2. Materials and Methods

2.1 Bacterial isolates

A combination of *S.* Typhimurium ST19, ST313, and ST34 isolates were used in this study. They are enumerated in **Appendix A, Table 1**. Many of the isolates used were kindly provided by the Institute of Tropical Medicine, Antwerp and the International Vaccine Institute.

2.1.1 Growth medium and growth conditions

Prior to experimentation, all isolates were grown on Isosensitest agar (Oxoid, CM0471) and subjected to ciprofloxacin M.I.C.E. (Oxoid, MA0104F) or ETEST (BioMerieux, 412311) to determine baseline ciprofloxacin susceptibility. Isolates were maintained on Isosensitest agar and streaked fresh weekly from frozen stocks. To prepare for experiments, isolates were always inoculated into 10 ml Isosensitest broth (Oxoid, CM0473) from plates followed by overnight shaking at 37°C for 16-18 h.

2.1.2 Ciprofloxacin susceptibility testing by MIC ETEST

Isolates were streaked from frozen stocks onto Isosensitest plates and grown at 37°C. Three serial streaks on fresh plates were subsequently performed. For M.I.C.E. or ETEST application, a few colonies from each plate were inoculated in $\sim$3 ml PBS and vortexed well to create a slightly cloudy solution. 100 μl of the solution was spotted on Isosensitest plates and spread well before gently laying down the MIC test strip. Inoculated and control plates were incubated overnight at 37°C and then visually analysed. Each *S.* Typhimurium isolate was tested a minimum of two times to ensure an accurate reading.

2.2 Time kill curves

Four *S.* Typhimurium isolates were chosen for the initial time kill curve analysis. These were D23580, SL1344, VNS20081, and 5390_4[80,370,57,176]. Initially, colonies from plates were inoculated into 10 ml of Isosensitest broth and these were shaken at 200 rpm at 37°C overnight. 10 μl of the subsequent culture was then added to 990 μl of 1x PBS to make a 1:100 dilution for the inoculum. 100 μl of this preparation was added to 10 ml of Isosensitest containing different levels (0x, 1x, 2x, 4x MIC) of ciprofloxacin according to the predetermined MIC of each isolate (**Table 2.1**). The starter inoculum was between 1 and 5 x 10^5 CFU/ml. Cultures were incubated shaking at 37°C and aliquots were taken to determine colony forming units (CFU) at 0, 2, 4, 6, 8, and 24 h. For this analysis, serial dilutions were made using samples of each culture, and a total of 50 μl of each dilution was plated using 10 μl spots of inoculum onto L-agar. CFUs were counted and determined as CFU/ml. Means and standard deviations (SD) of three replicates per isolate were calculated.

Table 2.1 *S.* Typhimurium isolates and respective ciprofloxacin MIC linked concentrations used to generate growth curves.

Isolate	0x MIC	1x MIC	2x MIC	4x MIC
D23580	–	0.03 µg/ml	0.06 µg/ml	0.12 µg/ml
SL1344	–	0.015 µg/ml	0.03 µg/ml	0.06 µg/ml
VNS20081	–	1.0 µg/ml	2.0 µg/ml	4.0 µg/ml
5390_4	–	0.5 µg/ml	1.0 µg/ml	2.0 µg/ml

2.2.1 Ciprofloxacin-degradation kill curves

Experiments were performed to determine whether ciprofloxacin was degraded or inactivated during the initial growth curve analysis. Here, the initial 24 h time kill curves were performed as described above. At 24 h, cultures were centrifuged and sterile-filtered through a 0.2 μm membrane, and the filtered medium was transferred to fresh growth tubes. Overnight bacterial cultures were then inoculated at a concentration of 1:10000 to the medium and CFU were determined at 0, 2, 4, 6, 8, and 24 h using conditions identical to those used in the initial growth curve analysis. Note: no additional ciprofloxacin was added to medium.

2.3 Spontaneous *gyrA* mutant generation and validation

To isolate spontaneous nalidixic acid mutant lines from *S.* Typhimurium isolates SL1344 and D23580, bacterial cultures were grown overnight in L-broth, and 100 μl of this was spread onto L-agar containing 100 μg/ml nalidixic acid for initial spontaneous mutant generation. After overnight incubation at 37°C, single colonies that had grown were re-plated on L-agar also containing 100 μg/ml nalidixic acid. Any colonies that were present on these agar plates were then streaked serially onto agar plates harbouring increasing concentrations of nalidixic acid up to 400 μg/ml, then these were switched to plates containing ciprofloxacin, harbouring from 0.1 μg/ml ciprofloxacin up to 1.0 μg/ml ciprofloxacin. Once colonies were able to grow stably on 1.0 μg/ml ciprofloxacin, overnight cultures were grown for genomic DNA purification and were purified using the Promega Wizard DNA Purification Kit (Promega, A1120). The DNA extraction protocol is detailed in **Appendix A**.

Following purification, DNA was PCR-amplified to check for mutations in the *gyrA* gene using primers: 5'-GAGATGGCCTGAAGC-3' for nucleotides 108 to 127 and 5'- TACCGT-CATAGTTATCCA CG -3' for nucleotides 435 to 454, forward and reverse, respectively[371].

2.4 *S.* Typhimurium D23580 bacteria grown for 24 h in ciprofloxacin medium for whole genome sequencing

One of the challenges in the ciprofloxacin growth curve experiments was to determine whether any SNPs or other mutations due to ciprofloxacin exposure had accumulated in the 24 h growth period from the initial inoculum. To determine this bacteria were isolated before and at the end of the culture period, and their DNA was isolated and sequenced. To prepare DNA, bacterial cultures of *S.* Typhimurium D23580 were initially grown overnight in 10 ml of broth. As in the time kill curve experiments, 10 ml of fresh Isosensitest broth containing 0x, 1x, 2x, or 4x ciprofloxacin MIC were inoculated with overnight cultures at 1:10000. Bacteria were grown for 24 h and then DNA was extracted. To ensure that the DNA extracted was not capturing a skewed population and to obtain a comprehensive sequence analysis, DNA was acquired in three ways, as described in detail in **Appendix A**.

After all bacterial pellets were collected, bacterial DNA was extracted using the Promega Wizard DNA Purification kit as described above. DNA was quantified on a Qubit 4 Fluorometer (Q33226) using the Qubit dsDNA HS Assay Kit (Q32851), then frozen at -80°C prior to whole genome sequencing.

2.5 Whole genome sequencing: library creation and sequencing

Library preparation for Illumina sequencing was undertaken at the Wellcome Sanger Institute using automated systems using the IHTP WGS NEB Ultra II library kit.

Libraries were sequenced on an Illumina HiSeq platform (Illumina, San Diego, USA) using standard running protocols, as described in greater detail in **Appendix A**.

2.6 Read mapping, variant detection, and SNP analysis

Illumina adapter content was removed from the reads using Trimmomatic v.0.33. Read mapping was undertaken using the WSI bacterial mapping pipeline, which uses bwa, and reads were assembled using the closest reference strain: D23580 (FN424405.1), SL1344 (FQ312003.1) or VNB151 (LT795114.1)[370,178,57]. samtools mpileup and bcftools were used to create a BCF file. Recombinant genomic regions were virtually masked from the alignment using an in-house script and verified using Gubbins, after which SNP sites were pulled out from the final alignment using snp-sites to generate a VCF file. SNPs were grouped as coding or non-coding using an R script from Van Puyvelde *et al.*, and the SNPs located in coding regions were manually evaluated[176].

2.7 Opera Phenix confocal phenotyping of bacteria

Several different experimental setups were assessed using the Opera Phenix; however, the general methodology was as follows. PerkinElmer CellCarrier-96 Ultra Microplates (PerkinElmer, 6655308) were coated with 50 μl Vitronectin (Stem Cell Technologies, 07180) in CellAdhere Dilution Buffer (Stem Cell Technologies, 07183) at 1:20, and these were incubated overnight at 37°C.

The following day, wells were aspirated and washed 1x with 50 μl CellAdhere buffer and then with 50 μl 1x PBS (Thermo, 10010023). 50 μl of bacteria culture was added to each well and incubated for standardised times to generate sufficient bacteria for imaging. After incubation, bacteria were aspirated from wells, and adherent bacteria were fixed using 50 μl 4% paraformaldehyde (PFA) (Alfa Aesar, J61899) for 10 min. Wells were washed 1x with PBS, and then 50 μl 2% BSA (Fisher Scientific, BP9700-100) in PBS was added to each well and incubated for 20 min at room temperature. BSA was aspirated and replaced with BSA containing either FITC- or Alexa-647-conjugated CSA antibody (BacTrace, 5330-0059; Novus Biologicals, NB110-16952AF647). Subsequent incubation was then for 1 h in the dark. Wells were aspirated and replaced with either 50 μl 1:100 DAPI (Sigma, D9542) or DAPI and 1:200 SYTOX Green (Thermo Fisher, A8- 0626) in HBSS (ThermoFisher, 14025092). The processed plates were incubated for 20 min in the dark, then washed 1x in PBS, which was replaced with 50 μl PBS, and then sealed with a foil seal ready for imaging.

2.7.1 Opera Phenix microscopy phenotyping of *S.* Typhimurium bacteria during ciprofloxacin exposure

S. Typhimurium D23580 and VNS20081 were screened at 2 h intervals over 24 h after ciprofloxacin exposures of 0x, 1x, 2x, and 4x as related to the MIC of each isolate. This was undertaken by inoculating overnight cultures of *S.* Typhimurium D23580 and VNS20081 independently at 1:1000 dilutions (100 μl) in 100 ml Isosensitest broth in a 200 ml flask. The rationale for using a 1:1000 inoculum was the need for greater volumes to concentrate the low CFU samples at early time points, and the aeration is greater in 200 ml flasks. Cultures were collected for imaging from 2 h – 24 h. Because of the low bacterial concentrations at

early timepoints, the amount of culture taken and the degree of concentration was modified per timepoint (**Table 2.2**). At each time point, a final volume of 100 μl of culture was used to fill two wells with 50 μl each, and the plates were incubated static at 37°C for 13 min. As per standard protocol (see section 2.7), the microbial culture was aspirated, then fixed with 4% PFA, and washed with 1x PBS. Wells were refilled with PBS and kept at 4°C until the next time point. At the end of the 24 h experiment, all wells were incubated with 2% BSA, then for 1 h with CSA-Alexa- 647 at 1:1000 in BSA. Wells were aspirated and then incubated with solutions harbouring DAPI and SYTOX Green for 20 min as described above. Wells were washed 1x; plates were sealed and imaged.

2.7.2 Opera Phenix phenotyping of *S.* Typhimurium ST313 isolates after ciprofloxacin exposure

24 *S.* Typhimurium isolates were chosen for screening (**Appendix A, Table 2**). Colonies from plates were inoculated in ∼2 ml Isosensitest broth in a 96-well, deep well plate (Nunc, 260251). The plate was sealed with a gas permeable seal (Sigma, A9224) and incubated shaking at 37°C at 100 rpm for 16-18 h. The following day, a ciprofloxacin-Isosensitest was prepared for inoculation of bacteria at 0.06 μg/ml, 0.25 μg/ml, and 1.0 μg/ml, corresponding to concentrations at which *S.* Typhimurium are considered sensitive, intermediate, or resistant, respectively. An additional aliquot of Isosensitest broth lacking ciprofloxacin was prepared to have a baseline readout. Ciprofloxacin-containing broth was prepared by adding 2x of each concentration to Isosensitest broth to a total volume of 5 ml. 25 μl of these 2x solutions was added to the wells of a pre-coated CellCarrier Ultra plate in duplicate. 25 μl of the overnight cultures were then added to each well. The plate was incubated statically at 37°C for 2 h and then processed for imaging as described in 2.7. For staining, the CSA-Alexa-647 antibody was used 1:1000 in BSA for 1 h. Wells were then incubated with DAPI at 1:100 and SYTOX Green at 1:200 for 20 min, after which the plate was washed and readied for imaging.

2.7.3 Opera Phenix image analysis

Images generated on the Opera Phenix were analysed using the Harmony software (Perkin Elmer). The analysis pipeline was refined from a more general pipeline developed within the

Table 2.2 Preparation of *S.* Typhimurium D23580 and VNS20081 cultures for imaging.

Time point	Isolate/conditions	Preparation
2 h	All	Centrifuged 10 ml (4°C, 4000 rpm, 7 min). Decanted supernatant, transferred pellet to 1.5 ml tube. Centrifuged 1.5 ml tube (23°C, 8000 rpm, 3 min). Aspirated supernatant and resuspended pellet in 100 µl PBS.
4 h	D23580 0x; VNS20081 0x and 1x	From 10 ml as above but resuspended in 250 µl.
4 h	D23580 1x, 2x, 4x; VNS20081 2x, 4x	From 10 ml with final resuspension in 100 µl.
6 h	D23580 1x, 2x, 4x; VNS20081 2x, 4x	From 10 ml with final resuspension in 100 µl.
6 h	D23580 0x; VNS20081 0x, 1x	Plated neat culture.
8 h – 14 h	D23580 1x, 2x, 4x; VNS20081 4x	From 10 ml with final resuspension in 100 µl.
8h – 14 h	D23580 0x; VNS20081 0x, 1x, 2x	Plated neat culture.
16 h	D23580 4x	From 10 ml with final resuspension in 100 µl.
16 h	D23580 0x, 1x, 2x; VNS20081 0x, 1x, 2x, 4x	Plated neat culture.
18 h	All	Plated neat culture.
20 h	D23580 4x	From 10 ml with final resuspension in 100 µl.
20 h	D23580 0x, 1x, 2x; VNS20081 0x, 1x, 2x, 4x	Plated neat culture.
22 h	D23580 4x	From 10 ml with final resuspension in 100 µl.
22 h	D23580 0x, 1x, 2x; VNS20081 0x, 1x, 2x, 4x	Plated neat culture
24 h	D23580 4x	From 10 ml with final resuspension in 100 µl.
24 h	D23580 0x, 1x, 2x; VNS20081 0x, 1x, 2x, 4x	Plated neat culture.

group for Gram-negative bacterial analysis. Inputted images underwent flatfield correction, and images were calculated using the DAPI and FITC channels. The output "Calculated Image" was used to find "Spots" in the image. The population "Spots" was used to select populations by removing border objects. The selected spots were then assessed for area and roundness, and only objects with an area > 1.6 μm were included for downstream analysis; these were labelled "bacteria". Bacteria were assessed for area, roundness, width, length, and the ratio of width to length. Independently and using the DAPI and FITC channels, multiple morphology properties were calculated: symmetry, threshold compactness, axial, radial, profile (width of 4 px), sliding parabola (curvature of 10), and texture SER (scale of 1 px with Kernal normalization). The mean and standard deviation of DAPI intensity was also calculated. By applying a linear classifier to the "bacteria" population, three categories were defined: "Single_cells", "Round_cells", or "Other", and 45 morphology and intensity characteristics were calculated. See **Appendix B, Table B.2** for full pipeline. The output of the Harmony analysis was tabulated by object, and the result table was visualized in R (v 3.6.1).

For the principal component analysis (PCA) conducted on the 24 *S.* Typhimurium ST313 isolates, analysis was performed using base R on the combined three biological replicates of the screen. Initial analysis was performed on all 24 isolates in the non-treated (0 μg/ml) condition. A separate PCA was conducted on all 24 isolates treated with 1 μg/ml ciprofloxacin. Following this, all susceptible isolates were excluded from the dataset, and a PCA was conducted on the 'intermediate' susceptibility isolates separately at each treatment (0, 0.06, 0.25, 1.0 μg/ml ciprofloxacin). The first three principal components of each analysis were visualized in R.

2.8 RNA extractions and RNA sequencing

After bacteria were grown according to experimental specifications, double the volume of RNAProtect Bacteria Reagent (Qiagen, cat no. 76506) was added to cultures and incubated for 10 min. Cultures were centrifuged at 3215 x *g* for 14 m at 4°C. Supernatant was decanted, and the pellets were resuspended in 400 μl Tris buffer (0.25 mM, pH 8.0) containing 10 mg/ml lysozyme, and incubated for 5 min. To this was added 700 μl RLT buffer containing 10 μl β-mercaptoethanol (Sigma, cat no. M6250) per ml, and samples were vortexed well.

1 ml 100% ethanol was immediately added and vortexed well. The Qiagen RNeasy Mini Kit (Qiagen, cat. no. 74104) was subsequently used to process samples. Briefly, samples were loaded onto columns and spun. Columns were washed with 700 μl RW1 Buffer, then 2x with 500 μl RPE Buffer, and eluted in 40 μl RNase-free water. Samples were frozen at -20°C if not immediately processed. Subsequently, samples were treated with DNase I using the Qiagen DNase Kit (Qiagen, cat no. 79254). Outputs of the DNase treatment were treated using phenol-chloroform by first increasing solution volume with RNase-free water to 400 μl. 400 μl of phenol-chloroform-isoamyl alcohol mixture (Sigma, cat. no. 77617) was then added to the samples, mixed by inversion, then centrifuged at 8000 x *g* for 5 min. Supernatant was transferred to a new tube and combined with 400 μl chloroform:isoamyl alcohol 24:1 (Sigma, cat no. C0549). Samples were mixed then centrifuged as above. The supernatant was transferred to a new tube and combined with 1 μl glycogen (Roche, cat no. 10901393001), 40 μl 3M sodium acetate, pH 5.5 (Ambion, cat no. AM9740), and 500 μl ice-cold 100% ethanol. Tubes were mixed by inversion and incubated at -20°C for 30 min before centrifugation at 4°C for 20 min at 16000 x *g*. Supernatant was decanted and replaced with 500 μl ice-cold 70% ethanol and centrifuged at 4°C for 5 minutes at 16,000 x *g*. Ethanol was decanted and pellets were air-dried before resuspension in 50 μl RNase-free water. Samples were frozen at -80°C prior to sequencing. All library preparation and RNA-sequencing were performed at the Wellcome Sanger Institute using standard protocols. Briefly, libraries were made using the NEB Ultra II RNA custom kit (NEB, cat no. E7530S) on an Agilent Bravo WS automation system. RiboZero was added to deplete ribosomal RNA. Libraries were pooled and normalized to 2.8 nM for sequencing. Sequencing was performed on an Illumina HiSeq 4000, using a minimum of two lanes per pool.

2.8.1 RNA extractions from 4 'pilot' isolates under 2x MIC ciprofloxacin exposure

Four *S.* Typhimurium isolates: D23580, SL1344, VNS20081, and 5390_4 were grown as overnight cultures and inoculated at a dilution of 1:1000 in 10 ml Isosensitest broth with and without 2x the MIC ciprofloxacin (see **Table 2.1** for concentrations). After 2 h growth, RNA was extracted from the cultures as described in section 2.8.

2.8.2 RNA extractions of *S.* Typhimurium D23580 under parallel treatment conditions

D23580 and a D23580 *gyrA* mutant derivative (D23580**gyrA*) were grown under standard overnight conditions. Seven different experimental conditions were established for 2 h growth (**Table 2.3**). Bacterial overnight cultures were inoculated by a dilution of 1:1000 in 10 ml Isosensitest set to the treatment condition and grown shaking at 200 rpm at 37°C for 2 h. At 2h, standard RNA extractions were performed.

Table 2.3 Treatment conditions for *S.* Typhimurium D23580 and D23580gyrA*.**

Strain	Treatment	Concentration
D23580	None	None
D23580	Ciprofloxacin	0.5x MIC (0.015 µg/ml)
D23580	Ciprofloxacin	2x MIC (0.12 µg/ml)
D23580	Mitomycin C	1 µg/ml
D23580	Azithromycin	1x MIC (8 µg/ml)
D23580*gyrA	None	None
D23580*gyrA	Ciprofloxacin	0.24x MIC (0.12 µg/ml)

2.8.3 Sucrose gradient separation of ciprofloxacin-treated D23580

To separate subpopulations of bacteria after ciprofloxacin exposure, a sucrose gradient procedure was developed. Bacterial overnight and sub-cultures were grown according to the particular experimental requirements. These cultures were either non-treated or exposed to 2x MIC for ciprofloxacin. As there was relatively low bacterial yield at 2 h, sub-cultures were inoculated 1:100 from overnight cultures into 10 ml of Isosensitest broth either containing 0 μg/ml or 0.06 μg/ml ciprofloxacin. These sub-cultures were incubated while shaking at 200 rpm at 37°C for 2 h. While cultures were growing, fresh sucrose solutions were prepared. The four concentrations of sucrose used were 25%, 50%, 60%, and 70%, and these were made by dissolving sucrose (Sigma, S7903) in 1x PBS (w/v). Solutions were sterile-filtered using 0.2 μm syringe filters (GE Healthcare, 6794-2502). 2 ml of each sucrose concentration was layered from 70% to 25% in open-top ultracentrifuge tubes (Beckman Coulter, 344059) immediately before use (**Figure 2.1**). At 2 h, cultures were removed from incubator and centrifuged for 14 min at 4000 x *g* at 4°C. The supernatant was aspirated off with a stripette (carefully because the pellets were small and unstable), leaving behind a small volume of

medium to avoid aspirating the pellet. Pellets were resuspended in the remaining medium and transferred to 1.5 ml tubes, which were centrifuged at 5000 x *g* for 2 min to re-pellet. The remaining supernatant was removed, and pellets were resuspended in 500 μl PBS. Using a Pasteur pipette, 500 μl of cells was carefully added to the top of the 25% layer of the sucrose column. Gradients were centrifuged for 9 min at 3000 x *g*, 4°C. After centrifugation, gradients were removed:

- There were two visible layers on the gradients loaded with non-treated cultures: 1. within 50% (thick band), 2. ∼60% (very thin band).
- There were three visible layers from the 2x MIC ciprofloxacin treated gradients: 1. within 50% (thin band), 2. within 60% (more distinct, diffuse band), 3. 60-70% interface (distinct but thin band).

The cloudy portion of each layer was carefully removed using a Pasteur pipette, beginning with the lowest-density layer (fresh pipettes were used for each lower layer to avoid mixing the layers).

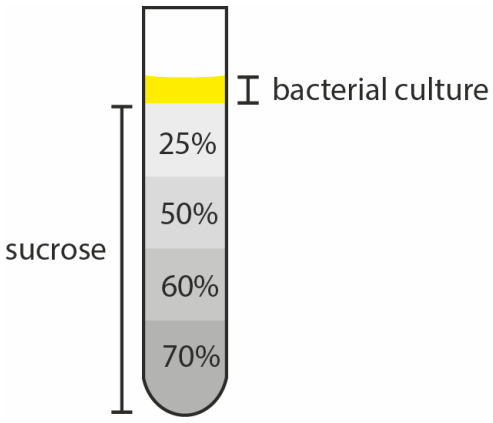

Figure 2.1 Assembly of sucrose gradient columns. Open-top ultra-centrifugation tubes were used to layer four concentrations of sucrose solution, on top of which bacterial cultures were added for sucrose density separation.

2.8.4 RNA extractions from *S.* Typhimurium D23580 after sucrose gradient separation

Subsequent to separation by sucrose gradients, isolated fractions were immediately added to 10 ml bacterial RNAProtect and processed using the standard RNA extraction protocol described above.

2.9 RNA sequencing analysis

Illumina sequence reads from *S.* Typhimurium D23580 and 5390_4 DNA were mapped to the reference D23580 genome (accession number FN424405.1) (Kingsley et al., 2009), VNS20081 was mapped to the sequenced isolate VNB151 (Mather et al., 2018), and SL1344 was mapped to reference sequence SL1344[80,57]. The Wellcome Sanger Institute pipeline tool DEAGO (Differential Expression Analysis Gene Ontology) a wrapper script for DESeq2 was used to determine differential gene expression starting with raw count files[372]. Using DESeq2, a Wald test was performed by comparing data from the treatment condition versus the non-treated equivalent. The p-value cut-off used was 0.05, and $\log_2$ fold change was calculated for treatment condition versus non-treated. Genetic networks and relationships were analysed using PheNetic, and GO terms were generated and compared[373].

Independently, data sets were analysed by the Monash University Bioinformatics Platform in Melbourne, Australia to provide comparative data sets. These data were analysed using the RNAsik pipeline, and differential gene expression comparisons were rendered using the Degust webtool using the limma-voom statistical test[374,375].

2.9.1 RNA sequencing analysis of gradient-separated bacteria

RNA-seq analysis was performed on the bacteria recovered from the gradients. These RNA-sequencing reads were processed using DEAGO as described in section 2.10. Pairwise comparisons were made between conditions:

- 50% fraction non-treated versus 50% fraction treated bacteria
- 50% non-treated versus 60% fraction treated
- 50% treated versus 60% treated
- 50% treated versus 60-70% treated

Heatmaps were made using R package heatmap.2, and other visualizations were performed using ggplot2. Network analysis was undertaken using PheNetic.

2.10 Light microscopy of gradient-separated *S.* Typhimurium D23580 bacteria

S. Typhimurium D23580 bacteria that had been gradient-separated after 2 h ciprofloxacin exposure were prepared for light microscopy to visualize gross morphological differences between non-treated and ciprofloxacin treated bacteria from the sucrose fractions. Bacterial samples were smeared on glass slides and heat-fixed. They were then stained using a standard Gram-staining protocol. Briefly, bacteria were first stained with crystal violet for 30 seconds followed by washing in H_2O for 5 seconds. The slides were then covered in Gram's iodine solution for 1 min before washing. Slides were decolorized by washing in 95% ethanol and acetone, then rinsed in H_2O for 5 seconds. Finally, slides were counterstained using safranin for 30 seconds and washed. After slides were air-dried, images were captured using an oil-immersion lens at 900x – 1000x.

2.11 Generation of *S.* Typhimurium D23580 single-gene knockout derivatives

Five genes identified in the RNA-seq analysis were chosen to be inactivated in *S.* Typhimurium D23580 by making targeted-gene knockouts. These genes were: *ybiI_2*, *sulA*,

malK, *cadA*, and *ddrA*. Targeted gene knockouts were generated using the lamda Red recombinase system[376]. Primer sequences were constructed for each gene (**Table 2.4**). Knockouts were designed and verified as described in **Appendix A**.

Table 2.4 Primer sequences for gene knockouts.

Gene	Forward	Reverse
ybiI_2	TGTGTAGGCTGGAGCTGCTTCG	CATATGAATATCCTCCTTAG
sulA	TGTGTAGGCTGGAGCTGCTTCG	CATATGAATATCCTCCTTAG
malK	TGTGTAGGCTGGAGCTGCTTCG	CATATGAATATCCTCCTTAG
cadA	TGTGTAGGCTGGAGCTGCTTCG	CATATGAATATCCTCCTTAG
ddrA	TGTGTAGGCTGGAGCTGCTTCG	CATATGAATATCCTCCTTAG

2.12 TraDIS screen on time kill curves of ciprofloxacin-exposed *S.* Typhimurium D23580

A previously validated *S.* Typhimurium D23580 Transposon Directed Insertion site Sequencing (TraDIS) library was used for the following experiment[181]. 50 μl of one batch of the total TraDIS library pool ($\sim$ 60000 mutants) was grown overnight in 10 ml of Isosensitest broth containing 15 μg/ml Kanamycin as three independent replicates. A 50 μl aliquot of this input library was separately frozen for genomic DNA processing. Multiple 50 ml tubes were prepared containing either Isosensitest broth or 0.06 μg/ml ciprofloxacin (2x MIC) in 10 ml Isosensitest broth. Three time points were determined for collection: 2 h, 10.25 h, 24 h on the basis of growth dynamics, and because of the potential for low bacterial yield at the earlier two timepoints, 12 tubes were prepared for the 2 h timepoint (6 per treatment condition), and 4 tubes were prepared for the 10.25 h time point (1 tube for non-treated and 3 tubes for 2x MIC). For the 24 h timepoint, only one tube per treatment was required. After $\sim$17.5 h pre-incubation, cultures were diluted 1:100 in PBS, and 100 μl of this was added to the readied testing tubes and incubated shaking at 200 rpm at 37°C. 1 ml of the overnight cultures was also spun down and the pellet frozen at -80°C for genomic DNA extraction. At 2 h post-treatment, sub-cultures were removed from the incubator. 100 μl was removed from a representative tube of non-treated and 2x MIC cultures for plating CFU at dilutions of 10^{-1}, 10^{-2}, and 10^{-3} on agar plates. Plates were incubated at 37°C overnight. The remainder of the sub-cultures were combined for centrifugation. Tubes were centrifuged at 4600 rpm for 20 min at 4°C. Supernatant was decanted, and the residual medium was

used to resuspend the pellet, which was then transferred to a 1.5 ml tube. This tube was centrifuged again at 8000 rpm for 3 min, and the supernatant was removed. The cell pellet was immediately frozen at -80°C. At 10.25 h and 24 h, the above protocol was repeated. Once all time point pellets were harvested, genomic DNA was extracted using the Promega Wizard Genomic DNA Purification Kit, eluted in 50 or 100 μl elution buffer depending on pellet size. Library preparation and sequencing were carried out by the Sequencing Pipelines at the Wellcome Sanger Institute. Similar to Ondari et al., gDNA was fragmented to $\sim$300 bp insert size using an E220 Evolution Sonicator (Covris), and the DNA fragments were end- repaired, A-tailed, and adaptor ligated using an Illumina DNA fragment library preparation kit (NEB Ultra II)[181]. Libraries were enriched by 10-20 PCR cycles using transposon specific primers 5'-ATCCCTATTTAGGTGACACTATAGAAGAGATGTGTA-3' and 3'- TTATGGGTAATACGACTCACTATAGGGAGATGTGTA-5'. Enriched libraries were purified by Agencourt AMPure XP beads and quantified using the Agilent DNA1000 chip. Libraries were sequenced on a HiSeq 2500 as 42 bp single reads across 2 lanes. Reads were processed using Bio::TraDIS toolkit v1.132190[377]. Reads were first filtered for the transposon tag sequence TAAGAGACAG. The filtered reads were mapped against the D23580 reference genome using SMALT, and transposon insertion sites and indices were calculated for each gene. Due to low percentage of genes mapped using the default settings, reads were mapped using a less stringent matching cut-off for the transposon tags. Gene essentiality (under-represented genes) was assessed by evaluating genes for which there were 0 or 1 insertions. Log_2 fold changes in mutant abundance between input pool and output were calculated using tradis_comparison.R within the Bio::TraDIS tookit. Pairwise contrasts were performed on treated versus non-treated bacteria for each time point.

2.13 TraDIS screen on *S.* Typhimurium D23580 injected into intestinal organoids

Intestinal organoids were prepared as described previously by Lees *et al.*, 2019[378]. Using the same *S.* Typhimurium D23580 TraDIS library, 50 μl of the library was inoculated in 10 ml LB containing 15 μg/ml Kanamycin and incubated shaking at 37°C overnight. The following day, the bacterial concentration was adjusted to a multiplicity of infection of 10:1 in 1 ml PBS. For each biological replicate, 60 organoids were microinjected with bacteria

and incubated at 37°C for 90 minutes. The detailed protocol of organoid injections and processing is in **Appendix A**. The organoids were lysed, and serial dilutions were performed and plated to calculate CFU and grow bacteria for TraDIS screening. CFU were plated on L-agar plates and incubated at 37°C overnight. The following day, CFU were enumerated, and colonies were collected for DNA extractions, as described in section 2.3.

Sequencing and analysis were performed as in section 2.13, including running the pipeline with default and relaxed transposon tag matching criteria.

2.14 Phylogenetic analysis

108 *S.* Typhimurium previously sequenced isolates were selected to represent global *S.* Typhimurium with an emphasis on ST313 isolates[176]. S. Paratyphi A270 was used as an outgroup for the phylogenetic analysis. Following BCF file generation as described in section 2.6, a pseudo-genome was generated by substituting uncertain sites in the reference genome base calls with an "N". Prophage and recombinant regions of the genome were informatically removed as described in section 2.8, after which snp-sites was used to extract SNP sites to create a maximum likelihood phylogeny. 1000 bootstraps were performed using RAxML with the substitution model GTRCAT to generate a tree with supported nodes[379]. The tree was visualized using iTOL[380].

2.14.1 SNP analysis of *S.* Typhimurium ST313 isolates

Based on a prior analysis of a *S.* Typhimurium ST313 lineage with decreased ciprofloxacin susceptibility (DCS), a SNP comparison was performed on isolates within the lineage that had greatest and least ciprofloxacin susceptibility (Van Puyvelde, personal communication). Isolates were compared as two discrete groups: those with greater and lesser DCS, and only SNPs that were either present or absent amongst all isolates in a given group were included. The coding SNPs were analyzed using an R script from Van Puyvelde *et al.*, as performed previously in section 2.6, to determine whether they were non-synonymous or synonymous and the codon position[176].

The SNPs in the 82 *S.* Typhimurium ST313 isolates contained within the 108 selected *S.* Typhimurium were independently compared following the methods described in section 2.6, mapped to isolate D23580. As before, coding SNPs were analyzed to determine synonymous or non-synonymous mutation and codon position. These SNPs were then compared against those from the DCS lineage above to determine overlap of specific SNPs, and these SNPs were manually evaluated in greater detail. The overlapping SNPs were used as the basis for defining manual functional groups that relevant SNPs might belong to. To determine functional groups of SNPs found within the 82 *S.* Typhimurium ST313 isolates, a text-based search was executed on the annotation for each gene containing a SNP. All SNPs found in a functional group of interest were concatenated into a single text file, and duplicates were removed. SNPs in genes involved in drug transport were manually evaluated in greater detail.

2.15 Pangenome analysis

Pangenome analysis tool Roary was used to generate a pangenome from annotated assemblies of the selected 108 *S.* Typhimurium isolates using the default settings and percentage identity of 90%[381]. To perform a pangenome-wide association study, isolates were scored for presence or absence of a chosen trait and evaluated using Scoary (Brynildsrud, 2016). The first trait was susceptibility, and isolates were determined based on ciprofloxacin MIC to have 'intermediate' susceptibility (1) or or 'susceptible' susceptibility (0). Isolates with unknown ciprofloxacin MICs were excluded from the analysis. Scoary was executed to evaluate whether gene clusters were trait-specific.

The second trait tested for was known *gyrA* mutation or *qnrs* presence or absence. Isolates with a known *gyrA* mutation or *qnrs* were scored with a 1, and those without were scored 0, based on in silico AMR analysis (see section 2.15). *gyrB*, *parC*, or *parE* mutations were excluded from the analysis because these are uncommon in *S.* Typhimurium isolates. Scoary was executed for this trait, and gene clusters for this trait were evaluated manually.

2.16 *In silico* AMR analysis

Antibiotic resistance genes were identified from the assemblies of the Illumina sequence data using ariba v 2.11.1 with CARD database 1.1.8[382,383]. ResFinder was also used to verify ariba results and to further identify resistance genes on plasmids by direct uploading of contig files to the ResFinder web browser[384].

2.17 Biofilm growth conditions

Tryptone, yeast extract, and agar were combined with MilliQ water to 1 L and autoclaved (see **Table 2.5**). After sufficient cooling, Coomassie Blue and Congo Red were added to the melted agar and gently mixed. Plates were poured with ∼20 ml agar per plate. Plates were left for 2-4 hours to dry and then sealed in parafilm and stored at 4°C in the dark until use. Selected isolates (see **Table 2.6**) were grown overnight (16-18 h) from plates in 10 ml Isosensitest broth with shaking at 200 rpm at 37°C. 10 μl of culture was added to 990 μl 1x PBS and vortexed well. 5 μl of each isolate was spotted on each plate with a maximum of 6 spots per plate. Plates were left on bench until dry, then sealed with parafilm and incubated at 27°C without inversion for a minimum of 72 h. Colonies were subsequently taken directly for SEM analysis.

Table 2.5 Components of RDAR agar plates (1 L).

Reagent	Supplier	Quantity
Tryptone	Difco, 211921	10 g
Yeast Extract	Difco, 212750	5 g
Agar	Difco, DF0812	15 g
Coomassie Brilliant Blue	VWR, 0615-10G	2 ml of 10 mg/ml stock
Congo Red, Indicator Grade	Acros Organics, 110501000	2 ml of 20 mg/ml stock

Table 2.6 Isolates for RDAR growth and SEM.

Isolate	RDAR morphology (red/smooth or white/smooth)
D23580	Red/smooth
2643	Red/smooth
10018	White/smooth
1577	White/smooth
2735	Red/smooth
10433_3	White/smooth
9266_3	Red/smooth
8429_3	White/smooth
2101	White/smooth
6549_3	Red/smooth

2.18 Scanning electron microscopy (SEM) of bacteria grown under stress conditions

In one experiment, *S.* Typhimurium D23580 cultures were treated with 0x or 2x MIC equivalents of ciprofloxacin, grown for 2 h or 8 h in shaking liquid cultures at 37°C, 200 rpm, and then plated on Isosensitest plates. Following colonial growth, bacteria were processed for SEM.

In an independent experiment, bacteria were grown under biofilm-forming conditions as specified in section 2.16 above. Sections of each colony were then selected by Dave Goulding and Claire Cormie for SEM processing at the Wellcome Sanger Institute, as described in **Appendix A**.