

1 **TITLE:** A competence-regulated toxin-antitoxin system in *Haemophilus influenzae*

2 **RUNNING TITLE:** A competence-regulated toxin-antitoxin system

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23

24 **ABBREVIATED SUMMARY:**

25 The competence regulon of *Haemophilus influenzae* includes an unprecedented toxin/antitoxin gene pair. When not
26 opposed by antitoxin, the toxin completely prevents DNA uptake but causes only very minor decreases in cell growth
27 and competence gene expression. The TA gene pair was acquired by horizontal gene transfer, and the toxin gene has
28 undergone repeated deletions in other strains.

29

30 **ABSTRACT**

31 Natural competence allows bacteria to respond to environmental and nutritional cues by taking up free DNA from
32 their surroundings, thus gaining both nutrients and genetic information. In the Gram-negative
33 bacterium *Haemophilus influenzae*, the genes needed for DNA uptake are induced by the CRP and Sxy transcription
34 factors in response to lack of preferred carbon sources and nucleotide precursors. Here we show that one of these
35 genes, *HI0659*, encodes the antitoxin of a competence-regulated toxin-antitoxin operon ('*toxTA*'), likely acquired by
36 horizontal gene transfer from a *Streptococcus* species. Deletion of the putative toxin (*HI0660*) restores uptake to the
37 antitoxin mutant. The full *toxTA* operon was present in only 17 of the 181 strains we examined; complete deletion
38 was seen in 22 strains and deletions removing parts of the toxin gene in 142 others. In addition to the expected Sxy-
39 and CRP-dependent-competence promoter, *HI0659/660* transcript analysis using RNA-seq identified an internal
40 antitoxin-repressed promoter whose transcription starts within *toxT* and will yield nonfunctional protein. We
41 propose that the most likely effect of unopposed toxin expression is non-specific cleavage of mRNAs and arrest or
42 death of competent cells in the culture. Although the high frequency of *toxT* and *toxTA* deletions suggests that this
43 competence-regulated toxin-antitoxin system may be mildly deleterious, it could also facilitate downregulation of
44 protein synthesis and recycling of nucleotides under starvation conditions. Although our analyses were focused on
45 the effects of *toxTA*, the RNA-seq dataset will be a useful resource for further investigations into competence
46 regulation.

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49 INTRODUCTION

50 Bacterial toxin-antitoxin gene pairs were originally discovered on plasmids, where they function to promote plasmid
51 persistence by killing any daughter cells that have not inherited the plasmid. Typically, one gene of the pair encodes a
52 relatively stable toxic protein that blocks cell growth, and the other encodes a labile antitoxin (RNA or protein) that
53 blocks the toxin's activity and limits its expression [1,2]. Toxin-antitoxin gene pairs have also been discovered on
54 many bacterial chromosomes, where they are thought to be relatively recent introductions that in some cases have
55 been co-opted to regulate cellular functions or provide other benefits [3]. Here we describe one such system, which
56 is induced in naturally competent cells and whose unopposed toxin completely prevents DNA uptake and
57 transformation.

58 Many bacteria are naturally competent, able to take up DNA from their surroundings and—when sequence similarity
59 allows—recombine it into their genomes [4,5,6]. In most species, DNA uptake is tightly controlled, with protein
60 machinery specified by a set of co-regulated chromosomal genes induced in response to diverse cellular signals.
61 Genes in the competence regulon encode not only components of the DNA uptake machinery that moves DNA across
62 the outer membrane of the cell, but proteins that translocate DNA across the inner membrane, proteins that
63 facilitate recombination, and proteins of unknown function. *Haemophilus influenzae* has an unusually small and well-
64 defined competence regulon (26 genes in 13 operons) induced by signals of energy and nucleotide scarcity [7,8].
65 Induction of these genes begins in response to depletion of phosphotransferase sugars. The resulting rise in cyclic
66 AMP (cAMP) activates the transcription factor CRP, and the CRP/cAMP complex then stimulates transcription of
67 genes with canonical CRP-promoter elements (CRP-N genes). Most of these genes help the cell to use alternative
68 carbon sources, but one encodes the competence-specific transcriptional activator Sxy. However, efficient
69 translation of *sxy* mRNA occurs only when purine pools are also depleted [9,10]. If both signals are active, Sxy then
70 acts with CRP at the promoters of competence genes, stimulating their expression and leading to DNA uptake and
71 natural transformation. These competence promoters are distinguished by the presence of 'CRP-S' sites (formerly
72 called CRE sites), variants of standard CRP sites that depend on both CRP and Sxy for activation [11], but the specific
73 role of Sxy is not yet understood. Development of competence thus requires the CRP/cAMP complex twice, first for
74 *sxy* transcription (at its CRP-N promoter) and then for transcription of the competence genes (at their CRP-S
75 promoters).

76 All but one of the fifteen Sxy-regulated *H. influenzae* genes needed for DNA uptake encode typical competence
77 proteins — membrane-associated proteins homologous to known components of the Type IV pilus-based DNA
78 uptake machinery present in nearly all known naturally competent species [5]. The one exception is *H10659*, which
79 instead encodes a predicted 98 amino acid cytoplasmic protein with no similarity to known DNA uptake proteins. It
80 shares a competence-inducible CRP-S promoter with an upstream gene encoding another short cytoplasmic protein

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81 (*HI0660*, 119 aa) (**Fig. 1, top**). Although a knockout of *HI0659* eliminates detectable DNA uptake and transformation,
82 a knockout of *HI0660* has no effect [8].

83 Here we show that *HI0660* and *HI0659* comprise a horizontally transferred operon that encodes a toxin-antitoxin
84 pair, and that expression of the toxin in the absence of the antitoxin completely prevents DNA uptake and
85 transformation. Surprisingly, expression of this toxin has only modest effects on induction of competence genes and
86 on cell growth and viability.

87 RESULTS

88 **HI0659 and HI0660 act as a toxin-antitoxin system that affects natural competence.** Our original analyses of
89 competence-induced genes did not identify any close homologs of *HI0659* or *HI0660* [7,8]. However subsequent
90 recent database searches and examination of BLASTP results revealed that these genes' products resemble proteins
91 in the Type II toxin/antitoxin families, which typically occur in similar two-gene operons. If *HI0660* and *HI0659* do
92 encode a toxin-antitoxin pair, then Δ *HI0659*'s DNA uptake defect would likely be caused by unopposed expression of
93 a *HI0660*-encoded toxin protein that prevents DNA uptake, and knocking out this toxin gene would restore
94 competence to the *HI0659* (antitoxin⁻) mutant. We tested this by constructing an *HI0660*/*HI0659* double mutant (**Fig.**
95 **1**) and examining its ability to be transformed with antibiotic-resistant chromosomal DNA (MAP7) compared to wild
96 type or either single mutant. The double mutant had normal transformation (**Fig. 2, black bars**), showing that
97 mutation of *HI0660* suppresses the competence defect of an *HI0659* mutant, and also that neither *HI0660* nor
98 *HI0659* is directly needed for the development of competence. This supported the postulated antitoxin function of
99 *HI0660*, so we named the *HI0660* and *HI0659* genes *toxT* (toxin) and *toxA* (antitoxin) respectively.

100 **Figure 1: Structure of wildtype and mutant *toxTA* genes.** Top line: structure of the wildtype *toxTA* operon in strain
101 Rd KW20. Lower lines: grey bars indicate segments deleted in Δ *toxT*, Δ *toxA*, and Δ *toxTA* mutants.

102 **Figure 2: Transformation phenotypes of wildtype cells and *toxTA* mutants.** MIV-induced cultures were incubated
103 with MAP7 chromosomal DNA, and transformation frequencies were calculated as the novobiocin resistant (Nov^R)
104 colonies per colony forming unit (CFU). Bars represent the means of at least three biological replicates, with error
105 bars representing one standard deviation. Grey bars indicate values below the detection limit ($\sim 10^{-8}$ CFU/ml).

106 **Phylogenetic evidence for lateral transfer of *toxTA*:** Since toxin/antitoxin operons are often highly mobile [13], we
107 examined the distribution of *toxTA* homologs in other strains and species (Fig. 3). Complete or partial *toxTA* operons
108 were found at the same genomic location in most *H. influenzae* genomes and in the closely related *H. haemolyticus*
109 (see below), but there were no recognizable homologs in most other bacteria, including most other members of the
110 Pasteurellaceae. Instead, most identifiable homologs (with about 60% amino acid identity) were in a very distant

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111 group, the Firmicutes, mainly within the streptococci. Ninety-six of the top 100 BLASTP hits to ToxT outside the
112 Pasteurellaceae were to diverse *Streptococcus* species. This suggests that the *toxTA* operon may have been
113 transferred from a Firmicute into a recent ancestor of *H. influenzae* and *H. haemolyticus*. When we excluded
114 *Streptococcus* from the BLAST search, sporadic matches were found in a wide variety of other taxa. In addition, *toxTA*
115 operons with about 50% identity were found in one other small distant Pasteurellacean subclade (*Actinobacillus*
116 *sensu stricto*), and on two 11kb plasmids (pRGRH1858 and pRGFK1025) from an uncultured member of a rat gut
117 microbiome and an uncultivated *Selenomonas* sp. The genes flanking the *A. pleuropneumoniae* homologs are
118 unrelated to those flanking HI0659 and HI0660. The distribution of *toxTA* homologs across taxa is summarized in Fig.
119 3A.

120 **Figure 3: Distribution of *toxTA* homologs in bacterial genomes.** **A.** Summary of BLASTP results. Red dots indicate
121 one or more taxa containing homologs of both ToxT and ToxA. Bacterial phylogeny image from Wikimedia Commons
122 [14]. Inset: Pasteurellacean phylogeny from Redfield *et al.* 2006. **B.** Unrooted maximum likelihood phylogeny of
123 concatenated ToxT and ToxA homologs from selected species where both were detected. Numbers at nodes are
124 bootstrap values. Species abbreviations: *Apl*: *Actinobacillus pleuropneumoniae*; *Aeq*: *A. equuli*; *Asu*: *A. suis*;
125 *Haemophilus haemolyticus*; *Hin*: *H. influenzae*; *Ssa*: *Streptococcus salivarius*; *Ssu*: *S. suis*; *San*: *S. anginosus*; *Sco*: *S.*
126 *constellatus*; *Sol*: *S. oligofermentans*; *Spn*: *S. pneumoniae*; *Sth*: *S. thermophilus*.

127 To resolve the history of gene transfer events in the two Pasteurellaceae sub-clades, we created an unrooted
128 maximum likelihood phylogeny from the concatenated alignment of *toxT* and *toxA* amino acid sequences from
129 selected species where both genes were found (Fig. 3B). Although a *Haemophilus-Actinobacillus* clade has 99%
130 bootstrap support, the absence of homologs from all other Pasteurellaceae makes a single Pasteurellacean origin
131 unlikely, since it would require multiple deletions in other Pasteurellacean subclades or a near-simultaneous within-
132 clade lateral transfer. Since the *Actinobacillus* sequences are also more similar to *Streptococcus* sequences than to
133 the *Haemophilus* sequences, the two Pasteurallacean groups are more likely to have acquired their *toxTA* operons by
134 independent lateral transfers, from Firmicutes.

135 **Deletions in *H. influenzae* *toxT* are common:** Of the 181 *H. influenzae* genome sequences available in public
136 databases, 162 had recognizable *toxA* sequences. All of these encoded full length ToxA proteins, but all except 24
137 had one of two common deletions affecting *toxT*. The extents of these deletions are shown by the grey bars at the
138 bottom of Fig. 4. The most common deletion (n=97) removed 178 bp of *toxT* coding sequence but left both
139 promoters intact. The second (n=45) removed 306 bp of sequence including both *toxTA* promoters and the *toxT* start
140 codon. The 19 genomes that lacked recognizable *toxA* sequences all had the same 1015 bp deletion removing both
141 *toxT* and *toxA* but leaving the flanking genes intact. In place of the missing sequences were 87 bp with no high-
142 scoring BLAST alignments in GenBank. The average pairwise distance among the 162 *toxA* genes is 0.106, slightly

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143 higher than the average of all genes with one copy per strain (0.088). The d_N/d_S ratio of 0.037 is consistent with mild
144 purifying selection on *toxA* and is higher than that of the average gene (0.243). However, this may underestimate the
145 strength of selection, since most *toxA*s lack functional *toxT*, no strains with *toxT* only were seen, and the operon may
146 not be expressed due to upstream deletions. Both sequence divergence and the high frequency of *toxT* deletions
147 agree with expectations for a toxin/antitoxin system whose antitoxin protects against a toxin that is at least mildly
148 deleterious.

149 **Figure 4: Natural deletions in the *toxTA* operon.** Top line: structure of the wildtype *toxTA* operon in strain KW20.
150 Lower lines: grey bars indicate the spans of the three naturally occurring deletions among the 181 available
151 sequences, annotated with the number of sequences with each deletion

152 **Variation in *toxTA* is independent of strain-specific variations in DNA uptake and transformation.** Maughan and
153 Redfield [15] measured the ability of 34 *H. influenzae* strains to both take up DNA and become transformed, so we
154 examined this data for correlations with the presence of *toxTA* in the 19 of these strains with genome assemblies
155 whose *toxTA* genotypes we were able to determine. All but one of the 19 strains had a complete *toxA* coding
156 sequence but only five had intact *toxTA* operons. Of the rest, four had the large deletion that removed both *toxTA*
157 promoters, nine had the smaller deletion internal to *toxT*, and one had the 1015 bp complete deletion. There was no
158 obvious correlation between the *toxTA* genotypes and the DNA uptake or transformation phenotypes, but there was
159 insufficient data for a high-powered analysis using allelic variation.

160 **The *Actinobacillus pleuropneumoniae* *toxTA* operon does not control growth or competence:** The *A.*
161 *pleuropneumoniae* *toxTA* operon was originally reported to have the CRP-S promoter typical of competence operons
162 [16]. Although reexamination of the promoter region failed to identify a high-quality CRP-S site, we constructed *toxT*,
163 *toxA* and *toxTA* knockout mutants to investigate whether a *toxA* deletion would prevent competence. There were no
164 significant differences between the transformation frequencies of wildtype cells and *toxA* mutants (Fig. 2, blue bars),
165 and the rich-medium growth properties of all mutants in the wells of a BioScreen incubator were identical to those of
166 wildtype cells (Supp. Fig. A). Thus, we conclude that the *toxTA* homolog in *A. pleuropneumoniae* does not affect
167 competence. A previously reported RNA-seq study [17] of *A. pleuropneumoniae* serotype JL03 [18] found low-level
168 expression of the *toxTA* homologs in a log-phase culture in rich medium, but competent cultures have not been
169 examined.

170 **Growth and competence phenotypes of *H. influenzae* *toxTA* mutants:** Earlier investigation of DNA uptake and
171 transformation by the *H. influenzae* *toxA* knockout strain found that both were below the limit of detection (>100-
172 fold reduction and >10⁶-fold reduction respectively) after the standard competence-inducing treatment [8] (see also

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173 **Fig. 2**), although there was no apparent growth defect in rich medium. Supp. Fig B confirms the non-transformability
174 of Δ toxA cells both in log phase growth and at intermediate time points during competence induction.

175 A simple explanation for this defect would be that unopposed ToxT prevents competence, when not opposed by
176 ToxA, by killing or otherwise inactivating the (competence-induced) cells in which it is expressed. To detect effects of
177 unopposed expression of *toxT* toxin, we analyzed growth rates of wildtype and Δ toxA strains (Fig. 5) before, during,
178 and after transfer to the competence-inducing starvation medium MIV. The first 60 min of Fig. 5 show that
179 unopposed expression of *toxT* toxin slightly slows exponential growth in rich medium. A more detailed analysis of
180 growth of rich-medium cultures using BioScreen wells is provided in Supp. Fig. C; the growth defect of the Δ toxA
181 strain is barely detectable under this condition. This lack of a severe growth defect is not surprising; because the
182 *toxTA* promoter is regulated by a CRP-S site, its expression (and thus ToxT production) might be limited to competent
183 cells even in the absence of ToxA [7]. The grey-shaded portion of Fig. 5 shows cell growth after transfer to MIV
184 (samples between 70 min and 165 min). Cells transferred to MIV usually undergo only one or two doublings, and
185 deletion of *toxA* delays this but does not eliminate it (see also Supp. Fig. D). Cells returned to rich medium from MIV
186 might be delayed in their recovery, if unopposed toxin expression during competence development kills cells or halts
187 growth. However, both strains had similar recovery kinetics after a fraction of their culture was returned to rich
188 medium, although Δ toxA cells again grew slightly slower than wildtype (175-230 min in Fig. 5). Supp. Fig. E shows the
189 OD₆₀₀ readings corresponding to the CFU/ml data shown in Fig. 5. These agree well, showing that differences in cell
190 growth can be explained by the differences in cell division.

191 **Figure 5: Growth of *H. influenzae* wildtype and Δ toxA cells before, during and after competence induction.** Log-
192 phase cells in sBHI were transferred to MIV at t=65 min; a portion of each MIV culture was diluted 10-fold into sBHI
193 at t=170 min. The grey-shaded area indicates samples taken from MIV cultures. Blue: KW20, orange: Δ toxA. Supp.
194 Fig. E shows the corresponding OD₆₀₀ values.

195 Since cyclic AMP is required for normal induction of the competence genes, and addition of cAMP induces partial
196 competence during exponential growth [19], we also tested the effect of cAMP on the Δ toxA knockout. Addition of
197 cAMP did not rescue its transformation defect (Supp. Fig. F), so failure to transform is not caused by defective cAMP
198 production in the antitoxin mutant.

199 Since some chromosomal toxin-antitoxin systems have acquired beneficial roles in modulating cell growth [20], we
200 also examined whether the absence of toxin changed growth and competence under various conditions. The grey
201 line in Supp. Fig. C shows that, under BioScreen growth assay conditions, Δ toxT's growth was indistinguishable from
202 that of wildtype cells (blue line), and Fig. 2 shows that its MIV-induced competence is also unchanged. Supp. Fig. G
203 shows that the kinetics of Δ toxT competence development and loss during growth in rich medium were also

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204 indistinguishable from wildtype. We conclude that ToxT's normal expression in cells expressing antitoxin does not
205 detectably regulate growth or the development or loss of competence. Unfortunately, inferring the relationship
206 between competence and growth is complicated because many cells in 'competent' cultures are unable to transform.
207 In some species the non-transformable cells are known to have not induced their competence genes, but this has not
208 been investigated for *H. influenzae*.

209 **Transcriptional control of competence:** Since these phenotypic analyses gave little evidence of MIV-specific toxicity
210 or insight into the cause of the competence defect, we used RNA-seq to investigate how *toxTA* is regulated and how
211 mutations affect expression of competence genes. In these experiments, samples for RNA preparations were taken
212 from three replicate cultures at four time points, first when cells were growing in log phase in the rich medium sBHI
213 (t=0), and then at 10, 30 and 100 minutes after each culture had been transferred to MIV. We first examined how
214 competence induction in wildtype and regulatory-mutant cells changed expression of genes known to be regulated
215 by CRP and CRP+Sxy (CRP-N and CRP-S genes respectively). **Fig. 6** gives an overview of the results. The top row
216 shows competence-induced changes in transcript abundances in wildtype cells at 10, 30 and 100 minutes, and the
217 lower rows show that some of these changes do not occur in Δcrp and Δsxy cells. Each coloured dot represents a
218 gene, colour-coded by function. Its horizontal position indicates its level of expression in rich medium (T=0) and its
219 vertical position indicates how this expression changed in MIV (top row – wildtype cells, lower rows, Δcrp and Δsxy
220 cells). Thus in **Fig. 6A** the higher positions of the dark blue dots (genes regulated by CRP-N sites) and the red
221 diamond (the competence regulator *sxy*) indicate that they were strongly induced after 10 min in MIV. Induction of
222 *sxy* was followed at 30 and 100 minutes by strong induction of the known competence-regulon genes (higher
223 positions of CRP-S genes; light green dots) (**Fig. 6 B** and **C**). Consistent with prior studies [7], induction of all these
224 genes was blocked by deletion of the *crp* gene (**Fig. 6 D-F**), and induction of the competence regulon (CRP-S) genes
225 was blocked by deletion of *sxy* (**Fig. 6 G-I**). Analysis of other competence-associated changes in gene expression in
226 normal cells and in Δcrp and Δsxy mutants is provided in the Supplementary Materials.

227 **Figure 6: Competence-induced changes in expression of *H. influenzae* genes regulated by CRP and Sxy.** Each dot
228 represents a gene, colour-coded by function: pink diamond: *sxy*; light green dot: CRP-S-regulated; blue dot: CRP-N-
229 regulated; red dot: *purR* regulon; yellow dot: tryptophan regulon; dark green dot: tRNA; purple dot: ribosomal
230 proteins; grey dot: other or unknown functions. Each dot's horizontal position indicates the gene's relative level of
231 expression (as FPKM) in rich medium (t=0) and its vertical position indicates how this expression changed at later
232 time points (**A**: t=10; **B**: t=30; **C**: t=100) or in a mutant background at T=30 (**D-F**: Δcrp ; **G-I**: Δsxy).

233 **Transcriptional control of *toxTA*:** RNA-seq analysis confirmed that *toxTA* is regulated as a typical competence
234 operon. In wildtype cells, baseline RNA-seq expression of *toxT* and *toxA* was very low during log phase in rich
235 medium, with approximately tenfold induction after 30 minutes incubation in MIV (green lines and points in **Fig. 7**

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236 (*toxT*) and Supp. Fig. H (*toxA*). As expected, this increase was eliminated by knockouts of CRP and Sxy (brown and
237 blue lines and points in Fig. & and Supp. Fig H). Like other CRP-S genes, both *toxT* and *toxA* were also strongly
238 induced in rich medium by *sxy* and *murE* mutations known to cause hypercompetence [38,39] and by a newly
239 identified hypercompetence mutation in *rpoD* (Supp. Fig. I). Thus the *toxTA* operon is regulated as a typical member
240 of the competence regulon.

241 Figure 7: **Competence-induced changes in expression of *H. influenzae* *toxT*.** Sample FPKM values (dots) and means
242 (lines) for *toxT* (HI0660). Strains: wildtype: green; Δ crp: brown; Δ sxy: blue; Δ toxA: red; Δ toxT: grey; Δ toxTA: black.
243 The values for the Δ toxT and Δ toxTA samples are underestimates because most of the gene has been deleted in
244 these strains.

245 RNA-seq analysis also showed that the *toxTA* operon is regulated as a typical type II toxin-antitoxin operon. In such
246 operons, the antitoxin protein usually binds to the toxin protein, which protects cells from the toxin in two ways.
247 First, antitoxin binding inactivates the toxin. Second, it also activates the antitoxin component as a repressor of the
248 *toxTA* promoter [2,21]. *H. influenzae* ToxA has a HTH-XRE DNA-binding domain, which is commonly found in
249 promoter-binding antitoxins [1,13], and the RNA-seq analysis in Fig. 7 strongly suggests that it represses *toxTA*
250 transcription. The Δ toxA mutant retains an intact *toxTA* promoter and *toxT* coding sequence (see Fig. 1); it had 9-fold
251 increased baseline expression of *toxT* in log phase cells (red line and points in Fig. 7). Expression increased further
252 during competence development, with the same kinetics as in wildtype cells, suggesting independent contributions
253 from baseline repression by antitoxin and competence induction by CRP and Sxy. (Values for *toxA* expression are
254 shown by the red points and line in Supp Fig. H, but are underestimates because most of the gene has been deleted.)

255 Because most antitoxins have only weak affinity for DNA in the absence of their cognate toxin, ToxA was predicted to
256 repress *toxTA* only when bound to ToxT. Therefore, we were initially surprised that knocking out *toxT* or both *toxT*
257 and *toxA* did not increase RNA-seq coverage of residual *toxT* sequences (Fig. 7, grey and black lines) and that
258 knocking out both genes did not increase coverage of residual *toxA* (grey line in Supp Fig. H). These deletion mutants
259 retain all the *toxTA* upstream sequences and the *toxT* start codon, and enough sequence of the deleted genes to
260 identify them in the RNA-seq analysis. An explanation was suggested by a recent study of the *Escherichia coli* *hicAB*
261 toxin/antitoxin system [22], and confirmed by more detailed analysis of *toxTA* transcripts. The HicA (toxin) and HicB
262 (antitoxin) proteins have no detectable sequence homology to ToxT and ToxA, but their operon is similarly regulated
263 by Sxy and has the same atypical organization (toxin before antitoxin) [23]. Turnbull and Gerdes [22] showed that
264 the *hicAB* operon has two promoters. Promoter P1 has a CRP-S site regulated by CRP and Sxy, which is not repressed
265 by the HicB antitoxin. A secondary promoter P2 is very close to the *hicA* start codon; it is repressed by HicB
266 independently of HicA, and its shortened transcripts produce only functional HicB, not HicA. Promoter P1 of this
267 *hicAB* system thus resembles the CRP-S regulation of the *toxTA* operon, and the presence of a second antitoxin-

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regulated internal promoter similar to P2 would explain the high *toxTA* operon expression seen in the *toxA* knockouts. This finding prompted us to do a more detailed analysis of *toxTA* transcription patterns in wildtype and mutant cells to determine whether the *toxTA* transcripts expressed in the absence of *toxA* were similarly truncated. **Figure 8A** shows RNA-seq coverage of the *toxTA* promoter region and the 5' half of *toxT*, in wildtype cells (green) and in the *toxA* deletion mutant (purple). As expected, the predicted CRP-S promoter upstream of *toxTA* was only slightly active at T=10 but strongly induced at T=30 and T=100 (note log scale); its activity was not affected by deletion of *toxA*. Deletion of *toxA* instead caused strong constitutive transcription from a second promoter ('P2'), with reads beginning about 30 bp downstream of the *toxT* start codon. Transcripts with this 5' end are unlikely to produce active ToxT; the only other in-frame AUG in *toxT* is 30 bp from the end of the gene, and it and the first GUG (position 35) lack Shine-Dalgarno sequences. This supports the hypothesis that the *H. influenzae* *toxTA* operon is regulated similarly to the *E. coli* *hicAB* operon, with Sxy-induced expression from a CRP-S promoter and antitoxin-repressed transcription from a downstream 'P2' promoter whose transcript produces antitoxin but not toxin.

Figure 8: Sequencing read coverage of the *H. influenzae* *toxTA* promoter region. **A.** The green (KW20) and purple (Δ *toxA*) lines indicate mean coverage normalized by library size using DESeq2 [size factors] at each position; shaded areas indicate standard errors. The yellow horizontal arrow indicates the 5' half of *toxT* (note that in this figure transcription is from right to left). **B.** and **C.** Time course of normalized read coverage at two specific positions in the *toxTA* operon. **B.** Position 0 = *toxA* start codon. **C.** Position 100.

In the *E. coli* *hicAB* system, P2 is repressed by HicB antitoxin alone, binding of HicB to the P2 operator is destabilized when HicA toxin is abundant, and transcription from P2 in plasmid constructs is elevated when the chromosomal *hicAB* operon is deleted [22]. To look for parallels in *H. influenzae*'s *toxTA*, we more precisely measured transcription in wildtype and *toxA* mutant cells by scoring the coverage at two positions in the *toxTA* operon; each is indicated by a red vertical line at the bottom of Fig. 8A. Position 0 is the *toxT* start codon, 34 nt downstream from the CRP-S promoter (P_{CRP-S}) but upstream of the putative P2 promoter, and position 100 is 70 nt downstream from P2 (P2 and position 100 are deleted in Δ *toxT*). To eliminate read-mapping artefacts arising from failure of reads that span an insertion or deletion to align to the reference sequence, each mutant's reads were instead mapped onto its own *toxTA* sequence. Comparison of **Figures 8B and 8C** shows that coverage at position 100 was always higher than coverage at position 0, consistent with the presence of a second promoter between positions 0 and 100. **Fig. 8B** also shows that coverage at position 0 (expression from P_{CRP-S}) was reduced by all of the *toxA* deletions. This was unexpected, and suggests that this promoter may have unusual properties, since coverage of other CRP-S genes was not similarly affected. The *toxA* deletion caused the predicted increase in coverage at position 100 (**Fig. 8C**), but the *toxTA* deletion unexpectedly reduced rather than increased coverage at this position ~3-fold from the wildtype level, even though this construct retains the first 150 bp of the operon, including P2. This reduction was not accounted for

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300 by the reduction in expression from P_{CRP-S} , suggesting that high-level transcription from the *toxTA* P2 promoter only
301 occurs when ToxT is present and ToxA is absent. This could mean either that ToxT directly binds the P2 promoter to
302 induce transcription, which seems unlikely given its lack of DNA-binding domain, or that the presence of ToxT
303 disrupts binding of a secondary repressor of the operon, such as a noncognate antitoxin [2]. Alternatively, it is
304 possible that the reduced expression in the $\Delta toxTA$ mutant instead reflects reduced transcript stability in this
305 mutant.

306 **Unopposed ToxT does not block induction of the competence regulon:** Expression of the competence operons that
307 these regulators induce was also normal or near-normal in the $\Delta toxA$ mutant at 30 min after transfer to MIV, the
308 time when competence-induced gene expression is normally highest (Fig. 9). Modest decreases in expression were
309 seen for some operons, but these are not expected to cause the absolute competence defect, for two reasons. First,
310 competence gene expression levels at this time were very similar between the $\Delta toxA$ mutant, which cannot take up
311 any DNA or produce any transformants (dark blue bars), and the $\Delta toxT$ and $\Delta toxTA$ mutants (other blue bars), which
312 have normal competence. Second, an unrelated regulatory defect mutation, a knockout of *hfq*, causes a more
313 extreme reduction in gene expression at 30 min (brown bars) but this causes a much less extreme competence defect
314 (only 10-20 fold rather than $>10^6$ -fold; green line in Supp. Fig. B). Mutation of *toxTA* genes also did not substantially
315 change expression of the *sxy*, *crp* and *cya* genes encoding the competence regulon regulators Sxy, CRP, and
316 adenylate cyclase (Supp. Fig. J). At t=100 the $\Delta toxA$ mutant showed a stronger reduction in RNA-seq coverage of
317 competence genes (Supp. Fig. K), however this cannot explain the competence defect and its significance is unclear.

318 **Fig. 9. RNA-seq analysis of *H. influenzae* *toxTA* effects on competence-operon expression.** Bars show the relative
319 expression (as FPKM) of competence operons in four different mutants after 30 minutes of competence induction:
320 $\Delta toxA$: dark blue, $\Delta toxT$: blue, $\Delta toxTA$: light blue, Δhfq : brown. Expression of each operon is relative to its expression
321 in wildtype cells at the same time point (grey horizontal bar). Error bars indicate standard errors of samples from
322 three replicate cultures.

323 **Other ToxT and ToxA effects in competence-induced cells:** The severe competence defect of $\Delta toxA$ was also not
324 explained by expression changes in other genes. Supp. Table 3 lists, for each timepoint, the genes whose expression
325 in the $\Delta toxA$ mutant differed significantly from expression in all three strains with normal competence (wildtype,
326 $\Delta toxT$ and $\Delta toxTA$). The most consistent effect was overexpression of the HI0655-58 operon immediately
327 downstream from *toxTA*. Analysis of sequencing coverage in this region showed this to be caused by read-through
328 transcription from both *toxTA* promoters (CRP-S and P2). The operon includes genes encoding shikimate
329 dehydrogenase, an ABC transporter, and a hypothetical protein with putative topoisomerase I domains. Expression
330 of genes in this operon increased about 1.2-1.5-fold in MIV in wildtype cells and in other mutants with normal
331 competence, so their higher expression in $\Delta toxA$ is unlikely to be responsible for this strain's competence defect.

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332 Several other genes showed consistent changes relative to most or all competent strains, but these only sometimes
333 reached statistical significance. HI0231 (*deaD*) encodes a DEADbox helicase involved in ribosome assembly and
334 mRNA decay [24]; its expression in control strains fell rapidly on transfer to MIV, but levels in Δ *toxA* were about 50%
335 higher at all time points. HI0235 contains an ArfA ribosome-rescue domain [25]; its expression was up 3-5-fold at
336 t=30 and 2-3-fold at t=100 in the Δ *toxT* and Δ *toxTA* comparisons, but not in the KW20 comparison. HI0362 encodes a
337 CRP-regulated iron-transport protein that normally increases in MIV but did not increase in *toxA* deletion mutants.
338 HI0504 (*rbsB*, a ribose transporter component) is normally induced 20-fold more in MIV than other genes in its
339 operon, but this increase was smaller (only 10-fold) in Δ *toxA*. Expression of HI0595 (*arcC*, carbamate kinase)
340 normally falls 2-3-fold immediately after transfer to MIV, but the fall was greater in Δ *toxA*. 28 other genes were
341 significantly changed only at t=100, but their expression patterns and predicted functions were diverse and did not
342 suggest an explanation for Δ *toxA*'s lack of competence.

343 **Related toxins may suggest mechanism of action for ToxT:** Since examination of gene expression shed little light on
344 how the ToxT toxin prevents competence, and it has no close homologs with characterized functions, we considered
345 the modes of action of other Type II toxins. The most common type II toxins (e.g. RelE) act as translation-blocking
346 ribonucleases, but several alternative modes of action are also known, and some newly discovered toxins lack
347 identified activities [13]. The Pfam and TAfinder databases assign the *H. influenzae* ToxT protein to the ParE/RelE
348 toxin superfamily, whose characterized members include both gyrase inhibitors and ribonucleases that arrest cell
349 growth by cleaving mRNAs and other RNAs [2,26,27].

350 **Unopposed toxin does not inhibit gyrase:** If ToxT inhibited gyrase we would expect the RNA-seq data to show that
351 transfer to MIV caused increased expression of *gyrA* (HI1264) and *gyrB* (HI0567) and reduced expression of *topA*
352 (HI1365), since these genes have opposing activities and compensatory regulation by DNA supercoiling [28].
353 However, these genes' coverage levels were similar in wildtype and all *toxTA* mutants, during both exponential
354 growth and competence development.

355 **Unopposed toxin does not cleave competence-induced mRNAs at specific sites:** The best-studied homologs of the
356 *toxT* toxin act by cleaving mRNAs at positions near their 5' ends during their translation on the ribosome [29,30].
357 Because the resulting 'non-stop' mRNAs lack in-frame stop codons and cannot undergo the normal ribosome-release
358 process, this causes a general block to translation [31] which is predicted to arrest cell growth until normal
359 translation can be restored [32]. Thus we considered whether ToxT might prevent competence by one of two
360 mechanisms. First, ToxT might specifically cleave the 5' ends of competence-gene transcripts, eliminating their
361 function without significantly changing their overall RNA-seq coverage levels or otherwise interfering with essential
362 cell functions. Visual examination of RNA-seq coverage of all positions within the competence operons did not reveal
363 any anomalies that might indicate that the mRNA in Δ *toxA* cells had been inactivated either by cleavage at specific

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364 sites or by random cleavage near the 5' end [33]. As an example, Supp Fig. L compares read coverage across the
365 *comNOPQ* operon in wildtype and Δ *toxA* cultures after 30 min in MIV. To detect nonspecific cleavage we examined
366 the insert sizes of our RNA-seq sequencing libraries by comparing the spanning-length distributions of paired-end
367 sequencing reads among strains. Because independent library preparations had different insert sizes, comparisons
368 were limited to samples prepared at the same time. Fig. 10 shows that the Δ *toxA* samples from library batch 1 had
369 shorter fragment sizes than the KW20 samples from the same batch, and that the difference increased as the time
370 after competence induction increased. This supports the hypothesis that the extreme lack of competence in Δ *toxA*
371 cultures is due to non-specific ToxT cleavage of mRNAs. This mechanism is consistent with those of homologous
372 HigB/RelE toxins, which inhibit translation by cleavage of mRNA at the ribosomal A-site [34,35].

373 **Fig. 10. Distribution of insert-size differences between *H. influenzae* RNA-seq libraries prepared at the same time.**
374 Distribution of insert length differences between KW20 (kw20_A and kw20_B samples) and Δ *toxA* (antx_A samples)
375 after 0, 10, 30 and 100 minutes in MIV.

376

377 DISCUSSION

378 Our investigation into why a HI0659 knockout prevents competence has provided a simple answer: HI0659 encodes
379 an antitoxin (ToxA) needed to block the expression and competence-preventing activity of the toxin encoded by
380 HI0660 (ToxT). But this answer has generated a number of new questions that we have only been able to partially
381 answer. Why is competence controlled by a toxin/antitoxin system? How does it completely abolish DNA uptake
382 and transformation without causing significant cell death? Do its effects in wildtype cells confer any benefit to the
383 cells, either generally or specific to competence?

384 Several findings support the conclusion that HI0660 and HI0659 encode proteins that function as a toxin/antitoxin
385 pair. First is the similarity of the encoded ToxT and ToxA proteins to biochemically characterized toxin and antitoxin
386 proteins of the RelE/ParE families. Second, and the strongest evidence, is the restoration of normal DNA uptake and
387 transformation to antitoxin-knockout cells when the putative toxin is also knocked out. Third is the regulatory
388 similarity between this system and the *hicAB* system of *E. coli*.

389 How did the *toxTA* operon come to be in the *H. influenzae* genome and under competence regulation?

390 Phylogenetic analysis showed that *H. influenzae* acquired its *toxTA* operon by horizontal transfer, either into a deep
391 ancestor of the Pasteurellaceae or more recently by independent transfers into ancestors of *H. influenzae* and *A. pleuropneumoniae*. The closest relatives of the *H. influenzae* *toxTA* genes are in the distantly related Firmicutes,
393 with homologs especially common in *Streptococcus* species. Since the Streptococci and Pasteurellaceae share both

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394 natural competence and respiratory-tract niches in many mammals, there may have been frequent opportunities for
395 horizontal transfer between them.

396 We do not know how the *toxTA* operon came to be under CRP-S regulation. The *toxTA* operon's strong regulatory
397 parallels with the *E. coli hicAB* system suggest that toxin-antitoxin systems with similar regulation and function have
398 adopted similar roles in separate instances, a phenomenon which is more likely in toxin antitoxin systems as they
399 undergo frequent horizontal transfers and are often under strong selective pressure. The *sxy* gene and the CRP-S
400 promoters it regulates are not known outside of the Gamma-Proteobacteria sub-clade that contains the
401 Vibrionaceae, Enterobacteraceae, Pasteurellaceae and Orbaceae [11]. Thus, it would be interesting to examine the
402 regulation and function of the *toxTA* homologs outside the Pasteurellaceae to determine when and where it adopted
403 a regulatory role and the mechanism of the toxic activity. Examining these homologs could give insight into both the
404 mechanism of action of the *H. influenzae* *toxTA* system, and its evolutionary history.

405 **How does unopposed ToxT prevent DNA uptake and transformation?** The transformation defect caused by deletion
406 of the antitoxin gene *toxA* is very severe, so it was surprising that RNA-seq analysis detected only few and minor
407 changes in expression of competence genes. Instead, the best explanation is that ToxT is an mRNA-cleaving
408 ribonuclease, whose activity causes a general block to translation that prevents functioning of the induced
409 competence genes. The most direct evidence is the decrease in insert size distributions seen in Δ *toxA* mutants, but
410 this conclusion is also supported by the combination of regulatory similarities between the *toxTA* and *hicAB* systems
411 and by sequence similarities between the ToxT protein and HigB ribonuclease toxins.

412 **Why then does the Δ *toxA* mutant not suffer from growth arrest or toxicity?** Part of the explanation is that mRNAs
413 encoding functional ToxT are only expressed after cells have been transferred to competence-inducing starvation
414 medium, a condition that severely slows cell growth and division even in wildtype cells. Detecting the predicted
415 competence-specific toxicity is further complicated by the uneven distribution of transformability in competence-
416 induced cells. Co-transformation experiments using multiple unlinked markers consistently show that no more than
417 half, and sometimes as little as 10%, of the cells in a MIV-treated culture produce recombinants [6]. We do not know
418 whether only the transforming cells express the competence genes or all cells express them but some fail to correctly
419 assemble the DNA uptake or recombination machinery. If only a modest fraction of the cells in a competent culture
420 are expressing the toxin then any toxic effect on culture growth and survival will be more difficult to detect.

421 **Does this operon confer any benefit (or harm) on *H. influenzae*?** Why have a competence-regulating toxin/antitoxin
422 system at all, when it has no detectable effect on competence unless its antitoxin component is defective? Although
423 regulatory parallels with the *hicAB* system suggest that CRP-S regulation is not incidental, we found no direct
424 evidence of any toxin-dependent alteration to the normal development of competence. Production of Sxy is subject

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425 to post-transcriptional regulation by the availability of nucleotide precursors [9,10], and we have elsewhere proposed
426 that DNA uptake is an adaptation to obtain nucleotides when nucleotide scarcity threatens to arrest DNA replication
427 forks [6]. In this context, competence-induction of the *toxTA* operon may be a specialization to help cells survive, by
428 slowing or arresting protein synthesis until the nucleotide supply is restored.

429 On the other hand, the high frequency of deletions that remove either complete *toxTA* or both promoters (35%)
430 indicates that the operon is dispensable. And the even higher frequency of toxin-inactivating deletions in the
431 presence of intact antitoxin genes and CRP-S promoter (51%), coupled with the absence of any deletion that
432 inactivates antitoxin but preserves toxin, indicates that unopposed toxin is indeed harmful under some natural
433 circumstances.

434 We have examined the *toxTA* operon from many angles and answered our initial question of why *toxA* knockout
435 prevents competence in *H. influenzae*, but have raised new questions whose eventual answers we hope will give us
436 greater insight not just into the *toxTA* system, but competence regulation in general. A number of desirable follow-
437 up experiments could improve understanding of the *toxTA* system, particularly complementation experiments to
438 determine whether ToxA expression in Δ toxA can restore competence, and whether ToxT expression in Δ toxTA can
439 block competence. In future work, it would also be valuable to express ToxT and ToxA in *E. coli* on separate plasmids
440 to examine the system's toxicity and effect on competence.

441

442 METHODS

443 Bacterial strains, plasmids, and growth conditions

444 Bacterial strains used in this work are listed in Supp Table 1. *Escherichia coli* strain DH5 α [F80/*lacZ* #(*lacIZYA-argF*)
445 *endA1*] was used for all cloning steps; it was cultured in Luria-Bertani (LB) medium at 37°C and was made competent
446 with rubidium chloride according to the method provided in the QIAexpressionist manual protocol 2 (Qiagen). When
447 antibiotic selection was required, 100 μ g/mL ampicillin and 50 μ g/mL spectinomycin were used.

448 *Haemophilus influenzae* cells were grown in sBHI medium (Brain Heart Infusion medium supplemented with
449 10mg/mL hemin and 2mg/mL NAD) at 37°C in a shaking water bath (liquid cultures) or incubator (plates). *H.*
450 *influenzae* strain Rd KW20 [36], the standard laboratory strain, was used as the wild type for all experiments. Mutant
451 strains used in this study were marked deletion mutants in which the coding region of the gene was replaced by a
452 spectinomycin resistance cassette, as well as unmarked deletion mutants derived from these strains; the generation
453 of these mutant strains is described in [8]. Specifically, we used an unmarked deletion of HI0659 (HI0659-), marked

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454 and unmarked deletions of HI0660 (HI0660::spec, HI0660-), and a marked deletion of the whole operon
455 (HI0659/HI0660::spec). Knockout mutants of *cpr* and *sxy* have been described previously [37,38].

456 *Actinobacillus pleuropneumoniae* cells were grown in BHI-N medium (Brain Heart Infusion medium supplemented
457 with 100µg/mL NAD) at 37°C. *A. pleuropneumoniae* strain HS143 [40] was used as the wild type for all experiments.
458 Marked deletion mutants in which the gene of interest was replaced by a spectinomycin resistance cassette strains
459 were generated for this study as described below. The HS143 genome region containing the homologs of the
460 *Actinobacillus pleuropneumoniae* serovar 5b strain L20 APL_1357 and APL_1358 genes, plus approximately 1 kb of
461 flanking sequence on each side, was PCR-amplified, ligated into Promega pGEM-T Easy and transformed into *E. coli*.
462 Plasmid regions containing APL_1357, APL_1358, or both genes were deleted from the pGEM-based plasmid by
463 inverse PCR, and the amplified fragments were blunt-end ligated to the spectinomycin resistance cassette [41] from
464 genomic DNA of a *H. influenzae* comN::spec strain [8]. Plasmids linearized with Scal were transformed into
465 competent *A. pleuropneumoniae* HS143 and transformants were selected for spectinomycin resistance using
466 100µg/mL spectinomycin after 80 minutes of growth in nonselective medium.

467 Generation of competent stocks

468 To induce competence, *H. influenzae* and *A. pleuropneumoniae* were cultured in sBHI or BHI-N respectively and
469 transferred to competence-inducing medium MIV [42] when they reached an optical density at 600nm (OD₆₀₀) of
470 approximately 0.25 [43]. After incubation with gentle shaking at 37°C for a further 100 min (*H. influenzae*) or 150
471 min (*A. pleuropneumoniae*), cells were transformed or frozen in 16% glycerol at -80 °C for later use.

472 Transformation assays

473 **Transformation of MIV-competent cells:** Transformation assays were carried out as described by Poje and Redfield
474 [43]. MIV-competent *H. influenzae* or *A. pleuropneumoniae* cells were incubated at 37°C for 15 minutes with 1µg/ml
475 DNA, then DNaseI (10µg/mL) was added and cultures were incubated for 5 minutes to ensure no DNA remained in
476 the medium. *H. influenzae* cultures were transformed with MAP7 genomic DNA [44], which carries resistance genes
477 for multiple antibiotics, while *A. pleuropneumoniae* cultures were transformed with genomic DNA from an *A.*
478 *pleuropneumoniae* strain with spontaneous nalidixic acid resistance (generated in this lab). Cultures were diluted
479 and plated on both plain and antibiotic-containing plates (2.5ug/mL novobiocin for *H. influenzae* cultures, 20ug/mL
480 nalidixic acid for *A. pleuropneumoniae* cultures) and transformation frequencies were calculated as the ratio of
481 transformed (antibiotic-resistant) cells to total cells. For *A. pleuropneumoniae*, transformed cells were given 80
482 minutes of expression time in BHI-N before plating.

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483 **Time courses in rich medium:** *H. influenzae* cells from frozen stocks of overnight cultures were diluted in fresh sBHI
484 and incubated with shaking at 37°C. Periodically, the OD₆₀₀ was measured, and at predetermined optical densities
485 aliquots of the culture were removed and transformed with MAP7 DNA and plated as described above. **Bioscreen**
486 **Growth Analysis:** The Bioscreen C apparatus (BioScreen Instruments Pvt. Ltd.) was used to measure growth. Cells
487 frozen from overnight cultures were pre-grown at low density in sBHI, and 300µL aliquots of 100-fold dilutions were
488 placed into 20 replicate wells of a 100-well Bioscreen plate. Wells at the edges of the plate were filled with medium
489 alone as controls. Cells were grown in the Bioscreen at 37°C for 18 hours with gentle shaking, and OD₆₀₀ readings
490 were taken every 10 minutes. Readings were corrected by subtracting the OD₆₀₀ measured for medium-only
491 controls, and replicates for each strain were averaged at each time point to generate growth curves. Doubling times
492 were calculated for each strain from the subset of time points that represents exponential growth phase, as
493 determined by linearity on a semi-log plot of time versus OD₆₀₀.

494 **Competence growth and survival time course:** Cells were grown in sBHI to a density of ~2x10⁸ cfu/ml (OD₆₀₀ =
495 0.075) and transferred to MIV. After 100 min (time for maximum competence development, an aliquot of each
496 culture was diluted 1/10 into fresh sBHI for recovery and return to normal growth. A fraction of each culture was
497 incubated in a shaking water bath, and aliquots of the initial and 'recovery' sBHI cultures were also grown and
498 monitored in a Bioscreen incubator.

499 **Cyclic AMP competence induction:** *H. influenzae* cells in sBHI were incubated with shaking to an OD₆₀₀ of
500 approximately 0.05. Cultures were split and 1mM cAMP was added to one half. At an OD₆₀₀ of approximately 0.3,
501 aliquots were transformed with MAP7 DNA and plated as described above.

502 **Phylogenetic Analysis:** A nucleotide BLAST search (discontinuous MEGABLAST) and a protein BLAST search against
503 translated nucleotide databases (tBLASTn) were used to identify homologs of the HI0659 and HI0660 genes [45].
504 Protein sequences found by the tBLASTn search were retained for analysis if they showed greater than 60% coverage
505 and greater than 40% identity to the *H. influenzae* query sequence. For species with matching sequences in multiple
506 strains, the sequence from only one strain was kept.

507 For species in which homologs of HI0659 and HI0660 were found next to one another, amino acid sequences of
508 concatenated matrices were aligned by multiple-sequence alignment using MAFFT, version 7.220 [46], run from
509 modules within Mesquite version 3.02 [47]. The L-INS-I alignment method was used due to its superior accuracy for
510 small numbers of sequences. After inspection of the alignments, poorly-aligning sequences were removed from the
511 analysis, and alignment was repeated.

512 Phylogenetic trees were generated using the RAxML [48] maximum likelihood tree inference program, run via the
513 Zephyr package of Mesquite. For each gene, 50 search replicates were conducted, using the PROTGAMMAAUTO

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514 option to allow RAxML to automatically select the best protein evolution model to fit the data. Since these trees
515 were found to correspond exactly to a set of trees generated using the PROTGAMMAJTT model, this faster model
516 was used to generate a majority-rules consensus tree from 1000 bootstrap replicates for each gene.

517 **Analysis of natural deletions:** 181 publicly available *H. influenzae* genomes were downloaded from NCBI and the
518 Sanger centre. Genomes were re-annotated using Prokka v1.11 [49], and the pangenome was calculated using Roary
519 v3.5.1 [50] with a minimum blastp threshold of 75. The *toxA* gene cluster in the pangenome was identified by finding
520 the gene cluster that contained the *toxA* gene from Rd KW20, and the *hicA* cluster was identified by finding the gene
521 cluster that contained the *hicA* gene from PittAA. 2300 bp genome sequences centered on *toxA* and/or *hicA* were
522 extracted from all *H. influenzae* genomes containing recognizable *toxA* and/or *hicB* genes, and aligned by MAFFT. For
523 strains that lacked recognizable *toxA* or *hicB*, sequences adjacent to the genes that normally flanked each operon
524 were extracted. K_a/K_s and pairwise distance were calculated for each gene using SeqinR v 3.4-5 [51] with codon
525 aware gene alignments were made using Prank (v.100802).

526 **RNA-seq analysis:**

527 **Sample Preparation:** Cell cultures of *H. influenzae* strain Rd, Δ cpr and Δ sxy derivatives, and Δ toxTA mutants were
528 grown in sBHI to an OD₆₀₀ of 0.2 – 0.25, then transferred to MIV. Aliquots of cells were removed just prior to transfer
529 to MIV, and after 10, 30, and 100 minutes in MIV, and immediately mixed with Qiagen RNAProtect (#76526) to
530 stabilize RNA. Cells were pelleted and frozen, and RNA was later extracted from thawed pellets using the Qiagen
531 RNeasy Min-elute Cleanup Kit (#74204). Contaminating DNA was removed with Ambion Turbo DNase (#AM2238),
532 and ribosomal RNA was depleted using the Illumina Ribo-Zero rRNA Removal kit (#MRZMB126). Sequencing libraries
533 were prepared using TruSeq mRNA v2 library preparation kit, according to manufacturer's instructions (Illumina).
534 Libraries were pooled and sequenced on a HiSeq 2500, generating paired-end 100 bp reads.

535 **Data Analysis Pipeline:** FASTQ files were analysed using the FASTQC tool (Andrews, 2015) to confirm read quality.
536 Reads were aligned to the *H. influenzae* Rd KW20 reference genome sequence using the Burrows-Wheeler Alignment
537 tool (BWA) algorithm bwa mem [52]. Differential expression analysis was performed using the DESeq2 package,
538 v.1.6.3 (Love *et al.*, 2013). Specifically, the function DESeqDataSetFromMatrix() was used to generate a
539 dataset to compare reads from each mutant strain reads from the wild-type control based on their strain, sample
540 time point, and the interaction between the two parameters. The function DESeq() was called to determine which
541 genes were differentially expressed based on these parameters, using p-values adjusted for a B-H false-discovery rate
542 [53] of 0.1 as a cut-off to determine significance, after normalizing total read counts and variances.

543

544 **Data availability:** RNA-seq data were deposited under BioProject 293882

545

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664 **Supplementary Figure Captions:**

665 **Supplementary Figure A: Bioscreen analysis of growth of *A. pleuropneumoniae* wildtype and *toxTA* deletion
666 strains in rich medium.**

667 **Supplementary Figure B. Development of competence by *H. influenzae* strains in MIV competence medium.**

668 **Supplementary Figure C: Bioscreen analysis of growth of *H. influenzae* wildtype and *toxTA* deletion strains in rich
669 medium.**

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670 **Supplementary Figure D. Growth of *H. influenzae* wildtype and Δ toxA strains after transfer to MIV competence**
671 **medium.**

672 **Supplementary Figure E: Growth and MIV recovery of *H. influenzae* KW20 and Δ toxA measured by OD₆₀₀.**

673 **Supplementary Figure F: Effect of cAMP on *H. influenzae* toxTA mutants.**

674 **Supplementary Figure G: Transformation frequencies of wildtype and Δ toxT cells growing in rich medium.**

675 **Supplementary Figure H: Competence-induced expression of *H. influenzae* toxA.**

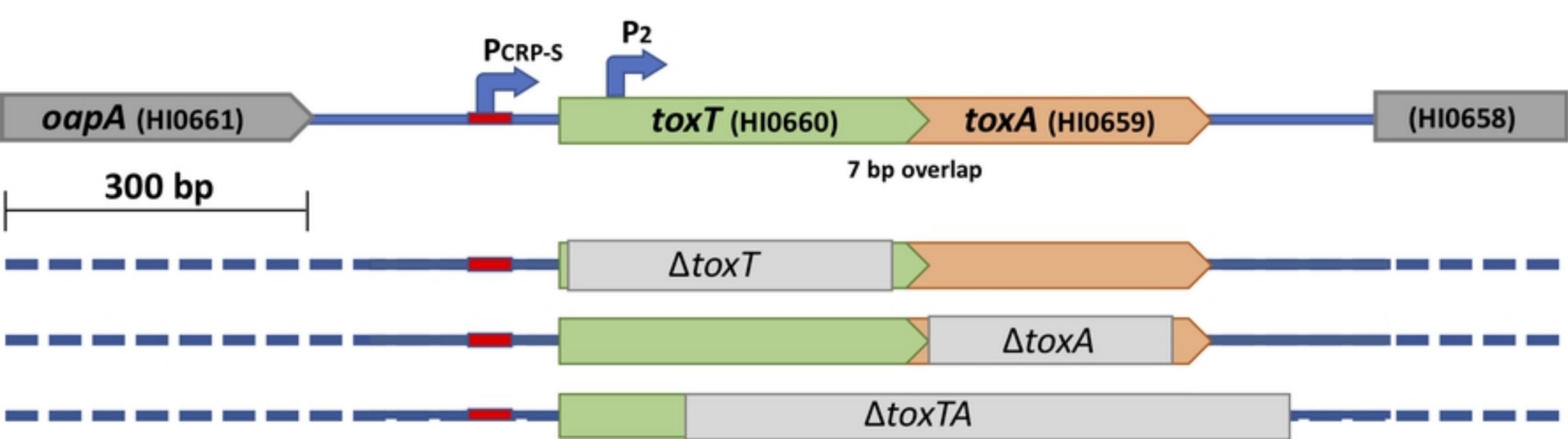
676 **Supplementary Figure I: Effect of hypercompetence mutations on expression of *H. influenzae* toxA and toxT.**

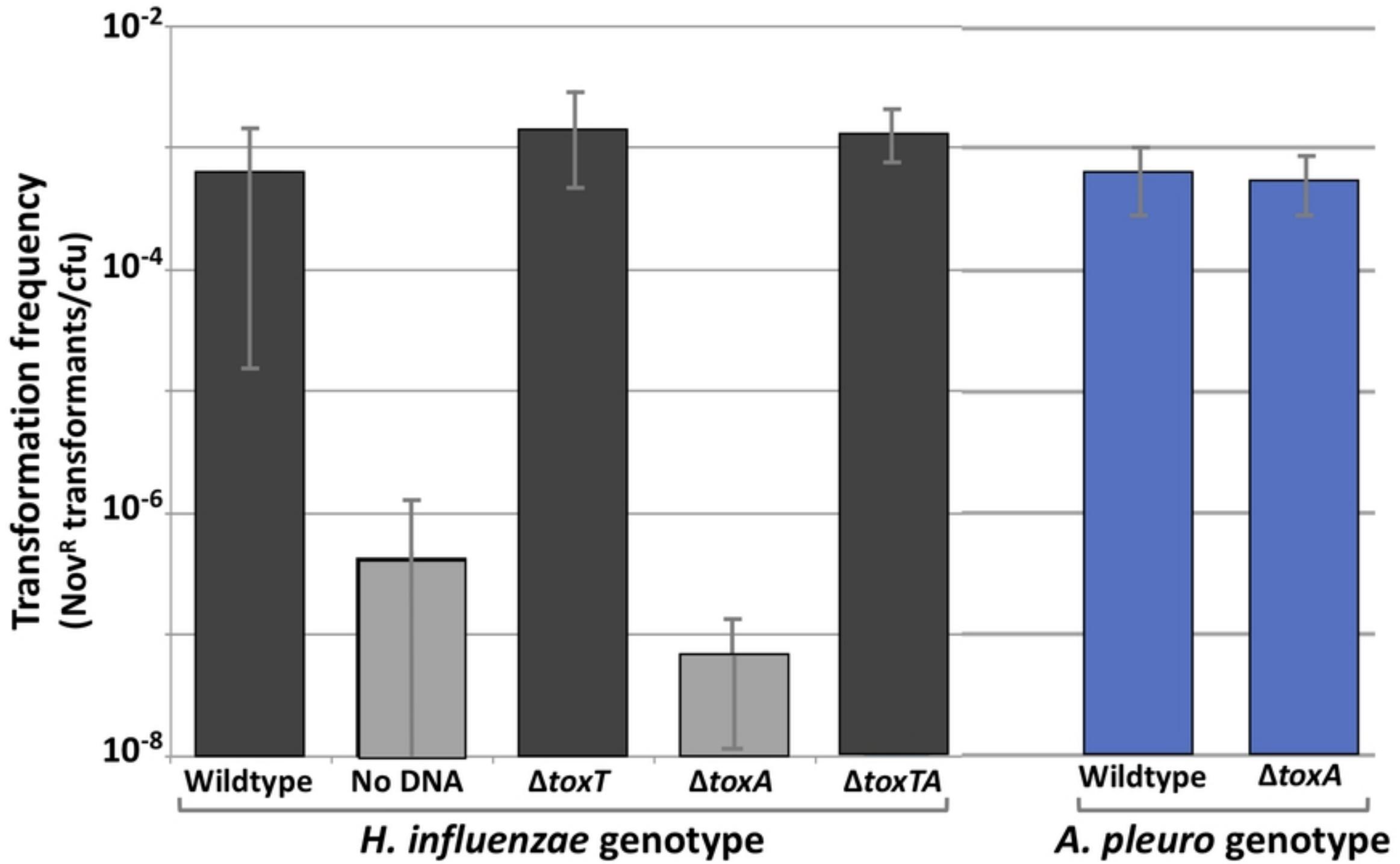
677 **Supplementary Figure J: Effects of Δ toxTA mutations on expression of competence regulator genes.**

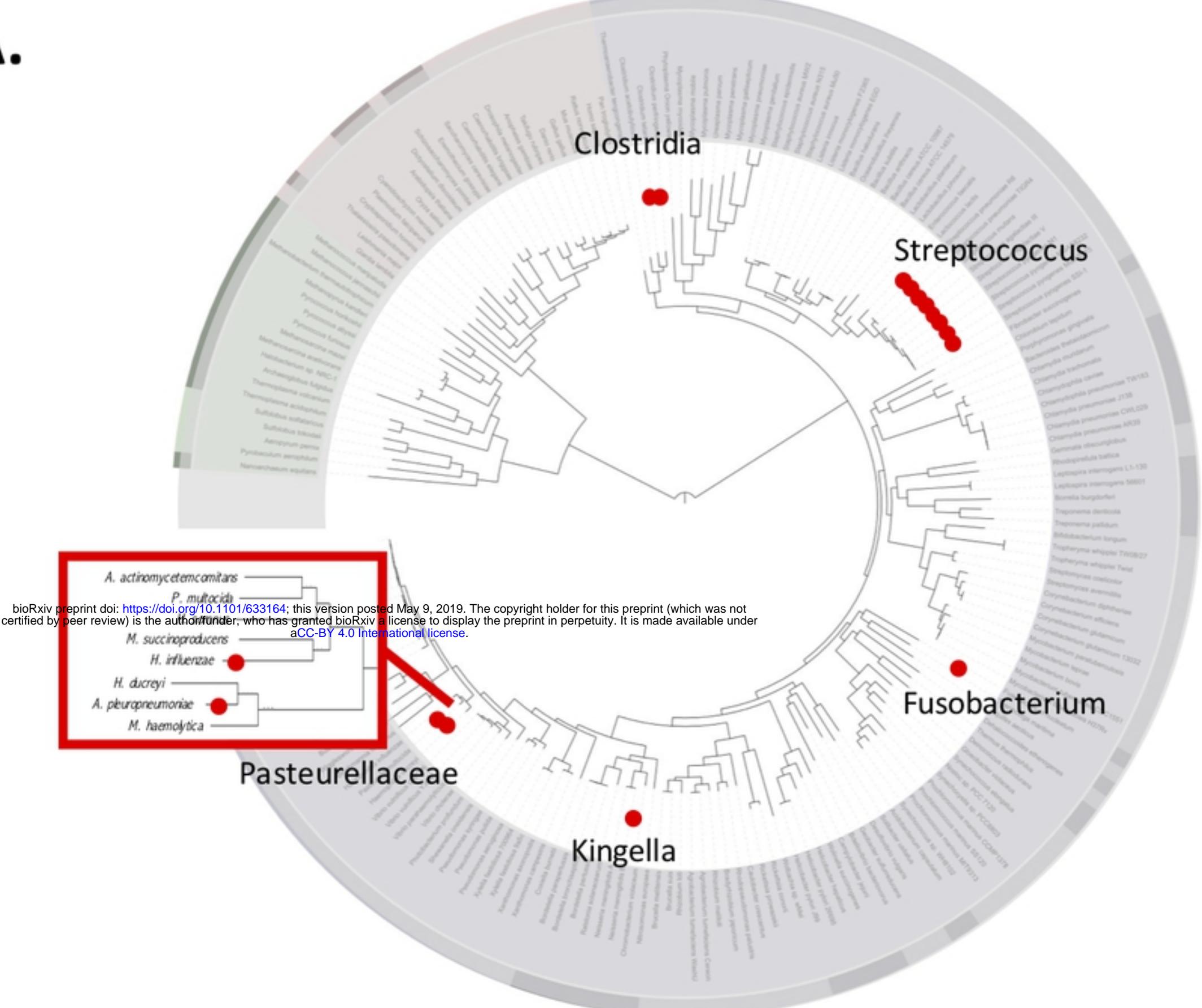
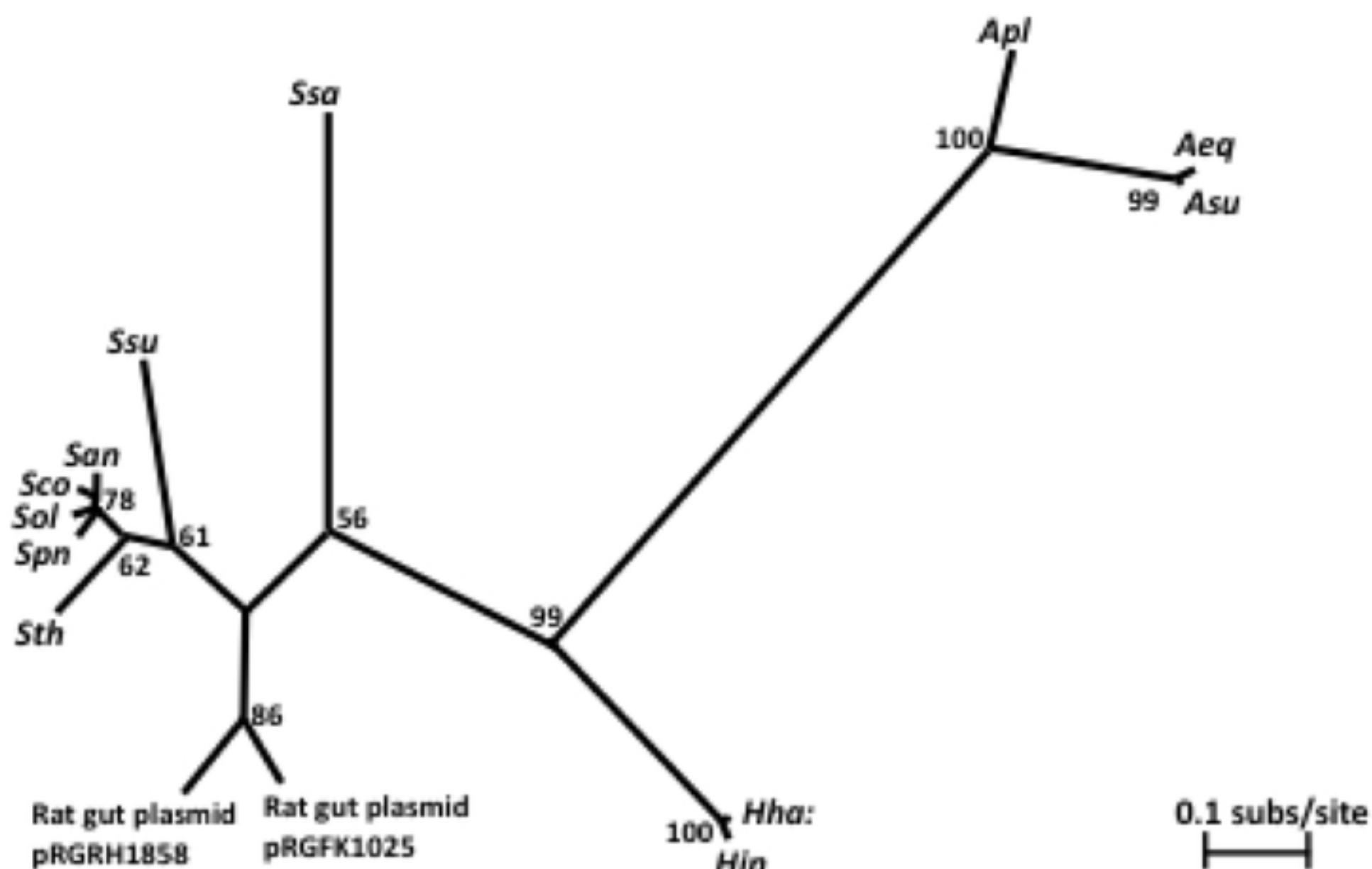
678 **Supplementary Figure K: Changes in expression levels of competence operons at t=100.**

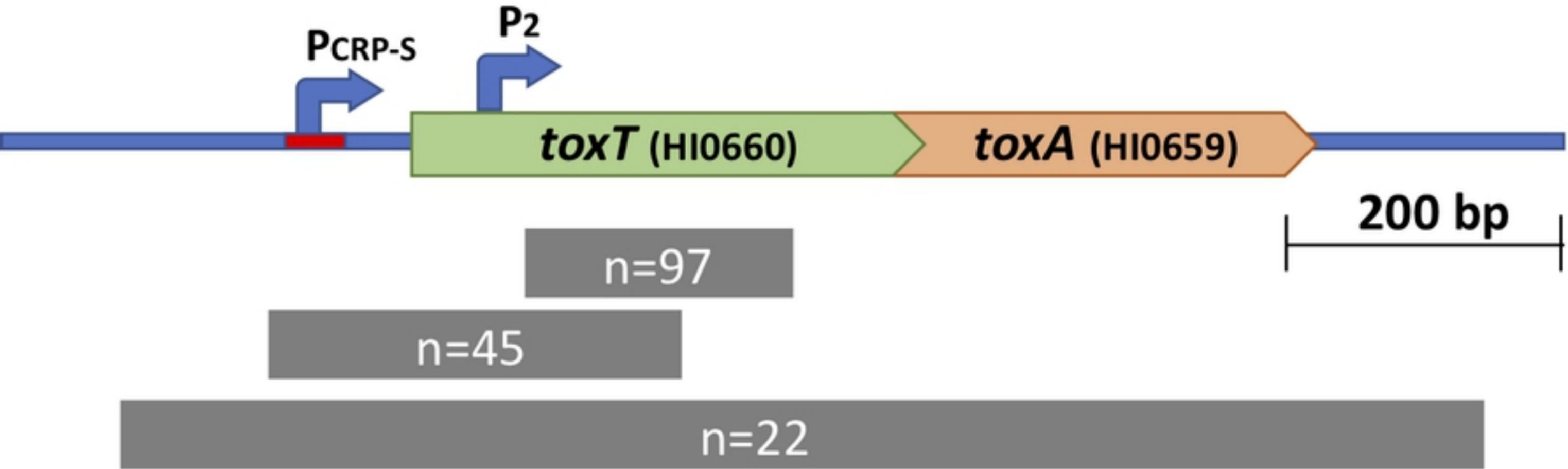
679 **Supplementary Figure L: RNA-seq coverage of *H. influenzae* comNOPQ after 30 min of competence induction.**

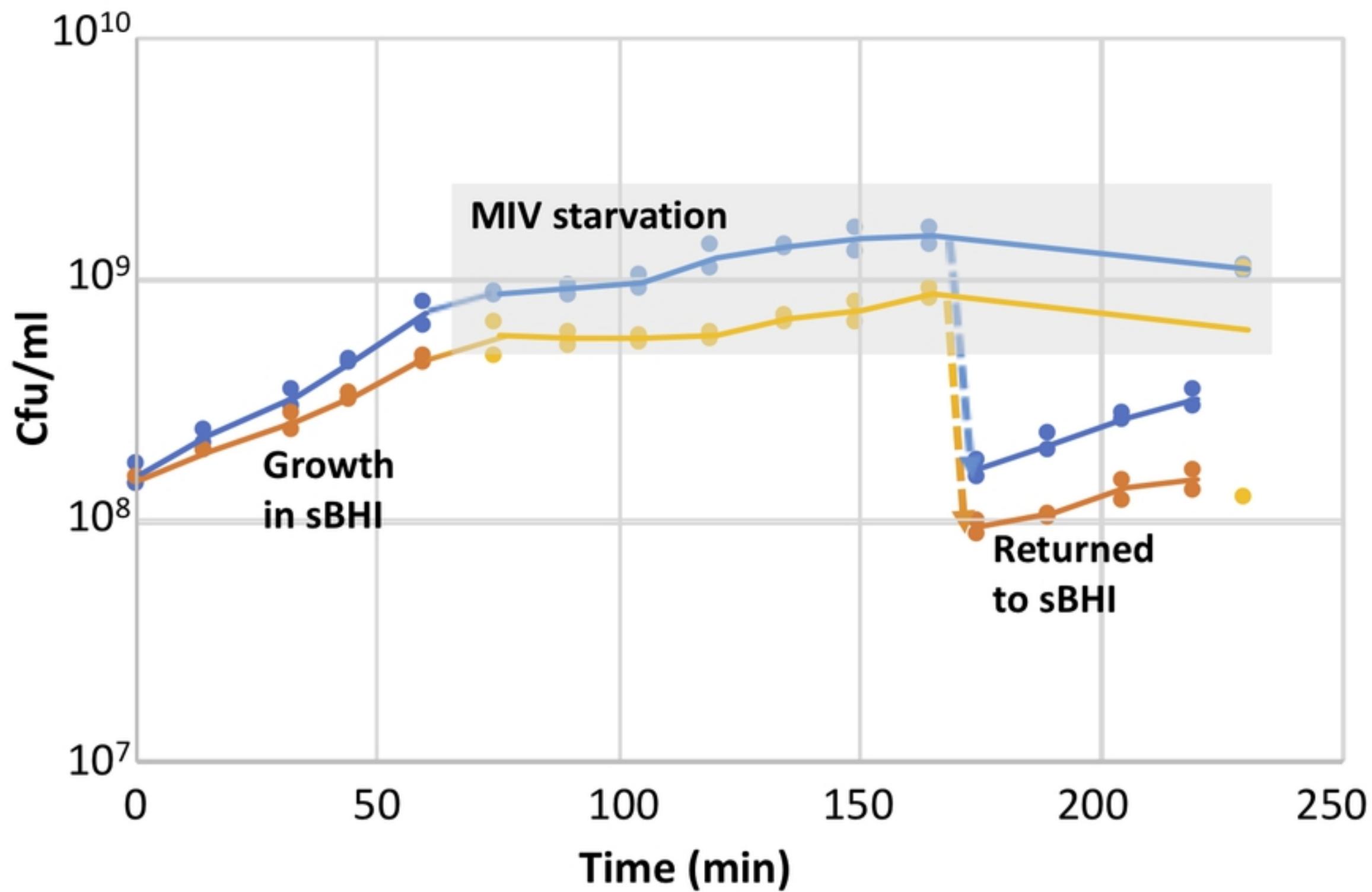
680



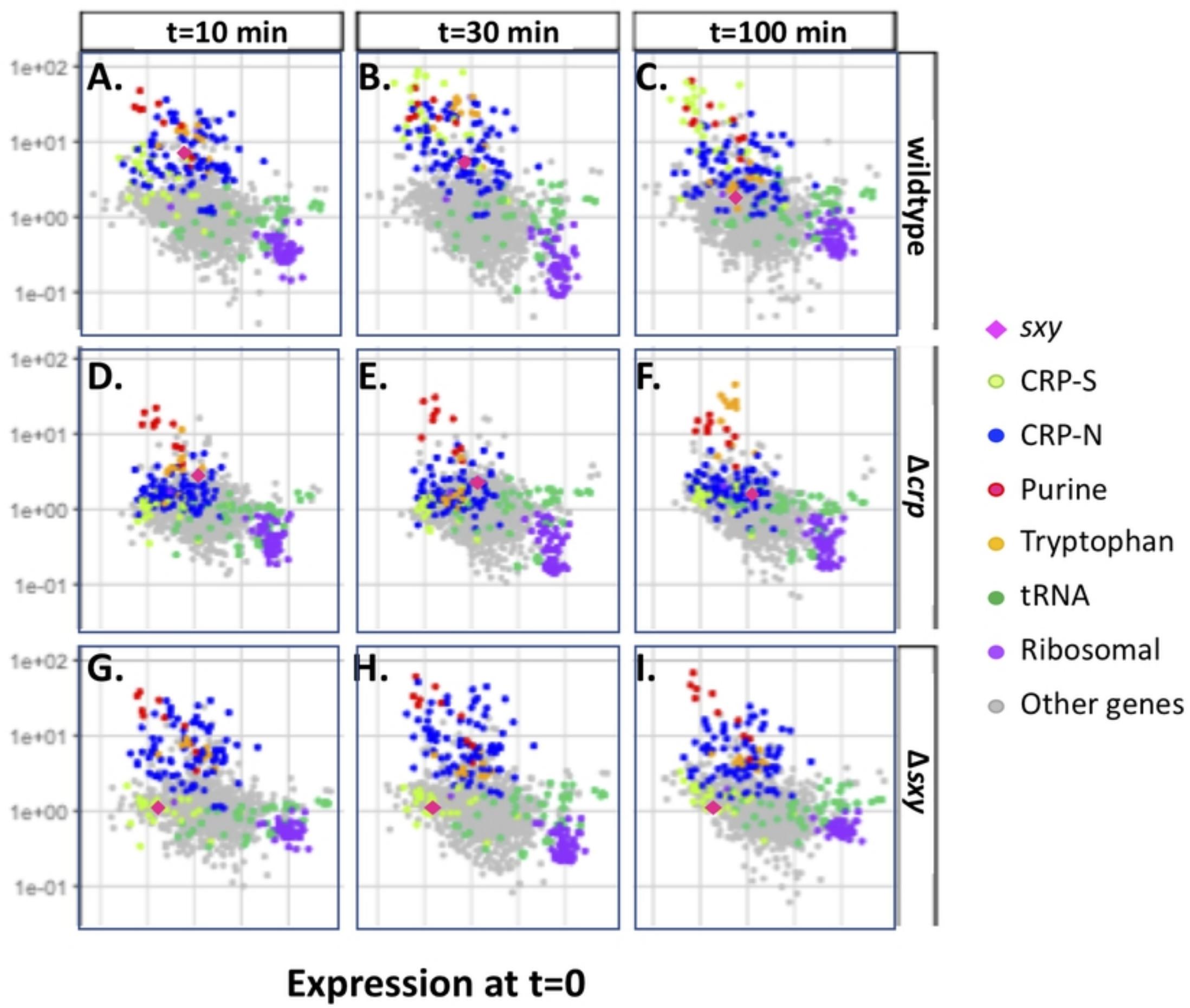


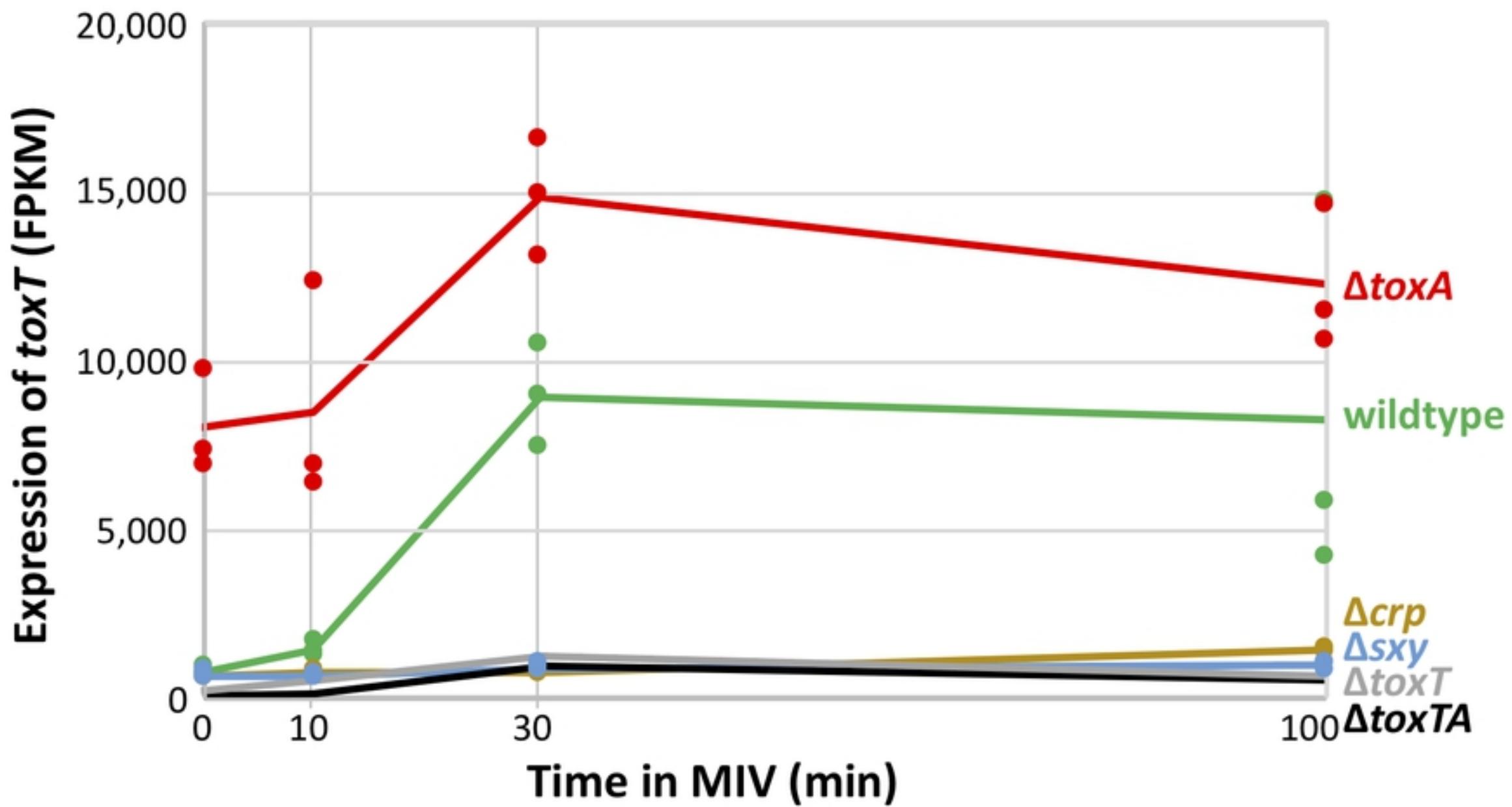
A.**B.**



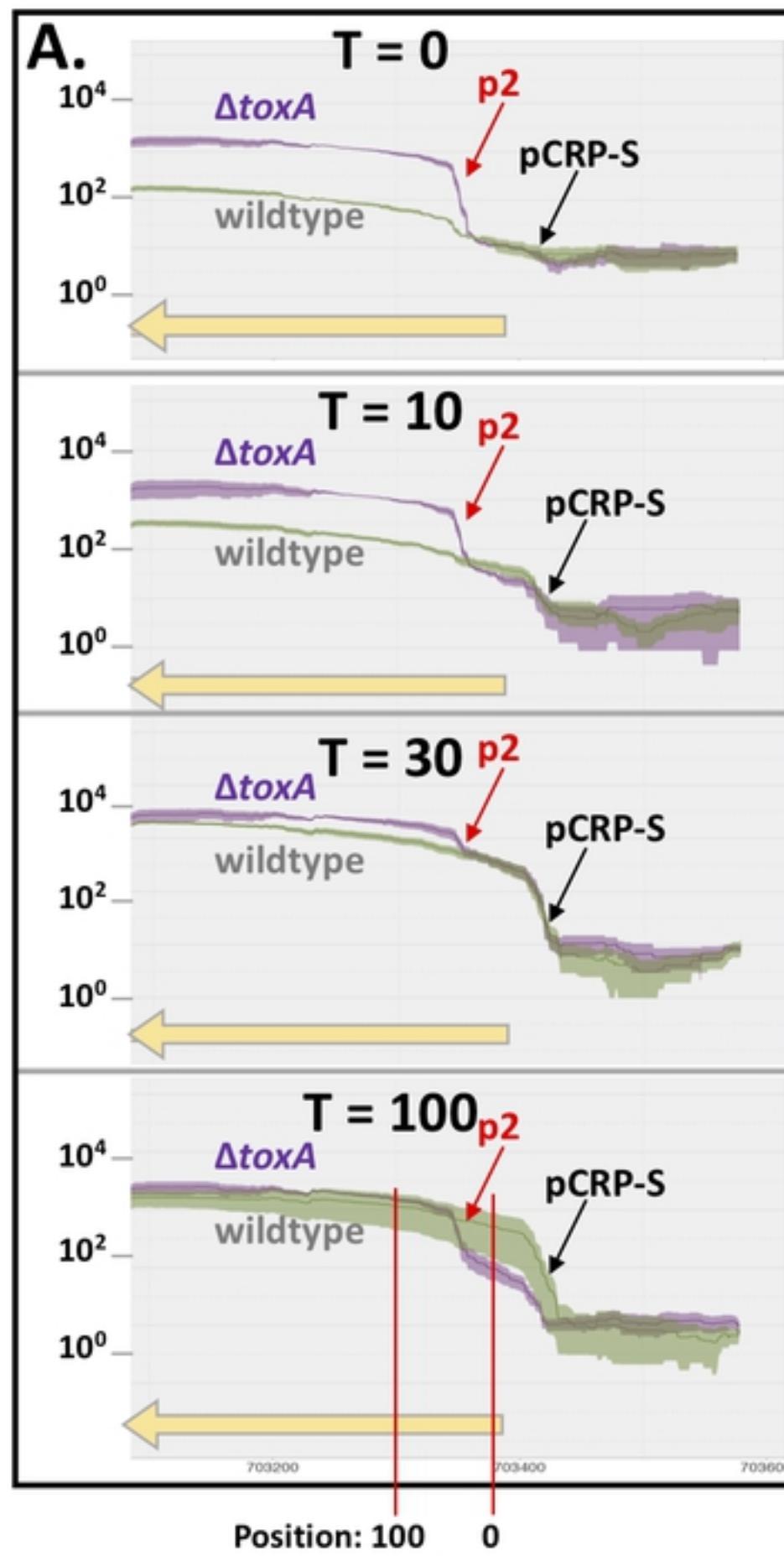


Fold change relative to $t=0$

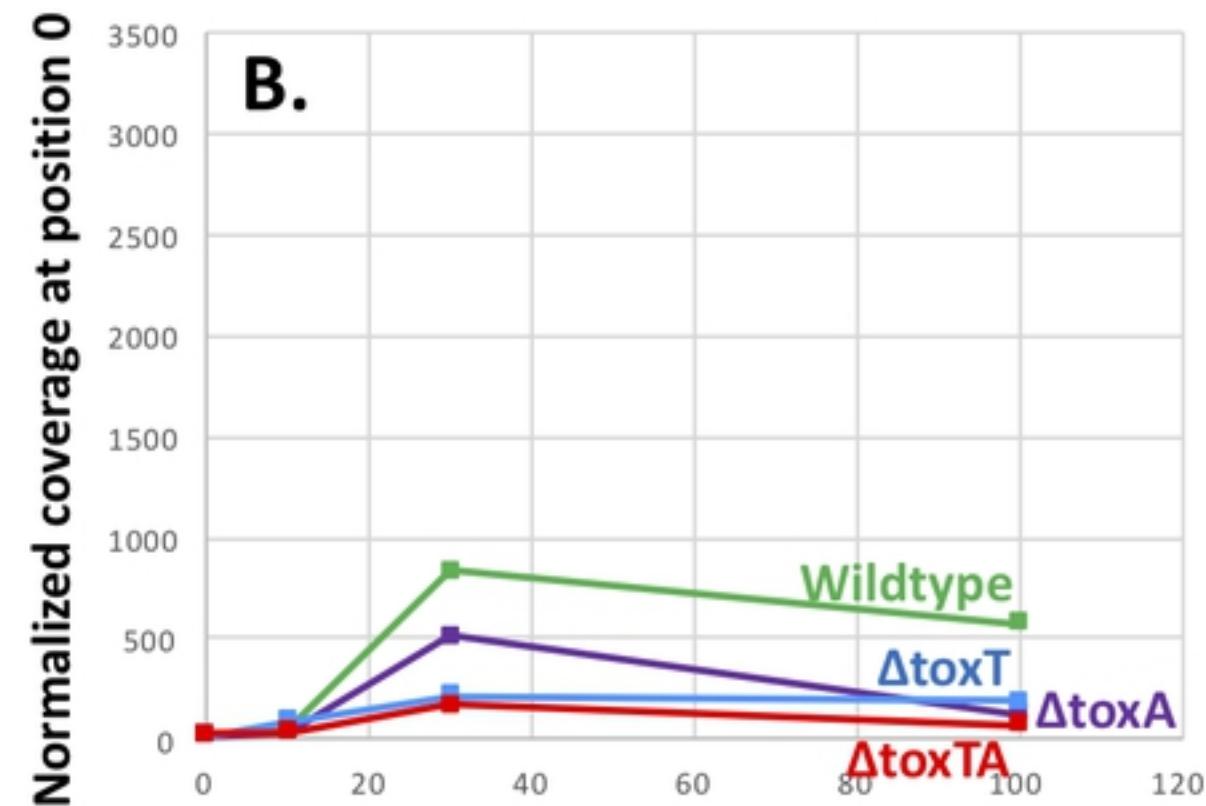




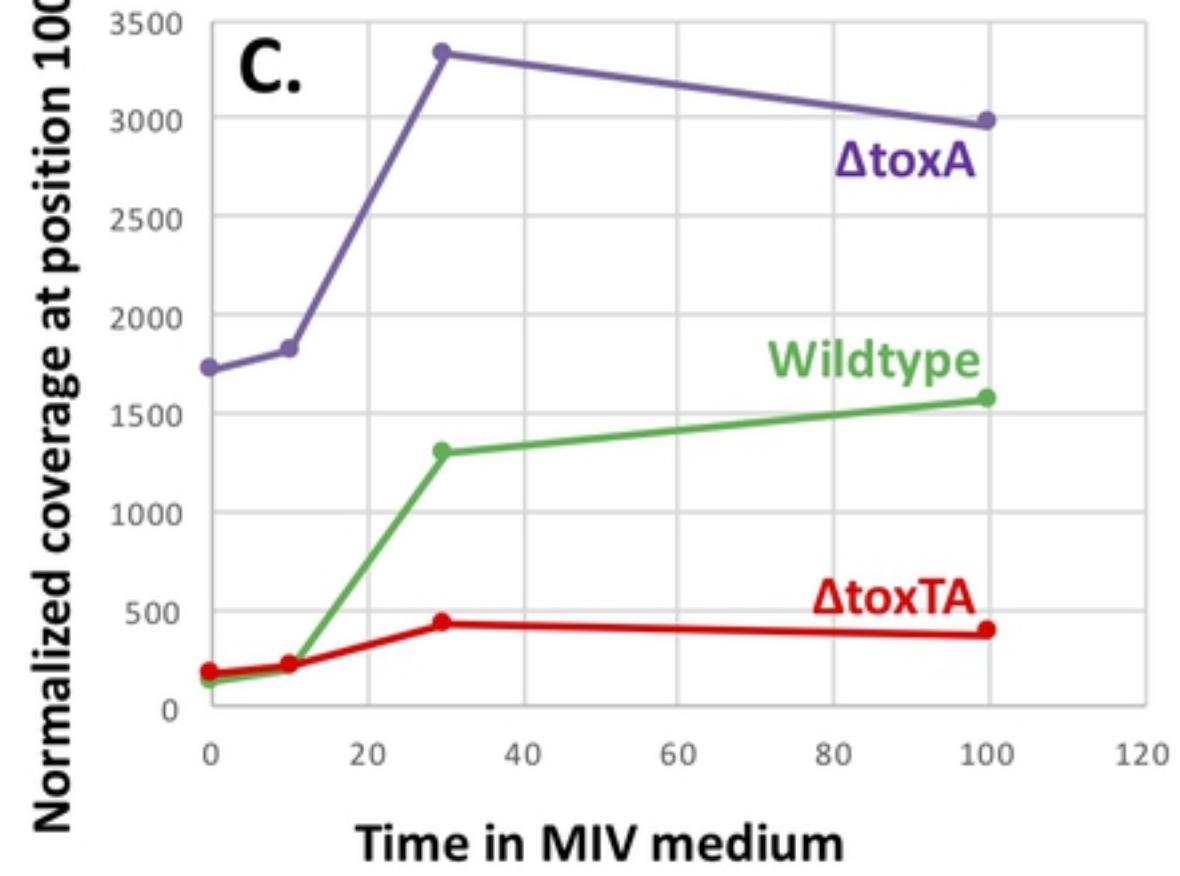
Mean normalized RNA-seq coverage (\log_{10})

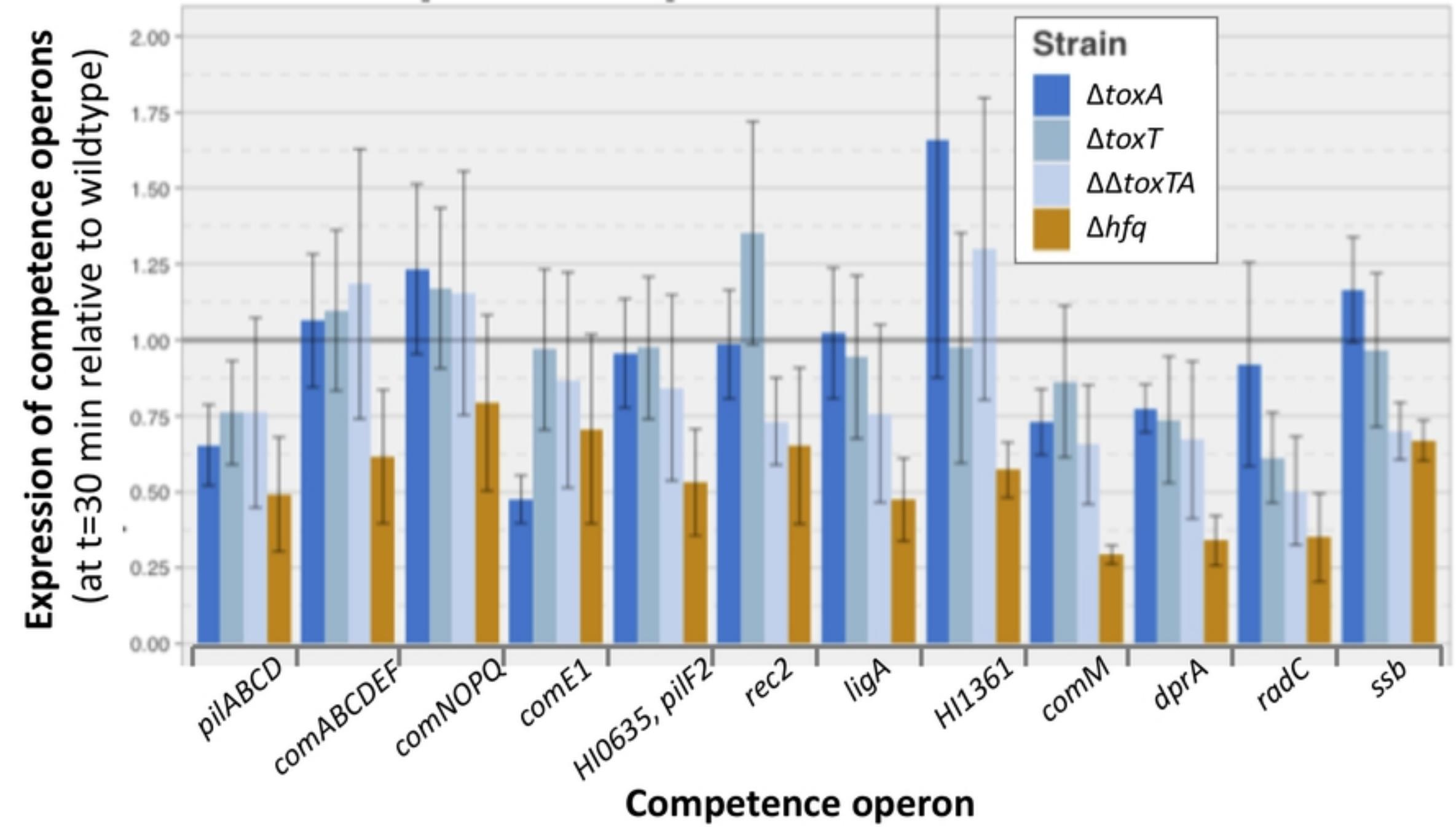


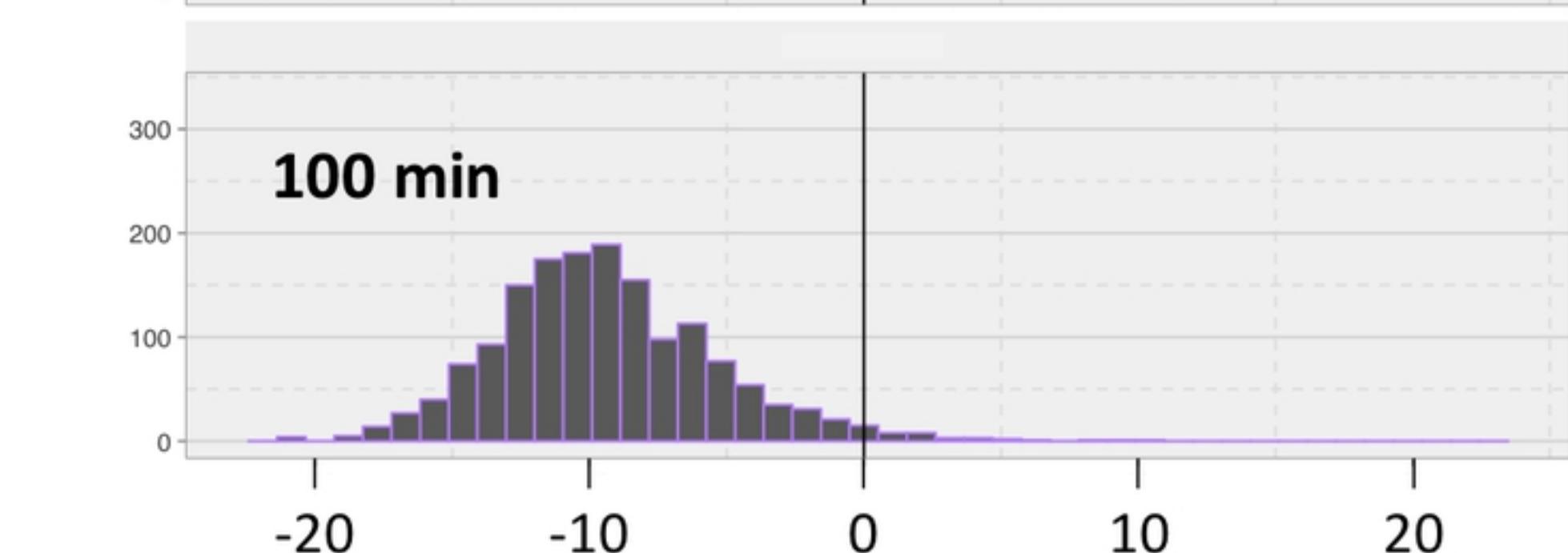
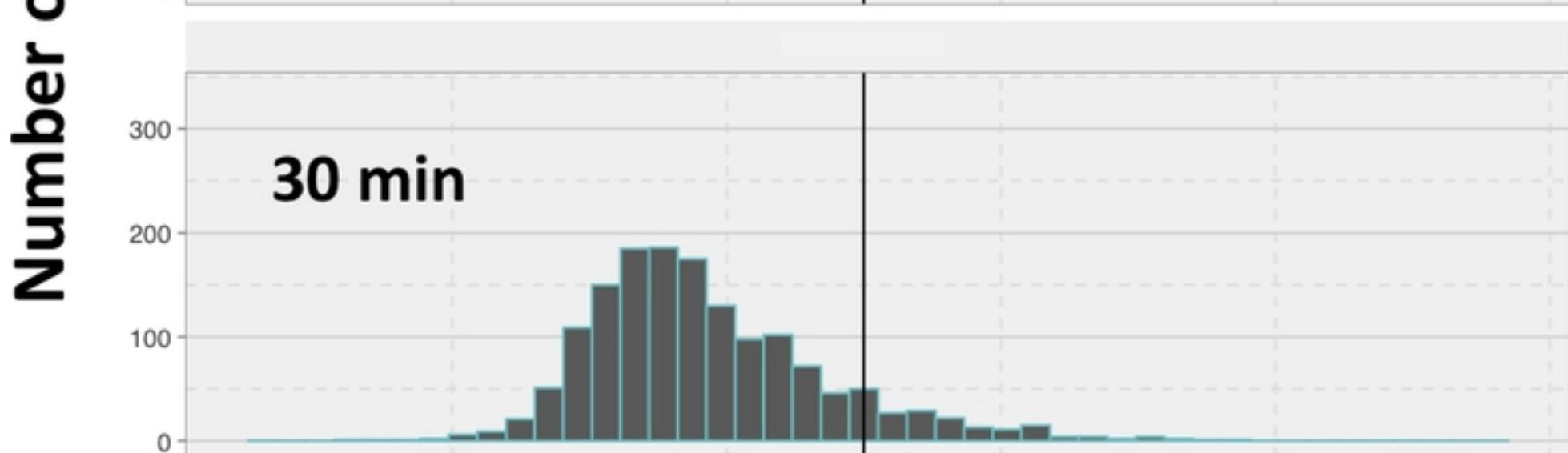
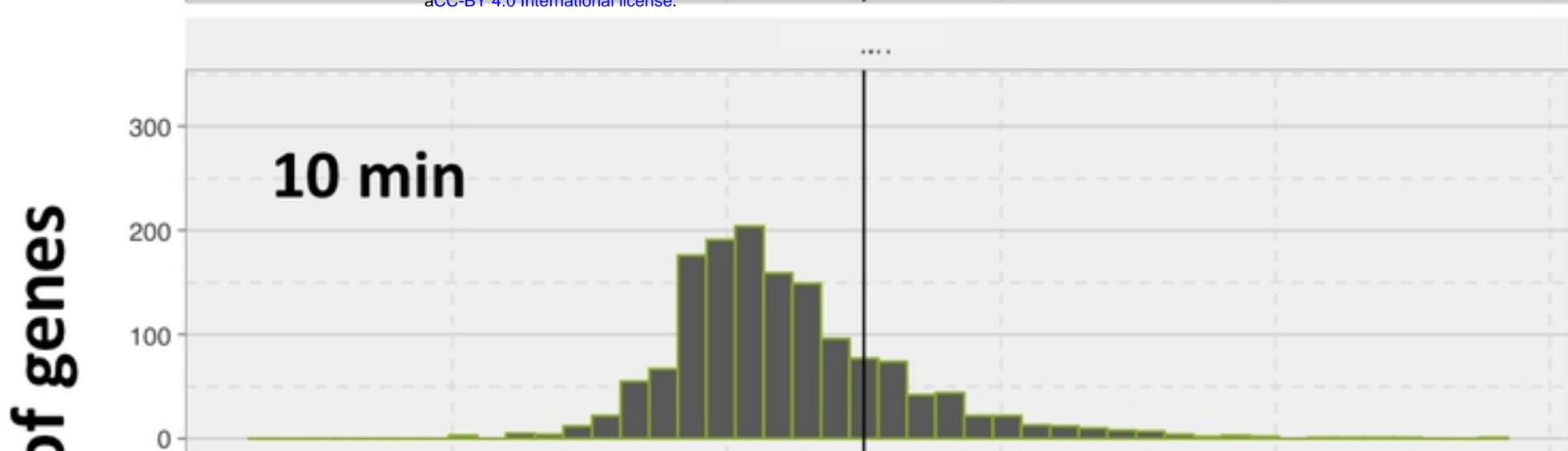
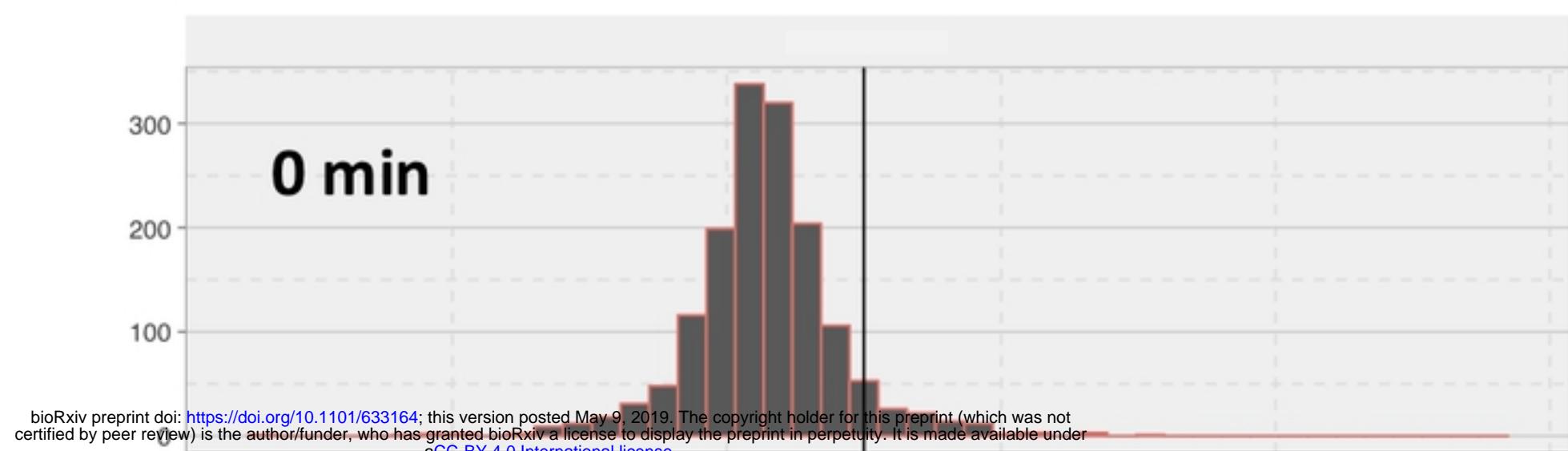
Normalized coverage at position 0



Normalized coverage at position 100







Insert size difference between Δ toxA and KW20 (bp)