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A genome-wide mutagenesis screen identifies multiple genes contributing to Vi capsular expression in *Salmonella* Typhi

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15 **Abstract**

16 **A transposon-based, genome-wide mutagenesis screen exploiting the killing**
17 **activity of a lytic ViII bacteriophage was used to identify *Salmonella* Typhi genes that**
18 **contribute to Vi polysaccharide capsule expression. Genes enriched in the screen**
19 **included those within the *viaB* locus (*tviABCDE*, *vexABCDE*) as well as *oxyR, barA/sirA***
20 **and *yrfF*, which have not previously been associated with Vi expression. The role of**
21 **these genes in Vi expression was confirmed by constructing defined null mutant**
22 **derivatives of *S.* Typhi and these were negative for Vi expression as determined by**
23 **agglutination assays with Vi-specific sera or susceptibility to Vi-targeting**
24 **bacteriophage. Transcriptome analysis confirmed a reduction in expression from the**
25 ***viaB* locus in these *S.* Typhi mutant derivatives and defined regulatory networks**
26 **associated with Vi expression.**

27

28 **INTRODUCTION**

29 Bacteria express an array of surface-associated macromolecular structures that
30 facilitate their interaction with the environment (1). In pathogenic bacteria, the expression of
31 such surface components can change rapidly, as the infecting bacteria move between and
32 within their hosts (2). Surface molecules on pathogens include proteins, flagella, fimbriae,
33 glycolipids and polysaccharides that can directly facilitate bacterial survival through
34 processes such as adhesion, nutrient scavenging and resisting immune attack or bacteriophage
35 killing (3, 4). Thus, the expression of surface structures is a complex process that can involve
36 multiple, co-ordinately expressed genes associated with biosynthesis and localisation.

37 Bacterial surface antigens play a key role as targets determining the specificity of
38 killing by antibodies or bacteriophage (5, 6). The antigens and macromolecular structures that
39 are targeted by these killing systems include outer membrane proteins, lipopolysaccharides
40 and carbohydrate capsules (4). We know from *in vitro* killing assays that otherwise sensitive
41 bacteria can escape killing by either modifying the structure of a target or by altering
42 expression (7). Indeed, it is likely that the immune system and bacteriophage have exerted
43 significant selection on surface antigens such that bacteria have evolved pathways to facilitate
44 escape. Surface antigens are attractive as candidate subunit vaccines or as targets for
45 therapeutic bacteriophage. In this context, it is important to determine how bacteria escape

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46 killing as a means to predict how such escape variants might emerge in control programmes,
47 such as rolling out new vaccines or introducing bacteriophage therapies.

48 *Salmonella enterica* serovar Typhi (*S*. Typhi) the etiological agent of typhoid fever,
49 expresses a surface-associated polysaccharide, Vi, that has been implicated in virulence (8-
50 10). Many key genes associated with Vi biosynthesis, which is a homopolymer of variably O-
51 acetylated α-1,4-linked *N*-acetylgalactosaminuronate (11), are encoded on the *viaB* locus
52 (*tviABCDE* and *vexABCDE* genes). Importantly, Vi is a protective antigen on which some
53 human typhoid vaccines are based (9, 12). Vi, was also targeted previously in bacteriophage
54 therapy clinical studies using Vi specific bacteriophage (13). We previously characterized a
55 related set of Vi-specific bacteriophage that has found general utility for typing *S*. Typhi
56 clinical isolates (5). Although these bacteriophages are genetically diverse, they all encode
57 tail fibre components that specifically target Vi to initiate infection. Here we use one of these
58 Vi phage, ViII, to drive selection in a whole genome mutagenesis screen based on a
59 technique we named TraDIS (14) to identify genes that, when inactivated by a transposon
60 insertion, decrease ViII-associated killing.

61 **MATERIALS AND METHODS**

62 **Bacteria, growth conditions and transposon libraries.** A transposon mutant library
63 based on *S*. Typhi BRD948(pHCM1) and the transposon EZ-Tn5 was exploited in the
64 selection of Vi-negative mutant derivatives. BRD948(pHCM1) *aroA htrA* is an attenuated
65 derivative of Ty2 harbouring the antibiotic resistance plasmid pHCM1 (14). This so called
66 TraDIS library, which harbours at least 1.1 million transposon mutants and was described in
67 detail previously (14), was stored at -80^{0}C. *S*. Typhi bacteria were routinely cultured on LB
68 agar or in LB-broth containing aromatic supplements (tryptophan, tyrosine and phenylalanine
69 at a final concentration of 40μg/ml and 4-aminobenzoic acid and 2,3-dihydroxybenzoic acid
70 at a final concentration of 10μg/ml). Antibiotics were added at final concentration of 50μg/ml
71 for ampicillin and 30μg/ml for Kanamycin and Chloramphenical. All defined mutant
72 derivatives constructed during this study were derived from *S*. Typhi BRD948 lacking
73 pHCM1. Specific mutations were generated via the Red recombinase system described
74 originally by Wanner *et al*. using plasmids pKD3 and pKD4 for chloramphenicol or
75 kanamycin selection respectively (15). The *S*. Typhi defined BRD948 mutant derivatives
76 generated in this study harboured mutations in *yrfF*, *rcsB*, *oxyR*, *sirA*, *envZ*, *barA*, *greA*, *efp*,

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77 *ihfA*, *ihfD*, *actP*, *ppiB* or *phoN*. The PCR oligonucleotides used to generate these mutants and
78 determine their genotypes are detailed in Table S1.

79 **Selection of Vi phage resistant mutants from the *S.* Typhi(pHCM1) transposon**
80 **TraDIS library.** A transposon mutant library based on *S.* Typhi BRD948(pHCM1) was
81 infected with ViII bacteriophage (5, 16) at an MOI of ~10 and the infection was allowed to
82 proceed for 20 minutes at 37^{0}C. One ml of LB-broth containing 5 mM EGTA was then added
83 to stop further phage infections and the mix was incubated at 37^{0}C to allow survivors to grow
84 before aliquots were plated out onto L agar plates containing kanamycin at 30µg/ml. After
85 incubation at 37^{0}C overnight, each plate harboured ~4,000 colonies. Approximately 100 of
86 these colonies were randomly selected from different plates and they were tested in slide
87 agglutination assays using anti-*Salmonella* O-4, O-9 and Vi antiserum (Murex). Slide
88 agglutination assays were carried out using antisera against Salmonella O-4, O-9 and Vi
89 antigens (Staten Serum Institut, Denmark) as specified in the manufacturer's instructions.
90 Colonies to be tested were prepared by growing the test bacteria overnight at 37^{0}C on L Agar
91 plates containing the aromatic nutrients required for growth (17). All tested colonies, but not
92 the wild type BRD948 (which was Vi- and O-9 positive), were Vi negative but O-9 positive
93 by agglutination. Subsequently, colonies from five plates (~20,000 colonies) were collected
94 into diluent, pooled and DNA was prepared from a 5ml aliquot using the method of Hull *et*
95 *al*. (18). This pooled DNA was then sequenced using an Illumina HiSeq instrument according
96 to the manufacturers instruction with modifications as described previously (14). Sequence
97 reads were parsed for exact match to the terminal 10 bp of the transposon Tn5
98 (TAAGAGACAG). Matching sequence reads had this sequence removed and these were
99 converted to fastq format. The modified fastq reads were mapped to the *S.* Typhi Ty2 genome
100 using MAQ (14). The map position of the first base was used as the precise insertion site of
101 the transposon and the distribution within genes and the number of reads at each position was
102 used to estimate the number of transposon insertions in the input library and the output
103 library after selection. The statistical significance was calculated as previously described
104 (14).

105 **Vi ELISA.** Costar 3590 EIA 96 well plates were treated overnight with 50µl of
106 monoclonal anti-Vi agglutination serum (Staten Serum Institut) diluted 1 in 100 in 1x coating
107 buffer (Kirkegaard and Perry laboratories, KPL). This was removed the following day and
108 the plates washed three times with KPL wash buffer and blotted dry. 100µl of KPL blocking
109 buffer was added to each well and left at 37^{0}C for 2 hours prior to three more washes with 1x

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110 wash buffer and dry blotting. Bacterial cultures under test were diluted to an OD600 of 0.55
111 using formalized PBS. 300µl of this bacterial suspension was added to the first well of a
112 further microtitre plate and doubly diluted across the plate using 150µl of KPL blocking
113 buffer as diluent. 50µl aliquots of the *S*. Typhi dilutions were transferred to the washed and
114 blocked plates, and were left on these anti-Vi antibody coated plate for 2 hours at 37^0C.

115 Plates were washed three times to remove the bacterial suspension and 50µl of a 1 in
116 100 dilution of rabbit polyclonal anti-Vi serum (Remel ZC18) in KPL 1x blocking buffer was
117 added to each well and left for 2 hours at 37^0C. The plate was then washed three times and
118 50µl anti-rabbit HRP conjugate (diluted 1 in 1000 in 1x blocking buffer) added to all wells
119 and left for a further 2 hours at 37^0C. Finally, the wells were washed three times, the wells
120 dried and 50µl of KPL Sure Blue TMB solution added. When sufficient blue colour had
121 developed, the reaction was stopped by the addition of 50µl 1M HCl to each well. The plates
122 were read at 450nm on a Bio-Rad microplate spectrophotometer.

123 **Testing of *S*. Typhi mutant derivatives for Vi expression and Vi phage**
124 **sensitivity**. *S*. Typhi BRD948 and selected mutant derivatives were tested for sensitivity to a
125 range of Vi phage including ViI, ViII, ViIII, ViIV, ViV and ViVI bacteriophage (5, 16). Each
126 mutant was grown overnight in 3mls of L broth left shaking overnight at 37^0C. Molten 0.35%
127 L agar was cooled to 42^0C and 3mls was added to falcon tubes containing 100ul of a culture
128 of wild type *S*. Typhi BRD948 aliquot or a mutant derivative and this mix was poured
129 immediately onto L agar plates containing kanamycin. 10µl of each phage preparation was
130 spotted onto the solidified top agar and the plates were left overnight at 37^0C. The next day
131 any phage killing activity was recorded and compared to a *S*. Typhi BRD948 control
132 infection.

133 **RNA transcriptome analysis.** L broth cultures of *S*. Typhi BRD948 or mutant
134 derivatives were grown to an OD600 of 0.3, mixed with RNA protect (Qiagen, USA) for 30
135 min at room temperature and the inactivated bacteria harvested and the pellet stored at -80^0C.
136 RNA was isolated from these pellets using Qiagen kits and total RNA was prepared using
137 Qiagen RNeasy Miniprep columns followed by DNAase treatment and Phenol-Chloroform
138 extraction. Ethanol was then used to precipitate the RNA and the RNA pellet was washed
139 with 70% Ethanol followed by drying. These pellets were re-suspended in 50µl of ultrapure
140 water and 1µl aliquots were checked for RNA quality and quantity using the Agilent
141 Technologies RNA 6000 Nano Assay protocol using an Agilent 2100 Bioanalyzer. A custom-
142 made oligonucleotide array (Agilent) represented of genes annotated for *S*. Typhi Ty2 was

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143 used for microarray analysis. The design of the oligonucleotide array is available from
144 Agilent database submissions (ID 25337671). 50 ng of total RNA for each sample was
145 amplified and labelled with Cyanine 3-CTP following the manufacturer's protocol (Agilent
146 Low Input Quick Amp WT Labeling Kit, one-color) (Agilent, 5190-2943). Labelling
147 efficiency was assessed using the Nanodrop-8000 Spectrophotometer (Thermo Scientific).
148 Cy-3 Labelled cRNA was hybridized on to Agilent custom 8x15k *S.* Typhimurium and *S.*
149 Typhi microarrays for 17 hours at 65^0C. After hybridization, the microarray slides were
150 washed and scanned using the Agilent DNA High Resolution Microarray Scanner (Agilent,
151 G2505C) following the manufacturer's protocol. Raw image data was processed using
152 Agilent's Feature Extraction software (v10.7.3.1).
153 Data from the Agilent array was analysed using Agilent Feature Extraction Software (v10.1)
154 (http://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Product&SubPageTy
155 pe=ProductDetail&PageID=1379). Array features were calculated using AFE default settings
156 for the GE2-v5_10_Apr08 protocol. The analysis was performed using scripts written in R
157 language (version 2.11.1 [2010-05-31]) with the aid of a targets file and an annotation file to
158 complement the information generated in the output. Using the application available from the
159 Bioconductor's LIMMA library,
160 http://www.bioconductor.org/packages/release/bioc/html/limma.html, a linear model fit was
161 applied to the data that was generated. Differentially expressed genes were tabulated for each
162 contrast using the method of Benjamini and Hochberg to correct the p-values (14). Files data
163 was sorted by significance (column: adj.P.Val). The adj.P.Val cut-off used in the microarrays
164 identified significant genes whose value was less than or equal to 0.02. A positive LogFC
165 indicates greater expression of the gene in the mutant. One output file per contrast is given
166 with unfiltered data that includes all probes on the array. This file was then used for
167 downstream analysis.
168

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169 **RESULTS**

170 **The exploitation of TraDIS to identify transposon insertion mutant derivatives**
171 **with reduced sensitivity to ViII bacteriophage mediated killing.** We hypothesised that Vi
172 expression at the surface of *S.* Typhi involves multiple genes, including some that had not
173 previously been identified. To test this hypothesis we used a sequencing-based screen, known
174 as TraDIS (14), that exploits large transposon libraries. A complex transposon library
175 generated in a Vi-positive *S.* Typhi BRD948(pHCM1), harbouring over 10^6 transposon
176 mutants, was infected with a Vi-specific, lytic bacteriophage ViII, in order to enrich for Vi-
177 negative survivors. The insertion sites for all the transposons in the input and surviving pools
178 was determined by Illumina sequencing from a primer specific for sequences at the end of the
179 transposon using DNA from both the input and surviving pool as template (14). We then
180 compared the distribution of insertion sites in the input transposon library with that in the
181 recovered *S.* Typhi following selection (Table 1).

182 Initial analysis of the output library generated following exposure to ViII phage identified
183 a limited number of genes with significant numbers of transposon insertions (Table 1). This
184 indicated that relatively few non-essential genes in the genome were required for productive
185 Vi phage propagation and suggested that the phage killing was indeed acting as a strong
186 selection. Importantly, insertion sites within genes of the *viaB* locus (*tviABCDE* and
187 *vexABCDE*), which are directly involved in Vi biosynthesis and secretion (19), were highly
188 represented in the sequence of the output library. These included the positive regulator of
189 *viaB* locus expression (*tviA*), biosynthesis genes (*tviB* to *tviE*) and transport genes (*vexA*-
190 *vexE*). Figure 1A illustrates the extensive insertions that mapped to within the *viaB* locus
191 using DNA from bacteria that were recovered after the ViII phage infection. It is noteworthy
192 that genes adjacent to the *viaB* locus are virtually free of transposon insertions. Selection of a
193 dense set of transposon insertion mutants within the *viaB* locus provided evidence that the
194 method employed to recover mutants that survive ViII phage challenge and the subsequent
195 mapping of the insertions themselves was successful.

196 In total, 37 genes had significantly more transposon insertions than would be expected
197 several of which, such as *ompR* and *envZ* (20-22) and *rcsB* and *rcsC* (23, 24), were
198 previously known to play a role in regulating Vi expression (25, 26). In addition, a number of
199 other novel genes not previously linked to Vi expression were identified in the screen (Fig.
200 1B-E). Transposon insertions were over-represented in the *barA*/*sirA* encoded two-
201 component regulatory system (Figure 1D and 1E). SirA is the cognate response regulator for

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202 the sensor kinase, BarA (6) . Unlike *ompR* and *envZ*, *barA* and *sirA* are located at different
203 regions of the genome in *S*. Typhi. The *yrfF* gene, also previously annotated as *igaA,* was one
204 of the most highly represented genes in terms of the density of transposon insertions (27) (Fig
205 1F). In *S*. Typhimurium, YrfF has been described previously as an attenuator, acting via
206 repression of RcsB and RcsC (27, 28). Mutations in *yrfF* are lethal in isolates of the *S*.
207 Typhimurium serovar (29) but we did not find this to be the case in *S*. Typhi BRD948. The
208 *oxyR* gene, which encodes a LysR-family member, was also identified in the screen (30, 31)
209 as were the transcription elongation factor encoding gene *greA* (32, 33) and elongation factor
210 P encoding gene *efp* (34).

211 **The construction of defined *S*. Typhi BRD948 mutant derivatives.** A number of
212 the genes with significant over-representation of transposon insertion mutations in the
213 TraDIS screen were selected for further analysis by constructing defined deletion mutations
214 in *S*. Typhi BRD948 (see Materials and Methods). We were unable to generate null deletion
215 mutations in several genes including *dnaK* and *parC* and these were not analysed further. *S*.
216 Typhi BRD948 mutant derivatives were first tested for their ability to agglutinate Vi antisera
217 (Table 2). The *S*. Typhi BRD948 derivatives harbouring mutations in *ihfA*, *ihfD*, *actP*, *ppiB,*
218 *efp, greA* and *phoN* exhibited a small or intermediate reduction in agglutination with anti-Vi
219 antisera but were still deemed to be positive. BRD948 derivatives harbouring all other
220 mutants listed in Table 2 were not obviously reproducibly agglutinated with anti-Vi
221 antiserum but these were well agglutinated with anti-O9.

222 The mutant derivatives of BRD948 were next tested for their ability to be infected by
223 the broader Vi phage types ViI (a Myoviridae), ViII (a Siphoviridae) and ViIII to ViVII
224 (Podoviridae). As expected, *S*. Typhi BRD948 derivatives that were agglutinated with anti-Vi
225 antisera such as *efp, ihfA and greA,* were also susceptible to lysis by other Vi-specific phage
226 (Table 2). *S*. Typhi BRD948 harbouring mutations in *envZ*, *rcsB*, *yrfF*, *barA*, *sirA* and *oxyR*
227 were significantly impaired in their ability to support Vi phage propagation irrespective of the
228 phage type.

229 **RNA transcriptome analysis.** A number of the genes identified in the screen located
230 outside of the *viaB* locus encode known or candidate transcriptional regulators and/or cognate
231 environmental sensors. Consequently, we performed microarray-based transcriptomic
232 analysis on independent *S*. Typhi BRD948 derivatives harbouring mutations in these genes to
233 define regulatory networks linked to Vi expression. As expected the *viaB* locus itself was
234 expressed at a lower level by all of these mutant derivatives (Fig 2). The *S*. Typhi BRD948

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235 derivatives harbouring mutations in *rcsB*, *oxyR* or *yrfF* exhibited a profound decrease in
236 expression across the *viaB* locus with a decrease of over 4 Logs compared to the wild type *S*.
237 Typhi BRD948. *S*. Typhi BRD948 derivatives harbouring mutations in *barA*, *sirA* or *greA*
238 had a smaller but significant decrease in *viaB* expression.

239 The number of genes up or down regulated in comparison to wild type *S*. Typhi
240 BRD948 ranged between 80 to over 200 genes in each of the mutant derivatives
241 (Supplementary Tables 3a-f). In addition to *viaB*, a number of other gene categories appeared
242 in more than one of the dysregulated gene lists. Flagella biosynthesis genes exhibited altered
243 expression in several of the *S.* Typhi BRD948 mutant derivatives including *rcsB*, *barA*, *oxyR*
244 and *yrfF*. A number of genes involved in sugar metabolism or transport genes including
245 ribose exhibited altered expression, for example in the *barA*, *sirA* and *oxyR S.* Typhi BRD948
246 mutant derivatives. A number of virulence associated genes and several Type III secretion
247 effector genes were linked to *viaB* expression, including the *srfABC* cluster, *sopE* (*rcsB* and
248 *barA*) and *prgH1* (*sirA* and *oxyR*). A number of other regulator genes were also linked
249 through these networks most notably in the *barA* mutant (Supplementary Table 2a).
250
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252 **DISCUSSION**

253 Here we describe the use of a high throughput, sequence-based mutagenesis screen
254 (TraDIS) to identify genes that are required for efficient Vi expression in *S*. Typhi. ViII
255 phage mediated selection of a *S*. Typhi transposon insertion library containing over one
256 million independent insertions, yielded different classes of Vi phage resistant mutant
257 derivatives. Insertion mutations in genes previously known to be involved in Vi expression
258 were enriched, along with insertions in several genes not previously linked to Vi expression.
259 In our screen each of the genes within the *viaB* locus were identified with a very broad
260 representation of insertion sites following selection (Table 1, Fig. 1). Other key known Vi-
261 regulatory genes such as *ompR* were also identified, along with the regulatory gene *rcsB*.

262 It has been shown previously that the *viaB* locus is differentially regulated compared
263 to flagellin and SPI-1 proteins such as SipB and SipC via RcsB with a role for the *viaB*
264 regulatory protein TviA (26, 35). Here we also identified several regulatory genes that have
265 not previously been associated with Vi expression. Of particular interest was the gene *yrfF*
266 that, according to the pfam database, likely encodes a member of an integral inner membrane
267 protein family (36). This protein is predicted to harbor five transmembrane helices distributed
268 along the protein sequence linked by four soluble loops that could mediate protein/protein
269 interactions via an ankyrin type motif. Interestingly, *yrfF* is also known as 'the intracellular
270 growth attenuator' (*igaA*) in recognition of a role in regulating bacterial growth inside
271 fibroblasts. *yrfF* can interact with the *rcsBCD* regulatory system in other enteric bacteria (27,
272 36, 37). We were able to introduce a large deletion into the *yrfF* gene of *S*. Typhi BRD948,
273 indicating that the phenotype of a *yrfF*/*igaA* mutant in *S*. Typhi differs from that in *S*.
274 Typhimurium, since mutation in this gene is lethal in isolates of the latter (29). Significantly,
275 *E. coli* harbouring mutations in *yrfF*/*igaA* are also viable (38). In *S*. Typhimurium,
276 compensatory deletions in the *rcsBCD* cluster are required to enable an *yrfF* mutant to retain
277 viability (37).

278 Mutations in the regulatory genes *barA* and *sirA* also impacted on the expression of
279 the *viaB* locus in our screen and follow up analysis. *barA* and *sirA* are linked functionally as a
280 two component regulatory system, even though the genes are located at different genomic
281 positions on the *S*. Typhi chromosome. The microarray data showed a decrease in *viaB* locus
282 gene expression of 1 or 2 Logs and reduced but detectable level of Vi polysaccharide
283 expression was detected using anti-Vi antibody with ELISA. The environmental signals
284 recognised by the *barA*/*sirA* system have not been fully defined but are known to include

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285 osmolarity (39). BarA and SirA have previously been demonstrated to have a regulatory role
286 in SPI-1 gene expression (39) and we now report another virulence locus the *viaB*, as part of
287 this regulon (26).

288 The Lys-R family transcriptional regulator OxyR, activates gene expression in
289 response to oxidative stress. OxyR plays a role in influencing bacteriophage susceptibility in
290 *S*. Typhimurium, through mechanisms involving phase variation in genes associated with
291 lipopolysaccharide synthesis (40). Linking Vi expression to the OxyR regulon may have
292 significance for our understanding of the mechanisms of gene regulation exploited by *S*.
293 Typhi as it moves though the tissues of the host during infection or establishes the carrier
294 state in organs such as the gall bladder. Interestingly, *S*. Typhi BRD948 still expresses
295 agglutinable Vi when grown under anaerobic conditions, suggesting other environmental
296 cues may be playing a role in the *oxyR*-mediated regulation of Vi (our unpublished results).
297 More work will be required to reveal the complexity of the link between BarA-SirA, YrfF,
298 OxyR and RcsA-RcsB to Vi expression. This intricate system mirrors observations made
299 concerning the regulation of SPI-1, a further horizontally acquired gene locus that is also
300 regulated by SirA and OmpR (2, 41).

301 Previous studies have shown that the *viaB* locus is encoded on a potentially mobile
302 element known as *Salmonella* Pathogenicity Island 7 (SPI-7) within *S*. Typhi, *S*. Paratyphi C
303 and individual isolates of *S*. Dublin (42). It is intriguing that this genetic system, encoded on
304 horizontally acquired DNA, has integrated into ancestral regulatory networks in *S*. Typhi. It is
305 perhaps significant that many of the transposon insertions impacting Vi expression outside of
306 *viaB* were in regulatory genes. Clearly, Vi expression is modulated in a complex but highly
307 integrated way in *S*. Typhi. Vi antigen itself is a key component of human typhoid vaccines
308 so it will be critical to monitor typhoid endemic sites where such vaccines are introduced for
309 any escape mutants (9, 43). Vi is known to potentiate the virulence of *S*. Typhi but it is not
310 essential for infection (44). Further, Vi-negative *S*. Typhi have been reported from clinical
311 typhoid cases in some parts of the world (43). Finally, we believe that this high throughput
312 and high density genetic screen could have value for mapping similar genetic networks
313 involved in the expression of surface components on other bacteria. Such screens could find
314 particular utility in antibody killing assays employed in pre- or post- clinical testing of
315 vaccines where bacterial surface antigens are essential components. Such assays could have
316 value for identifying potential genetic escape routes from vaccination.
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319

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323

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Figure 1

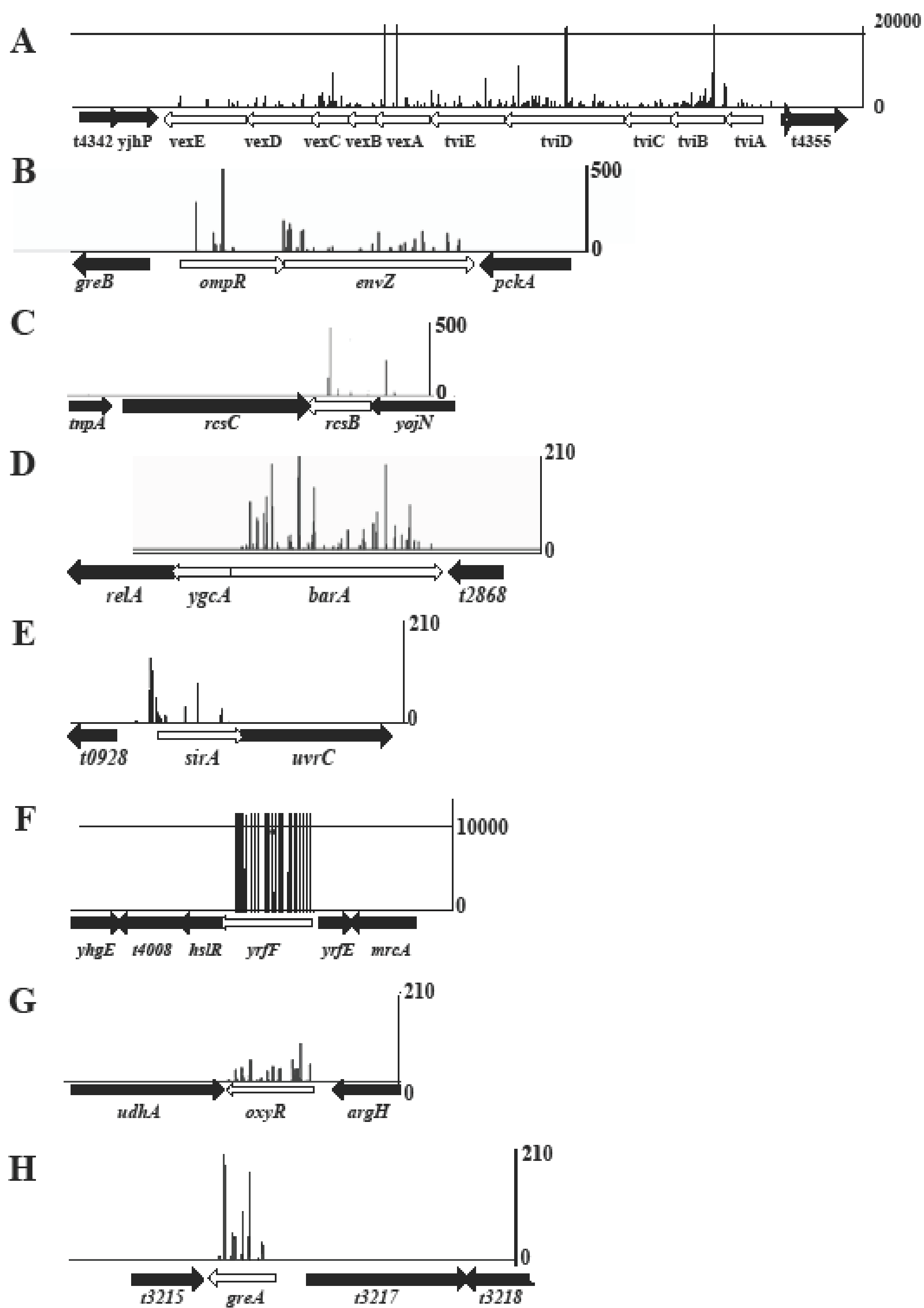

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Figure 2

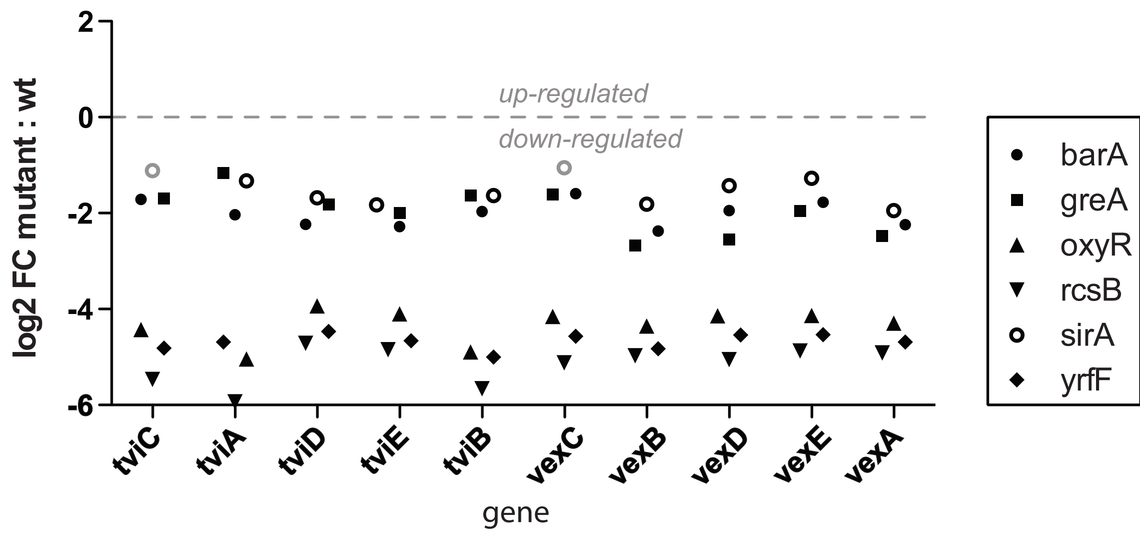

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Table 1- A summary of the genes with statistically significant transposon insertions recovered from the *S*. Typhi TraDIS library pool after infection by bacteriophage ViII.

Sys ID	Gene name	Input inserts	Output inserts	Input reads	Output reads	Log2 read ratio	P-value	Function
t4352	*tviB*	307	285	11914	1646207	-7.10	3x10-51	Vi polysaccharide biosynthesis, UDP-glucose/GDP-mannose dehydrogenase
t4349	*tviE*	280	209	4659	592697	-6.96	1.28E-49	Vi polysaccharide biosynthesis TviE
t4353	*tviA*	89	78	1721	184485	-6.66	2.79E-46	Vi polysaccharide biosynthesis regulator
t4350	*tviD*	627	540	22768	1870584	-6.35	6.18E-43	Vi polysaccharide biosynthesis
t4011	*yrfF*	190	112	1263	94290	-6.11	1.99E-40	putative membrane protein
t4344	*vexE*	141	81	3246	219685	-6.04	1.20E-39	Vi polysaccharide export protein
t4351	*tviC*	224	164	5856	332427	-5.80	2.68E-37	Vi polysaccharide biosynthesis protein, epimerase
t4347	*vexB*	168	131	5269	286393	-5.74	1.17E-36	Vi polysaccharide export inner-membrane protein
t4345	*vexD*	218	159	6661	325843	-5.59	3.05E-35	Vi polysaccharide export inner-membrane protein
t4348	*vexA*	206	164	9690	470499	-5.59	3.34E-35	Vi polysaccharide export protein
t4179	*actP*	94	5	1216	49412	-5.23	6.59E-32	Sodium:solute symporter family protein
t4346	*vexC*	177	142	18227	620241	-5.08	1.54E-30	Vi polysaccharide export ATP-binding protein
t4225	*phoN*	208	3	5341	87540	-4.01	8.23E-22	nonspecific acid phosphatase precursor
t4209	*dcuB*	71	5	2371	39400	-4.00	9.93E-22	anaerobic C4-dicarboxylate transporter
t1220	*ihfA*	8	2	60	2186	-3.84	1.51E-20	integration host factor alpha-subunit
t4362	-	246	2	4107	55798	-3.73	8.40E-20	putative membrane protein
t4004	*ompR*	47	16	272	4392	-3.59	7.64E-19	two-component response regulator OmpR
t4356	-	271	7	5186	60540	-3.52	2.44E-18	hypothetical protein
t4268	-	169	4	2629	30184	-3.47	5.12E-18	putative exported protein
t3216	*greA*	32	16	527	5488	-3.16	5.74E-16	transcription elongation factor
t0012	*dnaK*	5	3	14	862	-3.08	1.77E-15	DnaK protein
t4005	*envZ*	77	35	602	3708	-2.44	7.98E-12	two-component sensor kinase EnvZ
t4386	*efp*	51	17	300	1689	-2.16	2.12E-10	elongation factor P
t2867	*barA*	140	65	1780	8225	-2.15	2.50E-10	sensor protein
t3205	*nusA*	18	5	108	743	-2.02	1.03E-09	L factor

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t4313	-	27	1	220	968	-1.74	1.95E-08	putative membrane protein
t0929	*sirA*	36	18	263	964	-1.55	1.22E-07	invasion response-regulator
t3500	*oxyR*	66	30	717	1853	-1.26	1.73E-06	hydrogen peroxide-inducible regulon activator
t3474	*rpoB*	5	1	9	159	-1.25	1.87E-06	DNA-directed RNA polymerase, beta-subunit
t1627	*topA*	66	12	280	591	-0.86	4.06E-05	DNA topoisomerase I, omega protein I
t2325	*ppiB*	8	3	50	153	-0.75	8.91E-05	peptidyl-prolyl cis-trans isomerase B
t3095	*parC*	1	1	12	83	-0.71	1.23E-04	topoisomerase IV subunit A
t1238	-	78	2	788	1284	-0.64	1.96E-04	conserved hypothetical protein
t0013	*dnaJ*	38	12	234	419	-0.64	2.01E-04	DnaJ protein
t0595	*rcsB*	28	6	180	318	-0.58	2.96E-04	regulator of capsule synthesis B component
t1952	*ihfB*	16	2	102	183	-0.49	5.32E-04	integration host factor beta-subunit
t4219	-	46	2	2902	4090	-0.48	5.51E-04	hypothetical protein

Sys ID refers to the gene number in the sequenced *S*. Typhi Ty2 genome (Accession number NC_004631). The gene name and function are taken from this annotation. ‘Input inserts’ and ‘input reads’ refer to the *S*. Typhi TraDIS pool at T=0 (just before phage addition) while ‘output inserts’ and ‘reads’ refers to the recovered pool after treatment with *S*. Typhi ViII phage. The data was analysed to identify those genes that are statistically over represented in the output pool (Log2 read ratio and P-value)

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Table 2- Summary of Vi expression levels in the *S.* Typhi BRD948 and the various mutant derivatives.

Gene	**Slide agglutination profile**	**Type I**	**Type II**	**Type IV**	**Type VI and VII**	**Vi ELISA Titre**
yrlF	O9 +++ Vi-	-	-	-	-	<2
rcsB	O9 +++ Vi -	-	-	-	-	<2
oxyR	O9 +++ Vi -	-	-	-	-	<2
sirA	O9 +++ Vi +/-	+/-	-	-	+/-	ND
envZ	O9 +++ Vi +	+	+	+	+	128
barA	O9 +++ Vi +	+	+	+	+	256
greA	O9 +++ Vi ++	++	++	++	++	256
efp	O9 +++ Vi ++	++	++	++	++	512
ihfA	O9 +++ Vi +++	++/+++	++/+++	++/+++	+++	1024
ppiB	O9 - Vi +++	++	++	++	++	ND
ihfB	O9 +/- Vi +++	+++	+++	+	+++	ND
actP	O9 - Vi +++	++++	++++	++++	++++	ND
phoN	O9 - Vi +++	++++	++++	++++	++++	ND
BRD948	O9 - Vi +++	++++	++++	++++	++++	>2048

++++ indicates response equivalent to wild type *S.* Typhi BRD948. The number of + symbols indicates relative strength of the response. – no response. ND is not determined.