

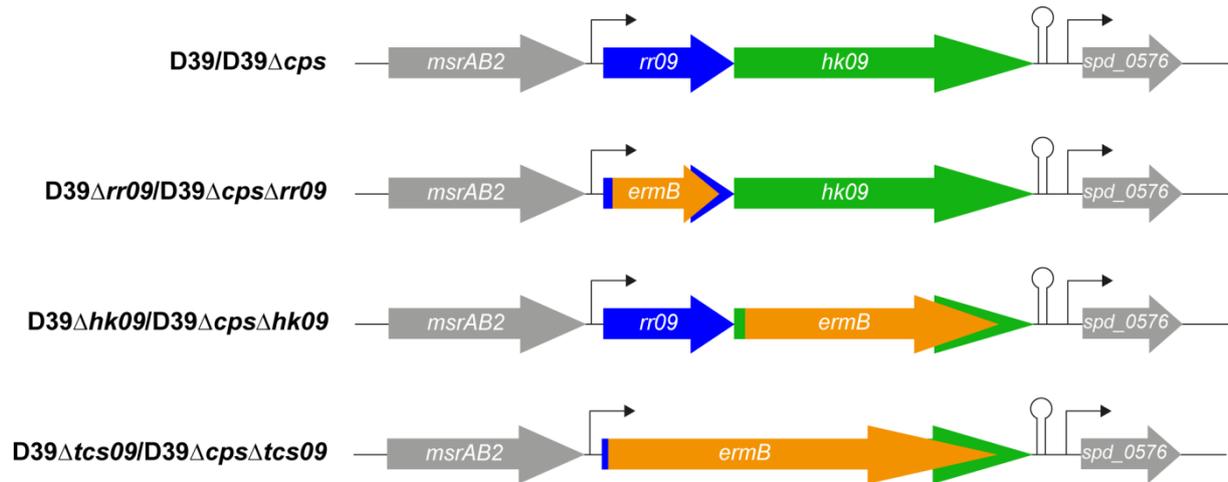
**Table S2.** Inline barcode sequences.

BC ID	Barcode sequence 5'-3'	Oligonucleotide sequence
L01	CCAAGTCG	ACCAAGTCGAGATCGGAAGAGCGTCGTGTA
L02	GCAGCCAC	AGCAGCCACAGATCGGAAGAGCGTCGTGTA
L03	GTAAGTGC	AGTAACTGCAGATCGGAAGAGCGTCGTGTA
L04	TCATCGTG	ATCATCGTGAGATCGGAAGAGCGTCGTGTA
L05	TTACCACG	ATTACCACGAGATCGGAAGAGCGTCGTGTA
L06	AGAATTAT	AAGAATTATAGATCGGAAGAGCGTCGTGTA
L07	GGCCCAAG	AGGCCCAAGAGATCGGAAGAGCGTCGTGTA
L08	TACAACAT	ATACAACATAGATCGGAAGAGCGTCGTGTA
L09	TGAACCAG	ATGAACCAGAGATCGGAAGAGCGTCGTGTA
L10	ATATGGAC	AATATGGACAGATCGGAAGAGCGTCGTGTA
L11	CAACTCGC	ACAACCTCGCAGATCGGAAGAGCGTCGTGTA
L12	CGGAGGGC	ACGGAGGGCAGATCGGAAGAGCGTCGTGTA
L13	ACATTATT	AACATTATTAGATCGGAAGAGCGTCGTGTA
L14	ATCACTTG	AATCACTTGAGATCGGAAGAGCGTCGTGTA
L15	CCCGTCTT	ACCCGTCTTAGATCGGAAGAGCGTCGTGTA
L16	CTCGGTAC	ACTCGGTACAGATCGGAAGAGCGTCGTGTA
L17	GTCTGGCG	AGTCTGGCGAGATCGGAAGAGCGTCGTGTA
L18	TCCCGCGG	ATCCCGCGGAGATCGGAAGAGCGTCGTGTA
L19	CGGCACTT	ACGGCACTTAGATCGGAAGAGCGTCGTGTA
L20	GAGATTGT	AGAGATTGTAGATCGGAAGAGCGTCGTGTA
L21	TACAGATG	ATACAGATGAGATCGGAAGAGCGTCGTGTA
L22	TGGGAGAC	ATGGGAGACAGATCGGAAGAGCGTCGTGTA
L23	CCCTACAG	ACCCTACAGAGATCGGAAGAGCGTCGTGTA
L24	CTCTAACT	ACTCTAACTAGATCGGAAGAGCGTCGTGTA
L25	AAGTGTTG	AAAGTGTTGAGATCGGAAGAGCGTCGTGTA
L26	GAGCCATC	AGAGCCATCAGATCGGAAGAGCGTCGTGTA
L27	GGTCCTCT	AGGTCCTCTAGATCGGAAGAGCGTCGTGTA
L28	TACCGGCC	ATACCGGCCAGATCGGAAGAGCGTCGTGTA
L29	CCCTCGGC	ACCCTCGGCAGATCGGAAGAGCGTCGTGTA
L30	CTGGATCG	ACTGGATCGAGATCGGAAGAGCGTCGTGTA
L31	TTTCTAAC	ATTTCTAACAGATCGGAAGAGCGTCGTGTA
L32	CCGGTACC	ACCGGTACCAGATCGGAAGAGCGTCGTGTA

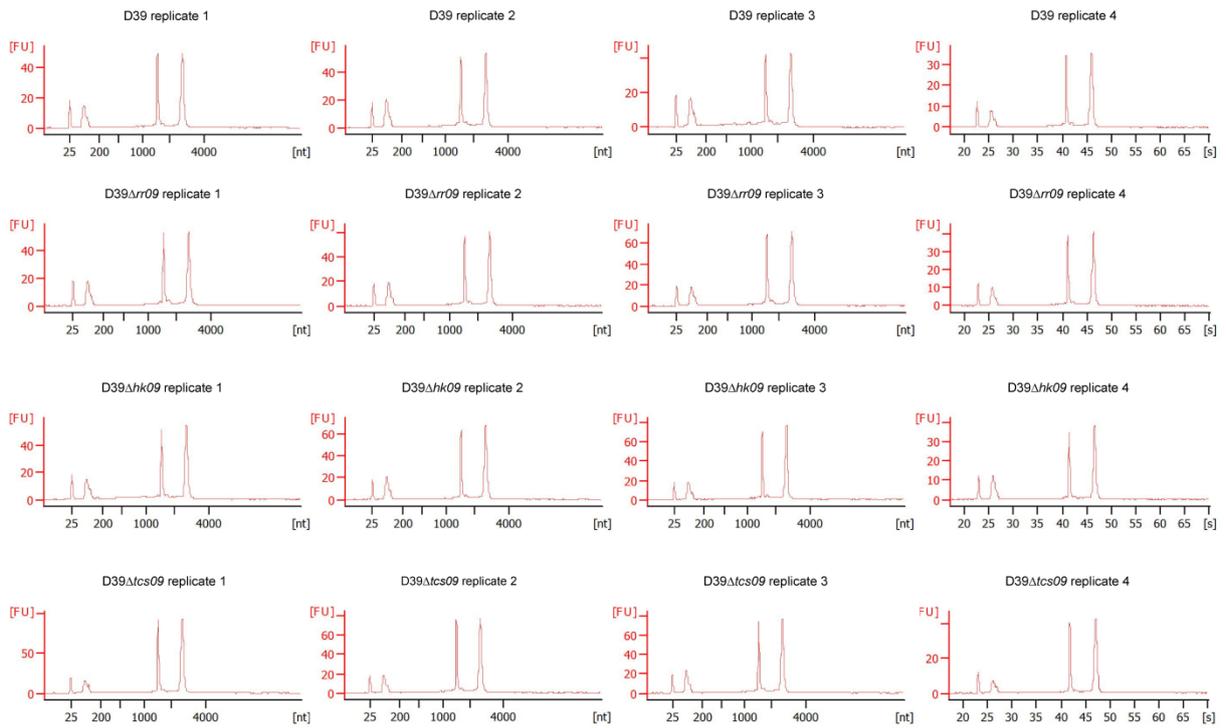
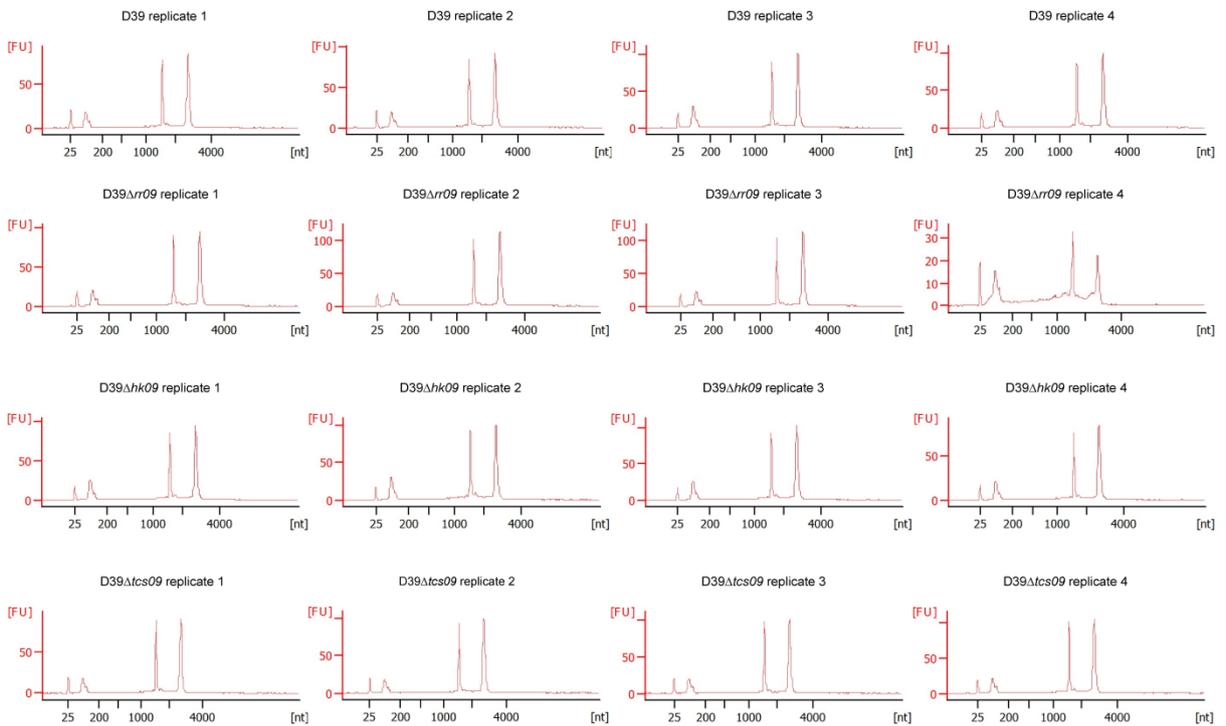
**Table S3.** Primers used for RNA preparation for RNA-sequencing.

Index code	Barcode sequence 5'-3'	Primer sequence 5'→3'
AR2		TACACGACGCTCTTCCGAT
3Tr3		iCiGiCCAGACGTGTGCTCTTCCGATCTrGrGrG
P5		AATGATACGGCGACCACCGAGATCTACACTCTTTC CCTACACGACGCTCTTCCGATCT
X01	AAGTAGAG	CAAGCAGAAGACGGCATAACGAGATAAGTAGAGGT GACTGGAGTTCAGACGTGTGCTCTTCCGATCT

*iC = iso-dC; iG = iso-dG; rG = ribonucleotide G*

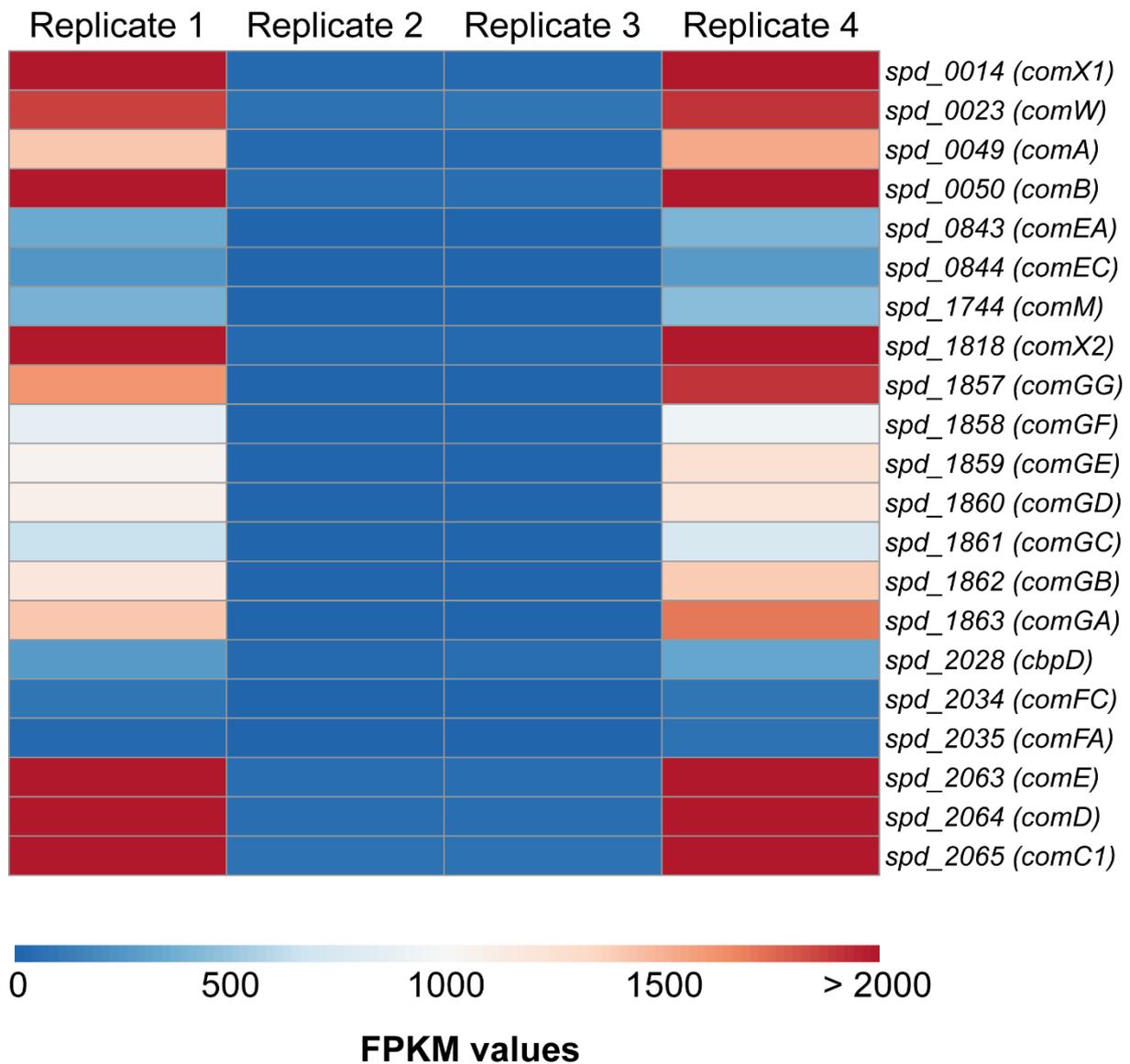


**Figure S1:** Genomic organization of the *tcs09* gene cluster in *S. pneumoniae* D39 wild-type and isogenic *tcs09*-mutants. The *rr09* gene is shown in blue and the *hk09* gene in green within its operon organization, the genes upstream and downstream of *rr09* and *hk09* are shown in grey and the inserted *ermB*- cassette for *tcs09* deletions is indicated by orange. The large and filled arrows represent their relative gene size and orientation in the genome. Transcriptional start sites are indicated with a thin arrow and terminators with lollypops.

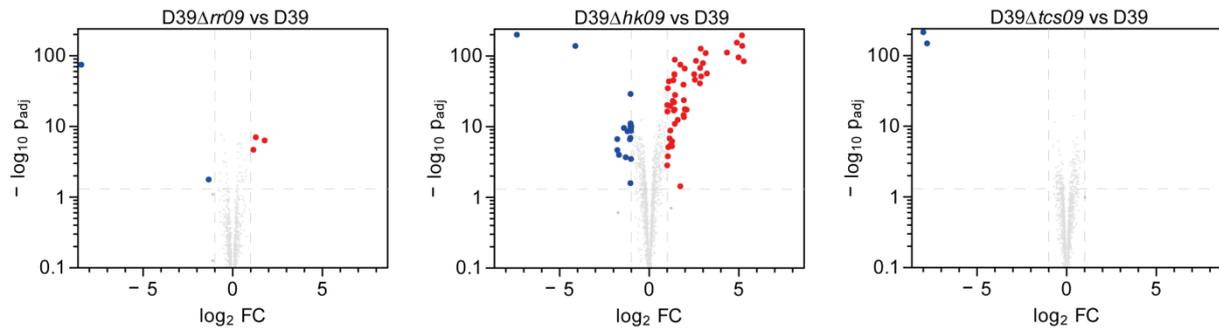
**A****RNA-seq****B****qPCR**

**Figure S2.** RNA integrity check with the Agilent Bioanalyzer. Shown are the electrophoretic representations of the individual RNA samples used for (A) RNA-seq and (B) qPCR. The curves consist of added marker and the individual ribosomal RNAs, which are used to calculate the RNA quality.

## D39Δ*tcs09*

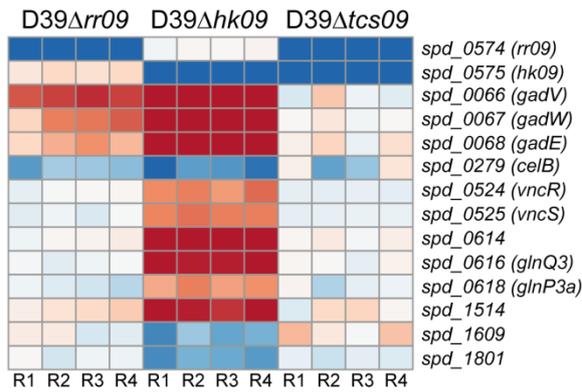


**Figure S3.** Expression profile of competence genes in D39Δ*tcs09*. Heat map showing FPKM values of competence genes in individual replicates of D39Δ*tcs09*. Red boxes show high values in the replicates, blue boxes low values.

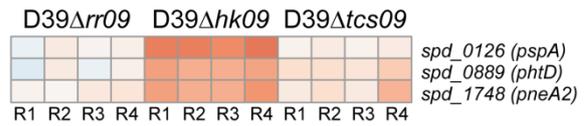


**Figure S4.** Volcano plots of the differentially expressed genes of *D39Δrr09*, *D39Δhk09* and *D39Δtcs09* identified by RNA-seq. Histograms represent the two-dimensional distribution of identified genes by fold change and p-value. Genes with a  $-\log_{10} p_{adj}$ -value  $\geq 1.3$  and a  $\log_2$  fold change  $\geq 1$  or  $\leq -1$  were set significant. Significantly downregulated genes are shown in blue and significantly upregulated genes in red.

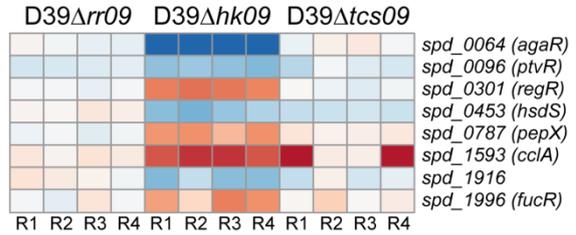
### Environmental Information Processing



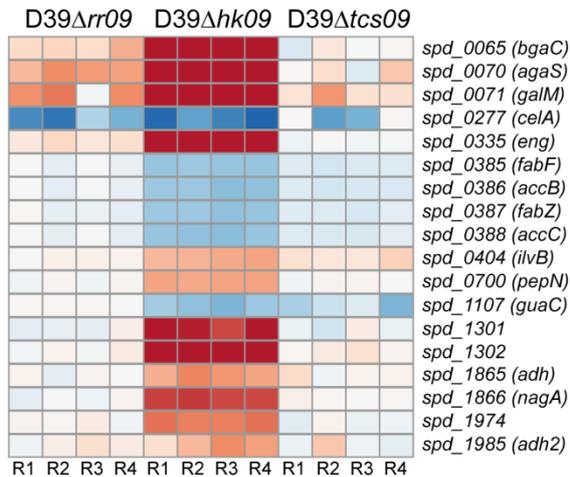
### Cellular Processes



### Genetic Information Processing

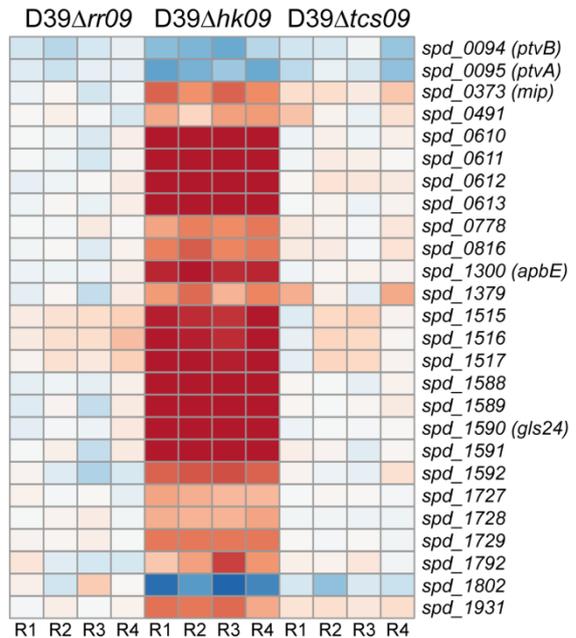


### Intermediary Metabolism

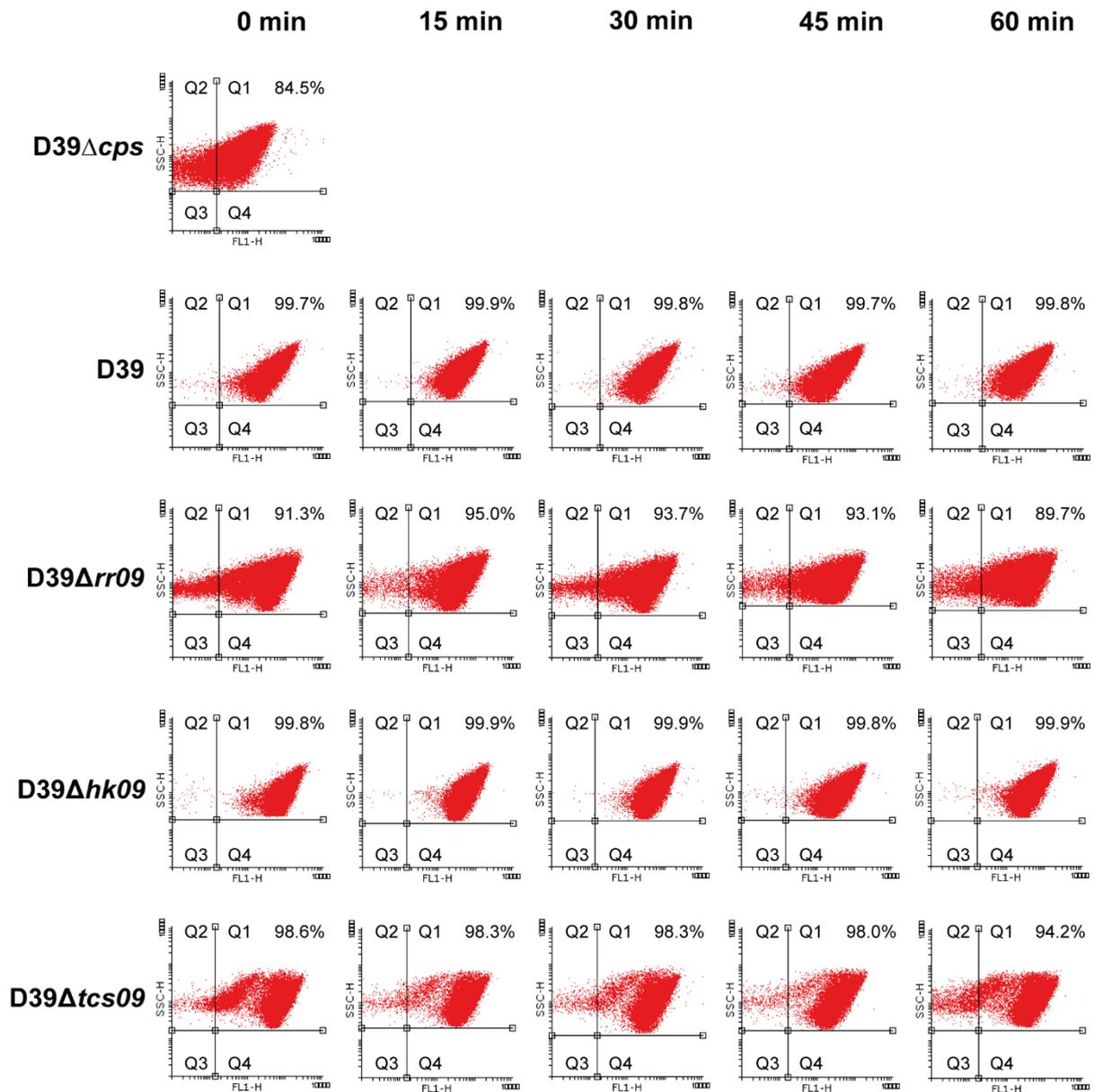


log<sub>2</sub> fold change

### Unknown Function



**Figure S5.** Expression profiles of 67 significantly regulated genes in D39 and *tcs09*-mutants. Heat maps showing the log<sub>2</sub> fold changes in individual replicates of D39Δ*rrr09*, D39Δ*hkk09* and D39Δ*tcs09*. Red boxes show upregulation, whereas blue boxes show downregulation in the corresponding replicate.



**Figure S6.** Analysis of capsule polysaccharide expression in flow cytometry.  $2 \times 10^8$  bacteria of the parental strains *D39* and their isogenic  $\Delta rr09$ -,  $\Delta hk09$ - and  $\Delta tcs09$ -mutants were incubated up to 60 min in PBS and afterwards capsule expression was analyzed with anti-serotype 2 and secondary Alexa conjugated antibody as described. As a control,  $2 \times 10^8$  bacteria of the nonencapsulated *D39Δcps* was used in the analysis. Shown are the scatter plots of one representative measurement per time point. The percentages indicate the proportion of fluorescent cells within the selection of all cells in Q1. Q1-4: quadrant 1-4, SSC-H: side scatter height