Overexpression of the fratricide immunity protein ComM leads to growth inhibition and morphological abnormalities in *Streptococcus pneumoniae*.

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Abstract

The important human pathogen *Streptococcus pneumoniae* is a naturally transformable species. When developing the competent state, it expresses proteins involved in DNA-uptake, DNA-processing and homologous recombination. In addition to the proteins required for the transformation process, competent pneumococci express proteins involved in a predatory DNA-acquisition-mechanism termed fratricide. This is a mechanism by which the competent pneumococci secrete a muralytic fratricin termed CbpD, which lyse susceptible sister cells or closely related streptococcal species. The released DNA can then be taken up by the competent pneumococci and be integrated into their genomes. To avoid committing suicide, competent pneumococci produce an integral membrane protein, ComM, which protects them against CbpD by an unknown mechanism. In the present study we show that overexpression of ComM results in growth inhibition and development of severe morphological abnormalities, such as cell elongation, misplacement of the septum and inhibition of septal cross-wall synthesis. The toxic effect of ComM is tolerated during competence because it is not allowed to accumulate in the competent cells. We provide evidence that an intramembrane protease called RseP is involved in the process of controlling the ComM levels, since Δ*rseP* mutants produce higher amounts of ComM compared to wild type cells. The data presented here indicate that ComM mediates immunity against CbpD by a mechanism that is detrimental to the pneumococcus if exaggerated.

Introduction

One of the major factors that drives the rapid spread of antibiotic resistance in the important human pathogen *Streptococcus pneumoniae* is its ability to become natural competent for genetic transformation (Hakenbeck, 1995, Chi *et al.*, 2007, Dowson *et al.*, 1993, Sibold *et al.*, 1994). In the competent state *S. pneumoniae* can actively take up naked DNA from its surroundings and integrate this new DNA into its own genome by homologous recombination (Claverys *et al.*, 2009). The competent state in *S. pneumoniae* involves the transient expression of two sets of genes: the early competence genes (*com*-genes) and the late *com*-genes. The early *com*-genes comprise more than 20 genes including *comAB* and *comCDE* required for competence induction, while the more than 80 late *com*-genes include genes required for DNA-uptake, DNA processing and recombination (Peterson *et al.*, 2000, Peterson *et al.*, 2004, Rimini *et al.*, 2000). Expression of the early *com*-genes is induced by a *comC* encoded peptide called competence stimulating peptide (CSP). Pneumococci produce pre-CSP at a constitutive low level. Mature CSP is formed during translocation out of the cells via the ComAB secretion complex (Håvarstein *et al.*, 1995b, Håvarstein *et al.*, 1995a, Hui and Morrison, 1991). Outside the cells a critical high concentration of CSP triggers competence development via a classical two-component system pathway comprising the transmembrane kinase ComD and the response regulator ComE (Martin *et al.*, 2013). Phosphorylated ComE activates expression of the early *com*-gene *comX*, which encodes the alternative sigma factor that promotes transcription of the late *com*-genes (Lee and Morrison, 1999).

One biological very important trait of competent pneumococci is that they have the ability to acquire DNA from other pneumococci and closely related species such as *Streptococcus mitis* and *Streptococcus oralis* via a predatory mechanism called fratricide (Johnsborg *et al.*, 2008,

Steinmoen *et al.*, 2003, Håvarstein *et al.*, 2006). Of particular importance is the horizontal transfer of genes encoding low-affinity penicillin binding proteins (targets for β-lactam antibiotics), which are the main cause of penicillin resistance in pneumococci. For example, genes encoding low-affinity penicillin binding proteins found in *S. mitis* are frequently taken up by competent *S. pneumoniae* that live in the same niche (Johnsborg *et al.*, 2008, Chi *et al.*, 2007, Dowson *et al.*, 1993, Hakenbeck, 1995). The fratricide mechanism is based on specific killing of non-competent streptococci by a murein hydrolase, a so-called fratricin, which is expressed and secreted by the attacker cells during competence (Berg *et al.*, 2012). In *S. pneumoniae* the late *com*-gene *cbpD* encodes such a fratricin (Eldholm *et al.*, 2009, Eldholm *et al.*, 2010, Kausmally *et al.*, 2005). CbpD (choline binding protein D), which is found to be essential for competence induced target cell lysis in *S. pneumoniae* (Kausmally *et al.*, 2005), contains a muralytic N-terminal cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain followed by two Src homology 3b (SH3b) domains and a choline binding domain at the C-terminal end. The choline binding domain targets CbpD onto the choline decorated teichoic acids in the cell wall of target cells, while the SH3b domains are thought to be involved in peptidoglycan binding, positioning the muralytic CHAP domain so that it can create damage to the cell wall of CbpD-susceptible cells. (Eldholm *et al.*, 2010, Steinmoen *et al.*, 2002). Interestingly, the muralytic domains of fratricins are highly conserved, while the cell wall binding domains have great diversity (Berg *et al.*, 2012). The reason for this is probably correlated to differences in the cell surface of target species among the different streptococci from which homologous DNA can be acquired. Genome sequencing shows that all streptococci known to develop natural competence contain a muralytic fratricine that most probably is expressed during competence. This suggests that the predatory fratricide mechanism must be very important for the biology and evolution of streptococci (Berg *et al.*, 2012, Straume

et al., 2015). Although the biological function of fratricide is not completely understood, most data indicate that it is a mechanism for competent cells to acquire homologues DNA from related bacteria during stress rather than functioning as a killing mechanism to eliminate competing bacteria. By having a large gene-pool available, streptococci have a big advantage in order to adapt quickly to challenges in the environment.

A critical part of fratricide is for the competent cells to avoid committing suicide from their own fratricin. In *S. pneumoniae* this is accomplished by expression of the early *com*-gene *comM* which encodes a 23.5 kDa integral membrane protein predicted to have 6 or 7 transmembrane segments. Gene deletion studies show that ComM is the only protein required for developing immunity against CbpD in *S. pneumoniae*. Being an early *com*-gene, transcription of *comM* elevates immediately after competence induction, peaking at around 5 min before it is quickly downregulated, reaching basal expression level after 15 - 20 min (Alloing *et al.*, 1998, Peterson *et al.*, 2000, Peterson *et al.*, 2004, Dagkessamanskaia *et al.*, 2004). Full immunity is acquired approximately 5 min after entering the competent state, which is just before expression of the late *com*-gene *cbpD* is initiated (Håvarstein *et al.*, 2006). Even though the immunity role of ComM in fratricide was identified 10 years ago, the activity of ComM and/or what cellular process(es) it affects is still a mystery. In fact, very little is known about the effect ComM has on the pneumococcus except for providing immunity against CbpD during competence. In this work we show that expression of ComM is a mixed-blessing for *S. pneumoniae*. The mechanism by which ComM gives immunity is actually harmful for the pneumococci if exaggerated. We show that competence induced ComM expression is transient, preventing that it accumulates to toxic levels. Low expression levels of ComM render the pneumococci immune to CbpD, while cells expressing higher levels of ComM displayed reduced growth rate and developed morphological abnormalities

such as increased cell size, misplacement and inhibition of the septal cross-wall synthesis resulting in growth arrest.

Methods

Cultivation and transformation of *S. pneumoniae*.

S. pneumoniae was grown in liquid C medium (Lacks and Hotchkiss, 1960) or on Todd-Hewitt (Difco) agar plates at 37°C. Agar plates were incubated in a closed chamber with Anaerogen™ 3.5L bags (ThermoFisher) to create anaerobic growth conditions. When necessary antibiotics were added to the growth medium with the following concentrations: kanamycin (400 $\mu g\ ml^{-1}$), streptomycin (200 $\mu g\ ml^{-1}$), chloramphenicol (2.5 $\mu g\ ml^{-1}$). To create transformants of *S. pneumoniae*, exponentially growing cells at $OD_{550} = 0.05$ were mixed with the transforming DNA (100-200 ng) and induced to competence by adding CSP (250 ng ml^{-1}). The cells were incubated for 2 h at 37°C before transformants were selected on Todd-Hewitt agars plates containing the appropriate antibiotic. When examining the growth of pneumococcal strains, the cells were grown in a 96-wells microplate with a clear bottom, and OD_{492} was measured every 5 minutes using a Synergy H1 Hybrid Reader (BioTek). All strains of *S. pneumoniae* used in this study are listed in Table S1.

Construction of *S. pneumoniae* mutants.

To create genetic knockouts or to introduce other mutations in the genome of *S. pneumoniae*, pneumococci were transformed with amplicons containing an antibiotic resistance markers or with

amplicons containing genetically modified target genes. The DNA amplicons were constructed by overlap extension PCR (Higuchi *et al.*, 1988) following the same protocol as described in previous publications (Berg *et al.*, 2013, Johnsborg *et al.*, 2008). All primers used in this work are listed in the supplementary Table S2. Briefly, the ~1000 bp region upstream and downstream of a target gene were fused to the 5' and 3' end, respectively, of a desired antibiotic resistance cassette by overlap extension PCR. This amplicon was then transformed into *S. pneumoniae* to knock out the target gene by homologous recombination. In this study the majority of mutants were created by using the Janus system (Sung *et al.*, 2001), which allows insertion and deletion of the Janus in a streptomycin resistant background. Gene mutations were introduced into the genome by substituting the Janus with a mutated version of a desired gene. Knockout mutants were screened by PCR and all mutations that were introduced in the genome of *S. pneumoniae* were confirmed by sequencing.

Ectopic expression of ComM.

In order to control the expression of ComM without inducing competence in *S. pneumoniae*, we took advantage of the ComRS gene expression/depletion system described by Berg *et al.*, (2011). The ComRS system is based on the response regulator ComR and the inducer peptide ComS, which regulate transcription of competence genes in *Streptococcus thermophilus*. The principle behind this system is that ComS is taken up from the extracellular space via the Ami oligopeptide transporter system. Inside the cells, ComS binds to ComR, which then activates transcription from a specific set of promoters (Fontaine *et al.*, 2010). The ComRS system used to drive ectopic gene expression in *S. pneumoniae* is based on the ComR-regulated promoter P_{comX} from *S. thermophilus*.

Since the ComRS system derives from a different organism than *S. pneumoniae*, and the conserved motifs found in ComR-regulated promoters are not present in promoters found in the *S. pneumoniae* genome, it is very unlikely that the ComRS system influences the expression of pneumococcal genes other than those placed behind the P_{comX} promoter. The *comM* gene was therefore placed behind the ComS-inducible P_{comX} promoter, and the endogenous $comM_{wt}$ gene was deleted by using the Janus cassette (Sung *et al.*, 2001). Gene expression from the P_{comX} promoter is highly dependent upon the amount of ComS inducer present in the growth medium. By varying the concentrations of ComS in the growth medium when cultivating strains containing the *comM* gene behind P_{comX}, ComM was expressed at different levels. ComM ectopically expressed by using the ComRS system will be referred to as $ComM^{ect}$.

Electron microscopy.

The strains SPH399, SPH400, SPH401 and SPH402 were grown to an OD_{550} = 0.3 in the presence of 0 and 1 µM ComS. Two ml cell culture were fixed by adding 2 ml fix solution (4% paraformaldehyde [w v^{-1}] and 5% glutardialdehyde [v v^{-1}] in 1 x PBS, pH 7.4) in a 1:1 ratio. After incubation at room temperature for 1 h, the cells were fixed overnight at 4°C. The fixed cells were washed three times in sodium cacodylat buffer (0.1 M, pH 7.4).

For scanning electron microscopy, fixed cells were dehydrated with 70% and 90% ethanol for 10 min each, and then with 100% ethanol overnight. Following dehydration with ethanol, samples were subjected to critical point drying with liquid CO_2. Then, samples were coated with Au-Pd and examined in a ZEISS EVO 50 EP scanning electron microscope.

171 For transmission electron microscopy, fixed cells were post-fixed for 1 h at room
172 temperature using 1% OsO_4 (w v^{-1}) and 1.5% $K_3[Fe(CN)_6]$ (w v^{-1}) dissolved in dH_2O. Following
173 three washing steps in dH_2O (3x10 min), cells were pre-stained for 30 min using 1% uranyl acetate.
174 Next, cells were washed for 3 x 10 min in water, and dehydrated with a gradient series of ethanol
175 comprising 10 min sequential incubations in 70%, 90% and 100% ethanol. Finally the cells were
176 stepwise infiltrated in LR White resin as follows: LR White resin : EtOH in ratios 1:3 for 30 min,
177 1:1 overnight, 3:1 for 4 h, and finally 100% LR White resin overnight followed by embedding in
178 100% LR White resin at 60°C overnight. Thin sections were cut with a diamond knife mounted on
179 an ultra-microtome (LEICA, EM UC 6). The sections were counterstained with 1% $KMNO_4$ for
180 10 min. After staining, the grids were washed thoroughly in dH_2O. The sections were examined in
181 a FEI MORGAGNI 268 electron microscope.
182

183 **Labelling *S. pneumoniae* with BodipyFL® vancomycin and GFP-CbpD**

184 SPH399 cells were grown in the presence of 0 or 1 µM ComS to an OD_{550} = 0.3 before they were
185 fixed by adding paraformaldehyde and glutardialdehyde to a final concentration of 2% (w v^{-1}) and
186 2.5% (v v^{-1}), respectively. After incubation at 4°C for 1 h, the cells were washed three times with
187 PBS. Then the cells were labeled with a 1:1 mixture of fluorescent BodipyFL® vancomycin
188 (Thermo Scientific) and vancomycin (Sigma) in PBS at a final concentration of 1 µg ml^{-1} for 10
189 min. The cells were washed two times in PBS before microscopic examination using a Zeiss LSM
190 700 DIC microscope. Excitation was performed at 488 nm using a 525 nm emission filter.

191 GFP-CbpD was expressed and purified as described by Eldholm *et al.*, (2010). SPH399
192 cells grown in the presence of 0 or 1 µM ComS were fixed in 4% paraformaldehyde for 1 h at 4°C.

The fixed cells were washed 3 times with PBS before they were incubated with 15 µg GFP-CbpD (dissolved in PBS with 0.05% Tween-20) for 10 min. After three washes (3 x 1 min) in PBS containing 0.05% Tween-20, the binding pattern of GFP-CbpD on the cells was detected by fluorescence microscopy as described above.

Immunodetection of ComM.

Endogenous ComM was detected in the strains RH1 and SPH415 (Δ*rseP*) while expression of $ComM^{ect}$ was detected in strain SPH399 and SPH400 (Δ*rseP*). RH1 and SPH415 were inoculated in C medium to an OD_{550} = 0.05. When reaching OD_{550} = 0.2 the cells were induced to competence by adding CSP to a final concentration of 250 ng ml^{-1}. Cells from 30 ml fractions were harvested at 4°C by centrifugation at time 0, 5, 10, 15, 20, 30 and 60 min after induction. All subsequent steps were done with the samples kept on ice. The cells were resuspended in 1 ml ice cold 10 mM Tris-HCl (pH 7.4) and lysed by fastprep for 3x20 sec at 6.5 m s^{-1}. The sample volume was increased to 25 ml by adding ice cold 10 mM Tris-HCl (pH 7.4) and whole cells and large cell debris were removed by centrifugation for 10 min at 5000 x *g*. The supernatant were centrifuged at 30 000 x *g* for 30 min to collect micelles and membranes containing ComM as described by Fjellbirkeland *et al.*, (1997). The membrane pellets were dissolved in 100 µl SDS-sample buffer and boiled for 5 min before the samples were separated in an SDS-PAGE (15% separation gel) as described by Laemmli (1970). For the expression of $ComM^{ect}$, strain SPH399 and SPH400 were inoculated to an OD_{550} = 0.05 in C medium containing 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.015 and 0 µM ComS. When reaching OD_{550} = 0.3 the 5 ml cell cultures was collected at 5000 x *g*. The cell pellets were mixed with 100 µl of SDS-sample buffer and total protein extracts were separated by

SDS-PAGE. After electrophoresis the proteins were electroblotted onto a polyvinylidene fluoride (PVDF) membrane. After incubation for 1 h in 5% skimmed milk in TBS-T buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl and 0.05% Tween 20) the membrane was washed 1x5 min with TBS-T followed by incubation for 1 h with primary anti-ComM antibody diluted 1: 1000 in TBS-T. The primary antibody was produced (by ProSci Inc.) in rabbits immunized with the peptide NH_2-NYLYTRKQEVHSVLASKK-COOH which constitutes the amino acids 52-69 in the ComM protein. Excess anti-ComM antibody was removed by washing the membrane 3x10 min in TBS-T. Then the membrane was incubated for 1 h with the alkaline phosphatase conjugated secondary antibody (Anti-Rabbit IgG from Sigma) which was diluted 1: 4000 in TBS-T. After washing 4x10 min in TBS-T the membrane was developed by incubating it in the substrate BCIP®/NBT-Blue Liquid System for Membranes (Sigma). ComM specific bands appeared blue on the membrane just above 20 kDa.

β-galactosidase assay.

To quantify the degree of immunity against CbpD, the amount of β-galactosidase released from lysed cells expressing the native $ComM^{ect}$ (strain SPH403) and different point mutated versions of $ComM^{ect}$ (SPH404-414) was determined. Strain SPH403-414 were grown in the presence of 1, 0.03 or 0 µM ComS. Cells grown with 0 µM ComS was used as a control for non-immune cells. When reaching OD_{550} = 0.2 the cells were induced to competence by adding CSP to a final concentration of 250 ng ml^{-1}. The amount of released β-galactosidase was measured 30 min after CSP induction. The assay was carried out as previously described by Steinmoen *et al.,* (2002) based on the protocol of Miller (1972).

Luciferase assay

All strains assayed for P_{comM} or P_{comX}-driven luciferase (*luc*) reporter activity were grown in C medium to an OD_{550} ~ 0.3. The bacterial cultures were then diluted to OD_{550} = 0.05 in C medium and transferred to a 96-well Corning NBS clear-bottom plate. D-luciferin (Thermo Scientific) was added to the wells to a final concentration of 10 mM. The plate was incubated in a Synergy H1 Hybrid Reader (BioTek®) at 37ºC, and OD_{492} and luminescence were measured automatically every 5 min throughout the experiment. CSP (250 ng ml^{-1}) or ComS (1 µM) was added to the P_{comM}-*luc* and P_{comX}-*luc* cells, respectively, to induce expression of the *luc* gene.

Results

ComM is short-lived during competence in *S. pneumoniae*.

The transcription of *comM* is induced during the early stage of competence (early *com*-gene) to give the pneumococcus protection against the fratricin CbpD, which is expressed approximately 10 - 15 min into the competent state (Peterson *et al.*, 2000, Peterson *et al.*, 2004). ComM must therefore render the cells immune against CbpD within this time frame. It is known that the transcription of early *com*-genes reaches maximum levels 5 min after competence induction before it quickly declines to basal levels after 15 – 20 min (Peterson *et al.*, 2004, Alloing *et al.*, 1998, Peterson *et al.*, 2000). It is not known, however, whether the level of ComM protein follows the same expression pattern, i.e. being present mainly before CbpD is expressed, or if it must remain in the cells to provide protection when fratricide takes place. In order to determine this we monitored the expression of ComM in wild type cells during competence by immunoblotting. *S.*

pneumoniae cells were induced to competence at OD_{550} = 0.2, and samples were collected at time 0 and 5, 10, 15, 20, 30 and 60 min after induction. Attempts to detect the native ComM in whole cell extracts did not succeed most probably because the levels of ComM were below the detection limit of our primary antibody (data not shown). However, since ComM is predicted to be an integral membrane protein, we expected to find ComM in the membrane fraction. By concentrating membranes from 30 ml of cell culture, ComM was detected (Fig. 1a). The maximum level of ComM in *S. pneumoniae* cells was reached 5 min after competence induction. From 10 min onwards the level started to decline, and ComM was hardly detected 20 min after competence induction. Thus, the presence of ComM is clearly transient during competence as nearly all ComM is removed from the cells after 20 min. Since *cbpD* transcription peaks ~10-15 min into the competent state, it seems that the majority of ComM has been removed before CbpD expression reaches a maximum rate.

Higher levels of ComM are produced in RseP deficient cells.

We reasoned that the rapid decrease in ComM levels in *S. pneumoniae* probably involves one or more specific proteases. Since ComM is an integral membrane protein, it was reasonable to assume that proteases possibly involved in modulating the levels of ComM also would be associated with the cell membrane. In an attempt to identify proteins contributing to ComM stability, we deleted four candidate genes encoding membrane-associated proteases (*htrA* [*spr2045*], *htpX* [*spr1162*], *ftsH* [*spr0012*] and *spr0242*). Of these, only the *htrA*, *htpX* and *spr0242* mutants were viable and could be examined with regard to ComM expression. Deletion of HtrA or HtpX did not influence the level of ComM (data not shown), however, deletion of Spr0242 turned out to increase the

amount of ComM during competence (Fig. 1b and c). The gene *spr0242* encodes a protein that has homology with a membrane protease called RseP in *Escherichia coli* (49% homology). Spr0242, hereafter also called RseP, is predicted to have 4 transmembrane segments, with a ~17 kDa cytoplasmic domain and a ~15 kDa extracellular domain. The RseP homolog in *E. coli* is a so-called RIP protease (regulated intramembrane proteolysis) that functions as a site 2 metallo protease. It performs the second and final cleavage of the anti σ^E protein RseA leading to activation of the stress induced σ^E factor (Alba *et al.*, 2002, Kanehara *et al.*, 2002, Li *et al.*, 2009). Similarly, in *Bacillus subtilis* the RseP ortholog YluC cleaves an anti-sigma factor to activate the stress induced σ^W (Schobel *et al.*, 2004). So far, a corresponding stress related function has not been identified for the *S. pneumoniae* RseP protein. In the Δ*rseP* mutant SPH415 ComM could be detected 60 min after competence induction, while it was barely detectable after 20 min in the wild type (Fig. 1a, b and c). To examine whether deletion of RseP results in elevated transcription from the P_{comM} promoter, we placed the *luc* reporter gene behind the P_{comM} promoter in both RseP proficient and deficient cells. The luciferase activity was then monitored during competence. As Fig. 1d shows, deletion of RseP did not result in elevated transcription from the P_{comM} promoter. The expression profile of the ComM protein was transient in the Δ*rseP* mutant, as observed for RseP proficient cells. However, ComM reached higher levels in the Δ*rseP* strain, suggesting that RseP somehow participates in the turnover of ComM. To further strengthen the observation that higher concentrations of ComM is produced in RseP deficient cells, we created a mutant that expressed *comM* ectopically ($comM^{ect}$). The strains used in this experiment lacked their native $comM_{wt}$ gene, but expressed a copy of $comM^{ect}$ ectopically using the ComRS gene expression/depletion system previously described by Berg *et al.,* (2011) (Induction of the ComRS system is not affected by deletion of *rseP*, see Fig. S1). Ectopic expression of $comM^{ect}$ produced

304 higher levels of full-length ComM^{ect} in RseP deficient cells (SPH400) compared to RseP proficient
305 cells (SPH399) when the *comM^{ect}* gene was expressed at similar levels (Fig. 2). ComM^{ect} was
306 expressed at increasing levels by adding ComS inducer ranging from 0 – 1 µM to the growth
307 medium. In addition to full-length ComM^{ect}, several ComM-derived degradation products are seen
308 for both strains, particularly the three bands at ~20 kDa, ~17 kDa and ~5 kDa. Hence, absence of
309 RseP somehow results in higher levels of ComM^{ect}, but it is not the main contributor for ComM^{ect}
310 removal. Due to cross-reaction of the primary antibody with an unspecific protein of similar size
311 as ComM^{ect}, a weak band was seen at the same position as ComM^{ect} for cells grown with 0 µM
312 ComS.

313

314 **High levels of ComM inhibits growth of *S. pneumoniae*.**

315 The fact that ComM is quickly removed from the cells made us wonder whether high levels of
316 ComM are toxic to the cells. To test if high concentrations of ComM would lead to other
317 phenotypic changes in *S. pneumoniae* beyond that of the immunity function, growth was monitored
318 in cells that overexpressed ComM^{ect} using the ComRS gene expression/depletion system described
319 above. Overexpression of ComM^{ect} using 1 µM ComS inducer had a slight negative effect on the
320 growth rate of *S. pneumoniae* with a wild type genetic background, and the cells entered the
321 stationary phase at a lower OD_{492} than cells not expressing ComM^{ect} (Fig. 3). In addition, autolysis
322 took place earlier in the stationary phase, but progressed at a lower speed compared to cells not
323 expressing ComM^{ect}. The negative effect of ComM^{ect} overexpression on growth was much more
324 pronounced in the Δ*rseP* mutant. These cells displayed significant growth reduction and stopped
325 growing at OD_{492} = 0.6 before starting to autolyze. These results clearly show that high levels of

326 ComM^{ect} are toxic to the cells leading to growth arrest and cell death. Hence, while ComM
327 expression provides immunity against CbpD during competence, uncontrolled and prolonged
328 expression is clearly harmful to the cell. To rule out the possibility that overexpression of any
329 membrane protein results in growth inhibition, we overexpressed an attenuated version of ComM
330 (see below) containing two alanine substitutions (D119A and R194A). Pneumococci
331 overexpressing this version of ComM did not display any reduced growth rate (Fig. S2).

332

333 **Analysis of point mutated versions of ComM.**

334 ComM is found in the cell membrane (see above) and is predicted to contain 6-7 transmembrane
335 segments (Fig. 4) depending on which prediction algorithm that is used. Clearly, ComM carries
336 out its immunity function within or close to the cell membrane, but the exact molecular mechanism
337 is not known. Amino acid sequence alignments between ComM and homologous proteins found
338 in other streptococcal species show that ComM contains several conserved residues, of which all
339 but one (Leu28) are located in the transmembrane segments (supplemental material). Interestingly,
340 ComM also displays some resemblance to so-called CAAX-proteases first identified in eukaryotes
341 (also called Abi family) (Kjos *et al.*, 2010). CAAX-proteases are known to cleave off the –AAX
342 motif (A represents an aliphatic amino acid, and X represents any amino acid) at the C-terminus
343 of proteins after the cysteine in the CAAX motif has been conjugated with an isoprenoid molecule
344 (Manolaridis *et al.*, 2013, Pryor *et al.*, 2013, Boyartchuk *et al.*, 1997, Tam *et al.*, 1998, Schmidt *et*
345 *al.*, 1998, Reiss *et al.*, 1990, Seabra *et al.*, 1992). However, ComM does not contain the conserved
346 $EE(X)_3R$ and $F(X)_3H$ motifs found in the catalytic site of CAAX proteases (Plummer *et al.*, 2006,
347 Dolence *et al.*, 2000, Manolaridis *et al.*, 2013). The third conserved motif $H(X)_3N/D$, found to take

part in the catalytic function of CAAX proteases, is on the other hand, present in ComM (H191, see supplemental material for sequence alignment). Based on the assumption that some of the conserved residues could be part of a catalytic site, we chose to point mutate several of the conserved residues to alanine or leucine to see if the immunity function of these ComM versions was lost (Fig. 4). The point mutated versions of ComM were expressed ectopically in a Δ*comM*$_{wt}$ background using the ComRS system at 0.03 µM or 1 µM ComS inducer. The degree of immunity was determined by measuring the level of β-galactosidase released from CbpD-sensitive cells 30 min after CSP induction (Table 1). A concentration of 0.03 µM ComS inducer produces ComM^{ect} levels that give immunity corresponding to wild type cells, while 1 µM ComS results in overproduction of ComM^{ect}. At 0.03 µM ComS the ComM^{ect} protein provides 95±2% protection against CbpD, while ComM^{ect} containing the point mutations N82A, S111A, D119A, H191A, R194A, D119A/H191A or D119A/R194A did not give any protection. Similar expression levels of the G117L, P125A, E185A and S187A versions of ComM^{ect} resulted in 1±3, 18±3, 23±6 and 15±2% immunity, respectively. When overexpressed (1 µM ComS), on the other hand, all the mutated versions of ComM^{ect} still had to various degrees some remaining immunity function. The ComM$_{D119A/R194A}$ protein displayed lowest functionality giving only 14±5% immunity when overexpressed. Interestingly, when the native ComM^{ect} is overexpressed, on the other hand, the cells appeared to lose some of their immunity (91±2% immunity). However, a closer examination revealed that this was in fact the result of cells that had lysed because of the toxic effect of ComM^{ect}, and not from CbpD activity (data not shown).

370 ***S. pneumoniae* overexpressing ComM^{ect} develops morphological abnormalities.**

371 Overexpression of ComM^{ect} is evidently toxic to pneumococcal cells since it leads to reduced
372 growth (Fig. 3). To see whether the cells with reduced growth also obtained other phenotypical
373 changes, we examined the morphology of *S. pneumoniae* cells overexpressing (1 μM ComS)
374 ComM^{ect} by electron microscopy. Since higher levels of ComM^{ect} are expressed in an RseP
375 negative strain, we assumed that any morphological changes in the wild type background would
376 be even more distinct in an RseP mutant. ComM^{ect} was therefore overexpressed both in an RseP
377 positive (SPH399) and an RseP negative (SPH400) strain. Scanning electron microscopy revealed
378 that wild type cells overproducing ComM^{ect} became elongated with pointy poles, and many cells
379 contained multiple and/or misplaced septa (Fig. 5a and b). Labelling with the fluorescent
380 vancomycin Bodipy FL®, which binds to the D-Ala-D-Ala moiety of stem peptides in newly
381 synthesized peptidoglycan confirmed this result (Fig. 6a). In addition, since CbpD is known to
382 bind the septal area of susceptible cells, we wanted to examine whether the multiple septa in
383 ComM^{ect}-overproducing cells served as equally good binding sites for CbpD. Binding of a GFP
384 fused CbpD (the catalytic CHAP domain of CbpD was replaced by GFP) to ComM^{ect}-
385 overproducing cells, showed that CbpD is not prevented from binding to the multiple septa (Fig.
386 6b).

387 For the Δ*rseP* mutant ComM^{ect} overexpression resulted in an even more dramatic change
388 in morphology. In addition to becoming elongated with multiple and misplaced septa, in many
389 cases two dividing cells displayed an irregular cell circumference, i.e. two cells looking like head-
390 pointing bowling pins (Fig. 5c and d). To confirm that the change in morphology was a
391 consequence of ComM^{ect} activity, and not just due to toxic accumulation of an integral membrane
392 protein, the almost inactive ComM$_{D119A/R194A}$ protein was overexpressed in the same genetic

backgrounds (SPH401 and SPH402). These cells had a normal cell shape, although some chain formation was observed (Fig. 5e and f).

Examination of $ComM^{ect}$-overproducing cells by transmission electron microscopy revealed that they contain several initiated but uncompleted septal cross-walls (Fig. 7). Asymmetrical cell division was also apparent in many of the cells (Fig. 7b). The uncompleted cross-walls were in many cases irregular in thickness, typically starting with a thicker wall at the cell periphery that tapered off towards the cell center. This was particularly evident for the RseP negative cells (Fig. 7d). Also, the part of the RseP negative cells containing aborted cross-walls had a larger diameter than normal cells, i.e., 660 ± 19 nm compared to 470 ± 11 nm, respectively (n=20). Overexpression of the attenuated $ComM_{D119A/R194A}$ mutant protein did not result in any morphological changes to the cells (Fig. 7e and f).

Discussion

When *S. pneumoniae* becomes competent for genetic transformation, a predatory mechanism called fratricide is activated (Berg *et al.*, 2012, Straume *et al.*, 2015, Johnsborg and Håvarstein, 2009). It involves competence induced expression of the secreted murein hydrolase CbpD, which lyse non-competent pneumococci or other closely related streptococci in order for the competent pneumococci to get access to their genomes. (Håvarstein *et al.*, 2006, Johnsborg *et al.*, 2008, Kausmally *et al.*, 2005, Eldholm *et al.*, 2010, Steinmoen *et al.*, 2003). In fratricide the immunity protein ComM plays a critical role for the competent pneumococci, as it provides protection against CbpD. During normal competence development, we observed that ComM was present in the cells for a very limited time (~15 min). As an early *com*-gene, *comM* is expressed

approximately 5-10 min before *cbpD* expression is initiated (Peterson *et al.*, 2000, Peterson *et al.*, 2004). Presumably, this will give ComM enough time to make the cells immune. It was unexpected that the level of ComM had started to decrease before CbpD expression had reached maximum rate (10-15 min after competence induction). This suggests that ComM does not need to be present in the cells at the same time as CbpD in order to give immunity. Most likely, ComM somehow makes the cells immune before CbpD is transported to the extracellular milieu. Considering that ComM is an integral membrane protein while CbpD attacks the cell wall of *S. pneumoniae* from the outside (Eldholm *et al.*, 2010), it is very unlikely that the two proteins are in physical contact. Hence, ComM probably mediates immunity indirectly, and not through direct interaction with CbpD. The most obvious hypothesis is that ComM mediates immunity by changing the cell wall structure of newly synthesized peptidoglycan in the septal area. ComM might modify a specific part of the stem peptides, i.e. the substrate that is recognized and cleaved by the catalytic domain of CbpD. Alternatively, ComM might introduce changes in peptidoglycan or teichoic acids that block attachment of CbpD to the cell wall. However, the fact that a GFP-fused CbpD bound equally well to the septum of $ComM^{ect}$-overexpressing cells as to wild type cells argues against this (Fig. 6b). It is, however, not possible to use fluorescence microscopy to detect whether changes in the structure of peptidoglycan or teichoic acids disturb the positioning of the catalytic CHAP domain relative to its stem peptide substrate.

The transient expression of ComM observed during competence made us speculate whether one or more proteases could be involved in ComM degradation. Since ComM is an integral membrane protein, we reasoned that membrane proteases were most likely to be involved. Deletions of candidate proteases identified RseP to have an effect on the ComM level during competence, i.e. higher levels of ComM were detected in a Δ*rseP* mutant. Consequently, the

presence of ComM was prolonged by 30-40 min in the Δ*rseP* mutant (Fig. 1b and c). Also, higher levels of ComM^{ect} was observed when it was expressed ectopically in an RseP mutant compared to RseP proficient cells (Fig. 2). The role of RseP in *S. pneumoniae* is not known, but in *E. coli* and some other bacterial species such as *B. subtilis*, *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Xanthomonas campestris* pv. *campestris*, RseP is found to take part in the activation mechanism of a stress response sigma factor (Alba *et al.*, 2002, Bordes *et al.*, 2011, Kanehara *et al.*, 2002, Muller *et al.*, 2009, Wood and Ohman, 2009, Schobel *et al.*, 2004). In *E. coli* RseP is one of two proteases that cleave the transmembrane anti sigma-factor RseA. The membrane anchored protease DegS makes the first cut in RseA, while RseP functions as a site 2 protease, making the second cut in RseA leading to the release of σ^E, which activates transcription of stress response genes (Li *et al.*, 2009, Alba *et al.*, 2002, Kanehara *et al.*, 2002). Homologs of DegS and σ^E are found in the *S. pneumoniae* R6 genome (HtrA [Spr2045] and RpoE [Spr0437] sharing 29% and 23% identity with their *E. coli* counterparts, respectively), but a homolog to the anti sigma-factor RseA is not found. The RseP homolog YluC found in *B. subtilis* has also been reported to cleave the membrane protein FtsL, which is an essential part of the cell division machinery. Mutants with reduced FtsL functionality display an accelerated cell division process in both *E. coli* and *B. subtilis* (Bramkamp *et al.*, 2006, Tsang and Bernhardt, 2015). Whether RseP is involved in activation of stress related genes or regulation of cell division proteins in *S. pneumoniae* are not known, but it is reasonable to believe that its main function goes beyond regulation of ComM expression. Why then is more ComM produced in cells that do not express RseP? There are two possible explanations: (i) ComM is truly a substrate for proteolytic cleavage by RseP. In *E. coli* RseP has been reported to cleave a broad range of transmembrane sequences that are not related to the RseA protein (Akiyama *et al.*, 2004). Alternatively, (ii) it could be the

result of an indirect effect, e.g. that RseP is part of the apparatus responsible for general protein recycling, or that it somehow positively regulates the activity of another protease that has ComM as its true substrate. Furthermore, although we demonstrated that the P_{comM} promoter activity is unchanged in a Δ*rseP* mutant (Fig. 1d), increased stability of *comM* transcripts in this mutant cannot be completely ruled as an explanation for the increased ComM levels we observed. Based on the data obtained in this work we cannot tell which of the above explanations that are true, and further studies must be done to find out the mechanism behind this.

ComM evidently carries out its immunity function close to or within the cell membrane, but whether it acts as an enzyme, is involved in protein protein interactions or binds to a specific ligand is not known. ComM shares some homology with the Abi family proteins (CAAX proteases), which are recognized by three conserved motifs: $EE(X)_3R$, $F(X)_3H$ and $H(X)_3N/D$. In eukaryotic CAAX proteases these motifs are involved in the proteolytic cleavage of target proteins having a CAAX moiety at their C-terminus (Manolaridis *et al.*, 2013, Tam *et al.*, 1998, Pryor *et al.*, 2013). Prokaryotes, on the other hand, do not express proteins with the CAAX motif. Instead some prokaryotic Abi proteins have been found to be involved in protein secretion and bacteriocin immunity (Ellermeier and Losick, 2006, Kjos *et al.*, 2010, Lux *et al.*, 2007, Frankel *et al.*, 2010), but the majority of Abi proteins, which are widespread among bacteria, remains uncharacterized. ComM's resemblance with Abi family proteins made us hypothesize that it might function as an enzyme. Although ComM does not have the typical $EE(X)_3R$ and $F(X)_3H$ motifs, it contains the $H(X)_3N/D$ motif and several other conserved amino acids that could be part of a catalytic site (see supplemental material). In the present study, we did amino acid substitutions of several of the conserved amino acids in ComM. Should any of these substitutions render ComM completely inactive, it would be a strong indication that it has enzymatic activity. What we found was that

484 alanine substitutions of Asn82, Ser111, Asp119, His191 or Arg194 (His191 and Arg194 are part
485 of the $H(X)_3N/D$ motif) resulted in a $ComM^{ect}$ version that gave no protection against CbpD when
486 expressed at levels for which the native $ComM^{ect}$ gave full protection (0.03 μM ComS inducer).
487 However, the same mutated versions of $ComM^{ect}$ could partly protect against CbpD when
488 overexpressed (1 μM ComS). This result was puzzling, assuming that ComM really is an enzyme,
489 since substitutions of residues involved in catalytic reactions should fully inactivate the protein
490 independent of expression levels. One explanation could be that the point mutated ComM proteins
491 have lost their catalytic activity, but not the ability to bind their substrate. When overexpressed it
492 is possible to imagine that more ComM substrate would be made unavailable to the cell by being
493 bound to the excess of attenuated ComM proteins, which in turn could produce a partial protection
494 against CbpD. Such a mechanism takes for granted that ComM normally inactivates or inhibits its
495 target to give CbpD immunity. On the other hand, if ComM is not an enzyme, the point mutations
496 have either reduced its functionality by interfering with the overall protein structure or decreased
497 its affinity for an interaction partner(s), which is partly compensated for by the high ComM
498 concentrations. Misfolding of ComM is unlikely, however, since the substitutions are conservative
499 (alanine is commonly found in the membrane-spanning regions of transmembrane proteins). Based
500 on the results showing that single alanine substitutions of conserved amino acids, often found to
501 be part of catalytic sites, rendered ComM inactive when expressed at native levels, and the fact
502 that ComM contains the conserved $H(X)_3D$ motif found in the catalytic site of CAAX proteases,
503 we believe that ComM acts as an enzyme. However, other functions cannot be ruled out due to
504 the overexpression results discussed above.

505 The normal expression level of ComM during competence seemed to be relatively low
506 since it could only be detected in concentrated membrane fractions (Fig. 1). Under these normal

expression levels of ComM the cells develops immunity against CbpD, and we could not see any other phenotypical changes to the pneumococcal cells. However, when the expression level of ComM was turned up by ectopic expression, we observed that the immunity function of $ComM^{ect}$ can have detrimental effects on the viability of *S. pneumoniae*. The cells displayed reduced growth rate and adopted grossly abnormal morphology (Fig. 3, 5 and 7). This effect was even more pronounced in the ΔRseP strain. Even though the levels of $ComM^{ect}$ were higher in this strain, we cannot rule out the possibility that these cells also are less suited to cope with stress in general since RseP is required to activate stress genes in other bacteria (see above). The most distinct phenotype observed was that the cells become elongated and contained multiple septa, showing that they struggle to complete the old septum before a new one is initiated (Fig. 5 and 7). Indeed, TEM analyses revealed that the old cross-wall was not cleaved down the middle to separate daughter cells, and newly synthesized septa were aborted. New division sites were also misplaced, resulting in two elongated cells with two smaller ones in between. These results show that the coordination of septal and lateral cell wall synthesis were interfered by high concentrations of $ComM^{ect}$. The huge negative effect on cell morphology seen in $ComM^{ect}$ overproducing cells could explain why ComM is expressed for such a short period of time during competence. It ensures that ComM does not accumulate to levels that would do excessive damage to the cells. One might speculate that ComM would need to interact with proteins or other membrane associated components involved in the cell division machinery in order to induce such morphological changes. In fact, we have recently shown that ComM is not functioning properly in *S. pneumoniae* cells where lateral cell wall synthesis is inhibited (reduced PBP2b functionality), i.e. the cells became hypersensitive to CbpD during competence, even though ComM was expressed (Straume *et al.,* 2016). However, to pinpoint whether ComM acts directly on the cell division machinery

needs to be addressed in future studies. It might not only tell us the mechanism of ComM, but could potentially give important clues about central processes in the pneumococcal cell division machinery.

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Tables

Table 1. Levels of immunity provided by mutated versions of $ComM^{ect}$.

Mutation(s)	% immunity[a]	
	0.03 µM[b] ComS	1 µM ComS
$ComM^{ect}$	95±2	91±2
$ComM_{N82A}$	0	78±0.5
$ComM_{S111A}$	0	78±6
$ComM_{G117L}$	1±3	57±4
$ComM_{D119A}$	0	60±5
$ComM_{P125A}$	18±3	75±5
$ComM_{E185A}$	23±6	92±0.5
$ComM_{S187A}$	15±2	91±3
$ComM_{H191A}$	0	59±12
$ComM_{R194A}$	0	63±2
$ComM_{D119A/H191A}$	0	29±8
$ComM_{D119A/R194A}$	0	14±5

[a] Immunity of 100% was defined as the β-galactosidase released from non-competent cells

[b] Inducer concentration giving immunity similar to competent wild type cells

Figure legends.

Fig. 1. Immunoblot detection of ComM expressed from its native promoter during competence in *S. pneumoniae*. ComM was detected in the membrane fraction derived from wild type cells (panel a, strain RH1) and a Δ*rseP* mutant (panel b, strain SPH415). Membranes were isolated from cells at time points of 0, 5, 10, 15, 20, 30 and 60 min after competence was induced (indicated on top). The arrows indicate the position of ComM. The arrowheads indicate bands corresponding to two unspecific proteins that cross-reacted with the primary antibody. The lower appears to be a competence induced protein. RH406 cells (Δ*comM*) induced to competence for 5 min were used as ComM negative controls. Equal amounts (~50 µg) of total membrane protein were separated in each lane. The relative levels of ComM are presented in panel c. The levels of ComM in panels a and b were normalized for each sample by dividing the signals measured (AzureSpot analysis software) for the ComM-specific bands with the signals obtained for the upper cross-reacting protein of the corresponding samples. By assuming that the levels of the cross-reacting protein remains stable during competence, the relative ComM levels were estimated. Panel d: The transcriptional activity from the P_{comM} promoter does not change in a Δ*rseP* mutant (▲, SPH425) during competence compared to RseP proficient cells (●, SPH424). RseP proficient and deficient cells having the *luc* reporter gene behind the P_{comM} promoter in their genomes were induced to competence (indicated by an arrow), and the luciferase activity were measured (solid lines). Orange and black lines represent competence induced and non-competent cells, respectively. Growth curves are represented by open symbols.

Fig. 2 Immunoblot detection of ectopically expressed $ComM^{ect}$ found in whole cell extracts of RseP proficient (SPH399) and RseP negative cells (SPH400). Strain SPH131 and SPH427

(Δ*rseP*), which contain the ComRS system, but without the *comM* gene, were used as negative controls. Full-length ComM^{ect} is indicated by the arrows, while ComM^{ect} degradation products are indicated by arrowheads. Unspecific proteins cross-reacting with the primary anti-ComM antibody are indicated by stars. The concentrations of ComS inducer used to drive ComM^{ect} expression are indicated on top.

Fig. 3. Overexpression of ComM^{ect} in *S. pneumoniae* leads to reduced growth. Panel a. ComM^{ect} was overexpressed in RseP proficient cells (SPH399, circles) and in a Δ*rseP* background (SPH400, triangles) using the ComRS system by having 1 μM ComS inducer in the growth medium (dotted lines). Cells without ComS in the growth medium was used as control (solid lines). Artificially high expression levels of ComM^{ect} resulted in a modest growth reduction in the RseP proficient cells, while a much severe growth inhibition was observed in the RseP negative strain.

Fig. 4. Schematic presentation of the predicted topology of ComM. Most topology prediction servers predict ComM to have six transmembrane segments shown here as green cylinders. The positions where point mutations were introduced into ComM and the peptide epitope used to generate antibodies are indicated. Amino acid alignment of the transmembrane segments IV and VI of ComM homologues found in different streptococcal species is shown at the figure bottom. Alanine substitutions of both the conserved Asp119 and Arg194 (indicated in yellow) resulted in a highly attenuated version of ComM.

Fig. 5. Morphological examination of *S. pneumoniae* cells that overproduce ComM^{ect}. ComM^{ect} was overexpressed in the RseP positive strain SPH399 (a and b) and the RseP negative strain SPH400 (c and d) by using the ComRS system having 1 µM ComS inducer in the growth medium. Panel b and d show enlarged views of a selection of cells from a and c, respectively. SPH399 cells exposed to high doses of ComM^{ect} adopted severe morphological abnormalities. They became elongated with pointy poles. The septum was also misplaced in many cells (indicated by arrows). The abnormal morphology was even more pronounced for strain SPH400 in which more ComM^{ect} is produced due to the lack of RseP. In addition to misplaced septa, SPH400 cells appeared asymmetrical consisting of one enlarged and one small part. Overexpression of the attenuated ComM$_{D119A/R194A}$ in an RseP positive (SPH401) and RseP negative (SPH402) background resulted only in the formation of some short chains (e and f, respectively). Wild type cells (RH1) are shown in panel g. Scale bars represent 1 µm.

Fig.6. Labelling of *S. pneumoniae* with Bodipy FL (a) or GFP-CbpD (b). Strain SPH399 was grown in the presence of 0 or 1 µM ComS (indicated) to drive ComM^{ect} expression. For cells not expressing ComM^{ect} (0 µM ComS), Bodipy FL bound to a single division zone at mid-cell while cells overexpressing ComM^{ect} (1 µM ComS) contained multiple septa. GFP-CbpD bound to the multiple septa of ComM^{ect} overexpressing cells equally well as to the septum of CbpD sensitive cells (0 µM ComS). Scale bars, 2 µm.

Fig. 7. Transmission electron microscopy showing the effect of high doses of ComM^{ect} on septum progression in *S. pneumoniae*. Strain SPH399 (ComM^{ect}) grown with 0 or 1 µM ComS is shown in panel a and b, respectively, while SPH400 (ΔRseP, ComM^{ect}) grown under similar conditions

767 is shown in panel c and d. As controls, $ComM_{D119A/R194A}$ was expressed in both RseP-positive
768 (SPH401) and- negative (SPH402) cells using 1 µM ComS in the growth medium (panel e and f,
769 respectively). Wild type cells (RH1) are shown in panel g. Arrows indicate uncompleted septa.
770 Scale bars, 0.5 µm. Panel h: Immunoblot detection of $ComM^{ect}$/$ComM_{D119A/R194A}$ in the strains
771 SPH399, SPH400, SPH401 and SPH402 grown in the presence of 0 or 1 µM ComS (indicated by
772 – and +, respectively). The position of $ComM^{ect}$/$ComM_{D119A/R194A}$ is indicated by the arrowhead.

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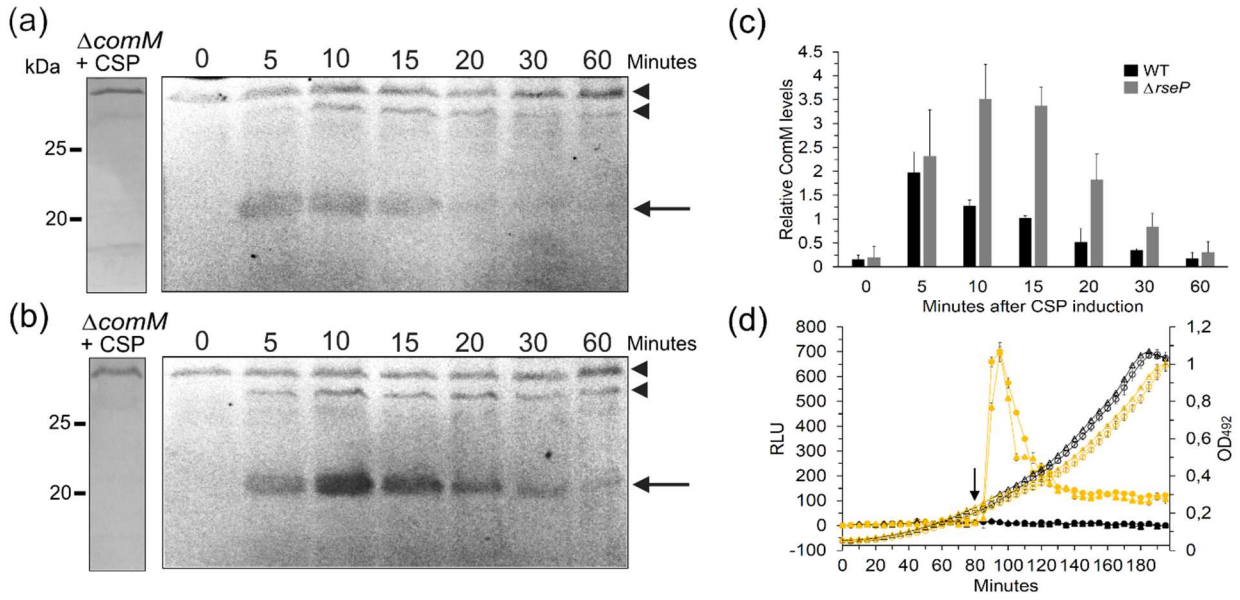
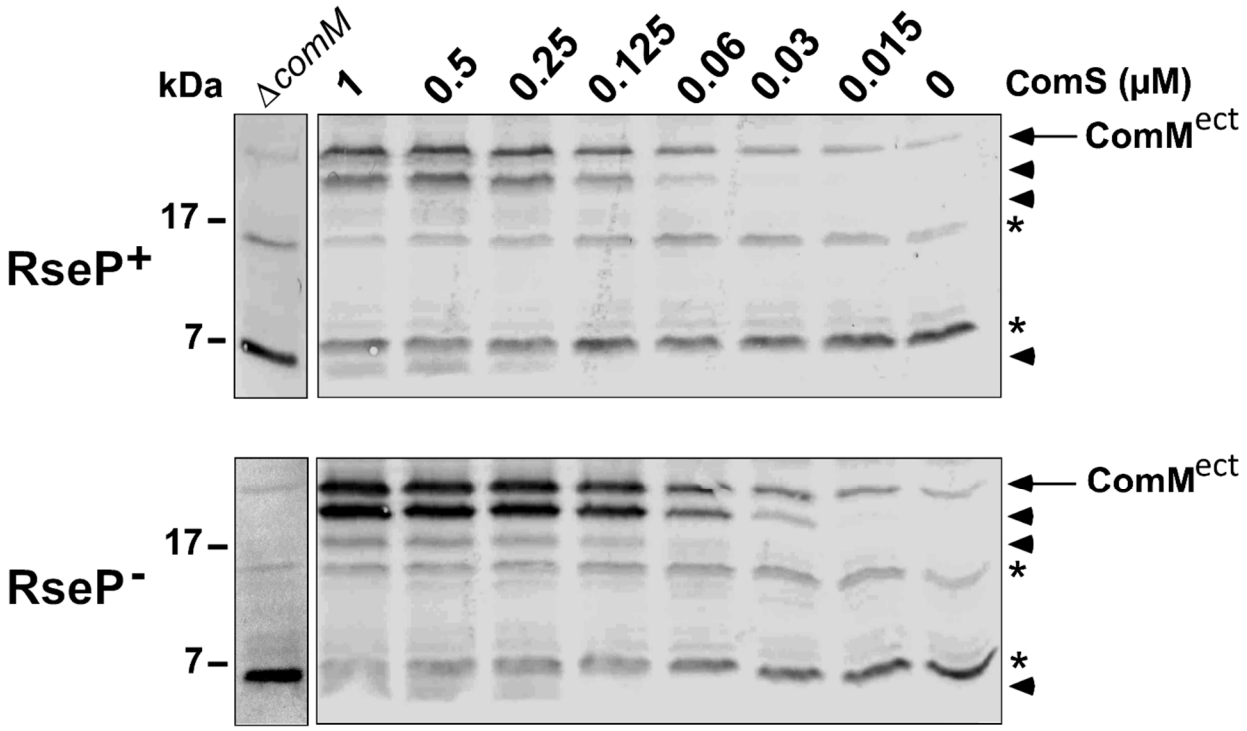
Figures

Fig. 1



781 Fig. 2



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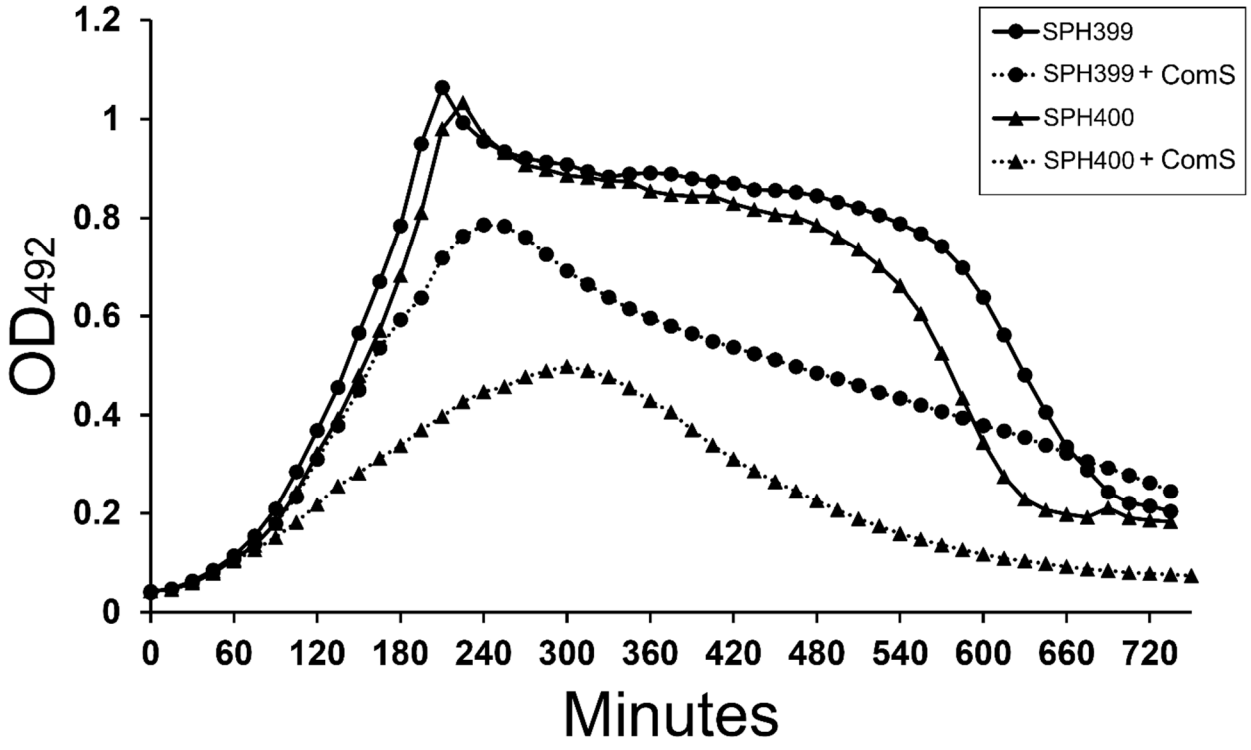
784 Fig.3.



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786 Fig. 4

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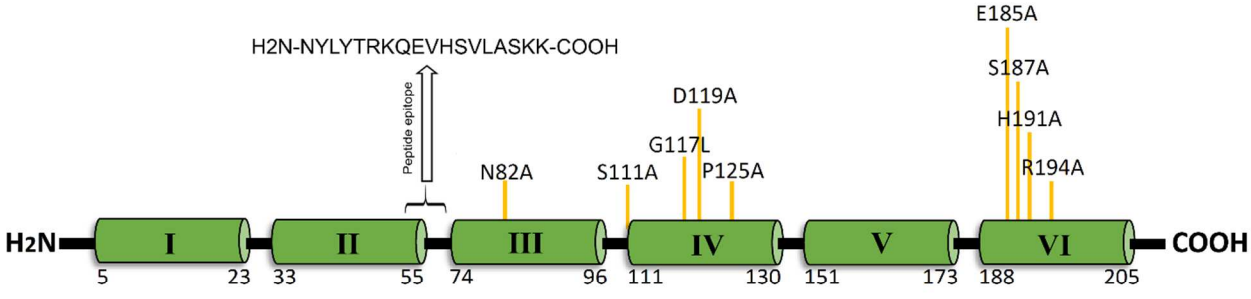

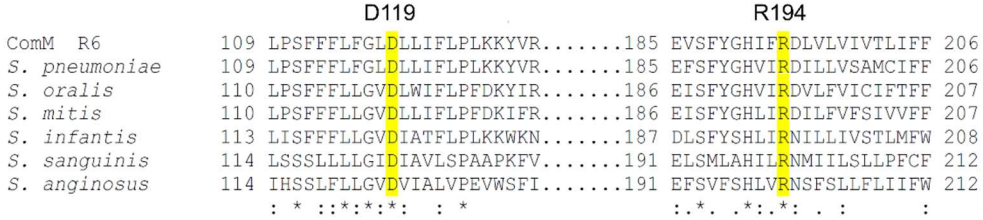

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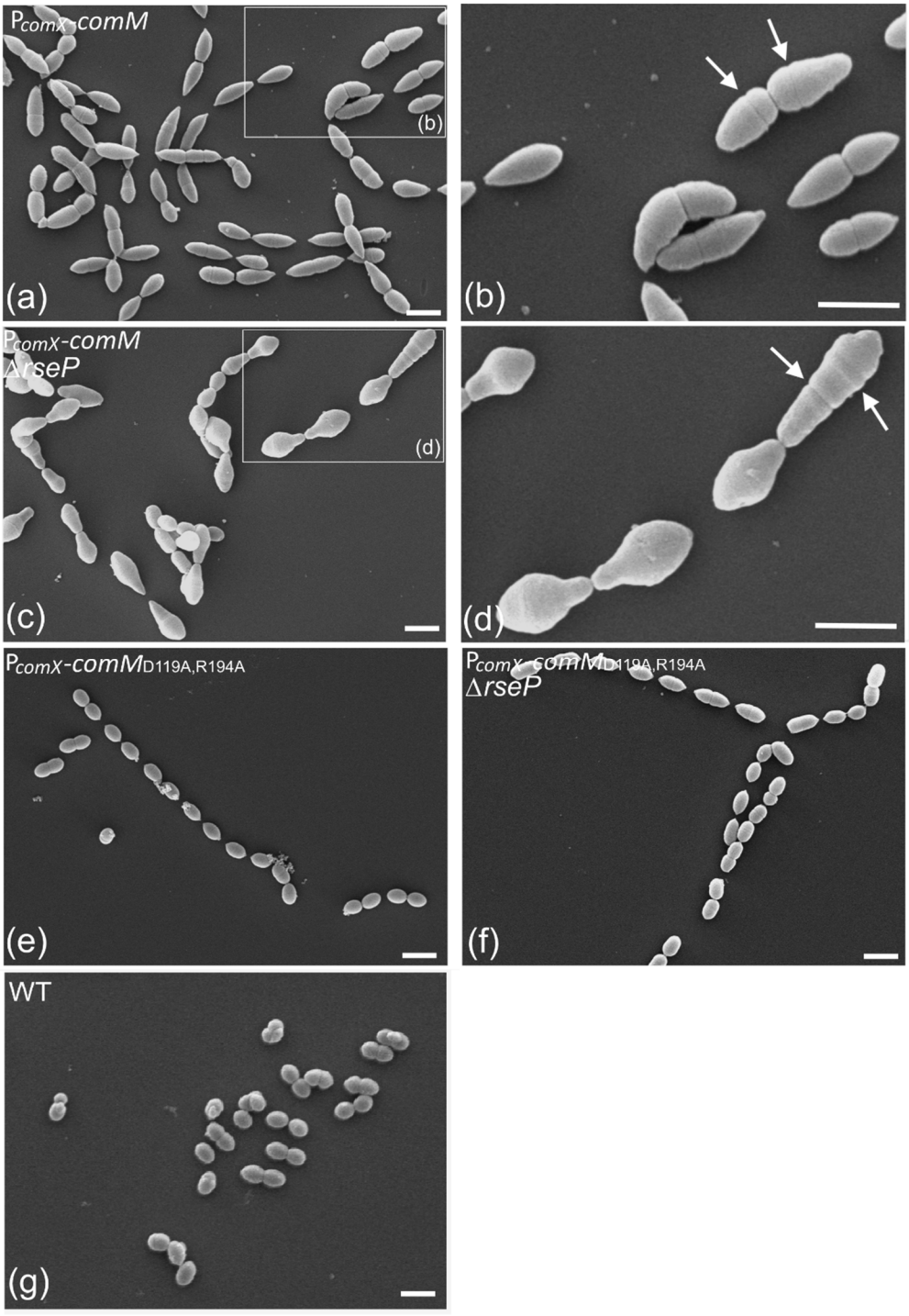
789 Fig. 5



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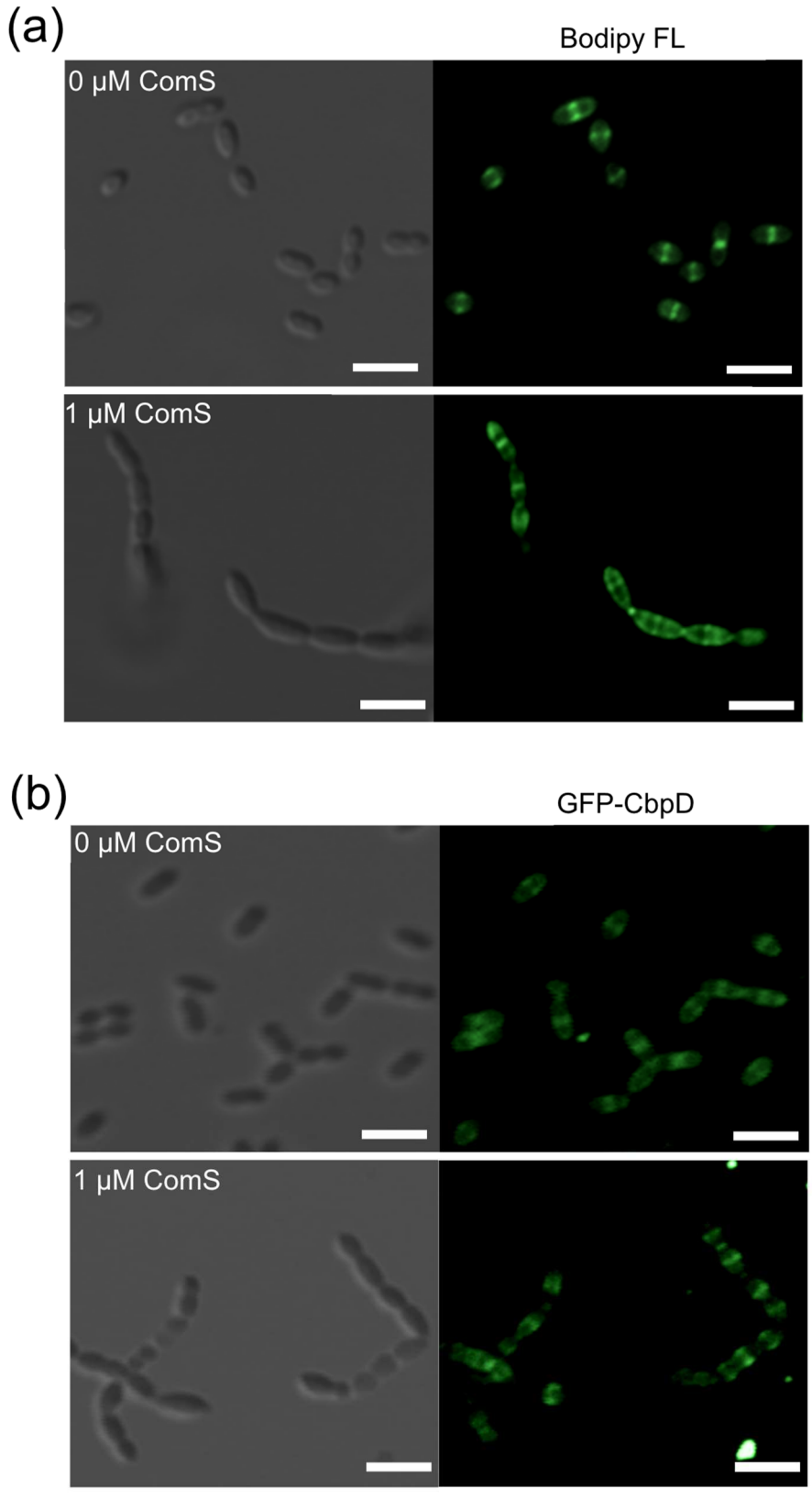
792 Fig. 6



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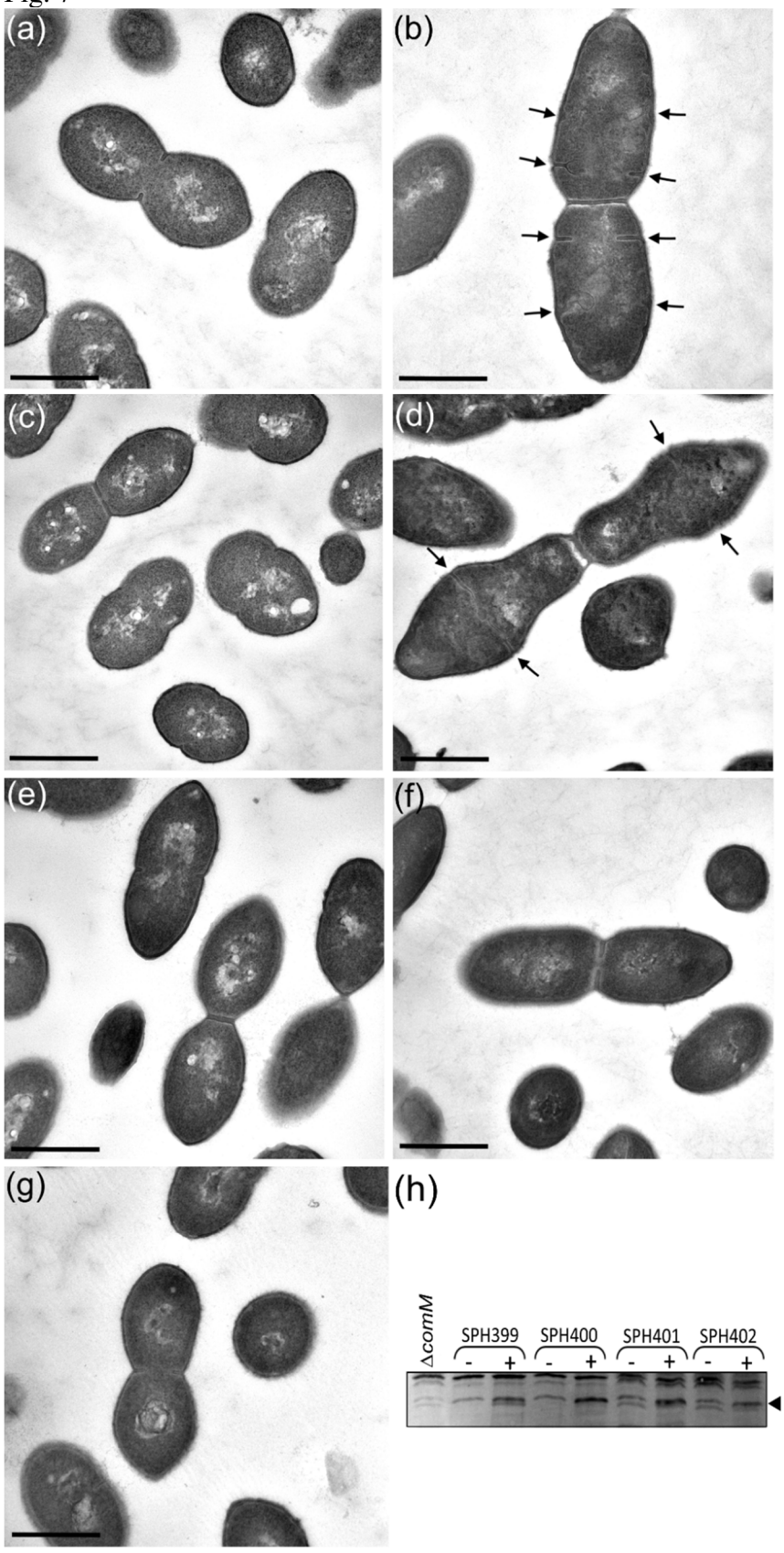
795 Fig. 7



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Supplementary material.

Table S1. *S. pneumoniae* strains used in the present study.

Strains	Relevant characteristics	Source
R704	R6 derivative, *comA*::*ermAM*; Ery^r	J. P. Claverys[a]
RH1	R704, but Δ*ebg*::*spc*; Ery^r Spc^r	(Johnsborg *et al.*, 2008)
RH406	Contains a Δ*comM*::janus cassette.	(Johnsborg *et al.*, 2008)
SPH130	Δ*comA*::*ermAM*, P$_{comX}$-*luc*; Ery^r, Sm^r	(Berg *et al.*, 2011)
SPH131	Δ*comA*::*ermAM,* P1::P$_{comR}$::*comR*, P$_{comX}$::Janus. Ery^r, Kan^r	(Berg *et al.*, 2011)
SPH399	Δ*comA*::*ermAM*, Δ*comM$_{wt}$*, P$_{comX}$-*comM*, Ery^r, Spc^r, Sm^r	This study
SPH400	Δ*comA*::*ermAM*, Δ*comM$_{wt}$*, Δ*rseP*, P$_{comX}$-*comM*, Ery^r, Spc^r, Sm^r	This study
SPH401	Δ*comA*::*ermAM*, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{D119A/R194A}$*, Ery^r, Spc^r, Sm^r	This study
SPH402	Δ*comA*::*ermAM*, Δ*comM$_{wt}$*, Δ*rseP*::janus, P$_{comX}$-*comM$_{D119A/R194A}$*, Ery^r, Spc^r, Kan^r	This study
SPH403	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH404	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{N82A}$*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH405	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{S111A}$*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH406	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{G117L}$*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH407	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{D119A}$*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH408	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{P125A}$*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH409	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{E185A}$*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH410	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{S187A}$*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH411	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{H191A}$*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH412	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{R194A}$*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH413	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{D119A/H191A}$*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH414	Δ*comA*::*ermAM*, Δ*ebg*::*spc*, *hirL*::*lacZ*-Cm^r, Δ*comM$_{wt}$*, P$_{comX}$-*comM$_{D119A/R194A}$*, Ery^r, Spc^r, Cm^r, Sm^r	This study
SPH415	R704, but Δ*ebg*::*spc*, Δ*rseP*::janus; Ery^r, Spc^r, Kan^r	This study
SPH424	Δ*comA*::*ermAM*, P$_{comM}$-*luc, ΔcbpD*; Ery^r, Sm^r	This study
SPH425	Δ*comA*::*ermAM*, P$_{comM}$-*luc, ΔcbpD,* Δ*rseP* ; Ery^r, Sm^r	This study

SPH426	Δ*comA*::*ermAM*, P_{comX}-*luc,* Δ*rseP* ; Ery^r, Sm^r	This study
SPH427	Δ*comA*::*ermAM,* P1::P_{comR}::*comR*, P_{comX}, Δ*rseP*	This study

[a] Gift from J. P. Claverys

Table S2. List of primers used in the present study.

Primer name	Sequence 5'→ 3'	Source
Primers used to amplify Janus		
Kan484.F	GTTTGATTTTTAATGGATAATGTG	(Johnsborg *et al.*, 2008)
RpsL41.R	CTTTCCTTATGCTTTTGGAC	(Johnsborg *et al.*, 2008)
Primers used to amplify P_{comX} and the ΔP_{comX}::Janus amplicon		
khb31	ATAACAAATCCAGTAGCTTTGG	(Berg *et al.*, 2011)
khb33	TTTCTAATATGTAACTCTTCCCAAT	(Berg *et al.*, 2011)
khb34	CATCGGAACCTATACTCTTTTAG	(Berg *et al.*, 2011)
khb36	TGAACCTCCAATAATAAATATAAAT	(Berg *et al.*, 2011)
Primers used to amplify the Δ$comM_{wt}$::janus amplicon		
ComMF	CTGCTCGCCTATTAGATGAC	(Johnsborg *et al.*, 2008)
ComM1R	CCCCACGCTCTTGGCTAC	(Johnsborg *et al.*, 2008)
Primers used to create the P_{comX}-*comM* amplicon		
KHB137 F	ATTTATATTTATTATTGGAGGTTCAATGAAATCAATGAGA ATCTTATTTTTG	This study
KHB138 R	ATTGGGAAGAGTTACATATTAGAAACTAAAAGAAAATGA GCGTAACAATG	This study
Primers used to create point mutations in *comM*		
ds91F E185A	GTCCCAAATTCGGTTAAGAAGGCAGTTTCCTTTTATGGTC ATATTTTC	This study
ds92R E185A	TGCCTTCTTAACCGAATTTGGGAC	This study
ds93F S187A	CAAATTCGGTTAAGAAGGAAGTTGCCTTTTATGGTCATAT TTTCCGAGATC	This study
ds94R S187A	GGCAACTTCCTTCTTAACCGAATTTG	This study
ds101F P125A	GCCTTGAAAAAATACGTGCGC	This study
ds102R P125A	GCGCACGTATTTTTTCAAGGCTAAAAAAATCAGCAAATCT AGCC	This study
ds103F G117L	TTGGCTAGATTTGCTGATTTTTTTACC	This study
ds104 R G117L	GGTAAAAAAATCAGCAAATCTAGCAAAAATAGGAAAAA GAAGGATGGC	This study
R6_comM_N82A_F	GCTTTGTTAGGAGCTGTTCTTGTTTTGT	This study
R6_comM_N82A_R	AACAGCTCCTAACAAAGCAATTAAGAGTA	This study
R6_comM_S111A_F	GCTTTCTTTTTCCTATTTGGGCTAGATTT	This study
R6_comM_S111A_R	TAGGAAAAAGAAAGCTGGCAATAAAAAGTCAACTAA	This study
R6_comM_D119A_F	GCTTTGCTGATTTTTTTACCCTTGAAAAAATAC	This study
R6_comM_D119A_R	GTAAAAAAATCAGCAAAGCTAGCCCAAA	This study
R6_comM_H191A_F	GCTATTTTCCGAGATCTTGTATGGGTCAT	This study
R6_comM_H191A_R	ATGACCAATACAAGATCTCGGAAAATAGCACCATAAAAG GAAACTTCCTTCTTAACC	This study
R6_comM_R194A_F	GCTGATCTTGTATTGGTCATTGTTACG	This study
R6_comM_R194A_R	ACAATGACCAATACAAGATCAGCGAAAATATG	This study
Primers for creating the P_{comM}-*luc* amplicon		
gs531	TATGTTTTTGGCGGATCTCATCTTCTCTCTCCCTTCCTACC	This study

gs532	GCGGAAAGCCCAAATTGTAAAGAAAGCCTGTTTTTTATG GATG	This study
KHB78 fwd *luc*	ATGAGATCCGCCAAAAACATA	This study
gs64 rev *luc*	TTACAATTTGGGCTTTCCGC	This study

References.

Berg, K. H., Biørnstad, T. J., Straume, D. & Håvarstein, L. S. 2011. Peptide-regulated gene depletion system developed for use in *Streptococcus pneumoniae*. *J Bacteriol,* 193**,** 5207-15.

Johnsborg, O., Eldholm, V., Biørnstad, 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**,** 245-53.

Amino acid sequence alignment of ComM with homologous proteins found in other streptococcal species. The transmembrane segments predicted for the R6 ComM are highlighted in yellow.

```
ComM  R6                ----------------------------------------MKSMRILFLLALIQISLSSCF      21
S.mitis                 ----------------------------------------MKSMRVLFLLALIQISLSSCF      21
S.pseudopneumoniae      ----------------------------------------MKSMRILFLLVLIQISLSSCF      21
S.oralis                MHKRAVFWSVEHKKRTLEAETIISNSSQGIIPYREGKEMVKSIRLLLLIAVIQISFSSCL      60
S.tigurinus             ----------------------------------------MKSIRVLLLLAVAQILFGSCL      21
Streptococcus sp. C300  ----------------------------------------MVKSIRLLLLIAVIQISFSSCL      22
S.infantis              ------------------------------------MEKVIKSIRLLLFFALIQLALCSCI      25
S.parasanguinis         ------------------------------------MEKMNKSIQLLLLISLVYLGCCSCL      25
S.anginosus             ------------------------------------MRKIKNILQMSLLIVLTQITLVLLT      25
S.intermedius           ------------------------------------MKNIKNILQMSLLIVLTQITLVLLT      25
S.sanguinis             ------------------------------------MLKLEKIIQLLLLAMLTQTGLLLML      25
                                                               : ::: ::  :

ComM  R6                LWKECILSFKQSTAFFIGSMVFVSGICAGVNYLYTRKQEVHSVLASK---KSVKLFYSML      78
S.mitis                 LWKECILSFKQSTAFFIGSMVFVSGICAGVNYLYTRKQEVHSVLASK---KSVKLFYSIL      78
S.pseudopneumoniae      LWKECILSFKQSTAFFIGSMVFVSGICAGVNYLYTRKQEVHSVLASK---KSVKLFYSML      78
S.oralis                LWKESFLSLKQTNAYFLILIVGISVLCAGINYFHTADQSRHSILHVQ---KKVSLVYCLL      117
S.tigurinus             LWKESFLSLREGNVYFLILIVGVSGFCAGINYFHTLGQKSHSILRVQ---KKVSLVYILL      78
Streptococcus sp. C300  LWKESFLSLKQTNAYFLILIVGISVLCAGINYFHTADQSRHSILHVQ---KKVSLVYCLL      79
S.infantis              FWSGTSLALKQSCFYFLLALLSLSGSCAFMHYLSSHNLKRDRLIDRS---RFIFLFYSMM      82
S.parasanguinis         VWPGIALTLKQSCFYFLLLLMLLAGICTFIHYLSGHDHVNSQLHNRS---RSFFLLYSSM      82
S.anginosus             TPLPKSLTFQQSSFVFLFILFFAGLLYFR--YFSRELTNFKSEILTARYWPLLRLSYLMM      83
S.intermedius           TPLSKSLTFQQSSFVFLFILFFCGLLYFR--YFSRELTDFKSEILVARYWPLLRFSYLMM      83
S.sanguinis             HPMPHRLVFSQANLLFMVGLLGLLFCCAF--YFSRELQEIKGSLRQSSNYRHLLFLYFLM      83
                              * : :    *:  :.          *:                  . : *  :

ComM  R6                LLINLLGAVLVLSDNLFIKNTLQQELVDFLLPSFFFLFGLDLLIFLPLKKYVRDFLA-ML      137
S.mitis                 LLINLLGAVLVLSDNLFIKNTLQQELVDFLLPSFFFLFGLDLLIFLPLKKYMRDLLA-ML      137
S.pseudopneumoniae      LLINLLGAVLVLSDNLFIKNTLQQELVDFLLPSFFFLFGLDLLIFLPLKKYMRDLLA-ML      137
S.oralis                LVVNLLATCLVLSESIQTTSKLQQELVDLFLPSFFFLLGVDLLIFLPFDKIFRDIEN-HL      176
S.tigurinus             LVVNLLATCLVLTESIQTTSKLQQDLVDLFLPSFFFLLGVDLWIFLPFDKYIRDMDN-HL      137
Streptococcus sp. C300  LVVNLLATCLVLSESIQTTSKLQQELVDLFLPSFFFLLGVDLLIFLPFDKIFRDIEN-HL      138
S.infantis              IVVNLCGVGLVLSDTIVTVNLLQKEAVDLILPSFFFLFGVDLVTFLPLKKWRRLQKN--S      140
S.parasanguinis         VIINVAGVCFILFESISTDTLLQKEIVDLLLISFFFLFGLDIATFLPLNKWRKLPTH--K      140
S.anginosus             VFINAIGAYLMILEGMTAASEGQQLLLSLFVHSSLFLLGVDIVALVPELRSFIIHFFVSK      143
S.intermedius           VFINAIGAYLMIVEGLAEVSESQKLFMSLFIHSSLFLLGVDVVALVPEVRSYVVRFLFLK      143
S.sanguinis             ILVNAAGVLLLCGKEAQSGQAVEQMLTESFLSSSLLLLGIDIAVLSPAAPKFVDSDASLR      143
                        :.:*  .. ::  .        ::   . :: * ::*:*:*:  : *

ComM  R6                DRKKTVLVTILATLLFLRNPMTIVSLLIYIGLGLFFAAYLVPNSVKKEVSFYGHIFRDLV      197
S.mitis                 DRKKTVLVTILATLLFLRNPMTIVSLLMYIGLGLFFAAYLVPNSVKKEVSFYGHIFRDLV      197
S.pseudopneumoniae      DRKKTVLVTILATLLFLRNPMTIVSLLMYIGLGLFFAAYLVPNSVKKEVSFYGHIFRDLV      197
S.oralis                NKKKTVVISVLATMVFLRNPLVISSIFFYISVGFLCARFLFPKCIQREISFYGHLIRDIL      236
S.tigurinus             NKKRTVVISVLGTIIFLRNPVTISSILLYIGLGFLCARFLFPKSVQREISFYGHVIRDVL      197
Streptococcus sp. C300  NKKKTVVISVLATMVFLRNPIVISSILLYISVGFLCARFLFPKCIQREISFYGHLIRDIL      198
S.infantis              SSKEVRFSIIISILIFLRNPITILSIAFYIGLGALFLSFLFPKNLRQEVSFYSHLIRDIL      200
S.parasanguinis         KTGRLTT-LIFLSLFFLHNPLTIFSVSFYILLGVSFLNFLFPKTLRQDLSFYSHLIRNIL      199
S.anginosus             DQARTSFVIGSLLFILLRNPADLTCFIVYTGLGSLLSFLISKPTIRLEFSIFSHLVRNSF      203
S.intermedius           GQARASFIIGSLIFIVLRNPADLVCFIIYTGLGLLLSFIIPKSSLRLEFSVFSHLVRNSV      203
S.sanguinis             YRKSWGIALGLLCFSLAKNPQETMCFLSYLVLGLVFAHLLRPYFQRLELSMLAHILRNMI      203
                                  : . :**    ..  *  :*      :     : :.*. .*:.*: .

ComM  R6                LVIVTLIFF    206
S.mitis                 LVIATLIFF    206
S.pseudopneumoniae      LVIATLIFF    206
S.oralis                FVFSIVVFF    245
S.tigurinus             FVICIFTFF    206
Streptococcus sp. C300  FVFSIVVFF    207
S.infantis              FVVCTFLLW    209
S.parasanguinis         LVLS-----    203
S.anginosus             SLLFLIIFW    212
S.intermedius           SLIFLLIFW    212
S.sanguinis             ILSLLPFCF    212
                         :
```

Multiple sequence alignment of ComM against CAAX proteins found in other streptococcal species. The motifs $EE(X)_3R$, $F(X)_3H$ and $H(X)_3N/D$ found to take part in the catalytic reaction of eukaryotic CAAX proteases are highlighted in cyan.

```
S.pneumoniae            ------------------MKEKNMWKELLNRAGWILVFLLAVLLYQVPLVVTS---ILTL       39
S.mitis                 -------------------------MTCWKRLMWGGCAFLALALYVLPMVFQQ---KAET       32
S.sanguinis             MLAVKNCLYKKRKLSYTRKEKEEDMKAILKKLEYILLTLFVLFLSQIPFIFIR---QM--       55
S.gordonii              -------------------------MTCWKRLMWGGCVFLALALYVLPMVFQQ---KAET       32
S.oligofermentans       -------------------------MTCWKRLIWVGCAFLALGLYVLPMLFQQ---VAII       32
S.cristatus             -------------------------MTWLKRLVWVGCAFLAMALYVLPMLFQQ---IAII       32
Streptococcus sp.DD04   -------------------------MTWWRRLIWVGCVFLALALYMLPMLSQQ---LAIT       32
S.sinensis              -------------------------MTWWRRLIWVGCVFLALALYMLPMLFQQ---LAIT       32
Streptococcus sp.DD11   ------------------------------------------------------------       0
S.dentasini             ------------------------MKGILKKLIAAIAIIGLFVVSQTPDLLMIIWKKENP       36
S.australis             ------------------MMENWMEKKWVSNGLWLLIGIPLLLATQLPMVALIL----GI       38
ComM R6                 --------------------------------MKSMRILFLLALIQISLSSCFLWKECIL       28


S.pneumoniae            K-EV---ALLQSGLIVAGLSIVVLALFIMGARKTKLASFNFSFFRAKDLARLGLSYLVIV       95
S.mitis                 Y-QILKQWTIGIGILL--IF-LALLVFIVVAKKIGILSQSGKVFQKGDGKRISLSILGMF       88
S.sanguinis             T-SSEKSFSAGQTIFVLVVYLLIVFFVLRMAKQEELLSLDLSFFKWSSFGWLAVSNVVMI       114
S.gordonii              Y-QISKQWTIGIGILL--IF-LALLIFIVVAKKIGILSQSGKFFQKGDGKRISLSILGMF       88
S.oligofermentans       Y-QFPKQWTIDLGLLL--II-LILLVFIVVAKKTGILSPSGKIFQKGDGKRIALGLLGML       88
S.cristatus             Y-QFPKQWTIGLGLLL--II-LILLVFVVVAKKTGILSPSGKIFQKGDGKRIALGLLGMI       88
Streptococcus sp.DD04   Y-QFPKQWSIGLGLLL--IF-LTLLVFVAVAKKAGILSQSGKIFQKGDGKRIALGLLGML       88
S.sinensis              Y-QFHKQWTIGLGLLL--IF-LTLLVFVVVAKKAGILSQSGKIFQQGDGKRIALGLLGML       88
Streptococcus sp.DD11   -----------------------------MAKQEGLLSLDFSFFGWSSVGWLALSYVMMF       31
S.dentasini             K-LDTWQWLL----IMILQIIVVIGFYLLARRKELISSGVKHWLSWKTFTVVSLGFIALF       91
S.australis             E-NAWSNWTINSLILG--VTMLLVYLLWWFMKWSPLDPLDFSRITGRDIGRNFLYFLLLL       95
ComM R6                 SFKQSTAFFIGSMVFVSGICAGVNYL---YTRKQEVHSVL------ASKKSVKLFYSMLL       79
                                                    :   :               :    :.


S.pneumoniae            GSNILGSILLQL-SNETTTANQSQINDMVQNSSLISSFFLLALLAPICEEILCRGIVPKK       154
S.mitis                 LISILGTALLRWLNGEVTTANQASLIEEFKRGNGILLPIMLGVLAPVVEEIIFRGILPLK       148
S.sanguinis             GVNMLGAIIMLLEGQAISTANQDALNALFQHVPKILLVVGAVIQAPILEEVVFRGLIPQK       174
S.gordonii              LISILGTALLRWVNGEVTTANQASLIEEFQRGNGILLPIILGVLAPIVEEIIFRGILPLK       148
S.oligofermentans       LISVLGTVLLRWLHGEATTANQASLMEEFRRGDIILLSIMLGVLAPIAEEIIFRGIIPLK       148
S.cristatus             LISVLGTVLLRWLHGEVTTANQASLMEEFRRGDIILLSIMLGVLAPIAEEIIFRGIIPQK       148
Streptococcus sp.DD04   LISVLGTVLLRWLHGEVTTANQASLMEEFRRGDMISFPIMLGVLAPIAEEIIFRGIIPLK       148
S.sinensis              LISVLGTVLLQWLHGEVTTANQASLMEEFRRGDMISFPIMLGVLAPIAEEIIFRGIIPLK       148
Streptococcus sp.DD11   GVSILGIVIMMMEGQGIDTANQEALKQMFKNVPSILLVMGAVIQAPILEEVAFRGLIAEK       91
S.dentasini             IIKLVGGIILTL-EGKTSTNNQEMINQLFENSSLLVMFMTIVIIAPLTEELIFRGLIPKL       150
S.australis             ANNIVGATVLRN-IGETTTANQETIQGLSSLAPQLAMGLLIVVVAPLGEEIICRAVIPRL       154
ComM R6                 LINLLGAVLVLSDNLFIKNTLQQELVDFLLPSFFFLFGLDLLIFLPLKKY--VRDFLAML       137
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S.pneumoniae            IFRGKENLGFVVGTIVFALLHQPSNLPSLLIYGGMSTVLSW-TVYKTQRLEMSILLHMIV       213
S.mitis                 IFKGYEGWGYIVGGLLFALFHGPTNIVSFVIYGGSSVILTL-LAYRTRRLEVSIAVHMIN       207
S.sanguinis             IFTKHYVWGLVVGVILFGLFHGPTNIGSFVIYAGMGAVLAA-VAYIFKRLEMSILAHMLR       233
S.gordonii              IFKGYEGWGYIVGGLLFALFHGPTNIMSFVIYGGSSVILTL-LAYRTRRLEVSIAVHMIN       207
S.oligofermentans       IFKGYESWGYIIGGLLFAIFHGPTNIMSFVIYGGASVILTL-LACRTRRLEVSIAVHMIN       207
S.cristatus             IFKGYESWGYIIGGLLFAIFHGPTNIMSFVIYGGASVILTL-LAYRTRRLEVSIAVHMIN       207
Streptococcus sp.DD04   IFKGYESWGYIIGGLLFAIFHGPTNIMSFVIYGGSSVILTL-LAYRTRRLEVSIAVHMIN       207
S.sinensis              IFKGYESWGYIIGGLLFAIFHGPTNIMSFVIYGGASVILTL-LAYRTRRLEVSIAVHMIN       207
Streptococcus sp.DD11   IFAKHSIWGLLVSSILFGLFHGPTNIGSFVLYAGIGGVLAF-VVYISKRLEMAVLAHMLR       150
S.dentasini             FSKRFEGLGFAVGALLFGLLHGPSDIGSFVLYVGMGAVLAV-ICYRFKHLEYSILTHALN       209
S.australis             IFKGHEKIGYLVGALVFAYLHTPSNLGSWIIYGGMSLILTW-VAYRYKRVEYSILLHFTM       213
ComM R6                 -DRKKTVLVTILATL--LFLRNPMTIVSLLIYIGLGLFFAAYLVPNSVKKEVSFYGHIFR       194
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S.pneumoniae            NGIAFCLLALVVIMSRTLGISV       235
S.mitis                 NGLPAIIMLLIGIFGMEV----       225
S.sanguinis             NGVAVLIMILTGLVNK------       249
S.gordonii              NGLPAIIMLLIGIFGMEV----       225
S.oligofermentans       NGLPAILMLLIPILGVEV----       225
S.cristatus             NGLPAILMLLIPILGVEV----       225
Streptococcus sp.DD04   NGLPAILMLLITIFGVEV----       225
S.sinensis              NGLPAILMLLIPIFGVEV----       225
Streptococcus sp.DD11   NGVAALLMLLMS----------       162
S.dentasini             NALGFAALLISHLMGS------       225
S.australis             NAFAFLITILVSFLPA------       229
ComM R6                 DLVLVIVTLIFF----------       206
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Transcription from the P_{comX} promoter of the ComRS system is not influenced by deletion of the *rseP* gene.

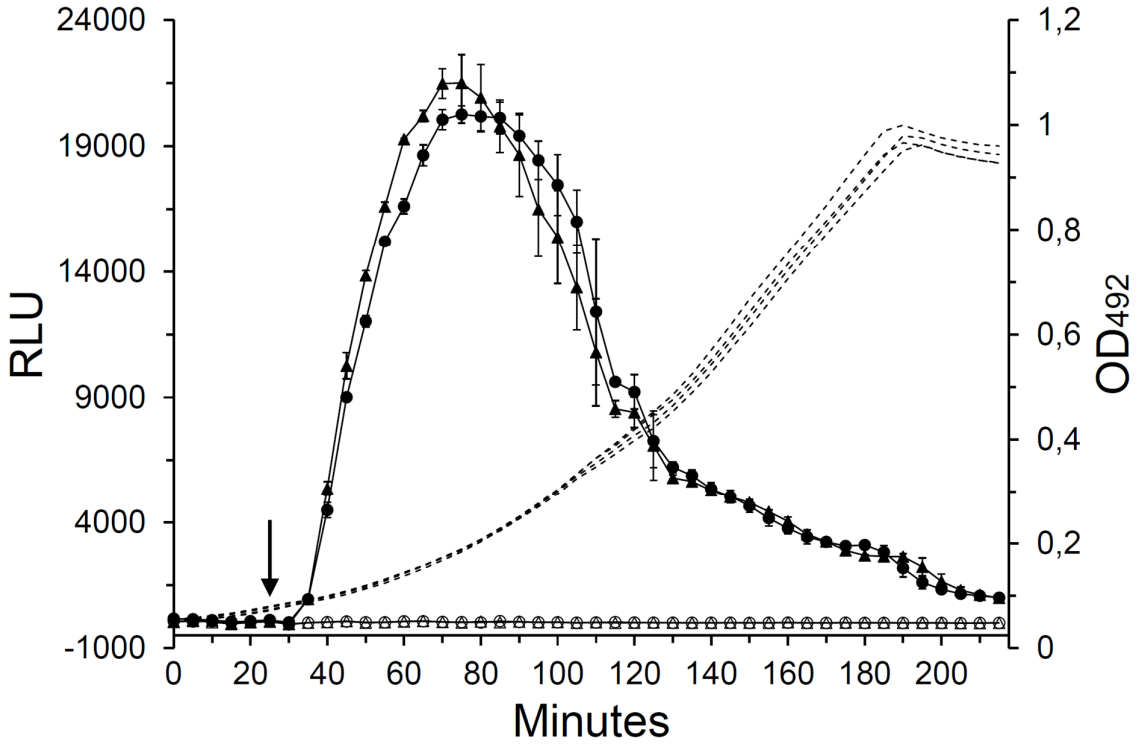

Fig S1. *S. pneumoniae* strain SPH130 (circles) and SPH426 (Δ*rseP* [triangles]) contain the *luc* reporter gene behind P_{comX} of the ComRS system. Filled symbols represent the relative luminescence measured for cells induced with a final concentration of 1 µM ComS, while open symbols represent the luminescence of non-induced cells. An arrow indicates when ComS was added to the cells. Growth curves are shown as dotted lines.

Overexpression of ComM$_{D119A, R194A}$ does not inhibit growth of *S. pneumoniae*.

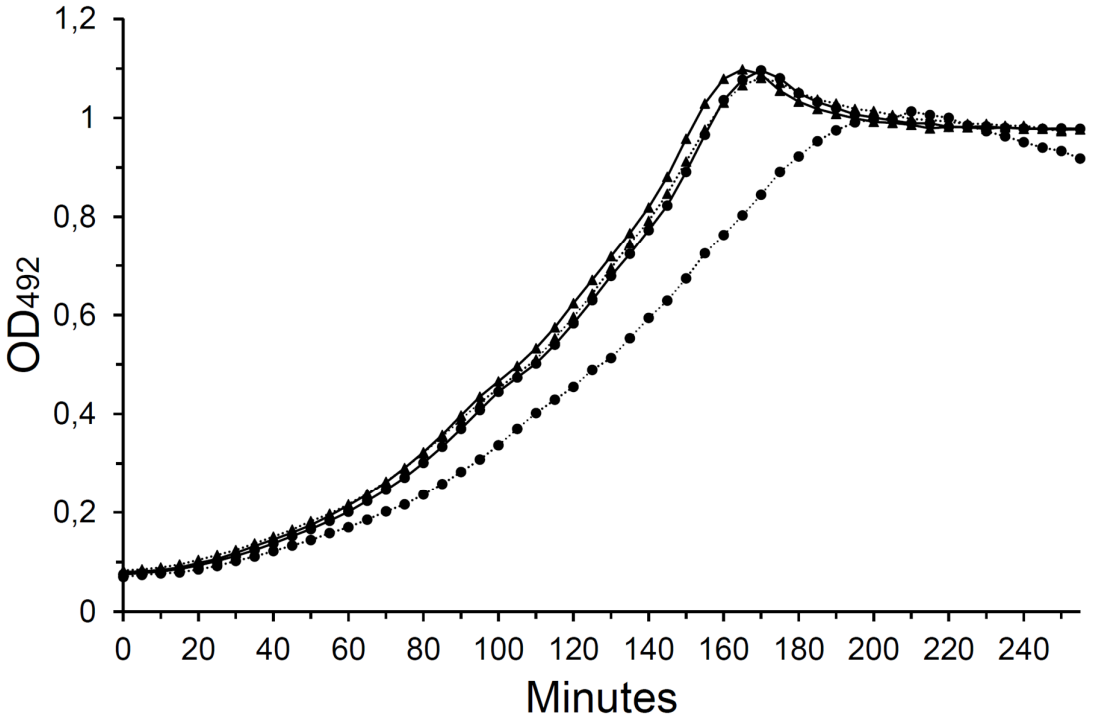

Fig S2. Growth curves of *S. pneumoniae* overexpressing ComM or the attenuated ComM$_{D119A,R194A}$ version. Strain SPH399 (●) and SPH401 (▲) were grown in the presence of 0 µM (solid lines) and 1 µM (dotted lines) ComS inducer.