J. Bacteriol. doi:10.1128/JB.00780-18

Refining the pneumococcal competence regulon by RNA-sequencing

Jelle Slager[1], Rieza Aprianto[1] and Jan-Willem Veening[1,2]

[1] Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, Centre for Synthetic Biology, University of Groningen, Nijenborgh 7, 9747 AG Groningen, the Netherlands

[2] Department of Fundamental Microbiology, Faculty of Biology and Medicine, University of Lausanne, Biophore Building, CH-1015 Lausanne, Switzerland

Abstract

Competence for genetic transformation allows the opportunistic human pathogen *Streptococcus pneumoniae* to take up exogenous DNA for incorporation into its own genome. This ability may account for the extraordinary genomic plasticity of this bacterium, leading to antigenic variation, vaccine escape, and the spread of antibiotic resistance. The competence system has been thoroughly studied and its regulation is well-understood. Additionally, over the last decade, several stress factors have been shown to trigger the competent state, leading to the activation of several stress response regulons. The arrival of next-generation sequencing techniques allowed us to update the competence regulon, the latest report of which still depended on DNA microarray technology. Enabled by the availability of an up-to-date genome annotation, including transcript boundaries, we assayed time-dependent expression of all annotated features in response to competence induction, were able to identify the affected promoters and produced a more complete overview of the various regulons activated during competence. We show that 4% of all annotated genes are under direct control of competence regulators ComE and ComX, while the expression of a total of up to 17% of all genes is, either directly or indirectly, affected. Among the affected genes are various small RNAs with an as-yet-unknown function. Besides the ComE and ComX regulons, we were also able to refine the CiaR, VraR (LiaR) and BlpR regulons, underlining the strength of combining RNA-seq with a well-annotated genome.

Importance

Streptococcus pneumoniae is an opportunistic human pathogen responsible for over a million deaths every year. Although both vaccination programs and antibiotic therapy have been effective in prevention and treatment of pneumococcal infections, respectively, the

sustainability of these solutions is uncertain. The pneumococcal genome is highly flexible, leading to vaccine escape and antibiotic resistance. This flexibility is predominantly facilitated by competence, a state allowing the cell to take up and integrate exogenous DNA. Thus, it is essential to obtain a detailed overview of gene expression during competence. This is stressed by the fact that several classes of antibiotics can lead to competence. Previous studies on the competence regulon were performed with microarray technology and limited to an incomplete set of known genes. Using RNA-sequencing, combined with an up-to-date genome annotation, we provide an updated overview of competence-regulated genes.

Introduction

Streptococcus pneumoniae (the pneumococcus) is a mostly harmless human commensal found in the nasopharynx. However, when the pneumococcus leaves the nasopharynx and ends up in other niches, it can cause severe diseases, such as sepsis, pneumonia and meningitis (1). Especially among individuals with an underdeveloped or weakened immune system, these diseases lead to over a million deaths per year (2). Although both vaccination and antibiotic therapy have been used successfully for prevention and treatment of infections, respectively, the pneumococcus remains a threat to human health. This persistence is largely due to the remarkable genomic plasticity of the pneumococcus, allowing the acquisition of antibiotic resistance and evasion of the host immune response. Horizontal gene transfer, underlying the vast majority of such diversification strategies, is facilitated by pneumococcal competence. The competent state allows cells to take up exogenous DNA and integrate it into their own genome (i.e. transformation). During competence, various functionalities are activated, including DNA repair, bacteriocin production and several stress-response regulons (3, 4). This diversity of activated functions is relevant in light of the fact that a broad

spectrum of antimicrobial compounds (causing various forms of stress) can induce competence development (5–7), through at least three distinct mechanisms: HtrA substrate competition (8, 9), *oriC*-proximal gene dosage increase (6) and chaining-mediated autocrine-like signaling (7). Other parameters that affect competence development include pH, oxygen, phosphate and diffusibility of the growth medium (10–12). The fact that various forms of stress induce competence, including several stress-response regulons, has led to the hypothesis that competence in the pneumococcus may function as a general stress response mechanism (13, 14).

Among the genes activated during competence are the CiaR and VraR (LiaR) regulons. Although the underlying molecular mechanisms of activation are unknown, both regulons have been associated with cell wall damage control (3, 15). Indeed, a growth lag during competence (4) and the reduced fitness of both *ciaR* and *vraR* mutants (3, 15) indicate that competence represents a significant burden for a pneumococcal cell. It seems plausible that the production and insertion of the DNA-uptake machinery (16) into the rigid cell wall has a significant impact on cell wall integrity. The CiaR regulon seems to be responsible for resolving such issues and preventing subsequent lysis (3, 17). An additional dose of competence-related cell wall stress is caused by fratricide, where competent cells kill and lyse non-competent sister cells and members of closely related species. Specifically during competence, pneumococci produce a fratricin, CbpD, and the corresponding immunity protein, ComM (18). Secreted CbpD, aided by the action of autolysins LytA and LytC, can kill non-competent, neighboring cells, which then release their DNA and other potentially valuable resources. Eldholm et al. showed that the VraR regulon represents a second layer of protection, on top of ComM, by which competent cells prevent CbpD-mediated lysis (15).

ComM is also crucial in causing cell division arrest during competence by inhibiting initiation of division and by interfering with the activity of StkP (19).

The activation of competence (**Figure 1A**) depends on the action of two key transcriptional regulators, ComE and ComX. The competence regulon is divided into early (i.e. ComE-dependent) and late competence (i.e. ComX-dependent) genes. Specifically, early competence involves, among others, the *comCDE* and *comAB* operons. A basal expression level of *comCDE* (20) ensures the production of the small peptide ComC, which contains a double-glycine leader and is processed and exported into the extracellular milieu by the bipartite transporter ComAB (21, 22). The resulting 17-residue matured peptide is referred to as competence-stimulating peptide (CSP) (23) and can interact with ComD, the membrane histidine kinase component of the two-component system ComDE (24). Upon CSP binding, ComD autophosphorylates and, subsequently, transfers its phosphate group to its cognate response regulator ComE (25). Finally, phosphorylated ComE dimerizes and binds specific recognition sequences to activate the members of the early-*com* regulon (26, 27). This regulon contains both aforementioned *comAB* and *comCDE* operons, creating a positive-feedback loop that self-amplifies once a certain threshold of extracellular CSP is reached. Since CSP can interact with ComD on the producer cell as well as on other pneumococcal cells, competence is a quorum-sensing system. Although cell-to-cell contact is not required for the spread of competence in a population (7, 28), it does lead to more efficient signaling (29) and larger recombination events (30).

Additional members of the early-*com* regulon are *comX1* and *comX2*, two identical genes that encode the alternative sigma factor ComX (σ^X) (31). The rapid accumulation of ComX during early competence leads to the activation of promoters with a ComX-binding motif, resulting in the expression of the late-*com* regulon (3, 4, 32, 33). Finally, within 20

minutes after the initiation of competence, the process is largely shut down through a combination of different mechanisms (**Figure 1B**; (34–36)). Early-*com* genes are repressed by late competence protein DprA and unphosphorylated ComE (27). As a second layer of control, sigma factor competition between ComX and RpoD (σ^A; induced during late competence) is probably responsible for the shutdown of late-*com* genes (36).

While the backbone of this regulatory system is quite well-understood, there are many other factors that complicate the matter, including the system's sensitivity to growth medium acidity and potential sRNA-mediated control of ComC expression (37).

To fully understand the implications of competence activation in the pneumococcus, it is important to know which genes are, directly or indirectly, differentially expressed during competence. Several comprehensive studies, based on DNA microarray technology, have been performed to determine the competence regulon, resulting in more than 100 reported competence-associated genes (3, 4, 38). All of these studies showed a high level of agreement on a certain core regulon, but discrepancies remained. Moreover, the recent identification of early-competence protein BriC (39, 40) illustrates that the description of the competence regulon can still be refined. In order to generate a more complete and nuanced overview of the competence regulon, we utilized data from PneumoExpress (39), a resource containing data on the pneumococcal transcriptome in various infection-relevant conditions. We used RNA-seq data sets from *S. pneumoniae* D39V (41) cells just prior to (t=0) and 3, 10 and 20 minutes after the addition of exogenous CSP. More importantly, compared to previous genome-wide assays of the competence regulon, which were based on DNA microarrays, our data set has higher sensitivity and precision and has a larger dynamic range (42). Secondly, the recent reannotation of the pneumococcal genome has revealed previously non-annotated protein-encoding sequences and small RNAs (41). DNA microarray studies are limited to the

target sequences present on the array and a new data set was therefore required to obtain information on the expression of these new elements. Finally, the new annotation also contains information on transcription start sites (TSSs) and terminators (41), which allows both for a more accurate search of transcription regulatory motifs (e.g. ComE- or ComX-binding sites) and for the integration of operon information into the interpretation of transcriptome data.

As expected, our results largely confirmed previous microarray-based studies and we observed distinct time-dependent expression patterns of ComE- and ComX-regulated genes. In addition, we provide an overview of the transcription start sites most likely to be responsible for the observed transcriptome changes, adding up to, among others, 15 ComE-regulated, 19 ComX-regulated, 18 CiaR-regulated and 4 VraR-regulated operons. We identified 7 new non-coding RNAs, affected by several regulators, among the differentially expressed genes, but their role in competence requires future studies.

Results

Competence induction disrupts the pneumococcal transcriptional landscape

Differential gene expression analysis revealed that many genes (13-17%; from gene-based or promoter-based analysis, respectively) are affected by the induction of competence (**Figure 2**): a total of 288 genes undergo a change in expression of more than twofold. Out of these, 192 genes are exclusively upregulated, 94 are exclusively downregulated and 2 genes are upregulated at one time point and downregulated at another. When using a stricter fold change cutoff of fourfold, 141 genes are still significantly affected, 119 of which are up- and 22 are downregulated. As can be seen in **Figure 2**, upregulated genes tend to be affected

more strongly and consistently, while not a single gene is significantly downregulated in all three time points.

Identification of ComE-, ComX- and CiaR/VraR-regulated WGCNA clusters

Weighted gene co-expression network analysis (WGCNA; see **Materials and Methods**) of all genes, based on their regularized log (rlog) expression levels across the 22 conditions included in PneumoExpress (39), yielded 36 clusters (**Table S2**). Using these results, we verified whether some of the clusters corresponded to specific regulons known to be affected during competence. Indeed, one of these clusters (cl. 29, n=26) contained 20 out of 25 genes that have been previously reported to be regulated by ComE (3, 4, 38), including *briC* (SPV_0391), which was only recently identified as a member of the competence regulon (39, 40). Aggarwal et al. showed that the double-glycine peptide encoded by *briC* is only conserved in pneumococci and related streptococci and is important for late biofilm development (40). The authors also found that BriC is secreted (in part by ComAB), but its exact mode of action is yet to be elucidated.

The five ComE-regulated genes that did not end up in this cluster include *blpA*, *blpY*, *blpZ* and *pncP* (SPV_0472-75), which are part of the BlpR regulon and whose promoters are likely to have lower affinity for ComE (43, 44). The fifth off-cluster ComE-regulated gene is *ybbK* (SPV_1984). A second cluster (cl. 11, n=56) contained 41 out of 51 members of the reported ComX regulon (3, 4, 38), confirming the power of the WGCNA approach, while simultaneously highlighting the general reliability of previous descriptions of the competence regulon. Less clearly, 13 out of 32 known CiaR-regulated genes (37, 45, 46) and 5 out of 14 VraR-regulated genes (15) clustered together (cl. 33, n=22). The fact that genes from the CiaR and VraR regulon cluster less clearly may, in part, be explained by the more diverse

nature of their regulation. For example, the heat-shock *hrcA-grpE* operon is not only regulated by VraR, but also by HrcA itself, accounting for a different expression dynamic across the diverse conditions sampled for PneumoExpress (39). Additionally, the two TSSs of *tarIJ-licABC* (SPV_1127-23) (37) and the downregulatory effect of CiaR on the *manLMN* operon (SPV_0264-62) (46) may prevent clear clustering.

Time-resolved expression profiles of several regulons during competence

We visualized the typical time-resolved expression patterns of the various regulons, where we plotted the fold changes, relative to t=0, of all genes that were i) previously reported to be activated by the corresponding regulator, and ii) fell into the associated WGCNA cluster (see above). We refer to these sets of genes as 'high-confidence (HC) members' of their respective regulons. It is clear from these plots that ComE-regulated genes peak early and rapidly drop in expression level afterwards (**Figure 3**, top left). This is in line with previous studies, which showed that early competence is actively shut down through the action of late-competence protein DprA. By specifically binding to active, phosphorylated ComE, DprA causes a shift towards a state where regulated promoters are, instead, bound by dephosphorylated ComE, leading to a shutdown of transcription (**Figure 1B**; (36, 47)).

Similar to the ComE regulon, the expression of ComX-regulated genes also increases rapidly, with high fold changes after 3 min. However, unlike the ComE regulon, the expression of these genes remains stable for a longer period of time, with most still increasing their level until 10 min. after CSP addition (**Figure 3**, top right). The following decrease in expression level is, in part, indirectly linked to the shutdown of early competence, since both production and stabilization by ComW (34) of ComX depend on the activity of phosphorylated ComE. However, a recent modelling approach suggested that another

shutdown mechanism was required to explain the observed rate of late competence shutdown (36). The authors argue, convincingly, that competition between sigma factors ComX (σ^X) and RpoD (σ^A) for interaction with RNA polymerase and/or stabilizing factor ComW would be a suitable explanation for the discrepancies between the model and experimental data. Indeed, the fact that *rpoD* transcription is upregulated up to tenfold during competence (3, 4) would make this a credible hypothesis, although *rpoD* upregulation could also simply serve to restore the expression levels of RpoD-controlled genes.

Constituting a more indirect consequence of competence induction, the CiaR-mediated response is generally weaker and delayed, compared to the ComE and ComX regulons (**Figure 3**, bottom left). Interestingly, the activation of this regulon also seems to be quite transient, with a fast drop in expression from 10 to 20 min. after CSP addition.

Finally, the expression profile of all reported VraR-regulated genes (15), regardless of their clustering behavior throughout infection-relevant conditions, was similar to that of the CiaR regulon (**Figure 3**, bottom right).

Shared expression trends within operons allow switching from gene to promoter level analysis of transcriptional regulation

Transcriptome studies are typically performed on a per-gene level, reporting for each individual gene whether or not it is differentially expressed between the studied conditions. That type of information is certainly relevant when trying to assess what changes occur in a cell or population when confronted with a certain change in environment or identity. However, to find out *how* these changes have come about, it is also interesting to consider which transcripts or, rather, which promoters have been affected. To this end, we used our previously created map of the pneumococcal transcriptional landscape (39, 41), where

possible, to identify which promoters are responsible for the observed differential expression of individual genes. As an example, we highlight a cluster of 22 genes (SPV_0192-213), encoding 21 ribosomal proteins and protein translocase subunit SecY (**Table 1**). Although expression of the entire operon was reported to be downregulated by Peterson et al. (4), we only observed significant upregulation for 10 genes (just above the 2-fold cutoff) and no significant effects for the rest of the operon. However, both from visual inspection and the fact that all genes cluster together in the WGCNA analysis, it is clear that the entire operon behaves as a single transcriptional unit, with a modest upregulation 3 minutes after CSP addition, followed by a drop in expression at 10 minutes. Therefore, regulation of a single TSS, at 195,877 (+), would suffice to explain the behavior of these 22 genes. Note that the observed gradient in fold changes across this operon was observed in several other operons as well (see **Table S5**). Although we do not fully understand this phenomenon, we speculate that differences in rates of mRNA degradation might play a role. Since (exo)ribonucleases have a specific directionality (i.e. 5'-3' or 3'-5' in Gram-positive bacteria), such differences could explain the observed gradients.

Early competence genes: the ComE and BlpR regulons

We reasoned that confining the analysis of promoter regions to only those upstream of HC members (defined above) of a regulon were likely to yield a more accurate consensus binding site of the corresponding regulator, compared to when such a consensus were based on all known regulated genes, as is the common procedure. Therefore, combining transcription start site (TSS) data on these selected genes (**Table S3**) as reported previously (41) and known characteristics of regulatory sites (e.g. typical distance from TSS), we redefined the binding motifs for ComE, ComX and CiaR (See **Materials and Methods** for details). The identified

242 ComE-binding sequence (**Figure 3**, top left; **Figure 4A**) strongly resembles previous reports
243 (26, 27) and consists of two imperfect repeats. The spacing between these repeats is 11-13 nts
244 (mode=11), while the spacing between the right motif and the TSS is 42-44 nts (mode=42).
245 Clearly, the second repeat is most conserved and is likely to be most important for ComE
246 recognition. In summary, this yields the following consensus ComE-binding motif:
247 [TNYWVTTBRGR]-[N_{11}]-[ACADTTGAGR]-[N_{42}]-[TSS] (**Figure 4A**).

248 As Martin et al. previously described (27), the internal spacing and the right arm of
249 the binding sequence in P_{comX} deviate from the consensus (**Figure 4A**). Indeed, from our data,
250 this deviation seems to lead to a somewhat lower expression level from P_{comX} after induction
251 (**Table 2**), relative to other members of the ComE regulon. This reduction is partially
252 compensated by the presence of two copies of *comX* on the chromosome. In contrast, we
253 found no indication that mismatches with the consensus ComE-binding sequence found in the
254 promoter region of *comAB* resulted in lower expression of those genes (**Table 2**), which was
255 suggested by Martin et al. (27) to explain an earlier observation that *comAB* transcript levels
256 were rate-limiting in the development of competence (48). Indeed, although higher ComAB
257 levels may indeed accelerate competence development, the fact that a duplication of *comC*
258 (22, 25) leads to competence upregulation suggests that ComAB is not exporting CSP at
259 maximum capacity in wild-type cells.

260 As reported before, the *comAB* genes are preceded by a BOX element (49), an
261 imperfectly repeated DNA element occurring 127 times in the D39V genome (41).
262 Interestingly, the BOX element is located downstream of the ComE-regulated TSS and is
263 therefore part of the *comAB* transcript. It was shown by Knutsen et al. that the BOX element
264 upstream of *comAB* is important for the fine-tuning of competence (50), but the underlying
265 mechanism is unknown. We previously detected several RNA fragments terminated between

266 the BOX element and the start codon of *comA*, leading us to annotate it as a ncRNA, *srf-03*
267 (41). Although some BOX elements were reported to contain putative protein-encoding
268 sequences, we did not find any uninterrupted coding sequence in this specific case. It seems
269 unlikely that the BOX element functions as an RNA switch, since *srf-03* and *comAB*
270 displayed the same time-dependent expression patterns with relatively low expression at t=0
271 (**Table 2**). Since removing this BOX element from P_{comA} leads to reduced *comAB* expression
272 (50), it is tempting to speculate that the prematurely terminated transcript, *srf-03*, plays a role
273 in competence regulation. However, a similar effect on transcription was observed when the
274 BOX element in front of *qsrAB* (also ComE-regulated) was removed (50) and we did not find
275 any evidence for premature termination between that BOX element and the start of *qsrA*.

276 Analysis of all upregulated promoters resulted in the detection of five additional
277 operons putatively regulated by ComE (the complete proposed ComE regulon is listed in
278 **Table 2**), including *ybbK*, a known early-*com* gene. The weaker induction of this gene and
279 the fact that its expression did not cluster with other early-*com* genes can be explained by the
280 fact that the ComE site is located 10 nts too far from the TSS, compared to a canonical
281 ComE-regulated gene (**Figure 4A**).

282 Three other ComE-induced operons (*blpT*; *blpABC*; *pncW-blpYZ-pncP*) are known to
283 be part of the BlpR regulon and their activation is the result of crosstalk, where ComE can
284 recognize the binding sites of BlpR, but with lower efficiency (43, 44). Indeed, close
285 inspection of the corresponding promoter regions shows marked differences with those of
286 other ComE-regulated genes, consistently deviating from the consensus ComE-binding site at
287 specific positions (**Figure 4A**). The same discrepancies were observed in the promoter
288 regions of *blpK* and, to a lesser extent, *blpSRH*, the two remaining *blp* operons. These
289 operons were not differentially expressed during competence, probably due to the poorer

resemblance to the ComE-binding consensus. Additionally, both *blpK* and *blpSRH* are constitutively expressed, such that any minor inducing effect by ComE would be negligible. A multiple sequence alignment of the five known *blp* operons in strain D39V revealed a conserved sequence very similar to, but slightly more extended than, the putative BlpR-binding site postulated by De Saizieu et al. (51). The here-reported binding site can be seen as an imperfect tandem 19-21 bp repeat: [NYAATTCAAGANGTTTYRATG]-[ACAATTCAAG(NN)ATTTGRANN]-[N_{33}]-[TSS]. More specifically, the region can be written as X_1-Y_1-X_2-Y_2, where X (resembling the ComE-binding site) and Y are 10 and 9-11 bps in length, respectively, having a highly conserved 'TT' (or 'TTT' in Y) at their centers (**Figure 4B**). Interestingly, the promoter region of the final operon putatively regulated by ComE, SPV_2249-SPV_0817, resembles the putative BlpR recognition site (**Figure 4B**) and we speculate that these genes are actually part of the BlpR regulon. This idea is supported by the very modest induction (2.3- and 2.5-fold, respectively) of this operon during competence. Additionally, SPV_0817 encodes a probable CAAX protease (41) that could be speculated to be involved in immunity against self-produced bacteriocins (52, 53).

Finally, only one feature from the WGCNA cluster associated with ComE regulation remained that could not be directly linked to a ComE-binding site. This feature, a pseudogene (SPV_2414), is part of an ISSpn7 insertion sequence (54) and represents a truncated version of the gene encoding the corresponding transposase. Since the D39V genome contains eight additional sites with a ≥95% sequence identity to this insertion sequence, clearly undermining mapping fidelity, and no significant differential expression was observed in any competence time point, we ruled out SPV_2414 as a member of the ComE-regulon.

312 **Late competence genes: the ComX regulon**

313 Directly following the strong, ComE-mediated increase in *comX* expression, the
314 late-competence regulon is activated. Based on the promoter sequences of HC members of
315 the late-*com* regulon (**Table S3**, **Figure 5**), the ComX recognition sequence was re-evaluated.
316 Not surprisingly, the identified motif (**Figure 3**, top right) does contain a near-perfect match
317 to the previously reported 8-nucleotide consensus sequence (3, 32, 33): [TMCGAATA].
318 However, our analysis shows that the region relevant to ComX binding is likely much wider
319 than that: with a thymine-rich stretch upstream and a, less-conserved, adenine-rich stretch
320 downstream of the reported 8 nucleotides, the actual recognition site is extended to 20-30
321 basepairs. In summary, this yields the following consensus ComX-binding motif:
322 [TTTTTNHNNNYTHTTMCGAATADWNWRRD]-[TSS] (**Figure 5**).

323 Besides the 16 HC ComX-regulated operons, we identified three additional promoters
324 containing the here-reported motif (**Table 3**). Firstly, SPV_0027-30, an operon encoding,
325 among others, a dUTP pyrophosphatase (*dut*) and DNA repair protein RadA (*radA*), was
326 already previously reported to be part of the late-*com* regulon (3, 4), but did not cluster with
327 the HC ComX regulon. A secondary TSS, 11 nucleotides downstream of the ComX-regulated
328 TSS, could be responsible for the lower correlation with other ComX-regulated genes.
329 Similarly, a second previously reported late-*com* gene, SPV_0683, may be under the control
330 of a secondary, not yet identified TSS, besides the here-reported ComX-activated TSS (**Table**
331 **3**), as supported by its relatively high expression level prior to CSP addition and sequencing
332 coverage observed in PneumoBrowse (41). A third ComX-binding site was found
333 downstream of *prs1* (SPV_0033) and immediately upstream of a novel ncRNA, *srf-01*
334 (SPV_2081), which we identified recently (41). While this addition to the known competence
335 regulon seemed interesting at first, the partial overlap between the ncRNA and a nearby IS

element (containing pseudogene SPV_0034) led us to question the functionality of this novel element (**Figure S1**). Additionally, another pseudogene (SPV_2082) was located on the other side of the IS element, also under the control of ComX (**Table 3**). A multiple genome alignment of several pneumococcal strains (not shown) revealed that the ComX-binding element downstream of *prsA1* (i.e. *prs1*), was conserved in, among other strains, *S. pneumoniae* INV200 (GenBank: FQ312029.1), but was followed in that strain by a set of pseudogenes (**Figure S1**). A BLASTX search showed that the two pseudogenes are probably derived from a protein-encoding gene mostly annotated as encoding a recombination-promoting nuclease or transposase. Interestingly, this gene was highly similar to SPV_2082 and an additional pseudogene, SPV_2340, located elsewhere on the D39V chromosome and also ComX-regulated. We speculate that the presence of a Repeat Unit of the Pneumococcus (RUP) (55) upstream of SPV_2082, in combination with the action of IS elements, might have enabled several duplication and/or reorganization events of the SPV_2082 locus. While these findings suggest that, in pneumococcal strains with an intact copy of this gene, it might be relevant to transformation and horizontal gene transfer, we do not expect *srf-01* (or pseudogenes SPV_2082 and SPV_2340, for that matter) to have a role in competence.

A second ncRNA, *srf-29*, is located upstream of and partially overlaps with *cbpD* (SPV_2028), a known late-*com* gene. A high-confidence terminator (41) inside the coding region of *cbpD* marks the 3'-end of *srf-29*. It is not clear whether *srf-29* represents an uncharacterized RNA switch regulating *cbpD* expression, produces a functional sRNA, or simply is an artefact produced by a premature terminator (see PneumoBrowse coordinates 2008356-2008242 (-)).

Since TSS and terminator information permits a promoter-based interpretation of our data, we observed examples of complex operon structures, wherein TSSs or imperfect

terminators inside the operon can lead to differences in expression between different genes in the same operon (41, 56, 57). A striking example is the *cinA-recA-dinF-lytA* operon (SPV_1740-37), shown in **Figure 6**, which is under control of ComX, with only an inefficient terminator (27%) between *recA* and *dinF*. However, the presence of three internal TSSs, upstream of *recA*, *dinF* and *lytA*, respectively, leads to very different basal expression levels at t=0 (**Table 3**). Due to these differences, the extent to which competence induction affects the expression of the four genes decreases from the 5'- to the 3'-end of the operon (**Figure 6**). Finally, Campbell et al. identified a transcription start site inside of and antisense to *dinF*, that was induced during competence and they provisionally named the associated hypothetical gene *tyg* (**Figure 6**; (32)). Although not discussed by Campbell and coworkers, both Håvarstein (58) and Claverys and Martin (59) argued that the peculiar positioning of the *tyg* TSS is reason for doubts regarding the functionality of any transcript produced. However, as Claverys and Martin concede, it cannot be ruled out that *tyg* has a role in mRNA stability of the *cinA-recA-dinF-lytA* operon. With Cappable-seq (60), we did indeed detect a transcription start site (41), accompanied by a consensus ComX recognition site (**Figure 6**). Since we did not detect a clearly demarcated associated transcript, we artificially annotated a 250 nucleotide long transcript, starting at the *tyg* TSS, to allow differential expression analysis. The time-dependent expression trend of this transcript during competence seemed to follow that of other late-com genes (39). However, the extremely low detected expression level prior to and, even, after CSP addition precluded any further statistical analysis regarding differential expression or clustering. We would like to reiterate that TSS and terminator data regarding other competence-specific operons can be retrieved from PneumoBrowse (41).

The expression pattern of 44 out of the 56 members of the ComX-associated WGCNA cluster (cl. 11) can now be linked to a ComX-regulated TSS. Bearing in mind that

the clustering was performed based on expression throughout all 22 infection-relevant conditions studied in PneumoExpress (39), only five other cluster members (SPV_0553, SPV_0957-59 and SPV_2317) showed an expression pattern similar to ComX-regulated genes in competence conditions specifically. While the TSS for SPV_0553 has not been determined, this gene is surrounded by two Repeat Units of the Pneumococcus (55) and one BOX element (49) and nothing resembling a ComX-binding site was found near it. Secondly, SPV_2317 represents a novel ncRNA (*srf-19*), potentially an RNA switch, that is preceded by a predicted RpoD site, rather than a ComX site. The last cluster member, operon SPV_0957-59, contains *rpoD* (SPV_0958). In light of the proposed role of RpoD in the shutdown of late competence (see above), it would be interesting if its upregulation was directly induced by ComX. However, analysis of the promoter region of the operon yielded no indication of a ComX-binding site and the mechanism of *rpoD* induction in competence continues to elude us.

The CiaR regulon is induced during competence and contains a novel non-coding RNA

Besides early and late-*com* genes, terminology reserved for the ComE- and ComX regulons, respectively, many other genes are more indirectly affected by the addition of CSP. Although a small portion of these genes can already be seen to be affected after 3 minutes, we will refer to all of these genes collectively as 'delayed', as these changes occur at least after the activation of the ComE regulon and most likely also downstream of the ComX regulon. Among the delayed genes are nearly all members of the CiaR regulon (**Table 4**) and promoter analysis of the HC members of the CiaR regulon (**Table S3**, **Figure S2**) returned the CiaR recognition site as previously reported (37, 45, 46): [TTTAAG]-[N_5]-[TTTAAG]-[N_{22}]-[TSS]; **Figure 3**, bottom left). Analysis of other affected promoters turned up three

407 additional monocistronic operons (*ccnC*, SPV_0098 and SPV_0775), all of which have
408 already previously been reported to be CiaR-regulated. While SPV_0098 is expressed from
409 two different TSSs (45) and *ccnC* (SPV_2078) expression is affected by transcriptional read-
410 through from the upstream ComE-regulated *comW* operon, it is not clear why SPV_0775
411 does not cluster with other CiaR-regulated genes.

412 Since CiaR-binding sites were found on the opposite strand for *dltXABCD*
413 (SPV_2006-02; upregulated) and *manLMN* (downregulated), we speculate that operon
414 SPV_0478-83 (i.a. *rimP*, *infB*, *nusA* and *rbfA*), encoding several proteins involved in
415 translation, is also regulated by CiaR (**Figure S2**). Intriguingly, another new member of the
416 CiaR regulon is *srf-21* (SPV_2378), a novel, uncharacterized non-coding RNA (41). The TSS
417 from which this ncRNA is expressed was already part of the reported CiaR regulon, but was
418 linked to the overexpression of the downstream *axe1* gene (SPV_1506). Inspection of the
419 transcriptional layout of the region (**Figure 7A**) shows that *srf-21* and *axe1* are separated by a
420 relatively efficient terminator and a second TSS. Nonetheless, *axe1* overexpression might still
421 be attributed to read-through from *srf-21*. We did not find any similar ncRNAs in RFAM and
422 BSRD databases (61, 62) and, since *axe1* is expressed from its own TSS, it seems unlikely
423 that *srf-21* functions as an RNA switch. Preliminary minimum free energy (MFE) secondary
424 structure prediction with RNAfold (63) and target prediction with TargetRNA2 (64) did not
425 provide us with any clear hints with regard to the function of this ncRNA. Firstly, the
426 predicted MFE structure (**Figure 7B**) might only represent a transient conformation, since it
427 makes up less than 1% of the modelled ensemble. Secondly, sRNA target prediction
428 produced many potential targets (**Table S4**). Some candidate regions are less likely to be
429 targeted, because they are located more than 20 nucleotides upstream of the start codon or
430 even upstream of the TSS, ruling out a possible interaction between *srf-21* and the transcript

in question. However, future work will be necessary to reveal whether any of the remaining genes (e.g. *queT*, *pezA* or *cps2H*) are regulated by *srf-21* or, indeed, whether this ncRNA might have a completely different mode of action.

It is noteworthy that four reported promoters of the CiaR regulon were not found to be significantly affected during competence. Firstly, the *tarIJ-licABC* operon is under the control of two TSSs and thereby apparently less sensitive to CiaR control. Finally, three out of five csRNAs, described by Halfmann et al. (37), did not appear to be significantly affected (**Table 4**). It should be noted, however, that the statistics on these short transcripts is rather poor and the current data can neither support nor contradict their upregulation in competence. However, we do believe that the data presented by Halfmann et al. regarding CiaR-regulation seem perfectly convincing, and the promoter regions of each of the five csRNAs contain a clear match with the consensus CiaR-binding site (**Figure S2**).

Other known regulons affected during competence

Regardless of whether or not competence should be regarded as a stress response mechanism in itself, it is clear that the activation of competence, at least indirectly, leads to a multifactorial stress response. Still unknown in previous descriptions of the competence regulon, the VraR (LiaR) regulon has been described by Eldholm et al. to be activated in response to competence-induced cell wall damage (15). Based on three pneumococcal promoters and six *L. lactis* promoters (**Table S3**, **Figure S3**), we rebuilt the consensus motif described by Eldholm et al. (**Figure 3**, bottom right) and observed that all of these motifs are located 32-34 nucleotides upstream of the corresponding TSS. In contrast, we could confirm the reported presence of a VraR-binding site upstream of *hrcA* (15), but this site is 81 nucleotides removed from its target TSS. However, the fact that this promoter region also

carries two HrcA-binding sequences could account for this difference in spacing. Finally, we suggest that SPV_1057 (spr1080 in R6) and SPV_1160 (spr1183) are not regulated by VraR, contrary to the report by Eldholm and coworkers. Firstly, both of these genes lacked a detected TSS and neither was found to be differentially expressed in our study. Secondly, the reported recognition site for SPV_1057 is actually located downstream of the gene, inside a repeat region (ISSpn7 element). Finally, as recognized by Eldholm et al., SPV_1160 represents a 5'-truncated version of a gene putatively encoding the ATP-binding component of an ABC transporter. These observations, combined with the fact that the reported VraR-binding site is located only 24 nucleotides upstream of the annotated start of SPV_1160, led us to conclude that SPV_1160, like SPV_1057, is not regulated by VraR. This limits the VraR regulon to 15 genes, distributed over 4 operons (**Table 5**). Eldholm et al. showed that specifically CbpK (PcpC; SPV_0357) and a putative phage shock protein (SPV_0803) are important in preventing competence-mediated lysis, forming an additional layer of protection besides ComM (15).

Besides VraR and CiaR, also the well-characterized HrcA (3, 65) and CtsR (3, 66) regulons are activated in competent cells, as previously reported (3). The only addition to be made here regarding the HrcA regulon is the annotation of a gene encoding a protein of unknown function (SPV_2171), not previously annotated in D39 or R6 strains. This gene is located between *dnaK* (SPV_0460) and *dnaJ* (SPV_0461) and therefore regulated by both VraR and HrcA (**Table 5**). Expression of almost the entire CtsR regulon was found to be upregulated. Only for *clpP* (SPV_0650) was the observed upregulation below the employed cutoff, possibly because its basal expression level (t=0) is 4- to 40-fold higher than that of other *clp* genes. Finally, the upregulation of an uncharacterized two-component regulatory

Journal of Bacteriology

system (SPV_2020-19), with unknown consequences, could be attributed to transcriptional read-through from the upstream *ctsR-clpC* operon.

Together, CtsR and HrcA account for the overproduction of several subunits of the Clp protease system and of several chaperone proteins (e.g. DnaK, DnaJ, GroES-GroEL) during competence (**Table 5**). Both of these protein functions are aimed at the reduction of stress caused by misfolded proteins and might be required to ensure proper folding of the many competence proteins.

Two other regulons seemed overrepresented in the set of differentially expressed genes ($p < 10^{-4}$, hypergeometric test). Firstly, transcription of all six genes predicted to be regulated by GntR (SPV_1524), as based on homology to *Streptococcus pyogenes* Spy_1285 (67), was found to be upregulated 10 and 20 minutes after addition of CSP. These six genes are distributed over two operons (SPV_0686-88 and SPV_1524-26). Secondly, expression of a significant number (31) of RpoD-regulated genes was downregulated, mostly after 10 and 20 minutes, which may readily be explained by the competition for RNA polymerase of RpoD (σ^A) with the alternative sigma factor ComX (σ^X).

Other differentially expressed genes

A total of 367 genes (i.e. 17% of all annotated genes) are either found to be differentially expressed or, at least, to be under the control of a TSS that appears to be differentially regulated at some point during competence (**Table S5**). The response of a large portion (204 genes) of these can be ascribed to the action of one of the transcriptional regulators discussed above. While 56% of the latter group display a maximum absolute change in expression of more than fourfold, only 29 of the remaining 163 genes (16%), distributed over 14 operons, meet the same criterion. These data show that the bulk of strong induction or repression can

be explained by a small set of regulators. Among the 29 strongly differentially expressed genes with no known regulators, our data confirmed the upregulation of *rpoD*, in line with the role that RpoD might play in late competence shutdown (36).

Functional analysis did not reveal any Gene Ontology or KEGG classes overrepresented among upregulated genes. Only classes related to ribosomes and translation seemed overrepresented, but the realization that all affected genes from these classes were part of a single operon (shown in **Table 1**) led us to discard them due to lack of evidence (see **Materials and Methods**). Similarly, most potential hits among downregulated genes were discarded. Only classes related to thiamine metabolism (GO:0009228, KEGG:ko00730) remained. The five genes in question are distributed over four operons: *adk* (SPV_0214), *thiM-1-thiE-1* (SPV_0623-24), *thiD* (SPV_0632), and *sufS* (SPV_0764). Two of these four operons are regulated by a TPP riboswitch, an RNA element that, when bound to thiamine pyrophosphate (TPP), prevents transcription of the downstream operon (68). We suspect, therefore, that the temporary growth lag accompanying competence development (3) leads to a transient accumulation of TPP, which then represses transcription of operons under control of a TPP riboswitch. Indeed, both other D39V operons led by a TPP riboswitch show a similar expression trend 10-20 minutes after competence induction. Firstly, *ykoEDC-tenA-thiW-thiM-2-thiE-2* (SPV_0625-31) is already minimally expressed prior to CSP addition, preventing significant downregulation (not shown). The second operon, SPV_2027-*thiXYZ* (SPV_2027-24), was excluded from gene enrichment analysis since it was part of the ComX regulon (**Table 3**). Since the hypothesized accumulation of TPP seems to occur with a delay, relative to the activation of the ComX regulon, expression of these genes is first upregulated (3-10 min) and then downregulated (20 min), even relative to the basal expression level.

Comparison to previous reports of the competence regulon

Finally, we compared our findings with previous, microarray-based studies by Peterson et al. (4) and Dagkessamanskaia et al. (3). Although both studies give a remarkably complete overview, our approach allowed us to refine and nuance the description of the competence regulon even further (**Table S5**). The higher sensitivity and accuracy of Illumina sequencing, the improved genome annotation, and the application of a promoter-based analysis (rather than gene-based) allowed us to expand the set of genes under direct control of ComE and ComX to 40 and 55 genes, respectively (combined: 4% of all genes). Especially several genes with putative BlpR-binding sites (**Table 2**), and therefore a generally weaker response, were missing from previous reports. Additionally, the previously reported *briC* operon (40) is now included in the ComE regulon and we confirmed that, while undetected by Dagkessamanskaia et al., *ybbK* and *def2* (early) and *radC* (late) are indeed part of the *com* regulon (**Tables 2** and **3**). Remaining discrepancies could be explained either by transcriptional read-through or the absence of certain elements (e.g. ncRNAs) from the TIGR4 and R6 genome annotation files used by Peterson et al. and Dagkessamanskaia et al., respectively.

Not surprisingly, larger discrepancies were found for genes displaying delayed differential expression, since their fold changes (mostly) are considerably smaller and therefore more sensitive to technical variation and differences in experimental conditions (e.g. growth medium, pH or time of induction). In contrast with the previous studies, we recovered nearly the entire CiaR regulon as differentially expressed in our data, confirming the higher sensitivity of RNA-seq, compared to microarray-based technology.

Discussion

546 Competence for genetic transformation is defined as a state in which a bacterial cell can take
547 up exogenous DNA and incorporate it into its own genome, either in the form of a plasmid or
548 via homologous recombination. Since the very first demonstrations of bacterial
549 transformation were provided by Griffith and Avery et al. (69, 70) in *S. pneumoniae*, the
550 pneumococcal competence system has been widely studied as soon as the required tools
551 became available. Therefore, much knowledge has been assembled about how this state is
552 regulated, which environmental triggers affect its development and what downstream
553 consequences it has. Indeed, several studies have been performed to compile a
554 comprehensive list of all competence-regulated pneumococcal genes. The most recent of
555 these studies (3, 4), although of very high quality and invaluable to the research field, date
556 from nearly fifteen years ago. Since then, the fields of transcriptome analysis and genome
557 sequencing and annotation have been revolutionized by second- (e.g. Illumina) and third-
558 generation (e.g. PacBio) sequencing techniques. Therefore, we have analyzed Illumina-based
559 RNA-seq data (39), using the recently sequenced and deeply annotated *S. pneumoniae* D39V
560 strain (41), to refine the previously reported pneumococcal competence regulon.
561 Additionally, rather than just reporting affected individual genes, we used previously
562 determined transcript boundaries (TSSs and terminators; (41)) to identify the affected
563 promoters, which may be used to gain more insights into the regulatory processes at work
564 during competence.

565 In short, we report that ComE directly regulates 15 early-*com* transcripts (40 genes),
566 including 4 transcripts (10 genes) that are part of the BlpR regulon (**Table 2**, **Figure 4**).
567 Alternative sigma factor ComX (σ^X) was found to control 19 late-*com* transcripts (55 genes),
568 in addition to the previously described *tyg* TSS, inside and antisense to *dinF* (**Table 3**). We
569 should note that four genes from the early and late-*com* regulons (e.g. *blpC*) did not meet

570 fold-change and/or statistical cutoff values, but were part of operons that were clearly
571 regulated. For each of these genes, the observed expression trends correlated with those of
572 other operon members.

573 Our data confirmed that, as shown in the previous studies (3, 4), the activation of the
574 early and late-competence regulons indirectly resulted in the activation of several other
575 regulons, most of which are implicated in pneumococcal stress response. Firstly, out of the
576 newly compiled CiaR regulon (18 transcripts, 38 genes), based on the work of Halfmann et
577 al. (37), 4 operons were not found to be significantly affected (**Table 4**). Other affected
578 regulons were those under control of VraR (LiaR), HrcA, CtsR and GntR (**Table S5**).
579 Additionally, transcription of genes led by a TPP riboswitch was downregulated, suggesting a
580 transient increase in intracellular thiamine pyrophosphate levels in competent cells.

581 Expression of many other genes, including *rpoD*, is up- or downregulated through
582 unknown mechanisms. In total, approximately 140 transcripts (containing 367 genes, 17% of
583 all genes) undergo some extent of differential expression. For several reasons (e.g. fold
584 changes near cutoff, expression from multiple TSSs or poor statistics due to low expression),
585 79 genes from these operons did not individually meet the detection criteria, leaving 288
586 differentially expressed genes (13%) when following the traditional, gene-based analysis
587 approach (**Figure 2**). Among the affected genes are several small, non-coding transcripts.
588 Some of these ncRNAs, like the CiaR-activated csRNAs (see below), have been
589 characterized and we showed that *srf-01* is unlikely to be functional. For others, e.g. *srf-03*
590 (upstream of *comAB*) and *srf-21* (upstream of *axe1*), future work is required to determine
591 their role, if any, during competence.

592 Given the fact that so many different functionalities are activated during competence,
593 including stress response systems such as the Clp protease and several chaperone proteins,

Claverys et al. proposed to refer to the system more neutrally as 'X-state' (for ComX). We would argue, however, that the primary response to a high extracellular CSP level is the activation of the ComE and ComX regulons, which mostly encode proteins relevant to transformation. Firstly, the expression of fratricin CbpD (SPV_2028), along with immunity protein ComM (SPV_1744), allows for the lysis of neighboring non-competent cells, which may offer access to both nutrients and DNA (71, 72). The upregulation of the gene encoding autolysin LytA (*lytA*) would fit in nicely here, since the simultaneous deletion of *lytC* and *lytA* abolishes competence-induced lysis completely (71). However, basal level *lytA* expression was reported to be already sufficient for the observed lysis rate (73). Next, the DNA uptake machinery comes into play, involving many proteins encoded by the late-*com* genes, as reviewed by Claverys et al. (16); most of ComGC-ComGG (SPV_1861-57), ComEA/C (SPV_0843-44) and ComFA (SPV_2035) are, together with constitutively expressed endonuclease EndA (SPV_1762) and through the action of prepilin peptidase CclA (SPV_1593) and proteins ComGA/B (SPV_1863-62), assembled into a pilus-like structure (74). The import of DNA is followed by DNA processing and recombination, involving DNA protection protein DprA (SPV_1122), recombinase RecA (SPV_1739), ssDNA-binding SsbB (SPV_1711) and other late-competence proteins (75–78). For the reasons discussed above, combined with the fact that the vast majority of other differentially expressed genes display a more modest change in expression (**Table S5**), we decided to keep calling the system competence for genetic transformation. We do, however, agree that the downstream effects of competence development should not be ignored, as discussed below.

The genome of *Streptococcus pneumoniae* D39V contains 13 two-component regulatory systems (TCSs) (41, 79). Interestingly, the regulons of two TCSs (ComDE and BlpRH) are activated during competence, while the regulons of another two TCSs (CiaRH

618 and VraRS) are activated shortly after. Expression of a fifth, uncharacterized TCS
619 (SPV_2020-19) is also slightly upregulated, possibly due to transcriptional read-through from
620 the *ctsR-clpC* operon. The additional activation of stress-related regulons of HrcA and CtsR
621 give support to the hypothesis, as proposed by Prudhomme at al. (5), that competence
622 activation serves as a general stress response in the pneumococcus, which lacks a LexA-
623 mediated SOS response that is common in many other bacteria. The activation of competence
624 in response to various types of stress (5–8) provides even more relevance to this idea. On the
625 other hand, Dagkessamanskaia et al. showed that a deletion of the CiaR regulon causes an
626 extended growth lag, as well as a stronger activation of the HrcA and CtsR regulons, after
627 competence induction (3). Both observations are in line with the notion that the development
628 of competence is accompanied by a significant burden to the cell. To our knowledge, the
629 exact mechanism underlying CiaR-mediated prevention of autolysis is unknown. However,
630 Halfmann et al. showed that the deletion of CiaR-induced non-coding csRNA4 and csRNA5
631 led to an enhanced-lysis phenotype (37). Furthermore, D-alanylation of lipoteichoic acids by
632 the DltXABCD proteins (45) might contribute to cell wall integrity during or after the
633 transient block in cell division that accompanies competence (19). Finally, HtrA can play a
634 role in the cleaning up of potential misfolded or excess proteins that cause stress to the
635 pneumococcus (9).

636 We consider it likely that the main benefit of the activation of the CiaR and other
637 stress response regulons during competence is to deal with the stress invoked by competence
638 itself, by building the large membrane- and cell-wall-protruding DNA-uptake machinery and
639 the production of cell wall hydrolases such as the fratricin CbpD. It is not unthinkable,
640 however, that the activation of the many different stress response regulons renders it
641 beneficial to a pneumococcal cell to become competent even in specific stressful conditions

642 that do not require DNA uptake or recombination machineries. Whether or not such
643 conditions played a role in the (co-)evolution of competence and downstream processes is
644 open to speculation. In this respect, it is interesting to note that the production of competence-
645 induced bacteriocins was found to be important to prevent 'intruder' pneumococci from
646 colonizing (80).

647 The apparent severity of the stress imposed on a competent cell emphasizes the need
648 to shut down competence after a short transformation-permissive time window. In addition to
649 the known role of DprA in early competence shut-down, Weyder et al. proposed that the
650 upregulation of *rpoD* is responsible for the, less-efficient, shut-down of late competence (36).
651 Related to this, RpoD-regulated genes are, to some extent, overrepresented among
652 downregulated genes during competence. While this explains the need for upregulation of
653 *rpoD*, the underlying mechanism is still unknown. Other aspects that might play a role in the
654 shutting down of competence are the CiaR-mediated upregulation of HtrA, which has been
655 shown to degrade extracellular CSP (9), and the recently discovered CiaR-regulated non-
656 coding csRNAs (*ccnA-E*) (37, 81–83), which were shown to repress ComC translation in an
657 additive fashion.

658 Finally, although several of the activated regulons are quite well-understood, still a
659 large portion of affected genes are differentially expressed through unknown mechanisms. It
660 seems plausible that many of these are due to the sudden and severe shift in metabolic state.
661 For example, the higher translational demands during competence could lead to the
662 upregulation of genes encoding ribosomal proteins (**Table 1**). Similarly, the hypothetical
663 transient increase in TPP concentrations, leading to riboswitch-mediated downregulation of
664 four operons, could be accompanied by the accumulation or depletion of other, unknown
665 metabolites, with potential transcriptional consequences.

Materials and Methods

Samples studied here are a subset of the data set presented in PneumoExpress (samples *C+Y*; *CSP, 3 min*; *CSP, 10 min*; *CSP, 20 min*; (39)) and detailed procedures regarding bacterial growth, RNA isolation, sequencing and mapping of reads are reported therein. The key points of these methods are summarized below.

Culturing and harvesting of *S. pneumoniae* D39V

Eight tubes with 2 mL C+Y medium (pH 6.8, non-permissive for natural competence; (39)) without antibiotics were each inoculated with wild-type *S. pneumoniae* D39V cells (initial OD_{600nm} ~ 0.004) and incubated at 37°C (standing culture in ambient air). When the cultures reached an OD_{600nm} of 0.05, two cultures were harvested for RNA isolation (t=0). To the other six, 100 ng/mL synthetic competence-stimulating peptide (CSP-1), purchased from GenScript (Piscataway, NJ), was added. Duplicate samples were harvested 3, 10 and 20 minutes after CSP-1 addition. Before harvesting, cultures were pre-treated with a saturated ammonium sulfate solution as described before (84), to prevent protein-dependent RNA production and degradation ((85); patent). Afterwards, cells were harvested by centrifugation (20 min, 4 °C, 10,000 × g) and cell pellets were snap-frozen with liquid nitrogen and stored at -80 °C.

Total RNA isolation, library preparation, sequencing and mapping of reads

RNA was isolated using phenol-chloroform extraction, followed by DNase treatment and another round of phenol-chloroform extraction (39). The quantity and quality of total RNA were estimated by Nanodrop, while a 1% bleach gel (86) was employed to confirm the presence of rRNA bands (23S, 2.9 kbp and 16S, 1.5 kbp) and absence of genomic DNA.

Subsequently, RNA quality was again checked using chip-based capillary electrophoresis (Agilent Bioanalyzer). Stranded cDNA library preparation was performed, without depletion of ribosomal RNA, using the TruSeq® Stranded Total RNA Sample Preparation Kit (Illumina, US). Sequencing was performed on an Illumina NextSeq 500, in 75 nucleotide single-end mode. The raw FASTQ data are accessible at http://www.ncbi.nlm.nih.gov/geo/ with accession number GSE108031 (samples B05-B11).

After a quality check with FastQC v0.11.5 (87), reads were trimmed using Trimmomatic 0.36 (88). Alignment of trimmed reads to the reference *S. pneumoniae* D39V genome (GenBank CP027540; (41)) was performed with STAR (89).

Read quantification and differential gene analysis

The aligned reads were then counted (90) according to the D39V annotation file (GenBank CP027540; (41)) in a strand-specific fashion, allowing mapping to multiple sites (-M), for which fractional counts are reported (--fraction), and allowing reads to overlap multiple features (-O) to account for polycistronic operons.

Subsequently, we analyzed the libraries in R-studio (R v3.4.2). We performed differential gene expression analysis on rounded raw count by DESeq2 (91). Normalized expression levels are presented as TPM (transcripts per million) (92) and can be found in **Table S1**. Genes with a more than twofold absolute change of expression and a corresponding *p*-value of below 0.001 were considered to be significantly differentially expressed.

When possible, PneumoBrowse (https://veeninglab.com/pneumobrowse; (41)) was used to trace back differential expression of individual genes to specific TSSs and promoter regions. As a starting point, the operon prediction from PneumoBrowse was used to define

groups of genes differentially expressed in competence. It is important to note that strong transcriptional responses such as those observed during competence may have significant downstream effects. Even in the presence of highly efficient transcriptional terminators, which were defined to be operon boundaries in PneumoBrowse, such read-through effects may be visible. Therefore, these co-expressed groups were refined by inspection of the raw data in PneumoBrowse and the consideration that minor read-through from a highly expressed gene can still be significant if the expression of the downstream gene is sufficiently lower.

Clustering and creation of position weight matrices

Using the weighted gene co-expression network analysis (WGCNA) R software package (93), genes were clustered based on their *rlog* (regularized log) expression value (**Table S2**), as output by DESeq2, across all 22 infection-relevant conditions analyzed in PneumoExpress (39). We noticed that the reported members of the ComE (26, 27), ComX (3, 4, 38) and CiaR (37, 45, 46) regulons each largely ended up in specific clusters (here: clusters 29, 11 and 33 for ComE, ComX and CiaR, respectively). Reported regulon members that properly clustered in these three identified main clusters, which will be referred to as 'training sets' (**Table S3**), were used to define the recognition motifs of these three regulators, in the form of position weight matrices (PWMs), and to determine the most common distance of such a motif from the TSS. Using the MEME suite (94), we analyzed the upstream regions of each training set for enriched sequence motifs. Firstly, since earlier work showed slightly different consensus sequences for the two tandem ComE-boxes that make up the ComE-site (27), we extracted the left ComE motif (CEM_L) by scanning the regions from 77 to 63 bps upstream and the right motif (CEM_R) by scanning the regions 56 to 42 bps upstream of TSSs in the training set.

The ComX-binding motif (CXM) was determined from the regions 35 bps upstream to the +1 site (TSS). When building CEM_L, CEM_R and CXM PWMs, each sequence in the training set was required to have exactly one match to the motif, in the transcription direction (i.e. on the locally defined 'plus'-strand).

CiaR has been described to bind to a direct repeat (37) and we scanned the regions 41 to 19 bps upstream, allowing for multiple hits per sequence in the training set. While some members of the CiaR regulon have binding sites on the opposite strand, no such genes were part of the training set and the CiaR-binding motif (CRM) was therefore also limited to the 'plus'-strand. Genes reported to belong to the VraR (LiaR) regulon (15) did not cluster together throughout the 22 conditions and for some of these genes the TSS was unknown. To be able to extract a VraR-binding motif (VRM), we combined upstream regions of the pneumococcal genes *spxA2* (SPV_0178), *vraT* (SPV_0350) and SPV_0803 with those of six *Lactococcus lactis* genes that were reported to be regulated by close VraR homolog CesR (15, 95): llmg_0165, llmg_0169, llmg_1115, llmg_1155, llmg_1650 and llmg_2164. Cappable-seq (60) was used to identify *L. lactis* TSSs (S.B. van der Meulen and O.P. Kuipers, unpublished). Importantly, we did not use the standard '0-order model of sequences' as a background model for motif discovery, but instead created background models based on the corresponding regions upstream of all known TSSs in the pneumococcal genome (e.g. -35 to +1 for ComX). Additionally, we defined summary consensus sequences using IUPAC nucleotide coding. Since the CiaR-binding motif reportedly consists of two perfect repeats, we determined the consensus based on the 16 motif occurrences in the CiaR training set (8 promoter sequences). Single base codes (A, C, G, T) were called when 75% (rounded up) of all promoters matched. Double base codes (R, Y, S, W, K, M) were called when 8/9 (ComE and VraR), 15/16 (ComX), 5/5 (BlpR) promoters matched either of the two encoded bases.

Triple base codes (B, D, H, V) were called when all promoters matched either of the three encoded bases. Note that, due to its degenerate appearance, the *blpRS* promoter was excluded when determining the BlpR-binding consensus.

Assigning putative regulons

After creating PWSs for ComE-, ComX-, CiaR- and VraR-binding sites, we used FIMO (96) to scan the 100 bps upstream of all known pneumococcal TSSs for matches to these motifs. Here, too, we used the appropriate background models (see above). A cutoff *q*-value of 0.01 was used for hits with ComX- and VraR-binding motifs. We defined a reliable ComE-binding site as CEM_L-[N_{11-13}]-CEM_R, using a cutoff *p*-value of 0.01 for each motif. Similarly, we defined a CiaR-binding site as CRM-[N_{5-6}]-CRM. Additionally, to assign a gene cluster to a given putative regulon, we also put a constraint on the position of the motif relative to the corresponding TSS, based on the typical spacing observed in the training sets. Thus, the allowed first nucleotide positions were [-77/-76/-75/-74/-73] for ComE, [-30/-29/-28] for ComX, [-40/-39/-38/-37/-36] for CiaR, and [-51/-50/-49/-48/-47] for VraR.

Putative binding sites for other regulatory proteins were copied from the propagated *S. pneumoniae* D39 regulons, as found in the RegPrecise database (67) and annotated in PneumoBrowse (41). RNA switches, annotated in D39V, were also taken into consideration as putatively responsible regulatory mechanisms.

Functional analysis of differentially expressed genes

Differentially expressed genes that could not be ascribed to the action of ComE, ComX, CiaR or VraR, were subjected to gene set enrichment analysis (functional analysis). For this, Gene Ontology and KEGG classifications were extracted from the GenBank file corresponding to

the latest annotation of *S. pneumoniae* D39V (41). Additionally, predicted transcription factor binding sites were used to assign genes to their putative regulons. A total of 448 hypergeometric tests were performed and a Bonferroni-corrected cutoff *p*-value of 0.0001 (i.e. 0.05 divided by 448) was used to determine whether certain regulons or Gene Ontology or KEGG classes were overrepresented among differentially expressed genes. We excluded overrepresented classes when all affected genes belonged to the same operon, since the activation of a single promoter does not confer any statistical evidence.

Data availability

The data analyzed here can be extracted from PneumoExpress (https://veeninglab.com/pneumoexpress-app). Raw RNA-seq data used to build PneumoExpress was deposited to the GEO repository: accession number GSE108031.

Acknowledgments

We are grateful to A. de Jong and S. Holsappel for (bio)informatics support; and S.B. van der Meulen for sharing *L. lactis* TSS data.

Work in the Veening lab is supported by the Swiss National Science Foundation (project grant 31003A_172861; a JPIAMR grant (50-52900-98-202) from the Netherlands Organisation for Health Research and Development (ZonMW); and ERC consolidator grant 771534-PneumoCaTChER.

Conflict of interest statement. None declared.

References

1. Henriques-Normark B, Tuomanen EI. 2013. The pneumococcus: epidemiology, microbiology, and pathogenesis. Cold Spring Harb Perspect Med 3:a010215.

2. GBD 2016 Lower Respiratory Infections Collaborators. 2018. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory infections in 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Infect Dis.

3. 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.

4. Peterson SN, Sung CK, Cline R, Desai BV, Snesrud EC, Luo P, Walling J, Li H, Mintz M, Tsegaye G, Burr PC, Do Y, Ahn S, Gilbert J, Fleischmann RD, Morrison DA. 2004. Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. Mol Microbiol 51:1051–1070.

5. Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys J-P. 2006. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science 313:89–92.

6. Slager J, Kjos M, Attaiech L, Veening J-W. 2014. Antibiotic-induced replication stress triggers bacterial competence by increasing gene dosage near the origin. Cell 157:395–406.

7. Domenech A, Slager J, Veening J-W. 2018. Antibiotic-induced cell chaining triggers pneumococcal competence by reshaping quorum sensing to autocrine-like signaling. Cell Rep 25:2390-2400.e3.

8. Stevens KE, Chang D, Zwack EE, Sebert ME. 2011. Competence in *Streptococcus pneumoniae* is regulated by the rate of ribosomal decoding errors. mBio 2.

9. Cassone M, Gagne AL, Spruce LA, Seeholzer SH, Sebert ME. 2012. The HtrA protease from *Streptococcus pneumoniae* digests both denatured proteins and the competence-stimulating peptide. J Biol Chem 287:38449–38459.

10. Chen J-D, Morrison DA. 1987. Modulation of competence for genetic transformation in *Streptococcus pneumoniae*. J Gen Microbiol 133:1959–1967.

11. Claverys J-P, Håvarstein LS. 2002. Extracellular-peptide control of competence for genetic transformation in *Streptococcus pneumoniae*. Front Biosci J Virtual Libr 7:d1798-1814.

12. Echenique JR, Chapuy-Regaud S, Trombe MC. 2000. Competence regulation by oxygen in *Streptococcus pneumoniae*: involvement of *ciaRH* and *comCDE*. Mol Microbiol 36:688–696.

13. Claverys J-P, Prudhomme M, Martin B. 2006. Induction of competence regulons as a general response to stress in Gram-positive bacteria. Annu Rev Microbiol 60:451–475.

14. Turlan C, Prudhomme M, Fichant G, Martin B, Gutierrez C. 2009. SpxA1, a novel transcriptional regulator involved in X-state (competence) development in *Streptococcus pneumoniae*. Mol Microbiol 73:492–506.

842 15. Eldholm V, Gutt B, Johnsborg O, Brückner R, Maurer P, Hakenbeck R, Mascher T,
843 Håvarstein LS. 2010. The pneumococcal cell envelope stress-sensing system LiaFSR is
844 activated by murein hydrolases and lipid II-interacting antibiotics. J Bacteriol
845 192:1761–1773.

846 16. Claverys J-P, Martin B, Polard P. 2009. The genetic transformation machinery:
847 composition, localization, and mechanism. FEMS Microbiol Rev 33:643–656.

848 17. Mascher T, Heintz M, Zahner D, Merai M, Hakenbeck R. 2006. The CiaRH system of
849 *Streptococcus pneumoniae* prevents lysis during stress induced by treatment with cell
850 wall inhibitors and by mutations in *pbp2x* involved in β-lactam resistance. J Bacteriol
851 188:1959–1968.

852 18. Wei H, Håvarstein LS. 2012. Fratricide is essential for efficient gene transfer between
853 pneumococci in biofilms. Appl Environ Microbiol 78:5897–5905.

854 19. Bergé MJ, Mercy C, Mortier-Barrière I, VanNieuwenhze MS, Brun YV, Grangeasse C,
855 Polard P, Campo N. 2017. A programmed cell division delay preserves genome
856 integrity during natural genetic transformation in *Streptococcus pneumoniae*. Nat
857 Commun 8:1621.

858 20. Martin B, Granadel C, Campo N, Hénard V, Prudhomme M, Claverys J-P. 2010.
859 Expression and maintenance of ComD-ComE, the two-component signal-transduction
860 system that controls competence of *Streptococcus pneumoniae*. Mol Microbiol
861 75:1513–1528.

862 21. Hui FM, Zhou L, Morrison DA. 1995. Competence for genetic transformation in
863 *Streptococcus pneumoniae*: organization of a regulatory locus with homology to two
864 lactococcin A secretion genes. Gene 153:25–31.

865 22. Alloing G, Martin B, Granadel C, Claverys JP. 1998. Development of competence in
866 *Streptococcus pneumoniae*: pheromone autoinduction and control of quorum sensing by
867 the oligopeptide permease. Mol Microbiol 29:75–83.

868 23. Håvarstein LS, Coomaraswamy G, Morrison DA. 1995. An unmodified heptadecapeptide
869 pheromone induces competence for genetic transformation in *Streptococcus*
870 *pneumoniae*. Proc Natl Acad Sci U S A 92:11140–11144.

871 24. Håvarstein LS, Gaustad P, Nes IF, Morrison DA. 1996. Identification of the streptococcal
872 competence-pheromone receptor. Mol Microbiol 21:863–869.

873 25. Pestova EV, Håvarstein LS, Morrison DA. 1996. Regulation of competence for genetic
874 transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and
875 a two-component regulatory system. Mol Microbiol 21:853–862.

876 26. Ween O, Gaustad P, Håvarstein LS. 1999. Identification of DNA binding sites for ComE,
877 a key regulator of natural competence in *Streptococcus pneumoniae*. Mol Microbiol
878 33:817–827.

879 27. Martin B, Soulet A-L, Mirouze N, Prudhomme M, Mortier-Barrière I, Granadel C,
880 Noirot-Gros M-F, Noirot P, Polard P, Claverys J-P. 2013. ComE/ComE~P interplay
881 dictates activation or extinction status of pneumococcal X-state (competence). Mol
882 Microbiol 87:394–411.

883 28. Moreno-Gámez S, Sorg RA, Domenech A, Kjos M, Weissing FJ, van Doorn GS,
884 Veening J-W. 2017. Quorum sensing integrates environmental cues, cell density and
885 cell history to control bacterial competence. Nat Commun 8:854.

886 29. Prudhomme M, Berge M, Martin B, Polard P. 2016. Pneumococcal competence
887 coordination relies on a cell-contact sensing mechanism. PLOS Genet 12:e1006113.

888 30. Cowley LA, Petersen FC, Junges R, Jimson D Jimenez M, Morrison DA, Hanage WP.
889 2018. Evolution via recombination: cell-to-cell contact facilitates larger recombination
890 events in *Streptococcus pneumoniae*. PLoS Genet 14:e1007410.

891 31. Lee MS, Morrison DA. 1999. Identification of a new regulator in *Streptococcus*
892 *pneumoniae* linking quorum sensing to competence for genetic transformation. J
893 Bacteriol 181:5004–5016.

894 32. Campbell EA, Choi SY, Masure HR. 1998. A competence regulon in *Streptococcus*
895 *pneumoniae* revealed by genomic analysis. Mol Microbiol 27:929–939.

896 33. Pestova EV, Morrison DA. 1998. Isolation and characterization of three *Streptococcus*
897 *pneumoniae* transformation-specific loci by use of a *lacZ* reporter insertion vector. J
898 Bacteriol 180:2701–2710.

899 34. Piotrowski A, Luo P, Morrison DA. 2009. Competence for genetic transformation in
900 *Streptococcus pneumoniae*: termination of activity of the alternative sigma factor ComX
901 is independent of proteolysis of ComX and ComW. J Bacteriol 191:3359–3366.

902 35. Weng L, Piotrowski A, Morrison DA. 2013. Exit from competence for genetic
903 transformation in *Streptococcus pneumoniae* is regulated at multiple levels. PloS One
904 8:e64197.

905 36. Weyder M, Prudhomme M, Bergé M, Polard P, Fichant G. 2018. Dynamic modeling of
906 *Streptococcus pneumoniae* competence provides regulatory mechanistic insights into its
907 tight temporal regulation. Front Microbiol 9:1637.

908 37. Halfmann A, Kovács M, Hakenbeck R, Brückner R. 2007. Identification of the genes
909 directly controlled by the response regulator CiaR in *Streptococcus pneumoniae*: five
910 out of 15 promoters drive expression of small non-coding RNAs. Mol Microbiol
911 66:110–126.

912 38. Rimini R, Jansson B, Feger G, Roberts TC, de Francesco M, Gozzi A, Faggioni F,
913 Domenici E, Wallace DM, Frandsen N, Polissi A. 2000. Global analysis of transcription
914 kinetics during competence development in *Streptococcus pneumoniae* using high
915 density DNA arrays. Mol Microbiol 36:1279–1292.

916 39. Aprianto R, Slager J, Holsappel S, Veening J-W. 2018. High-resolution analysis of the
917 pneumococcal transcriptome under a wide range of infection-relevant conditions.
918 Nucleic Acids Res 46:9990–10006.

919 40. Aggarwal SD, Eutsey R, West-Roberts J, Domenech A, Xu W, Abdullah IT, Mitchell
920 AP, Veening J-W, Yesilkaya H, Hiller NL. 2018. Function of BriC peptide in the
921 pneumococcal competence and virulence portfolio. PLoS Pathog 14:e1007328.

922 41. Slager J, Aprianto R, Veening J-W. 2018. Deep genome annotation of the opportunistic
923 human pathogen *Streptococcus pneumoniae* D39. Nucleic Acids Res 46:9971–9989.

924 42. Wang Z, Gerstein M, Snyder M. 2009. RNA-Seq: a revolutionary tool for
925 transcriptomics. Nat Rev Genet 10:57–63.

926 43. Kjos M, Miller E, Slager J, Lake FB, Gericke O, Roberts IS, Rozen DE, Veening J-W.
927 2016. Expression of *Streptococcus pneumoniae* bacteriocins is induced by antibiotics
928 via regulatory interplay with the competence system. PLoS Pathog 12:e1005422.

929 44. Wholey W-Y, Kochan TJ, Storck DN, Dawid S. 2016. Coordinated bacteriocin
930 expression and competence in *Streptococcus pneumoniae* contributes to genetic
931 adaptation through neighbor predation. PLoS Pathog 12:e1005413.

932 45. Denapaite D, Brückner R, Hakenbeck R, Vollmer W. 2012. Biosynthesis of teichoic
933 acids in *Streptococcus pneumoniae* and closely related species: lessons from genomes.
934 Microb Drug Resist Larchmt N 18:344–358.

935 46. Mascher T, Zähner D, Merai M, Balmelle N, de Saizieu AB, Hakenbeck R. 2003. The
936 *Streptococcus pneumoniae cia* regulon: CiaR target sites and transcription profile
937 analysis. J Bacteriol 185:60–70.

938 47. Mirouze N, Bergé MA, Soulet A-L, Mortier-Barrière I, Quentin Y, Fichant G, Granadel
939 C, Noirot-Gros M-F, Noirot P, Polard P, Martin B, Claverys J-P. 2013. Direct
940 involvement of DprA, the transformation-dedicated RecA loader, in the shut-off of
941 pneumococcal competence. Proc Natl Acad Sci 110:E1035–E1044.

942 48. Martin B, Prudhomme M, Alloing G, Granadel C, Claverys JP. 2000. Cross-regulation of
943 competence pheromone production and export in the early control of transformation in
944 *Streptococcus pneumoniae*. Mol Microbiol 38:867–878.

945 49. Martin B, Humbert O, Camara M, Guenzi E, Walker J, Mitchell T, Andrew P,
946 Prudhomme M, Alloing G, Hakenbeck R. 1992. A highly conserved repeated DNA
947 element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Res
948 20:3479–3483.

949 50. Knutsen E, Johnsborg O, Quentin Y, Claverys J-P, Håvarstein LS. 2006. BOX elements
950 modulate gene expression in *Streptococcus pneumoniae*: impact on the fine-tuning of
951 competence development. J Bacteriol 188:8307–8312.

952 51. Saizieu A de, Gardès C, Flint N, Wagner C, Kamber M, Mitchell TJ, Keck W, Amrein
953 KE, Lange R. 2000. Microarray-based identification of a novel *Streptococcus*
954 *pneumoniae* regulon controlled by an autoinduced peptide. J Bacteriol 182:4696–4703.

955 52. Kjos M, Snipen L, Salehian Z, Nes IF, Diep DB. 2010. The Abi proteins and their
956 involvement in bacteriocin self-immunity. J Bacteriol 192:2068–2076.

957 53. Pei J, Grishin NV. 2001. Type II CAAX prenyl endopeptidases belong to a novel
958 superfamily of putative membrane-bound metalloproteases. Trends Biochem Sci
959 26:275–277.

960 54. Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. ISfinder: the reference
961 centre for bacterial insertion sequences. Nucleic Acids Res 34:D32-36.

962 55. Oggioni MR, Claverys JP. 1999. Repeated extragenic sequences in prokaryotic genomes:
963 a proposal for the origin and dynamics of the RUP element in *Streptococcus*
964 *pneumoniae*. Microbiol Read Engl 145 (Pt 10):2647–2653.

965 56. Warrier I, Ram-Mohan N, Zhu Z, Hazery A, Echlin H, Rosch J, Meyer MM, van Opijnen
966 T. 2018. The transcriptional landscape of *Streptococcus pneumoniae* TIGR4 reveals a
967 complex operon architecture and abundant riboregulation critical for growth and
968 virulence. PLoS Pathog 14:e1007461.

969 57. Rosinski-Chupin I, Sauvage E, Sismeiro O, Villain A, Da Cunha V, Caliot M-E, Dillies
970 M-A, Trieu-Cuot P, Bouloc P, Lartigue M-F, Glaser P. 2015. Single nucleotide
971 resolution RNA-seq uncovers new regulatory mechanisms in the opportunistic pathogen
972 *Streptococcus agalactiae*. BMC Genomics 16:419.

973 58. Håvarstein LS. 1998. Identification of a competence regulon in *Streptococcus*
974 *pneumoniae* by genomic analysis. Trends Microbiol 6:297–299; discussion 299-300.

975 59. Claverys J-P, Martin B. 1998. Competence regulons, genomics and streptococci. Mol
976 Microbiol 29:1126–1127.

977 60. Ettwiller L, Buswell J, Yigit E, Schildkraut I. 2016. A novel enrichment strategy reveals
978 unprecedented number of novel transcription start sites at single base resolution in a
979 model prokaryote and the gut microbiome. BMC Genomics 17:199.

980 61. Kalvari I, Argasinska J, Quinones-Olvera N, Nawrocki EP, Rivas E, Eddy SR, Bateman
981 A, Finn RD, Petrov AI. 2018. Rfam 13.0: shifting to a genome-centric resource for non-
982 coding RNA families. Nucleic Acids Res 46:D335-342.

983 62. Li L, Huang D, Cheung MK, Nong W, Huang Q, Kwan HS. 2013. BSRD: a repository
984 for bacterial small regulatory RNA. Nucleic Acids Res 41:D233-238.

985 63. Gruber AR, Lorenz R, Bernhart SH, Neuböck R, Hofacker IL. 2008. The Vienna RNA
986 websuite. Nucleic Acids Res 36:W70–W74.

987 64. Kery MB, Feldman M, Livny J, Tjaden B. 2014. TargetRNA2: identifying targets of
988 small regulatory RNAs in bacteria. Nucleic Acids Res 42:W124-129.

989 65. Narberhaus F. 1999. Negative regulation of bacterial heat shock genes. Mol Microbiol
990 31:1–8.

991 66. Derré I, Rapoport G, Msadek T. 1999. CtsR, a novel regulator of stress and heat shock
992 response, controls *clp* and molecular chaperone gene expression in gram-positive
993 bacteria. Mol Microbiol 31:117–131.

994 67. Novichkov PS, Kazakov AE, Ravcheev DA, Leyn SA, Kovaleva GY, Sutormin RA,
995 Kazanov MD, Riehl W, Arkin AP, Dubchak I, Rodionov DA. 2013. RegPrecise 3.0--a
996 resource for genome-scale exploration of transcriptional regulation in bacteria. BMC
997 Genomics 14:745.

998 68. Winkler W, Nahvi A, Breaker RR. 2002. Thiamine derivatives bind messenger RNAs
999 directly to regulate bacterial gene expression. Nature 419:952–956.

1000 69. Griffith F. 1928. The significance of pneumococcal types. J Hyg (Lond) 27:113–159.

1001 70. Avery OT, Macleod CM, McCarty M. 1944. Studies on the chemical nature of the
1002 substance inducing transformation of pneumococcal types: induction of transformation
1003 by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J Exp Med
1004 79:137–158.

1005 71. Håvarstein LS, Martin B, Johnsborg O, Granadel C, Claverys J-P. 2006. New insights
1006 into the pneumococcal fratricide: relationship to clumping and identification of a novel
1007 immunity factor. Mol Microbiol 59:1297–1037.

1008 72. Veening J-W, Blokesch M. 2017. Interbacterial predation as a strategy for DNA
1009 acquisition in naturally competent bacteria. Nat Rev Microbiol 15:621–629.

1010 73. Moscoso M, Claverys J-P. 2004. Release of DNA into the medium by competent
1011 *Streptococcus pneumoniae*: kinetics, mechanism and stability of the liberated DNA.
1012 Mol Microbiol 54:783–794.

1013 74. Laurenceau R, Péhau-Arnaudet G, Baconnais S, Gault J, Malosse C, Dujeancourt A,
1014 Campo N, Chamot-Rooke J, Le Cam E, Claverys J-P, Fronzes R. 2013. A type IV pilus
1015 mediates DNA binding during natural transformation in *Streptococcus pneumoniae*.
1016 PLoS Pathog 9:e1003473.

1017 75. Bergé M, Mortier-Barrière I, Martin B, Claverys J-P. 2003. Transformation of
1018 *Streptococcus pneumoniae* relies on DprA- and RecA-dependent protection of incoming
1019 DNA single strands. Mol Microbiol 50:527–536.

1020 76. Desai BV, Morrison DA. 2006. An unstable competence-induced protein, CoiA,
1021 promotes processing of donor DNA after uptake during genetic transformation in
1022 *Streptococcus pneumoniae*. J Bacteriol 188:5177–5186.

1023 77. Burghout P, Bootsma HJ, Kloosterman TG, Bijlsma JJE, de Jongh CE, Kuipers OP,
1024 Hermans PWM. 2007. Search for genes essential for pneumococcal transformation: the
1025 RadA DNA repair protein plays a role in genomic recombination of donor DNA. J
1026 Bacteriol 189:6540–6550.

1027 78. Attaiech L, Olivier A, Mortier-Barrière I, Soulet A-L, Granadel C, Martin B, Polard P,
1028 Claverys J-P. 2011. Role of the single-stranded DNA–binding protein SsbB in
1029 pneumococcal transformation: maintenance of a reservoir for genetic plasticity. PLOS
1030 Genet 7:e1002156.

1031 79. Lange R, Wagner C, de Saizieu A, Flint N, Molnos J, Stieger M, Caspers P, Kamber M,
1032 Keck W, Amrein KE. 1999. Domain organization and molecular characterization of 13
1033 two-component systems identified by genome sequencing of *Streptococcus*
1034 *pneumoniae*. Gene 237:223–234.

1035 80. Shen P, Lees JA, Bee GCW, Brown SP, Weiser JN. 2019. Pneumococcal quorum sensing
1036 drives an asymmetric owner–intruder competitive strategy during carriage via the
1037 competence regulon. Nat Microbiol 4:198.

1038 81. Schnorpfeil A, Kranz M, Kovács M, Kirsch C, Gartmann J, Brunner I, Bittmann S,
1039 Brückner R. 2013. Target evaluation of the non-coding csRNAs reveals a link of the
1040 two-component regulatory system CiaRH to competence control in *Streptococcus*
1041 *pneumoniae* R6. Mol Microbiol 89:334–349.

1042 82. Laux A, Sexauer A, Sivaselvarajah D, Kaysen A, Brückner R. 2015. Control of
1043 competence by related non-coding csRNAs in *Streptococcus pneumoniae* R6. Front
1044 Genet 6:246.

1045 83. Tsui H-CT, Mukherjee D, Ray VA, Sham L-T, Feig AL, Winkler ME. 2010.
1046 Identification and characterization of noncoding small RNAs in *Streptococcus*
1047 *pneumoniae* serotype 2 strain D39. J Bacteriol 192:264–279.

1048 84. Aprianto R, Slager J, Holsappel S, Veening J-W. 2016. Time-resolved dual RNA-seq
1049 reveals extensive rewiring of lung epithelial and pneumococcal transcriptomes during
1050 early infection. Genome Biol 17:198.

1051 85. Korfhage C, Wyrich R, Oelmüller U. August 2002. Ammonium sulfate for neutralization
1052 of inhibitory effects. WO2002059360A2.

1053 86. Aranda PS, LaJoie DM, Jorcyk CL. 2012. Bleach gel: a simple agarose gel for analyzing
1054 RNA quality. Electrophoresis 33:366–369.

1055 87. Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data.
1056 Babraham Bioinformatics.

1057 88. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina
1058 sequence data. Bioinforma Oxf Engl 30:2114–2120.

1059 89. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M,
1060 Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinforma Oxf Engl
1061 29:15–21.

1062 90. Liao Y, Smyth GK, Shi W. 2014. *featureCounts*: an efficient general purpose program
1063 for assigning sequence reads to genomic features. Bioinformatics 30:923–930.

1064 91. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion
1065 for RNA-seq data with DESeq2. Genome Biol 15:550.

1066 92. Wagner GP, Kin K, Lynch VJ. 2012. Measurement of mRNA abundance using RNA-seq
1067 data: RPKM measure is inconsistent among samples. Theory Biosci 131:281–285.

1068 93. Langfelder P, Horvath S. 2008. WGCNA: an R package for weighted correlation network
1069 analysis. BMC Bioinformatics 9:559.

1070 94. Bailey TL, Elkan C. 1994. Fitting a mixture model by expectation maximization to
1071 discover motifs in biopolymers. Proc Int Conf Intell Syst Mol Biol 2:28–36.

1072 95. Martínez B, Zomer AL, Rodríguez A, Kok J, Kuipers OP. 2007. Cell envelope stress
1073 induced by the bacteriocin Lcn972 is sensed by the Lactococcal two-component system
1074 CesSR. Mol Microbiol 64:473–486.

1075 96. Grant CE, Bailey TL, Noble WS. 2011. FIMO: scanning for occurrences of a given
1076 motif. Bioinforma Oxf Engl 27:1017–1018.

1077 97. Lanie JA, Ng W-L, Kazmierczak KM, Andrzejewski TM, Davidsen TM, Wayne KJ,
1078 Tettelin H, Glass JI, Winkler ME. 2007. Genome sequence of Avery's virulent serotype
1079 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated
1080 laboratory strain R6. J Bacteriol 189:38–51.

1081

1082

1083 **Tables**

Locus tag	Gene	TPM 0 min.	^{2}log fold change$^\alpha$ 3 min.	10 min.	20 min.
SPV_0192	*rpsJ*	4585	0.7	0.0	-0.1
SPV_0193	*rplC*	2992	0.9	-0.1	0.0
SPV_0194	*rplD*	2181	0.8	-0.1	-0.3
SPV_0195	*rplW*	2919	1.0	-0.2	0.2
SPV_0196	*rplB*	2611	1.0	-0.2	-0.1
SPV_0197	*rpsS*	4134	1.0	-0.5	0.1
SPV_0198	*rplV*	3838	1.0	-0.5	0.1
SPV_0199	*rpsC*	3158	1.0	-0.4	0.0
SPV_0200	*rplP*	4329	1.0	-0.6	0.0
SPV_0201	*rpmC*	3060	0.9	-0.7	0.0
SPV_0202	*rpsQ*	4348	1.1	-0.7	-0.1
SPV_0203	*rplN*	3640	1.1	-0.7	0.1
SPV_0204	*rplX*	3917	1.1	-0.7	0.0
SPV_0205	*rplE*	2848	1.1	-0.7	-0.2
SPV_0206	*rpsN*	2717	1.1	-0.6	-0.3
SPV_0207	*rpsH*	4013	1.3	-0.8	0.0
SPV_0208	*rplF*	4411	1.1	-0.8	-0.2
SPV_0209	*rplR*	3598	1.1	-0.9	-0.2
SPV_0210	*rpsE*	3477	1.1	-0.9	-0.2
SPV_0211	*rpmD*	4120	1.1	-0.9	-0.4
SPV_0212	*rplO*	2247	1.2	-0.7	-0.2
SPV_0213	*secY*	1920	1.0	-0.7	-0.3

1084

1085 **Table 1**. Expression trend of a 22-gene operon (SPV_0192-213) transcribed from TSS

1086 195,877 (+). $^\alpha$Purple cells indicate significance ($p < 0.001$, ^{2}log FC > 1; DESeq (91)).

1087

Gene	Product	TPM	^{2}log fold change			Notes
		0 min.	3 min.	10 min.	20 min.	
comX1	Competence-specific sigma factor	8	9.1	3.8	3.1	
tRNA-Glu-1	tRNA-Glu-UUC	387	2.8	0.1	-0.6	Secondary
comW	Competence positive regulator	17	9.1	4.7	3.0	
purA	Adenylosuccinate synthetase	619	4.4	1.0	0.0	Secondary
ccnC	csRNA3	155	4.4	1.2	0.0	Secondary; also CiaR regulon
srf-03	ncRNA of unknown function	39	7.1	2.0	1.5	
comA	CSP ABC transporter ATP-binding protein	21	9.3	4.1	3.4	
comB	CSP ABC transporter permease	24	9.5	4.4	3.3	
briC	Biofilm-regulating peptide	97	4.9	1.7	0.7	
*ydiL**	Putative membrane peptidase	67	5.3	2.4	1.0	
blpT	BlpT protein	8	3.9	0.7	1.5	BlpR regulon
*blpA**	Peptide ABC transporter permease/ATP-binding protein	2	5.1	2.1	2.5	BlpR regulon
*blpB**	Peptide ABC transporter permease	3	4.5	2.2	2.4	BlpR regulon
blpC	Peptide pheromone	3	4.4	2.7	1.9	BlpR regulon
pncW	Putative bacteriocin	13	4.0	1.1	2.0	BlpR regulon
blpY	Bacteriocin immunity protein	17	4.3	1.4	2.2	BlpR regulon
blpZ	Immunity protein	13	4.3	1.5	1.9	BlpR regulon
pncP	Putative protease	14	4.2	1.3	1.9	BlpR regulon
SPV_2249	Hypothetical protein	50	1.2	0.3	0.8	BlpR regulon
SPV_0817	CAAX amino terminal protease family protein	45	1.3	0.2	0.4	BlpR regulon
ribF	FMN adenylyltransferase/riboflavin kinase	147	5.2	1.2	0.7	
def2	Peptide deformylase	177	3.7	0.0	-0.1	
SPV_1380	Cell shape-determining protein	276	3.9	0.1	0.0	Secondary
SPV_1379	Hypothetical protein	183	3.3	0.0	-0.1	Secondary
yaaA	UPF0246 protein	85	1.2	0.0	-0.3	Secondary
qsrA	ABC transporter ATP-binding protein - Na+ export	223	4.5	0.6	0.2	Secondary
qsrB	ABC transporter permease - Na+ export	216	4.5	0.4	-0.2	Secondary
srf-22	ncRNA of unknown function	54	4.1	0.2	-0.7	Secondary
comM	Immunity factor	9	8.0	4.4	2.8	
tsaE	tRNA processing protein	280	3.1	0.3	0.2	Secondary
SPV_1742	Acetyltransferase	319	2.7	0.3	0.2	Secondary
lytR	LytR-CpsA-Psr family protein	355	2.8	0.1	0.1	Secondary
comX2	Competence-specific sigma factor	8	9.1	3.8	3.1	
tRNA-Glu-3	tRNA-Glu-UUC	387	2.8	0.1	-0.6	Secondary
ybbK	Putative membrane protease subunit	795	2.1	0.6	0.6	TSS too far from ComE-binding site
comC1	Competence-stimulating peptide precursor	36	8.8	5.1	4.6	
comD	Two-component system sensor histidine kinase	27	8.4	4.3	3.8	
comE	Two-component system response regulator	38	8.3	4.0	3.5	
tRNA-Glu-5	tRNA-Glu-UUC	63	5.8	1.9	1.5	Secondary
tRNA-Asn-2	tRNA-Asn-GUU	181	5.1	0.8	0.4	Secondary

1088

1089 **Table 2**. ComE-regulated genes, distributed over 15 operons, as indicated by grey/white
1090 colored blocks. Members of the BlpR regulon are included, as indicated under 'Notes'.
1091 Secondary (under 'Notes') indicates either read-through after incomplete termination or the
1092 influence of an additional TSS. For complete information, including TSS positions, see **Table**
1093 **S5**. Purple cells indicate significance ($p < 0.001$, $^{2}\log FC > 1$; DESeq (91)). *Pseudogene.

1094

Gene	Product	TPM	^{2}log fold change			Notes
		0 min.	3 min.	10 min.	20 min.	
dut	Deoxyuridine 5'-triphosphate nucleotidohydrolase	78	2.7	1.5	-0.2	
SPV_0028	Hypothetical protein	94	2.6	1.6	-0.2	
radA	DNA repair protein	96	2.5	2.4	-0.1	
SPV_0030	Carbonic anhydrase	388	0.8	1.1	0.1	Secondary
srf-01	ncRNA of unknown function	71	3.7	3.3	0.9	Putative pseudogene
SPV_2082*	Hypothetical protein	22	6.6	6.3	2.6	
SPV_0034*	IS1167 transposase	3	3.6	3.7	1.1	
cibA	Two-peptide bacteriocin peptide	8	12.0	12.9	7.4	
cibB	Two-peptide bacteriocin peptide	11	10.2	11.0	5.5	
cibC	CibAB immunity factor	10	10.6	12.3	6.5	
SPV_2121	Hypothetical protein	124	4.6	4.4	0.6	
SPV_0186	Competence-damage induced protein	283	2.4	2.3	0.1	Secondary
SPV_0683	Hypothetical protein	136	4.2	4.9	0.8	
comEA	Late competence DNA receptor	4	9.8	9.2	3.7	
comEC	Late competence DNA transporter	3	9.5	9.8	5.0	
SPV_2256	Hypothetical protein	156	4.0	4.8	1.4	Secondary
SPV_2257*	ABC transporter ATP-binding protein	91	3.7	5.0	1.3	Secondary
SPV_0846	Hypothetical protein	68	3.9	5.4	1.5	Secondary
coiA	Competence protein	1	8.2	7.4	1.2	
pepF1	Oligoendopeptidase F	252	1.3	1.2	0.0	Secondary
SPV_0867	O-methyltransferase family protein	136	1.4	1.5	0.3	Secondary
radC	DNA repair protein	3	9.8	9.8	2.6	
dprA	DNA protecting protein	6	10.1	8.8	4.6	
SPV_2340*	Hypothetical protein	222	2.5	2.6	0.2	Secondary
pgdA	Peptidoglycan N-acetylglucosamine deacetylase	397	1.4	1.7	0.1	Secondary
SPV_1308	Oxidoreductase of aldo/keto reductase family, subgroup 1	182	0.9	1.4	0.3	Secondary
cclA	Type IV prepilin peptidase	2	9.3	9.0	3.7	
ssbB	Single-stranded DNA-binding protein	9	10.7	11.9	6.8	
cinA	ADP-ribose pyrophosphatase/nicotinamide-nucleotide amidase	78	5.8	6.5	2.5	
recA	DNA recombination/repair protein	391	3.0	3.8	0.7	Secondary
dinF	MATE efflux family protein	288	2.2	3.8	0.4	Secondary
lytA	Autolysin/N-acetylmuramoyl-L-alanine amidase	703	1.4	3.2	0.4	Secondary
tyg$^{\alpha}$	n/a	<1	6.7	6.8	4.2	
rmuC	DNA recombination protein	314	2.1	1.8	-0.3	
yhaM	3'->5' exoribonuclease	312	1.7	1.7	-0.4	
SPV_1828	Hypothetical protein	142	6.5	6.0	1.4	
nadC	Quinolinate phosphoribosyltransferase	72	1.7	3.2	0.3	Secondary
SPV_1825*	IS630-Spn1 transposase	162	0.5	1.7	0.0	Secondary
SPV_1824	ABC transporter, permease	34	0.9	2.7	0.2	Secondary
comGA	Late competence protein	14	9.4	10.4	5.1	
comGB	Late competence protein	8	9.4	10.8	5.4	
comGC	Late competence protein	6	9.3	11.2	5.3	
comGD	Late competence protein	4	10.1	12.1	6.3	

comGE	Late competence protein	3	10.3	12.3	6.5	
comGF	Late competence protein	2	9.9	12.2	6.5	
comGG	Late competence protein	8	8.7	11.0	5.5	
SPV_2427*	S-adenosylmethionine-dependent methyltransferase	2	10.3	13.0	7.2	
srf-29	ncRNA of unknown function	3	9.3	8.8	3.0	
cbpD	Choline-binding protein D	6	9.9	10.0	3.8	
SPV_2027	Cytoplasmic thiamin-binding component of thiamin ABC transporter	89	1.0	1.2	-0.7	Secondary
thiX	Thiamin ABC transporter transmembrane component	88	0.8	1.2	-1.1	Secondary
thiY	Thiamin ABC transporter substrate-binding component	134	0.8	0.9	-1.1	Secondary
thiZ	Thiamin ABC transporter ATPase component	128	0.6	1.0	-1.2	Secondary
comFA	DNA transporter ATPase	3	9.0	8.1	2.9	
comFC	Phosphoribosyltransferase domain protein	3	8.6	8.5	2.7	
hpf	Ribosome hibernation promotion factor	624	0.9	1.4	-0.3	Secondary

Table 3. ComX-regulated genes, distributed over 19 operons, as indicated by grey/white colored blocks. Secondary (under 'Notes') indicates either read-through after incomplete termination or the influence of an additional TSS. For complete information, including TSS positions, see **Table S5**. Purple and green cells indicate significance ($p < 0.001$, $|^2\log FC| > 1$; DESeq (91)). [α]The *tyg* TSS was previously found to be ComX-regulated (32). An artificial 250 nucleotide transcript starting on this TSS was added to the annotation file to allow differential expression analysis. *Pseudogene.

		TPM	^{2}log fold change			
Gene	**Product**	**0 min.**	**3 min.**	**10 min.**	**20 min.**	**Notes**
ccnC	csRNA3	155	4.4	1.2	0.0	Also ComE regulon
SPV_0098	Glycosyltransferase, group 2 family	218	-0.5	1.0	0.3	
ccnE	csRNA5	32	0.6	0.8	0.2	
ccnA	csRNA1	27	-2.0	-1.4	-1.0	
ccnB	csRNA2	2	-0.6	2.8	-1.5	
ccnD	csRNA4	25	0.4	1.9	0.0	
manL	Mannose-specific PTS IIAB components	3379	0.0	-3.3	-1.0	
manM	Mannose-specific PTS IIC component	3296	0.1	-3.1	-1.1	Also CcpA-binding site
manN	Mannose-specific PTS IID component	4750	0.2	-3.3	-1.0	
rimP	Bacterial ribosome SSU maturation protein	228	-0.2	1.0	0.8	
nusA	Transcription termination/antitermination protein	206	0.1	1.0	0.9	
SPV_0480	Putative transcription termination proten	117	0.5	1.2	1.1	CiaR-binding motif on opposite strand
SPV_0481	L7Ae family ribosomal protein	103	0.6	1.4	1.4	
infB	Translation initiation factor 2	277	0.8	0.7	0.9	
rbfA	Ribosome-binding factor A	194	1.0	0.5	0.8	
ciaR	Two-component system response regulator	225	0.2	3.2	0.3	
ciaH	Two-component system sensor histidine kinase	164	0.3	3.2	0.2	
SPV_0775	Acetyltransferase	28	0.4	4.8	1.0	
prsA	Putative parvulin type peptidyl-prolyl isomerase	267	1.3	3.9	0.6	Potentially also ComX regulon
SPV_0913	Extracellular protein	55	2.0	5.9	1.3	
rlmCD	23S rRNA (uracil(1939)-C(5))-methyltransferase	38	-0.4	2.0	0.4	Secondary
tarI$^{\alpha}$	Ribitol-5-phosphate cytidylyltransferase	308	0.6	0.4	0.2	
tarJ$^{\alpha}$	Ribulose-5-phosphate reductase	362	0.4	0.3	0.1	
licA$^{\alpha}$	Choline kinase	319	0.6	0.2	0.1	
licB$^{\alpha}$	Choline permease	397	0.6	0.0	-0.1	
licC$^{\alpha}$	Cholinephosphate cytidylyltransferase	422	0.8	0.0	0.0	
srf-21	ncRNA of unknown function	325	0.7	3.2	0.3	
axe1	Acetyl xylan esterase 1/cephalosporin-C deacetylase	58	0.8	4.2	0.2	Secondary
SPV_1769	Membrane protein	497	0.0	1.9	-0.4	
malQ	4-alpha-glucanotransferase (amylomaltase)	69	0.1	4.2	-0.2	
malP	Maltodextrin phosphorylase	83	0.2	4.2	-0.4	
dltX	D-alanyl-lipoteichoic acid biosynthesis protein	277	-0.6	1.9	0.2	
dltA	D-alanine--poly(phosphoribitol) ligase subunit 1	392	-0.3	1.6	0.1	
dltB	D-alanyl transfer protein	322	0.0	1.6	0.0	CiaR-binding motif on opposite strand
dltC	D-alanine--poly(phosphoribitol) ligase subunit 2	429	0.0	1.5	0.1	
dltD	Poly(glycerophosphate chain) D-alanine transfer protein	326	0.3	1.5	0.0	
htrA	Serine protease	49	1.5	7.0	1.5	
parB	Chromosome partitioning protein	57	0.9	6.5	1.4	Secondary

1104

1105 **Table 4**. CiaR-regulated genes, distributed over 18 operons, as indicated by grey/white

1106 colored blocks. Secondary (under 'Notes') indicates either read-through after incomplete

termination or the influence of an additional TSS. For complete information, including TSS positions, see **Table S5**. Purple and green cells indicate significance ($p < 0.001$, $|^2\log FC| > 1$; DESeq (91)). [α]Operon has two different detected TSSs. The TSS at 1,159,217 (-) is under control of CiaR.

Gene	Product	TPM 0 min.	^{2}log fold change 3 min.	10 min.	20 min.	Regulon	Notes
spxA2	Transcriptional regulator SpxA2	772	0.0	1.4	0.0	VraR	
SPV_0179	Hypothetical protein	163	-0.2	1.7	0.1	VraR	Secondary
clpL	Clp protease ATP-binding subunit ClpL	24	-0.2	6.6	3.7	CtsR	
vraT	Cell wall-active antibiotics response protein VraT	62	0.1	2.4	-0.1	VraR	
vraS	Two-component system sensor histidine kinase VraS	52	0.0	2.2	0.0	VraR	
vraR	Two-component transcriptional regulator VraR	48	0.1	2.2	-0.3	VraR	
*alkD**	DNA alkylation repair enzyme AlkD	43	0.0	2.2	0.0	VraR	
SPV_0355	Hypothetical protein	118	-0.2	1.2	0.5	VraR	Secondary
*cbpG**	Choline-binding protein CbpG	156	-0.2	1.1	0.4	VraR	Secondary
cbpK	Choline-binding protein CbpK	151	0.1	1.0	0.3	VraR	Secondary
hrcA	Heat-inducible transcription repressor HrcA	650	-0.2	2.7	0.1	VraR, HrcA	
grpE	Heat shock protein GrpE	600	-0.3	2.7	-0.1	VraR, HrcA	
dnaK	Chaperone protein DnaK	1005	-0.6	2.5	-0.2	VraR, HrcA	
SPV_2171	Hypothetical protein	679	-0.8	2.3	-0.1	VraR, HrcA	
dnaJ	Chaperone protein DnaJ	551	-0.6	2.1	-0.1	VraR, HrcA	
clpE	Clp protease ATP-binding subunit ClpE	168	0.2	1.4	0.9	CtsR	
SPV_0803	Putative phage shock protein C	316	0.1	1.7	-0.1	VraR, HrcA	
groES	Heat shock protein 60 family co-chaperone GroES	1099	0.5	2.5	0.6	CtsR, HrcA	
groEL	Heat shock protein 60 family chaperone GroEL	1146	0.3	2.2	0.6	CtsR, HrcA	
ctsR	Transcriptional regulator CtsR	162	-0.2	1.3	1.2	CtsR	
clpC	Clp protease ATP-binding subunit ClpC	257	-0.1	1.0	0.9	CtsR	
SPV_2021	Hypothetical protein	220	0.3	1.1	1.0	CtsR?	
SPV_2020	Two-component system response regulator	167	0.1	0.9	1.1	CtsR?	
SPV_2019	Two-component system sensor histidine kinase	86	0.4	1.1	1.2	CtsR?	

Table 5. Competence-induced genes that are part of the VraR, CtsR and/or HrcA regulons. Operons are indicated by grey/white colored blocks. Secondary (under 'Notes') indicates either read-through after incomplete termination or the influence of an additional TSS. For complete information, including TSS positions, see **Table S5**. Purple cells indicate significance ($p < 0.001$, $|^2\log FC| > 1$; DESeq (91)). *Pseudogene.

Figure legends

Figure 1. Overview of the regulatory network controlling competence in *Streptococcus pneumoniae*. (**A**) Positive-feedback loop responsible for activation of competence, adapted from Slager et al., 2014 (6). HtrA-mediated degradation of CSP is relevant to the competence-inducing effect of certain antibiotics (8). (**B**) Early competence is shut down through the action of late competence protein DprA. Binding of DprA to phosphorylated ComE leads to an increase of unphosphorylated ComE dimers repressing *comCDE* expression. Late competence shutdown is the result of the competition for ComW and RNA polymerase binding between sigma factors ComX (σ^X) and RpoD (σ^A). Although expression of *rpoD* is strongly upregulated during competence, the underlying mechanism is unknown.

Figure 2. Venn diagrams of differentially expressed genes, created with http://eulerr.co. Diagrams show how many genes were significantly up- (purple) or downregulated (green), using a cutoff fold change of 2 (left) or 4 (right). Differential expression 3, 10 and 20 minutes after addition of CSP is indicated by solid, dashed and dotted lines, respectively.

Figure 3. Regulons affected during competence. Top of each panel: expression profiles of genes previously reported to be ComE-, ComX-, CiaR-, or VraR-activated. Each line shows the fold changes (vs. t=0) of a single gene from the corresponding regulon. Except for the VraR regulon (all genes shown) only genes that fell into the appropriate WGCNA cluster (cl. 29 for ComE, cl. 11 for ComX, cl. 33 for CiaR) were included. Bottom of each panel: position weight matrices of the recognition sites for ComE, ComX, CiaR and VraR, as determined with MEME (94), from the promoters of the HC members of the corresponding

1142 regulons (ComE, ComX and CiaR) or from three pneumococcal and six *L. lactis* promoters
1143 (VraR; (15)).

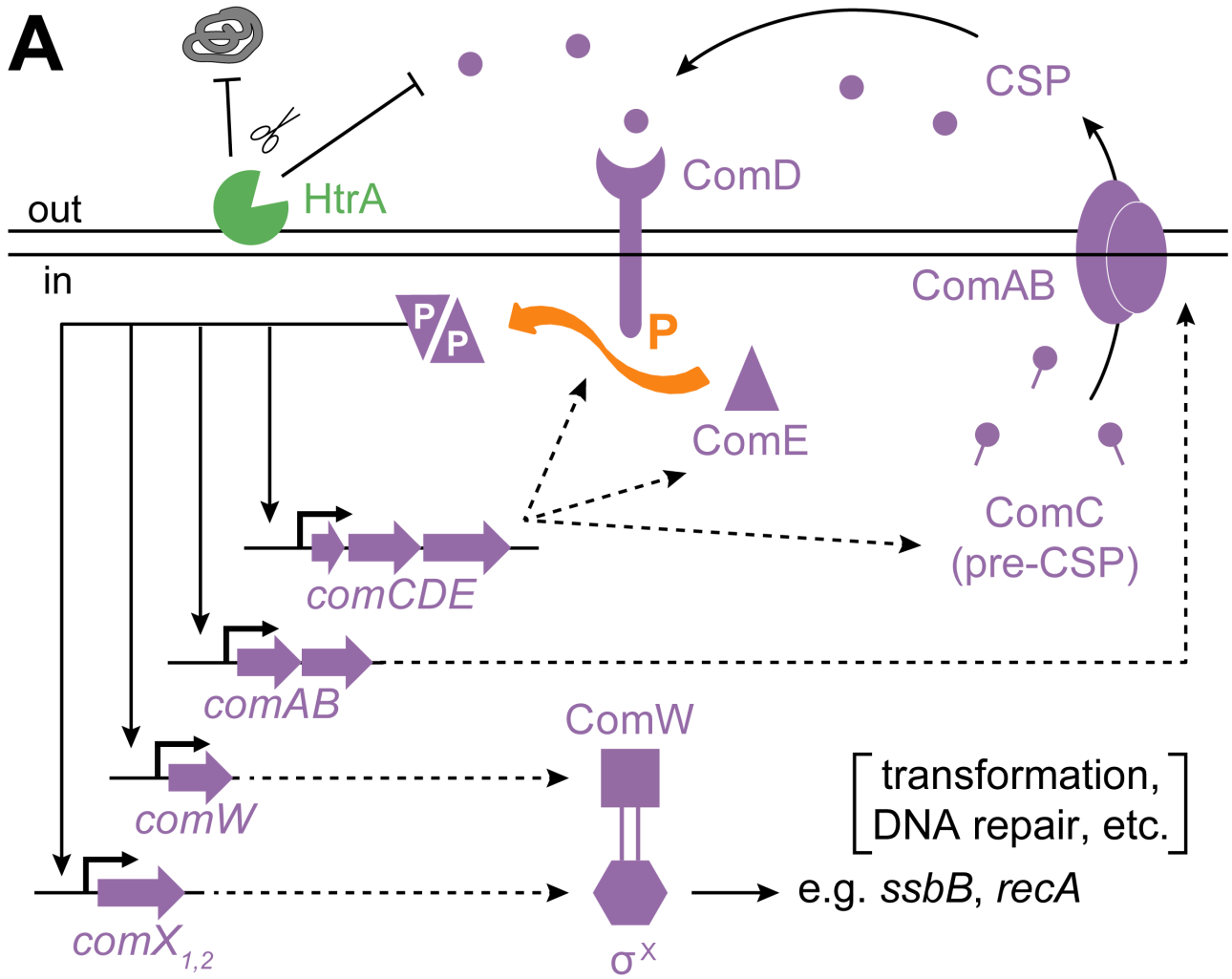
1144 **Figure 4**. (**A**) ComE-binding sequences on the *S. pneumoniae* D39V genome. Consensus
1145 sequence was determined as described in **Materials and Methods**. Multiple possible
1146 nucleotides are indicated, according to IUPAC nomenclature, by R (A/G), Y (C/T), W (A/T),
1147 B (C/G/T), D (A/G/T), V (A/C/G) or N (any). Nucleotides and spacings colored red deviate
1148 from this consensus. HC: high-confidence. (**B**) Putative BlpR-binding site consensus. The
1149 blue box corresponds to the internal spacer indicated in panel A. Consensus sequence
1150 (IUPAC nomenclature, as in **A**) was determined as described in **Materials and Methods**,
1151 where letters colored green indicate where the BlpR consensus is incompatible with the
1152 ComE consensus. [α]Promoters of *comX1* (SPV_0014) and *comX2* (SPV_1818) are identical.
1153 [β]These operons were not differentially expressed in response to CSP addition. [γ]The genes
1154 encoding the export machinery of signaling peptide BlpC and bacteriocins BlpK and PncW,
1155 *blpA* and *blpB*, are frameshifted, eliminating the regulatory positive-feedback loop of the Blp
1156 system. [δ]First gene both annotated in D39V and D39W (97).

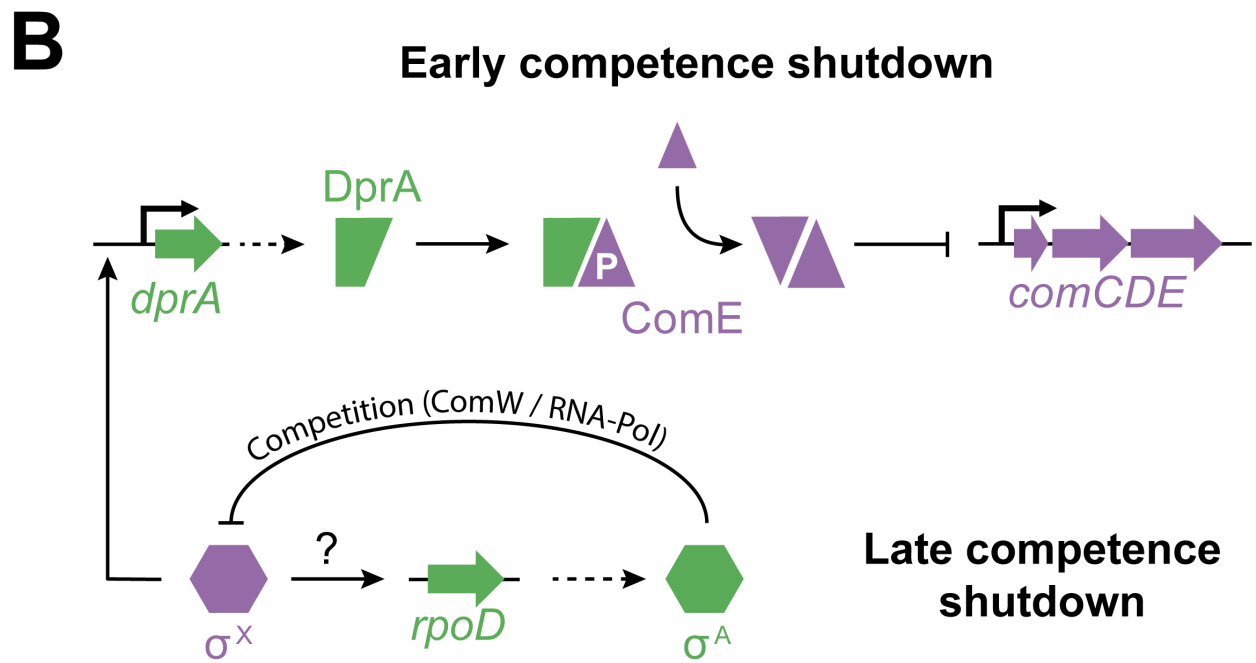
1157 **Figure 5**. ComX-binding sequences on the *S. pneumoniae* D39V genome. Consensus
1158 sequence was determined as described in **Materials and Methods**. Multiple possible
1159 nucleotides are indicated, according to IUPAC nomenclature, by R (A/G), Y (C/T), W (A/T),
1160 M (A/C), D (A/G/T), H (A/C/T) or N (any). HC: high-confidence. [α]First gene both annotated
1161 in D39V and D39W (97), if available. [β]*tyg* was described by Campbell et al. (32), its TSS
1162 was detected in PneumoBrowse (41), but no CDS or ncRNA has been reported (41, 59).

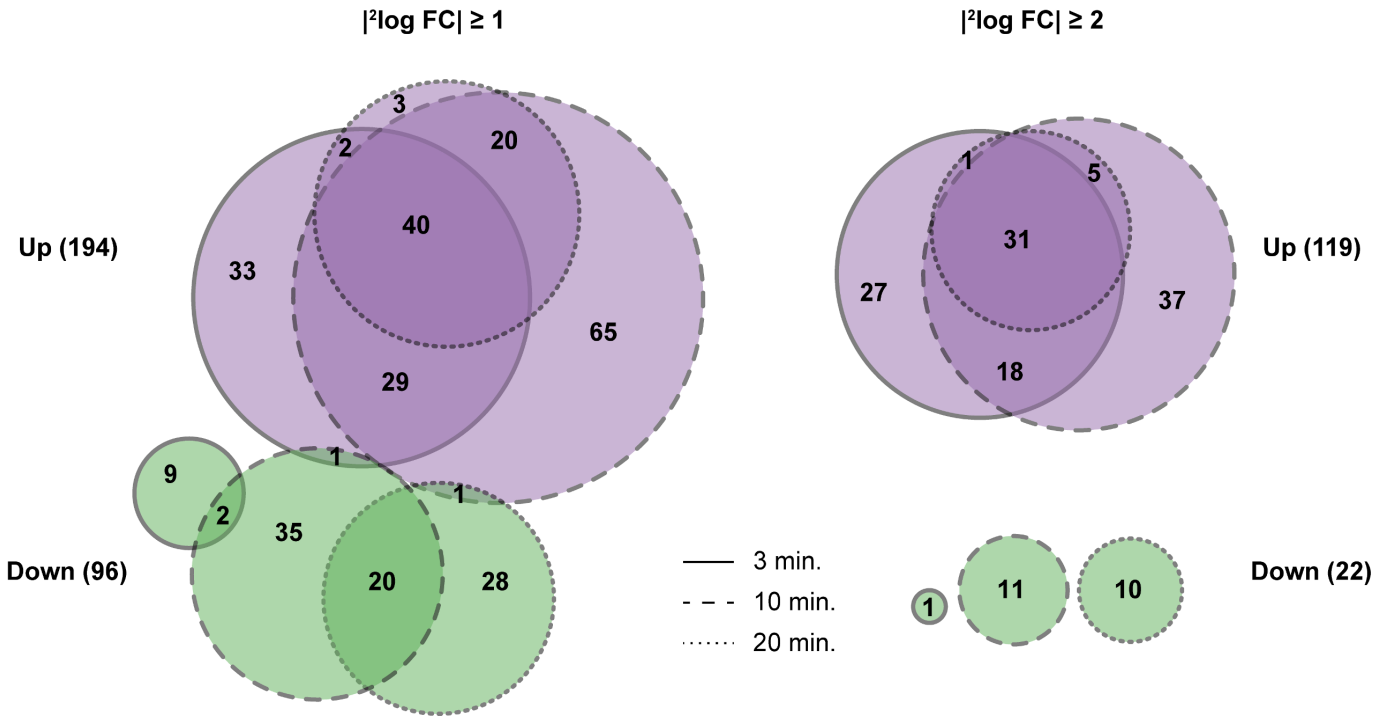
1163 **Figure 6**. Top: overview of the complex *cinA-recA-dinF-lytA* operon, with an imperfect
1164 internal terminator and TSSs upstream of each gene, leading to four overlapping operons.
1165 The TSS upstream of *cinA* is preceded by a ComX-binding site and addition of CSP indeed

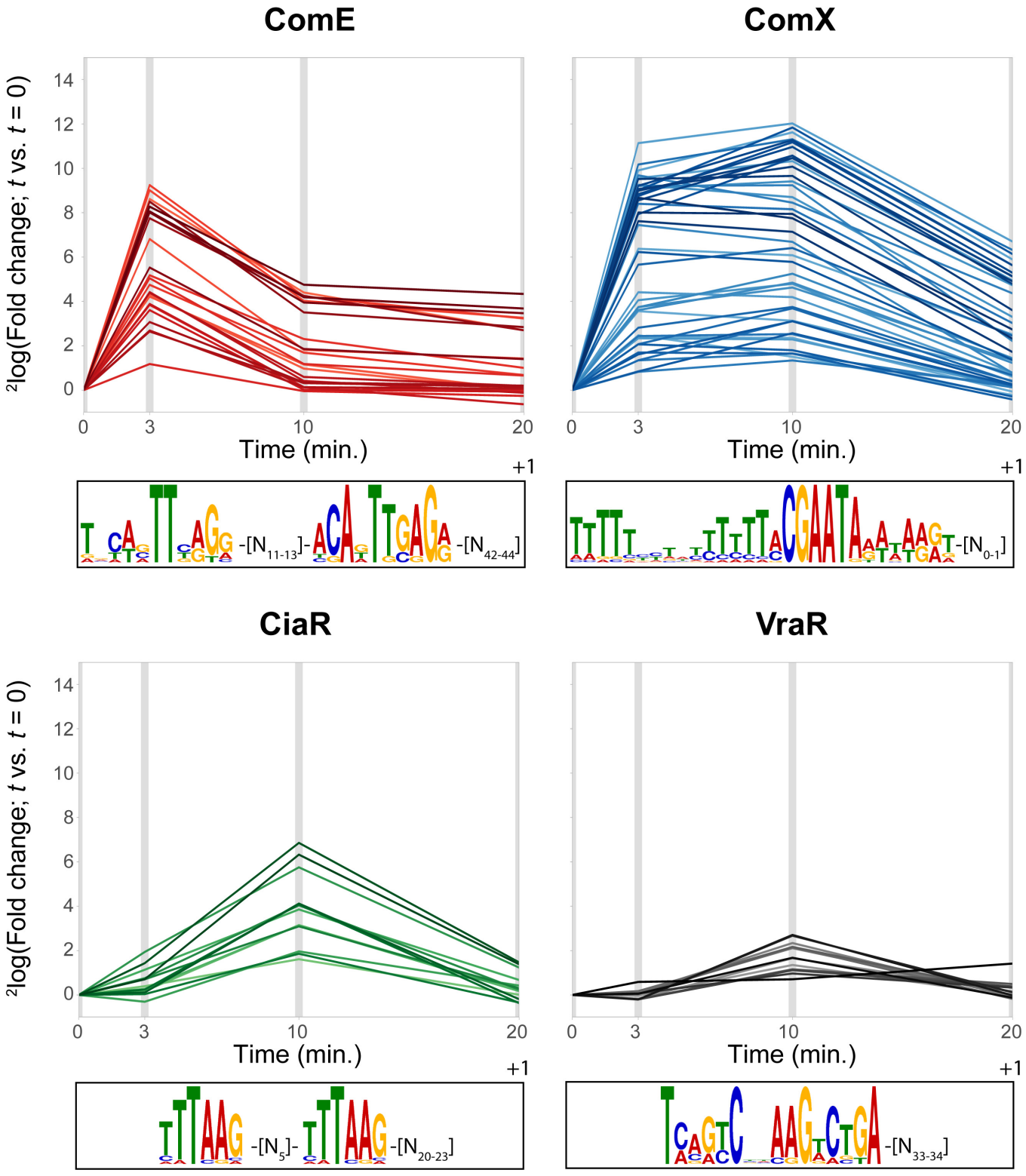
affects expression of all four genes in the operon. Bottom: ^{2}log(fold change) relative to t=0 (i.e. basal expression). However, the effect size decreases with every gene, due to differences in basal expression from the internal TSSs. Additionally, a ComX-regulated TSS, is found inside of and antisense to *dinF*, giving rise to the hypothetical transcript *tyg* (32), with an unknown 3'-end.

Figure 7. Non-coding RNA *srf-21* (SPV_2378) is part of the CiaR-regulon. (**A**) Overview of the genomic context of *srf-21*. CiaR-dependent upregulation of downstream gene *axe1* might be due to read-through from *srf-21*. The CiaR-binding sequence is indicated by a boxed 'C'. (**B)** MFE secondary structure of *srf-21*, as predicted by RNAfold (63).









A

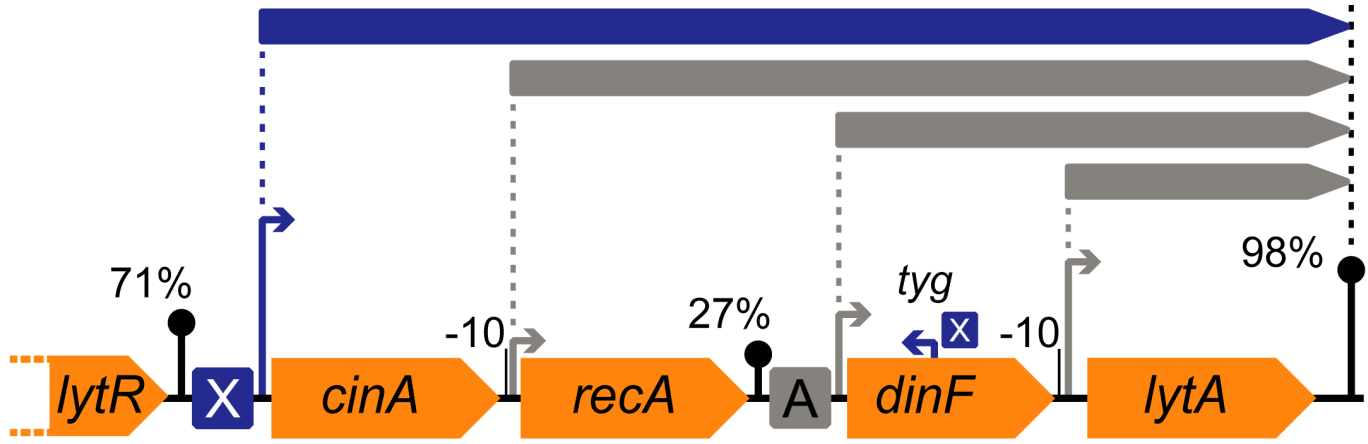
	TSS	First gene[δ]	
HC ComE regulon	14240 (+)[α]	*comX1* (SPV_0014)	TGCAGTTTAGG - 13 - AGAATGGAGA - 42 - TSS
	22142 (+)	*comW* (SPV_0023)	TCTTGTTGAGG - 11 - ACAATTCAGG - 42 - TSS
	41719 (+)	*comA* (SPV_0049)	TGCAGTTGGGA - 11 - TCATTTGGGA - 44 - TSS
	394420 (+)	*briC* (SPV_0391)	TGCAATTCAGG - 11 - CCAGTTGAGG - 43 - TSS
	961366 (-)	*ribF* (SPV_0994)	TACACTTCATG - 11 - ACATTTCAGA - 43 - TSS
	1400846 (-)	*def2* (SPV_1381)	TCATCTTCGGA - 12 - ACAGTTGAGG - 42 - TSS
	1551527 (-)	*qsrA* (SPV_1528)	AACAATTCAGG - 11 - ACAGTTGAGG - 42 - TSS
	1738327 (-)	*comM* (SPV_1744)	GTTTGTTTAGC - 11 - ACATTTGAGA - 44 - TSS
	2043461 (-)	*comC1* (SPV_2065)	TACACTTTGGG - 11 - ACAGTTGAGA - 43 - TSS
		Consensus ComE	**TNYWVTTBRGR - 11 - ACADTTGAGR - 42 - TSS**
	1958186 (-)	*ybbK* (SPV_1984)	GACAATTCACC - 11 - ACATTTCAGG - 52 - TSS
BlpR regulon[γ]	477186 (-)	*blpT* (SPV_0466)	CGCAATTCAAG - 11 - ACAATTAAAG - 42 - TSS
	483648 (-)	*blpA* (SPV_0472)	TACCATTCAGG - 11 - ACTATTCAAG - 42 - TSS
	483870 (+)	*blpY* (SPV_0473)	GCTAATTCAAG - 11 - ACAATTCAAG - 42 - TSS
	39464 (+)[β]	*blpK* (SPV_0046)	GCTAATTCAAG - 11 - CCAATTCAAG - 42 - TSS
	477290 (+)[β]	*blpS* (SPV_0467)	TTTTATTCAAA - 9 - GTCATTGAAA - 43 - TSS
	835420 (+)	SPV_0817	TATAGTTTAAG - 11 - ACAATTCATG - 42 - TSS

B

BlpR regulon[γ]	477186 (-)	*blpT* (SPV_0466)
	483648 (-)	*blpA* (SPV_0472)
	483870 (+)	*blpY* (SPV_0473)
	39464 (+)[β]	*blpK* (SPV_0046)
	477290 (+)[β]	*blpS* (SPV_0467)
	835420 (+)	SPV_0817
		Consensus BlpR

```
GCAATTCAAG ACATTTCAATG ACAATTAAAG --ATTTGAATA
ACCATTCAGG AAGTTTTAATG ACTATTCAAG --ATTTCATAA
CTAATTCAAG ACGTTTCGATG ACAATTCAAG --ATCTGGATG
CTAATTCAAG ACGTTTCGATG CCAATTCAAG --ATTTGGATG
TTTATTCAAA TC-TTT-AATT GTCATTGAAA TGTCTTGAATT
ATAGTTTAAG ATGTTTTGATG ACAATTCATG --ATTTGAAGA
NYAATTCAAG ANGTTTYRATG ACAATTCAAG --ATTTGRANN
X1         Y1          X2         Y2
```

	TSS	First gene[α]	
HC ComX regulon	31219 (-)	SPV_0034	TTTTTCAAACTTTTTCCGAATAAATAGAT - A
	138189 (-)	*cibA* (SPV_0133)	TTTTCCTTTTCTTTTCCGAATATAAAAGT - G
	189781 (+)	SPV_0186	TTTTTCTTGTCTTTTACGAATAGAAAGAA - A
	857941 (+)	*comEA* (SPV_0843)	TTTTTTCTTCCTCTTACGAATAATCTAAG
	879640 (+)	*coiA* (SPV_0865)	TCTTTTTTCTTTTTTACGAATGATATAGA
	975625 (-)	*radC* (SPV_0975)	TTTTCCATCCTTCTCACGAATAATAAAGT - G
	1154757 (-)	*dprA* (SPV_1122)	TTTTTACGAACTTTTACGAATAGATAGAT - G
	1329816 (-)	*pgdA* (SPV_1309)	TATTAGCTTTCTTTTCCGAATAAATAGAT - A
	1609620 (+)	*cclA* (SPV_1593)	TTTCTCCCATTTTTTACGAATAGATAAGT - A
	1712878 (-)	*ssbB* (SPV_1711)	TTTTTTTGATTTCCTACGAATAGATAAGT - A
	1735554 (-)	*cinA* (SPV_1740)	TTTTTTCAAAAAAATACGAATAGATAGGT - A
	1776166 (-)	*rmuC* (SPV_1778)	AAGTGTACGCTTCTAACGAATATATAGAT - A
	1814090 (-)	SPV_1828	TTTTTATTTTTCATTACGAATAATATAGA - TG
	1843500 (-)	*comGA* (SPV_1863)	TTTTTCTAACTCTTTGCGAATAGTATAGG - TG
	2008356 (-)	*cbpD* (SPV_2028)	CTTTTTCTTTTTTCTCCGAATAATTTAGG - TG
	2015211 (-)	*comFA* (SPV_2035)	AAAGTGCTAGTTTTTACGAATAAAGAAGT - A
		Consensus ComX	**TTTTTNHNNNYTHTTMCGAATADWNWRRD - TSS**
	24736 (+)	*dut* (SPV_0027)	TTTTTCGTGCTTTTTCCGAATAAATAAGA - TA
	29658 (+)	*srf-01* (SPV_2081)	TTTTTCGTCTTTTTTCCGAATAAATAGAT - A
	698401 (+)	SPV_0683	AATCTACAAGATTTTACGAATAAACTAAT - G
	1732125 (+)	*tyg*[β]	TTCTGGCACACTTTTACGAATATAATAAA - AG



Basal expr. (TPM)		**78**	**391**	**288**	**703**
Log_2 FC	3 min.	**5.8**	**3.0**	**2.2**	**1.4**
	10 min.	**6.5**	**3.8**	**3.8**	**3.2**
	20 min.	**2.5**	**0.7**	**0.4**	**0.4**