTCA	This study
CbpDDSCbpFlink	ACTATAATGGCGAATGGGTTCAATAAAAAATTGGAGTAGGAGAAATTTCCT	This study
CbpDFstop	AGTAAACAGTGATGGAGAACGAGTATAG	This study
CbpDlinkerR	CCATTTTCCGTTTACTTTTTGCCAATCGACAGTTGAACTATTATTGGATCCA	This study
CbpDR	CCAAGGGTTTGCTCGCAT	(Eldholm *et al.*, 2009)
CbpDRXhoI	AGCAATCTCGAGCTATACTCGTTCTCCATCACTGT	This study
CbpD-StopR	CTATACTCGTTCTCCATCACTGT	This study
CbpFCBDGF	CGGCATGGACGAGCTGTACAAGGATTGGCAAAAAGTAAACGGAAAATGGT	This study
CbpFCBDF	GATTGGCAAAAAGTAAACGGAAAAT	This study
CbpFCBDRXhoI	AGCAATCTCGAGTTATTGAACCCATTCGCCATTATAGT	This study
CbpFstopR	TTATTGAACCCATTCGCCATTATAGT	This study
GfpFNheI	AGCAATGCTAGCGTGAGCAAGGGCGAGGA	This study
GfpR	ACTATTCCCAACACTGCCACTATCCTTGTACAGCTCGTCCATG	This study
LinkDelF	CTAGAGGGAGTGACTTCTTCACAAGGTTGGAAGAAAATAAATGGTAGTTGGT	This study
LinkDelR	TTGTGAAGAAGTCACTCCCTCTA	This study
SH3F	GATAGTGGCAGTGTTGGGAATA	This study
SH3F-CHAPtail	CGATGACAGGATTTATTCATTTTAAAGATTTGGATAGTGGCAGTGTTGGGAATAGT	This study
SH3R-DStail	GCAGGAAATTTCTCCTACTCCAATTTTCTATATATAGCGACGACTTCCGT	This study
SH3RXhoI	CTCGAGCTATATATAGCGACGACTTCCGT	This study
SH3W234AF	CGGCTATAAGGCGTTGAGTTATACTGCCTATAATGGAAGCTAT	This study
SH3W234AR	GGCAGTATAACTCAACGCCTTATAGCCGTCTTTTTCAAGTATCTGATCATAATGAACC TTCTCCC	This study
SH3W310A-F	GAAAAAGACGGCTATAAGGCGTTGAGTTATACGGCT	This study
SH3W310A-R	AGCCGTATAACTCAACGCCTTATAGCCGTCTTTTTC	This study

References:

Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. and Warren, G. L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* **54**: 905-921.

Eldholm, V., Johnsborg, O., Haugen, K., Ohnstad, H. S. and Havarstein, L. S. (2009) Fratricide in *Streptococcus pneumoniae*: contributions and role of the cell wall hydrolases CbpD, LytA and LytC. *Microbiology* **155**: 2223-2234.

Jones, T. A., Zou, J. Y., Cowan S. W. and Kjeldgaard, M. (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr A* **47 (Pt 2)**: 110-119.

Laskowski, R. A., Moss D. S. and Thornton, J. M. (1993) Main-chain bond lengths and bond angles in protein structures. *J Mol Biol* **231**: 1049-1067.