Quinolone-resistant gyrase mutants demonstrate decreased susceptibility to triclosan due to de-repression of general stress response pathways

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Synopsis

Objectives

Cross-resistance between antibiotics and biocides is a potentially important driver of multidrug resistance. A relationship between susceptibility of *Salmonella* to quinolones and triclosan has been observed. This study aimed to (1) investigate the mechanism underpinning this (2) determine if the phenotype is conserved in *Escherichia coli*, and (3) evaluate the potential for triclosan to select for quinolone-resistance.

Methods

Wild-type *E. coli*, *Salmonella enterica* serovar Typhimurium and *gyrA* mutants were used. These were characterised by determining antimicrobial susceptibility, DNA gyrase activity and sensitivity to inhibition. Expression of stress response pathways (SOS, RpoS, RpoN and RpoH) was measured as was the fitness of mutants. The potential for triclosan to select for quinolone-resistance was determined.

Results

All gyrase mutants showed increased triclosan MICs and altered supercoiling activity. There was no evidence for direct interaction between triclosan and gyrase. Identical substitutions in GyrA had different impacts on supercoiling in the two species. For both, there was a correlation between altered supercoiling and expression of stress responses. This was more marked in *E. coli* where an Asp87Gly GyrA mutant demonstrated greatly increased fitness in the presence of triclosan. Exposure of parental strains to low concentrations of triclosan did not select for quinolone resistance.

Conclusions

Our data suggest *gyrA* mutants are less susceptible to triclosan due to up-regulation. The impact of *gyrA* mutation differs between *E. coli* and *Salmonella*. The impacts of

47 *gyrA* mutation beyond quinolone resistance has implications for the fitness and
48 selection of *gyrA* mutants in the presence of non-quinolone antimicrobials.
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Introduction

Antimicrobials, including biocides and antibiotics, are crucial for the prevention and treatment of diseases but their efficacy is under threat due to bacterial drug-resistance.[1] Interactions between antibiotics of different classes have long been recognised and can be synergistic or antagonistic.[2-4] We and others have observed that there is an association between resistance to quinolone drugs and susceptibility to the biocide triclosan, although a mechanistic basis to explain this link has not been described.[5-9]

Mechanisms of resistance to antimicrobials can be specific to single agents or can confer reduced susceptibility to multiple agents, including those with very different chemistry and unrelated targets.[10] In Gram-negative bacteria most quinolone antibiotics primarily target the essential topoisomerase DNA gyrase. High levels of resistance are conferred by mutations within a portion of the *gyrA* gene known as the QRDR (quinolone-resistance-determining region) that reduce binding efficiency of the drug to the enzyme–DNA complex.[11] Additional mutations in genes encoding other topoisomerase subunits, de-repression of multidrug efflux pumps and acquisition of mobile quinolone resistance genes can also contributors to quinolone resistance.[11]

Triclosan is a biocide which has been commonly incorporated into a wide range of domestic products to provide antimicrobial activity. Unusually for a biocide, triclosan has a specific cellular target, the enzyme FabI, which catalyses an essential step in fatty acid biosynthesis. High-level resistance to triclosan is mediated by mutations in the gene encoding FabI, resulting in a mutant protein which is not bound efficiently by triclosan.[12, 13] As for quinolones, resistance to triclosan is multifactorial with other mechanisms, including multidrug efflux and changes to core metabolism, being shown to contribute.[14-17]

Recently, we demonstrated that a quinolone-resistant mutant of *Salmonella* Typhimurium SL1344 (carrying a substitution within GyrA of aspartic acid for glycine at position 87) exhibited a broad, low-level, decrease in susceptibility to various antimicrobials.[5] The effect was seen for this mutant with a substitution of Asp87Gly in GyrA, but not for the more commonly observed Ser83Phe substitution in the same background. Interestingly, both mutants were less susceptible to triclosan (MIC of triclosan of 0.25 mg/L against both GyrA mutants compared to 0.06 mg/L against the parental strain) and this was the largest MIC change seen for any drug.[5] The decreased susceptibility to quinolones and triclosan was not a result of increased efflux in the *gyrA* mutants (accumulation and transcriptomic data show these strains had less, rather than more, efflux activity than the parental strain).[5] The link between quinolone and triclosan resistance is important as triclosan has become ubiquitous in the environment, foodstuffs and even human tissues in the last 20 years.[18-24] If *gyrA* mutants have a competitive advantage over wild-type strains in the presence of triclosan, then triclosan found in the environment may promote the survival or emergence of quinolone-resistant mutants. Infection in people with quinolone-resistant bacteria carries a significantly higher risk of mortality than infection with susceptible strains.[25]

This study aimed to determine if the impact of GyrA substitutions was similar in *E. coli* and *Salmonella* which, whilst being closely-related species, have been shown to maintain different baseline levels of supercoiling.[26] Secondly, we aimed to investigate the mechanisms of cross-resistance between quinolones and triclosan.

Methods

Strains

E. coli MG1655 and *S.* Typhimurium SL1344 were used as parental wild-type strains (Table 1). Mutants with changes in *gyrA* in *Salmonella* were described previously.[5] *E. coli* MG1655 carrying substitutions of Ser83Phe and Asp87Gly in GyrA, were gifts of Will Parks, John Innes Centre.[27] All *gyrA* mutations were confirmed by sequencing. Strain L1234 was used as a positive control in fluorescent experiments, this is a SL1344 derivative lacking chromosomal *ramRA* but carrying plasmid pMW82-*ramA*-*gfp* producing constitutive fluorescence.[28]

Antimicrobial susceptibility testing

The MICs of antibiotics and triclosan were determined following the EUCAST recommended agar dilution methodology. Differences in ability to grow in the presence of different antimicrobials were also determined by measuring absorbance over time at 600 nm in a FluoSTAR Optima plate reader (BMG Labtech). Strains were grown overnight in 5 mL cultures of LB broth before being diluted in fresh broth to an OD_{600} of 0.1; 100 µL of these suspensions were then used to inoculate wells of a microtitre tray containing antimicrobials diluted in 100 µL of LB broth at twice the desired final concentration. Absorbance was measured every 10 minutes for 12 hours. All experiments were repeated on at least three separate occasions and each experiment included two biological and two technical replicates per strain.

Biolog phenotypic microarrays were used to determine differences in the ability of strains to respire in the presence of antimicrobials using plates PM11-20 as previously described.[29]

Enzymes and supercoiling assays

E. coli gyrase subunits, wild-type and mutant, were expressed in strains JMtacA and JMtacB and purified as described previously.[30] *Salmonella* gyrase genes were amplified by PCR from SL1344 and mutant strains described previously [5] and cloned into plasmid pET28a, which was transformed into *E. coli* BL21(DE3) pLysS. Cultures (10 mL) were grown overnight at 37ºC from glycerol stocks and added to 1 L of LB + 30 mg/L kanamycin, 30 mg/L chloramphenicol or LB + 30 mg/L kanamycin, 50 mg/L spectinomycin and incubated at 37ºC. Protein expression was induced with IPTG at OD_{600} = 0.6, and cultures incubated for a further 3.5 h at 37ºC. Cells were centrifuged and pellets re-suspended in 5 mL 10% glycerol, 50 mM Tris·HCl pH 8.0, 2 mM 2-mercaptoethanol, and stored at -80ºC. Re-suspended cell pellets were thawed on ice, before being lysed by disruption at 25 kPsi using a French Press. Lysates were centrifuged and supernatants loaded onto a HisTrap™ FF Ni^{2+} column (5 mL/min, GE Healthcare) previously equilibrated in Buffer A (10% (w/v) glycerol, 20 mM HEPES pH 7.5, 20 mM Imidazole, 300 mM NaCl, 2 mM 2-mercaptoethanol, EDTA-free protease inhibitors). Protein was eluted on a gradient of Buffer A + 1 M imidazole over 20 mins and the flow-through collected. Eluted fractions were pooled and dialysed into Buffer A without imidazole. Samples were taken for identification of purified protein and activity before storage at -80ºC. To remove the his-tag, proteins were incubated with 5-15 units of thrombin per mg of protein. These were incubated at 8ºC for approximately 20 h before being applied to a HisTrap™ FF Ni^{2+} column. Flow-through fractions were collected and pooled, and 100 µL of a protease inhibitor cocktail (Sigma Aldrich) was added before being dialysed into Buffer A without imidazole. Protein samples were concentrated in 50 kDa pore size centrifugal filter units (Milipore), which also assisted in removal of thrombin. Proteins were aliquoted and stored at -80ºC.

Mutations were introduced into the GyrA expression plasmids using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instruction. DNA supercoiling assays with *E. coli* gyrases were as described previously.[31] *Salmonella* gyrases were assayed under the same conditions.

Competitive fitness assays

The competitive fitness of *gyrA* mutants when co-cultured with their parental strain was assessed.[5] Briefly, for each competition experiment, four separate overnight cultures of parental and mutant strain were grown in 10 mL of LB broth at 37°C overnight. A viable count of each sample was determined and four competition lineages established by introducing 100 µL each of one parental and one mutant lineage into 10 mL of pre-warmed LB broth. A sample was taken at time '0', diluted and the viable count of both parent and mutant strain determined by plating samples on LB agar with or without the presence of 64 mg/L of nalidixic acid. Each lineage was then incubated and sampled at 12 h time points. After 12 hours 100 µL of each mixed culture was used to inoculate a fresh broth. This was repeated until seven passage cycles were completed. At each time point the ratio of parent:mutant in each lineage was determined. Each experiment was replicated in the presence and absence of 0.03 mg/L of triclosan.

Selection of quinolone resistant mutants by triclosan

In order to determine whether triclosan would promote the emergence of *gyrA* mutants by exerting a selective pressure for these strains, an *in-vitro* evolution experiment was used. *E. coli* MG1655 was repeatedly passaged in media alone, or in the presence of 0.03 mg/L of triclosan. This concentration was used as under these conditions we

found in the competition assays that the *E. coli* Asp87Gly mutant strongly outcompeted its parent.

Six independent lineages of MG1655 were grown overnight as in the competition experiments above. Each lineage (A-E) was divided into two 10 mL cultures by adding 100 µL of each overnight culture into fresh broth, half had no drug and half contained 0.03 mg/L triclosan. These were then grown in a shaking incubator at 37°C, with shaking at 200 rpm until stationary phase was reached. Samples were taken as in the competition experiments and a sample of each lineage added to new broth. The passaging was repeated ten times. In each sample numbers of quinolone-resistant mutants were determined as in the competition assays and the frequency of nalidixic acid-resistant mutants was calculated. Twenty mutants recovered from either drug-free or triclosan-containing media experiments were randomly selected and the QRDR of *gyrA* amplified and sequenced.

Measurement of stress response gene expression

In our previous publication we had identified up-regulation of general stress response pathways including the SOS response and RpoS, N and H regulons in gyrase mutants.[5] To monitor expression of these pathways in each of the mutants under different conditions, reporter strains were made in plasmid pMW82.[32] A series of promoters known to respond to these stresses were amplified by PCR and cloned upstream of *gfp* which was then dependent upon their activation for transcription and subsequent protein expression. The promoters were *recA* (SOS response), *gabD* (*rpoS*), *glnA* (*rpoN*) or *odpA* (*rpoH*); each was conserved in both species. The constructs were introduced into all strains of interest and the fluorescence emitted by each measured in various conditions. All assays were completed at 37°C in LB media.

Replicate experiments were completed with the addition of various stressors to act as controls for the relevant stress responses: nalidixic acid (80 mg/L, an SOS response inducer), serine hydroxymate, (100 mg/L, a stringent response inducer of RpoS and RpoN), sucrose (20% final concentration, an inducer of RpoE), growth at 42°C (an inducer of RpoH) and chlorpromazine (50 mg/L, an inducer of the *ramA-gfp* reporter as a control for measuring induction of fluorescence).

Overnight cultures of each strain were diluted 1:100 in MOPS minimal medium (Teknova, USA) supplemented with 50 mg/L of ampicillin. Cultures were incubated at 37ºC until mid-log phase. Cells were then harvested by centrifugation and re-suspended in 500 µL of PBS before 200 µL of each was transferred into the wells of a microtitre tray. Fluorescence from GFP (Ex 492, Em 520) was then measured in two ways. Firstly, fluorescence was measured in a BMG FluoSTAR plate reader every 10 min for 16 hours (absorbance at 600 nm was also measured at each time point and fluorescence/absorbance values then compared between strains). Secondly, samples were also taken and analysed by flow cytometry to examine population dynamics by measuring expression from individual cells within a population. All experiments included three biological replicates of each strain in each condition.

For flow cytometry, strain L1234 was used as a positive control.[28] Data from this strain was used to set gates to identify GFP producing cells. Samples were then run until 50,000 cells within the GFP producing population were analysed. Data were analysed in two ways, (i) by calculating the proportion of cells within each sample population that expressed GFP, and (ii) by calculating the average fluorescence produced by each population. Flow cytometry used an Attune NxT instrument and software for data analysis (ThermoFisher, U.K).

Results

Gyrase mutants of E. coli and Salmonella have reduced susceptibility to triclosan

In both *E. coli* and *Salmonella,* substitutions at serine 83 and aspartic acid 87 of GyrA resulted in an eight-fold increase in ciprofloxacin MIC and between 64-128-fold increases in nalidixic acid MIC (Table 1). Both substitutions also increased the MIC of triclosan four-fold in both species. These results were supported by growth kinetics and Biolog Phenotype Microarrays where the ability of the mutants to grow or respire in the presence of triclosan or nalidixic acid were determined, respectively. In LB media alone, there was no significant change in growth rate between the parental strains and their gyrase mutants (Figure 1). However, in the presence of 0.03 mg/L of triclosan (0.5 X the MIC for the parental strains) both *E. coli* and *Salmonella* Asp87Gly mutants grew significantly better than the Ser83Phe mutants or the parental strains. The Phenotype Microarray data for strains grown in the presence of nalidixic acid showed that both *Salmonella* gyrase mutants were able to respire whereas the parental strain was inhibited. In the presence of nalidixic acid, the Asp87Gly mutant started respiration earlier than the Ser83Phe mutant (Figure 2).

Analogous substitutions within gyrase have different impacts on quinolone-sensitivity and supercoiling activity in E. coli and Salmonella.

To determine if there was a correlation between the drug susceptibility phenotypes and supercoiling activity of mutant gyrase enzymes, we purified mutant GyrA proteins from over-producing strains and compared their activities and quinolone susceptibilities to wild-type GyrA. We found that gyrase comprising the mutant *Salmonella* proteins showed the expected levels of resistance to ciprofloxacin (~5-fold increase in the IC_{50} for Phe83 and ~10-fold for Gly87; Table 3), and a ~3-fold (Phe83)

and ~6-fold drop in supercoiling activity compared with the wild-type enzyme (Table 3). The *E. coli* mutant enzymes were also tested and again showed increases in the ciprofloxacin IC_{50}. The Gly87 substitution resulted in a ~10-fold increase (as seen with the *Salmonella* enzyme) but the Phe83 enzyme showed a ~14-fold increase, greater than that seen with the equivalent substitution in *Salmonella*. Interestingly, the relative supercoiling activity of the Phe83 mutant from *E. coli* was only reduced 10% relative to the wild-type whereas in *Salmonella* there was a 70% reduction for the equivalent enzyme. The increases in the IC_{50} of ciprofloxacin were reflected in the ciprofloxacin MICs against each strain (Table 1). However, the subtle changes in enzyme sensitivity seen in these assays were not reflected in changed MIC values between the Phe83 and Gly87 substitutions. In addition, we also made and tested Phe83/Gly87 double mutant GyrA proteins; we found that these mutant enzymes from both species showed similar low supercoiling activity and no detectable inhibition by ciprofloxacin (Table 3).

Triclosan does not directly interact with DNA gyrase in vitro

One possible explanation for the cross resistance between quinolones and triclosan in the GyrA mutants is that triclosan is itself an inhibitor of DNA gyrase with gyrase representing a secondary target for this drug.[5] We tested this possibility using *in vitro* supercoiling assays in the presence of triclosan and found no significant inhibition of either *Salmonella* or *E. coli* gyrase by triclosan. This suggests that the alterations in triclosan susceptibility in bacterial strains bearing quinolone-resistance gyrase mutations are not related to triclosan binding to DNA gyrase.

Expression of stress response genes is up-regulated in Asp87Gly mutants

The lack of direct interaction of triclosan with DNA gyrase suggested an indirect mechanism by which *gyrA* mutation influences triclosan sensitivity. We previously observed that four stress response pathways were up regulated in the *Salmonella* Asp87Gly mutant.[5] To test the hypothesis that *gyrA* mutations altered expression of stress response pathways and thereby susceptibility to triclosan we used a set of promoter-*gfp* reporter fusions (*recA* – SOS response, *gabD* – *rpoS* responsive driven by the stationary phase response, *glnA* – *rpoN* responsive induced by nitrogen limitation, *opdA* – *rpoH* responsive driven by heat shock).

When compared to the parental strain, there was elevated expression of all four main stress response pathways in the *E. coli* Asp87Gly mutant (Figure 3). This elevated expression was maintained across all stages of the growth phase (Figure 3). However, there was no increase in fluorescence seen with the Ser83Phe mutant for any of the four reporters. Up-regulation of all four pathways in the *E. coli* Asp87Gly mutant was seen under all the various stress conditions tested with the exception of expression of *recA* in the presence of nalidixic acid. In this case *recA* expression was markedly induced in the parental strain when exposed to nalidixic acid but not in the *gyrA* mutants (data not shown). The two mutants are resistant to nalidixic acid and therefore the SOS response was not induced in these strains.

Data for the reporter constructs in *Salmonella* showed a different pattern and data from whole populations captured in the FluoSTAR showed no significant difference in stress response expression between the *gyrA* mutants and parent. Flow cytometry analysis of GFP expression by individual cells did reveal differences between the *Salmonella* strains. The percentage of individual cells within a population expressing each reporter gene ranged from ~20-25% and did not vary greatly between SL1344 or the isogenic *gyrA* mutant strains (Figure 4A). However, when the expression level

of GFP was measured there were significant differences. There was no increased expression of any of the reporters in the Ser83Phe mutant (Figure 4B). However, in the Asp87Gly mutant there was significantly increased expression of *gabD* and *glnA*. Both genes were expressed ~66% more than in SL1344 and at more than twice the levels seen in the Ser83Phe mutant. These data were collected from cells in mid-log growth phase with no stress present and therefore represent a constitutive up-regulation of the stationary and nitrogen stress responses in the Asp87Gly mutant.

An Asp87Gly substitution within DNA gyrase of E. coli but not Salmonella confers a strong competitive fitness benefit in the presence of a low level of triclosan

The reduced susceptibilities to triclosan of gyrase mutants suggested that these strains may have a fitness benefit when competed against the parental strains in a sub-inhibitory concentration of triclosan. Competition experiments were used to test this possibility where a 1:1 ratio of parent and mutant were inoculated and then repeatedly passaged for approximately 50 generations in the presence or absence of 0.5 X the triclosan MIC for the parental strains (0.03 mg/L).

Both *Salmonella* mutants were outcompeted by SL1344 in drug-free medium indicating that the mutant gyrase conferred a fitness cost revealed by extended growth in competition. The Ser83Phe mutant was outcompeted by SL1344 by the 5^{th} passage (Figure 5). The Asp87Gly mutant was also outcompeted by wild type although it never fell below ~5% of the population during the experiment. When the experiment with these strains was repeated in the presence of triclosan both strains were still outcompeted. Interestingly, the Ser83Phe mutant was relatively fitter in this condition than in drug-free media and was not excluded from the populations at the end of the experiments. The Asp87Gly mutant did not show a fitness benefit in the presence of

triclosan. Whilst the growth kinetics suggested faster growth for the Asp87Gly mutant in this concentration of triclosan, this was not reflected by increased competitive fitness in these experiments.

In contrast to the *Salmonella* mutants, the fitness of the *E. coli* mutants was less compromised in the absence of any drug and both were maintained at similar proportions to the original inoculum throughout the competition experiments. As for *Salmonella,* the Ser83Phe mutant was not fitter in the presence of triclosan. However, in the presence of triclosan, the Asp87Gly *E. coli* mutant had a dramatic increase in competitive fitness and strongly out-competed its parent strain dominating each population from as early as the second passage (Figure 5). This correlates with the growth kinetics where this mutant was able to maintain its velocity of growth better than any other strain in the presence of triclosan (Figure 1).

Triclosan exposure does not promote emergence of quinolone resistant mutants

As the *E. coli* GyrA Asp87Gly mutant demonstrated a marked competitive fitness benefit in the presence of triclosan compared to its parent, we determined whether prolonged triclosan exposure would select for the emergence of Asp87Gly mutants wild-type cells. To measure this, six independent cultures were inoculated and split into LB broth with or without triclosan (0.03 mg/L was used as in the competitive index experiment this strongly selected for the Asp87Gly mutant) and passaged repeatedly. After each passage the fraction of nalidixic acid-resistant colonies within the population was determined in each of the six lineages, and the genotype of the QRDR of *gyrA* determined for 20 of these from each condition (broth alone or triclosan).

Quinolone-resistant mutants were recovered from both lineages grown in the presence and absence of triclosan but there was no increase in the frequency of nalidixic acid-

resistant mutants in the triclosan exposed lineages (Figure 6B) compared to the control lineages (Figure 6A). Interestingly, in both cases nalidixic acid-resistant mutants were detected after approximately five passages; after being first detected the number of mutants increased but then decreased by passage nine. DNA sequencing and susceptibility testing of a random selection of mutants recovered from nalidixic acid containing plates confirmed that all strains tested were highly quinolone-resistant (MICs of nalidixic acid of 512 mg/L or greater and 0.06-0.12 mg/L for ciprofloxacin). All mutants sequenced carried mutations within the QRDR of *gyrA*. The Asp87Gly substitution was recovered in lineages passaged in both the presence and absence of triclosan. However, the population of Asp87Gly mutants did not expand after first being observed in the triclosan exposed lineages. The Ser83Phe substitution was most commonly recovered in both lineages representing the *gyrA* genotype of 90% of all mutants.

Discussion

We have previously identified an association in *Salmonella* between resistance to quinolone antibiotics mediated by mutations in *gyrA* and decreased susceptibility to the biocide triclosan.[5, 33] Here, we examined two possible mechanisms to explain the association between *gyrA* mutation and triclosan resistance: (1) that triclosan is itself able to bind gyrase and mutant alleles are insensitive to inhibition, and (2) that changed supercoiling activity of mutant gyrase proteins indirectly influences triclosan susceptibility by altering expression of stress response pathways.

To address the first hypothesis, experiments found no evidence for an interaction of triclosan with gyrase which makes the possibility that gyrase is a secondary target for triclosan unlikely. Interestingly, the supercoiling activity of mutant gyrase enzymes with substitutions at Ser83 and Asp87 of GyrA differed between the two species. In *Salmonella* the Ser83Phe substitution reduced activity of the enzyme to 30% of that of the wild-type and increased the IC_{50} of ciprofloxacin ~7-fold. In contrast, the same substitution in *E. coli* only reduced enzyme activity 10% but this resulted in a ~14-fold increase in IC_{50} of ciprofloxacin. The difference in the altered levels of supercoiling activity resulting from the same mutations in *Salmonella* versus *E. coli* was surprising. The sequence of GyrA is highly conserved between the two species with 92% identity and complete conservation of amino acid sequence across the enzyme's active site. It has been demonstrated that *E. coli* and *Salmonella* maintain a different level of basal supercoiling of chromosomal DNA suggesting that although GyrA (and other topoisomerases) are conserved between the two species there are significant functional differences.[26]

Whilst the triclosan MIC against both gyrase mutants was similar for both species, the Asp87Gly substitution had a greater impact than the Ser83Phe substitution. This was

seen by significantly faster growth rates observed for the Asp87Gly mutant compared with the Ser83Phe mutant and parent in the presence of a low level of triclosan.

The greater change in supercoiling activity in Asp87Gly mutants compared to Ser83Phe mutants and their relatively increased ability to grow in the presence of triclosan were consistent with our second hypothesis. Analysis of expression of four general stress responses in each mutant of both species found upregulation of all four pathways under all conditions tested in the Asp87Gly mutant of *E. coli* but not the Ser83Phe mutant. In *Salmonella* there was also a significant difference between the two mutants although this was less marked with greater expression of the RpoS and RpoN reporters seen in the Asp87Gly mutant. The impact of bacterial stress responses in antimicrobial tolerance has been established and some recent work has suggested specific impacts on triclosan susceptibility. RpoS has been associated with triclosan resistance; specific mutations in *rpoS* have been shown to be required for high-level resistance to triclosan in *Salmonella* and highly triclosan-resistant mutants carrying classical substitutions within FabI became hyper-sensitive to triclosan if *rpoS* was inactivated.[9, 34]

Triclosan is now commonly found in the environment, water, vegetables and even in people with concentrations detected in urine or plasma ranging from 2.7 to 48 µg/L. [18, 35] Therefore, as the gyrase mutants were cross-resistant to triclosan, we sought to examine the impact of triclosan at a sub-inhibitory concentration on the relative fitness of gyrase mutants compared to their parent. In competition assays, for both the *Salmonella* mutants there was a fitness cost which was evident in both drug-free media and in the presence of low level triclosan. Therefore, under these conditions the benefit of the mutants in the face of higher triclosan concentrations was not evident and the wild-type was still fitter in the presence of this concentration of triclosan (Figure

411 5). In contrast to *Salmonella*, the *E. coli* Asp87Gly mutant was able to strongly out-
412 compete its parental strain when grown in a low level of triclosan (Figure 5). However,
413 the Ser83Phe mutant in *E. coli* was unable to outcompete MG1655 in either drug-free
414 or triclosan-containing media.
415 To determine whether there was a sufficiently strong selective pressure for Asp87Gly
416 mutants to emerge from an *E. coli* wild-type population passaged in triclosan, we used
417 an evolution experiment (Figure 6). Whilst quinolone-resistant strains (including
418 Asp87Gly mutants) did emerge in the experiments, these were not enriched by
419 triclosan exposure. However, this experiment only used one triclosan concentration
420 and a relatively short time period; longer time periods or other selective conditions may
421 give different results.
422 Taken together the results presented here demonstrate that the association between
423 quinolone and triclosan resistance seen in both *E. coli* and *Salmonella* is not mediated
424 by triclosan interacting with gyrase. We postulate that the association is mediated by
425 up-regulation of stress responses in gyrase mutants as a result of altered supercoiling.
426 There was a greater phenotypic impact of substitution of Asp87Gly than Ser83Phe in
427 both species tested but a significantly greater impact of this substitution on triclosan
428 resistance and stress response expression was seen in *E. coli* compared to
429 *Salmonella*. This has implications for the relative fitness of different *gyrA* mutations in
430 different species and suggests some *gyrA* mutants may be ‘primed’ to deal with non-
431 quinolone antimicrobial stress exposures. We predicted that this would influence the
432 competitive fitness and prevalence of these strains. However, our short term evolution
433 experiment did not show expansion of quinolone-resistant mutants from populations
434 exposed to triclosan. Given the increasing prevalence of triclosan and other
435 antimicrobials in the environment a greater understanding of the impact they can have

on bacteria and how exposure to these non-antibiotic antimicrobials may impact the selection and spread of clinically relevant antibiotic resistance is needed.

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Transparency declaration

None to declare

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Tables

Table 1. Strains, GyrA substitutions and antimicrobial susceptibility.

Strain	GyrA genotype	Minimum Inhibitory Concentration (mg/L) Nalidixic Acid	Ciprofloxacin	Triclosan
E. coli				
MG1655	Wild-type	8	0.008	0.06
I980	Ser83Phe	512	0.06	0.25
I1042	Asp87Gly	512	0.06	0.25
S. Typhimurium				
SL1344	Wild-type	4	0.015	0.06
L821	Ser83Phe	512	0.12	0.25
L825	Asp87Gly	512	0.12	0.25

551 **Table 2. Primers used in this study**

Primer	Sequence	Description
Ec*gyrA* F	TACACCGGTCAACATTGAGG	*E. coli gyrA* QRDR sequencing
Ec*gyrA* R	TTAATGAATGCCGCCGTCGG	*E. coli gyrA* QRDR sequencing
Stm*gyrA* F	CGTTGGTGACGTAATCGGTA	*Salmonella gyrA* QRDR sequencing
Stm*gyrA* R	CCGTACCGTCATAGTTATCC	*Salmonella gyrA* QRDR sequencing
recA F	AAAGGATCCATGAAGAAGCCAAAGCGCAGAT	*recA* promoter amplimer for cloning into pMW82
recA R	CGCTCTAGATGAACCGGATAGTGAATCGT	
gabD F	AAAGGATCCCTTATCGGCTGTGCAGGACT	*gabD* promoter amplimer for cloning into pMW82
gabD R	CGCTCTAGATGCACAGCGAGTTTTTCATC	
glnA F	AAAGGATCCTCGATCACAACTTTGCCTCA	*glnA* promoter amplimer for cloning into pMW82
glnA R	CGCTCTAGAGGTCGTCGTGGTAACGAGAT	
opdA F	CCGGAAGGATCCATGTTAAAGGGGCGGCTTAT	*opdA* promoter amplimer for cloning into pMW82
opdA R	ACATGCTCCGGTTTAATTGC	

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555 **Table 3. Comparison of supercoiling activities and quinolone susceptibilities of**

556 **wild-type and quinolone-resistant gyrase mutants from *S.* Typhimurium and *E.***

557 ***coli*.**

Organism	GyrA	Relative supercoiling activity*	Ciprofloxacin IC_{50} µM
S. Typhimurium	Wild type	100	7
	Phe83	30	35
	Gly87	15	70
	Phe83+Gly87	30	>100
E. coli	Wild type	100	0.5
	Phe83	90	7
	Gly87	15	5
	Phe83+Gly87	20	>100

558 *For each enzyme activity is relative to the wild type (100%)

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Figure Legends.

Figure 1. Growth kinetics of strains in the presence and absence of triclosan. Growth kinetics of the parent and gyrase mutant strains in LB broth over a 12-hour period. Each line represents an average of eight biological replicates. Black lines indicate parental strains, blue lines Ser83Phe mutants and red lines Asp87Gly mutants. Panels A and B show data for *E. coli* and C and D data for *Salmonella*. Panels A and C are data from growth with no drug and panels B and D show data for growth in the presence of 0.03 mg/L of triclosan (0.5 X the MIC against the parental strains).

Figure 2. Biolog data for respiration in the presence of quinolones. Data from Biolog phenotypic microarrays showing the respiration rates of *Salmonella* parent, gyrase and *fabI* mutant strains over a 26-hour period. Each line represents an independent replicate. Black lines indicate SL1344, blue lines the GyrA Ser83Phe mutant and red lines the GyrA Asp87Gly mutant. Panel A shows data for nalidixic acid (wells E11 on the PM11C Biolog plate) and B shows data for ofloxacin (wells H12 on the PM11C Biolog plate).

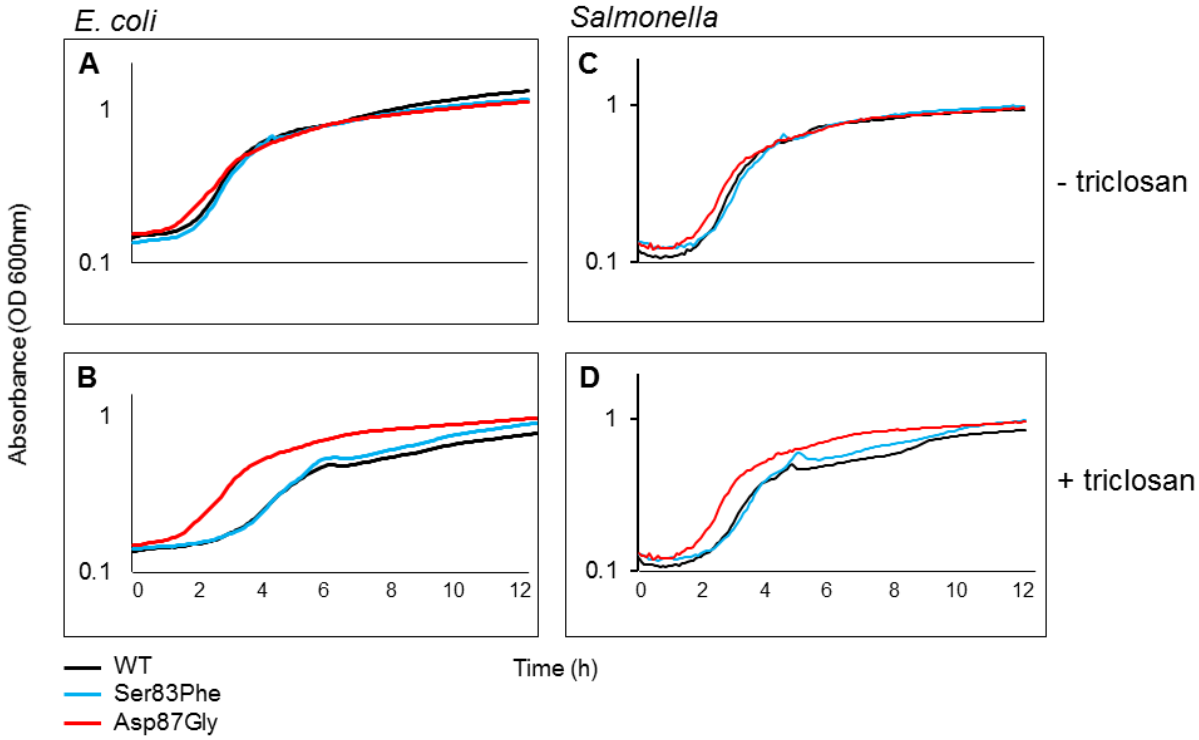
Figure 3. Expression of stress response pathways in *E. coli* strains in drug free media. Black lines indicate average fluorescence from 8 replicates (two biological, four technical) of MG1655, blue lines show data from the Ser83Phe mutant and red lines data from the Asp87Gly mutant. Panel A shows expression from the *recA* (SOS) reporter, panel B the *glnA* (*rpoN*) reporter, panel C the *gabD* (*rpoS*) reporter and panel D shows data from the *opdA* (*rpoH*) reporter. Data is expressed relative to the level of expression from the parental strain at the first recording point.

Figure 4. Expression of stress response genes in *Salmonella* gyrase mutants. Panel A shows the percentage of total cells within populations of SL1344 (WT) and isogenic gyrase mutants which were positive for expression of GFP. Panel B shows the average fluorescence of GFP positive cells. Bars indicate averages of three independent replicate cultures, for each 50,000 cells were counted. Values statistically different to the corresponding wild-type ($p<0.05$) are marked by an asterisk.

Figure 5. Competition assays of gyrase mutants vs parental strains in the presence and absence of triclosan. Data show the ratio of parent to *gyrA* mutant strains in competition assays over six days grown in drug-free media (left hand panels) and in the presence of 0.03 mg/L of triclosan (right hand panels). Data are averages from four independent experiments and in each panel the wild-type is represented by the striped bars and the corresponding mutant by the open bars. Data from *Salmonella* are in the top four panels and data from *E. coli* are in the bottom four panels.

Figure 6. Emergence of nalidixic acid resistant mutants in the presence and absence of triclosan. Average frequency of nalidixic acid-resistant mutants within populations ('y' axis) against passage number ('x' axis) of MG1655 which were grown in LB broth in the absence (panel A), or presence (panel B) of 0.03 mg/L of triclosan.

Data shown are average frequencies calculated from six independent lineages in each condition.



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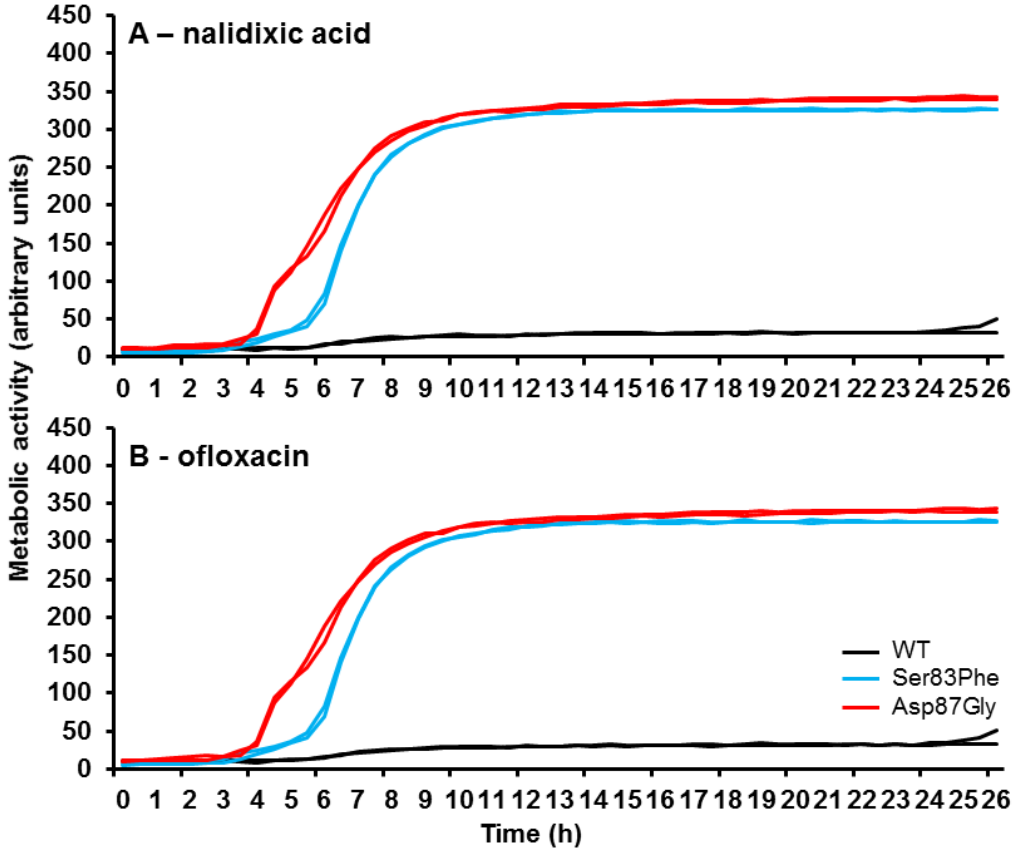

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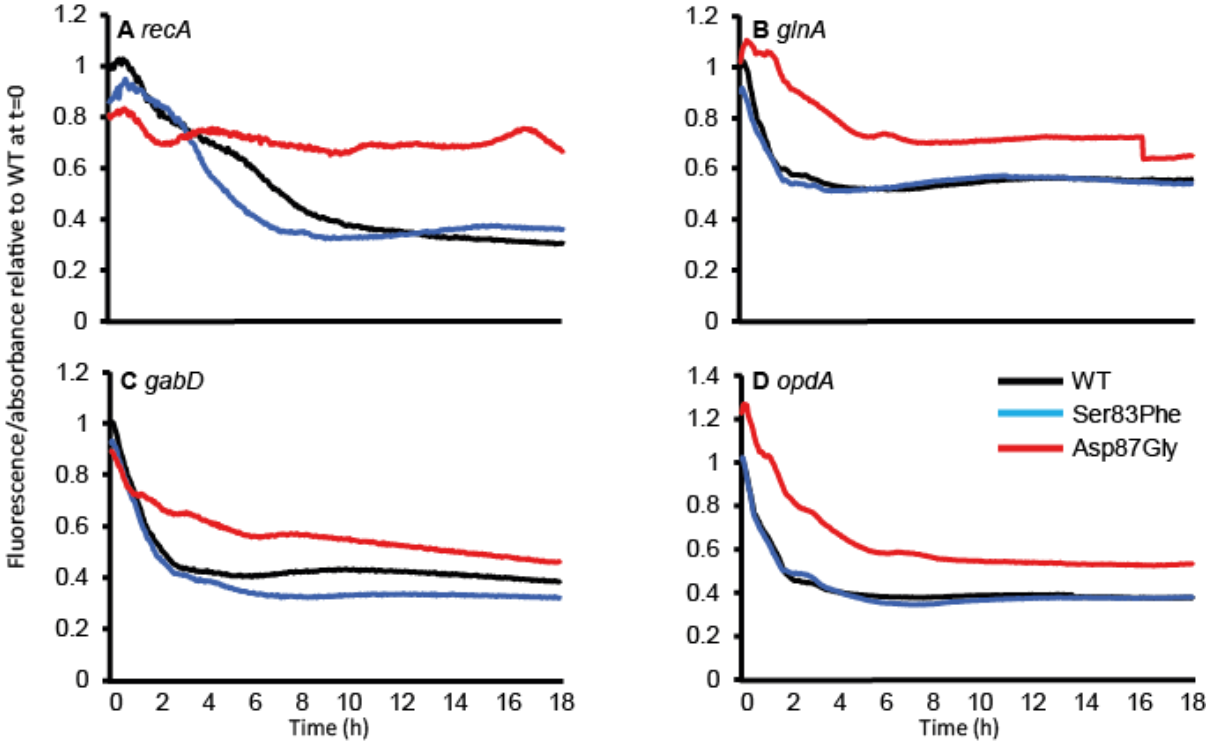

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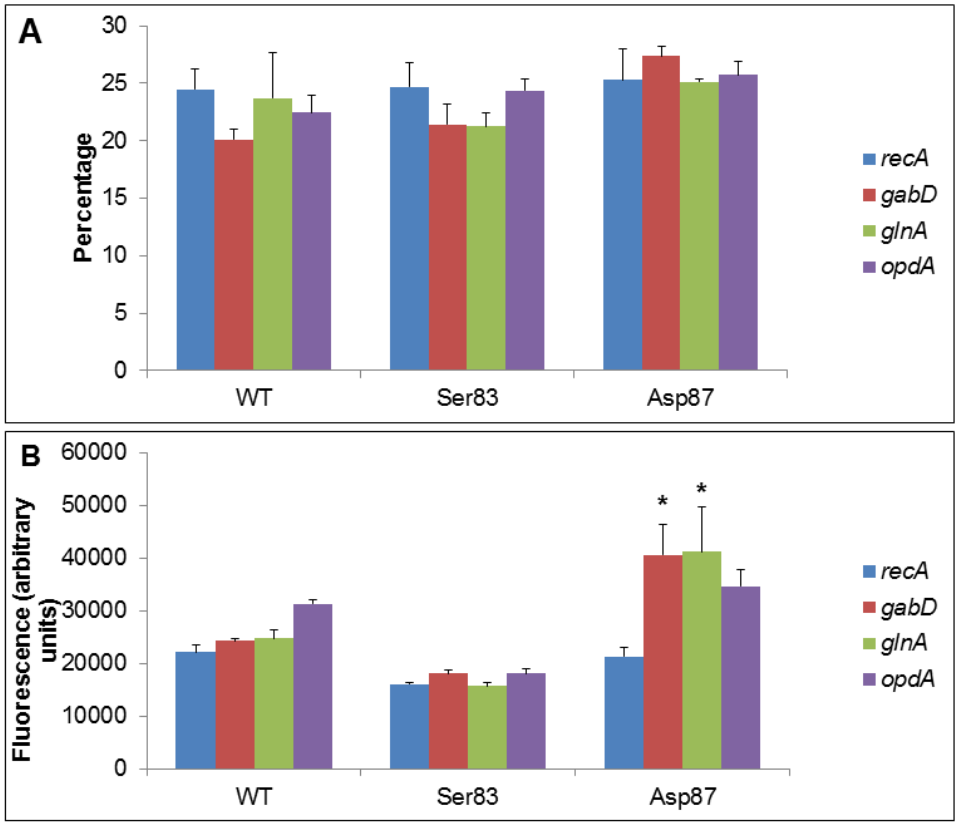

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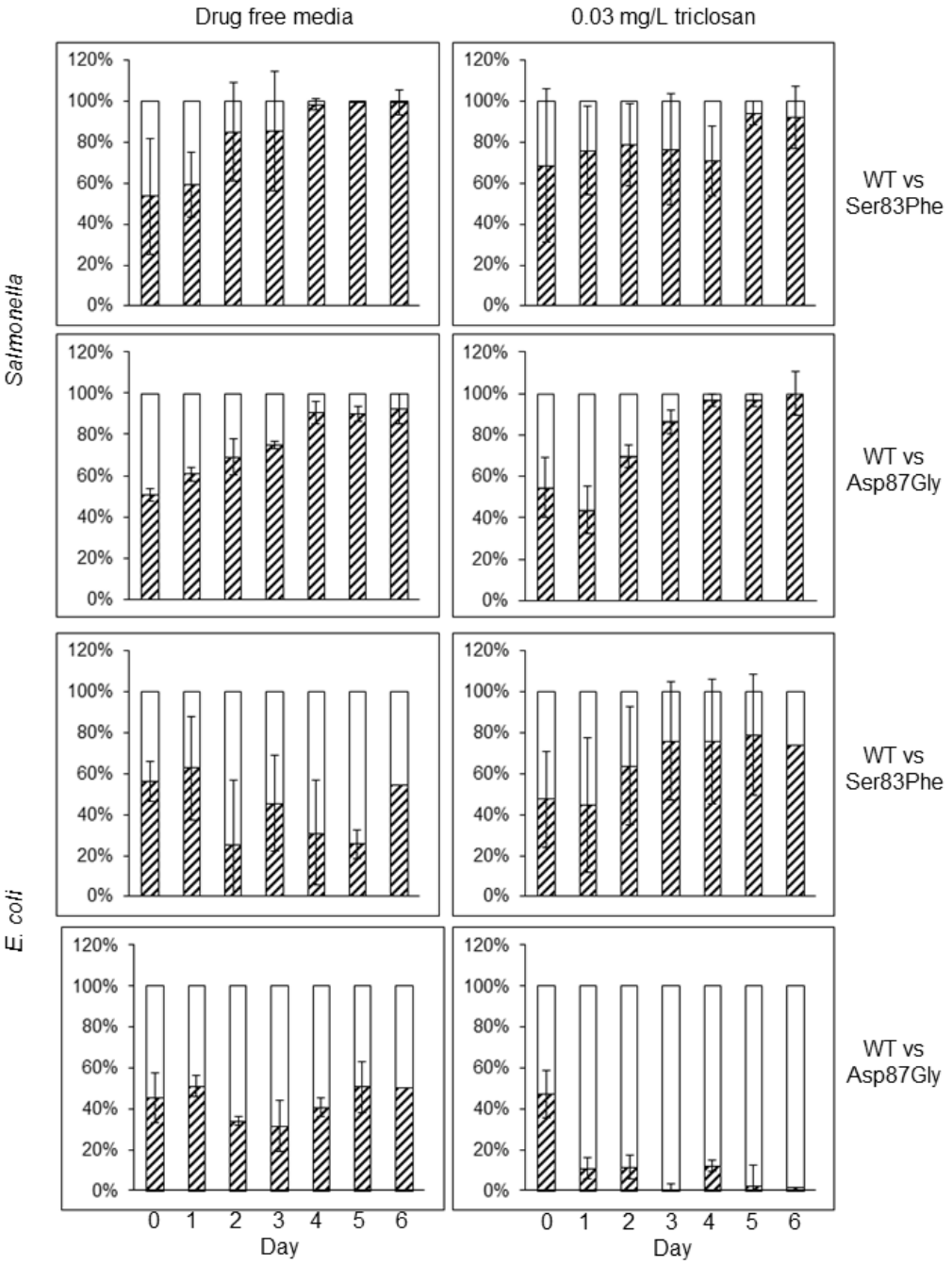

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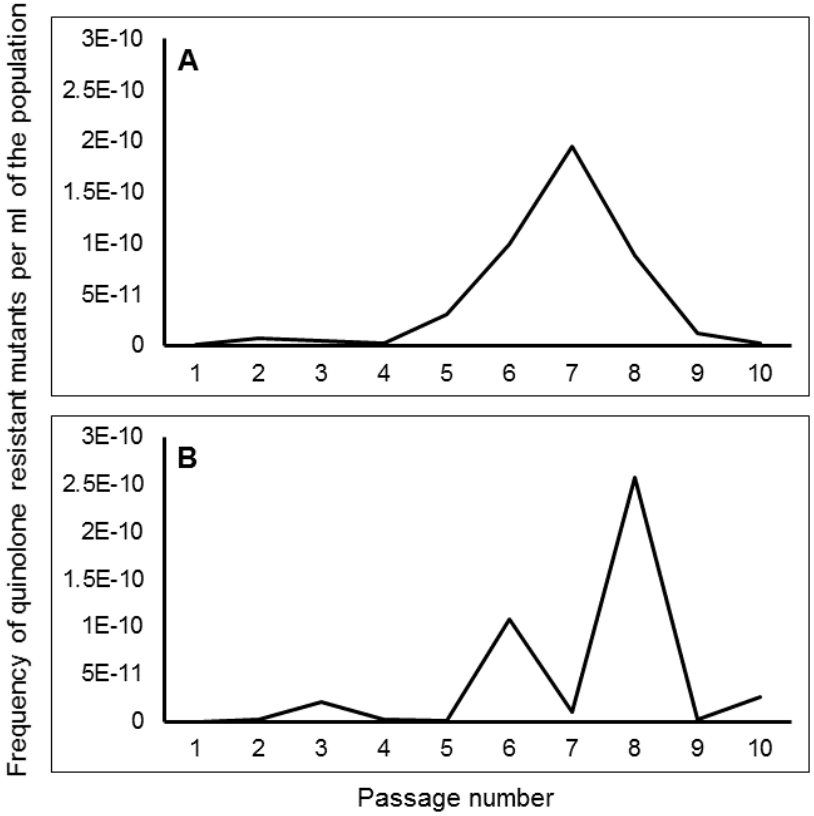

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