

1 **PinR mediates the generation of reversible population diversity in *Streptococcus***
2 ***zooepidemicus***

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28 **Abstract:**

29 Opportunistic pathogens must adapt to and survive in a wide range of complex ecosystems.
30 *Streptococcus zooepidemicus* is an opportunistic pathogen of horses and many other animals,
31 including man. The assembly of different surface architecture phenotypes from one genotype is likely
32 to be crucial to the successful exploitation of such an opportunistic lifestyle. Construction of a series of
33 mutants revealed that a serine recombinase, PinR, inverts 114 bp of the promoter of SZO_08560,
34 which is bordered by GTAGACTTTA and TAAAGTCTAC inverted repeats. Inversion acts as a switch,
35 controlling the transcription of this sortase-processed protein, which may enhance the attachment of
36 *S. zooepidemicus* to equine trachea. The genome of a recently sequenced strain of *S. zooepidemicus*,
37 strain 2329 (Sz2329), was found to contain a disruptive internal inversion of 7 kb of the FimIV pilus
38 locus, which is bordered by TAGAAA and TTTCTA inverted repeats. This strain lacks *pinR* and we
39 hypothesized that this inversion may have become irreversible following the loss of this recombinase.
40 Active inversion of FimIV was detected in three strains of *S. zooepidemicus*: 1770 (Sz1770), B260863
41 (SzB260863) and H050840501 (SzH050840501), all of which encoded *pinR*. A deletion mutant of
42 Sz1770 that lacked *pinR* was no longer capable of inverting its internal region of FimIV. Our data
43 highlight redundancy in the PinR sequence recognition motif around a short TAGA consensus and
44 suggest that PinR can reversibly influence the wider surface architecture of *S. zooepidemicus*,
45 providing this organism with a bet-hedging solution to survival in fluctuating environments.

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55 **Abbreviations:**

56 ACT, Artemis Comparison Tool

57 AFHP, acute fatal haemorrhagic pneumonia

58 DMEM, Dulbecco's modified Eagle's medium

59 ST, sequence type

60 *S. equi*, *Streptococcus equi* subspecies *equi*

61 *S. zooepidemicus*, *Streptococcus equi* subspecies *zooepidemicus*

62 THA, Todd Hewitt Agar

63 THAE, Todd Hewitt Agar containing at 0.5 µg ml⁻¹

64 THB, Todd Hewitt Broth

65 THBE, Todd Hewitt Broth containing at 0.5 µg ml⁻¹

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82 **Introduction:**

83 The Gram-positive organism *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) is the
84 most frequently isolated opportunistic pathogen of horses, associated with respiratory disease in
85 young horses (Lindahl *et al.*, 2013; Velineni *et al.*, 2014; Wood *et al.*, 1993; Wood *et al.*, 2005) and
86 uterine infections in mares (Hong *et al.*, 1993; Rasmussen *et al.*, 2013; Smith *et al.*, 2003). The
87 bacterium is also associated with disease in a wide range of other animal hosts including dogs (Abbott
88 *et al.*, 2010; Chalker *et al.*, 2003; Pesavento *et al.*, 2008) and humans (Abbott *et al.*, 2010; Balter *et*
89 *al.*, 2000). The *S. zooepidemicus* group contains a wide variety of strain types, reflecting the diverse
90 array of hosts and tissues that this species of bacteria can infect, and there are 324 distinct sequence
91 types (ST) currently listed on the multilocus sequence typing (MLST) online database
92 <http://pubmlst.org/szooepidemicus/> [last accessed 24th November 2014], (Webb *et al.*, 2008).
93 However, *S. zooepidemicus* strains of the same ST are frequently isolated from several host species,
94 highlighting that at least some strains are equipped to exploit new pathogenic niches as and when the
95 opportunity arises.

96
97 Within the *S. zooepidemicus* group, *Streptococcus equi* subspecies *equi* (*S. equi*) is the causative
98 agent of strangles, which is the most frequently diagnosed infectious disease of horses worldwide. *S.*
99 *equi* is host-restricted and only causes strangles, which is characterized by abscessation of the lymph
100 nodes of the head and neck. Comparison of the genomes of *S. zooepidemicus* strain H70 (SzH70)
101 and *S. equi* strain 4047 (Se4047) provided evidence of functional loss in the genome of Se4047 due to
102 mutation and deletion, coupled with pathogenic specialization through the acquisition of mobile genetic
103 elements (Heather *et al.*, 2008; Holden *et al.*, 2009). The majority of *S. zooepidemicus* isolates (101 of
104 140 isolates tested), including SzH70, encode a 131 kDa putative sortase-processed surface protein,
105 SZO_08560, which contains a C-terminal LPXTG motif (Holden *et al.*, 2009). SZO_08560 contains
106 four Listeria-Bacteroides repeat Pfam domains (PF09479) with structural similarity to mucin-binding
107 proteins (Ebbes *et al.*, 2011), but the function of this protein remains unknown. The Se4047 genome
108 encodes only the final 112 amino acids of the orthologous protein (SEQ_1307a) and lacks an

109 orthologue of an adjacent gene, SZO_08550, which is predicted to encode a serine recombinase
110 (pfam00239), named PinR (COG1961). Examination of the SzH70 genome sequencing data revealed
111 five of fifty sequence reads that positioned 114 bp of the promoter region of SZO_08560 (-170 bp to -
112 55 bp) in the inverted 'B' orientation as opposed to the annotated reference 'A' orientation. This
113 sequence is bordered by GTAGACTTTA and TAAAGTCTAC inverted repeats and it is proposed that
114 inversion of this sequence by PinR switches transcription of SZO_08560 on or off, thereby modulating
115 the production of the SZO_08560 surface protein in a manner akin to phase variation in Gram-
116 negative bacteria such as *Escherichia coli* or *Bacteroides fragilis* (Abraham *et al.*, 1985; Cerdeno-
117 Tarraga *et al.*, 2005; Coyne *et al.*, 2003).

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119 We constructed a series of *S. zooepidemicus* deletion mutants to determine if PinR mediates the
120 inversion of the SZO_08560 promoter and investigate the wider recombinase-mediated regulation of
121 protein production in *S. zooepidemicus*.

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123 **Methods:**

124 **Bacterial isolates**

125 Full details of all of the isolates examined in this study are available in Table S1 and on the MLST
126 database (<http://pubmlst.org/szooepidemicus/>). SzH70 was isolated from a nasopharyngeal swab
127 taken from a healthy Thoroughbred racehorse in Newmarket, UK during 2000 and is ST-1 (Holden *et al.*
128 *et al.*, 2009). *S. zooepidemicus* strain 2329 (Sz2329) is an ST-118 strain that was isolated from a
129 tracheal wash recovered from a healthy Welsh mountain pony in the UK during 1996. *S.*
130 *zooepidemicus* strain 1770 (Sz1770) was recovered from a case of acute fatal hemorrhagic
131 pneumonia in a greyhound from Kent in 2008 and is ST-18. *S. zooepidemicus* strain B260863
132 (SzB260863) was isolated from an aborted fetus of equine origin in the UK during 2006 and is ST-13.
133 *S. zooepidemicus* strain H050840501 (SzH050840501) is an ST-195 strain that was recovered from
134 the blood of a man who died of septicemia in the UK during 2005. Unless otherwise stated, *S.*

135 *zooepidemicus* strains were grown on COBA strep select plates (bioMérieux), on Todd Hewitt Agar
136 (THA) (Oxoid) or in Todd Hewitt Broth (THB) (Oxoid) at 37 °C in an atmosphere containing 5 % CO₂.
137

138 **Allelic replacement mutagenesis**

139 Internal gene deletions and rearrangements were introduced into SzH70 or Sz1770 through an allelic
140 replacement strategy using the pG⁺host9 plasmid (Maguin *et al.*, 1996), which has been described
141 previously for the deletion of *prtM* in *Se4047* (Hamilton *et al.*, 2006). Briefly, approximately 500 bp
142 fragments of DNA that flanked the desired sequence to be modified were generated by PCR using the
143 primers listed in Table S2, and cloned into the p⁺Ghost9 plasmid via *EcoRI* and *SalI* restriction sites.
144 To complement gene disruptions, full copies of *pinR* or SZO_08560 under the control of their native
145 promoters, were cloned into the *AgeI* and *PvuI* restriction sites of the pGHost9ΔSZO07770 construct
146 that was previously utilized to insert a novel control qPCR target sequence into SzH70 (Webb *et al.*,
147 2013). The sequences of the insertions into each plasmid were obtained on both strands using an
148 ABI3100 DNA sequencer with BigDye fluorescent terminators and the primers listed in Table S2.

149
150 In order to generate each modified strain, SzH70 or Sz1770 was transformed with the relevant
151 pG⁺host9 plasmid and transformants were subjected to two rounds of homologous recombination as
152 described previously (Hamilton *et al.*, 2006). The first recombination event, leading to the integration of
153 the plasmid into the bacterial chromosome, was achieved by growing transformants in THB containing
154 erythromycin at 0.5 µg ml⁻¹ (THBE) at 28 °C overnight and then increasing the temperature to 37 °C for
155 3 hours. Integrants were selected following growth on Todd Hewitt agar containing erythromycin at 0.5
156 µg ml⁻¹ (THAE) overnight at 37 °C. Integrants were inoculated into THB and grown at 37 °C overnight
157 followed by dilution into THB and incubation at 28 °C for a further 48 hours. Incubation at the
158 permissive temperature (28 °C) allowed plasmid replication and facilitated the second recombination
159 event. Bacteria were plated on THA and grown at 37 °C to promote the loss of free plasmid. Putative
160 mutant colonies were sub-cultured onto fresh THA and THAE plates to confirm their erythromycin
161 sensitivity. The presence of the relevant mutant allele in the chromosome of putative mutants was

162 determined by PCR using the primers listed in Table S2 followed by DNA sequencing on an ABI3100
163 DNA sequencer with BigDye fluorescent terminators. A schematic of the mutants generated in this
164 study is shown in Figure 1.

165

166 **Preparation of chromosomal DNA**

167 Chromosomal DNA was purified from a single colony using GenElute spin columns according to
168 manufacturer's instructions (Sigma).

169

170 **Isolation of total bacterial RNA and preparation of cDNA**

171 An overnight culture was diluted 1/20 in fresh THB and grown to an $OD_{600\text{ nm}}$ of 0.3. The culture was
172 mixed with two volumes of RNA protect (Qiagen) and cells were harvested by centrifugation at 4 °C at
173 5000 x g for 10 minutes followed by 8000 x g for 10 minutes. Supernatant was poured off and the
174 pellet re-suspended in 200 μ l tris-EDTA buffer (Fluka), 3 mg lysozyme (Sigma) and 500 U of
175 mutanolysin (Sigma). The cells were vortexed repeatedly for 45 minutes, 700 μ l of RLT buffer
176 (Qiagen) was added and the sample vortexed for 10 seconds. 0.05 g of acid washed glass beads
177 (Sigma) was added and the sample vortexed for 5 minutes to complete cell lysis. The sample was
178 centrifuged at 16100 x g and RNA was extracted from the supernatant using an RNeasy midi kit
179 (Qiagen) with the inclusion of two on-column DNase 1 treatment steps according to the manufacturer's
180 instructions. RNA was quantified using a NanoDrop 1000 V3.7.1 spectrophotometer and reverse
181 transcribed using a Verso cDNA kit according to the manufacturer's instructions (Thermo Scientific).

182

183 **qPCR for quantification of transcripts and the orientation of the invertible region**

184 The number of copies of DNA or cDNA of interest were quantified by qPCR with the primers listed in
185 Table S2. Reactions contained 10 μ l Kapa SYBR fast (Kapa Biosystems), 0.3 μ M forward primer, 0.3
186 μ M reverse primer, 6 μ l 1/10 dilution of DNA or cDNA. Reactions were made up to 20 μ l with water
187 and thermocycled on an ABI StepOnePlus instrument at 95 °C for 3 minutes followed by 40 cycles of
188 95 °C for 30 seconds and 60 °C for 10 seconds with a SYBR read taken at the end of each cycle, then

189 95 °C for 15 seconds. A melt curve was performed from 60 °C to 95 °C with SYBR reads every 0.3 °C
190 to differentiate potential non-specific amplification products and data analyzed using StepOnePlus
191 Software v2.1. No template and no reverse transcription controls were used as negative controls and
192 standard curves with a DNA reference were performed for each primer pair. The experiments were
193 repeated in triplicate and data were normalized by comparison with the house-keeping gene *gyrA*.
194 Amplified FimIV DNA fragments were purified using a PCR purification kit (Qiagen), and the
195 sequences obtained on both strands using an ABI3100 DNA sequencer with BigDye fluorescent
196 terminators using the original PCR primers. Sequence data were assembled using SeqMan 5.03
197 (DNASTar Inc.).

198

199 **Quantification of *in vitro* growth rate**

200 Mutant strains were inoculated into THB containing 10 % fetal calf serum (THBS) in triplicate and the
201 growth of each strain was monitored by measuring the OD_{600nm}.

202

203 **Air-interface infection model**

204 Air-interface respiratory tract organ cultures were constructed using explants of equine trachea as
205 described previously (Hamilton *et al.*, 2006). The trachea used in this study were recovered from six
206 ponies that were euthanized for reasons unrelated to this project and processed on the same day to
207 maximize cell viability. Trachea were washed in Dulbecco's modified Eagle's medium supplemented
208 with 2 mM L-glutamine (DMEM) containing penicillin 100 U ml⁻¹; streptomycin 50 µg ml⁻¹; gentamicin
209 100 µg ml⁻¹ and amphotericin-B 2.5 µg ml⁻¹ (PAA) for four hours to remove commensal flora. Following
210 further washing in DMEM to remove residual antibiotics and amphotericin-B, the trachea were
211 dissected into pieces approximately 5 mm² and mounted on agarose platforms surrounded by 3 ml
212 DMEM supplemented with 2 mM L-glutamine, in six-well cell culture plates. Organ cultures were
213 maintained in a humidified 5 % CO₂ incubator at 37 °C. The viability of the air-interface organ cultures
214 was assessed using 1 µm polystyrene bead (Park Scientific) clearance. Contamination was monitored
215 by running a bacteriology loop around all four edges of the culture pieces and streaking onto strep

216 select plates. Any tissue pieces in which contamination was detected were discarded. Organ culture
217 pieces were infected with a 10 μ l suspension containing 1×10^6 colony forming units (c.f.u.) of SzH70
218 mutants, or were mock-infected with THB. Attachment of bacteria to the organ culture pieces was
219 quantified by measuring viable counts (six organ culture pieces per time point) of adherent bacteria at
220 two hours post-infection. Organ culture pieces were vortexed for 5 seconds in phosphate buffered
221 saline (PBS) to remove non-adherent bacteria and then homogenized before plating serial ten-fold
222 dilutions onto THA and enumerating colonies. Data are presented from six independent experiments.

223

224 **Whole genome sequencing**

225 Sz2329 has previously been shown to lack *pinR* and SZO_08560 by PCR screening of a diverse
226 population of *S. zooepidemicus* (Holden *et al.*, 2009). Lack of *pinR* raised the possibility that novel
227 invertible sequences could be fixed in the genome, facilitating their identification. Therefore, the
228 genome of Sz2329 was sequenced to 25-fold coverage using a Genome Sequencer-FLX (454 Life
229 Sciences, Roche Applied Sciences, IN, USA). Two sequencing libraries were prepared from genomic
230 DNA, the first a fragment (~250 bp read length) and a second 3,000 bp insert, long-tag paired end
231 library (~100 bp) to provide scaffolding. The reads were assembled with Newbler (v2.0.01.14) using
232 default assembly parameters. Comparison with the genome sequence of SzH70 (FM204884) (Holden
233 *et al.*, 2009) was facilitated by using the Artemis Comparison Tool (ACT) (Carver *et al.*, 2005). The
234 sequence and annotation of the Sz2329 genome has been deposited in the EMBL database under
235 accession number JTJH00000000.

236

237 **Statistical analysis**

238 A two-sided student's *t*-test was used to compare continuous data where assumptions of a normal
239 distribution and equal variance were satisfied. A Kruskal Wallis test was performed to determine the
240 significance of growth curve data. A paired student's *t*-test was used to compare the attachment of
241 wild-type and mutant strains of SzH70 to explants of equine trachea, accounting for variation between
242 the six different trachea.

243

244 **Results:**

245 **PinR is responsible for inversion of the region upstream of SZO_08560**

246 We generated a series of mutant and complementation strains in SzH70 (Figure 1) to determine if
247 PinR mediates inversion of the promoter of SZO_08560. Each deletion was confirmed by PCR and
248 sequencing across the deletion site. The amount of the invertible promoter region in both the A and B
249 orientations was quantified by qPCR. Wild-type SzH70 contained 96 % of SZO_08560 promoter
250 copies in the A orientation and 4 % in the B orientation (Figure 2). Deletion of *pinR* ($\Delta pinR$ A) fixed the
251 promoter in the A orientation, no copies of the promoter in the B orientation were identified in this
252 mutant. Both *pinR* and the SZO_08560 promoter were deleted ($\Delta pinR$) and then the SZO_08560
253 promoter was re-introduced in the B orientation to produce a mutant strain ($\Delta pinR$ B) that only
254 contained the SZO_08560 promoter in the B orientation. Complementation of the *pinR* deletion in
255 strains $\Delta pinR$ A and $\Delta pinR$ B by insertion of a copy of *pinR* under the control of its native promoter into
256 the pseudogene SZO_07770 ($\Delta pinR$ A c and $\Delta pinR$ B c, respectively) restored inversion of the
257 promoter of SZO_08560 in the $\Delta pinR$ A c strain such that 0.8 % of promoter copies were in the B
258 orientation. However, inversion of the promoter of SZO_08560 was not restored in the $\Delta pinR$ B c
259 strain (Figure 2). The $\Delta pinR$ A and $\Delta pinR$ A c strains were found to have a significantly reduced
260 growth rate when compared with SzH70 and the other mutant strains ($P = 0.006$) (Figure S1).

261

262 **The orientation of the invertible region determines SZO_08560 transcription**

263 To determine if the promoter of SZO_08560 was more active in the A or B orientation, total RNA
264 isolated from each mutant strain was reverse transcribed and used to quantify the transcription of
265 SZO_08560 by qPCR. Data were normalized based on the number of *gyrA* transcripts in each
266 triplicate sample. The transcription of SZO_08560 in wild-type SzH70 was found to be equivalent to
267 that of *gyrA* in this strain (Figure 3). Deletion of *pinR* such that the promoter of SZO_08560 was fixed
268 in the A orientation ($\Delta pinR$ A) caused a reduction of SZO_08560 transcription to 0.7 % of wild-type
269 levels ($P < 0.0001$). However, fixation of the promoter of SZO_08560 in the B orientation increased

270 SZO_08560 transcription to 189 % of wild-type levels ($P < 0.0001$). Deletion of SZO_08560 abolished
271 its transcription, which was restored to 50 % of wild-type transcription levels by complementation
272 through the insertion of a copy of SZO_08560 under the control of its native promoter in the B
273 orientation into the pseudogene SZO_07770 (strain $\Delta 08560$ c).

274

275 **Deletion or increased transcription of SZO_08560 alone did not significantly affect attachment**
276 **of *S. zooepidemicus* to equine tissues**

277 The number of bacteria recovered from explants of equine trachea two hours post-infection with the
278 wild-type SzH70 strain did not significantly differ from the number recovered from those infected with
279 the mutant strains (Figure 4). However, the reduction in the amount of $\Delta 08560$ and $\Delta 08560$ c strains
280 recovered relative to SzH70 approached statistical significance ($P = 0.0859$ and $P = 0.0883$,
281 respectively). A higher number of bacteria were recovered from those explants infected with the $\Delta pinR$
282 B mutant, which transcribes the most SZO_08560 relative to SzH70, although this was also not
283 statistically significant ($P = 0.67$).

284

285 **PinR inverts sequences distant to the promoter of SZO_08560**

286 Analysis of the draft genome sequence of Sz2329 using the ACT confirmed that this strain contained a
287 deletion of *pinR* and the majority of its SZO_08560 homologue, which was identical to that previously
288 identified in Se4047 (Holden *et al.*, 2009). The assembled Sz2329 draft genome contained one
289 example of altered locus architecture consisting of an inversion of a 7,137 bp region containing the
290 major and minor pilin genes, but not the AraC-like regulator or associated sortases of FimIV (Figure
291 5). The inversion occurred in 100 % of the sequencing reads covering this region, which was
292 represented in a single contig. The inverted region of FimIV is flanked by a six-base inverted repeat
293 (TAGAAA), which partially (TAGA) matches the 10 base inverted repeat (GTAGACTTTA) that flanks
294 the invertible promoter region upstream of SZO_08560 in SzH70 (Holden *et al.*, 2009).

295

296 To determine if inversion of the FimIV locus was actively occurring in other strains of *S.*
297 *zooeidemicus*, PCR primers were designed to amplify a product when the FimIV region was in either
298 the original orientation as annotated in the SzH70 genome, or inverted orientation. A collection of ten
299 FimIV-containing strains were screened by PCR for the occurrence of amplification products
300 suggesting the presence of DNA in both orientations (Table S1). Active inversion of the FimIV
301 sequence was identified in *S. zooeidemicus* strains Sz1770, SzB260863 and SzH050840501. Only
302 the inverted FimIV PCR product was amplified from strain Sz2329. The PCR products were purified
303 and sequenced, confirming that the inverted region in FimIV was flanked by the same inverted repeat
304 (TAGAAA) in all strains.

305

306 To determine if PinR was mediating FimIV inversion, *pinR* was deleted from Sz1770 by allelic
307 replacement mutagenesis. Deletion of *pinR* was confirmed by PCR and sequencing across the
308 deletion site. The number of original and inverted copies of FimIV in the $\Delta pinR$ mutant, wild-type
309 Sz1770 and Sz2329 were quantified by qPCR and normalized to *gyrA*. Wild-type Sz1770 contained
310 0.01 % (1:10,000) of FimIV copies in the inverted orientation (Figure 6). Deletion of *pinR* from strain
311 Sz1770 prevented inversion of the FimIV region, yielding 100 % of qPCR products in the original
312 orientation.

313

314 **Discussion**

315 The surface architecture of *S. zooeidemicus* is likely to be crucial to its ability to adapt and interact
316 with mammalian hosts and the wider environment in order to fulfill the requirements of its opportunistic
317 lifestyle. The organism must survive outside a host, in drinking water or on soil, grass and other
318 surfaces in competition with a vast array of other micro-organisms, whilst remaining in a state of
319 readiness to infect a susceptible new host should the opportunity arise. The population of *S.*
320 *zooeidemicus* infects many different mammalian hosts and tissues. Indeed, individual strains are
321 themselves capable of infecting multiple hosts and zoonotic transmission, for example from an
322 infected dog to a veterinary nurse, has been demonstrated (Abbott *et al.*, 2010). *S. zooeidemicus*

323 persists in the tonsils or on the mucosal surfaces of recovered horses in the face of a mature immune
324 response, increasing the likelihood of onward transmission (Lindahl *et al.*, 2013). Therefore, the ability
325 of *S. zooepidemicus* to modulate its surface is likely to be essential to its long-term survival.

326

327 Here we present evidence that the inversion of the promoter of SZO_08560 is performed by PinR and
328 demonstrate that inversion acts as a switch, controlling transcription of SZO_08560. SZO_08560
329 contains an N'-terminal signal sequence, C'-terminal LPXTG sortase-processing motif and four
330 Listeria-Bacteroides repeat Pfam domains (PF09479) with structural similarity to mucin-binding
331 proteins (Ebbes *et al.*, 2011). Whilst the exact receptor bound by SZO_08560 remains unknown, the
332 reduced ability of SZO_08560 mutants to attach to explants of equine trachea, which approached
333 statistical significance, suggests that SZO_08560 is likely to play a role in the attachment of *S.*
334 *zooepidemicus* to host tissue.

335

336 The $\Delta pinR$ A mutant lacks *pinR* with the SZO_08560 promoter orientated in the A direction and had a
337 slow growth rate. One explanation for the slow growth of this strain is interference of the transcription
338 of SZO_08540 or SZO_08530 by the SZO_08560 promoter, which could be enhanced by its closer
339 proximity to these coding sequences following the deletion of *pinR*. SZO_08540 encodes a conserved
340 hypothetical protein, whilst SZO_08530 encodes RpsP, the 30S ribosomal protein S16. Interestingly,
341 the $\Delta pinR$ B mutant, which lacks *pinR*, whilst orientating the SZO_08560 promoter in the B direction
342 had a normal growth rate, as did the $\Delta pinR$ mutant, which lacks both *pinR* and the SZO_08560
343 promoter (Figure S1). Therefore, the inversion of the SZO_08560 promoter from the A to the B
344 orientation in strain $\Delta pinR$ A c, which contains a complementing copy of *pinR* may be preferred as it is
345 likely to yield strains with a normal growth rate. However, the inversion of the SZO_08560 promoter
346 from the B to the A orientation in strain $\Delta pinR$ B c was not detected, most likely as the resultant
347 mutants would have a slower growth rate.

348

349 Analysis of the Sz2329 genome sequence, which lacks *pinR*, identified a disruptive internal inversion
350 of the FimIV locus, which was bordered by short inverted repeats that shared a four-base motif
351 (TAGA) with the SZO_08560 promoter. The FimIV locus encodes an AraC-like regulator, three
352 putative sortase enzymes, a putative exported protein and three putative sortase-processed proteins
353 that are predicted to form a surface pilus structure (Holden *et al.*, 2009). Screening of a panel of *S.*
354 *zooepidemicus* isolates identified three strains with active FimIV inversion. The deletion of *pinR* in one
355 of these strains, Sz1770, stopped FimIV inversion revealing a wider role for PinR in the global
356 regulation of bacterial surface components and highlighting redundancy in the DNA sequences of the
357 inverted repeats. Our data suggest that the ancestor of Sz2329 contained a functional copy of *pinR*
358 and was actively inverting the FimIV region until the loss of *pinR* fixed this region in the position that it
359 was in at the time. FimIV was present in 81 (58%) of 140 isolates of *S. zooepidemicus* that were
360 tested and is missing from the *S. equi* genome [1], indicating that its loss from the genomes of some
361 strains may be beneficial in the particular environments that they occupy. It is interesting that inversion
362 of the FimIV region was not observed in strain SzH70, despite this strain actively inverting the
363 SZO_08560 promoter via PinR, suggesting that co-factors may assist PinR to invert alternative
364 substrates. Variation in the sequence of inverted repeats and size of the inverted regions of DNA
365 confounds the *in silico* identification of substrate sites and further research is required to identify the
366 range of PinR substrates and the consequences of inversion on the properties of the variants
367 produced.

368

369 The data presented here suggest that PinR plays an important role in modulating the surface
370 architecture of *S. zooepidemicus* forming a mixture of distinct phenotypes, which provides this
371 organism with a bet-hedging solution to survival in fluctuating environments (Stewart & Cookson,
372 2012). PinR of *S. zooepidemicus* shares >60 % predicted amino acid identity with putative resolvases
373 including those encoded by strains of *Streptococcus anginosus*, *Streptococcus pneumoniae* (strain
374 GA17545), *Streptococcus constellatus*, *Streptococcus suis*, *Streptococcus mitis*, *Streptococcus ovis*,
375 *Streptococcus pseudopneumoniae*, *Peptoniphilus indolicus*, *Eubacterium saphenum*, *Parvimonas*

376 *micra*, *Eggerthia catenaformis*, *Gemella bergeri*, *Gamella cuniculi*, *Bulleidia extracta*, *Enterococcus*
377 *faecium*, *Erysipelotrichaceae* bacterium, *Clostridiales* bacterium, *Gardnerella vaginalis*, *Coprobacillus*
378 sp., *Catenibacterium* sp. and *Mogibacterium* sp., suggesting that serine recombinase-mediated
379 modulation of surface architecture is a mechanism that is widely adopted by other Gram-positive
380 bacteria. It is intriguing to note that PinR shares 27% amino acid sequence identity and conserved
381 serine residue with the site-specific recombinase of *Bacteroides fragilis*, FinA (also known as Mpi),
382 which modulates the production of several surface components in this Gram-negative bacterium by
383 inversion of promoter sequences (Cerdeno-Tarraga *et al.*, 2005; Coyne *et al.*, 2003). Our data provide
384 the first evidence to suggest that the reversible ON-OFF phenotype known as phase variation can be
385 mediated by a recombinase in streptococci.

386

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390

391

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475

476

477

478 **Figure 1. Schematic of the modified strains produced during this study.** A) The structure of the
479 *pinR*/SZO_08560 region for wild-type SzH70 is shown. The $\Delta pinR$ A mutant lacks *pinR*, which was
480 predicted to fix the invertible region in the A orientation. The $\Delta pinR$ mutant lacks both *pinR* and the
481 invertible region. The $\Delta pinR$ B mutant was generated from the $\Delta pinR$ mutant by introducing the
482 invertible region in the B orientation. The $\Delta 08560$ mutant lacks SZO_08560. The direction of the
483 SZO_08560 promoter is indicated by the black arrow. B) The structure of the SZO_07770 region for
484 wild-type SzH70 is shown. The $\Delta pinR$ A c and $\Delta pinR$ B c mutants were generated by inserting a copy
485 of *pinR* under the control of its native promoter into the $\Delta pinR$ A and $\Delta pinR$ B mutants, respectively.
486 The $\Delta 08560$ c mutant was generated by inserting a copy of SZO_08560 downstream of the invertible
487 region in the B orientation into the $\Delta 08560$ mutant. The top DNA strand is shown by the solid black
488 line and bottom strand by the broken black line. The inverted repeats are shown in red and blue
489 boxes. The direction of the SZO_08560 promoter is indicated by the black arrow.

490

491 **Figure 2. Orientation of the invertible region as determined by qPCR.** The Log10 of the number of
492 copies of the invertible region in the A or B orientation are shown following normalisation of the
493 samples based on the amount of *gyrA*. Error bars indicate the standard deviation.

494

495 **Figure 3. Transcription of SZO_08560 in the mutant strains.** The number of transcript copies of
496 SZO_08560 were quantified by qPCR and normalised relative to the amount of *gyrA*. Error bars
497 indicate the standard deviation.

498

499 **Figure 4. Attachment of SzH70 and mutant strains to explants of equine trachea.** Error bars
500 indicate 95% confidence intervals.

501

502 **Figure 5. Partial inversion of the FimIV locus in strain Sz2329 relative to the SzH70 reference**
503 **genome visualised using the Artemis Comparison Tool (Carver *et al.*, 2005).** The coloured bars
504 separating each genome (blue and red) represent similarity matches identified by reciprocal TBLASTX

505 analysis, with a score cut-off of 100. Blue lines link matches in the same orientation; red lines link
506 matches in the reverse orientation.

507

508 **Figure 6. Inversion of FimIV pre- and post-deletion of *pinR*.** Graph showing the Log10 of mean
509 copies of FimIV in the original annotated orientation of the SzH70 genome, and disrupted inverted
510 orientation normalised to the number of copies of *gyrA*. Error bars indicate the standard deviation.

511

512 **Table S1. List of strains used in this study.** AFHP: acute fatal haemorrhagic pneumonia.

513

514 **Table S2. List of oligonucleotide primers used in this study.** Restriction sites in primers used to
515 clone target sites for gene deletion are underlined.

516

517 **Figure S1. Growth of SzH70, $\Delta pinR$ A, $\Delta pinR$, $\Delta pinR$ B, $\Delta 8560$, $\Delta pinR$ A c and $\Delta pinR$ B c strains**
518 **in Todd Hewitt Broth.** Error bars indicate the standard deviation.

519

Figure 1
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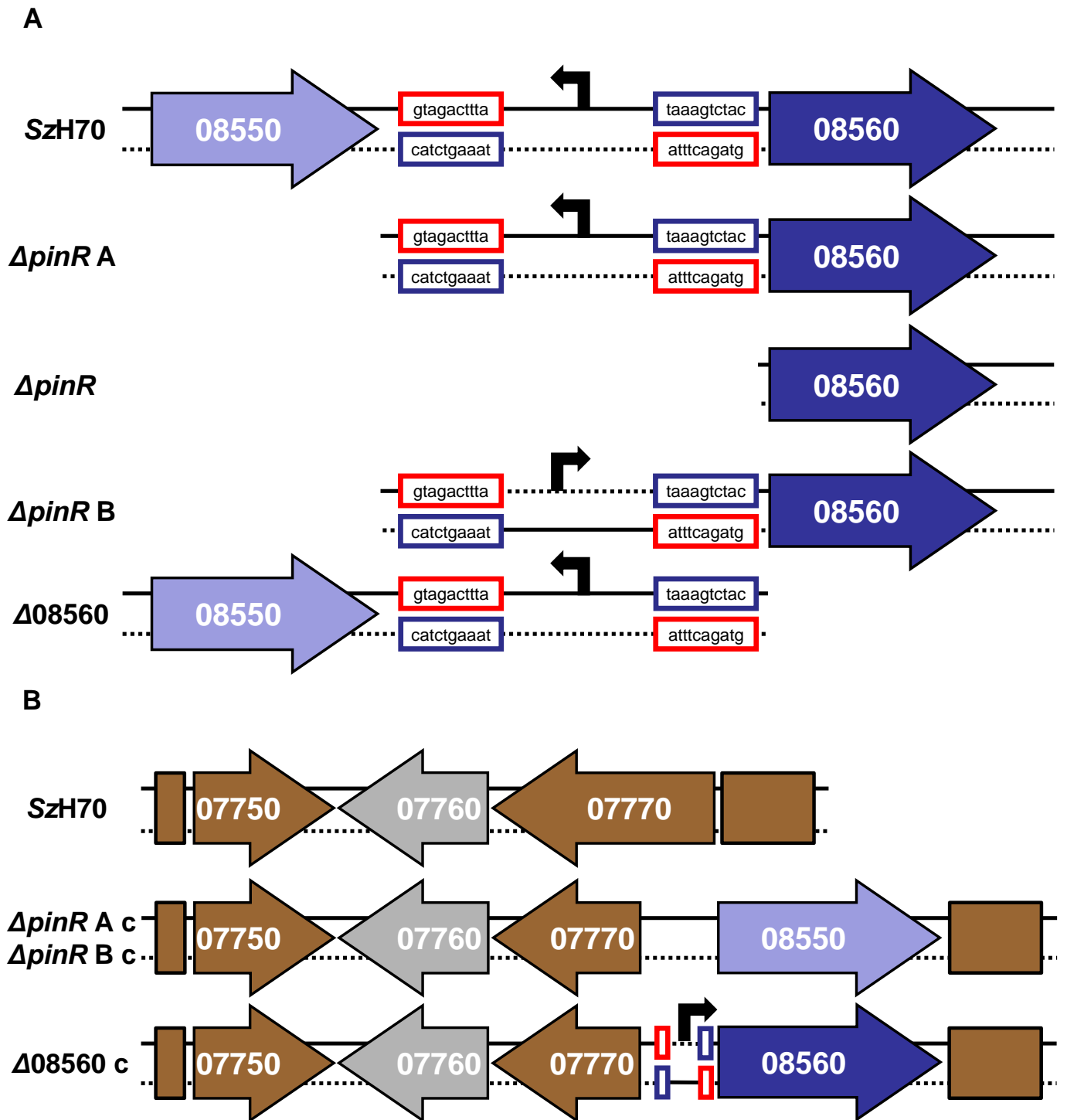


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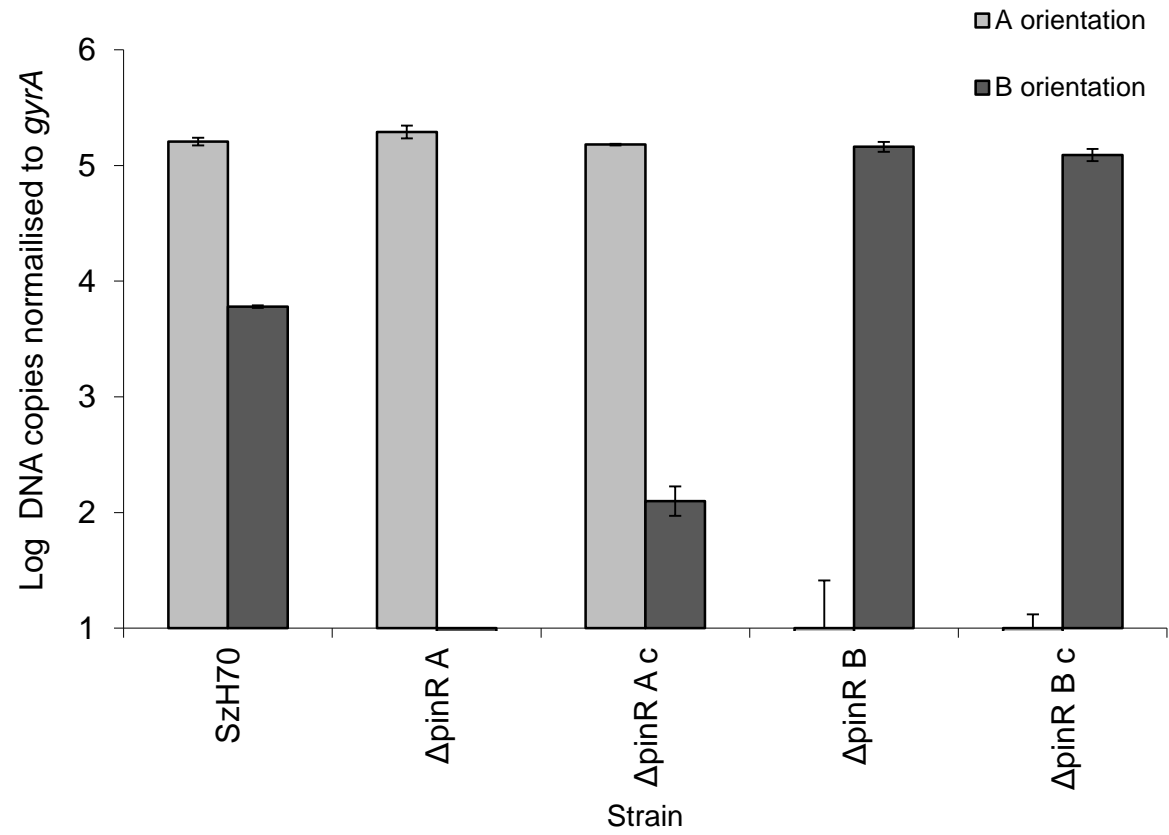


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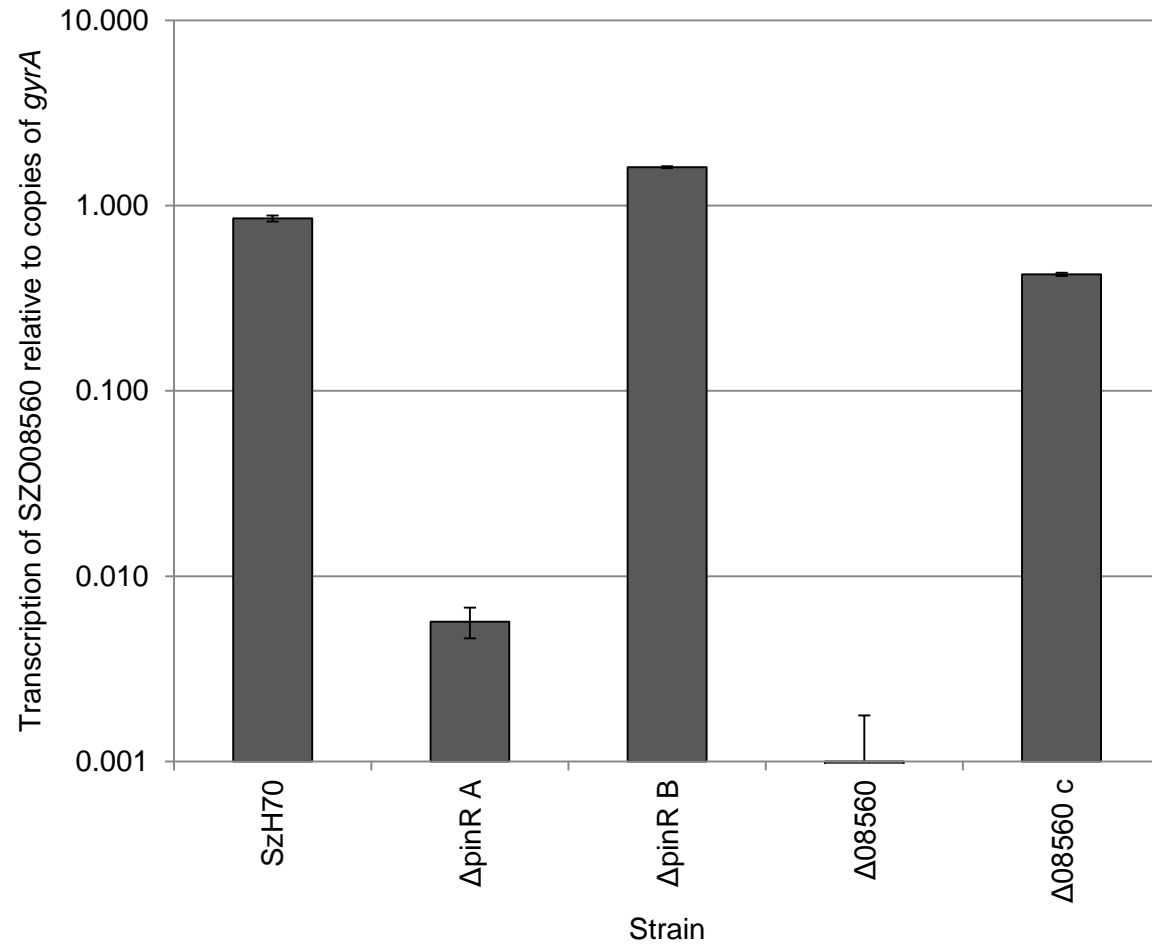


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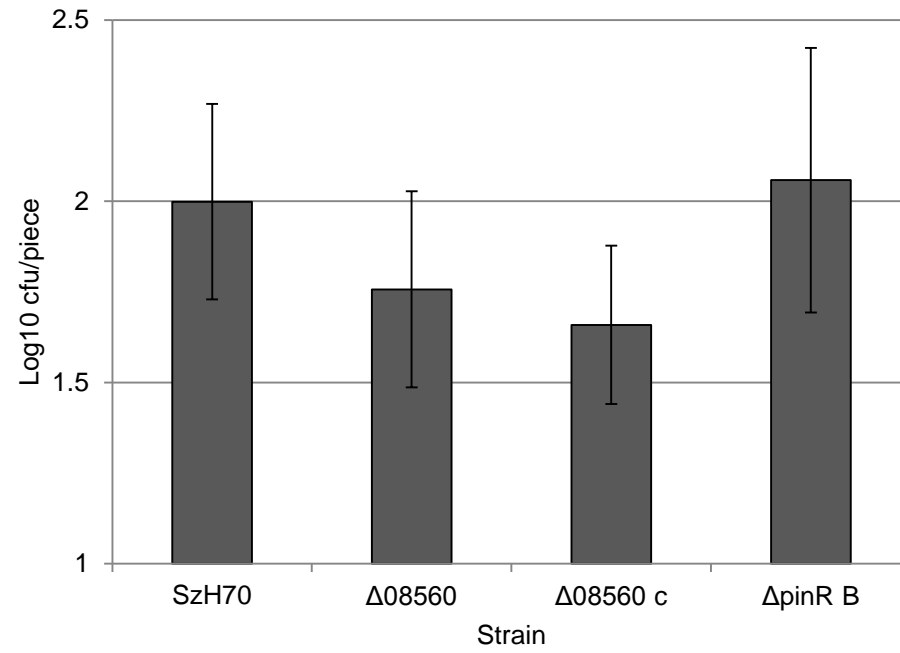


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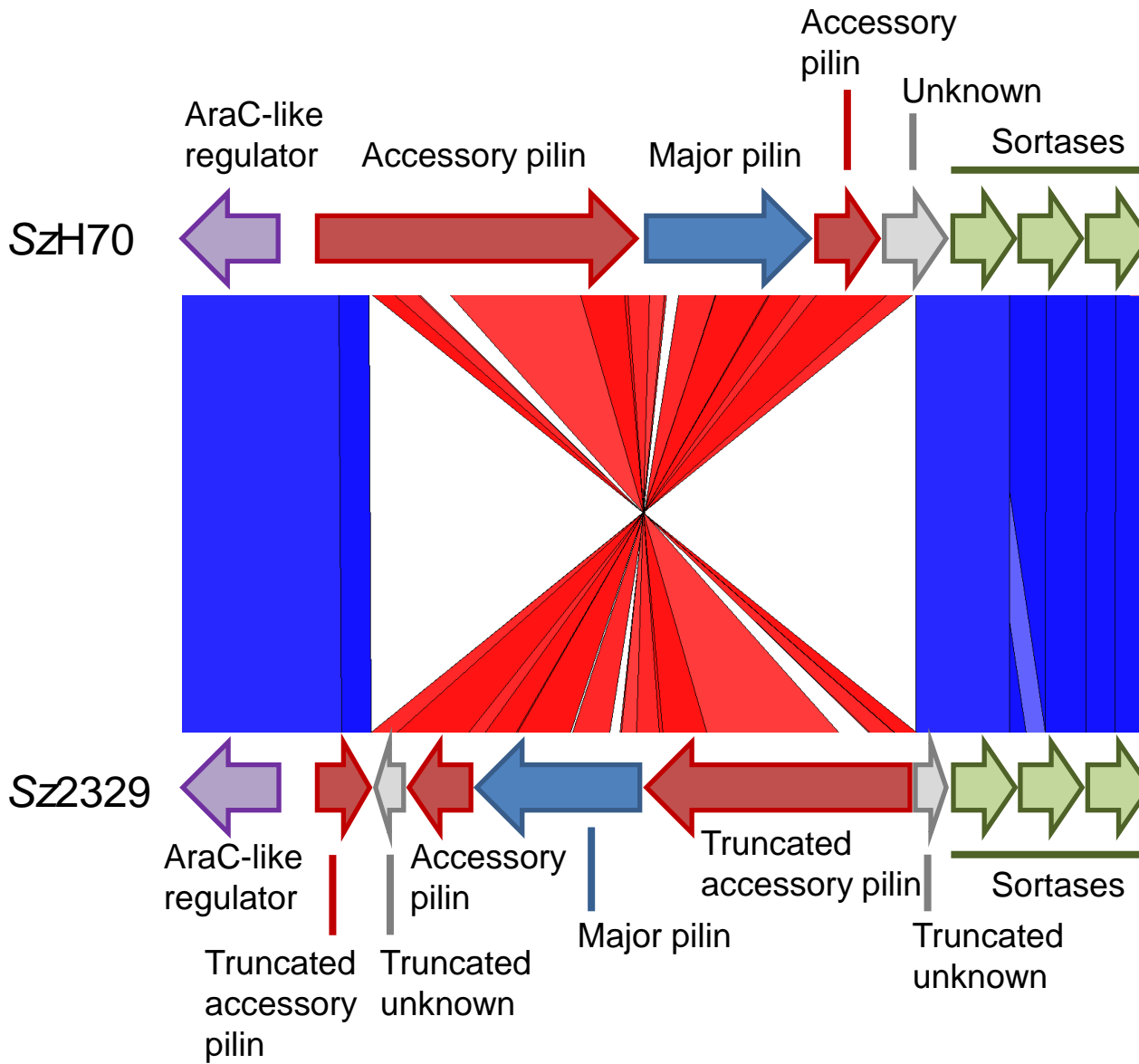
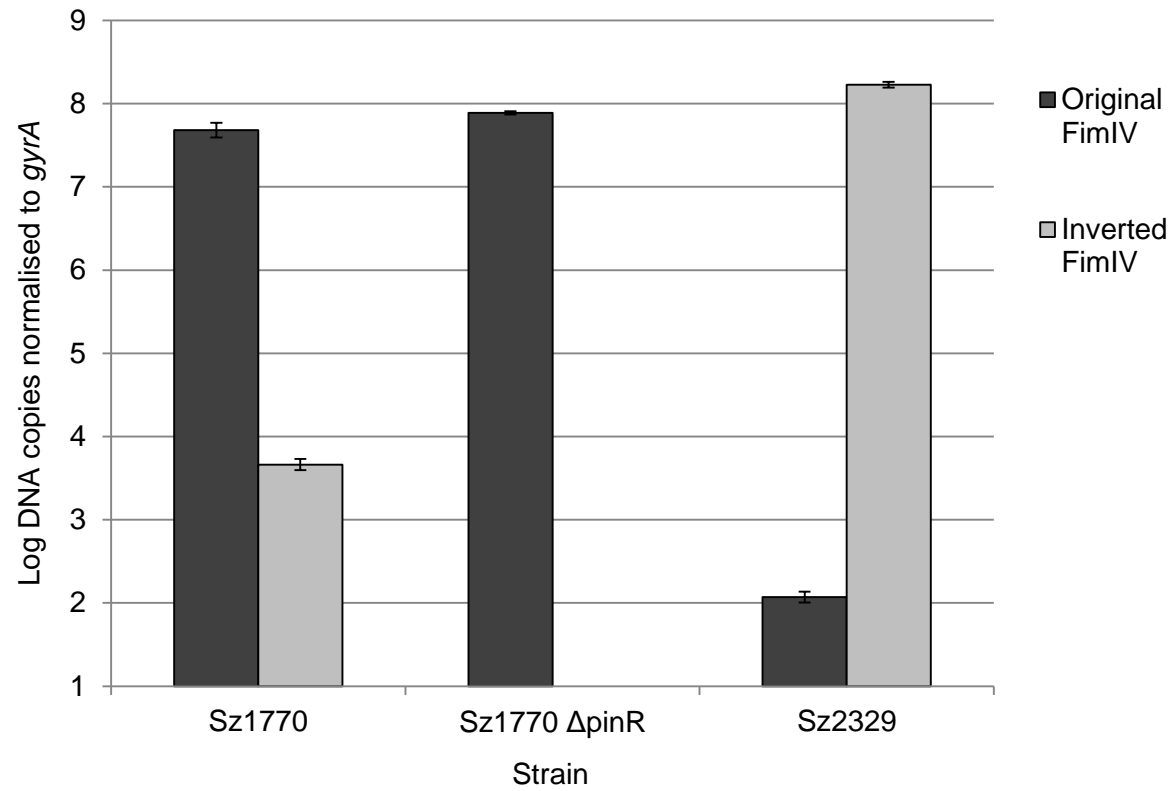


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