

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

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

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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.

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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.

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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 therapies¹. 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^{2,3}.

73 Previously, we reported the antivirulence activity of aurodox, a metabolite produced from
74 *Streptomyces goldiniensis*⁴⁻⁷. 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*⁶, 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)².

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 assays⁸. 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 EHEC⁶ 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 EHEC⁹.
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 phylogroup¹⁰. 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*¹¹ and *Edwardsiella ictaluri*¹² as they are significant aquacultural pathogens.

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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.

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187 version arising from this submission.

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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.

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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

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Figure 1

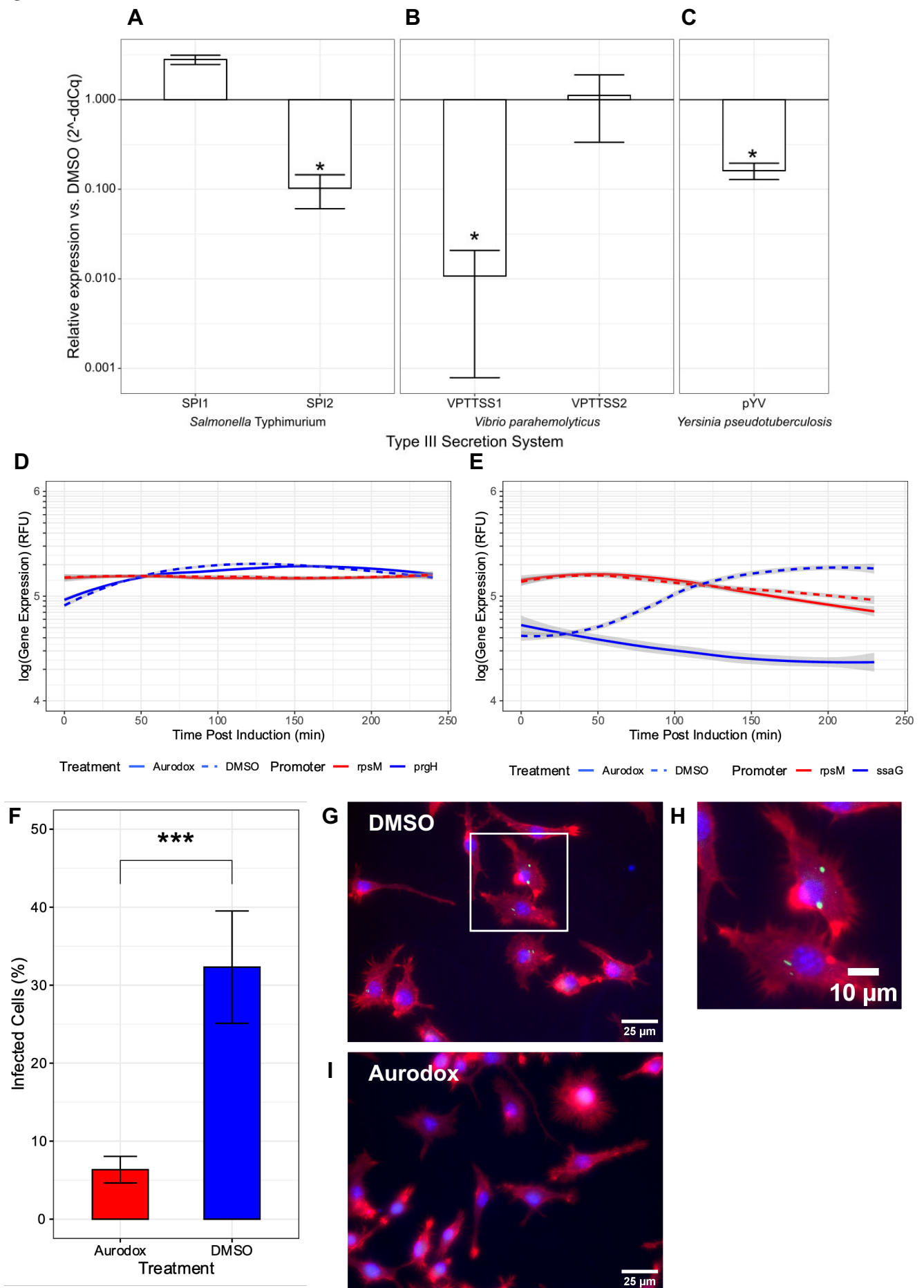
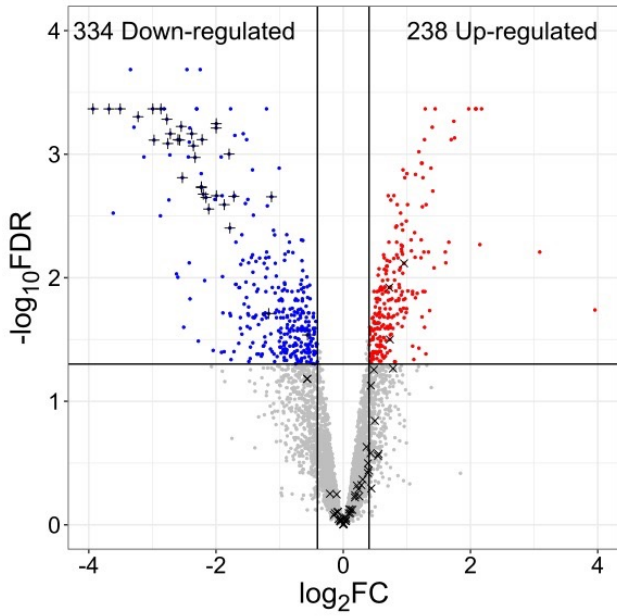
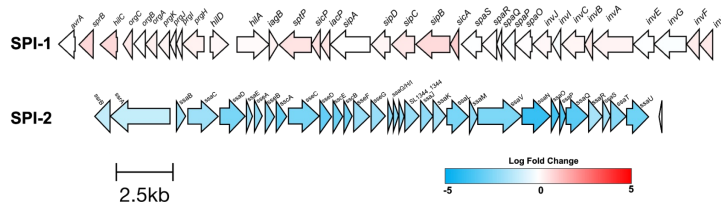


Figure 2

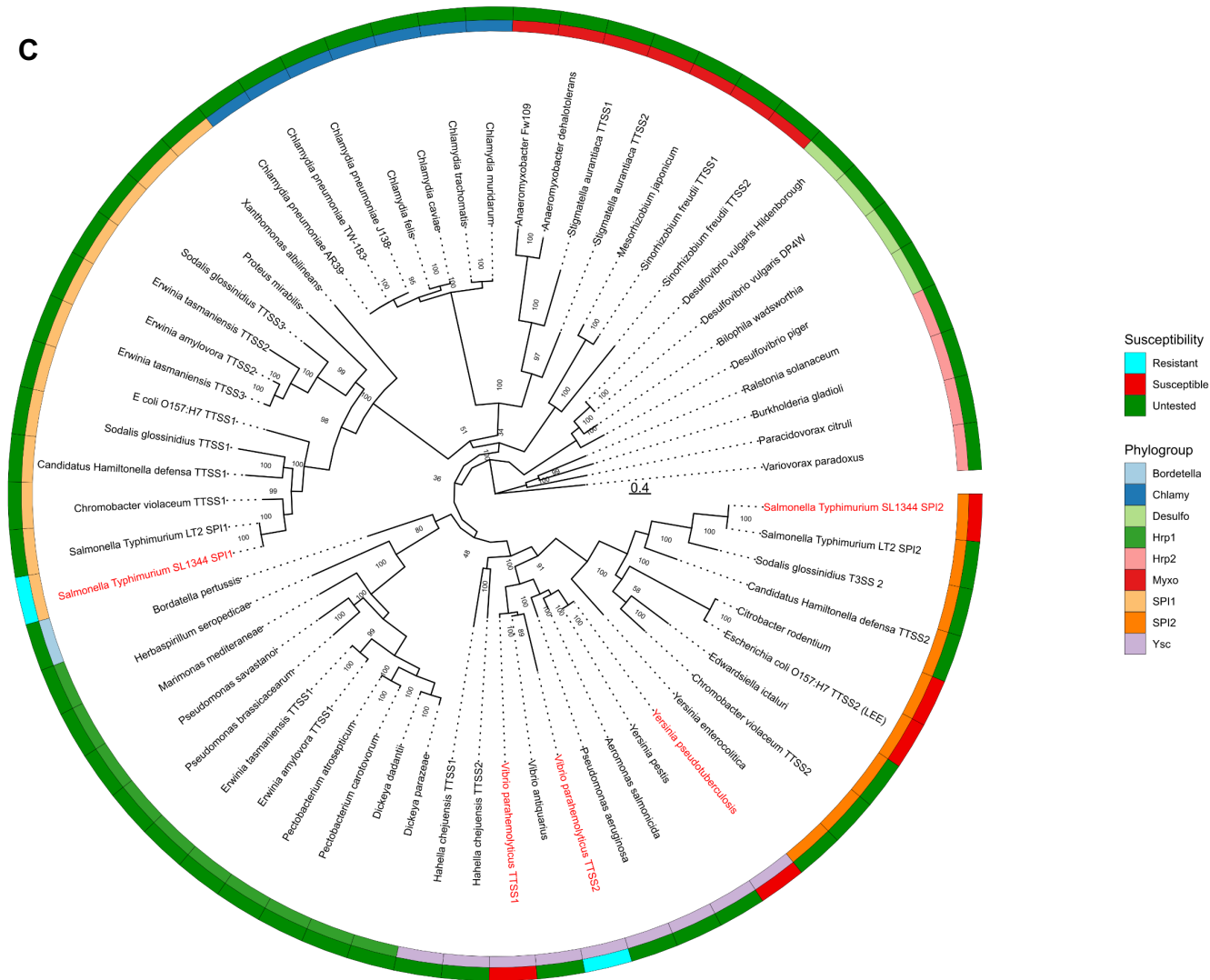
A



B



C



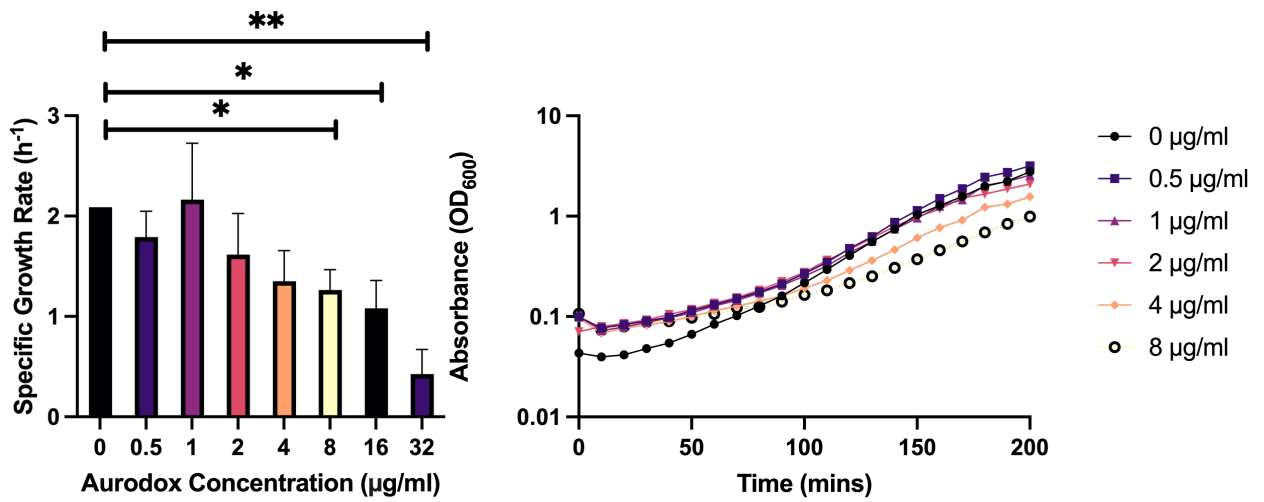


Figure S1: Growth of *Salmonella* Typhimurium is not affected by aurodox

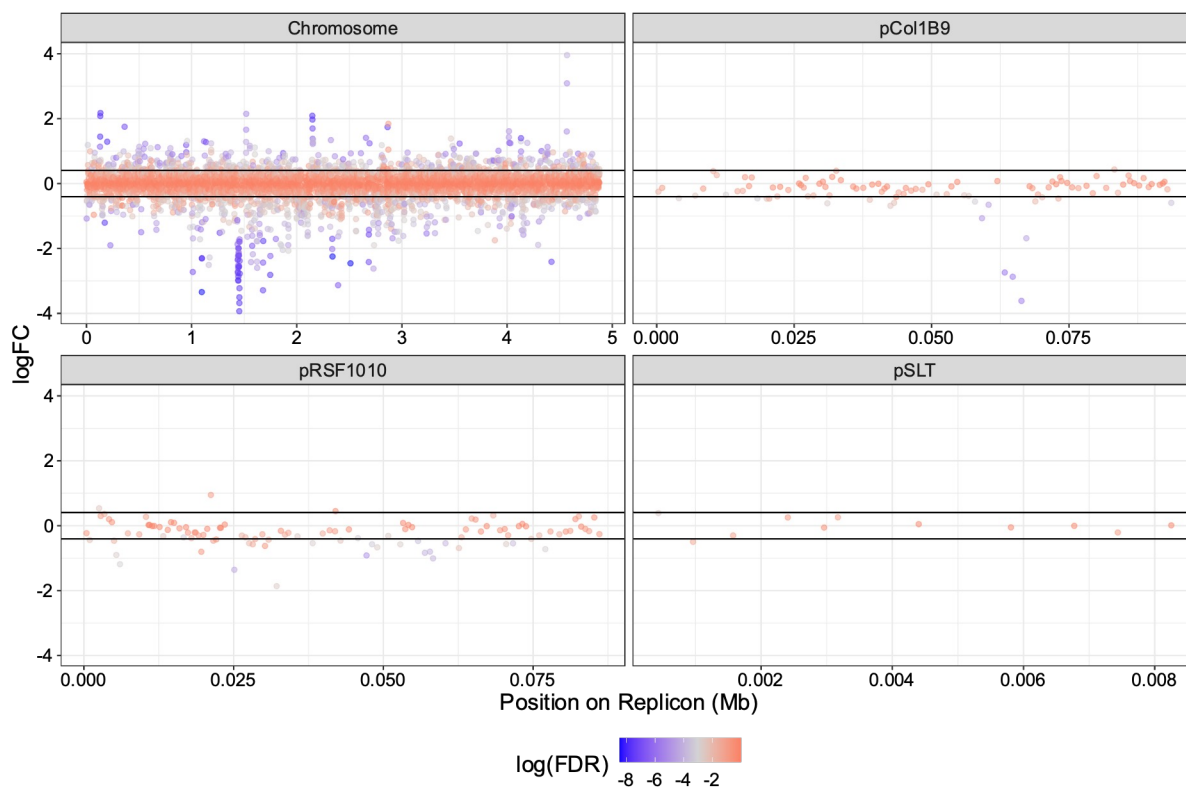


Figure S2: Aurodox affects gene expression at both the chromosome and plasmid level

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.

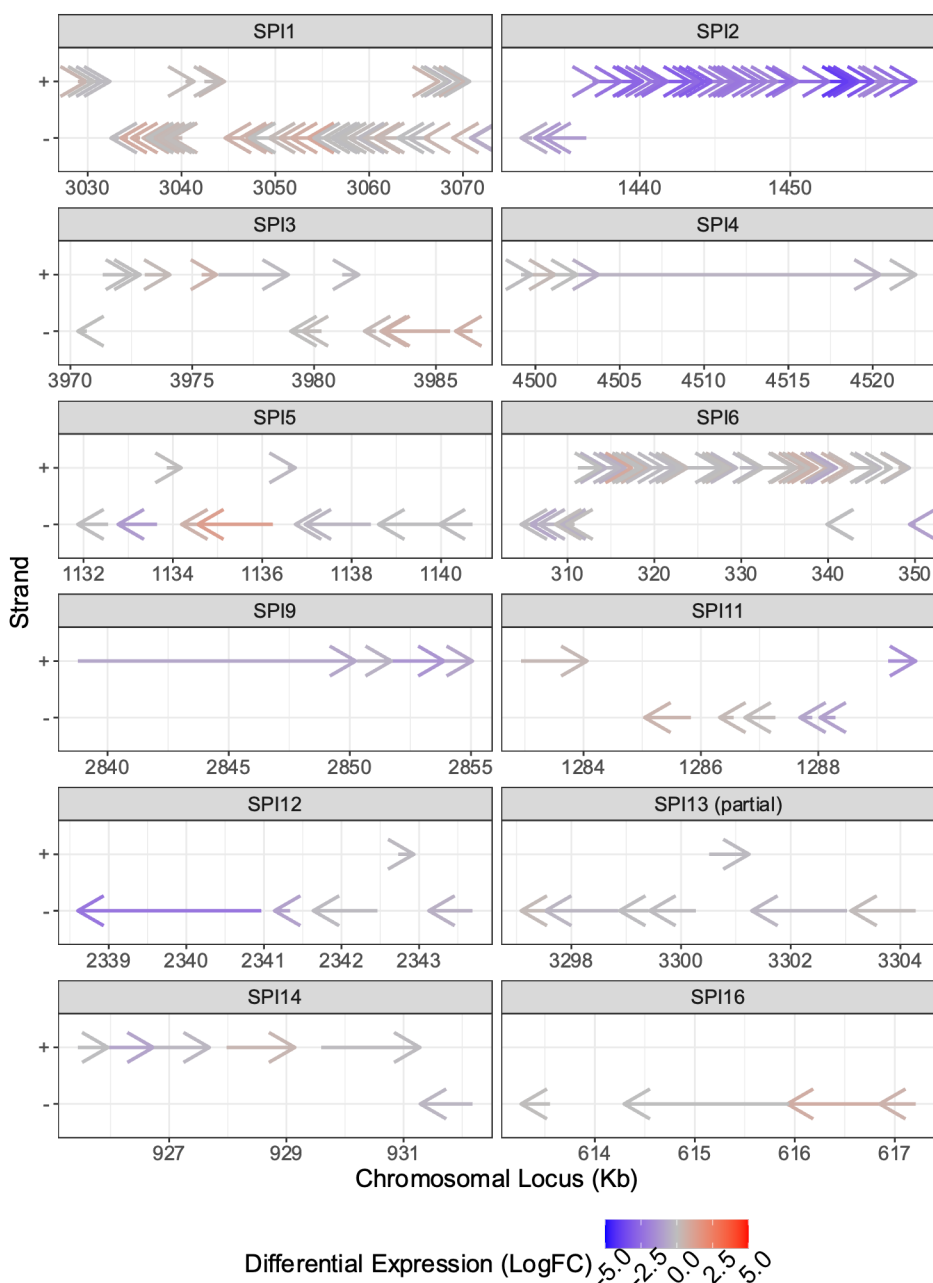


Figure S3: The effect of aurodox on pathogenicity island gene expression is constrained to SPI-2

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

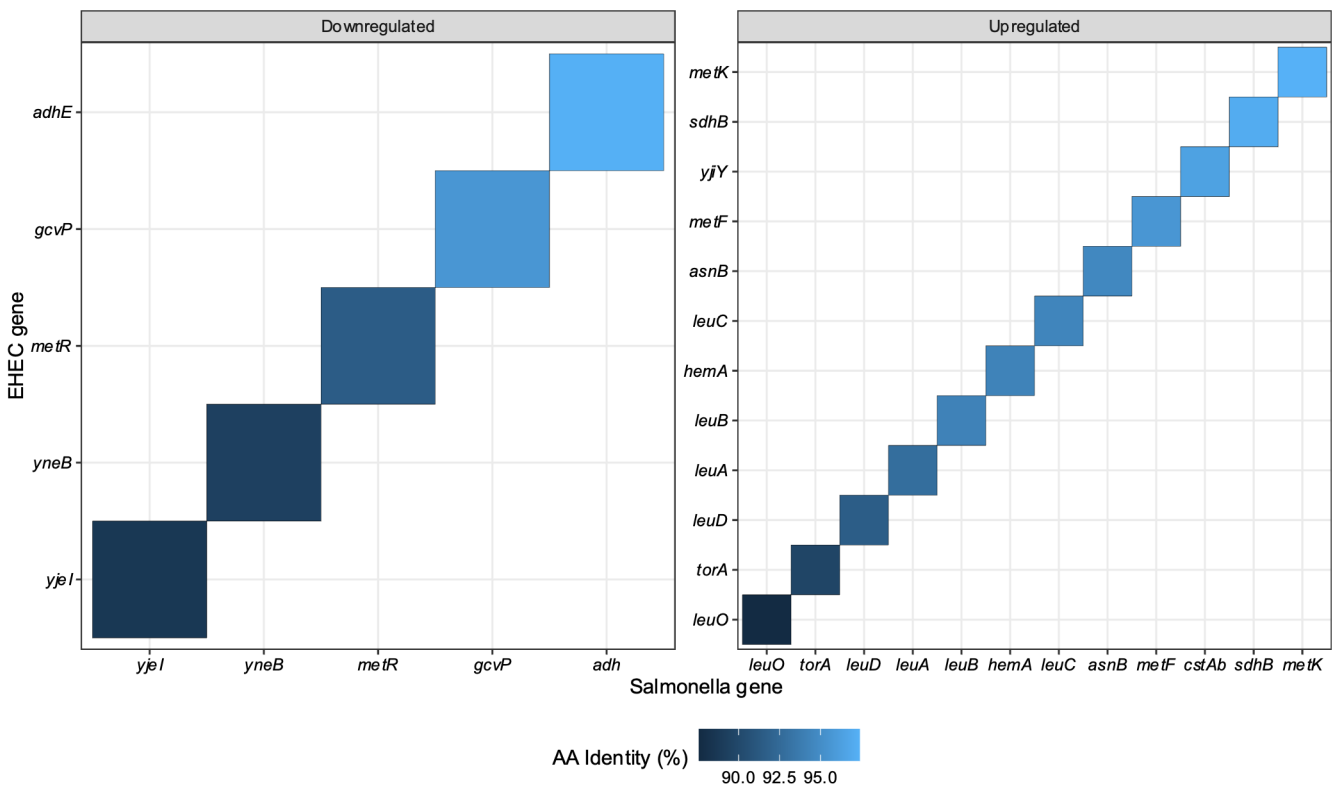


Figure S4: Orthologous genes differentially expressed in EHEC and *Salmonella* Typhimurium post aurodox treatment. Upregulated and downregulated genes identified from EHEC or *S. Typhimurium* in response to aurodox were analysed using the BLAST+ Reciprocal best hits tool