

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

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

3 **AUTHORS:** Hailey Findlay Black¹, Scott Mastromatteo¹, Sunita Sinha², Rachel L. Ehrlich³, Corey Nislow⁴, Joshua
4 Chang Mell³, Rosemary J. Redfield^{1*}

5 * Corresponding author: redfield@zoology.ubc.ca, (778) 960-4950

6

7 **AUTHOR AFFILIATIONS:**

8 1 Department of Zoology, University of British Columbia, Vancouver BC, Canada

9 2 Sequencing + Bioinformatics Consortium, Office of the Vice-President, University of British Columbia, Vancouver
10 BC, Canada

11 3 Department of Microbiology & Immunology, Center for Genomic Sciences, Drexel University College of Medicine,
12 Philadelphia PA, USA

13 4 Department of Pharmaceutical Sciences, University of British Columbia, Vancouver BC, Canada

14

15 **AUTHOR CONTRIBUTIONS:**

16 - Performed the experiments: HFB, SM, SS, RJR

17 - Carried out the analyses: HFB, SM, SS, RLE, JCM, RJR

18 - Wrote the paper: HFB, SM, JCM, RJR

19 - Contributed reagents and supplies: CN, RJR

20

21 **ABBREVIATED SUMMARY:**

22 The competence regulon of *Haemophilus influenzae* includes a toxin/antitoxin gene pair. The toxin completely
23 prevents DNA uptake when not opposed by antitoxin, without obviously compromising cell growth or viability. The
24 TA gene pair was acquired by horizontal gene transfer, and the toxin gene has undergone repeated deletions.

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26

27 **ABSTRACT**

28 Natural competence allows bacteria to respond to environmental and nutritional cues by taking up free
29 DNA from their surroundings, thus gaining nutrients and genetic information. In the Gram-negative
30 bacterium *Haemophilus influenzae*, the DNA uptake machinery is induced by the CRP and Sxy transcription
31 factors in response to lack of preferred carbon sources and nucleotide precursors. Here we show that
32 *H10659*—which is absolutely required for DNA uptake— encodes the antitoxin of a competence-regulated
33 toxin-antitoxin operon ('*toxTA*'), likely acquired by horizontal gene transfer from a *Streptococcus* species.
34 Deletion of the toxin restores uptake to the antitoxin mutant. In addition to the expected Sxy- and CRP-
35 dependent-competence promoter, transcript analysis using RNA-seq identified an internal antitoxin-
36 repressed promoter whose transcription starts within *toxT* and will yield nonfunctional protein. We present
37 evidence that the most likely effect of unopposed toxin expression is non-specific cleavage of mRNAs and
38 arrest or death of competent cells in the culture, and we show that the toxin gene has been inactivated by
39 deletion in many *H. influenzae* strains. We suggest that this competence-regulated toxin-antitoxin system
40 may facilitate downregulation of protein synthesis and recycling of nucleotides under starvation conditions,
41 or alternatively be a simple genetic parasite.

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43

44 INTRODUCTION

45 Toxin-antitoxin systems are bacterial gene pairs that were originally discovered on plasmids, where they function to
46 promote plasmid persistence by killing any daughter cells that have not inherited the plasmid. Typically, one gene of
47 the pair encodes a relatively stable toxic protein that blocks cell growth, and the other encodes a labile antitoxin
48 (RNA or protein) that blocks the toxin's activity and regulates its expression (Yamaguchi *et al.*, 2011, Goeders and Van
49 Melderer, 2014). Similar toxin-antitoxin gene pairs have been discovered on many bacterial chromosomes, where
50 they are thought to be relatively recent introductions that in some cases have been co-opted to regulate cellular
51 functions (Van Melderer and Saavedra de Bast, 2009). Here we describe one such system, which is induced in
52 naturally competent cells and whose unopposed toxin completely prevents DNA uptake and transformation.

53 Many bacteria are naturally competent, able to take up DNA from their surroundings and—when sequence similarity
54 allows—recombine it into their genomes (Ambur *et al.*, 2016, Johnston *et al.*, 2014, Mell and Redfield, 2014). In most
55 species, this DNA uptake is tightly controlled, with protein machinery specified by a set of co-regulated chromosomal
56 genes induced in response to diverse cellular signals. In addition to components of the DNA-uptake machinery,
57 competence-regulon genes encode proteins that translocate DNA across the inner membrane, proteins that facilitate
58 recombination, and proteins of unknown function. *Haemophilus influenzae* has an unusually small and well-defined
59 competence regulon (26 genes in 13 operons) induced by signals of energy and nucleotide scarcity (Redfield *et al.*,
60 2005, Sinha *et al.*, 2012). Induction of these genes begins in response to depletion of phosphotransferase sugars,
61 when rising levels of cyclic AMP (cAMP) first stimulate transcription of genes regulated by the transcriptional
62 activator CRP. One of these induced genes encodes the competence-specific transcriptional activator Sxy, but
63 efficient translation of *sxy* mRNA occurs only when purine pools are also sufficiently depleted (Macfadyen *et al.*,
64 2001; Sinha *et al.*, 2013). Sxy then acts with CRP at the promoters of competence genes, stimulating their expression
65 and leading to DNA uptake and natural transformation. These competence promoters are distinguished by the
66 presence of 'CRP-S' sites (formerly called CRE sites), variants of standard CRP sites that depend on both CRP and Sxy
67 for activation (Cameron and Redfield, 2006).

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68 All but one of the fifteen *H. influenzae* genes needed for DNA uptake encode typical competence proteins—
69 membrane-associated proteins homologous to known components of the Type IV pilus-based DNA uptake machinery
70 present in nearly all known naturally competent species (Johnston *et al.*, 2014). The one exception is *HIO659*, which
71 instead encodes a 98 amino acid cytoplasmic protein with no similarity to known DNA uptake proteins. It shares a
72 competence-inducible CRP-S promoter with an upstream gene encoding another short cytoplasmic protein (*HIO660*,
73 119 aa) (**Fig. 1, top**). Although a knockout of *HIO659* eliminates detectable DNA uptake and transformation, a
74 knockout of *HIO660* has no effect (Sinha *et al.* 2012).

75 Here we show that *HIO660* and *HIO659* comprise a horizontally transferred operon that encodes a toxin-antitoxin
76 pair, and that expression of the toxin in the absence of the antitoxin completely prevents DNA uptake and
77 transformation. Surprisingly, this unopposed toxin expression has only slight effects on induction of competence
78 genes, and on cell growth and viability. The *HIO660* toxin is unusual in that its overexpression in the absence of
79 *HIO659* is not lethal to cells growing in rich medium, which may be explained by our observation that transcription in
80 the absence of *HIO659* occurs mainly from a second internal promoter that would not produce functional protein.

81 **RESULTS**

82 ***HIO659* and *HIO660* act as a toxin-antitoxin system.** Our original analyses of competence-induced genes did not
83 identify any close homologs of *HIO659* or *HIO660* (Redfield *et al.* 2005, Sinha *et al.* 2012). However recent database
84 searches and examination of BLAST results revealed that these genes' products resemble proteins in the Type II
85 toxin/antitoxin families, which typically occur in similar two-gene operons. If *HIO660* and *HIO659* do encode a toxin-
86 antitoxin pair, then $\Delta HIO659$'s DNA uptake defect would likely be caused by unopposed expression of a *HIO660*-
87 encoded toxin protein that prevents DNA uptake, so knocking out this toxin gene should restore competence to the
88 *HIO659* (antitoxin) mutant. We tested this by constructing an *HIO660/HIO659* double mutant (Fig. 1) and examining
89 its ability to be transformed with antibiotic-resistant chromosomal DNA. The double mutant had normal
90 transformation (**Fig. 2**), showing that mutation of *HIO660* suppresses the competence defect of an *HIO659* mutant,
91 and also that neither *HIO660* nor *HIO659* is directly needed for the development of competence. This supported the

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92 postulated antitoxin function of *H10660*, so we named the *H10660* and *H10659* genes *toxT* (toxin) and *toxA* (antitoxin)
93 respectively.

94 **ToxT does not modulate growth and/or competence development in normal cells:** The ToxT protein must have a
95 strong effect on competent cells, since earlier work found that a *toxA* knockout strain had no detectable DNA uptake
96 or transformation under standard competence-inducing conditions (Sinha *et al.* 2012, see also **Fig. 2**). DNA uptake by
97 the *toxA* mutant was below the limit of detection (100-fold reduction), but the $>10^6$ -fold reduction in transformation
98 frequency provided a more sensitive measure of the magnitude of the defect. A simple explanation for this
99 phenotype would be that unopposed ToxT prevents competence by killing or otherwise inactivating the cells in which
100 it is expressed; however, growth rates were very similar between wildtype and the three *toxTA* mutant strains
101 growing in rich medium (**Supp. Fig. A**). However, because the *toxTA* promoter is regulated by a CRP-S site, its
102 expression (and thus ToxT production) might be limited to competent cells even in the absence of ToxA. This
103 prompted us to look for evidence of competence-dependent toxicity. Cells with and without *toxA* had similar CFU/ml
104 values in standard transformation assays, but this is not a very sensitive test of competence-dependent toxicity since
105 cells in the competence-inducing starvation medium MIV are already growth-arrested. Furthermore, only 10%-50%
106 of cells in a competent culture are typically transformable (Mell *et al.*, 2014), and the extent of competence gene
107 expression in the non-transformable fraction is not known.

108 A more detailed time course analysis compared wildtype and $\Delta toxA$ mutant cell numbers (CFU/ml; **Fig. 3**) and culture
109 densities (OD₆₀₀; **Supp. Fig. B**) during growth in rich medium, during the development of competence, and during
110 recovery from MIV starvation medium in rich medium. $\Delta toxA$ cells grew slightly slower than wildtype during the
111 initial log phase growth in rich medium. In both cultures, transfer to the starvation medium MIV slowed growth to
112 the same extent (both OD and in CFU/ml) (grey-shaded area in both figures). If unopposed toxin expression during
113 competence development kills cells or halts growth, then returning cells to rich medium might reveal a stronger
114 growth defect. However, both strains also had similar recovery kinetics after a fraction of their culture was returned
115 to rich medium, although $\Delta toxA$ cells again grew slightly slower than wildtype.

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116 Since cyclic AMP is required for induction of the competence genes, and addition of cAMP induces partial
117 competence during exponential growth (Dorocicz *et al.*, 1993), we also tested the effect of cAMP on the $\Delta toxA$
118 knockout. Addition of cAMP did not rescue its transformation defect (Supp. Fig. C), so failure to transform is not
119 caused by defective cAMP production in the antitoxin mutant.

120 Because chromosomal toxin-antitoxin systems are often reported to have acquired roles in modulating cell growth
121 (Page and Peti, 2016), we examined the $\Delta toxT$ mutant for changes in growth and competence under various
122 conditions. The grey line in Supplementary Fig. A shows that $\Delta toxT$'s growth is indistinguishable from that of
123 wildtype cells (blue line), and Fig. 2 shows that its MIV-induced competence is also unchanged. Supp. Fig. D shows
124 that the kinetics of competence development and loss during growth in rich medium is also indistinguishable from
125 wildtype. Also unchanged is the gradual loss of competence when cells are left overnight in MIV medium (not
126 shown). We conclude that $ToxT$'s normal expression in wildtype cells does not detectably regulate competence
127 development or loss. Since these phenotypic analyses did not show direct evidence of MIV-specific toxicity, we used
128 RNA-seq to investigate how a $toxT$ deletion affects expression of $toxT$ and $toxT$, and how these changes affect cells.

129 **Transcriptional control of competence:** The $toxTA$ operon has a typical CRP-S-type regulatory motif upstream of the
130 $toxT$ coding sequence, and previous global analysis of transcription using microarrays (Redfield *et al.*, 2005) showed
131 that it is competence-induced. We have now investigated this regulation in more detail as part of a comprehensive
132 RNA-seq analysis of competence-associated gene expression in wildtype and mutant cells. In these experiments,
133 samples for RNA preparations were taken from three replicate cultures at four time points, first when cells were
134 growing in log phase in the rich medium sBHI (t=0), and then at 10, 30 and 100 minutes after each culture had been
135 transferred to the competence-inducing starvation medium MIV.

136 We first examined how competence induction changed expression of known CRP-regulated (CRP-N) and CRP+Sxy-
137 regulated (CRP-S) genes. Fig. 4 gives an overview of the results. Each coloured dot represents a gene, colour-coded
138 by function. Its horizontal position indicates its level of expression in rich medium (T=0) and its vertical position
139 indicates how this expression changed at later time points (**A**: T=10; **B**: T=30; **C**: T=100) or in a mutant background at

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140 T=30 vs. T=0 (**D**; Δcrp ; **E**: Δsxy). Thus in Fig. 4A the higher positions of the green dots (genes regulated by CRP-N sites)
141 and the red diamond (the competence regulator *sxy*) indicate that they were strongly induced after 10 min in MIV.
142 Induction of *sxy* was followed (T=30 and T=100) by strong induction of the known competence-regulon genes (higher
143 positions of CRP-S genes; blue dots) (Fig. 4B and C). Consistent with prior studies (Redfield *et al.*, 2005), induction of
144 all these genes was blocked by deletion of the *crp* gene (Fig. 4D), and induction of the competence regulon (CRP-S)
145 genes was blocked by deletion of *sxy* (Fig. 4E).

146 To identify all genes whose wildtype expression differed between sBHI and MIV, DESeq2 was used to compare RNA-
147 seq expression values in wild-type RNA samples before and after competence induction (T=0 and T=30). Of the 1747
148 genes examined, 325 had significantly different expression (adjusted p-value < 0.05 after performing a Wald test and
149 Benjamini-Hoschberg correction in DESeq2). To focus on genes with large changes in expression, we imposed an
150 additional requirement that expression be changed by at least 4-fold, the same threshold used in the previous
151 microarray study (Redfield *et al.*, 2005). This higher stringency gave 123 genes significantly decreased and 71
152 significantly increased, for a total of 194/1747 or 11% of all genes tested (listed in Supp. Table 2). Of these, 130 were
153 among the 192 genes previously identified as differentially expressed in the microarray study.

154 Many of these changes are likely due to the absence of many essential nutrients from MIV medium. The significant
155 induction of 9 of the 10 genes regulated by PurR is expected, since MIV lacks nucleotide precursors; the tenth PurR-
156 regulated gene, HI1616, was also strongly induced in all replicates. MIV also lacks tryptophan, and all 11 Trp-regulon
157 genes regulated by TrpR were significantly induced. The nutritional downshift is also likely to be responsible for the
158 induction of many permeases and transporters, and for the significant downregulation of 39 of the 51 genes
159 encoding ribosomal proteins (*rps* and *rpl* genes). The other 22 ribosomal protein genes were also downregulated,
160 the majority with adjusted p-values < 0.05. Expression of 16S and 23S rRNAs was not measured since these
161 molecules had been depleted from the samples during sequencing library preparation, but expression of the tRNA^{Ala},
162 tRNA^{Leu}, and tRNA^{Gly} genes encoded within the six rRNA operons was also significantly reduced.

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163 To clarify the roles of competence-regulating genes in the observed MIV-induced gene expression changes, RNA-seq
164 coverage values from *sxy* and *crp* knockout strains were compared to values for KW20 sampled at the same four sBHI
165 and MIV timepoints. To evaluate changes at all timepoints simultaneously, a likelihood ratio test was performed
166 using DESeq2 to identify genes that behaved differently between strains. Significant genes were flagged after
167 adjusting for multiple hypothesis testing. *Sxy* is the competence-specific regulator, and deleting it significantly
168 reduced expression of 24 of the known competence genes (Redfield *et al.* 2005, Sinha *et al.*, 2013). Two other
169 previously identified competence genes, HI0250 (*ssb*) and HI1631, also had reduced expression, but these did meet
170 the significance cutoff. *ssb* is an essential gene (Sinha *et al.*, 2012); its high baseline expression increased 40% on
171 transfer to MIV and returned to normal in the *sxy* knockout. Expression of HI1631 was reduced 6-fold by the *sxy*
172 knockout, but high variability in KW20 expression led to an insignificant adjusted P-value.

173 **Transcriptional control of *toxTA*:** RNA-seq analysis confirmed that *toxTA* is regulated as a typical competence
174 operon. In wildtype cells, baseline RNA-seq expression of *toxT* and *toxA* was very low during log phase in rich
175 medium, with approximately tenfold induction by incubation in MIV (Fig. 5 (*toxT*) and Supp. Fig. E (*toxA*), green lines
176 and points). As expected, this increase was eliminated by knockouts of CRP and *Sxy* (brown and blue lines and
177 points). Like other CRP-S genes, both *toxT* and *toxA* were also induced in rich medium in the presence of mutations
178 known to cause hypercompetence (Redfield 2005) (RNA-seq data not shown). Thus the *toxTA* operon is regulated as
179 a typical member of the competence regulon.

180 RNA-seq analysis also showed that the *toxTA* operon is regulated as a typical type II toxin-antitoxin operon. In such
181 operons, the antitoxin protein usually protects cells from the toxin in two ways. First, it inactivates the toxin protein
182 by forming a complex with it that has no toxin activity. Second, this toxin-antitoxin complex binds to the *toxTA*
183 promoter and represses transcription (Overgaard, 2008, Goeders and Van Melder, 2014). *ToxA* has a HTH-XRE
184 DNA-binding domain, which is commonly found in promoter-binding antitoxins (Makarova *et al.*, 2009, Yamaguchi *et*
185 *al.*, 2011), and the RNA-seq analysis in Fig. 5 confirmed that it represses *toxTA* transcription. The Δ *toxA* mutant,
186 which retains an intact *toxTA* promoter and *toxT* coding sequence (see Fig. 1), had 9-fold increased baseline
187 expression of *toxT* in log phase cells (red line and points in Fig. 5). Expression increased further during competence

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188 development, with the same kinetics as in wildtype cells, suggesting independent contributions from baseline
189 repression by antitoxin and competence induction by CRP and Sxy. (Values for *toxA* expression are shown by the red
190 points and line in Supp Fig. E, but are underestimates because most of the gene has been deleted.)

191 Since antitoxin is predicted to repress *toxTA* only when bound to toxin, we were initially surprised that knocking out
192 *toxT* or both *toxT* and *toxA* did not increase RNA-seq coverage of *toxT* (Fig. 5, grey and black lines) and that knocking
193 out both genes did not increase coverage of *toxA* (grey line in Supp Fig. E). These mutants retain all the upstream
194 sequences and the *toxT* start codon, and enough sequence of the deleted genes to identify them in the RNA-seq
195 analysis.) An explanation was suggested by a recent study of the *Escherichia coli hicAB* toxin/antitoxin system
196 (Turnbull and Gerdes, 2017), and confirmed by more detailed analysis of *toxTA* transcripts. The HicA (toxin) and HicB
197 (antitoxin) proteins have no detectable sequence homology to ToxT and ToxA, but their operon is also Sxy-regulated
198 and has the same atypical organization (toxin before antitoxin) (Sinha 2009). Turnbull and Gerdes show that the
199 *hicAB* operon has two promoters. Promoter P1 has a CRP-S site regulated by CRP and Sxy, which is not repressed by
200 the HicB antitoxin. A secondary promoter P2 is very close to the *hicA* start codon; it is repressed by HicB
201 independently of HicA, and its shortened transcripts produce only functional HicB, not HicA. Promoter P1 of this
202 *hicAB* system thus resembles the CRP-S regulation of the *toxTA* operon, and the presence of a second antitoxin-
203 regulated internal promoter similar to P2 would explain the high *toxTA* operon expression seen in the *toxA*
204 knockouts.

205 This finding in the *hicAB* system prompted us to do a more detailed analysis of *toxTA* transcription patterns in
206 wildtype and mutant cells to determine whether the *toxTA* transcripts expressed in the absence of *toxA* were
207 similarly truncated. Figure 6A shows RNA-seq coverage of the *toxTA* promoter region and the 5' half of *toxT*, in
208 wildtype cells and in the *toxA* deletion mutant (note that transcription of *toxTA* is from right to left). As expected, the
209 predicted CRP-S promoter upstream of *toxTA* was active only at T=30 and T=100; its activity was not affected by
210 deletion of *toxA*. Deletion of *toxA* instead caused strong constitutive transcription from a second promoter ('P2'),
211 with reads beginning about 30 bp downstream of the *toxT* start codon. Transcripts produced from this start point are
212 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

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213 first GUG (position 35) lack Shine-Dalgarno sequences. This supports the hypothesis that the *H. influenzae toxTA*
214 operon is regulated similarly to the *E. coli hicAB* operon, with the antitoxin repressing transcription from a
215 downstream 'P2' promoter whose transcript produces antitoxin but not toxin.

216 In the *E. coli hicAB* system, P2 is repressed by HicB antitoxin alone, binding of HicB to the P2 operator is destabilized
217 when HicA toxin is abundant, and transcription from P2 in plasmid constructs is elevated when the chromosomal
218 *hicAB* operon is deleted (Turnbull and Gerdes, 2017). To see if this also happens in *H. influenzae's toxTA*, we
219 measured transcription in wildtype and *toxTA* mutant cells more accurately by scoring the coverage at two positions
220 in the *toxTA* operon (indicated by red vertical lines at the bottom of Fig. 6A). Position 0 is the *toxT* start codon, 34 nt
221 downstream from the CRP-S promoter (P_{CRP-S}) but upstream of the putative P2 promoter, and position 100 is about
222 70 nt downstream from P2 (P2 and position 100 are deleted in $\Delta toxT$). To eliminate read-mapping artefacts arising
223 from failure to align reads that span an insertion or deletion, each mutant's reads were mapped onto its own *toxTA*
224 sequence rather than the reference sequence. Comparison of Figures 6B and 6C shows that coverage at position 100
225 was always higher than coverage at position 0, consistent with the presence of a second promoter between positions
226 0 and 100. Fig. 6B also shows that coverage at position 0 (expression from P_{CRP-S}) was reduced by all of the *toxTA*
227 deletions. This was unexpected, and suggests that this promoter may have unusual properties, since coverage of
228 other CRP-S genes was not similarly affected. The *toxA* deletion caused the predicted increase in coverage at
229 position 100 (Fig. 6C), but the *toxTA* deletion unexpectedly reduced rather than increased coverage at this position
230 ~3-fold from the wildtype level, even though this construct retains the first 150 bp of the operon, including P2. This
231 reduction was not accounted for by the reduction in expression from P_{CRP-S} , suggesting that high-level transcription
232 from the *toxTA* P2 promoter only occurs when ToxT is present and ToxA is absent. This could mean either that ToxT
233 directly binds the P2 promoter to induce transcription, which seems unlikely given its lack of DNA-binding domain, or
234 it could mean that the presence of ToxT disrupts binding of a secondary repressor of the operon, such as a
235 noncognate antitoxin (Goeders and Van Melderen, 2014).

236 **ToxT does not prevent induction of the competence regulon:** To investigate how deletion of the *toxTA* antitoxin
237 causes severe defects in DNA uptake and transformation, we first examined changes in expression of the genes that

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238 regulate the competence regulon. Comparison of the RNA-seq data for wildtype cells (orange) and *toxT*, *toxA* and
239 *toxTA* mutants (yellow, blue and grey) ruled out direct inhibition of competence gene expression by a *toxT*-encoded
240 toxin. Unopposed expression of *toxT* (in the $\Delta toxA$ mutant) only slightly reduced induction of the *sxy* transcript
241 needed for induction of the competence regulon (Supp. Fig. F-A, blue line). Importantly, similar modest reductions
242 were also seen in the other *toxTA* mutants (grey and yellow lines), which have normal competence. The mRNA levels
243 of *crp* and *cya* (CRP (HI0957) and adenylate cyclase (HI0604)) were also not changed by $\Delta toxA$ (Supp. Fig. F-B and F-
244 C).

245 The competence operons induced by these regulators also retained normal or near-normal expression in the $\Delta toxA$
246 mutant at 30 min after transfer to MIV, the time when competence-induced gene expression is highest (Fig. 7). As
247 noted above for *sxy*, competence gene expression levels at this time were very similar between the $\Delta toxA$ mutant,
248 which cannot take up DNA or transform, and the $\Delta toxT$ and $\Delta toxTA$ mutants, which take up DNA and transform
249 normally (Supp. Fig. G-A and G-B). Although expression of most of the competence operons was substantially
250 reduced in $\Delta toxA$ cells at the t=100 min time point (Supp. Fig. G-C), this effect was too little and too late to explain
251 the mutant cells' complete lack of competence.

252 **Other ToxT and ToxA effects in competence-induced cells:** Since changes in competence gene expression could not
253 readily explain the severe competence defect of $\Delta toxA$, we extended our investigation to genes not known to be
254 involved in competence. Supp. Table 3 lists, for each timepoint, the genes whose expression was significantly
255 different in the $\Delta toxA$ mutant than in all three strains with normal competence (wildtype, $\Delta toxT$ and $\Delta toxTA$). In rich
256 medium (T=0) the only statistically significant effect of $\Delta toxA$ on gene coverage was about 1.5-fold increased
257 expression of three genes in the HI0654-0658 operon, which are directly downstream from *toxA* (see Fig. 1) and thus
258 may experience read-through from the *toxTA* P2 promoter (which was constitutively active in the $\Delta toxA$ mutant).
259 The operon includes genes encoding shikimate dehydrogenase, an ABC transporter, and a hypothetical protein with
260 putative topoisomerase I domains. Expression of genes in this operon increased about 1.2-1.5-fold in MIV in wildtype
261 cells and in other mutants with normal competence, suggesting that read-through also occurs from the *toxTA* CRP-S
262 promoter. Their normal induction in competence suggests that their higher expression in $\Delta toxA$ is unlikely to be

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263 responsible for this strain's competence defect, but it may cause the slight $\Delta toxA$ growth defect described above
264 (Supp. Fig. A). The absence of other detectable changes in gene expression is consistent with the postulated lack of
265 functional ToxT protein produced from the P2 promoter.

266 ToxT effects are also not expected at the 10 min time point, since the Sxy-dependent CRP-S promoter is not yet
267 active. Only two genes were significantly changed in $\Delta toxA$: HI0655 (see above) and HI0231 (*deaD*), which encodes a
268 DEADbox helicase involved in ribosome assembly and mRNA decay (Iost and Dreyfus, 2006). In all strains, this gene's
269 expression falls rapidly on transfer to MIV, but levels in $\Delta toxA$ were about 50% higher at all time points.

270 At the 30 min time point, seven genes' expression levels were significantly altered by deletion of *toxA*. Most were
271 only changed by about 2-fold, but two genes had large increases at both the t=30 and t=100 time points and may be
272 relevant to the competence defect: Deletion of *toxA* increased HI0235 expression 3-5-fold at t=30 and 2-3-fold at
273 t=100 (in the $\Delta toxT$ and $\Delta toxTA$ comparisons, but not in the KW20 comparison. Its protein has strong similarity to the
274 ArfA ribosome-rescue domain (Garza-Sanchez *et al.*, 2011); the significance of this is discussed below. HI0362
275 encodes a CRP-regulated iron-transport protein that normally increases in MIV but does not increase in *toxA* deletion
276 mutants.

277 Global RNA-seq analysis did not reveal any obvious candidate genes. Although many more genes were significantly
278 changed by $\Delta toxA$ at the 100 min time point, only four of these were also changed at t=30. Two of these, HI0235 and
279 HI0362, were described above. Additionally, In all competent strains, HI0504 (*rbsB*, a ribose transporter component),
280 was induced 20-fold more in MIV than other genes in its operon, but this increase was only 10-fold in $\Delta toxA$ (at t=30
281 as well as t=100). Expression of HI0595 (*arcC*, carbamate kinase) normally falls 2-3-fold immediately after transfer to
282 MIV, but the fall was greater in $\Delta toxA$. 28 other genes were significantly changed only at t=100, but their expression
283 patterns and predicted functions were diverse and did not suggest an explanation for $\Delta toxA$'s lack of competence.

284 Overall, this gene expression analysis did not reveal any promising mechanisms through which unopposed *toxT*
285 expression could prevent competence.

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286 **Related toxins may suggest mechanism of action:** Since examination of gene expression shed little light on how the
287 ToxT toxin prevents competence, as an alternative approach we considered the modes of action of well-studied
288 relatives of ToxT. The most common type II toxins act as translation-blocking ribonucleases, such as RelE, but several
289 alternative modes of action are also known, and some newly discovered toxins lack identified activities (Makarova *et*
290 *al.*, 2009). The Pfam and TAFinder databases assign the *H. influenzae* ToxT protein to the ParE/RelE toxin superfamily,
291 whose characterized members include both ribonucleases and gyrase inhibitors (Goeders and Van Melderen, 2014).
292 Because *toxTA* shares regulatory features, gene order, and chromosomal location with *E. coli's hicAB*, we gave special
293 consideration to the possibility that their toxins also share a mechanism; the HicA toxin is a ribonuclease that arrests
294 cell growth by cleaving mRNAs and other RNAs (Jorgensen *et al.*, 2009).

295 **Unopposed toxin does not inhibit gyrase:** If ToxT inhibited gyrase we would expect the RNA-seq data to show that
296 transfer to MIV caused increased expression of *gyrA* (HI1264) and *gyrB* (HI0567) and reduced expression of *topA*
297 (HI1365), since these genes have opposing activities and compensatory regulation by DNA supercoiling (Gmuender *et*
298 *al.*, 2001). However, these genes' coverage levels were similar in wildtype and all *toxTA* mutants, during both
299 exponential growth and competence development.

300 **Unopposed toxin does not cleave competence-induced mRNAs site-specifically:** The best-studied homologs of the
301 *toxT* toxin act by cleaving mRNAs at random positions near their 5' ends during their translation on the ribosome
302 (Hurley, 2011, Goeders *et al.*, 2013). Thus we considered whether ToxT might prevent competence by one of two
303 mechanisms. First, ToxT might specifically cleave the 5' ends of competence-gene transcripts, eliminating their
304 function without significantly changing their overall RNA-seq coverage levels or otherwise interfering with essential
305 cell functions. Visual examination of RNA-seq coverage of all positions within the competence operons did not reveal
306 any anomalies that might indicate that the mRNA in $\Delta toxA$ cells had been inactivated either by cleavage at specific
307 sites or by random cleavage near the 5' end (Gordon *et al.*, 2017). As an example, Supp Fig. H compares read
308 coverage across the *comNOPQ* operon in wildtype and $\Delta toxA$ cultures after 30 min in MIV.

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309 **Unopposed toxin may nonspecifically cleave mRNAs:** A second mechanism we considered was that ToxT might
310 nonspecifically cleave mRNAs. This would result in a large population of mRNAs lacking in-frame stop codons ('non-
311 stop' mRNAs). Because these cannot undergo the normal ribosome-release process, this would cause a general block
312 to translation (Tollervey, 2006). This block in turn is predicted to arrest cell growth until normal translation can be
313 restored (Pandey and Gerdes, 2005). To indirectly detect such cleavage, we examined the insert sizes of our RNA-seq
314 sequencing libraries by comparing the spanning length distributions of paired-end sequencing reads among strains.
315 Because independent library preparations had different insert sizes, comparisons were limited to samples prepared
316 at the same time. Fig. 8 shows that the $\Delta toxA$ samples from library batch 1 had shorter fragment sizes than the
317 KW20 samples from the same batch, and that the difference increased as the time after competence induction
318 increased. This supports the hypothesis that the extreme lack of competence in $\Delta toxA$ cultures is due to non-specific
319 ToxT cleavage of mRNAs.

320 Additional support for a generally toxic effect on translation comes from the *toxA* deletion's effects on genes known
321 to rescue ribosomes that have stalled on non-stop mRNAs. *H. influenzae* has two rescue systems: In the first, an
322 abundant small RNA named transfer-messenger RNA (tmRNA), binds with its protein cofactor SmbB to arrested
323 ribosomes, detaches both the non-stop mRNA and the incomplete protein, and tags the protein for degradation. In
324 the second rescue system, ArfA recruits ribosome release factor 2 (HI1212) to the ribosome and causes it to cleave
325 the nascent peptidyl-tRNA (Keiler, 2015). Translation of *arfA* is increased when tmRNA activity is reduced (Garza-
326 Sanchez *et al.*, 2011). Consistent with these expectations, tmRNA (HI1281.2) is downregulated in the $\Delta toxA$ mutant,
327 however, as noted above, the *arfA* homolog HI0235 is upregulated several fold (Christensen *et al.* 2003a, 2003b).

328 **Sxy regulation of TA systems:** Jaskolska and Gerdes (2015) and Sinha *et al.*, (2009) reported that three other *E. coli*
329 TA operons are regulated by Sxy, so we examined the promoter sequences and expression levels of the other seven
330 *H. influenzae* TA operons. None of the promoters had strong matches to the *H. influenzae* CRP-S consensus
331 (Cameron and Redfield, 2008, Sinha *et al.*, 2009) and their RNA abundance levels showed no evidence of
332 competence-regulated expression or dependence on Sxy.

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333 **Phylogenetic evidence for lateral transfer of the *toxTA*:** Since toxin/antitoxin operons are highly mobile (Makarova
334 *et al.*, 2009), we examined the distribution of the *toxTA* operon in other strains and species (Fig. 9). *toxTA* operons
335 are present at the same genomic location in most *H. influenzae* genomes (see below) and in the closely related *H.*
336 *haemolyticus*, but there are no recognizable homologs in most other bacteria (including most other members of the
337 Pasteurellaceae). Instead, most identifiable homologs (with about 60% identity) are in a very distant group, the
338 Firmicutes, especially *Streptococcus* (96 of the top 100 BLAST hits to ToxT outside the Pasteurellaceae are to diverse
339 *Streptococcus* species). This suggests that the *toxTA* operon may have been transferred from a Firmicute into a
340 recent ancestor of *H. influenzae* and *H. haemolyticus*. When we excluded *Streptococcus* spp. from the BLAST search,
341 sporadic matches were found in a wide variety of other taxa. In addition, *toxTA* operons with about 50% identity
342 were found in one other small Pasteurellacean clade (*Actinobacillus sensu stricto*), and on two 11kb plasmids
343 (pRGRH1858 and pRGFK1025) from an uncultured member of a rat gut microbiome and an uncultivated *Selenomonas*
344 sp. The distribution is summarized in Fig. 9A.

345 To resolve the history of gene transfer events in the two Pasteurellaceae sub-clades, we created an unrooted
346 maximum likelihood phylogeny of concatenated *toxT* and *toxA* homologs from selected species where both genes are
347 present (Fig. 9B). Although there is 99% bootstrap support for a *Haemophilus-Actinobacillus* clade, the absence of
348 homologs from all other Pasteurellaceae makes a single Pasteurellacean origin unlikely, since it would require there
349 to have been multiple deletions in other Pasteurellacean subclades, or a second lateral transfer. Since the
350 *Actinobacillus* sequences are also more distant from the *Haemophilus* sequences than from the *Streptococcus*
351 sequences, the two Pasteurellacean groups may instead have acquired their *toxTA* operons by independent lateral
352 transfers, probably from Firmicutes, since these homologs have the highest identity to the Pasteurellacean
353 sequences. The alternative hypothesis of a single Pasteurellacean origin requires that acquisition was followed by
354 multiple deletions, though the analysis in the next paragraph makes this less implausible.

355 **Deletions in *H. influenzae toxT* are common:** 181 *H. influenzae* genome sequences were available for examination.
356 (Supp table #???) Of these, 162 had recognizable *toxA* sequences. All of these encoded full length ToxA proteins, but
357 all except 24 had one of two common deletions affecting *toxT*. The extents of these deletions are shown by the dark

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358 grey bars at the bottom of Fig. 10. The most common deletion (n=93) removed 178 bp of *toxT* coding sequence but
359 left both promoters intact. The second (n=45) removed 306 bp of sequence including both *toxTA* promoters and the
360 *toxT* start codon. The 19 genomes that lacked recognizable *toxA* sequences all had the same 1015 bp deletion
361 removing both *toxT* and *toxA* but leaving the flanking genes intact. In place of the missing sequences were 87 bp that
362 have no homologs in GenBank. The average pairwise distance among the 162 *toxA* genes is 0.106, which is slightly
363 higher than 0.088, the average of all genes with at most one copy per strain. The d_N/d_S ratio of 0.037 is consistent
364 with mild purifying selection on *toxA* and is higher than the average gene, which is 0.243. However, the strength of
365 selection may be underestimated, since most *toxAs* lack functional *toxT* and/or may not be expressed due to
366 deletions. Both sequence divergence and the high frequency of *toxT* deletions agree with expectations for a
367 toxin/antitoxin system whose antitoxin protects against a toxin that is at least mildly deleterious.

368 As mentioned above, the *E. coli* HicA and HicB protein sequences have little sequence similarity to ToxT and ToxA,
369 and our wildtype *H. influenzae* strain (KW20, Rd) lacks a *hicAB* operon, but Syed and Gilsdorf (2007) found that 69/79
370 other *H. influenzae* strains were positive for *hicAB* by dot-blot analysis, so we examined the *hicAB* genes in our set of
371 181 *H. influenzae* genomes. Like *toxTA*, the *hicAB* operons in most *H. influenzae* strains have intact antitoxin (*hicB*)
372 genes but deletions in their toxin (*hicA*) genes. Of the 181 strains examined, 122 were tagged as having *hicB*. All but
373 20 of these have a 250 bp deletion that removes both *hicAB* promoters and the first 50 bp of *hicA*. Many strains that
374 lack *hicAB* share a large deletion that removes a large multi-gene 7147 bp segment flanked by a 57 bp duplication,
375 but others have more complex structures that were not investigated further. Overall, the deletion pattern of the *H.*
376 *influenzae* *hicAB* genes resembles that of *toxTA*, with frequent deletions of the toxin gene but preservation of the
377 antitoxin.

378 **Might the variation in *toxTA* help explain the observed strain-specific variations in DNA uptake and**
379 **transformation?** Maughan and Redfield (2009) measured the ability of 34 *H. influenzae* strains to both take up DNA
380 and become transformed, so we examined this data for correlations with the presence of *toxTA* in the 19 of these
381 strains whose *toxTA* and *hicAB* genotypes we were able to determine. All but one of the 19 strains had a complete
382 *toxA* coding sequence but only five had intact *toxTA* operons. Of the rest, four had the large deletion that removed

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383 both *toxTA* promoters, nine had the smaller deletion internal to *toxT*, and one had the 1015 bp complete deletion.
384 There was no obvious correlation between the *toxTA* genotypes and the DNA uptake or transformation phenotypes,
385 but there was insufficient data for a high powered analysis.

386 **Does the *Actinobacillus pleuropneumoniae* *toxTA* operon affect competence?** The *A. pleuropneumoniae* *toxTA*
387 operon was originally reported to have the CRP-S promoter typical of competence operons (Bosse *et al.*, 2009).
388 Although reexamination of the promoter region failed to identify a high-quality CRP-S site, we constructed *toxT*, *toxA*
389 and *toxTA* knockout mutants to investigate whether a *toxA* deletion would prevent competence. There were no
390 significant differences between the transformation frequencies of wildtype cells and all *toxTA* mutants. Thus we
391 conclude that the *A. pleuropneumoniae* *toxTA* operon does not affect competence. Expression of the *toxT* gene in
392 the absence of the antitoxin had no detectable effect on growth or survival.

393

394 **DISCUSSION**

395 Our investigation into why a HI0659 knockout prevents competence has provided a simple answer: HI0659 encodes
396 an antitoxin (ToxA) needed to block the expression and competence-preventing activity of the toxin encoded by
397 HI0660 (ToxT). But this answer has generated a number of new questions that we have only partially answered.
398 Why is competence controlled by a toxin/antitoxin system? How does this system completely abolish DNA uptake
399 and transformation without causing significant cell death? How did this TA system come to be competence-
400 regulated? Does it confer any benefit to the cells, either generally or competence-specific?

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

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406 **How did the *toxTA* operon come to be in the *H. influenzae* genome and under competence regulation?** *H.*
407 *influenzae* acquired its *toxTA* operon by horizontal transfer, either into a deep ancestor of the Pasteurellaceae or
408 independently into more recent ancestors of *H. influenzae* and *A. pleuropneumoniae*. The closest relatives of the
409 *toxTA* genes are in the distantly related Firmicutes, with homologs especially common in *Streptococcus* species. Since
410 the Streptococci and Pasteurellaceae share both natural competence and respiratory-tract niches in many mammals,
411 there may have been frequent opportunities for horizontal transfer between them.

412 We do not know how the *toxTA* operon came to be under CRP-S regulation. The *toxTA* operon's strong regulatory
413 parallels with the *E. coli hicAB* system suggest two explanations. One hypothesis is a distant shared evolutionary
414 origin of the two systems, with selection maintaining regulation more strongly than protein sequence. Based on this
415 hypothesis, the strong sequence similarity between the Pasteurellacean and Streptococcal *toxTA* systems then
416 predicts that the regulatory features shared by the Pasteurellacean *toxTA* systems and the more-distant *hicAB*
417 system (a competence-regulated promoter producing both proteins and an antitoxin-regulated promoter producing
418 only antitoxin) could also be shared by the Streptococcal homologs. However, it is also possible that toxin-antitoxin
419 systems with similar regulation and function have adopted similar roles in separate instances, a phenomenon which
420 is more likely in toxin antitoxin systems as they undergo frequent horizontal transfers and are often under strong
421 selective pressure. The *sxy* gene and the CRP-S promoters it regulates are not known outside of the Gamma-
422 Proteobacteria sub-clade that contains the Vibrionaceae, Enterobacteraceae, Pasteurellaceae and Orbaceae
423 (Cameron *et al.*, 2006). Thus, it would be interesting to examine the regulation and function of the *toxTA* homologs
424 outside the Pasteurellaceae to determine when and where it adopted a regulatory role and the mechanism of the
425 toxic activity. Examining these homologs could give insight into both the mechanism of action of the *H. influenzae*
426 *toxTA* system, and its evolutionary history.

427 **How does unopposed ToxT prevent DNA uptake and transformation?** The transformation defect caused by deletion
428 of the antitoxin gene *toxA* is very severe, so it was surprising that RNA-seq analysis detected only few and minor
429 changes in expression of competence genes. Instead, the best explanation is that ToxT is an mRNA-cleaving
430 ribonuclease, whose activity causes a general block to translation that prevents functioning of the induced

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431 competence genes. The most direct evidence is the decrease in insert size distributions seen in $\Delta toxA$ mutants, but
432 this conclusion is also supported by the combination of regulatory similarities between the *toxTA* and *hicAB* systems
433 and by sequence similarities between the ToxT protein and Type II ribonuclease toxins.

434 **Why then does the $\Delta toxA$ mutant not suffer from growth arrest or toxicity?** Part of the explanation is that mRNAs
435 encoding functional ToxT are only expressed after cells have been transferred to competence-inducing starvation
436 medium, a condition that severely slows cell growth and division even in wildtype cells. Detecting the predicted
437 competence-specific toxicity is further complicated by the uneven distribution of transformability in competence-
438 induced cells. Co-transformation experiments using multiple unlinked markers consistently show that no more than
439 half, and sometimes as little as 10%, of the cells in a MIV-treated culture produce recombinants (Mell and Redfield,
440 2014). We do not know whether only the transforming cells express the competence genes or all cells express them
441 but some fail to correctly assemble the DNA uptake or recombination machinery. If only a modest fraction of the
442 cells in a competent culture are expressing the toxin then any toxic effect on culture growth and survival will be more
443 difficult to detect.

444 **Does this operon confer any benefit (or harm) on *H. influenzae*?** Why have a competence-regulating toxin/antitoxin
445 system at all, when it has no detectable effect on competence unless its antitoxin component is defective?

446 Regulatory parallels with the *hicAB* system suggest that CRP-S regulation is not incidental. We found no direct
447 evidence of any toxin-dependent alteration to the normal development of competence. Production of Sxy is subject
448 to post-transcriptional regulation by the availability of nucleotide precursors (Macfadyen *et al.*, 2001, Sinha *et al.*,
449 2013), and we have elsewhere proposed that DNA uptake is an adaptation to obtain nucleotides when nucleotide
450 scarcity threatens to arrest DNA replication forks (Mell and Redfield, 2014). In this context, competence-induction of
451 the *toxTA* operon may be a specialization to help cells survive, by slowing or arresting protein synthesis until the
452 nucleotide supply is restored.

453 However, the high frequency of deletions that remove either complete *toxTA* or both promoters (35%) indicates that
454 the operon is dispensable. And the even higher frequency of toxin-inactivating deletions in the presence of intact

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455 antitoxin genes and CRP-S promoter (51%), coupled with the absence of any deletion that inactivates antitoxin but
456 preserves toxin indicates that unopposed toxin is indeed harmful under some natural circumstances.

457 We have examined the *toxTA* operon from many angles and answered our initial question of why *toxA* knockout
458 prevents competence in *H. influenzae*, but have raised new questions whose eventual answers we hope will give us
459 greater insight not just into the *toxTA* system, but competence regulation in general.

460

461 **METHODS**

462 **Bacterial strains, plasmids, and growth conditions**

463 Bacterial strains used in this work are listed in Supp Table 1. *Escherichia coli* strain DH5 α [F80lacZ #(lacIZYA-argF
464 endA1)] was used for all cloning steps; it was cultured in Luria-Bertani (LB) medium at 37°C and was made competent
465 with rubidium chloride according to the method provided in the QIAexpressionist manual protocol 2 (Qiagen). When
466 antibiotic selection was required, 100 μ g/mL ampicillin and 50 μ g/mL spectinomycin were used.

467 *Haemophilus influenzae* cells were grown in sBHI medium (Brain Heart Infusion medium supplemented with
468 10mg/mL hemin and 2mg/mL NAD) at 37°C in a shaking water bath (liquid cultures) or incubator (plates). *H.*
469 *influenzae* strain Rd KW20 (Alexander and Leidy 1951), the standard laboratory strain, was used as the wild type for
470 all experiments. Mutant strains used in this study were marked deletion mutants in which the coding region of the
471 gene was replaced by a spectinomycin resistance cassette, as well as unmarked deletion mutants derived from these
472 strains; the generation of these mutant strains is described in Sinha et al. (2012). Specifically, we used an unmarked
473 deletion of HI0659 (HI0659-), marked and unmarked deletions of HI0660 (HI0660::spec, HI0660-), and a marked
474 deletion of the whole operon (HI0659/HI0660::spec). Knockout mutants of *crp* and *sxy* have been described
475 previously (Chandler, 1992, Williams et al., 1994)

476 *Actinobacillus pleuropneumoniae* cells were grown in BHI-N medium (Brain Heart Infusion medium supplemented
477 with 100 μ g/mL NAD) at 37°C. *A. pleuropneumoniae* strain HS143 (Blackall et al. 2002) was used as the wild type for

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478 all experiments. Marked deletion mutants in which the gene of interest was replaced by a spectinomycin resistance
479 cassette strains were generated for this study as described below. The HS143 genome region containing the
480 homologs of the *Actinobacillus pleuropneumoniae* serovar 5b strain L20 APL_1357 and APL_1358 genes, plus
481 approximately 1 kb of flanking sequence on each side, was PCR-amplified, ligated into Promega pGEM-T Easy and
482 transformed into *E. coli*. Plasmid regions containing APL_1357, APL_1358, or both genes were deleted from the
483 pGEM-based plasmid by inverse PCR, and the amplified fragments were blunt-end ligated to the spectinomycin
484 resistance cassette (Tracy *et al.*, 2008) from genomic DNA of a *H. influenzae* comN::spec strain (Sinha *et al.*, 2012).
485 Plasmids linearized with Scal were transformed into competent *A. pleuropneumoniae* HS143 and transformants were
486 selected for spectinomycin resistance using 100µg/mL spectinomycin after 80 minutes of growth in nonselective
487 medium.

488 **Generation of competent stocks**

489 To induce competence, *H. influenzae* and *A. pleuropneumoniae* were cultured in sBHI or BHI-N respectively and
490 transferred to competence-inducing medium MIV (Herriot *et al.* 1970) when they reached an optical density at
491 600nm (OD₆₀₀) of approximately 0.25 (Poje and Redfield 2003). After incubation with gentle shaking at 37°C for a
492 further 100 min (*H. influenzae*) or 150 min (*A. pleuropneumoniae*), cells were transformed or frozen in 16% glycerol
493 at -80 °C for later use.

494 **Transformation assays**

495 **Transformation of MIV-competent cells:** Transformation assays were carried out as described by Poje and Redfield
496 (2003). MIV-competent *H. influenzae* or *A. pleuropneumoniae* cells were incubated at 37°C for 15 minutes with
497 1µg/ml DNA, then DNaseI (10µg/mL) was added and cultures were incubated for 5 minutes to ensure no DNA
498 remained in the medium. *H. influenzae* cultures were transformed with MAP7 genomic DNA (Barcak *et al.* 1991),
499 which carries resistance genes for multiple antibiotics, while *A. pleuropneumoniae* cultures were transformed with
500 genomic DNA from an *A. pleuropneumoniae* strain with spontaneous nalidixic acid resistance (generated in this lab).
501 Cultures were diluted and plated on both plain and antibiotic-containing plates (2.5µg/mL novobiocin for *H.*

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502 *influenzae* cultures, 20ug/mL nalidixic acid for *A. pleuropneumoniae* cultures) and transformation frequencies were
503 calculated as the ratio of transformed (antibiotic-resistant) cells to total cells. For *A. pleuropneumoniae*, transformed
504 cells were given 80 minutes of expression time in BHI-N before plating.

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

516 **Competence growth and survival time course:** Cells were grown in sBHI to a density of ~2x10⁸ cfu/ml (OD₆₀₀ =
517 0.075) and transferred to MIV. After 100 min (time for maximum competence development, an aliquot of each
518 culture was diluted 1/10 into fresh sBHI for recovery and return to normal growth. A fraction of each culture was
519 incubated in a shaking water bath, and aliquots of the initial and 'recovery' sBHI cultures were also grown and
520 monitored in a Bioscreen incubator.

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

524 **Phylogenetic Analysis:** A nucleotide BLAST search (discontinuous MEGABLAST) and a protein BLAST search against
525 translated nucleotide databases (tBLASTn) were used to identify homologs of the HI0659 and HI0660 genes (Altshul

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526 *et al.* 1990). Protein sequences found by the tBLASTn search were retained for analysis if they showed greater than
527 60% coverage and greater than 40% identity to the *H. influenzae* query sequence. For species with matching
528 sequences in multiple strains, the sequence from only one strain was kept.

529 For species in which homologs of HI0659 and HI0660 were found next to one another, amino acid sequences of
530 concatenated matrices were aligned by multiple-sequence alignment using MAFFT, version 7.220 (Kato, 2013), run
531 from modules within Mesquite version 3.02 (Maddison and Maddison 2015). The L-INS-I alignment method was used
532 due to its superior accuracy for small numbers of sequences. After inspection of the alignments, poorly-aligning
533 sequences were removed from the analysis, and alignment was repeated.

534 Phylogenetic trees were generated using the RAxML (Stamatakis 2014) maximum likelihood tree inference program,
535 run via the Zephyr package of Mesquite. For each gene, 50 search replicates were conducted, using the
536 PROTGAMMAAUTO option to allow RAxML to automatically select the best protein evolution model to fit the data.
537 Since these trees were found to correspond exactly to a set of trees generated using the PROTGAMMAJTT model,
538 this faster model was used to generate a majority-rules consensus tree from 1000 bootstrap replicates for each gene.

539 **Analysis of natural deletions:** 181 publicly available *H. influenzae* genomes were downloaded from NCBI and the
540 Sanger centre. (Supp table #???) Genomes were re-annotated using Prokka v1.11 (Seemann, 2014), and the
541 pangenome was calculated using Roary v3.5.1 (Page *et al.*, 2015) with a minimum blastp threshold of 75. The *toxA*
542 gene cluster in the pangenome was identified by finding the gene cluster that contained the *toxA* gene from Rd
543 KW20, and the *hicA* cluster was identified by finding the gene cluster that contained the *hicA* gene from PittAA. 2300
544 bp genome sequences centered on *toxA* and/or *hicA* were extracted from all *H. influenzae* genomes containing
545 recognizable *toxA* and/or *hicB* genes, and aligned by the MAFFT server. For strains that lacked recognizable *toxA* or
546 *hicB*, sequences adjacent to the genes that normally flanked each operon were extracted. K_a/K_s and pairwise distance
547 were calculated for each gene using SeqinR v 3.4-5 (Charif and Lobry, 2007) with codon aware gene alignments were
548 made using Prank (v.100802).

549 **RNA-seq analysis:**

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550 **Sample Preparation:** Cell cultures of *H. influenzae* strain Rd, Δcrp and Δsxy derivatives, and $\Delta toxTA$ mutants were
551 grown in sBHI to an OD₆₀₀ of 0.2 – 0.25, then transferred to MIV. Aliquots of cells were removed just prior to transfer
552 to MIV, and after 10, 30, and 100 minutes in MIV, and immediately mixed with Qiagen RNAprotect (#76526) to
553 stabilize RNA. Cells were pelleted and frozen, and RNA was later extracted from thawed pellets using the Qiagen
554 RNeasy Min-elute Cleanup Kit (#74204). Contaminating DNA was removed with Ambion Turbo DNase (#AM2238),
555 and ribosomal RNA was depleted using the Illumina Ribo-Zero rRNA Removal kit (#MRZMB126). Sequencing libraries
556 were prepared using TruSeq mRNA v2 library preparation kit, according to manufacturer’s instructions (Illumina).
557 Libraries were pooled and sequenced on a HiSeq 2500, generating paired-end 100 bp reads.

558 **Data Analysis Pipeline:** FASTQ files were analysed using the FASTQC tool (Andrews, 2015) to confirm read quality.
559 Reads were aligned to the *H. influenzae* Rd KW20 reference genome sequence using the Burrows-Wheeler Alignment
560 tool (BWA) algorithm `bwa mem` (Li and Durbin, 2009). Differential expression analysis was performed using the
561 DESeq2 package, v.1.6.3 (Love *et al.*, 2013). Specifically, the function `DESeqDataSetFromMatrix()` was used to
562 generate a dataset to compare reads from each mutant strain reads from the wild-type control based on their strain,
563 sample time point, and the interaction between the two parameters. The function `DESeq()` was called to
564 determine which genes were differentially expressed based on these parameters, using p-values adjusted for a B-H
565 false-discovery rate (Benjamini and Hochberg, 1995) of 0.1 as a cut-off to determine significance, after normalizing
566 total read counts and variances.

567
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573

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574

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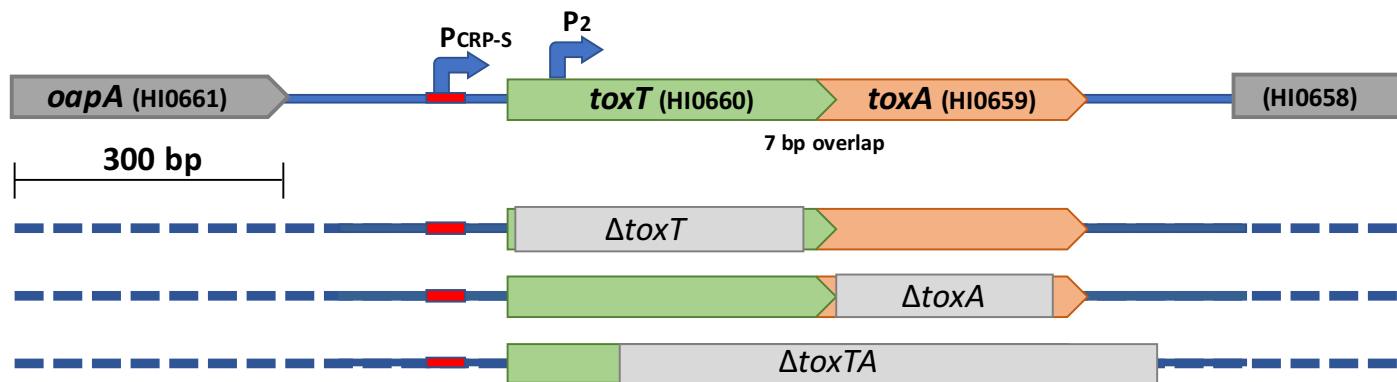
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681

682 **FIGURE LEGENDS**

683 Figure 1: **Structure of wildtype and mutant *toxTA* genes.** Top line: structure of the wildtype *toxTA* operon in strain
 684 KW20. Lower lines: light grey bars indicate segments deleted in $\Delta toxT$, $\Delta toxA$, and $\Delta toxTA$ mutants.

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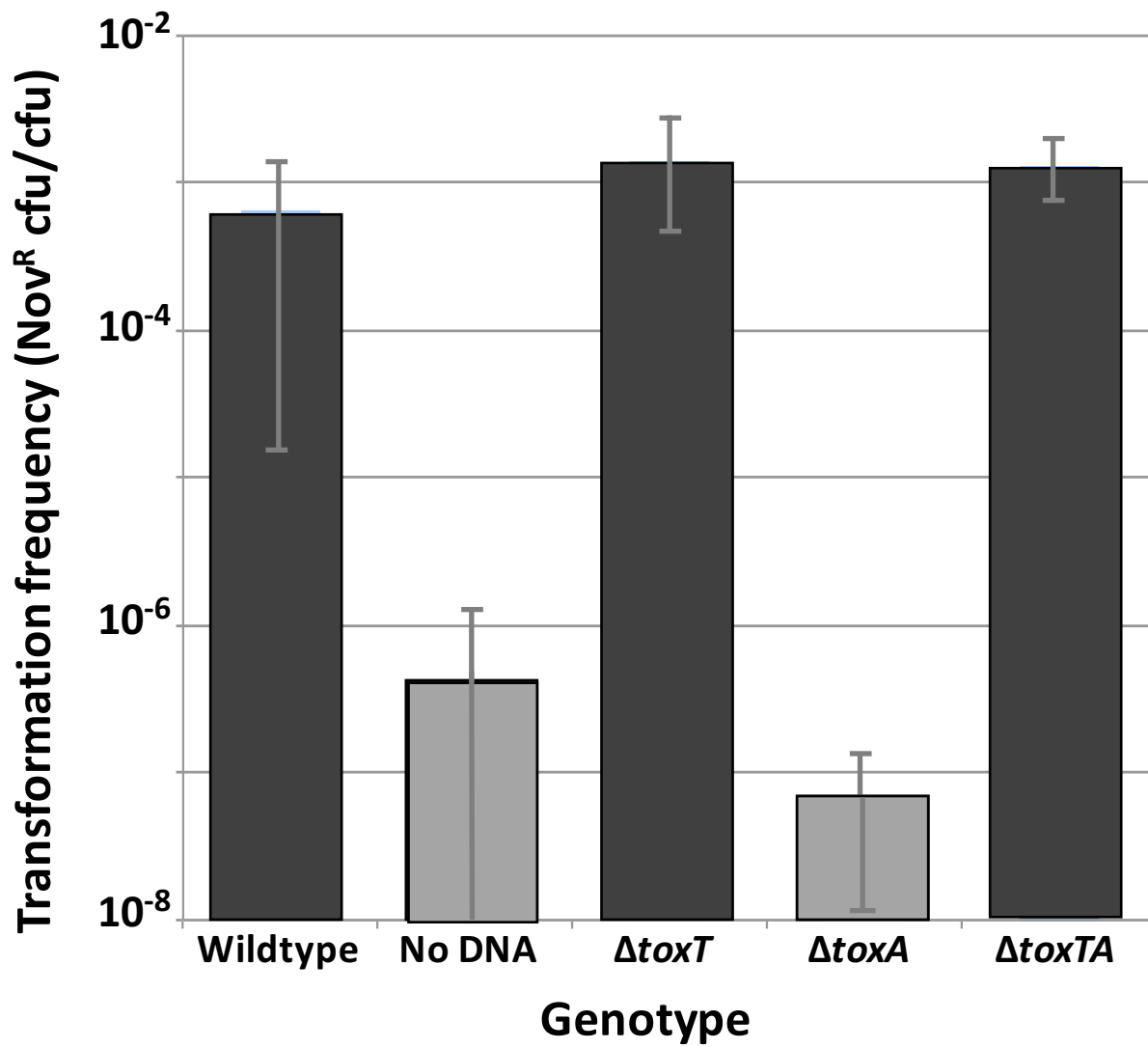


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688 Figure 2: Transformation phenotypes of wildtype cells and *toxTA* mutants. Bars represent the means of at least
689 three biological replicates, with error bars representing one standard deviation. Grey bars indicate values below the
690 detection limit.

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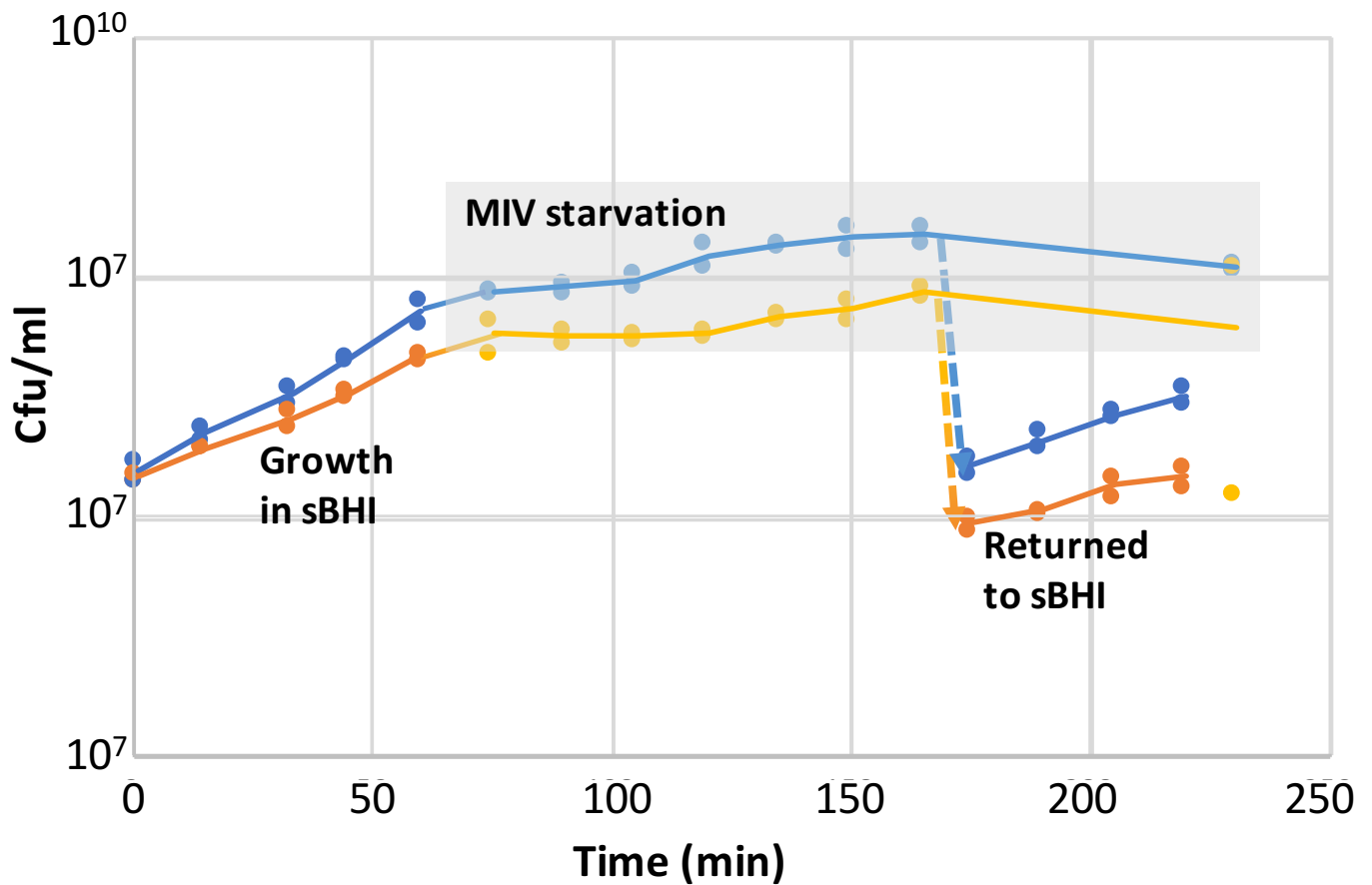
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695 Figure 3: **Growth and MIV recovery of log-phase KW20 and Δ toxA.** Log-phase cells in sBHI were transferred to MIV
696 at t=65 min; a portion of each MIV culture was diluted 10-fold into sBHI at t=170 min. The grey-shaded area indicates
697 samples taken from MIV cultures. Blue: KW20, orange: Δ toxA.

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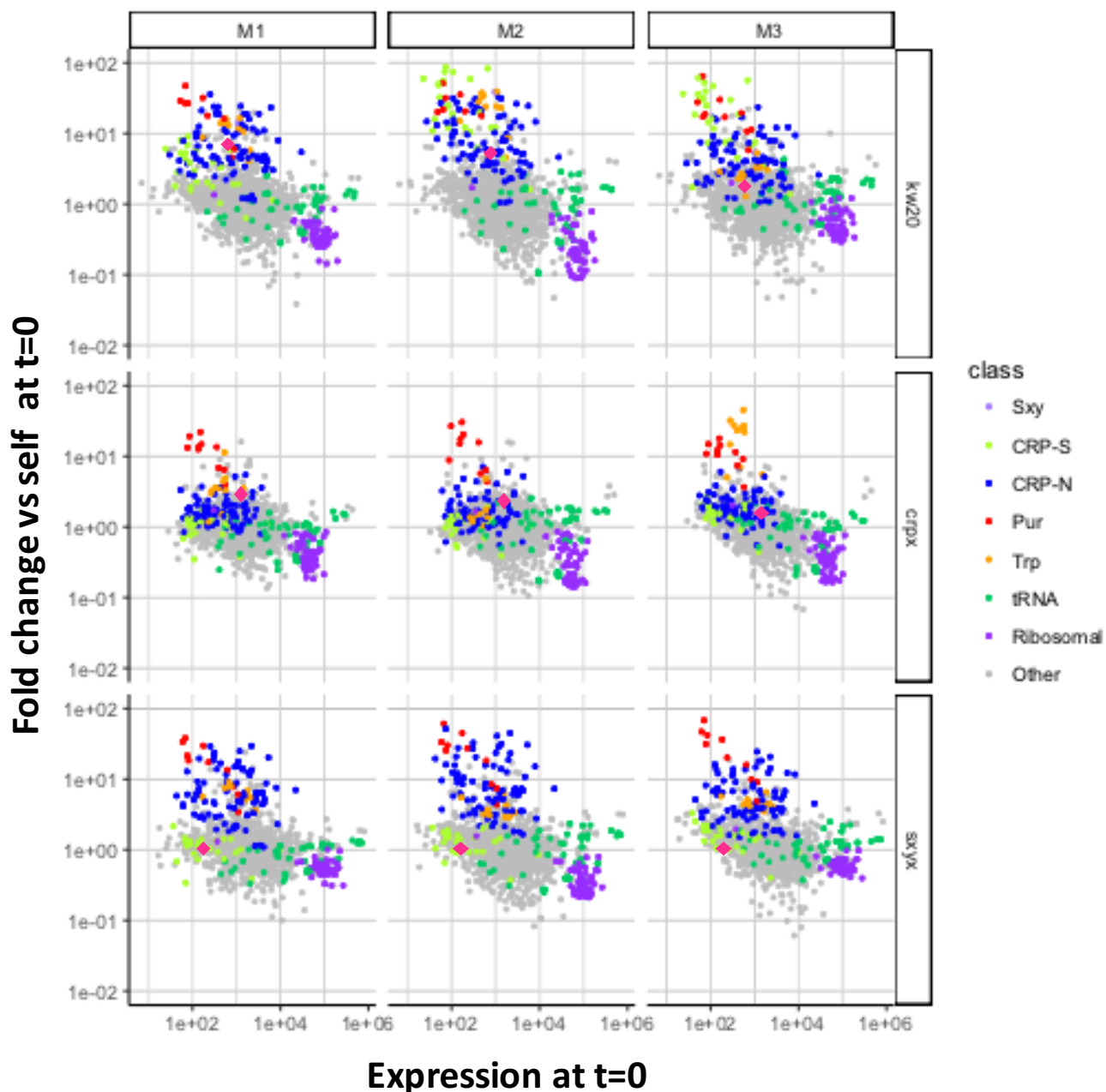
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703 Figure 4: **Changes in expression of genes regulated by CRP and Sxy.** Each circle represents a gene, colour-coded by
 704 function: red: *sxy*, green, CRP-N-regulated; blue, CRP-S-regulated; yellow, ribosomal; purple, purine synthesis;
 705 orange, tryptophan synthesis; grey, other or unknown function. Each circle's horizontal position indicates the gene's
 706 level of expression in rich medium (T=0) and its vertical position indicates how this expression changed at later time
 707 points (**A**: T=10; **B**: T=30; **C**: T=100) or in a mutant background at T=30 (**D**; Δcrp ; **E**: Δsxy).



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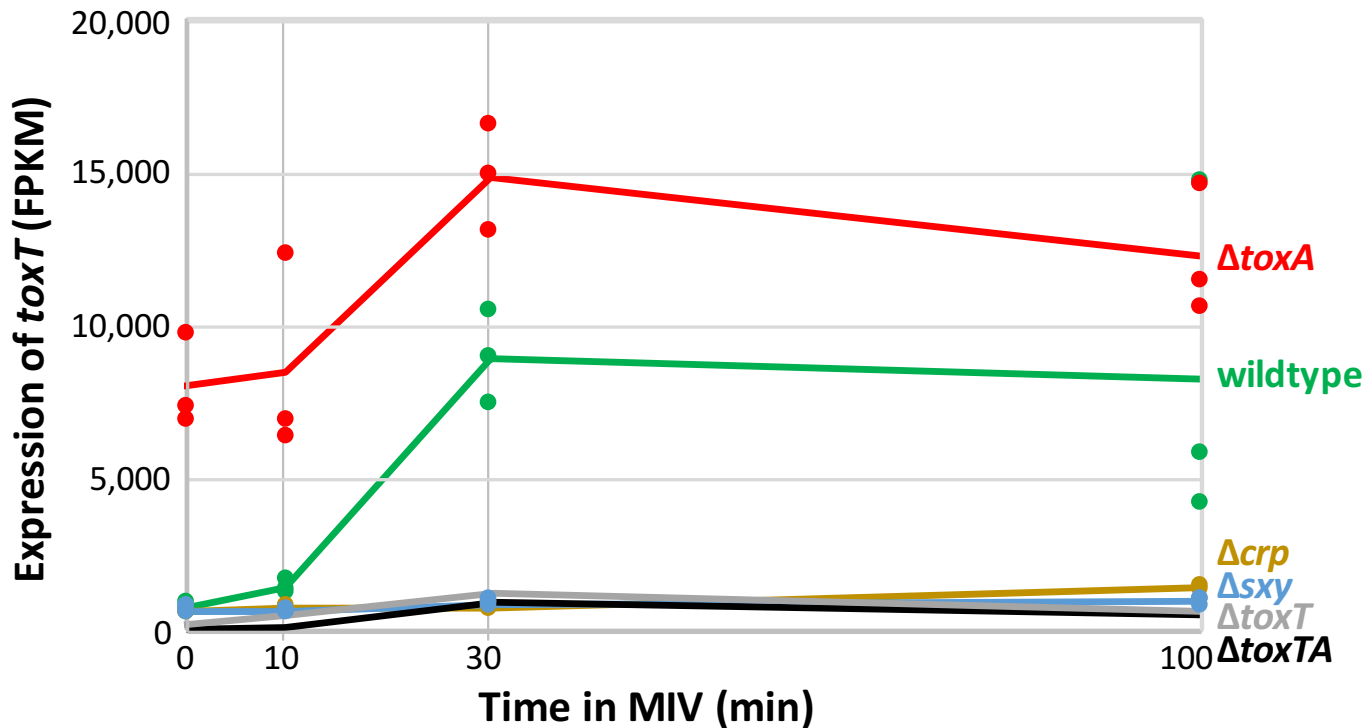
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711 Figure 5: **Competence-induced expression of *toxT***. Sample FPKM values (dots) and means (lines) for *toxT* (HI0660).

712 Strains: wildtype: green; Δcrp : brown; Δsxy : blue; $\Delta toxA$: red; $\Delta toxT$: grey; $\Delta toxTA$: black. The values for the $\Delta toxT$ and

713 $\Delta toxTA$ samples are underestimates because most of the gene has been deleted in these strains.

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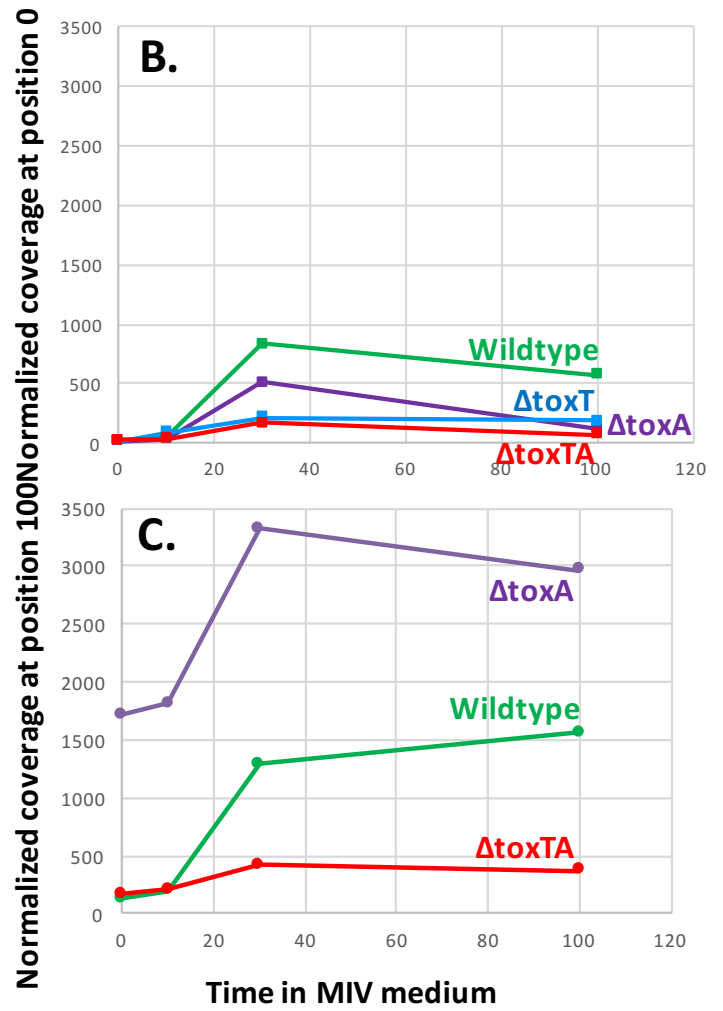
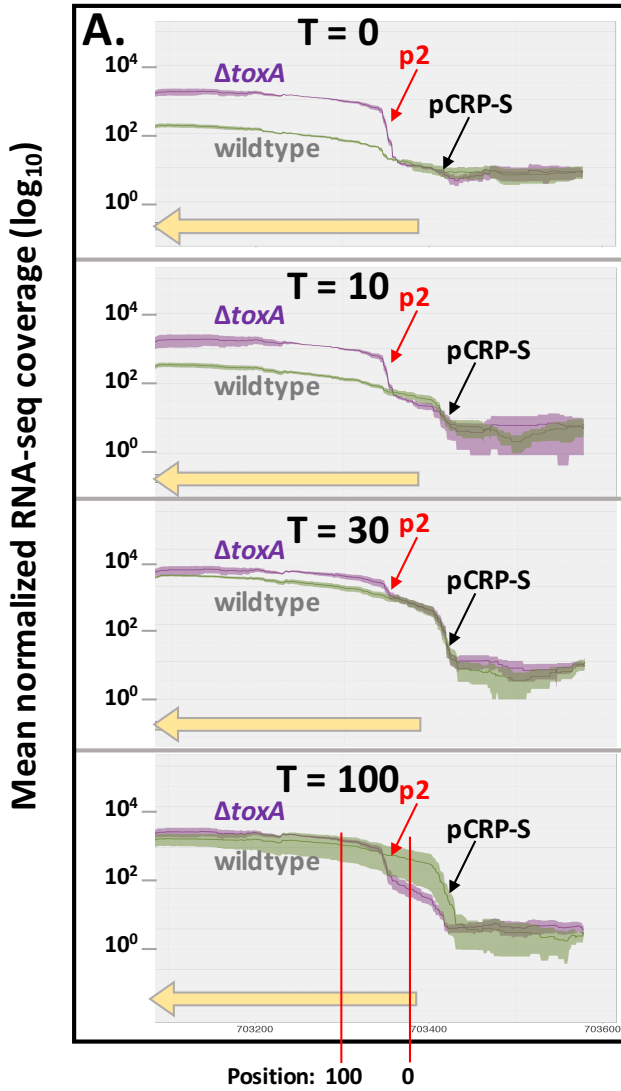


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717 Figure 6: Read coverage of the *toxTA* promoter region. **A.** The green (KW20) and purple (Δ *toxA*) lines indicate mean
 718 normalized coverage at each position, shaded areas indicate standard errors. The yellow bar indicates the 5' half of
 719 *toxT*. **B.** and **C.** Time course of normalized read coverage at two specific positions in the *toxTA* operon. **B.** Position 0 =
 720 *toxA* start codon. **C.** Position 100.

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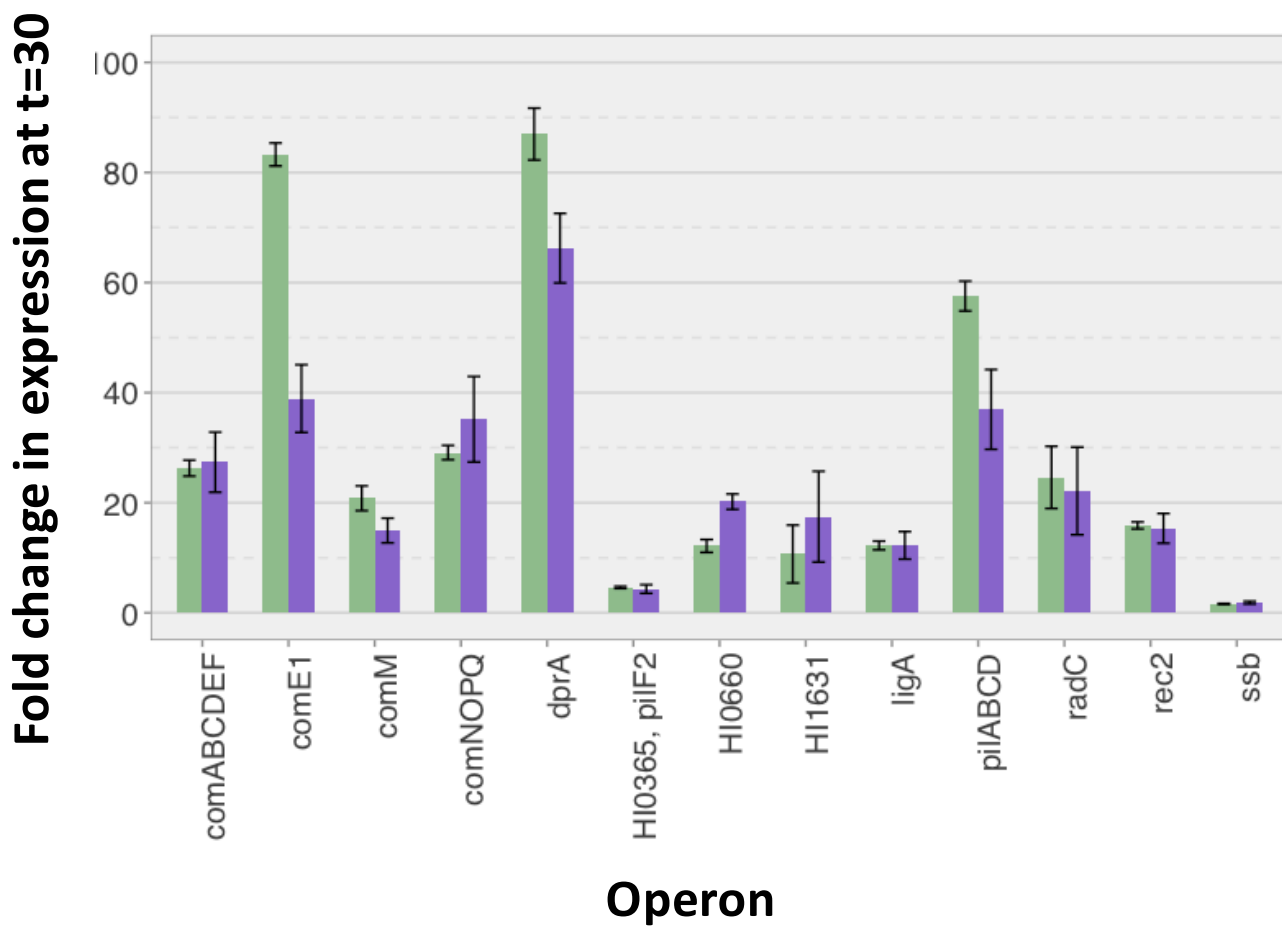


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724 Figure 7: Changes in competence operon expression levels after 30min in MIV. Fold changes in competence operon
725 expression levels in KW20 (green) and $\Delta toxA$ (purple) after 30 min in MIV, compared to 0 minute samples. Black lines
726 show standard errors.

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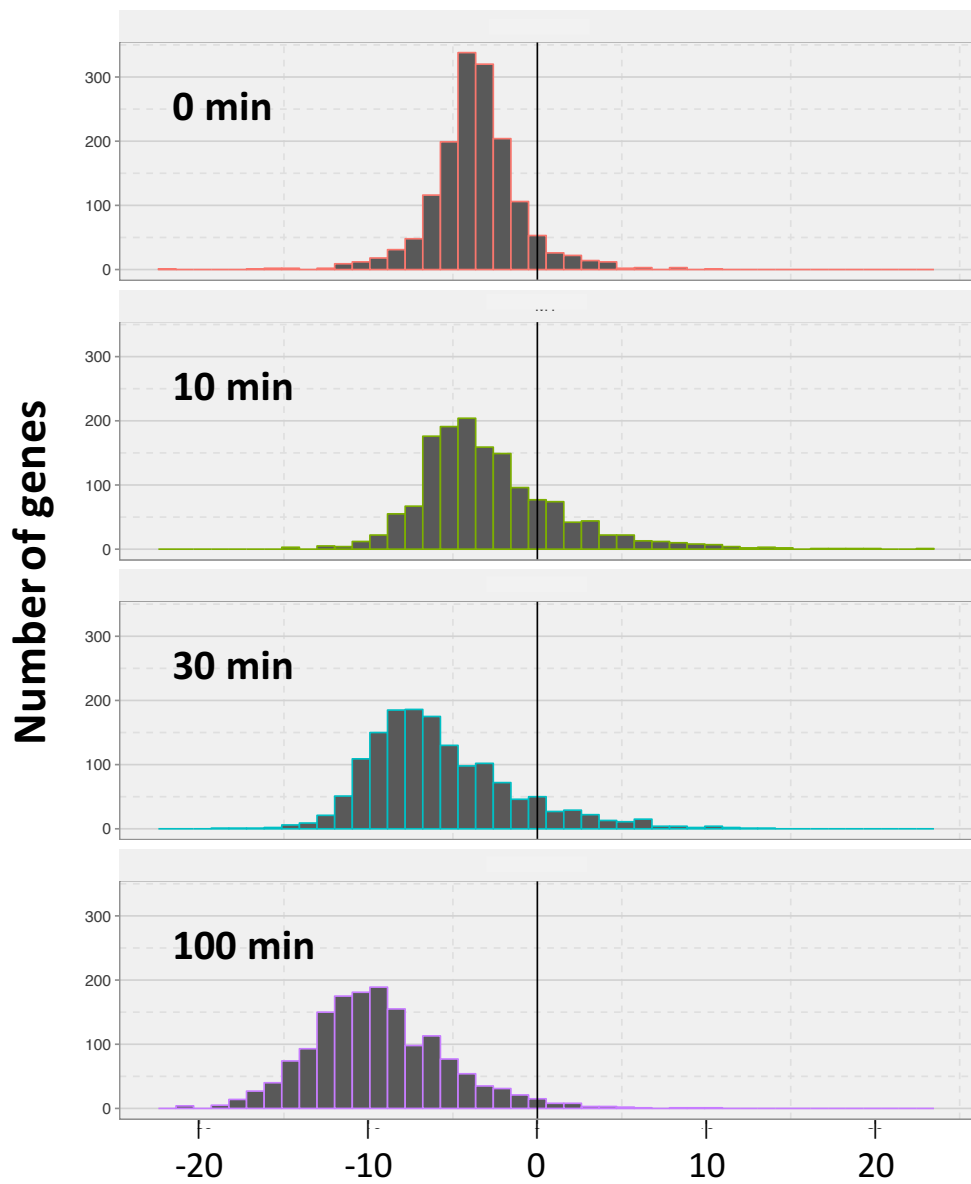


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730 Figure 8: **Distribution of insert-size differences between RNA-seq libraries prepared at the same time.** Distribution
731 of insert length differences between KW20 (kw20_A and kw20_B samples) and $\Delta toxA$ (antx_A samples) after 0, 10,
732 30 and 100 minutes in MIV.

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Insert size difference between $\Delta toxA$ and KW20 (bp)

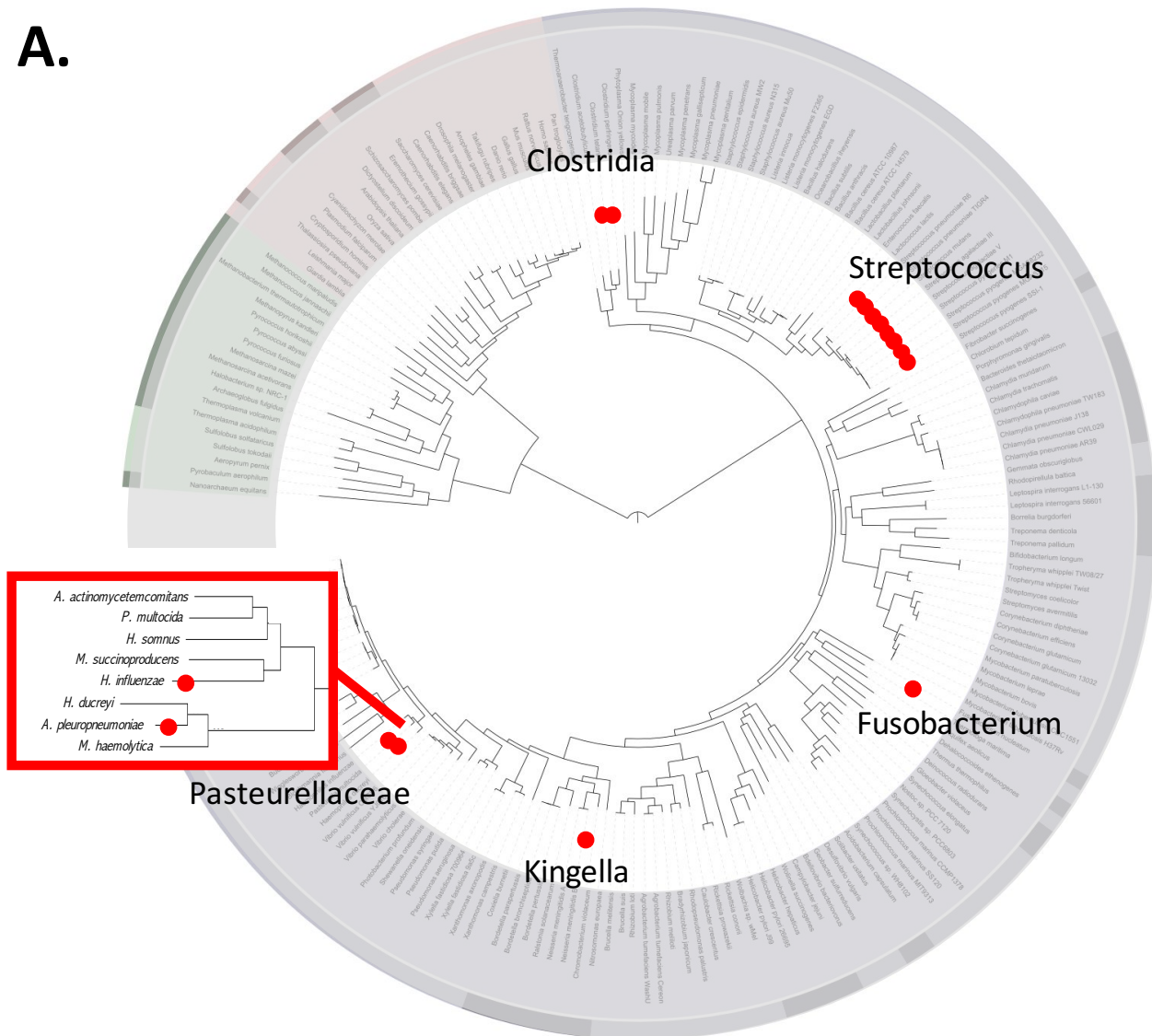
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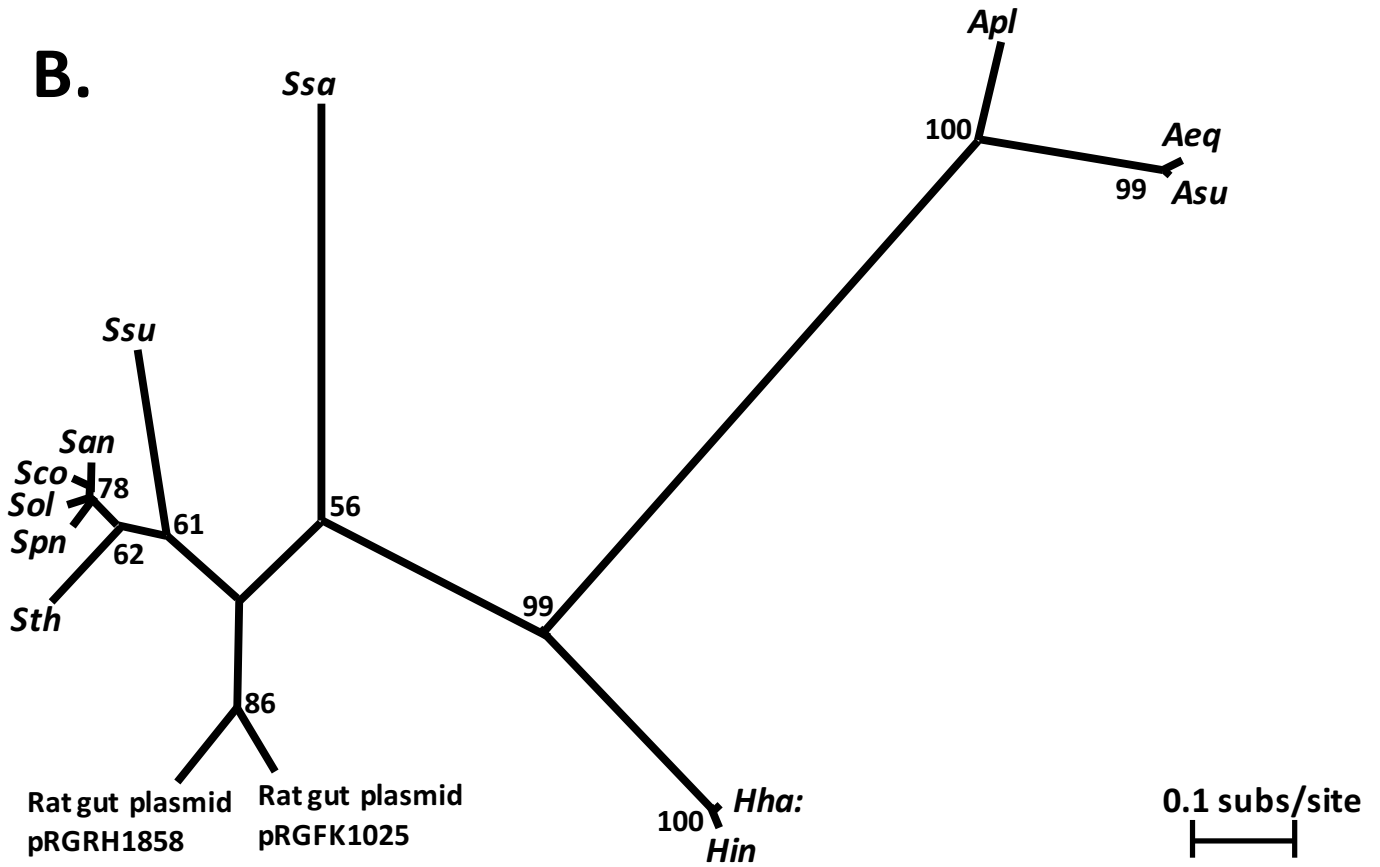
737 Figure 9: **Distribution of *toxTA* homologs in bacterial genomes.** **A.** Red dots indicate one or more taxa containing
 738 homologs of both ToxT and ToxA. Bacterial phylogeny image from Wikimedia Commons (Letunic 2007). Inset:
 739 Pasteurellacean phylogeny from Redfield *et al.* 2006. **B.** Unrooted maximum likelihood phylogeny of concatenated
 740 *toxT* and *toxA* homologs from selected species where both are present. Numbers at nodes are bootstrap values.
 741 Species abbreviations: *Apl*: *Actinobacillus pleuropneumoniae*; *Aeq*: *A. equuli*; *Asu*: *A. suis*; *Haemophilus haemolyticus*;
 742 *Hin*: *H. influenzae*; *Ssa*: *Streptococcus salivarius*; *Ssu*: *S. suis*; *San*: *S. anginosus*; *Sco*: *S. constellatus*; *Sol*: *S.*
 743 *oligofermentans*; *Spn*: *S. pneumoniae*; *Sth*: *S. thermophilus*.

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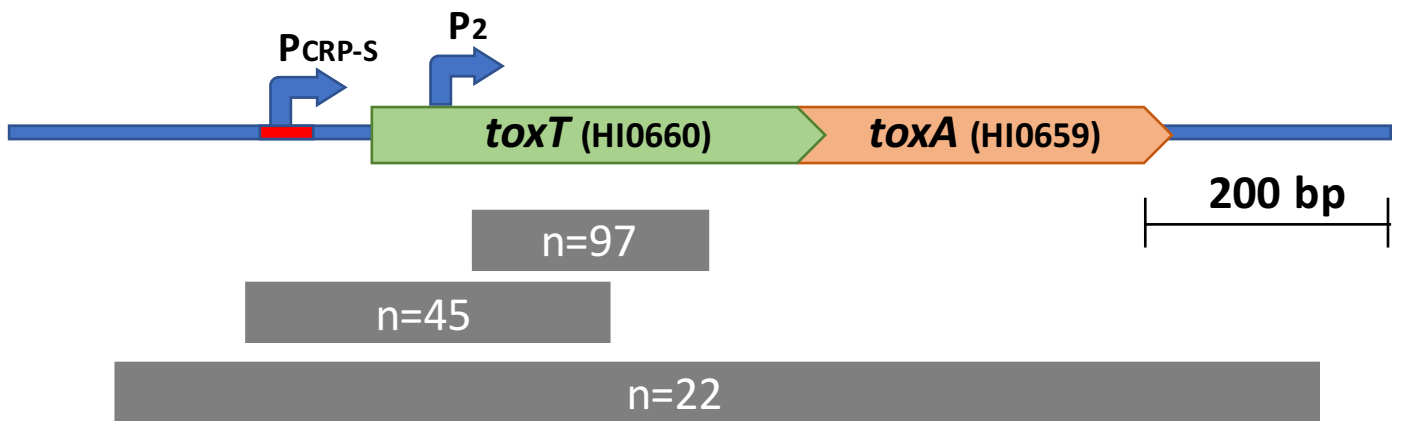
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748 Figure 10: **Natural deletions in the *toxTA* operon.** Top line: structure of the wildtype *toxTA* operon in strain KW20.

749 Lower lines: dark grey bars indicate the spans of the three naturally occurring deletions, annotated with number of

750 strains possessing each deletion.



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