Supplementary Materials and Methods

The materials and methods described here expand on those introduced in Chapter 2.

DNA extractions using the Promega Wizard DNA Purification Kit

Bacterial pellets were resuspended in 600 μl Nucleic Lysis Solution, pipetted, incubated at 80°C, then cooled. 3 μl RNAse Solution was added, mixed, and incubated at 37°C for 15-60 m. After cooling, 200 μl of Protein Precipitation Solution was added and vortexed then incubated on ice for 5 m before centrifugation at 13,000 x g for 3 m. Supernatant was transferred to a tube of 600 μl isopropanol and mixed. Cells were centrifuged and supernatant decanted. 600 μl of 70% ethanol was added and mixed then centrifuged again. Ethanol was aspirated and pellets were air-dried for 10-15 m. Pellets were rehydrated for 1 h at 65°C in 100 μl Rehydration Solution.

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

Detailed protocol of the three methods of bacterial growth of *S.* Typhimurium D23580 bacteria grown for 24 h in ciprofloxacin medium, following from **section 2.4**.

1. The first method used was intended to capture all bacterial DNA (from live and dead cells) in the culture at 24 h. One ml of each 0x or 1x ciprofloxacin MIC-treated culture was taken at time 0 and then after 24 h. Given a high density of bacteria, each 1 ml sample was spun down at 8000 x *g* for 3 min to collect bacteria before DNA was extracted as previously described . 10 ml of the 2x and 4x ciprofloxacin MIC-treated cultures were aliquoted into 50 ml Falcon tubes, and these were spun down at 4000 rpm for 7 min at 4°C. The supernatant was decanted, and the pellet was resuspended in the remaining medium. This was transferred to a 1.5 ml microfuge tube and spun again at 8000 rpm for 3 min, and then the supernatant was aspirated and pellet was ready for DNA extraction.

2. The second method of DNA extraction was intended to capture DNA from viable cells after 24 h growth as a population. For the 0x and 1x ciprofloxacin MIC 24 h cultures, 100 μl of the 24 h bacterial cultures were spread onto agar plates to do a plate sweep of all viable CFU[592]. For the 2x and 4x ciprofloxacin MIC cultures, 1000 μl was spread on agar plates. After overnight growth at 37°C, colonies were carefully scraped from the agar and resuspended in 1x PBS. This was spun down at 8000 rpm for 3 min, and the supernatant was aspirated off. The pellets were processed for DNA extraction.

3. The third method of 24 h growth was intended to identify and grow individual representative colonies viable to determine whether there are colony-specific stable genetic differences after ciprofloxacin-treatment. A dilution of 100 μl of each 24 h culture (1:100 of 0x and 1x, neat of 2x and 4x) was spread on agar plates. Plates were incubated at 37°C overnight. Three single colonies were taken from each plate and inoculated in 5 ml Isosensitest broth. Cultures were grown shaking at 200 rpm at 37°C for 6 h. 1 ml was removed and centrifuged at 8000 rpm for 3 min before the supernatant was aspirated. Pellets were processed for DNA extraction.

Whole genome sequencing: library creation and sequencing

Detailed protocol of library creation and sequencing at the Wellcome Sanger Institute, from **section 2.5**. Samples were quantified with Biotium Accuclear Ultra high sensitivity dsDNA Quantitative kit using the Mosquito LV liquid platform, Bravo WS and BMG FLUOstar Omega plate reader, and samples were cherrypicked to 200 ng /120 μl using Tecan liquid handling platform. Cherrypicked samples were sheared to 450 bp using a Covaris LE220 instrument. Post sheared samples were purified using Agencourt AMPure XP SPRI beads on the Agilent Bravo WS. Library construction was performed (ER, A-tailing and ligation) using 'NEB Ultra II custom kits' on an Agilent Bravo WS automation system. PCR was set up using KapaHiFi Hot start mix and IDT 96 iPCR tag barcodes on the Agilent Bravo WS automation system. The PCR cycles were as follows using 6 standard cycles:

- Incubate 95°C for 5 min
- Incubate 98°C for 30 sec
- Incubate 65°C for 30 sec

- 72°C for 1 min
- Cycle from 2, 5 more times
- Incubate 72°C for 10 min

The post PCR plate was purified using Agencourt AMPure XP SPRI beads (Beckman Coulter, A63882) on a Beckman BioMek NX96 liquid handling platform. Libraries were quantified with Biotium Accuclear Ultra high sensitivity dsDNA Quantitative kit using the Mosquito LV liquid handling platform, Bravo WS and BMG FLUOstar Omega plate reader. Libraries were pooled in equimolar amounts on a Beckman BioMek NX-8 liquid handling platform. Libraries were then normalised to 2.8nM ready for cluster generation on a c-BOT and loading on the requested Illumina sequencing platform.

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

Detailed methodology of *S.* Typhimurium D23580 single-gene knockout derivatives generation, from **section 2.11**. After primer design for the five single-gene knockouts (**Table 2.4**), copies of each gene were cloned independently into plasmid pKD4 carrying a kanamycin resistance cassette[376]. To amplify and purify the DNA fragments for each gene, AccuPrime Taq DNA Polymerase was used (Invitrogen, cat no. 12346094) for the PCR, with 30 cycles using an annealing temperature of 56°C for 5 cycles followed by an annealing temperature of 65°C for 25 cycles. This was followed by 10 minutes at 68°C. Once amplified, PCR products were purified by running on a 1% TAE agarose gel. DNA was extracted from gels using the Promega Wizard SV Gel and PCR Clean-Up System (Promega, cat no. A9281). The DNA pellet was washed in 70% ethanol and stored while semi-dry at -20°C or 4°C for subsequent use.

To produce electrocompetent *S.* Typhimurium D23580 cells, an overnight culture of D23580 was prepared in 5 ml LB and shaken at 37°C. 1 ml of the overnight culture was added the following day to 100 ml LB and incubated shaking at 37°C for $\sim$ 2 h until an OD600 of 0.3-0.5 was obtained. Cells were heat-shocked in a 42°C heat block for 15 min and then cooled on ice for 5 minutes. Cells were centrifuged at 4000 rpm for 10 min at 4°C. The cell pellets were resuspended in 100 ml of chilled 10% glycerol and centrifuged. The addition of glycerol and centrifugation were repeated before pellets were resuspended to a final volume of 180 μl in chilled 10% glycerol. The pSIM18 vector carrying the lambda

Red recombinase system was electroporated into *S.* Typhimurium D23580 using 200 ng pSIM18 DNA at 2.5 kV, 200 ohms, 25 μF (Ec2 on BioRad MicroPulser, using 0.2 gap cuvettes). 800 μl of warmed SOC medium was added to electroporation cuvettes, and cells were incubated for 2 h at 30°C to recover. Bacteria were then plated on L-agar containing 150 μg/ml hygromycin and incubated at 30°C overnight to grow. Six colonies were selected and re-grown on hygromycin plates. These colonies were stored at -80°C until use. The *S.* Typhimurium D23580::pSIM18 strain was used to create the mutant derivatives.

To prepare *S.* Typhimurium D23580::pSIM18 for electroporation, an overnight culture was grown overnight (in regular low-salt LB 10 ml with 150 μg/ml hygromycin) and then diluted 1:100 the next day in 200 ml of low salt LB plus 100 μg/ml Hygromycin B (Invitrogen) and incubated for 3 hrs, shaking at 30°C until the culture attained an OD590 of 0.4. To activate the recombinase, the culture was aliquoted into 4 x 50 ml volumes and placed at 42°C in a water bath for 15 minutes and then cooled on ice for 10 minutes. The culture was centrifuged for 10 min at 2500 x g (4000 rpm) and the pellet was washed twice in 50 ml aliquots of ice cold 10% glycerol (the culture was recentrifuged for 10 min at 2500 x g (4000 rpm)). The pellet was transferred to a 1.5 ml tube and the volume was increased to 1.4 ml with glycerol and spun for 20-30 sec at 8000 rpm. The cell pellet was finally resuspended in 320 μl of 10% glycerol and electroporated with 500 ng DNA for each mutant derivative in a precooled 2 mm electroporation cuvette under conditions specified above. Cells were then incubated in 700 μl of pre-warmed SOC outgrowth medium (New England Biolabs) at 37°C for 2 h. Separately, 20 μl of the cell pellet was incubated in 700 μl of SOC to use as a cellular control. 4 x 10 μl aliquots were plated onto 33 μg/ml Kanamycin (Km) and 50 μg/ml Chloramphenicol (Cm) (Life technologies)-containing L-agar plates and grown overnight at 37°C. The remaining volume was left at room temperature overnight and plated on 33 μg/ml Kanamycin (plus Chloramphenicol as before)-containing L agar plates (to allow more time for recombination overnight) and left to grow at 37°C.

Individual colonies that grew on the agar plates after approximately 18 hours were re-plated onto fresh plates and re-grown at 37°C. Upon growth, 6-12 colonies that grew well were inoculated in 10 ml L-agar for overnight growth at 42°C shaking at 200 rpm. The broth was plated out on Km Cm containing L-agar plates, and the same isolates were also streaked on hygromycin plates (600 μl of 50 mg/ml stock in 200 ml media). Plates were incubated overnight at 37°C. The following day, colonies should only have grown on Km Cm plates, and these colonies were re-inoculated for PCR. Colonies were prepared for PCR by boiling

for 5 min. The PCR master mix was as follows using the AccuPrime Taq DNA Polymerase (Invitrogen, 12346094):

- 5 μl of Accuprime High Fid Buffer I
- 1 μl of Forward Primer
- 1 μl of Reverse Primer
- 0.3 μl of Accuprime Taq DNA Polymerase
- 33 μl of sterile water

The PCR cycle used was: 94°C 3 min 1 cycle. 94°C 30 sec, 54°C 30 sec, 68°C 1 min- 30 cycles. 68°C 10 min 1 cycle.

4 μl of each sample was run on a 0.9% TAE agarose gel, and bands were assessed.

Intestinal organoid processing after infection with transposon mutant library

Detailed methodology of intestinal organoid processing, from **section 2.13**. Intestinal organoids were prepared as described previously by Lees *et al.*[378]. Using the same *S.* Typhimurium D23580 TraDIS library as described in section **section 2.12**, 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 OD600 was measured, and bacterial concentration was adjusted to a multiplicity of infection of 10:1 in 1 ml PBS and 500 μl phenol red. For each biological replicate, 60 organoids were microinjected with bacteria and incubated at 37°C for 90 minutes. Media was aspirated from organoid plates after incubation and replaced with 3 ml cell recovery solution for 45 minutes at 4°C. Organoids in recovery solution were aspirated from plates into a 15 ml tube containing 5 ml PBS. Tubes of organoids were centrifuged for 3 minutes at 1500 rpm. The supernatant was aspirated, and organoids were resuspended in BMS media containing gentamicin and incubated at 37°C for 1 h. Organoids were centrifuged for 3 minutes at 1500 rpm, and the supernatant was aspirated. 50 μl of 1% Triton-X-100 was added to 5 ml PBS and warmed to dissolve before use as lysis buffer. The organoids were washed 1x with PBS and centrifuged again before resuspension in 500 μl lysis buffer and pipetting 20x to break up organoids. Cells were left

at room temperature for 5 minutes to further lyse. An additional 1 min spin at 1200 rpm was performed to separate organoids and bacteria. Serial dilutions were performed and plated to calculate CFU and grow bacteria for TraDIS screening.

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

Detailed methodology of sample processing for scanning electron microscopy, from **section 2.17**. Colonies were first fixed on agar with 2.5% (1:10) GA and 2% (1:3) PFA in 0.05M sodium cacodylate buffer for 1 hour. Samples were rinsed in 0.05 M (or 0.1 M) sodium cacodylate buffer 3x 5 min. A secondary fix in osmium tetroxide solution in sodium cacodylate buffer for 3 hours was performed after removal of colony regions of interest. Alternation of osmium tetroxide and thiocarbohydrazide washes was then carried out:

- Buffer rinses 2x 20 min
- 1% aq thiocarbohydrazide 10 min
- Buffer rinses 3x 10min
- 1% osmium tetroxide 30 min
- dd.H_2O washes 3x 10 min
- 1% aq thiocarbohydrazide 10 min
- dd.H_2O washes 3x 10 min
- 1% osmium tetroxide 30 min
- dd.H_2O washes 3x 10 min

Samples were then dehydrated in an ethanol series of 30%, 50%, 70%, 90%, 3x 100% for 20 min each. A critical point dry then took place in a Leica CPD300. Samples were mounted onto aluminium stubs with silver dag, sputter coated in a Leica ACE600, and then dried in a vacuum drying cabinet. Samples were visualized using a Hitachi SU-8000 scanning electron microscope.

Isolate	Sanger Lane ID	Country	Year	MIC to ciprofloxacin (µg/ml)	Thesis chapter used in
D23580	Salmonella_enterica_subsp_enterica_serovar_Typhimurium_str_D23580_v1.2	Malawi	2004	0.03	3-7
SL1344		UK	1960s	0.015	3-5,7
VNS20081	7969_2#94	Vietnam	2009	1.0	3-5
5390_4	22709_8#181	DRC	2016	0.5	4,5,7
2735	22709_8#96	DRC	2008	0.25	7
10433_3	L10433_3_L001	DRC	2014	0.03	7
9412_3	L9412_3_L001	DRC	2014	0.03	7
2101	2101_R1_P_trim	DRC	2008	0.032	7
9266_3	9266_3_R1_P_trim	DRC	2014	0.032	7
12299_3	L12299_3_L001	DRC	2015	0.032	7
6948_3	26189_8#73	DRC	2002	0.032	7
10055_3	10055_3_R1_P_trim	DRC	2014	0.032	7
8866_3	8866_3_R1_P_trim	DRC	2014	0.023	7
12155_3	L12155_3_L001	DRC	2015	0.016	7
10393_3	10393_3_R1_P_trim	DRC	2014	0.032	7
8599_13	22709_8#227	DRC	2013	0.023	7
2643	22709_8#95	DRC	2008	0.023	7
319_8	25692_2#134	DRC	2008	0.25	7
10018	25692_2#162	DRC	2008	0.032	7
6452_11	22709_8#189	DRC	2011	0.5	7
453_08	22709_8#173	DRC	2008	0.023	7
1577	22709_8#86	DRC	2009	0.016	7
9085_3	22709_8#259	DRC	2014	0.016	7
1304	25692_2#149	DRC	2009	0.023	7
6088_3	22709_8#187	DRC	2012	0.032	7
6549_3	22709_8#190	DRC	2013	0.047	7
8314_12	22709_8#220	DRC	2012	0.19	7
9227_14	25692_2#79	DRC	2014	0.032	7
861	25692_2#141	DRC	2008	0.023	7
857	22709_8#76	DRC	2008	0.023	7
05_157	22709_8#243	DRC	2005	0.016	7
8429_3	22709_8#222	DRC	2014	0.023	7
8795_13	25692_2#71	DRC	2013	0.012	7
3382_3	22709_8#152	DRC	2011	0.023	7
7239_11	22709_8#198	DRC	2011	0.19	7
7236_11	25692_2#194	DRC	2011	0.032	7
6941_11	25692_2#188	DRC	2011	0.016	7
1303	25692_2#150	DRC	2009	0.012-0.016	7
5326_3	22709_8#179	DRC	2012	0.002	7

1563	25692_2#144	DRC	2009	0.023	7
1515	22709_8#84	DRC	2009	0.19	7
3484_3	25692_2#176	DRC	2011	0.023	7
P0494 S80	P0494_S80	Burkina Faso	2013	0.032	7
N0629	25692_2#82	Burkina Faso	2014	0.023	7
2407/N0487 H	25692_2#81	Burkina Faso	2014	0.023	7
P0114	25692_2#74	Burkina Faso	2013	0.032	7
N0084	25692_2#73	Burkina Faso	2013	0.047	7
AA00050	22709_8#65	Rwanda	1984	0.016	7
AA00279	22709_8#46	Rwanda	1984	0.016	7
AA00271	22709_8#47	Rwanda	1984	0.016	7
AA00065	25692_2#237	Rwanda	1984	0.023	7
AA00324	22709_8#44	Rwanda	1984	0.016	7
AA000104	25692_2#232	Rwanda	1984	0.016	7
N0442 S114	N0442_S114	Burkina Faso	2014	0.016	7
AA00512	22709_8#30	Rwanda	1984	0.023	7
AA00489	22709_8#36	Rwanda	1984	0.023	7
bkf00707	16404_4#76	Burkina Faso	2013	0.032	7
gnb00628	16404_4#79	Guinea-Bissau	2012	0.016	7
gnb00588	16404_4#43	Guinea-Bissau	2012	0.016	7
gha113848	16404_5#66	Ghana	2011	0.25	7
gnb01487	16399_1#19	Guinea-Bissau	2013	0.016	7
gha113018	16549_7#28	Ghana	2010	0.023	7
gha113289	16404_5#25	Ghana	2011	0.75	7
gha200597	16404_5#26	Ghana	2011	0.016	7
gha113988	16399_3#33	Ghana	2010	0.032	7
gha114575	16473_1#72	Ghana	2011	0.016	7
gha114348	16404_5#30	Ghana	2010	0.032	7
gha113920	16404_5#49	Ghana	2011	0.016	7

Table A.1 All *S.* Typhimurium isolates used in this thesis.

Isolate	Ciprofloxacin MIC
bkf00707	0.032
gnb00628	0.016
gnb00588	0.016
gha113848	0.25
gnb01487	0.016
gha113018	0.023
gha113289	0.75
gha200597	0.016
gha113988	0.032
gha114575	0.016
gha114348	0.032
gha113920	0.016
8599_13	0.023
2643	0.023
319_8	0.25
10018	0.032
6452_11	0.5
453_08	0.023
1577	0.016
9085_3	0.016
1304	0.023
6088_3	0.032
6549_3	0.047
8314_12	0.19

Table A.2 *S.* Typhimurium ST313 isolates phenotyped on the Opera Phenix.