

1 **Aurodox, a polyketide from *Streptomyces goldiniensis*, inhibits transcription of the type III**  
2 **secretion system of multiple Gram-negative pathogens**

3

4 David Mark<sup>1</sup>, Nicky O'Boyle<sup>2</sup>, Kabo R Wale<sup>1,3</sup>, Samantha K Tucker<sup>1</sup>, Rebecca E McHugh and  
5 Andrew J Roe<sup>1</sup>.

6 1. School of Infection and Immunity, University of Glasgow, Glasgow, G12 8TA, UK.

7 2. School of Microbiology, University College Cork, National University of Ireland, Cork T12  
8 K8AF, Ireland

9 3. Biological Sciences, University of Botswana, Gaborone, Botswana.

10

11

12 **Key words:** aurodox, polyketide, virulence, type three secretion system, infection

13

14

15 **Abstract**

16 Gram-negative pathogens pose a significant threat due to their propensity for causing various  
17 infections, often coupled with formidable resistance to conventional antibiotic treatments. In light  
18 of this challenge, the development of antivirulence (AV) compounds emerges as a promising  
19 alternative strategy, aiming to disrupt key virulence mechanisms rather than directly targeting  
20 bacterial viability. One such compound, aurodox, derived from *Streptomyces goldiniensis*, has  
21 exhibited promising AV properties in our prior studies. Specifically, aurodox caused a marked  
22 downregulation in the expression and function of the *E. coli* type 3 secretion system (T3SS), a  
23 needle-like injectosome structure which is deployed to translocate effector proteins from the  
24 cytoplasm to the host target cells.

25 However, the broader spectrum of aurodox's efficacy against T3SS across diverse pathogens  
26 remained unanswered, prompting the focus of this work. Using quantitative real-time PCR, we  
27 show that aurodox exerts inhibitory effects on selected T3SS in various pathogens, including  
28 *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, and *Vibrio parahaemolyticus*. However,  
29 aurodox was not a universal blocker of all secretion systems, showing selectivity in its mode-of-  
30 action, even within a single strain. This finding was verified using transcriptomics which  
31 demonstrated that aurodox selectively blocks the expression of the *Salmonella typhimurium* SPI-  
32 2 type T3SS whilst other pathogenicity islands, including the SPI-1 system were not inhibited. To  
33 delve deeper into the mechanisms governing aurodox's efficacy against these pathogens, we  
34 analysed transcriptomic datasets from both *E. coli* and *S. Typhimurium* treated with aurodox. By  
35 identifying orthologous genes exhibiting differential expression in response to aurodox treatment  
36 across these pathogens, our study sheds light on the potential mechanisms underlying the action  
37 of this rediscovered antibiotic.

38

39

40

41

42

43

44 **Importance**

45 New treatments to address antibiotic resistance pathogens are urgently needed. Aurodox, a linear  
46 polyketide produced by *Streptomyces goldiniensis* has previously been shown to be able to  
47 downregulate the expression of a critically important virulence factor, the type three secretion  
48 system of *E. coli* and also block host cell colonization. We have explored the wider ability of  
49 aurodox to block type three secretion in other species and show that it is capable of blocking the  
50 function of T3SSs in further pathogens thereby markedly expanding the known range of pathogens  
51 that this compound may be used to combat. This study also shows that aurodox specifically targets  
52 SPI-2, a subtype of T3SS crucial for Salmonella persistence within host cells whilst not affecting  
53 the SPI-1 system. This finding implies that aurodox likely works through a conserved mechanism  
54 and helps reveal insights underlying the action of this rediscovered antibiotic.

55

56

57

58

59

60

61

62

63

64

65

66 Gram-negative pathogens (GNPs) pose a serious threat to public health due to their ability to cause  
67 a range of infections whilst exhibiting high levels of resistance to antibiotic therapies<sup>1</sup>. One potential  
68 strategy to treat GNPs is the use antivirulence (AV) compounds. The mode of action of these  
69 compounds differs from traditional antibiotics as they are not designed to kill or inhibit the infecting  
70 organism, but simply to inactivate virulence mechanisms. One such AV target is the type 3  
71 secretion system (T3SS), a needle-like injectosome structure which is deployed to translocate  
72 effector proteins from the cytoplasm to the host target cells<sup>2,3</sup>.

73 Previously, we reported the antivirulence activity of aurodox, a metabolite produced from  
74 *Streptomyces goldiniensis*<sup>4-7</sup>. In our studies, aurodox abolished T3S in EHEC, EPEC and  
75 *Citrobacter rodentium*. As the activity of aurodox in these pathogens is dependent on the inhibition  
76 of the master virulence regulator, *ler*<sup>6</sup>, we had proposed that only pathogens carrying a LEE-  
77 encoded, *ler*-regulated T3SS would display susceptibility to the antivirulence effects of aurodox.  
78 To test this hypothesis, we undertook the screening of aurodox against additional GNPs pathogens  
79 that carry T3SSs that are phylogenetically distinct and not regulated by *ler*. A range of enteric  
80 pathogens encoding T3SSs were selected. These were *Salmonella enterica ssp enterica* serovar  
81 Typhimurium, which encodes two distinct T3SS: SPI-1 and SPI-2, *Yersinia pseudotuberculosis*  
82 which encodes a Ysc-type T3SS and *Vibrio parahaemolyticus*, which carries two T3SS (VPTTSS1  
83 and VPTTSS2)<sup>2</sup>.

84 Expression of specific T3SS effectors in each pathogen in response to aurodox was measured  
85 using qRT-PCR (Figure 1A-C). In *S. Typhimurium*, we observed downregulation of the SPI-2  
86 effector protein *sseB* (Figure 1A, >23-fold reduction in SPI-2 inducing media,  $p < 0.0001$ ), and  
87 marginal upregulation of the expression of the SPI-1 effector *sipC* (0.35-fold change in SPI-1  
88 inducing media,  $p = 0.08$ ). In *Vibrio parahaemolyticus*, aurodox treatment results in a significant  
89 reduction in the expression of *vopD* (Figure 1B, >90-fold reduction,  $p = 0.002$ ) however expression  
90 of *vopD2* remained unaffected (0.91-fold change,  $p = 0.97$ ). Finally, in *Yersinia pseudotuberculosis*,  
91 aurodox downregulated the expression of the Ysc-type effector *yopD* (Figure 1C, >6-fold reduction,  
92  $p = 0.015$ ). As we observed a range of activity across multiple enteric pathogens, these data reveal  
93 that aurodox activity is not limited to LEE-encoded, *ler*-regulated T3SSs.

94 The differential repression of SPI-1 and SPI-2 in *S. Typhimurium* was further analysed using  
95 transcriptional GFP reporter assays<sup>8</sup>. For SPI-1, the expression of the *prgH* promoter (which  
96 normally drives expression of a T3SS structural protein) was measured over a six-hour time course  
97 (Figure 1D). Similarly, a reporter driven by the *ssaG* promoter was used to measure the  
98 transcriptional response of the SPI-2 structural gene to aurodox treatment. Addition of aurodox  
99 does not affect *prgH* expression (SPI-1) during the assay, whereas in contrast, there was a marked  
100 downregulation of *ssaG* (SPI-2) (Figure 1E). As a control, the ribosomal promoter, *rpsM*, was used  
101 for comparison which showed less than 5% variation between aurodox treated and untreated cells.  
102 Importantly, the growth of *Salmonella* was not significantly affected until aurodox concentrations  
103 of 8  $\mu\text{g}\cdot\text{ml}^{-1}$  were used, which is higher than was required to inhibit SPI-2 (Figure S1). These data  
104 demonstrate that the T3SS-inhibitory activity of aurodox is not limited to LEE-encoded, *ler*-  
105 regulated T3SSs, and confirms that aurodox has a specific inhibitory effect on the SPI-2 T3SS in  
106 *S. Typhimurium*. Moreover, aurodox is not a “universal” blocker of T3SSs.

107 During *Salmonella Typhimurium* infection, the SPI-2 T3SS is responsible for maintaining  
108 *Salmonella* within *Salmonella*-containing vacuoles (SCVs) within intestinal epithelial cells and  
109 macrophages. To determine whether the inhibitory effects of aurodox observed for SPI-2 could  
110 suppress this activity, the compound was tested in an *in vitro* macrophage model. RAW 267.4  
111 macrophages were infected with *S. Typhimurium* constitutively expressing GFP to aid  
112 visualization. Aurodox treatment significantly reduced the number of RAW cells infected by  
113 *Salmonella*, with 32% of DMSO treated cells infected compared to 6% of aurodox treated cells (>5  
114 fold reduction,  $p < 0.00001$ ) (Figure 1F). Consistent with this change in infection levels,  
115 morphological changes associated with *Salmonella* invasion were reduced in response to aurodox  
116 treatment (Figure 1G-I). These results demonstrate that aurodox can exhibit its effect during the  
117 intracellular phase of *Salmonella* pathogenesis, resulting in a reduction in pathogen burden within  
118 the macrophage.

119 The differential effect on SPI-2 over SPI-1 raised the question of how aurodox affects transcription  
120 more widely across the genome. To investigate this, whole transcriptome sequencing of aurodox-  
121 treated *S. Typhimurium* was carried out. Triplicate cultures were grown in SPI-2-inducing media  
122 with either 5  $\mu\text{g}/\text{ml}$  aurodox or DMSO. RNA was extracted from each after one hour and converted

123 to cDNA for transcriptomic analysis. Transcripts were mapped to the reference genome and mean  
124 fold change and p values calculated. Overall, 11.5% of the genome was differentially expressed in  
125 response to aurodox treatment, with 334 genes downregulated and 238 upregulated when  
126 compared to the DMSO-treated control (Figure 2A). Differentially expressed genes were identified  
127 within the chromosome and three plasmids (pCol1B9, pRSF1010 and pSLT; Figure S2). This  
128 analysis revealed that all 32 genes encoded within the SPI-2 pathogenicity island were  
129 downregulated in response to aurodox treatment, with the entire pathogenicity island showing  
130 statistical significance based on *EDGE test-derived* P value (Figure 2B). These analyses confirm  
131 the results of qRT-PCR and GFP-reporter assays as they demonstrate that SPI-2 is downregulated  
132 in its entirety in response to aurodox.

133 Additionally, gene expression patterns of an additional 12 pathogenicity islands within *S.*  
134 *Typhimurium* were examined in response to aurodox. This revealed that SPI-2 is the only  
135 pathogenicity island which is transcriptionally downregulated in response to aurodox treatment  
136 (Figure S3). Three genes in SPI 1 (*sicA*, *sipB*, and *hilC*) were affected and showed a degree of  
137 upregulation.

138 To gain a clearer understanding of the mode-of-action of aurodox, we identified orthologous genes  
139 which were differentially expressed in response to aurodox in both EHEC<sup>6</sup> and *S. Typhimurium*  
140 (Figure S4). From this analysis of the whole transcriptome sequencing 17 orthologous genes were  
141 identified, with five genes commonly upregulated and 12 genes downregulated. Several genes  
142 which have previously been shown to be involved in virulence regulation were identified, including  
143 the alcohol dehydrogenase-encoding gene *adhE*. This finding was of interest because we  
144 previously showed that deletion of *adhE* can affect the expression of the T3SS in EHEC<sup>9</sup>.  
145 Additionally, multiple amino acid biosynthesis genes were upregulated including the *leu* operon  
146 encoding leucine biosynthesis and *met* operon encoding methionine. These analyses identified  
147 multiple orthologs which are differentially expressed. To establish a clear link between their altered  
148 expression and the observed modulation of virulence will require further experiments.

149 Of the five T3SSs examined in this study, aurodox was found to inhibit effector expression in three,  
150 all of which clustered within the SPI-2 and Ysc phylotypes. In addition, the LEE-encoded T3SSs  
151 from EPEC, EHEC and *C. rodentium* which were found to be downregulated by aurodox in our

152 previous study, can also be assigned to the SPI-2 phylogroup<sup>10</sup>. From a phylogenetic tree  
153 constructed using core T3SS proteins from multiple GNPs (Figure 2C), we observed that the SPI-  
154 2 and Ysc T3SS cluster within one clade. This finding suggests that phylogeny may well be a  
155 predictor of aurodox activity. To test this hypothesis, more expansive testing of species within the  
156 SPI-2/Ysc clade is required. This should include piscine isolates within this clade, including  
157 *Aeromonas salmonicida*<sup>11</sup> and *Edwardsiella ictaluri*<sup>12</sup> as they are significant aquacultural pathogens.

158

## 159 **Conclusion**

160 We have shown that aurodox selectively inhibits expression of specific T3SS in different  
161 pathogens. In this work we demonstrate activity against *Salmonella typhimurium*, *Yersinia*  
162 *pseudotuberculosis*, and *Vibrio parahaemolyticus*. However, aurodox does not universally block  
163 all T3SS and has a specific inhibitory effect on the SPI-2 T3SS in *Salmonella Typhimurium*. We  
164 also note a phylogroup-dependent activity and a preference for SPI-2 and Ysc phylogroups. The  
165 research highlights the potential of aurodox as a promising compound to combat antibiotic-  
166 resistant pathogens by targeting virulence mechanisms rather than bacterial viability.

167

168

169

170

171

172

173

174

175

176

177

178

179

180

181 **Acknowledgements**

182 We would like to acknowledge funding from the Wellcome Trust Confidence in Concept scheme  
183 for funding work by DM and NOB and the Wellcome Trust Integrated Infection Biology PhD  
184 Scheme for funding the PhD of SKT. We like would like to thank the Medical Research Council  
185 (MR/V011499/1) for funding REM and AJR. For the purpose of open access, the authors have  
186 applied a Creative Commons Attribution (CC BY) license to any Author Accepted Manuscript  
187 version arising from this submission.

188



189 **Figure legends**

190 Figure 1. Aurodox inhibits Type III secretion in multiple GNPs. (A-C) Q-RT-PCR data show that  
191 aurodox inhibited the expression of multiple T3SS effectors including *sseB* of the *Salmonella*  
192 Typhimurium SPI-2 TTSS, in SPI-2 inducing conditions (A), *vopD*, secreted by the *Vibrio*  
193 *parahemolyticus* TTSS1 (B), and *yopD* of *Yersinia pseudotuberculosis* (C). It did not, however,  
194 repress expression of *vopD2* of the *V. parahemolyticus* TTSS2 or *sipC* of *S. Typhimurium*'s SPI-  
195 1; \*P<0.05 by One-way ANOVA and Tukey's post-hoc test. Fig 1D & E: transcriptional reporter  
196 assays in *Salmonella* Typhimurium measuring GFP accumulation from the SPI-1 encoded *prgH*  
197 promoter (D) or SPI-2 encoded *ssaG* promoter (E). Bacteria were grown to early stationary phase  
198 in LB before being switched to inducing media ± aurodox. Aurodox prevented induction of *ssaG* in  
199 SPI-2 inducing media, but had no effect on the expression of *prgH*. (F-I) Infection assays showing  
200 the protective effect of aurodox against infection of RAW 246.7 Macrophage-like cells by  
201 *Salmonella* Typhimurium. After infection in the presence of DMSO, 32% of cells contained  
202 *Salmonella*-containing vesicles, compared to only 6% of Aurodox treated cells. (G) Representative  
203 image of DMSO treated cell, with SCVs highlighted (H). (I) Representative image of aurodox  
204 treated cells.

205  
206 Figure 2. The T3SS inhibiting effects of aurodox may be phylogenetically constrained. (A) Volcano  
207 plot showing the global effect of aurodox on the *S. Typhimurium* transcriptome. Genes denoted by  
208 a plus (+) are encoded on the SPI-2 pathogenicity island, whilst genes encoded by a cross (×) are  
209 encoded on SPI-1 (B) Maps of SPI-1 and SPI-2 illustrating the SPI-2-selectivity in aurodox  
210 mediated inhibition of Type III Secretion. (C) Maximum-likelihood phylogenetic tree of selected  
211 Type III secretion systems, including those tested against aurodox. We observe that all susceptible  
212 T3SSes were placed into a single clade containing the Ysc and SPI-2 phylogroups. Tree generated  
213 using IQ-tree, with ModelFinder and 1000 ultrafast bootstraps.

214  
215 Figure S1: Growth of *Salmonella* Typhimurium is not affected by aurodox.

216

217 Figure S2: Aurodox affects gene expression at both the chromosome and plasmid level. Map of  
218 the *Salmonella* Typhimurium SL1344 replicons. Inhibition of expression was seen on all replicons,  
219 except pSLT. We note that expression of SPI-2 effectors encoded on pCol1B9 occurred after  
220 aurodox treatment, suggesting polar effects from inhibition of the secretion system.

221

222 Figure S3: The effect of aurodox on pathogenicity island gene expression is constrained to SPI-2.  
223 Maps of the SPI pathogenicity islands encoded on the *Salmonella* Typhimurium chromosome. SPI-  
224 2 was the most strongly inhibited island by aurodox treatment.

225

226 Figure S4: Orthologous genes differentially expressed in EHEC and *Salmonella* Typhimurium post  
227 aurodox treatment. Upregulated and downregulated genes identified from EHEC or *S.*  
228 Typhimurium in response to aurodox were analysed using the BLAST+ Reciprocal best hits tool

229

230

231

232

233 **References**

- 234 1 Asokan, G. V., Ramadhan, T., Ahmed, E. & Sanad, H. WHO global priority pathogens list:  
235 A bibliometric analysis of medline-pubmed for knowledge mobilization to infection  
236 prevention and control practices in Bahrain. *Oman Medical Journal* **34**, 184-193 (2019).  
237 <https://doi.org/10.5001/omj.2019.37>
- 238 2 Notti, R. Q. & Stebbins, C. E. The Structure and Function of Type III Secretion Systems.  
239 *Microbiol Spectr* **4** (2016). <https://doi.org/10.1128/microbiolspec.VMBF-0004-2015>
- 240 3 Costa, T. R. D. *et al.* (2015).
- 241 4 McHugh, R. E. *et al.* Biosynthesis of aurodox, a Type III secretion system inhibitor from  
242 *Streptomyces goldiniensis*. *Applied and Environmental Microbiology* (2022).
- 243 5 McHugh, R. E., Munnoch, J. T., Roe, A. J. & Hoskisson, P. A. Genome sequence of the  
244 aurodox-producing bacterium *Streptomyces goldiniensis* ATCC 21386. *Access*  
245 *Microbiology* **4**, 1-5 (2022). <https://doi.org/10.1099/acmi.0.000358>
- 246 6 McHugh, R. E., O'Boyle, N., Connolly, J. P. R., Hoskisson, P. A. & Roe, A. J.  
247 Characterization of the mode of action of aurodox, a type III secretion system inhibitor from  
248 *streptomyces goldiniensis*. *Infection and Immunity* (2019).  
249 <https://doi.org/10.1128/IAI.00595-18>
- 250 7 Kimura, K. *et al.* A small-molecule inhibitor of the bacterial type III secretion system  
251 protects against in vivo infection with *Citrobacter rodentium*. *The Journal of antibiotics* **64**,  
252 197-203 (2011). <https://doi.org/10.1038/ja.2010.155>
- 253 8 Hautefort, I., Proenca, M. J. & Hinton, J. C. Single-copy green fluorescent protein gene  
254 fusions allow accurate measurement of *Salmonella* gene expression in vitro and during  
255 infection of mammalian cells. *Appl Environ Microbiol* **69**, 7480-7491 (2003).  
256 <https://doi.org/10.1128/AEM.69.12.7480-7491.2003>
- 257 9 Beckham, K. S. H. *et al.* The metabolic enzyme AdhE controls the virulence of *Escherichia*  
258 *coli*O157: H7. *Molecular Microbiology* **93**, 199-211 (2014).  
259 <https://doi.org/10.1111/mmi.12651>

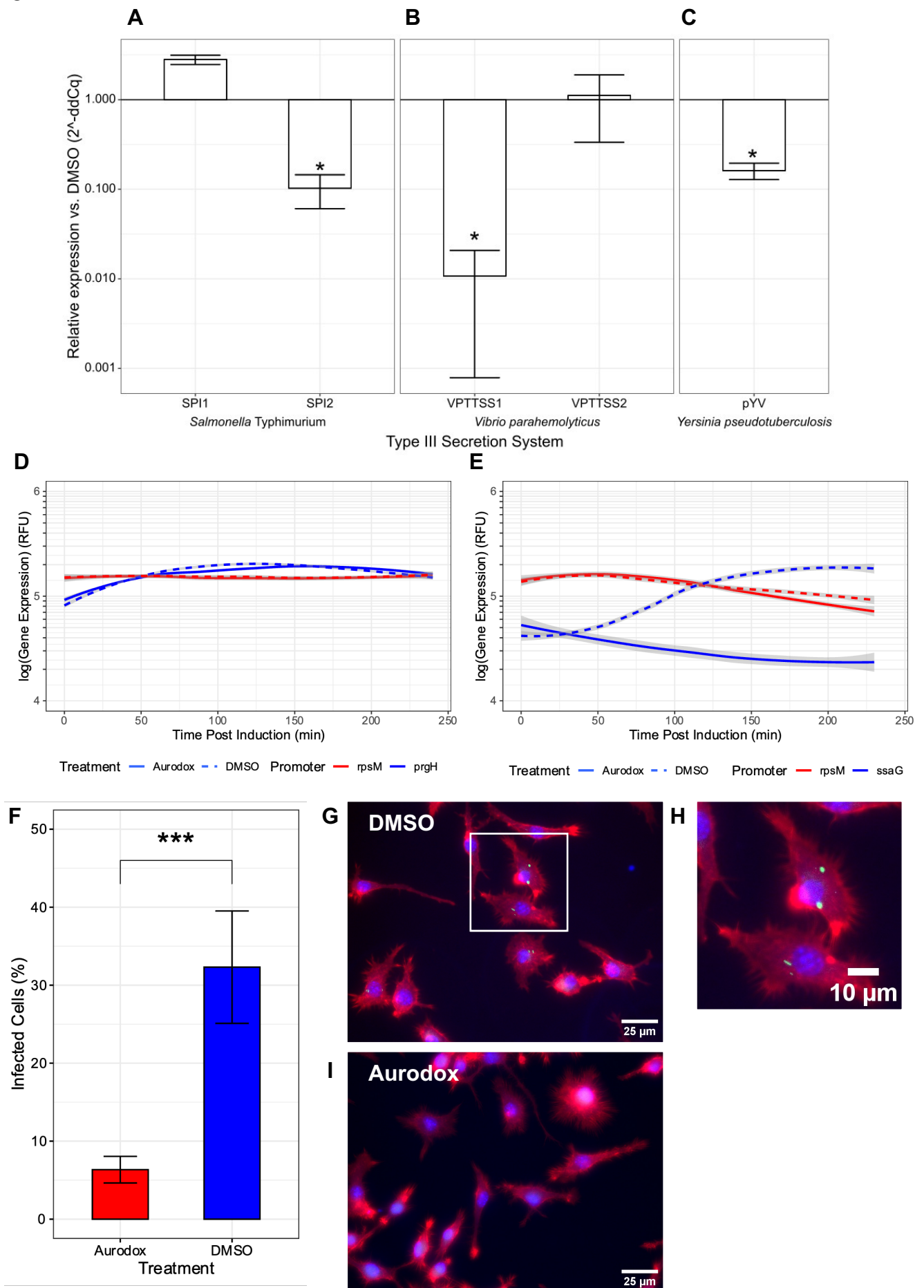
260 10 Abby, S. S. & Rocha, E. P. The non-flagellar type III secretion system evolved from the  
261 bacterial flagellum and diversified into host-cell adapted systems. *PLoS Genet* **8**,  
262 e1002983 (2012). <https://doi.org/10.1371/journal.pgen.1002983>

263 11 Frey, J. & Origgi, F. C. Type III Secretion System of  
264 Undermining the Host's Immune Response. *Frontiers in Marine Science* **3** (2016).  
265 <https://doi.org/ARTN> 130  
266 10.3389/fmars.2016.00130

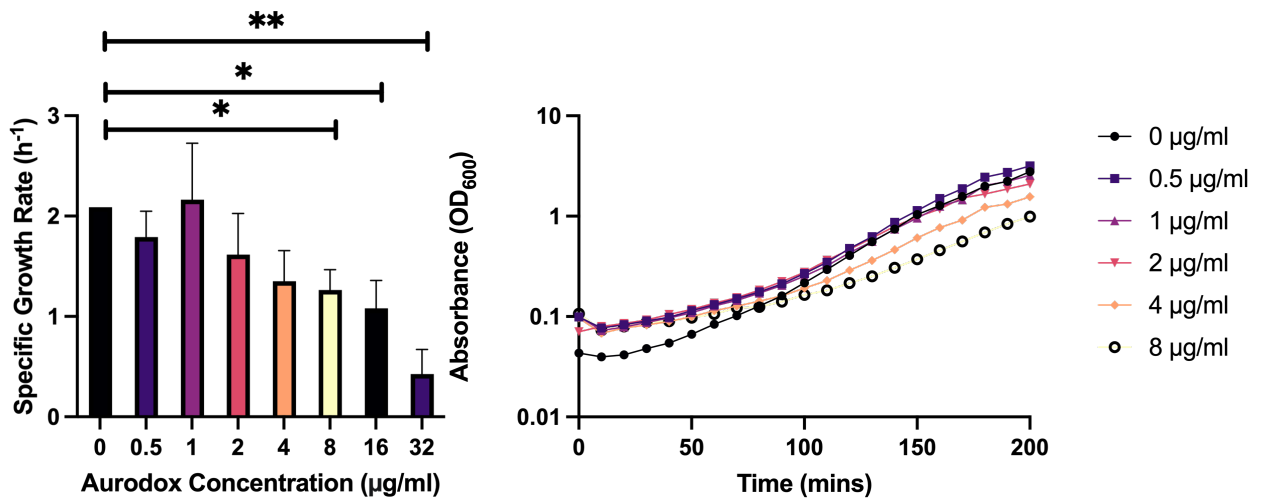
267 12 Rogge, M. L. & Thune, R. L. Regulation of the  
268 Type III Secretion System by pH and Phosphate Concentration through EsrA, EsrB, and EsrC.  
269 *Applied and Environmental Microbiology* **77**, 4293-4302 (2011).  
270 <https://doi.org/10.1128/Aem.00195-11>

271

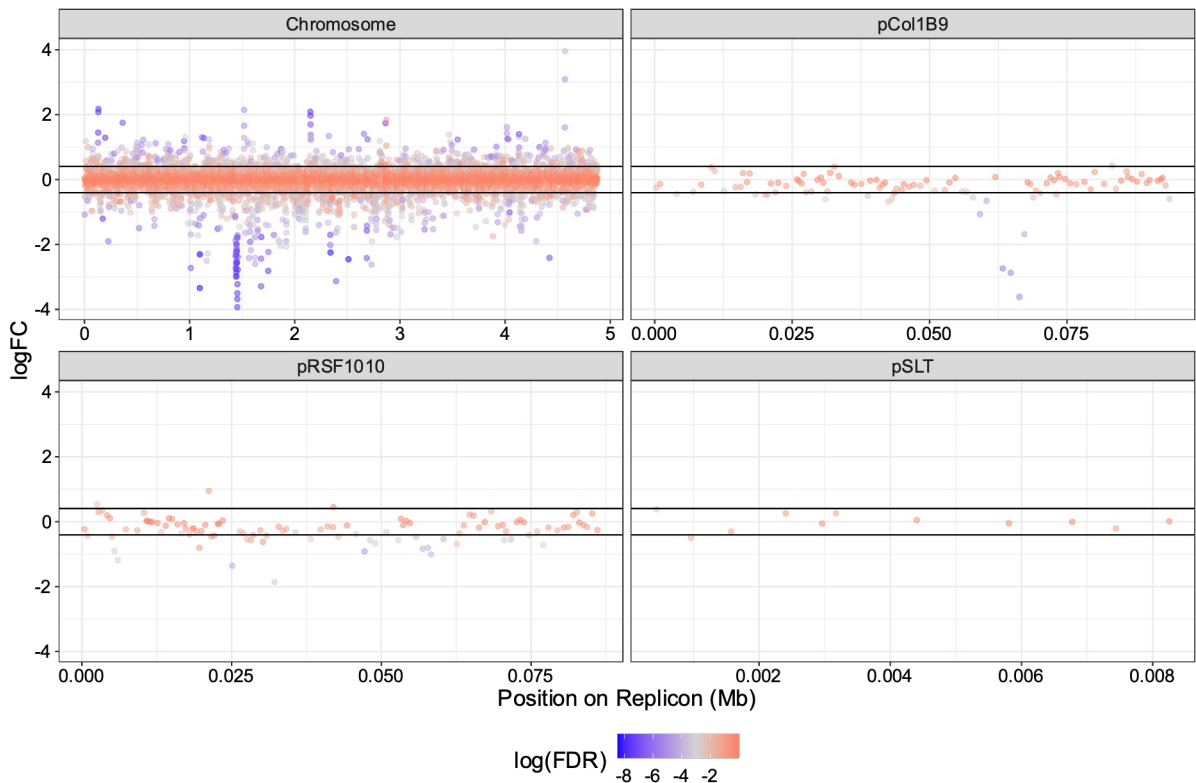
Figure 1







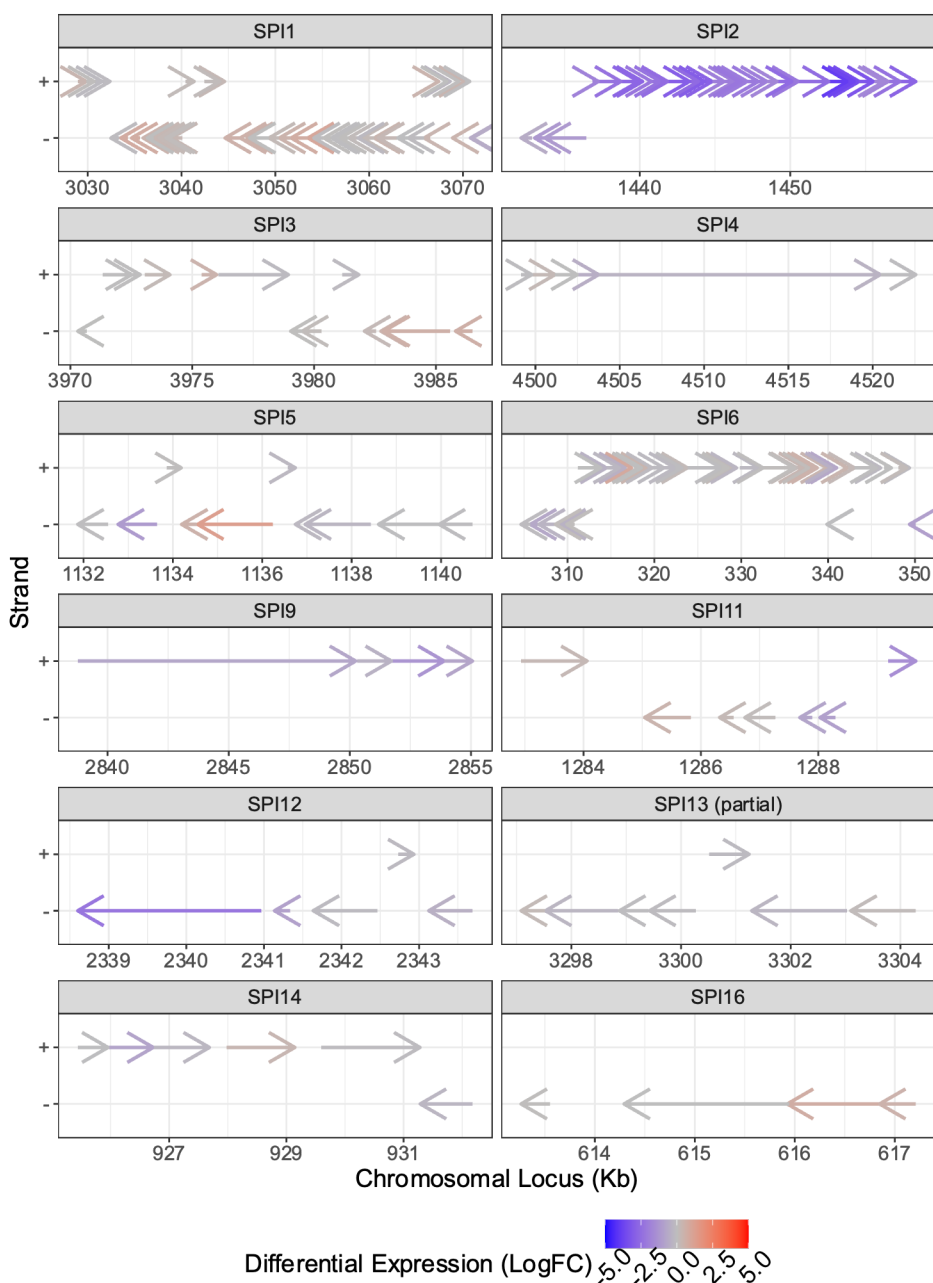
**Figure S1: Growth of *Salmonella* Typhimurium is not affected by aurodox**



**Figure S2: Aurodox affects gene expression at both the chromosome and plasmid level**

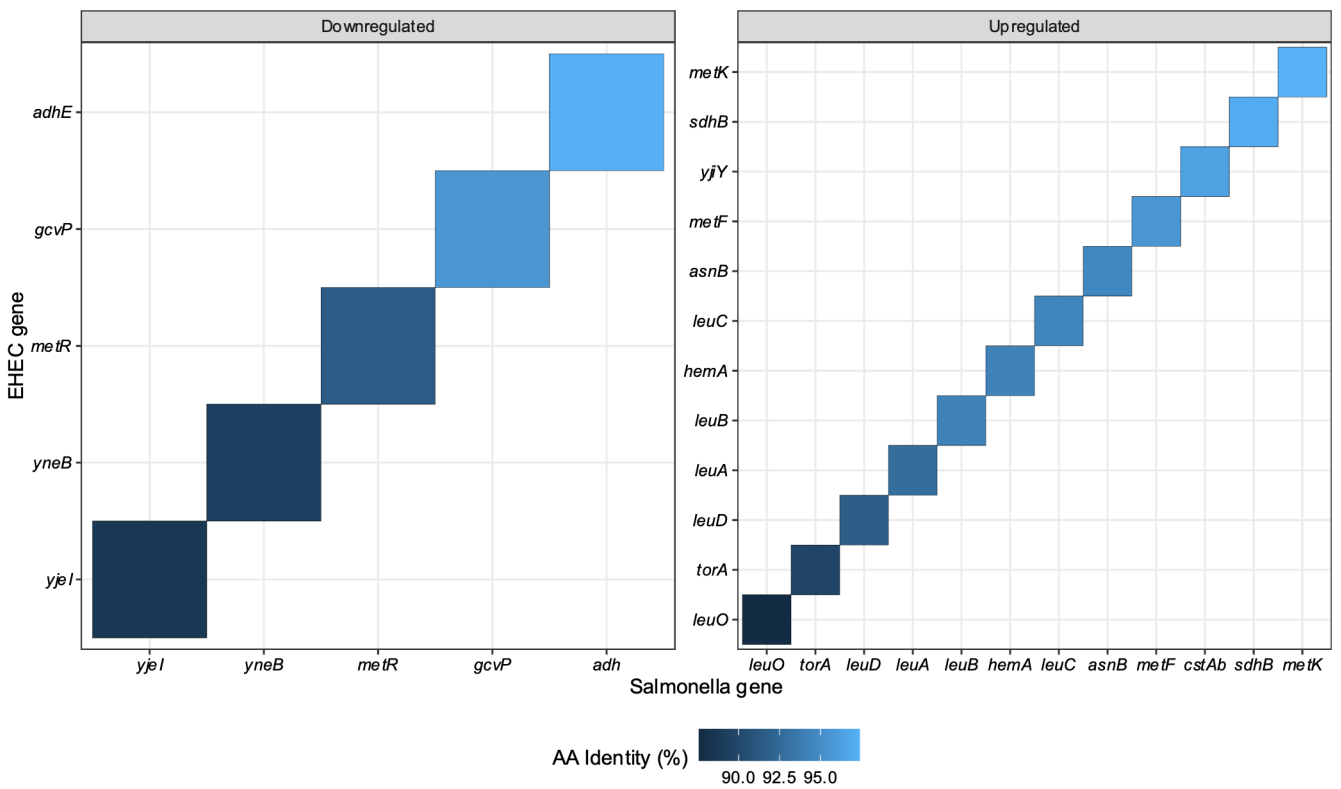
Map of the *Salmonella* Typhimurium SL1344 replicons. Inhibition of expression was seen on all replicons, except pSLT. We note that expression of SPI-2 effectors encoded on pCol1B9 occurred after aurodox treatment, suggesting polar effects from inhibition of the secretion system.





**Figure S3: The effect of aurodox on pathogenicity island gene expression is constrained to SPI-2**

Maps of the SPI pathogenicity islands encoded on the *Salmonella* Typhimurium chromosome. SPI-2 was the most strongly inhibited island by aurodox treatment.



**Figure S4: Orthologous genes differentially expressed in EHEC and *Salmonella* Typhimurium post aurodox treatment.** Upregulated and downregulated genes identified from EHEC or *S. Typhimurium* in response to aurodox were analysed using the BLAST+ Reciprocal best hits tool