

Expression of homologous RND efflux pump genes is dependent upon AcrB expression

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1 **Expression of homologous RND efflux pump genes is dependent upon AcrB expression:**

2 **Implications for efflux and virulence inhibitor design**

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Synopsis

21 **Objectives** Enterobacteriaceae have multiple efflux pumps which confer intrinsic resistance
22 to antibiotics. AcrB mediates clinically relevant multi-drug resistance and is required for
23 virulence and biofilm formation making it an attractive target for the design of inhibitors.
24 The aim of this study was to assess the viability of single transporters as a target for efflux
25 inhibition using *S. Typhimurium* as the model pathogen.

26 **Methods** The expression of RND efflux pump genes, in response to inactivation of single or
27 multiple homologues was measured using real-time RT-PCR. Phenotypes of mutants were
28 characterised by measuring antimicrobial susceptibility, dye accumulation and ability to
29 cause infection *in vitro*.

30 **Results** Expression of all RND efflux pump genes was increased when single or multiple *acr*
31 genes were inactivated, suggesting a feedback mechanism which activates transcription of
32 homologous efflux pump genes. When two or three *acr* genes were inactivated, the
33 mutants had further reduced efflux, altered susceptibility to antimicrobials (including
34 increased susceptibility to some, but conversely and counter-intuitively, decreased
35 susceptibility to some others) and were more attenuated in the tissue culture model than
36 mutants lacking single pumps.

37 **Conclusions** These data indicate that it is critical to understand which pumps an inhibitor is
38 active against and the effect of this on expression of homologous systems. For some
39 antimicrobials, an inhibitor with activity against multiple pumps will have a greater impact
40 on susceptibility but, an unintended consequence of this may be decreased susceptibility to
41 other drugs, such as aminoglycosides.

42

43

Introduction

44 Efflux is an important mechanism of multidrug resistance in bacteria, conferring decreased
45 susceptibility to a wide range of substrates including antibiotics, dyes, detergents and
46 biocides.¹ This makes them an attractive target for the design of inhibitors which could be
47 used to potentiate the use of existing antimicrobials.

48 Resistance nodulation division (RND) efflux transporters are found in the inner membrane of
49 Gram-negative bacteria and form a complex with an outer membrane channel and a
50 periplasmic adaptor protein (PAP) to form a tri-partite efflux pump system spanning both
51 the inner and outer membrane.^{2, 3} Substrates of these multi-protein complexes are
52 structurally diverse and include antibiotics, biocides, dyes, detergents and host-derived
53 molecules. Active efflux of substrates by RND systems is responsible for the intrinsic
54 resistance of Gram negative bacteria to multiple classes of structurally distinct
55 antimicrobials.¹

56 *Salmonella* have five RND efflux pump systems: AcrAB, AcrD, AcrEF, MdtABC and MdsABC.
57 Further RND efflux pumps are found in some other members of the Enterobacteriaceae
58 including MdtF in *Escherichia coli*.⁴ The transporter protein, AcrB, and its homologues in
59 other Gram-negative bacteria, is considered the most clinically relevant RND system
60 because it has the broadest substrate profile and is more abundant within the cell than
61 other efflux systems.¹ Inactivation of *acrB* increases susceptibility of laboratory mutants of
62 *E. coli*, *Salmonella enterica* and other Enterobacteriaceae to many antimicrobials, whereas
63 overexpression confers multi-drug resistance (MDR), including to clinically efficacious
64 agents. Such mutants have been selected *in vitro* and *in vivo*.⁵⁻⁸ Efflux via AcrB is driven by
65 the proton-motive force and forms a tri-partite complex with the PAP, AcrA, and the outer

66 membrane channel, TolC.⁹ The recent elucidation of the structure of AcrB in complex with
67 different substrates of varying molecular weights has revealed two large, discrete, multisite
68 binding pockets within AcrB, which may explain how AcrB can transport such structurally
69 varied substrates.^{10, 11}

70 Single deletions of RND efflux pump genes other than *acrB* have little or no effect on the
71 susceptibility of Enterobacteriaceae to most antimicrobial agents.¹² Antimicrobial
72 susceptibility of deletion mutants and strains with increased expression of certain RND
73 efflux pumps indicates that there is overlap, or redundancy between the antimicrobials,
74 biocides, dyes and detergents that can be transported by the different RND pumps of
75 *Salmonella*.^{12, 13} AcrF is the closest homologue of AcrB in *Salmonella* (80% identity) and
76 AcrEF overexpression can suppress antibiotic hyper-susceptibility in AcrB deficient strains.^{12,}
77 ¹³ AcrD (64% identity to AcrB) and MdtABC have similar substrate profiles including SDS,
78 novobiocin, deoxycholate, some β -lactams, copper and zinc.^{12, 14} In *E. coli* and *Salmonella*,
79 AcrD also transports aminoglycoside antibiotics.^{15, 16} MdsABC is found only in *Salmonella*
80 and in LB medium is expressed at lower levels than the other four RND efflux pumps.¹⁷
81 However, overexpression of *mdsAB*, encoding the pump and periplasmic adaptor protein,
82 or *MdsABC*, encoding all three components, were overexpressed in a strain lacking AcrB,
83 susceptibility to novobiocin, acriflavine, crystal violet, methylene blue, rhodamine 6G,
84 benzalkonium chloride and SDS was decreased compared to the mutant suggesting that
85 MdsB is capable of exporting these compounds.¹⁸

86 In addition to the role in resistance to antimicrobials, some RND efflux pumps are also
87 required for virulence of many Gram-negative pathogens.¹⁹ In *S. Typhimurium*, inactivation
88 of *acrB* attenuated invasion of tissue culture cells *in vitro* and colonisation in poultry.^{20, 21}

89 Inactivation of *acrAB* or *acrEF* has also been shown to attenuate the virulence of the
90 organism in mice.¹²

91 Regulation of RND efflux pumps is complex; transcriptional control is multi-layered and
92 some regulators control expression of more than one pump. In *Salmonella* and *E. coli*, the
93 regulation of *acrAB* is the best studied. The *acrA* and *acrB* genes are encoded in a single
94 operon and are co-regulated. At a local level *acrAB* is repressed by AcrR, which is encoded
95 alongside and divergently transcribed from *acrAB*. At a global level members of the
96 AraC/XylS family of DNA transcriptional activators, such as MarA, SoxS, Rob and RamA, all
97 influence expression of *acrAB-tolC* and have overlapping recognition sites.²²⁻²⁷ AcrD and
98 MdtABC are both under the control of the two-component regulatory systems BaeSR and
99 CpxAR which induce expression of AcrD and MdtABC in response to high levels of indole,
100 copper, zinc or envelope stress.^{28, 29} Expression of *acrEF* in *E. coli* is generally low due to
101 repression by the global regulator H-NS.³⁰ However, *acrEF* is also encoded alongside a gene
102 encoding a local repressor, AcrS (previously EnvR) which inhibits expression of *acrAB* and
103 acts as a regulatory switch between expression of *acrAB* and *acrEF*.³¹

104 Due to the functional redundancy of RND pumps, potential exists for the loss of certain
105 pump components to be compensated by increased expression of a homologous
106 component that could fulfil, at least to some extent, the same function. For example, Eaves
107 and colleagues³² showed that when *acrB* or *acrF* of *Salmonella* was inactivated, expression
108 of *acrD* increased (3.6 and 4.9 fold, respectively). To allow compensatory changes in
109 expression levels of efflux systems upon inactivation of homologous systems, there must be
110 a tightly controlled and integrated regulatory network that can respond to loss of efflux
111 function. Whilst the literature contains multiple examples of regulation of single efflux

112 systems or a small number of efflux systems, an integrated network of regulation is yet to
113 be elucidated.

114 The role of RND systems in both antimicrobial resistance and virulence makes them
115 attractive targets for the design of inhibitors. Using *S. Typhimurium* as a model, the aim of
116 the present study was to investigate the viability of single transporters such as AcrB as a
117 target for efflux inhibition by investigating the expression and roles of structurally similar
118 efflux pumps in antimicrobial resistance and virulence, and the extent of the redundancy
119 between RND efflux pumps. This information is crucial for the rational design of inhibitors
120 which inhibit all pumps so preventing resistance by compensatory over-production of
121 homologous RND efflux systems.

122

Materials and Methods

123 **Strains and growth conditions.** All strains were derived from *Salmonella enterica* serovar
124 Typhimurium SL1344.³³ *Salmonella* was used as a model organism in this study as it is an
125 important human pathogen that causes a significant number of infections annually. SL1344
126 is a widely studied pathogenic strain of *Salmonella* for which there are well validated
127 models of infection including an *in vitro* tissue culture model. Single gene inactivated
128 mutants were constructed as described previously.^{32, 34} Mutants with multiple efflux pumps
129 inactivated were created by P22 transduction between mutants in which single genes were
130 inactivated or deleted. All mutants were verified by PCR and DNA sequencing. All
131 experiments including MICs reveal that the phenotype of the marked and unmarked
132 mutants for the same gene is indistinguishable. LB broth (Sigma-Aldrich, UK) and MOPS
133 minimal medium (Teknova Inc., USA) were used throughout this study.

134 **RNA extraction and Real Time qRT-PCR.** Overnight cultures of *S. Typhimurium* SL1344 and
135 the test strains were grown in MOPS minimal medium at 37°C. From each strain three
136 biological replicate RNA preparations were made and quantified as described previously.^{22,}
137 ³⁵ Primers (Supplementary Table 1) were designed with an annealing temperature of 57.3°C
138 using Beacon Designer 4.0 (Premier Biosoft, USA). cDNA was synthesised from 2 µg total
139 RNA using superscript III cDNA synthesis kit (Invitrogen). Validation experiments were
140 carried out using 5 cDNA standards of different concentrations (10, 1, 0.1, 0.01, 0.001 ng/µl)
141 to determine PCR efficiency for the housekeeping gene 16S and each test gene. qRT-PCRs
142 were set up in biological triplicate and technical duplicate in a BIORAD PCR tray using 1 µl
143 neat cDNA for test genes and 1 µl of a 1:1000 dilution cDNA for 16S in a 25 µl reaction
144 containing 12.5 µl iQ SYBR green supermix (BIORAD, UK), 1 µl primers (500nm) and 9.5 µl

145 sterile water. qRT-PCR was carried out in a CFX-96 Real-time machine (BIORAD, UK) using
146 the following protocol; 95°C for 5 minutes followed by 40 plate read cycles of 95°C for 30 s,
147 57.3°C for 30 s and 72°C for 30 s. Data were analysed using CFX Manager (BIORAD, UK) and
148 expression ratios were calculated using the $\Delta\Delta\text{ct}$ method and normalised to expression of
149 16S.³⁶

150 **Determination of susceptibility to antimicrobials.** Biolog data was confirmed by measuring
151 growth in the presence of representative AcrB substrates. Briefly, overnight bacterial
152 cultures were diluted to 10^4 CFU/ml and grown in a 96 well plate in iso-sensitest broth in the
153 presence of selected drugs at 0.25 x the wild-type MIC.

154 The minimum inhibitory concentrations (MIC) of antibiotics, dyes and detergents were
155 determined for each strain according to the standardised agar doubling dilution method
156 procedure of the British Society of Antimicrobial Chemotherapy (BSAC) using iso-sensitest
157 agar.³⁷ The MIC was determined as the lowest concentration of antimicrobial that caused no
158 visible growth. Values stated are the mode value from at least three biological replicates
159 performed on at least three independent occasions. All antimicrobials tested were obtained
160 from Sigma, UK.

161 **Accumulation of Hoechst 33342 and norfloxacin.** The efflux activity of the mutants was
162 assessed by determining the accumulation of the fluorescent dye Hoechst 33342 and
163 norfloxacin (Sigma, UK) as described previously.^{38, 39} Differences in steady state
164 accumulation values between mutants and parental strains were analysed for statistical
165 significance using a two-tailed Student's t-test, where $P < 0.05$ was considered significant.
166 Data presented are the mean of three independent biological replicates \pm standard error of
167 the mean.

168 **Adhesion and invasion assays.** The ability of the strains to adhere to, and invade, INT-407
169 (human embryonic intestine cells) was measured as previously described.²¹ Each assay was
170 repeated a minimum of three times, with each experiment including four technical
171 replicates per bacterial strain. The results were analysed using Student's *t*-test and P values
172 of ≤ 0.05 were considered significant.

173

174

Results

175 ***In silico* analysis**

176 The *Salmonella* genome encodes five efflux systems of the RND family. The three most
177 similar pump proteins are AcrB, AcrD and AcrF (Table 1). The two remaining RND pumps,
178 MdsB and MdtB/C, which is a heteromultimer of MdtB and MdtC, are 46.7% and 48.6%
179 identical to AcrB, respectively. ClustalW alignments of all six RND type proteins revealed
180 that five residues (Asp407, Asp408, Lys940, Arg971 and Thr978) vital to proton transport
181 within AcrB of *E. coli*^{40, 41} are identical in all six *Salmonella* proteins indicating that energy
182 transduction is conserved in this family of proteins. However, greater variation was seen in
183 residues involved in substrate recognition or binding. For example, of six important residues
184 in the hydrophobic, phenylalanine rich binding pocket of AcrB (Phe178, 615, 610, 136, 617
185 and 628)⁴² all six were conserved in AcrF, two in AcrD, one in MdtC, one in MdsB and none
186 of these residues were found in MdtB.

187 **Expression of efflux pump genes is altered upon inactivation of homologous pumps.**

188 To determine whether the expression of each gene was altered upon inactivation of one or
189 more homologous genes, real-time quantitative RT-PCR was used to measure the level of
190 *acrB*, *acrD* and *acrF* transcription in the single, double and triple efflux mutants compared to
191 SL1344 (Table 2). As shown previously, in the *acrB* mutant both *acrD* and *acrF* had increased
192 expression.³² In the *acrD* mutant, expression of *acrB* was increased while *acrF* expression
193 was unchanged. In the *acrF* mutant both *acrB* and *acrD* had increased expression. The
194 expression of *mdsB* and *mdtB*, was also affected by loss of single RND pumps. The
195 expression of *mdtB* was increased in the *acrF* mutant while expression of the *Salmonella*
196 specific efflux pump *mdsB* was decreased upon inactivation of *acrB*, *acrD* or *acrF* (Table 2).

197 When two genes were inactivated (e.g. *acrB* and *acrF* or *acrB* and *acrD*) expression of *acrD*
198 or *acrF* was increased, although expression was lower than in the strain lacking only *acrB*
199 (L110). When *acrF* and *acrD* were inactivated expression of *acrB* was increased, this was
200 greater than that seen upon inactivation of *acrD* and similar to that in the *acrF* mutant.
201 Expression of the *mdtB* and *mdsB* efflux genes was increased in all *acr* gene double mutants
202 (L646, L1297 and L1395) but in the triple *acrBDF* mutant only *mdtB* expression was
203 increased. Expression of both *mdtB* and *mdsB* was highest in the *acrDF* mutant (L1395)
204 (Table 2).

205 The expression level of known regulators of RND efflux was also measured. The expression
206 of *ramA* and *marA* was increased when *acrB* was inactivated but not changed in the *acrD*
207 (L132) or *acrF* (L131) mutants (Table 2). Expression of *soxS* was increased in the *acrBF*
208 mutant (6.3 fold), in the *acrDF* mutant (2.2 fold) and in the strain lacking all three RND pump
209 genes. Expression of *rob* was not significantly altered in any of the mutants. The expression
210 of the genes encoding the repressor proteins AcrR and EnvR/AcrS was also measured.
211 Transcription of *acrR* was decreased in the *acrB* mutant and transcription of *envR* was
212 increased in the *acrBDF* mutant (Table 2).

213

214 **Inactivation of two or more RND efflux systems altered antimicrobial susceptibility.**

215 As previously described, inactivation of *acrB* led to multi-drug hyper-susceptibility while
216 single inactivation of either *acrD* or *acrF* did not significantly alter MICs of antibiotics, dyes
217 and detergents compared to the wildtype strain. Re-interrogation of previously published
218 data⁴³ from the Biolog Phenotype Microarray showed that the *acrD* and *acrF* mutants grew
219 better than SL1344 when exposed to four β -lactams, five macrolides, and five quinolones

220 (Supplementary Table S2). This observation was confirmed by measuring the growth kinetics
221 of the strains in the presence of representative AcrB substrates. However, the beneficial
222 effect of lacking *acrD* or *acrF* was lost when *acrB* was deleted in the same strain (L1297 and
223 L646, respectively) (Figure 1).

224 The antimicrobial susceptibility of the double mutant lacking AcrD and AcrF (L1395) was not
225 significantly different from that of SL1344 (Table 3). Furthermore, except for ethidium
226 bromide (for which the MIC value was lower) and the aminoglycosides (for which the MIC
227 values were increased), the susceptibility of the *acrBD* (L1297), *acrBF* (L646) and the triple
228 *acrBDF* (L1405) mutants to antibiotics, dyes and detergents was not significantly different to
229 that of the *acrB* mutant. Surprisingly, the MICs of the aminoglycoside antibiotics,
230 streptomycin, gentamicin and amikacin, were higher for the *acrBF* (L646) mutant and the
231 triple *acrBDF* mutant (L1405) than for the wild-type parental strain SL1344; the MIC of
232 tobramycin was also greater for L1405 than SL1344 (Table 3B). All *acrB* mutants (L110, L646,
233 L1297 and L1405) were more susceptible to the efflux inhibitors Phe-arg- β -naphthylamide
234 (PA β N) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) than the wild-type parental
235 strain (SL1344).

236 **Inactivation of two or more RND efflux systems decreased efflux activity.**

237 We previously showed that inactivation of *acrB* led to increased accumulation of the dye
238 Hoechst 33342.²⁰ Compared to SL1344, inactivation of *acrD* (L132) or *acrF* (L131) or
239 inactivation of both *acrD* and *acrF* (L1395) did not significantly alter the accumulation of
240 Hoechst 33342 (Figure 2). However, mutants with *acrB* and another *acr* gene inactivated
241 (*acrBD* (L1297) or *acrBF* (L646)) accumulated less Hoechst 33342 than the *acrB* mutant. The
242 *acrBDF* mutant (L1405) accumulated the highest level of Hoechst 33342 indicating the

243 lowest level of efflux. Accumulation of the fluoroquinolone antibiotic, norfloxacin, showed a
244 similar pattern although the *acrB* mutant accumulated a higher concentration than the
245 *acrBDF* mutant (Figure 3).

246 **Inactivation of two or more RND efflux systems attenuated the ability of *Salmonella* to**
247 **infect tissue culture cells.**

248 In addition to their role in antimicrobial resistance, in many Gram-negative bacterial
249 pathogens RND efflux pumps are required for the ability to cause infection.¹⁹ Inactivation of
250 *acrB* has been previously shown to attenuate invasion of *S. Typhimurium* into mammalian
251 cells growing in tissue culture.^{12, 20, 21} We now show that single gene inactivation of either
252 *acrD* (L132) or *acrF* (L131) also significantly attenuate the virulence of *Salmonella*. Adhesion
253 by L131 (*acrF::aph*) to human intestinal cells (INT-407) was 56.0% that of SL1344 and it only
254 invaded at 39.1% of wild-type level. L132 (*acrD::aph*) was even more attenuated; adhesion
255 was only 29.0% that of SL1344 and invasion was only 39.1% of the SL1344 level (Figure 4).

256 When two efflux pump genes were inactivated or deleted, the ability of *Salmonella* to
257 adhere to, and invade, INT-407 cells was attenuated more than that seen in single *acr*
258 mutants (Figure 4). Invasion of the *acrBD* double mutant was the lowest among all single
259 and double mutants at only 0.4% of wild-type level. The adhesion of the *acrDF* mutant,
260 L1395 (*acrD::cat, acrF::aph*), was 83.7% that of SL1344 adhesion, which is significantly
261 greater than mutants lacking only one of these systems. Despite this, the invasion level of
262 this mutant was only 17.8% that of the parental strain showing that these two mutations
263 have an additive effect upon invasive ability. When all three of the efflux genes were
264 inactivated in L1405, *Salmonella* was almost completely unable to adhere to or invade INT-
265 407 cells (adhesion = 0.16% of wild-type level, invasion = 0.004% of wild-type level).

Discussion

266

267 The role of RND efflux pumps in multi-drug resistance and virulence makes them attractive
268 targets for the design of efflux inhibitors. We have shown that expression of all RND efflux
269 pump genes can be altered when single or multiple *acr* genes are inactivated. These data
270 suggest that the bacterium can sense and respond to the levels of RND transporters and due
271 to overlapping substrate specificity this affords resilience to the bacterium to prevent
272 intracellular accumulation of toxic metabolites,⁴⁴⁻⁴⁷ or survival in toxic environments.

273 Critically, we can correlate alteration in efflux level and sensitivity to antimicrobials to the
274 compensatory changes in efflux pump gene expression in strains lacking specific RND
275 pumps. For example, in the Biolog phenotype microarray many of the compounds in which
276 the *acrD* and *acrF* mutant grew better are known substrates of AcrB which is over-expressed
277 in these mutants. Furthermore, this beneficial effect is lost when *acrB* is also inactivated.
278 Other than to the aminoglycosides, the susceptibility of the *acrBD* and *acrBF* mutants was
279 not significantly different to that of the mutant lacking only *acrB* and it is likely that this is
280 because the other three RND systems are overproduced in both cases.

281 AcrD, which is known to transport aminoglycosides, and MdtB, which has a similar substrate
282 profile to AcrD, are over-expressed in the *acrBF* mutant. This could explain the decreased
283 susceptibility to the aminoglycosides seen in this mutant. The *acrBDF* mutant also had
284 decreased susceptibility to the aminoglycosides. Expression of *mdsB* is increased in this
285 mutant but there is currently no evidence this pump can transport aminoglycosides.
286 Aminoglycosides enter bacterial cells by self-promoted uptake and it is possible that
287 changes in expression of genes encoding cell envelope components, including LPS, could be
288 responsible for this effect.⁴⁸

289 These data provide proof of principle that changes in expression of pumps in response to
290 inactivation of RND efflux pumps can alter susceptibility to clinically relevant antimicrobials.
291 We postulate the same will be true when the pump proteins themselves are inhibited and
292 recent evidence showing that the efflux inhibitors PA β N and NMP altered expression of RND
293 efflux pump genes in *E. coli* supports this.⁴⁹ Additionally, this highlights that any change in
294 the phenotype of strains with single or multiple genes inactivated should be interpreted
295 with caution as the phenotype represents, the engineered inactivation and any consequent
296 transcriptional changes.

297 The role of AcrAB-TolC in virulence of *S. Typhimurium* is well established and inactivation of
298 *acrB* causes decreased expression of genes in SPI-1, which are known to be required for
299 infection.^{12, 20, 21, 35} Nishino *et al.*, showed that inactivation of *acrD* did not confer significant
300 attenuation in the BALB/C mouse model of infection and inactivation of *acrEF* (encoding the
301 RND pump protein and the periplasmic adaptor protein) increased the host survival rate
302 with 20% of mice surviving to 21 days rather than none when infected with the wild-type
303 strain.¹² In the tissue culture model lack of either AcrD or AcrF caused a significant reduction
304 in the ability of *Salmonella* to infect INT-407 cells with the *acrD* mutant (L132) being more
305 attenuated than the *acrF* mutant (L131). There are several hypotheses to explain these data.
306 First, as inactivation of *acrB* is known to alter expression of genes found in SPI-1³⁵ it is
307 possible that inactivation of other RND pump genes also affects virulence gene expression.
308 Alternative explanations include that RND efflux pumps export substrates that are required
309 for infection or that absence of some RND efflux pumps causes damage or stress to the
310 bacterial cell membrane which compromises the ability to cause infection.

311 The effect of inactivating *acrB* plus one or two other efflux pump genes upon the ability to
312 cause infection was additive. The *acrDF* (L1395) mutant was less attenuated than either of
313 the single mutants (L131 and L132). One explanation for this is that *acrB*, *mdtB* and *mdsB*
314 are all overexpressed in this mutant and so are able to partially compensate for the
315 functions of the other two systems. The triple mutant lacking AcrB, AcrD and AcrF was
316 unable to adhere to, or invade the INT-407 cells. This could suggest that no other
317 transporter could compensate for the loss of these proteins or that inactivation of multiple
318 RND efflux pump genes causes greater changes in expression of virulence genes.

319 The role of efflux pumps in antibiotic resistance makes them targets for the design of
320 inhibitors. Due to the role of efflux pumps in virulence we also postulate that efflux
321 inhibitors will inhibit virulence as well as augment the activity of antibacterial drugs. Our
322 data show that inhibitors designed to inhibit all RND efflux systems will have a greater anti-
323 virulence effect on the organism.

324 The compensatory expression of efflux pump genes was associated with changes in
325 regulatory gene expression. We hypothesise that the bacterial cell is attempting to increase
326 expression of the inactivated/deleted genes by increasing expression of factors known to
327 regulate expression of RND efflux pump genes such as *ramA*, *marA*, *soxS* and *rob*.^{22-24, 26, 27,}

328^{43, 50} Our data suggest that these regulators are involved in the modulation of RND efflux
329 pump expression in the absence of homologous systems. Expression of *ramA* was increased
330 when *acrB* was inactivated,⁵¹ however, expression of *soxS* was increased when two or more
331 *acr* genes were inactivated. SoxS is also a transcription factor of the AraC/XylS family
332 involved in regulating the response to oxidative stress and genes including *acrAB* and *micF*.⁵²
333 Increased expression of *soxS* could suggest that lack of efflux by Acr pump proteins leads to

334 accumulation of toxic metabolites, as proposed by Rosner and Martin when *E. coli tolC* is
335 inactivated.^{44, 47}

336 The critical role of RND systems in both antimicrobial resistance and virulence of pathogenic
337 bacteria makes them attractive targets for the design of inhibitors. These molecules could
338 be used to re-sensitise the bacterium to antimicrobials whilst simultaneously attenuating
339 virulence of the infecting organism. Critically, our data indicate that care should be taken
340 when developing efflux pump inhibitors against the RND pumps to determine which pumps
341 are inhibited and to understand the effect of this on expression of homologous systems. In
342 terms of attenuating virulence, the effect of inhibition was additive so inhibition of multiple
343 pumps is a good strategy. However, the benefit of this strategy on increasing susceptibility
344 to antimicrobials may be more complex and the impact of this will depend on which drugs
345 are used to treat infections by a particular pathogen. For some antimicrobials, an inhibitor
346 with activity against multiple pumps will have a greater impact on susceptibility but, an
347 unintended consequence of this may be decreased susceptibility to other drugs, such as the
348 aminoglycosides.

349

350

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Transparency declaration

356 The authors declare no conflict of interest.

357

358 **Figure legends**

359 **Figure 1. Growth of efflux pump mutants in the presence of AcrB substrates.** Growth of the
360 efflux in the presence of (a) ciprofloxacin and (b) doxycycline at a concentration of 0.25 x
361 the MIC for wild-type. Data presented is the mean of three biological replicates.

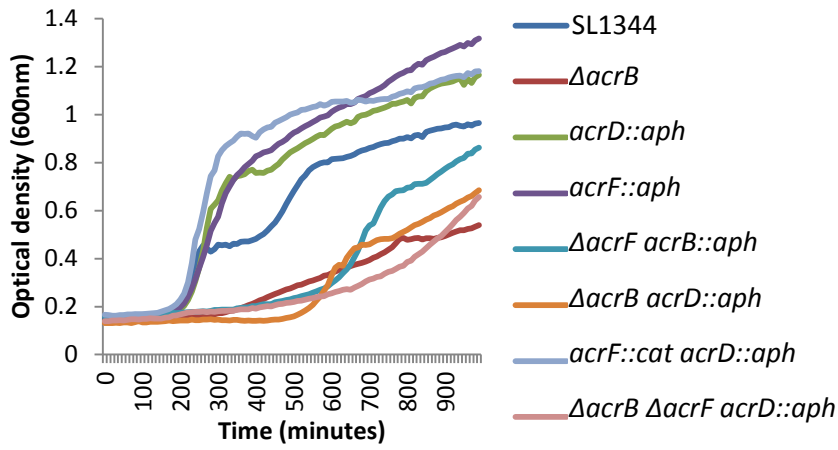
362 **Figure 2. Hoechst 33342 Accumulation in (A) single efflux mutants and (B) double and**
363 **triple efflux mutants.** The data presented are the mean of three separate experiments
364 presented as fold change compared to SL1344 at the end point of the assay \pm standard error
365 of the mean. Student's t-tests were performed to compare the Hoechst 33342 accumulation
366 of each strain to that of SL1344 and those returning *P* values of less than 0.05 are indicated
367 by *.

368 **Figure 3. Accumulation of norfloxacin.** Data shown is the mean of three biological replicates
369 +/- the standard error of the mean.

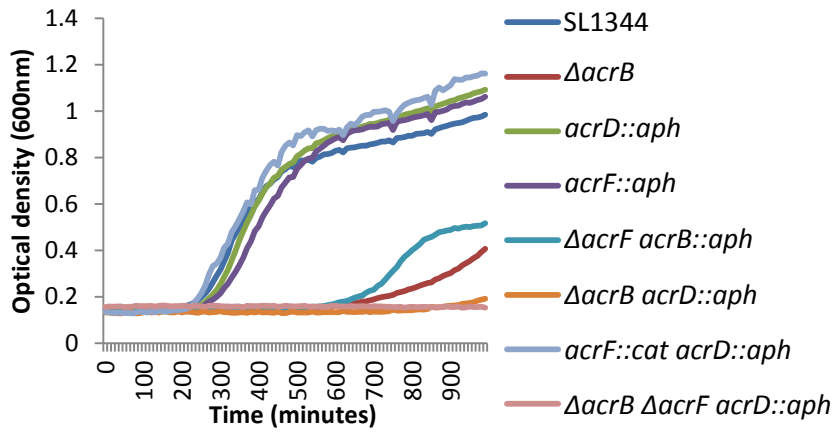
370 **Figure 4. Adhesion (A) and Invasion (B) of strains lacking AcrB, AcrD, AcrF and**
371 **combinations thereof to invade INT-407 cells *in vitro*.** Data shown is the mean of at least
372 three independent experiments. Student's *t*-tests were used to compare values for each
373 strain with that of the wild-type, SL1344. *P* values of ≤ 0.05 were considered significant and
374 are indicated by asterisks.

375

376 Figure 1.

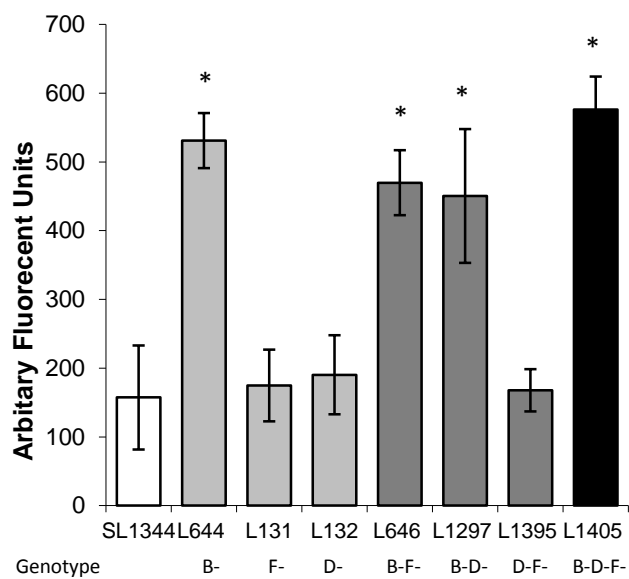


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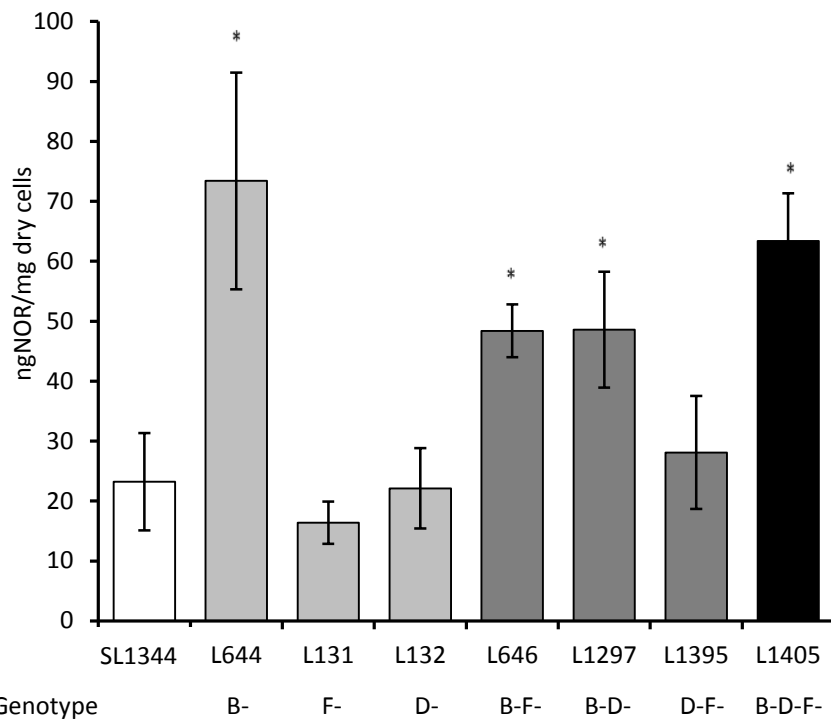
379 **Figure 2. Hoechst 33342 accumulation**



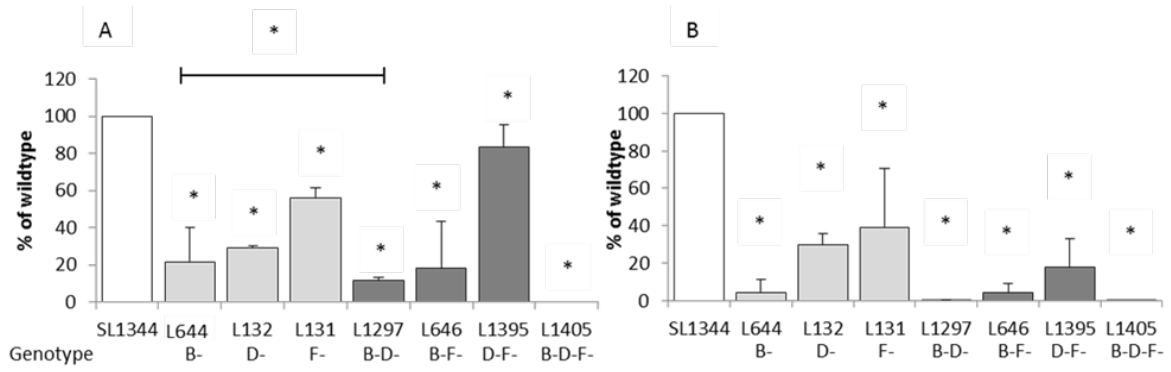
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382 **Figure 3. Accumulation of norfloxacin**



385 **Figure 4. Adhesion (A) and Invasion (B) of strains lacking AcrB, AcrD, AcrF and**
 386 **combinations thereof to invade INT-407 cells *in vitro***



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388

389 **Table 1. Percentage nucleotide identity and amino acid similarity between RND efflux**
 390 **pump genes and proteins of *Salmonella***

	<i>acrB</i> AcrB	/	<i>acrD</i> AcrD	/	<i>acrF</i> AcrF	/	<i>mdtB</i> MdtB	/	<i>mdtC</i> MdtC	/	<i>mdsB</i> MdsB
<i>acrB</i> /AcrB	-		70 / 79.1		74 / 90.4		55 / 46.7		55 / 48.6		59 / 63.4
<i>acrD</i> /AcrD	-		-		68 / 78.2		54 / 49.1		54 / 48.8		59 / 61.3
<i>acrF</i> /AcrF	-		-		-		54 / 47.4		53 / 48.5		57 / 63.1
<i>mdtB</i> /MdtB	-		-		-		-		62 / 66.0		56 / 49.0
<i>mdtC</i> /MdtC	-		-		-		-		-		56 / 49.2
<i>mdsB</i> /MdsB	-		-		-		-		-		-

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Table 2. Expression of RND efflux pump genes and regulators thereof, quantified by real time RT-PCR

		Fold change in gene expression										
		RND efflux pump genes					Known regulators of efflux					
Strain		<i>acrB</i>	<i>acrD</i>	<i>acrF</i>	<i>mdtB</i>	<i>mdsB</i>	<i>marA</i>	<i>ramA</i>	<i>rob</i>	<i>soxS</i>	<i>acrR</i>	<i>envR</i>
SL1344	WT	1.0	1.00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
L644	<i>ΔacrB</i>	-	13.4	16.0	1.0	<i>0.6</i>	2.0	2.8	1.4	2.6	0.4	1.2
L132	<i>acrD::aph</i>	1.8	-	1.0	0.9	<i>0.5</i>	1.2	0.8	1.4	1.3	0.3	0.6
L131	<i>acrF::aph</i>	3.4	3.4	-	1.8	<i>0.7</i>	0.9	1.0	1.3	1.4	0.9	1.5
L646	<i>ΔacrF acrB::aph</i>	-	4.6		4.5	3.0	2.5	1.6	1.2	6.3	0.4	0.8
L1297	<i>ΔacrB acrD::aph</i>	-	-	2.4	4.6	2.1	1.2	1.4	1.1	1.8	2.2	1.2
L1395	<i>ΔacrF acrD::aph</i>	3.8	-	-	6.0	5.9	1.0	1.3	1.4	2.2	0.4	1.0
L1405	<i>ΔacrB ΔacrF acrD::aph</i>	-	-	-	1.1	3.2	1.6	1.9	1.0	5.0	0.5	4.5

Bold text indicated statistically significant ($P \leq 0.05$) increased expression. Italic text indicates statistically significant decreased expression.

Table 3. Antimicrobial susceptibility of SL1344 and RND mutants thereof.

A. Minimum Inhibitory Concentration of antimicrobials to SL1344 and mutants thereof.

	Genotype	MIC (mg/L)													
		Amp	Chl	Cip	Tet	Nal	EtBr	Fus	Ami	Gent	Hyg	Strep	Tob	PAβN	CCCP
SL1344	Wild-type	2	4	0.015	1	4	>256	>256	4	0.5	32	8	2	>1024	64
L110	<i>acrB::aph</i>	0.25	1	<0.008	0.5	1	64	8	4	1	32	8	1	64	32
L644	Δ <i>acrB</i>	0.25	1	<0.008	0.5	1	64	8	4	1	32	8	1	64	32
L131	<i>acrF::aph</i>	2	4	0.015	2	4	>256	>256	4	1	32	16	2	>1024	64
L132	<i>acrD::aph</i>	2	4	0.015	1	4	>256	>256	4	1	32	16	2	>1024	64
L646	Δ <i>acrF acrB::aph</i>	2	1	<0.008	1	1	16	4	8	2	64	32	4	64	32
L1297	Δ <i>acrB acrD::aph</i>	0.25	1	<0.008	0.5	1	64	8	2	0.5	32	8	1	64	32
L1395	<i>acrF::cat acrD::aph</i>	2	16	0.015	2	4	>256	>256	4	1	64	16	2	>1024	64
L1405	Δ <i>acrB \Delta</i> <i>acrF acrD::aph</i>	0.12	1	<0.008	0.5	1	16	8	16	2	64	32	8	64	32

B. Fold change in MIC compared to Δ acrB (L644)

	Genotype	Amp	Chl	Cip	Tet	Nal	EtBr	Fus	Ami	Gent	Hyg	Strep	Tob	PA β N	CCCP
L646	<i>ΔacrF acrB::aph</i>	4			2		<i>-4</i>	<i>-2</i>	2	2	2	4	4		
L1297	<i>ΔacrB acrD::aph</i>								<i>-2</i>	<i>-2</i>	<i>-2</i>				
L1405	<i>ΔacrB ΔacrF acrD::aph</i>	<i>-2</i>					<i>-4</i>		4	2	2	4	8		

Amp, ampicillin; Chl, chloramphenicol; Tet, tetracycline; Nal, nalidixic acid; EtBr, Ethidium bromide; Fus, Fusidic Acid; Ami, Amikacin, Gent, Gentamicin; Hyg, Hygromycin; Strep, Streptomycin; Tob, Tobramycin; PA β N, Phe-Arg β -naphthylamide dihydrochloride; CCCP, Carbonyl cyanide 3-chlorophenylhydrazone. The *aph* gene used is *aph(3')*-1 which gives resistance to kanamycin, neomycin and paromycin.

Table 3A: Bold red font indicates an increase in the MIC of the same compound compared to SL1344. Italic blue font indicates a decrease in the MIC of the same compound compared to SL1344. Table 3B: Bold red font indicates an increase in MIC compared to the same compound for L644. Italic blue font indicates a decrease in the MIC of the same compound for Δ acrB. No value indicates no difference in MIC values.

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