

RESEARCH ARTICLE

Antivirulence DsbA inhibitors attenuate *Salmonella enterica* serovar Typhimurium fitness without detectable resistance

Rabeb Dhouib^{1,2} | Dimitrios Vagenas¹ | Yaoqin Hong^{1,2} | Anthony D. Verderosa^{1,2} | Jennifer L Martin^{3,4} | Begoña Heras⁵ | Makrina Totsika^{1,2}

¹Institute of Health and Biomedical Innovation, School of Biomedical Sciences, Queensland University of Technology, Herston, QLD, Australia

²Centre for Immunology and Infection Control, School of Biomedical Sciences, Queensland University of Technology, Herston, QLD, Australia

³Griffith Institute for Drug Discovery, Griffith University, Nathan, QLD, Australia

⁴University of Wollongong, Wollongong, NSW, Australia

⁵La Trobe Institute for Molecular Science, La Trobe University, Bundoora, VIC, Australia

Correspondence

Associate Professor Makrina Totsika,
Institute of Health and Biomedical
Innovation, Queensland University of
Technology, 300 Herston Rd, Herston,
QLD, 4006, Australia.
Email: makrina.totsika@qut.edu.au

Associate Professor Begoña Heras, La
Trobe Institute for Molecular Science, La
Trobe University, Bundoora, VIC, 3068,
Australia.
Email: b.heras@latrobe.edu.au

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ABSTRACT

Inhibition of the DiSulfide Bond (DSB) oxidative protein folding machinery, a major facilitator of virulence in Gram-negative bacteria, represents a promising antivirulence strategy. We previously developed small molecule inhibitors of DsbA from *Escherichia coli* K-12 (EcDsbA) and showed that they attenuate virulence of Gram-negative pathogens by directly inhibiting multiple diverse DsbA homologues. Here we tested the evolutionary robustness of DsbA inhibitors as antivirulence antimicrobials against *Salmonella enterica* serovar Typhimurium under pathophysiological conditions in vitro. We show that phenylthiophene DsbA inhibitors slow *S. Typhimurium* growth in minimal media, phenocopying *S. Typhimurium* isogenic *dsbA* null mutants. Through passaging experiments, we found that DsbA inhibitor resistance was not induced under conditions that rapidly induced resistance to ciprofloxacin, an antibiotic commonly used to treat *Salmonella* infections. Furthermore, no mutations were identified in the *dsbA* gene of inhibitor-treated *S. Typhimurium*, and *S. Typhimurium* virulence remained susceptible to DsbA inhibitors. Our work demonstrates that under in vitro pathophysiological conditions, DsbA inhibitors can have both antivirulence and antibiotic action. Importantly, our finding that DsbA inhibitors appear to be evolutionarily robust offers promise for their further development as next-generation antimicrobials against Gram-negative pathogens.

KEYWORDS

antimicrobial resistance, disulfide bond, enzyme inhibitors, experimental evolution, infection

Abbreviations: ASST, aryl-sulfate sulfotransferase; CFU, colony forming unit; CI, confidence interval; DMSO, dimethyl sulfoxide; DSB, disulfide bond; Glc, glucose; His, histidine; IC₅₀, half maximal inhibitory concentration; MDR, multidrug resistant; MIC, minimum inhibitory concentration; MUS, methylumbelliferyl sulfate; SEM, standard error of the mean; TRX, thioredoxin; UPEC, uropathogenic *Escherichia coli*; WHO, World Health Organization; WT, wild-type.

Rabeb Dhouib and Dimitrios Vagenas are contributed equally to this work.

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1 | INTRODUCTION

Tackling antimicrobial resistance is a global public health priority for the 21st century.^{1,2} The rapid emergence and widespread dissemination of antibiotic resistance mechanisms in bacteria are outpacing the discovery of new antibiotics and thwarting their development.³ Antibiotic alternatives or adjuvants for treating common infections that are now refractory to most, or even all, available antibiotics are urgently needed, particularly for multidrug-resistant Gram-negative pathogens.⁴⁻⁶ Virulence inhibition is an attractive antimicrobial approach: antivirulence drugs targeting major virulence pathways in bacteria (e.g., toxins, adherence, quorum sensing, and protein secretion) are currently at various stages of development.⁷⁻¹¹ Antivirulence drugs promise a number of clinical advantages over traditional antibiotics. One proposed benefit is that inhibiting bacterial pathogenicity (rather than blocking growth or viability) will lower selection pressure for drug resistance in bacterial populations, depending on the virulence target.^{8,12-15} However, for the majority of antivirulence drugs in development, this “evolution-proof” tenet remains to be experimentally validated.

Only a few studies to date have directly tested the evolutionary robustness of antivirulence drugs. Of note, extracellular siderophore (iron-scavenging) quenching in *Pseudomonas aeruginosa* proved to be evolutionarily robust, but resistant mutants were later reported to arise sporadically in studies utilizing human serum for bacterial growth.^{16,17} Similarly, the action of quorum sensing inhibitors was shown to be bypassed by *P. aeruginosa* mutants arising in vitro or in the lungs of chronic cystic fibrosis patients.^{16,17} Such contrasting results highlight the diverse evolutionary scenarios that can arise when inhibiting different virulence targets from different bacteria. Discrepancies in study findings might also be due to different assay protocols and testing conditions. These findings caution against drawing generalized conclusions on the evolutionary robustness of antivirulence drugs. Further studies are needed that directly test the evolution of resistance to antivirulence drugs under physiologically relevant conditions. Such studies provide important insights into the clinical advantages of these new classes of antibacterials.¹⁵

Here we investigate the evolutionary robustness of inhibiting DsbA enzymes in *Salmonella enterica* serovar Typhimurium. DsbA enzymes in *S. Typhimurium*, and several other Gram-negative pathogens, catalyze the formation of disulfide bonds (Dsb), which are involved in the structural bracing and functional folding of multiple virulence factors.¹⁸ Thus, Dsb enzymes play a central role in bacterial pathogenesis and constitute promising targets for antivirulence therapeutics.^{19,20} Recently, several compounds targeting both

enzyme components of the oxidative protein folding machinery in *Escherichia coli* K-12, EcDsbA (oxidase) and EcDsbB (isomerase), have been reported.²¹⁻²³ Further, we have recently shown that small molecule inhibitors of EcDsbA can attenuate the virulence of uropathogenic *E. coli* and *S. Typhimurium*.²⁴ Both of these Gram-negative pathogens encode, apart from a prototypical DsbA, accessory DsbA homologues that mediate different virulence phenotypes.^{25,26} *S. Typhimurium*, a common cause of food-borne illness and the major causative agent of diarrheal disease globally, encodes, in addition to the prototypical DsbA/DsbB pair, the accessory DsbL/DsbI pair and the plasmid-encoded DsbA homologue, SrgA.^{25,27} We have previously shown that DsbA inhibitors from two chemical classes (phenylthiophene and phenoxyphenyl derivatives) can inhibit each of these diverse DsbA homologues found in *S. Typhimurium*.²⁴

Here we followed an experimental evolution approach to assess the evolutionary robustness of these EcDsbA inhibitors against *S. Typhimurium*. These experiments were performed under nutrient-limiting growth conditions that mimic the metabolic stress encountered by the pathogen during host infection.^{28,29} Inhibition of DsbA in *S. Typhimurium* cultured in pathophysiological conditions incurred a fitness cost on pathogen growth that phenocopied isogenic *dsbA* null mutants cultured under the same conditions. No drug-specific adaptation was observed in the bacterial population following continuous exposure to DsbA inhibitors. All drug-exposed bacteria retained wild-type virulence in the absence of the drug, while became attenuated for DsbA-mediated virulence phenotypes in the presence of the drug. Our findings demonstrate no detectable resistance development to DsbA inhibitors in *S. Typhimurium* under physiologically relevant in vitro conditions. This outcome supports the tenet that DsbA inhibitors could be regarded as “evolution-proof” antimicrobials against critical-priority Gram-negative pathogens.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table S1. Bacterial cultures were routinely maintained at 37°C in solid or liquid Lysogeny Broth (LB, Lennox) or in M9-Glc-His medium (M9 salts supplemented with 0.2% [w/v] Glc and 0.135 mM His³⁰), supplemented when required with different concentrations of DsbA inhibitors F1 and F2 or dimethyl sulfoxide (DMSO), as the drug carrier control. Ampicillin (Amp, 100 µg/mL) and chloramphenicol (CPL, 34 µg/mL) were added to culture media where needed to maintain Dsb complementation plasmids (pSrgA, pDsbLI, and pDsbA) and pASST, respectively.

2.2 | Growth experiments

2.2.1 | a) In vitro assays

S. Typhimurium SL1344 growth assays were performed in M9 minimal medium supplemented with glucose (Glc) and L-Histidine (His) (M9-Glc-His) at 37°C with aeration (200 rpm), unless otherwise stated. Supplementation of culture media with antibiotics, DsbA inhibitors, or DMSO was performed as required for each strain (wild-type, mutants, and complemented mutants). Inhibitors F1 and F2 were tested at various concentrations ranging from 0.005 to 3 mM. Briefly, four independent SL1344 cultures grown overnight at 37°C in M9-Glc-His medium with shaking at 200 rpm were diluted to an OD_{600 nm} of 0.07. DsbA inhibitors were then added at the desired concentration, and control cultures containing a final concentration of 1.2% DMSO or unsupplemented M9-Glc-His were also prepared. Each culture was aliquoted three times into 96-well microtiter plates (200 µL/well) (Greiner, 650101) sealed with Breathe-Easy[®] membrane (Sigma-Aldrich) and incubated at 37°C for 20 h in a microplate reader (BMG LABTECH CLARIOstar[®] for DsbA inhibitors/BMG LABTECH SPECTROstar Nano for ciprofloxacin). Bacterial growth (OD_{600 nm}) was monitored every 15 min and plates were shaken continually (Meander corner) except during the reading event. Growth (OD_{600 nm}) over time data were statistically modeled as described below.

2.2.2 | b) Statistical modeling

Bacterial growth curves (OD_{600 nm} over time) were estimated using custom-written scripts in R³¹ based on the package “grofit”.³² Both Logistic and Gompertz models were generated and the best fit was judged using the Akaike's Information Criterion. In the majority of cases, the Logistic curves fitted the data better and thus these were used in further analyses. It should be noted that the formulation of the Logistic curve in “grofit” assigns biological meaning to each of the parameters, which is a major advantage, as the calculated parameters directly describe distinct phases of the bacterial growth cycle: (i) latency phase, (ii) maximum slope (i.e., exponential phase), and (iii) maximum of the curve (i.e., stationary phase). In order to get robust estimates of the confidence intervals for the Logistic curve parameters, a bootstrap procedure (n = 10,000 repeats) was used based on the “boot” package of R.³³ Figures were created using “ggplot2” with auxiliary functions from packages “grid” and “gridExtra”³⁴ as well as the “reshape” package for data manipulation.³⁵

2.3 | Experimental evolution

S. Typhimurium SL1344 wild-type strain was subjected to experimental evolution of resistance to DsbA inhibitors F1 and F2 or ciprofloxacin for 10 days. Four independent overnight cultures of SL1344 were grown and diluted as described above. F1 and F2 inhibitors at 1 mM or ciprofloxacin at 10 µg mL⁻¹ were then added to each culture with carrier and media-only controls with and without 1.2% DMSO, respectively, also prepared at the same time. Every culture was aliquoted twice into a 96-well microtiter plate and bacterial growth (OD_{600 nm}) was monitored over 20 h of growth with conditions as described above. At the end of each 20 h growth cycle, final OD_{600 nm} values were measured for each culture and bacteria were resuspended in fresh media (same formulation as the previous culture) by diluting to an OD_{600 nm} = 0.07. Bacterial growth was monitored as described above, and subcultures were repeated nine times resulting in a total of 10 sequential culture cycles. Growth curves were plotted and statistically modeled, as described above. At the end of each 20-hour culture cycle, samples were taken from each well, 10-fold serially diluted, and plated out on LB-agar plates to enumerate viable CFU. Stationary phase samples from each culture cycle were also used in downstream growth and virulence assays.

2.4 | Virulence assays

Swimming motility of *S. Typhimurium* SL1344 was conducted on soft agar as previously described.²⁴⁻²⁶ For ASST activity assays, SL1344 was transformed with pASST, cultured overnight in LB-CPL broth, and spot plated at an OD_{600 nm} = 1 onto LB agar plates containing 0.1 mM 4-methylumbelliferyl sulfate (4-MUS), CPL, and 1 mM F1 or DMSO. After overnight incubation at 37°C, the fluorescent product 4-methylumbelliferone generated by the cleavage of 4-MUS by ASST was quantified under UV light (320 nm) using Image LabTM software. Relative ASST inhibition was calculated as the ratio of fluorescence generated by F1-exposed SL1344 versus unexposed SL1344.

2.5 | STRUM analyses

To predict the mutability of *S. Typhimurium* DsbA, the three-dimensional structure of DsbA (PDB code 3L9S) was subjected to STRUM.³⁶ This computational tool predicts changes in the stability of the protein (Gibbs free energy changes) upon mutating each position in the protein to all other amino acids. The output from STRUM provides a predicted free energy change on all-to-all mutations ($\Delta\Delta G = \Delta G_{\text{mutant}} - \Delta G_{\text{native}}$). $\Delta\Delta G$ values >1 denote stabilizing mutations, while $\Delta\Delta G$

values <1 correspond to destabilizing mutations. STRUM also provides a predicted mutability score F_i that describes the total probability of mutations of each amino acid in the protein to every other amino acid. F_i is calculated from

$$F_i = \sum_0^{19} e^{\Delta\Delta G_{\text{native} \rightarrow \text{mutant}}}$$

Heat maps representing the F_i scores were plotted on to the surface of the DsbA crystal structure on a gradient from white (F_i less than 10), cyan (F_i values between 10 and 20), and light purple to dark purple representing increasing F_i values ($F_i >20$). The structure figures were created with PyMOL.³⁷

3 | RESULTS

3.1 | DsbA inhibitors reduce *S. Typhimurium* SL1344 fitness in physiologically relevant conditions

We have previously demonstrated that the phenylthiophene EcDsbA inhibitors F1 and F2 attenuate the virulence of *S. Typhimurium* SL1344 without affecting growth in standard laboratory culture conditions.²⁴ However, their impact on bacterial growth under nutrient deprivation remains unexplored. During infection, *S. Typhimurium* invade the intestinal epithelium and are taken up by macrophages where they survive and replicate.³⁸ Macrophages expose bacteria

to oxidative stress and antimicrobials, and metabolically challenge invading pathogens by starving them of intracellular nutrients.^{28,29} Thus, to assess the impact of DsbA inhibitors under nutrient-deprived conditions, we conducted *S. Typhimurium* growth experiments in minimal media M9-Glc-His supplemented with different concentrations of inhibitor F1 or F2. The growth of SL1344 was unaffected at DsbA inhibitor concentrations below 1 and 0.6 mM for F1 and F2, respectively (Figure 1). However, at higher inhibitor concentrations, the growth of SL1344 decreased in a dose-dependent manner, with complete growth inhibition (minimum inhibitor concentration; MIC) observed at approximately 1.8 mM and 1.5 mM of F1 and F2, respectively (Figure 1). IC₅₀ values (with 95% CI) were calculated from dose-response curves at 1.30 mM (1.24, 1.34) for F1 and 1.14 mM (0.99, 1.26) for F2. Taken together, these findings demonstrate that DsbA inhibitors can reduce the fitness of *S. Typhimurium* under physiologically relevant growth conditions.

3.2 | DsbA enzymes contribute to *S. Typhimurium* SL1344 growth in minimal media

To examine if reduced pathogen growth in minimal media was due to the specific inhibition of DsbA enzymes, we utilized a genetic approach and profiled the M9-Glc-His growth of a previously described set of SL1344 isogenic mutants lacking one,

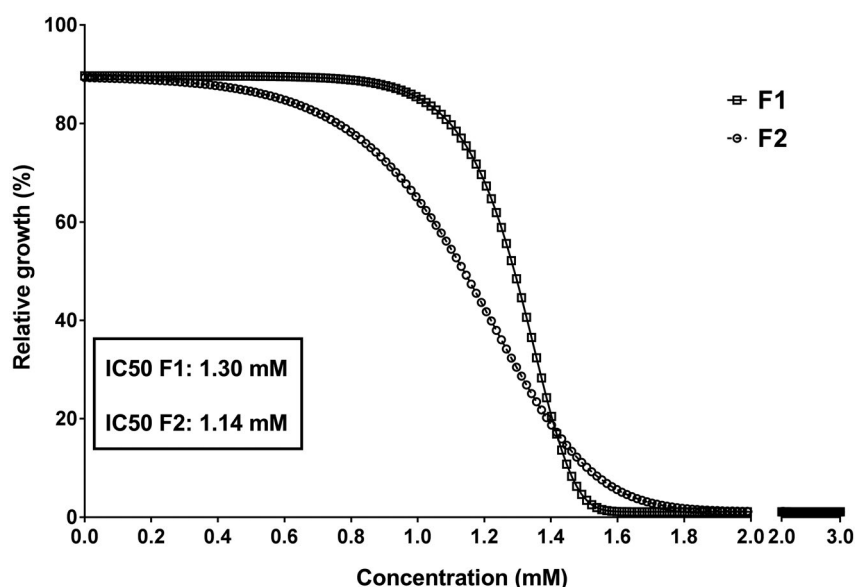


FIGURE 1 *S. Typhimurium* growth under physiologically relevant conditions in increasing concentrations of DsbA inhibitors. Best-fit curves of SL1344 growth in increasing concentrations of DsbA inhibitors F1 and F2 were generated by modeling SL1344 growth curve data (OD_{600 nm}) collected over 20 hours of culture at 37°C in minimal media (M9-Glc-His) containing different concentrations of F1 or F2 (0.005, 0.05, 0.1, 0.5, 0.75, 1, 1.5, and 3 mM). Growth inhibition curves of four independent culture repeats were conducted each with three technical replicates. IC₅₀ values (with 95% CI) for inhibitors F1 and F2 were estimated at 1.30 mM (1.24, 1.34) and 1.14 mM (0.99, 1.26), respectively.

two, or three *dsbA* homologues.²⁵ Mutants complemented *in trans* with each missing homologue or the empty vector were used alongside as controls (Figure S1). The growth parameters (growth rate [$\Delta OD_{600\text{ nm}}/\Delta h$], maximum $OD_{600\text{ nm}}$, and length of latency phase [h]) of SL1344 strains lacking *srgA* or *dsbLI* were not significantly different from the wild-type (WT) strain (Figure 2). However, deletion of *dsbA* resulted in a significantly lower growth rate, maximum $OD_{600\text{ nm}}$, and longer latency phase compared to WT in these growth conditions (Figure 2). In a genetic background already devoid of *dsbA*, deletion of either *srgA* (SL1344 *dsbA srgA*) or *srgA* and *dsbLI* (SL1344 *dsbA srgA dsbLI*) further reduced the growth rate and maximum $OD_{600\text{ nm}}$, and increased growth lag phase (Figure 2). Interestingly, when provided *in trans*, all three *dsbA* homologues could restore the growth defect of the triple mutant (SL1344 *dsbA srgA dsbLI*) to or close to WT levels (Figure 2).

Exogenously restoring protein oxidative folding by supplementing the growth media with a strong oxidant

(0.1 mM L-cystine) similarly restored the growth rate of the triple mutant to WT levels (Figure S2A). These findings demonstrate that in nutrient-limiting *in vitro* conditions, all three DsbA enzymes contribute to *S. Typhimurium* growth synergistically and to different extents, that is, in the absence of either *SrgA* or *DsbL*, *DsbA* can fully compensate SL1344 growth. However, in the absence of *DsbA* or any of the other homologues, growth is significantly reduced but to a different extent. Importantly, the growth rate of the triple mutant was (a) reduced by a similar level as that of the wild-type when cultured in the presence of *DsbA* inhibitors (0.52 [triple mutant] vs. 0.56 [F1]), and (b) was not further impacted by the addition of inhibitor F1 in the growth media (Figure S2B), indicating a lack of off-target effects. Taken together, these findings suggest that *DsbA* inhibitors can slow the growth of *S. Typhimurium* in physiologically relevant conditions by specifically inhibiting its *DsbA* enzymes.

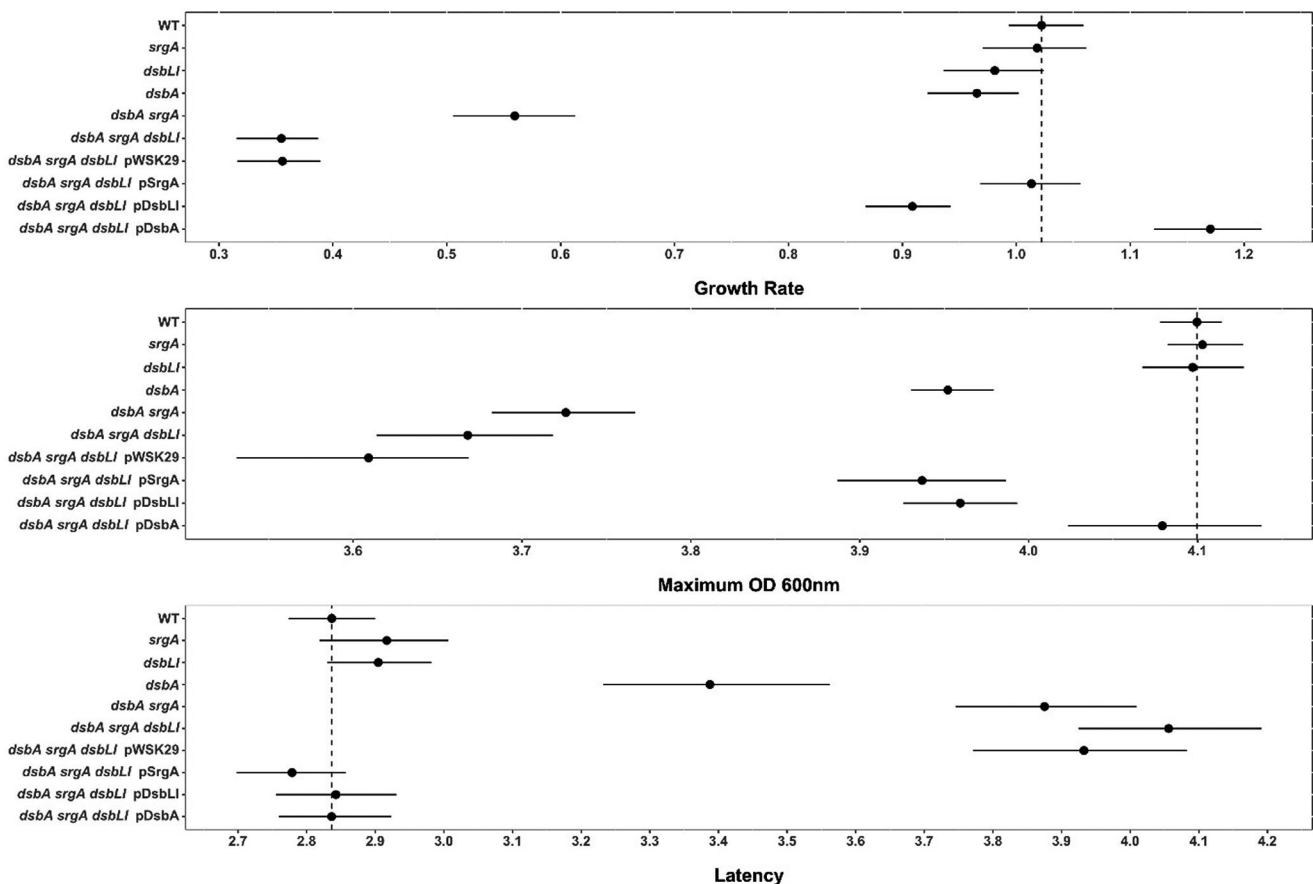


FIGURE 2 Growth parameters of *S. Typhimurium* SL1344 wild-type, isogenic single, double, and triple *dsbA* deletion mutants and complemented strains. (A) Growth rate, (B) maximum $OD_{600\text{ nm}}$, and (C) latency phase length of SL1344 strains cultured in minimal media (M9-Glc-His; supplemented with Amp as required) at 37°C with shaking. Strains lacking one (*dsbA*, *srgA*, or *dsbLI*), two (*dsbA srgA*), or all three (*dsbA srgA dsbLI*) genes and complemented mutants were assessed in at least three independent cultures, each tested in triplicate. Logistic curve parameters were estimated from bacterial growth curve data ($OD_{600\text{ nm}}$) collected over 20 hours of culture and are shown as means with 95% CI. Vertical dotted lines depict the mean parameter value for wild-type (WT) SL1344 as the reference strain. Statistically significant differences between WT and mutants exist where the mean (with 95% CI) does not cross the vertical dotted line of the WT.

3.3 | Passage of *S. Typhimurium* SL1344 in sub-MIC concentrations of DsbA inhibitors does not induce inhibitor resistance

The fitness cost of DsbA inhibitors for *S. Typhimurium* under physiologically relevant conditions would suggest that inhibitor-resistant mutants might be placed under positive selection in a hypothetical scenario of inhibitor clinical use. To explore this, we first utilized an *in silico* approach to probe the mutability of the *S. Typhimurium* DsbA enzyme using the structure-based computational tool STRUM,³⁶ which predicts changes in protein stability resulting from mutating each residue in the protein to all other amino acids. Our analysis showed that the probability of mutations at the His32 position of *S. Typhimurium* DsbA, in the Cys-Pro-His-Cys catalytic center, was considerably higher than for any other residue in the protein (Figure 3 and Figure S3). The Cys-X-X-Cys catalytic center is a conserved essential motif in thioredoxin-like redox-active proteins, and the X-X dipeptide of this motif determines specific redox properties and function.³⁹ The His residue has also been shown to interact with EcDsbA inhibitors that block enzyme activity.²² The STRUM results identifying His32 of *S. Typhimurium* DsbA as a highly probable site for mutation led us to investigate whether treatment with DsbA inhibitors could select for mutations at that site.

To test this scenario *in vitro*, we performed continuous SL1344 sub-cultures in M9-Glc-His medium containing F1 or F2 at sub-MIC concentrations for a 10-day period

(Figure S4). Passaging SL1344 in control media lacking DsbA inhibitors daily for 10 days did not result in altered growth, as expected (Figure 4). In the presence of DMSO (drug carrier control), some fluctuation in SL1344 growth rate (but not in maximum OD_{600 nm} value) was observed from day 7 onwards, but differences between cycles were not statistically significant (Figure 4A,B). As expected, culturing SL1344 in the presence of 1 mM F1 or F2 resulted in a slower growth rate compared to DMSO control (about 50% reduction upon the first growth cycle). While some fluctuation in growth rate was detected between subsequent sub-cultures in F1 or F2, no significant improvement in bacterial growth was observed incrementally from one cycle to the next, that would be indicative of a growth rate recovery due to the emergence and selection of mutants resistant to either inhibitor tested (Figure 4B).

This finding was further confirmed by enumerating viable CFU recovered after each growth cycle, which demonstrated a very similar trend to that seen for the maximum OD_{600 nm} (Figure 4C). In addition, no sequence changes were detected in the *dsbA* gene of all inhibitor-treated cultures (data not shown). Importantly, in passaging experiments conducted under the same growth conditions, SL1344 quickly gained resistance to the antibiotic ciprofloxacin with a cumulative growth rate increase of 64% observed between cycle 1 and cycle 10 (Figure S5). These results suggest that continuous treatment of *S. Typhimurium* with DsbA inhibitors under pathophysiological *in vitro* conditions does not induce resistance, in contrast to the antibiotic treatment.

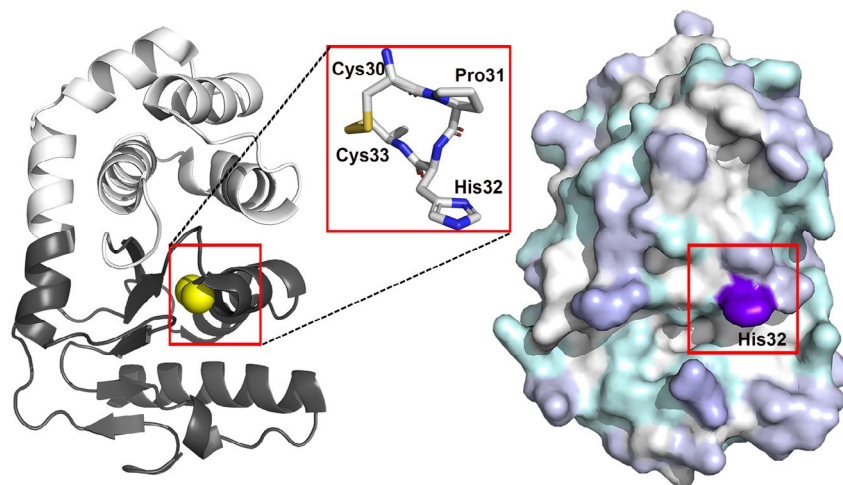
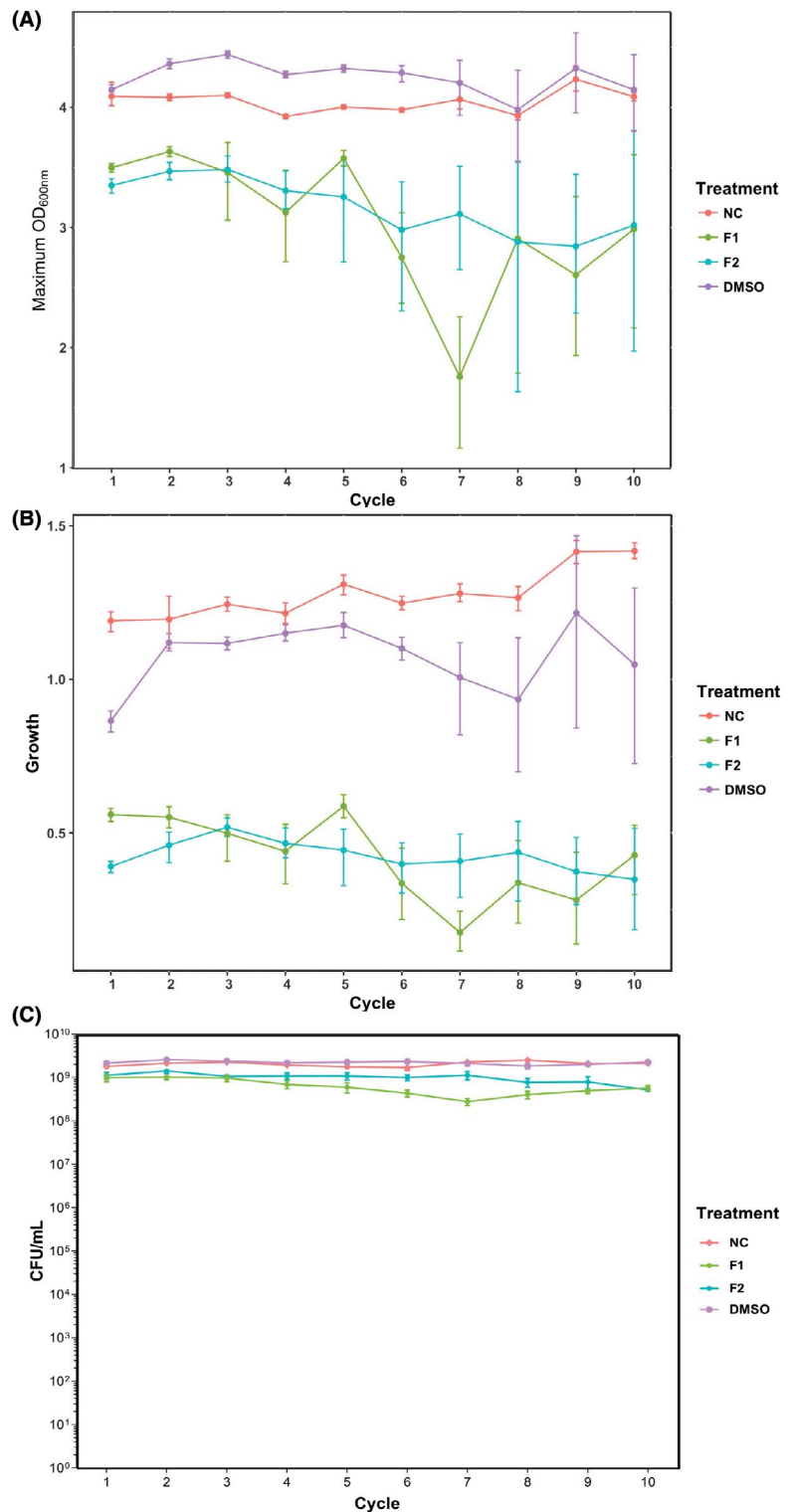


FIGURE 3 3D Structure and mutation mapping of *S. Typhimurium* DsbA enzyme. (Left panel) Ribbon representation of *S. Typhimurium* DsbA (PDB entry 3L9S) with the thioredoxin (TRX)-fold and inserted helical domain shown in dark and light grey, respectively, and the sulfur atoms of the catalytic site shown as yellow spheres. Inset shows a close-up view of the ³⁰Cys-³¹Pro-³²His-³³Cys active site. (Right panel) Mutation score (*Fi*) of *S. Typhimurium* DsbA. The *Fi* score was calculated with STRUM³⁶ and describes the probability of mutations in each residue to all other amino acids (the higher the mutation score, the greater the probability of mutation). Calculated *Fi* scores (Figure S3 and Figure S4) for each *S. Typhimurium* DsbA residue were mapped onto the protein surface and depicted using a color gradient: white (low *Fi* score), through to cyan (intermediate *Fi* score), light purple (high *Fi* score), and dark purple (very high *Fi* score).

FIGURE 4 Growth parameters of *S. Typhimurium* sequentially cultured in the presence of sub-MIC DsbA inhibitor concentrations in physiologically relevant conditions. SL1344 (A) growth rate and (B) maximum OD_{600 nm} in each of 10 sequential growth cycles in M9-Glc-His media supplemented with 1 mM DsbA inhibitor (F1 or F2), the drug carrier control (DMSO), or nothing (NC). Logistic curve parameters were estimated from growth data (OD_{600 nm}) of four independent SL1344 cultures collected over 20 hours and are shown as means with 95% CI. (C) Viable bacteria were determined at the end of each SL1344 growth cycle in the different conditions (F1, F2, DMSO, and NC) and quantified as colony forming units (CFUs) recovered per mL of culture. CFU data are shown as mean \pm SEM calculated from at least three independent cultures, each with three or four technical repeats.



3.4 | Virulence of *S. Typhimurium* SL1344 remains susceptible to DsbA inhibitors after passage in sub-MIC concentrations

To further confirm that inhibitor-treated SL1344 cultures did not show resistance to DsbA inhibitors, we assessed their susceptibility to virulence inhibition in assays that report specifically on DsbA enzyme in vivo function. We first compared

the capacity of F1-exposed and unexposed SL1344 cultures to functionally fold the flagellar motor protein component FlgI, which results in SL1344 swimming motility that can be inhibited by F1.^{24,25} We hypothesized that if any SL1344 cultures had gained resistance to F1, DsbA-mediated folding of FlgI could still occur in the presence of inhibitors and motility would be observed in this condition. Inhibitor-exposed and unexposed SL1344 cultures were inoculated onto LB

semi-solid agar supplemented with 0.4% DMSO or 1 mM F1. Motility was observed for all F1-exposed cultures, with swimming zone diameters reaching the same level as unexposed SL1344, and in the presence of F1 motility was equally inhibited in all cultures (Figure 5A).

We have previously shown that functional folding of the bacterial ASST enzyme is catalyzed by DsbA enzymes in SL1344 and can be inhibited by F1.^{24,25} In the cell-based ASST enzymatic assay, the cleavage of the substrate 4-methylumbelliferyl sulfate (MUS) by ASST is monitored by fluorescence. Using this assay, we found that ASST activity was equally inhibited by the presence of F1 in F1-exposed SL1344 cultures or in unexposed SL1344 cultures (Figure 5B). Taken together, these findings demonstrate that continuous exposure of *S. Typhimurium* to DsbA inhibitors in physiologically relevant conditions does not alter the pathogen's virulence susceptibility to these inhibitors. When considered together with the unaltered growth phenotype

following sequential sub-MIC exposure, our results strongly indicate that DsbA inhibitors do not induce detectable population resistance in *S. Typhimurium* under the conditions tested and could thus be regarded as “evolution-proof” early antivirulence drug candidates.

4 | DISCUSSION

Signs of a post-antibiotic era are already evident, with many infections increasingly becoming untreatable by all available antibiotic classes. Dr Mariângela Simão, the WHO assistant director general for access to medicines and health products, stated that antibiotic resistance is now an “invisible pandemic”.⁴⁰ Indeed, the evolution and rapid spread of antibiotic resistance typically outpace their discovery and costly development for clinical use.³ For instance, the first daptomycin-resistant *S. aureus* clinical isolate was reported only 3 years

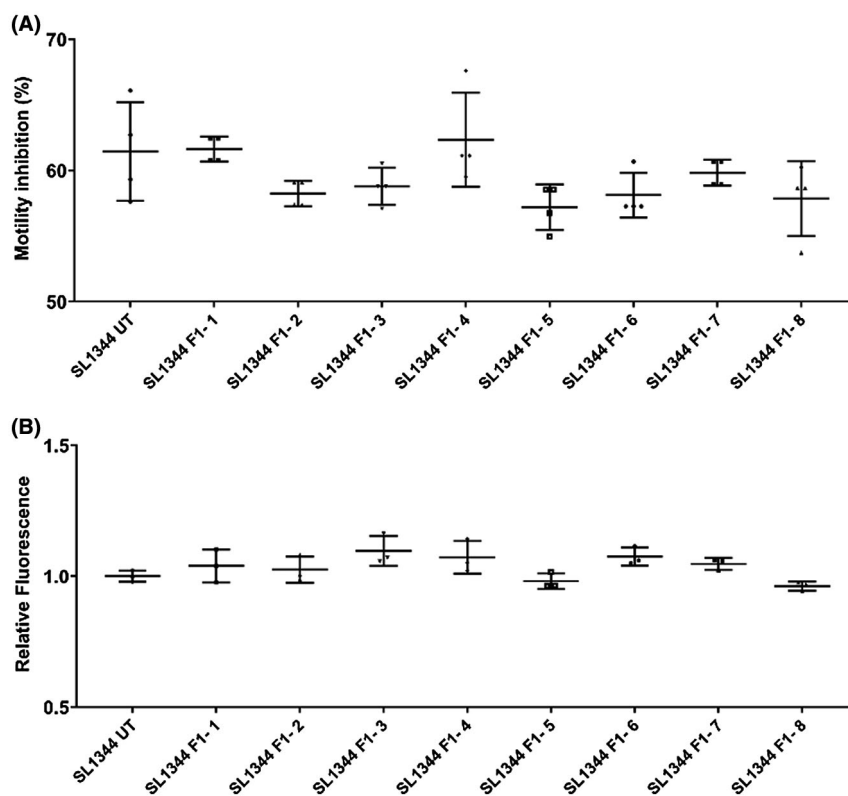


FIGURE 5 *S. Typhimurium* virulence remains sensitive to DsbA inhibition following sequential growth in sub-MIC inhibitor concentrations. (A) SL1344 motility inhibition by DsbA inhibitor F1: Eight SL1344 replicate cultures treated with 1 mM F1 over 10 sequential growth cycles (SL1344 F1-1 to SL1344 F1-8) or untreated controls (SL1344 UT) were inoculated onto LB soft agar plates (0.3% agar) containing 1 mM F1 or DMSO (carrier control). The diameter of bacterial swimming zones was recorded after 12 h of incubation at 37°C and motility inhibition was calculated as the ratio of motility zone diameter in F1 over DMSO plates. Data are shown as % motility inhibition of four independent replicates (dots) with the mean (horizontal line) and standard error of the mean shown as error bars. (B) SL1344 ASST activity in the presence of F1: eight SL1344 replicate cultures treated with 1 mM F1 over 10 sequential growth cycles (SL1344 F1-1 to SL1344 F1-8) or untreated controls (SL1344 UT) were transformed with pASST and inoculated onto LB-CPL agar containing 0.1 mM MUS and 1 mM F1. ASST cleaves MUS to release a fluorescent product that was quantified under UV light (320 nm) using Image LabTM software. Relative ASST activity was calculated as the fluorescence ratio of F1-treated SL1344 over untreated SL1344. Data are shown as relative fluorescence of three independent replicates (dots) with group means marked as horizontal lines and standard error of the mean shown as error bars.

after the drug was introduced,⁴¹ and in less than a decade, resistance had spread to all clinically important multidrug-resistant Gram-positive pathogens.⁴²

Treatment of bacteria with antibiotics triggers the SOS mutagenic response,⁴³ leading to increased mutation rates, and thus promoting the emergence of resistant mutants.^{44,45} Epistatic mutations elsewhere in the chromosome often quickly arise to stabilize the resistant mutants, resulting in stable clones with high competitive fitness.⁴⁶ It is now also evident that exposure to low-level antibiotics present in many environments can facilitate mutational changes in bacteria to acquire high-level resistance.⁴⁷ Most antibiotics are natural compounds (or their synthetic analogues) produced by microorganisms to outcompete other microorganisms. Defensive mechanisms for these compounds have often already evolved before a drug's clinical use, with resistance genes widely present in the environment, even at remote uninhabited areas (e.g., in Antarctic soils).⁴⁸ Several decades of heavy antibiotic usage in clinics and agriculture have consistently selected a large number of drug-resistance genes, which are frequently widely spread by mobile genetic elements. Lineages such as *S. Typhimurium* ST313,⁴⁹ *E. coli* ST131,⁵⁰ and *S. aureus* ST22⁵¹ are of particular concern, having acquired multiple antibiotic resistance genes, and causing large numbers of difficult to treat infections worldwide. With antimicrobial resistance growing at alarming rates globally and antibiotic discovery and development heavily thwarted, the need for effective measures is more pressing than ever. To address this challenge, new approaches toward antibiotic design are needed; in particular, ones that take into consideration the evaluation of mutational frequencies and fitness-associated costs of resistance emergence at the early stages of development. Targeting virulence is one promising approach.⁵²

Antivirulence drugs, unlike antibiotics, target bacterial virulence factors. Due to their different mode of action, antivirulence drugs are considered attractive alternatives that could even surpass antibiotics, in the sense that their antimicrobial action will not be rendered ineffective by resistance development.^{6,14,15} The proposed evolutionary robustness for antivirulence drugs mainly stems from the theory that targeting bacterial virulence factors incurs a smaller fitness cost, thus reducing the SOS mutagenic response and evolution of resistant mutants. This tenet is still actively debated.^{12,53-55} Emerging experimental evidence^{16,17,56,57} appears to support the theoretical prediction that evolution of resistance to antivirulence drugs will largely depend on the virulence factor targeted, and will thus differ considerably for different antivirulence drugs.^{14,15} Antivirulence drug candidates thus need to be tested individually before any general conclusions can be drawn as to their clinical value.

With a central role in the biogenesis of virulence factors in many bacteria, the DSB machinery for oxidative protein folding is a promising antivirulence target¹⁹ and several

inhibitors of DsbA and DsbB have been reported.²⁰ Our team has demonstrated that small molecule inhibitors developed against the prototypic DsbA enzyme from *E. coli* K-12²² can also block DsbA homologues in UPEC and *S. Typhimurium* strains and can attenuate virulence without affecting pathogen growth under standard laboratory culture conditions.²⁴ A similar lack of growth defects was reported for the same pathogens using isogenic *dsb* gene deletion mutants.^{25,26} In the present study, we show that EcDsbA enzyme inhibitors can attenuate the virulence of *S. Typhimurium* while also slowing pathogen growth under pathophysiological conditions. Interestingly, pharmacological inhibition of DsbA or deletion of *dsbA* had similar effects on *S. Typhimurium* fitness in minimal media. The lack of further growth defects caused by the DsbA inhibitor on *S. Typhimurium* lacking *dsbA* confirmed this was an inhibitor on-target effect. Our findings are supported by previous reports documenting other culture conditions where DsbA enzymes were found to contribute to bacterial growth. For example, an *E. coli* K-12 mutant lacking *dsbA* had reduced growth in minimal M63 Glc media,⁵⁸ and failed to grow under anaerobic conditions in the same media.⁵⁹ Likewise, in the absence of its two DsbA homologues, the opportunistic human pathogen *Serratia marcescens* exhibited growth rate reduction in minimal media with limited aeration, but not in standard growth conditions.⁶⁰ Thus, DsbA inhibitors could have combined antivirulence-antibiotic activity against different pathogens under specific conditions; such dual action would largely depend on the various conditions encountered by the pathogen at different host niches and stages of infection.

For *S. Typhimurium*, the ability to replicate in nutrient-deprived environments, such as inside macrophages, is key for its survival, pathogenesis, and subsequent dissemination.^{38,61} While the pathogen will encounter several other environmental stimuli while residing inside macrophages that are not completely mimicked by our study conditions,^{28,29,62} nutrient deprivation as that experienced during growth in minimal media requires DsbA and thus provides a selective condition well-suited to screening for bypass mutations. In addition, *Salmonella* possess several virulence proteins that require folding by DsbA homologues and are key for its pathogenesis. These include Type 3 secretion system components and effectors of the *Salmonella* Pathogenicity Island (SPI-) 2, as well as proteins mediating pathogen adhesion, dissemination, and survival within the host (e.g., fimbriae, flagella).^{19,24} Further, DsbA is a prerequisite for *Salmonella* chronic carriage in the gallbladder, as it folds RcsC, a key mediator of biofilm formation that is critical for long-term persistence.^{63,64} Our findings suggest that the combined effect of DsbA inhibitors on *Salmonella* growth and virulence attenuation could facilitate effective bacterial clearance by macrophages, although this tenet remains to be tested. Given macrophages are essential players in the first line of defense

against *Salmonella*,⁶⁵ enhancing early control of the pathogen in the host could reduce transmission or prevent systemic infection, such as life-threatening invasive salmonellosis.⁶⁶ While the inhibitors used in this study have levels of toxicity toward human epithelial cells that prevent this type of testing (Dhouib and Totsika, unpublished), elaborated compounds with reduced cytotoxicity and improved potency would allow host-pathogen-inhibitor investigations in the future. This would be a key step in the development of DsbA inhibitor candidates with more clinical relevance.

Moreover, we are encouraged that we did not detect selection of spontaneous resistant mutants following continuous pathogen exposure to sub-MIC DsbA inhibitor concentrations, despite in silico prediction of a mutational hotspot in DsbA's CXXC catalytic center. Theoretical mutability predictions for DsbB, the redox partner of DsbA, show a high mutability score in key DsbB-DsbA interaction residues (Figure S6), but no spontaneous resistant mutants were obtained in a study investigating resistance to DsbB pyridazinone inhibitors by direct selection.⁶⁷ Selection of resistant *dsbB* mutants was only observed when the mutation rate was artificially increased by error-prone PCR mutagenesis, though even then selected mutants only conferred modest pyridazinone resistance (2–5 fold increase in IC₅₀).⁶⁷ Conversely, continuous sub-MIC exposure to antibiotics, as reported here and in previous studies, does drive high-level resistance in bacteria.^{47,68} Collectively, these studies indicate that the frequency with which resistance arises to inhibitors of the DsbAB oxidative protein folding pathway is very low, at least under laboratory experimental evolution conditions. Employing cell- and animal-based infection models in future will allow further evaluation of the evolutionary robustness of DsbA inhibition under relevant spatial and temporal selection encountered during infection.

In conclusion, our study has established a hierarchical contribution of different DsbA enzymes to *Salmonella* growth under pathophysiological conditions. Furthermore, we show that inhibition of DsbA in this pathogen attenuates growth and virulence, without detectable resistance development. These findings, thus, support the case for further development of DsbA inhibitors as a novel and effective strategy to control multidrug-resistant Gram-negative bacteria on WHO's pathogens priority list.¹

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHOR CONTRIBUTIONS

RD and DV were involved in study design, data collection and interpretation, statistical analyses, and writing of the manuscript; YH and ADV assisted with data collection and interpretation, and writing of the manuscript; JLM was involved in conception of the study and securing study funds; BH contributed to study conception, data analysis and interpretation, securing study funds, and writing of the manuscript; MT was involved in conception, design and coordination of the study, data interpretation, securing study funds, and writing of the manuscript. All authors critically reviewed the manuscript and have approved the publication of this final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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