Quinolone-resistant gyrase mutants demonstrate decreased susceptibility to triclosan

Mark A. Webber[1,2]*, Michelle M. C. Buckner[1], Liam S. Redgrave[1], Gyles Ifill[3]†, Lesley A. Mitchenall[3], Carly Webb[1], Robyn Iddles[1], Anthony Maxwell[3] and Laura J. V. Piddock[1]

[1]*Institute of Microbiology & Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham B152TT, UK;* [2]*The Quadram Institute, Norwich Research Park, Norwich NR47UH, UK;* [3]*Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Norwich NR47UH, UK*

*Corresponding author. Tel: !44-1603-255233; E-mail: mark.webber@quadram.ac.uk
†Present address: Department of Microbiology & Immunology, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada.

Objectives: Cross-resistance between antibiotics and biocides is a potentially important driver of MDR. A relationship between susceptibility of *Salmonella* to quinolones and triclosan has been observed. This study aimed to: (i) investigate the mechanism underpinning this; (ii) determine whether the phenotype is conserved in *Escherichia coli*; and (iii) evaluate the potential for triclosan to select for quinolone resistance.

Methods: WT *E. coli*, *Salmonella enterica* serovar Typhimurium and *gyrA* mutants were used. These were characterized 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 of stress responses. The impact of *gyrA* mutation differs between *E. coli* and *Salmonella*. The impacts of *gyrA* mutation beyond quinolone resistance have implications for the fitness and selection of *gyrA* mutants in the presence of non-quinolone antimicrobials.

Introduction

Antimicrobials 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 recognized and can be synergistic or antagonistic.[2–4] We and others have observed that there is an association between resistance to quinolone antibiotics resulting from topoisomerase mutation and susceptibility to the biocide triclosan, although a mechanistic basis to explain this link has not been described.[5–9]

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

Triclosan is a biocide that 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 *fabI* gene, resulting in a mutant protein that is not bound efficiently by triclosan.[12,13] As for quinolones, resistance to

Table 1. Strains, GyrA substitutions and antimicrobial susceptibility

Strain	GyrA genotype	Relative supercoiling activity[a]	Ciprofloxacin IC$_{50}$ (1M)	MIC (mg/L)		
				nalidixic acid	ciprofloxacin	triclosan
E. coli						
MG1655	WT	100	0.5	8	0.008	0.06
I980	Ser83Phe	90	7	512	0.06	0.25
I1042	Asp87Gly	15	5	512	0.06	0.25
Salmonella Typhimurium						
SL1344	WT	100	7	4	0.015	0.06
L821	Ser83Phe	30	35	512	0.12	0.25
L825	Asp87Gly	15	70	512	0.12	0.25

[a]For each enzyme activity is relative to the WT (100%).

triclosan is multifactorial, with other mechanisms, including multi-drug 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 Asp87Gly) exhibited a broad, low-level, decrease in susceptibility to various antimicrobials.[5] The effect was seen for this mutant, but not for the more commonly observed Ser83Phe substitution in the same background. Interestingly, both mutants were less susceptible to triclosan (MIC of triclosan 0.25 mg/L for both GyrA mutants compared with 0.06 mg/L for 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 *gyrA* mutants.[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 WT 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 *Salmonella* carries a significantly higher risk of mortality than infection with susceptible strains.[25]

This study aimed to determine whether the impact of GyrA substitutions was similar in *Escherichia coli* and *Salmonella*.[26] Secondly, we aimed to investigate the mechanisms of cross-resistance between quinolones and triclosan.

Methods

Strains

E. coli MG1655 and *Salmonella* Typhimurium SL1344 were used as WT strains (Table 1). *gyrA* mutants were as described previously.[5,27] SL1344-derivative L1234 was used as a positive control in fluorescence experiments as previously described.[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 antimicrobials were determined by measuring absorbance over time in a FluoSTAR Optima plate reader (BMG Labtech).[5] All experiments were repeated on three occasions and each 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.[29]

Enzymes and supercoiling assays

Full-length gyrase subunits of each species were expressed and reconstituted for supercoiling assays. *E. coli* gyrase subunits, WT and mutant, were expressed in strains JMtacA (*gyrA*) and JMtacB (*gyrB*) 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, protein expression induced and proteins isolated as previously described.[30]

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

Competitive fitness assays

The competitive fitness of *gyrA* mutants when co-cultured with their parental strain was assessed as previously described.[5] For each competition experiment, four separate 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 were established by introducing 100]L each of one parental and one mutant lineage into 10 mL of pre-warmed LB broth. A sample was taken at time 0 and diluted, and the viable count of both parent and mutant strain determined by plating samples on LB agar with or without 64 mg/L nalidixic acid. Each lineage was incubated and sampled at 12 h timepoints. After 12 h, 100]L of each mixed culture was used to inoculate a fresh broth. This was repeated for seven passage cycles. At each timepoint the ratio of parent:mutant in each lineage was determined. Experiments were replicated in the presence and absence of 0.03 mg/L 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 medium alone or in the presence of 0.03 mg/L triclosan. This concentration and *E. coli* were used as in competition assays containing triclosan the

E. coli Asp87Gly mutant outcompeted its parent rapidly (more than the *Salmonella* equivalent mutant).

Six independent lineages of MG1655 were grown overnight as above. Each lineage (A–F) 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 at 37°C, with shaking at 200 rpm until stationary phase was reached. Samples were taken as in competition experiments and a sample of each lineage was added to new broth. The passaging was repeated 10 times. In each sample, numbers of quinolone-resistant mutants were determined as in 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* was amplified and sequenced (all primers used are in Table S1, available as Supplementary data at *JAC* Online).

Measurement of stress response gene expression

In our previous publication we identified up-regulation of general stress response pathways 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*. 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 was measured in various conditions. All assays were completed at 37°C in LB medium. 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 ampicillin. Cultures were incubated at 37°C 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 (excitation 492 nm, emission 520 nm) was then measured in two ways. Firstly, fluorescence was measured in a BMG FluoSTAR plate reader every 10 min for 16 h (absorbance at 600 nm was also measured at each timepoint and fluorescence/absorbance values were 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 were used to set gates to identify GFP-producing cells. Samples were then run until 50000 cells within the GFP-producing population had been analysed. Flow cytometry used an Attune NxT instrument and software for data analysis (ThermoFisher, UK).

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 8-fold increase in ciprofloxacin MIC and between 64- and 128-fold increases in nalidixic acid MIC (Table 1). Both substitutions also increased the MIC of triclosan 4-fold in both species. These results were supported by growth kinetics and Biolog Phenotype Microarrays. In LB medium alone, there was no significant change in growth rate between the parental strains and their gyrase mutants (Figure S1). However, in the presence of 0.03 mg/L triclosan (0.5% the MIC for the parental strains) both *E. coli* and *Salmonella* Asp87Gly mutants grew 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 S2).

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

To determine whether there was a correlation between drug susceptibility and supercoiling activity, we purified mutant GyrA proteins and compared their activities and quinolone susceptibilities with that of the WT. 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 Ser83Phe and 10-fold for Asp87Gly), and an ~3-fold (Ser83Phe) and ~7-fold (Asp87Gly) drop in supercoiling activity compared with the WT enzyme (Table 1). The *E. coli* mutant enzymes were also tested and again showed increases in the ciprofloxacin IC_{50}. The Asp87Gly substitution resulted in a 10-fold increase (as seen with the *Salmonella* enzyme) but the Ser83Phe enzyme showed a 14-fold increase, greater than that seen with the *Salmonella* equivalent. Interestingly, the relative supercoiling activity of the Ser83Phe mutant from *E. coli* was only reduced by 10%, 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 for each strain (Table 1). However, the subtle changes in enzyme sensitivity seen in these assays were not reflected in changed MIC values between the Ser83Phe and Asp87Gly substitutions. In addition, we also made and tested Ser83Phe/Asp87Gly double-mutant GyrA proteins; these mutant enzymes from both species showed similar low supercoiling activity and no detectable inhibition by ciprofloxacin.

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 inhibition of either *Salmonella* or *E. coli* gyrase by triclosan.

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 susceptibility. 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.

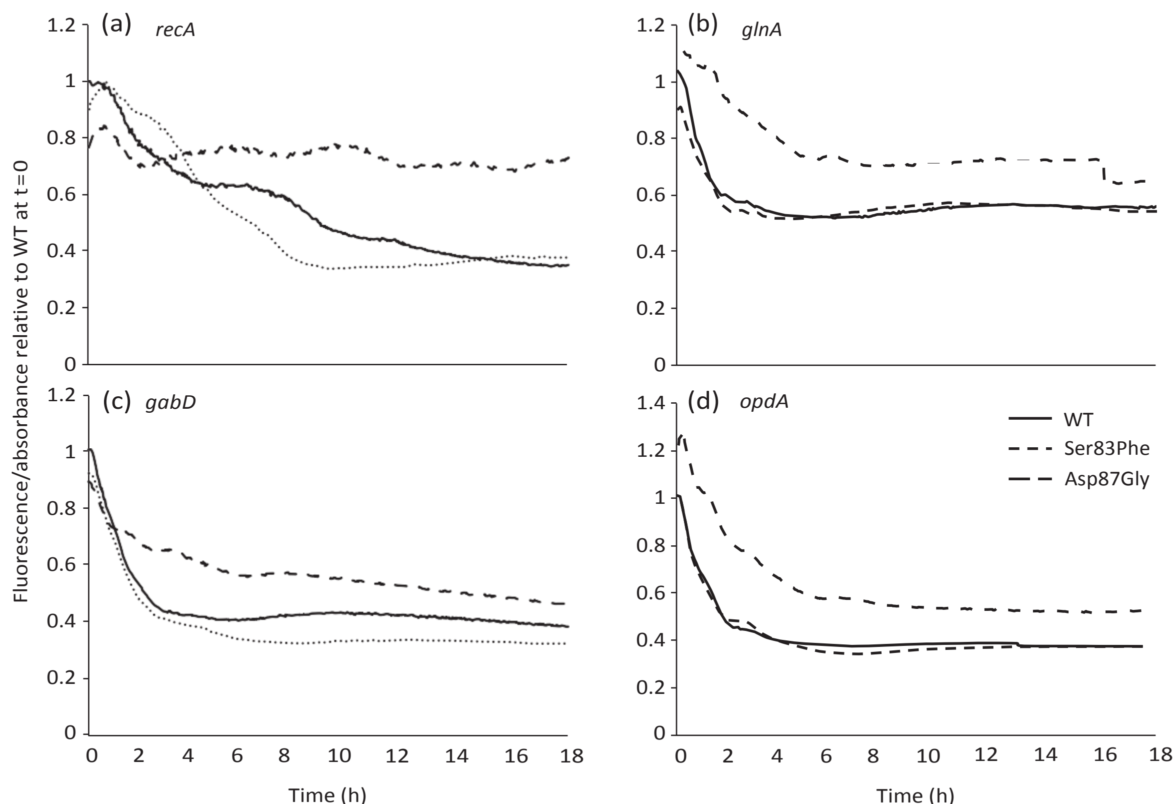

Figure 1. Expression of stress response pathways in *E. coli* strains in drug-free medium. Continuous lines indicate average fluorescence from eight replicates (two biological, four technical) of MG1655, dotted lines show data from the Ser83Phe mutant and dashed lines data show from the Asp87Gly mutant. Panel (a) shows expression from the *recA* (SOS) reporter, panel (b) shows expression from the *glnA* (*rpoN*) reporter, panel (c) shows expression from the *gabD* (*rpoS*) reporter and panel (d) shows expression from the *opdA* (*rpoH*) reporter. Data are expressed relative to the level of expression from the parental strain at the first recording point.

When compared with the parental strain, there was elevated expression of all four main stress response pathways in the *E. coli* Asp87Gly mutant (Figure 1). 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.

Data from *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% to 25% and did not vary greatly between SL1344 or the isogenic *gyrA* mutant strains (Figure 2a). 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 2b). 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.

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

Competition experiments were used to test the hypothesis that gyrase mutants would show increased fitness in the presence of triclosan. A 1:1 ratio of parent and mutant was inoculated and then repeatedly passaged for 50 generations in the presence or absence of 0.5% the triclosan MIC for the parental strains (0.03 mg/L). We chose this concentration in order to see whether a concentration of triclosan below that needed to inhibit a WT strain would promote expansion of gyrase mutants.

Both *Salmonella* mutants were outcompeted by SL1344 in drug-free medium. The Ser83Phe mutant was outcompeted by SL1344 by the fifth passage (Figure 3). The Asp87Gly mutant was also outcompeted by WT, 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 mutants were still outcompeted. 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.

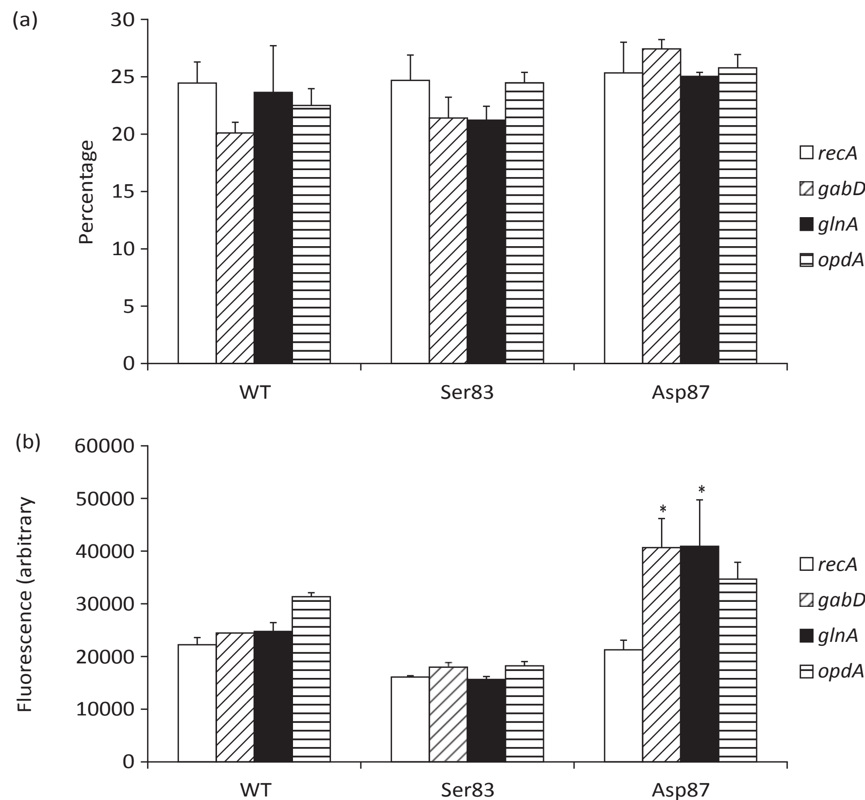

Figure 2. Expression of stress response genes in *Salmonella* gyrase mutants. (a) Percentage of total cells within populations of SL1344 (WT) and isogenic gyrase mutants that were positive for expression of GFP. (b) Average fluorescence of GFP-positive cells. Bars indicate averages of three independent replicate cultures; for each, 50000 cells were counted. Values statistically different from the corresponding WT ($P \leq 0.05$) are marked by an asterisk.

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 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 outcompeted its parent strain, dominating each population from as early as the second passage (Figure 3).

Triclosan exposure does not promote emergence of quinolone-resistant mutants

As the *E. coli* GyrA Asp87Gly mutant demonstrated a competitive fitness benefit in the presence of triclosan, we determined whether prolonged triclosan exposure would select for the emergence of Asp87Gly mutants from WT cells. To measure this, six independent cultures were inoculated and split into LB broth with or without triclosan (0.03 mg/L, as in the competition experiments this 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 lineage, and the genotype of the QRDR of *gyrA* determined for 20 of these from each condition.

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 triclosan-exposed lineages (Figure 4b) compared with control lineages (Figure 4a). Interestingly, in both cases nalidixic acid-resistant mutants were detected after approximately five passages 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 mutants were quinolone resistant. All mutants had 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 Ser83Leu 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: (i) that triclosan is itself able to bind gyrase and mutant alleles are insensitive to inhibition; and (ii) that changed supercoiling activity of mutant gyrase proteins indirectly influences triclosan susceptibility by altering expression of stress response pathways.

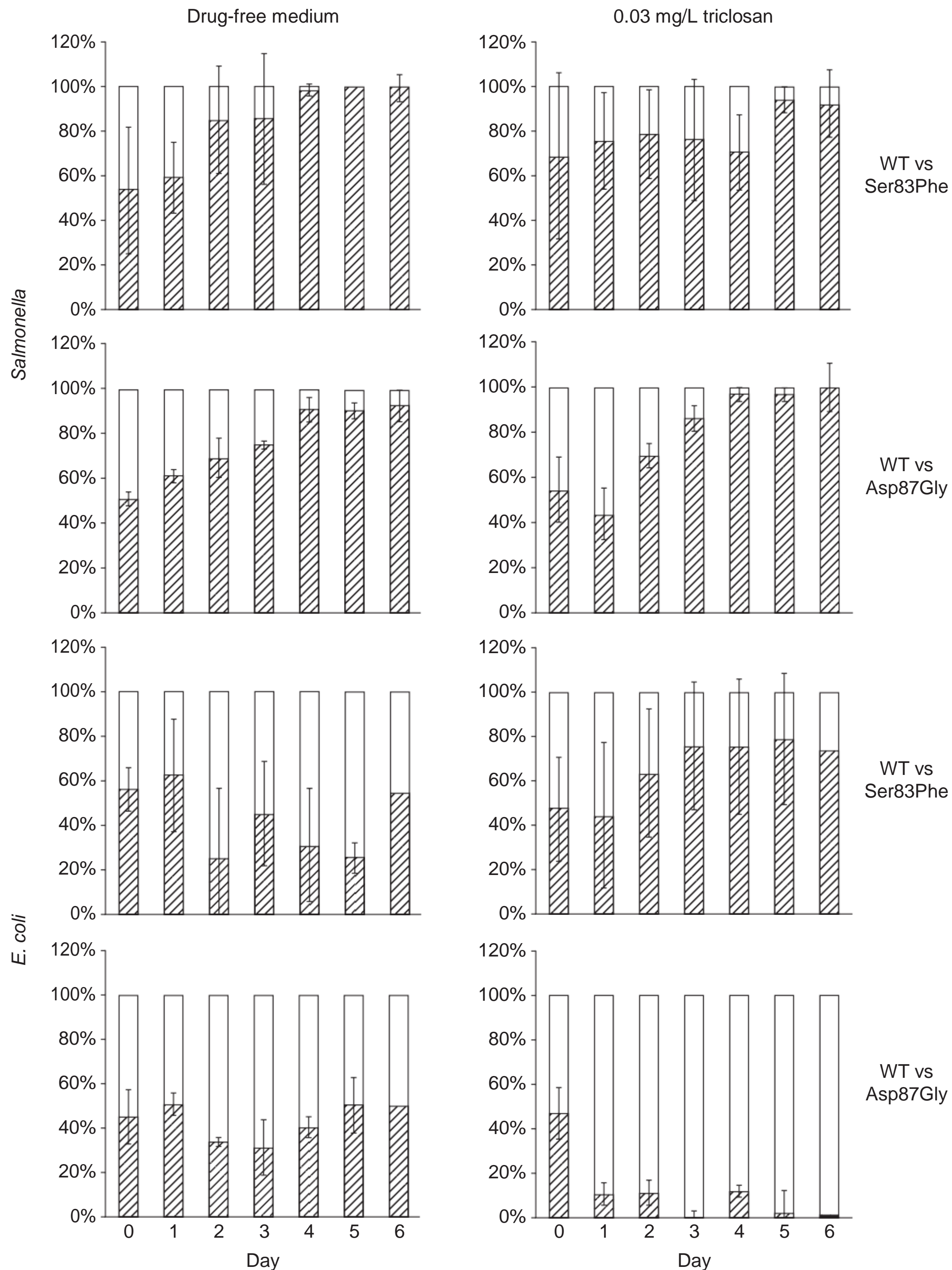

Figure 3. Competition assays of gyrase mutants versus parental strains in the presence and absence of triclosan. Data show the ratio of parent to *gyrA* mutant strains in competition assays over 6 days grown in drug-free medium (left-hand panels) and in the presence of 0.03 mg/L triclosan (right-hand panels). Data are averages from four independent experiments and in each panel the WT is represented by hatched bars and the corresponding mutant by open bars. Data from *Salmonella* are in the top four panels and data from *E. coli* are in the bottom four panels.

Experiments found no evidence for an interaction of triclosan with gyrase, which makes the first hypothesis 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 the activity of the enzyme to 30% of that of the WT and increased the IC_{50} of ciprofloxacin 5-fold. This is an impact on supercoiling activity similar to that previously described for substitution at

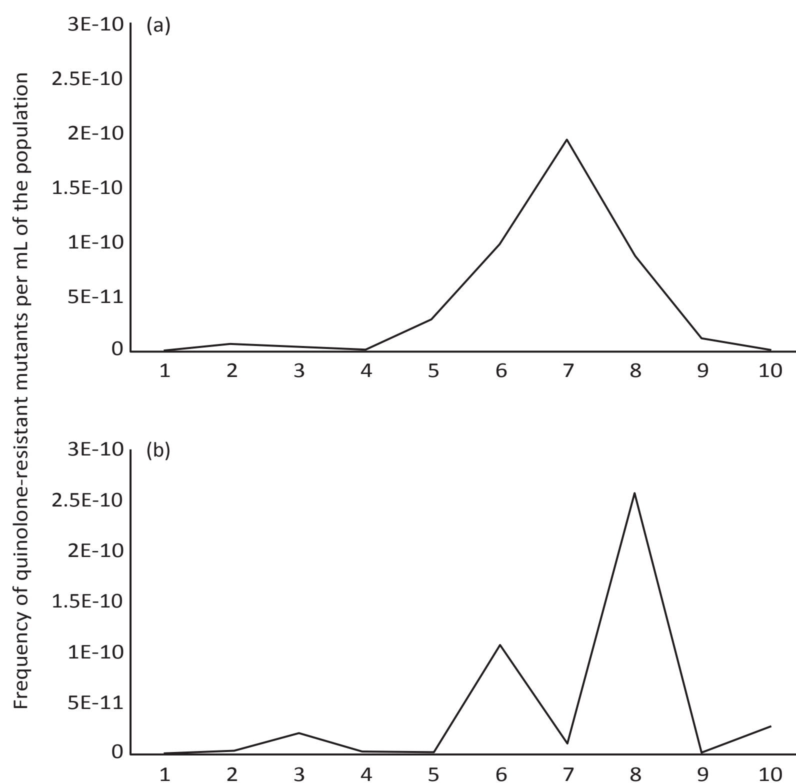

Figure 4. Emergence of nalidixic acid-resistant mutants in the presence and absence of triclosan. Average frequency of nalidixic acid-resistant mutants within populations (*y*-axis) against passage number (*x*-axis) of MG1655 that were grown in LB broth in the absence (a) or presence (b) of 0.03 mg/L triclosan. Data shown are average frequencies calculated from six independent lineages in each condition.

Ser83 in *E. coli*.[34] In contrast, the same substitution in *E. coli* only reduced enzyme activity by 10%, but this resulted in a 14-fold increase in the 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. There is, however, a difference in codon usage between the species within *gyrA* at the site that encodes Ser83—this favours the Ser83Leu substitution in *E. coli* rather than Ser83Phe, which is favoured in *Salmonella*. We sought here to examine the impact of the same *gyrA* allele in the two species so created the Ser83Phe mutation in *E. coli*. The bigger impact of this mutation in *E. coli* may be influenced by usage of a rare codon change. 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]

We observed a greater reduction in supercoiling activity associated with Asp87Gly mutants in both species; this is again similar to other observations in *E. coli*, although the relative impact of the substitution was greater in this study. We used *in vitro* assays whereas the comparator study analysed supercoiling changes *in vivo*, which may account for the difference in degree of impact seen.[34] The greater change in supercoiling activity in Asp87Gly mutants compared with 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 up-regulation 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.[9,35]

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 1g/L.[18,36] Therefore, as the gyrase mutants showed reduced susceptibility to triclosan, we sought to examine the impact of triclosan at a subinhibitory concentration on the relative fitness of gyrase mutants compared with their parent. In competition assays, for both the *Salmonella* mutants there was a fitness cost, which was evident in both drug-free medium and in the presence of low concentrations of triclosan. Under these conditions the benefit of the mutants in the face of higher triclosan concentrations was not evident and the WT was still fitter in the presence of this concentration of triclosan (Figure 3). In contrast to *Salmonella*, the *E. coli* Asp87Gly mutant could strongly outcompete its parental strain when grown in a low concentration of triclosan (Figure 3).

To determine whether there was a sufficiently strong selective pressure for Asp87Gly mutants to emerge from an *E. coli* WT population passaged in triclosan, we used an evolution experiment (Figure 4). Whilst quinolone-resistant strains (including Asp87Gly mutants) did emerge in the experiments, these were not enriched by triclosan exposure. However, this experiment only used one triclosan concentration and a relatively short time; longer time periods or other selective conditions may give different results. Whilst we saw a competitive advantage for the Asp87Gly mutant in our competition assays, these were initiated with this strain and the parent at a ratio of 50:50. In these evolution experiments the frequency of emergence of *gyrA* mutants was $\sim 1\%10^{\#8}$, and it may be that a much longer selection is needed to allow these rare mutants to expand in the population.

Taken together, the results presented here demonstrate that the association between quinolone and triclosan resistance seen in both *E. coli* and *Salmonella* is not mediated by triclosan interacting with gyrase. We postulate that the association is mediated by up-regulation of stress responses in gyrase mutants as a result of altered supercoiling. There was a greater phenotypic impact of substitution of Asp87Gly than Ser83Phe in both species tested, but a significantly greater impact of this substitution on triclosan susceptibility and stress response expression was seen in *E. coli* compared with *Salmonella*. This has implications for the relative fitness of different *gyrA* mutations in different species and suggests some *gyrA* mutants may be 'primed' to deal with non-quinolone antimicrobial stress exposures. We predicted that this would influence the competitive fitness and prevalence of these strains. However, our short-term evolution experiment did not show expansion of quinolone-resistant mutants from populations exposed to triclosan. Given the increasing prevalence of triclosan and other 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.

Acknowledgements

We thank Manal AbuOun for help with biology experiments. *E. coli* MG1655 carrying substitutions of Ser83Phe and Asp87Gly in GyrA were gifts of Will Parks, John Innes Centre.

Funding

This work was supported in part by a BBSRC Doctoral Training Grant to L. S. R. and a 1 year industry placement funded by the John Innes Centre to G. I. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1 and S2 are available as Supplementary data at *JAC* Online.

References

1 Department of Health, UK. *Chief Medical Officer Annual Report: Volume 2*. https://www.gov.uk/government/publications/chief-medical-officer-annual-report-volume-2.

2 Choi H, Lee DG. Synergistic effect of antimicrobial peptide arenicin-1 in combination with antibiotics against pathogenic bacteria. *Res Microbiol* 2012; 163: 479–86.

3 Cokol M, Chua HN, Tasan M *et al*. Systematic exploration of synergistic drug pairs. *Mol Syst Biol* 2011; 7: 544.

4 Pena-Miller R, Laehnemann D, Jansen G *et al*. When the most potent combination of antibiotics selects for the greatest bacterial load: the smile-frown transition. *PLoS Biol* 2013; 11: e1001540.

5 Webber MA, Ricci V, Whitehead R *et al*. Clinically relevant mutant DNA gyrase alters supercoiling, changes the transcriptome, and confers multidrug resistance. *MBio* 2013; 4: e00273-13.

6 Karatzas KA, Webber MA, Jorgensen F *et al*. Prolonged treatment of *Salmonella enterica* serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. *J Antimicrob Chemother* 2007; 60: 947–55.

7 Kastbjerg VG, Hein-Kristensen L, Gram L. Triclosan-induced aminoglycoside-tolerant *Listeria monocytogenes* isolates can appear as small-colony variants. *Antimicrob Agents Chemother* 2014; 58: 3124–32.

8 Tkachenko O, Shepard J, Aris VM *et al*. A triclosan-ciprofloxacin cross-resistant mutant strain of *Staphylococcus aureus* displays an alteration in the expression of several cell membrane structural and functional genes. *Res Microbiol* 2007; 158: 651–8.

9 Gantzhorn MR, Olsen JE, Thomsen LE. Importance of sigma factor mutations in increased triclosan resistance in *Salmonella* Typhimurium. *BMC Microbiol* 2015; 15: 105.

10 Blair JM, Webber MA, Baylay AJ *et al*. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 2015; 13: 42–51.

11 Redgrave LS, Sutton SB, Webber MA *et al*. Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. *Trends Microbiol* 2014; 22: 438–45.

12 Heath RJ, Yu YT, Shapiro MA *et al*. Broad spectrum antimicrobial biocides target the FabI component of fatty acid synthesis. *J Biol Chem* 1998; 273: 30316–20.

13 McMurry LM, Oethinger M, Levy SB. Triclosan targets lipid synthesis. *Nature* 1998; 394: 531–2.

14 Bailey AM, Constantinidou C, Ivens A *et al*. Exposure of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification. *J Antimicrob Chemother* 2009; 64: 973–85.

15 Mavri A, Smole Mozina S. Effects of efflux-pump inducers and genetic variation of the multidrug transporter *cmeB* in biocide resistance of *Campylobacter jejuni* and *Campylobacter coli*. *J Med Microbiol* 2013; 62: 400–11.

16 Webber MA, Coldham NG, Woodward MJ *et al*. Proteomic analysis of triclosan resistance in *Salmonella enterica* serovar Typhimurium. *J Antimicrob Chemother* 2008; 62: 92–7.

17 Webber MA, Randall LP, Cooles S *et al*. Triclosan resistance in *Salmonella enterica* serovar Typhimurium. *J Antimicrob Chemother* 2008; 62: 83–91.

18 Allmyr M, Adolfsson-Erici M, McLachlan MS *et al*. Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. *Sci Total Environ* 2006; 372: 87–93.

19 Asimakopoulos AG, Thomaidis NS, Kannan K. Widespread occurrence of bisphenol A diglycidyl ethers, *p*-hydroxybenzoic acid esters (parabens), benzophenone type-UV filters, triclosan, and triclocarban in human urine from Athens, Greece. *Sci Total Environ* 2014; 470–471: 1243–9.

20 Frederiksen H, Jensen TK, Jorgensen N *et al*. Human urinary excretion of non-persistent environmental chemicals: an overview of Danish data collected between 2006 and 2012. *Reproduction* 2014; 147: 555–65.

21 Mathews S, Henderson S, Reinhold D. Uptake and accumulation of antimicrobials, triclocarban and triclosan, by food crops in a hydroponic system. *Environ Sci Pollut Res Int* 2014; 24: 6025–33.

22 Mortensen ME, Calafat AM, Ye X *et al*. Urinary concentrations of environmental phenols in pregnant women in a pilot study of the National Children's Study. *Environ Res* 2014; 129: 32–8.

23 Toms LM, Allmyr M, Mueller JF *et al*. Triclosan in individual human milk samples from Australia. *Chemosphere* 2011; 85: 1682–6.

24 Mendez MO, Valdez EM, Martinez EM *et al*. Fate of triclosan in irrigated soil: degradation in soil and translocation into onion and tomato. *J Environ Qual* 2016; 45: 1029–35.

25 Helms M, Simonsen J, Molbak K. Quinolone resistance is associated with increased risk of invasive illness or death during infection with *Salmonella* serotype Typhimurium. *J Infect Dis* 2004; 190: 1652–4.

26 Cameron ADS, Stoebel DM, Dorman CJ. DNA supercoiling is differentially regulated by environmental factors and FIS in *Escherichia coli* and *Salmonella enterica*. *Mol Microbiol* 2011; 80: 85–101.

27 Parks W. The interaction between DNA gyrase and the peptide antibiotic Microcin B17. *PhD Thesis*. University of East Anglia, 2004. https://portal.uea.ac.uk/library.

28 Ricci V, Busby SJ, Piddock LJ. Regulation of RamA by RamR in *Salmonella enterica* serovar Typhimurium: isolation of a RamR superrepressor. *Antimicrob Agents Chemother* 2012; 56: 6037–40.

29 Bailey AM, Paulsen IT, Piddock LJ. RamA confers multidrug resistance in *Salmonella enterica* via increased expression of *acrB,* which is inhibited by chlorpromazine. *Antimicrob Agents Chemother* 2008; 52: 3604–11.

30 Maxwell A, Howells AJ. Overexpression and purification of bacterial DNA gyrase. *Methods Mol Biol* 1999; 94: 135–44.

31 Reece RJ, Maxwell A. Tryptic fragments of the *Escherichia coli* DNA gyrase A protein. *J Biol Chem* 1989; 264: 19648–53.

32 Bumann D, Valdivia RH. Identification of host-induced pathogen genes by differential fluorescence induction reporter systems. *Nat Protoc* 2007; 2: 770–7.

33 Webber MA, Whitehead RN, Mount M *et al*. Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. *J Antimicrob Chemother* 2015; 70: 2241–8.

34 Bagel S, Hullen V, Wiedemann B *et al*. Impact of *gyrA* and *parC* mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli*. *Antimicrob Agents Chemother* 1999; 43: 868–75.

35 Poole K. Stress responses as determinants of antimicrobial resistance in *Pseudomonas aeruginosa*: multidrug efflux and more. *Can J Microbiol* 2014; 60: 783–91.

36 Pirard C, Sagot C, Deville M *et al*. Urinary levels of bisphenol A, triclosan and 4-nonylphenol in a general Belgian population. *Environ Int* 2012; 48: 78–83.