The role of ppGpp in modulating the evolution of fluoroquinolone-sensitive *Shigella sonnei* to develop antibiotic resistance

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Abstract

Shigella species causes shigellosis that requires antibiotic treatment in severe cases. Sub-lethal antibiotic concentrations can promote resistance, but their effect on antibiotic-sensitive bacteria prior to resistance development is unknown. This study investigated the effects of sublethal norfloxacin challenges on a norfloxacin-sensitive strain *Shigella sonnei* UKMCC1015. We show that low levels of norfloxacin do not change the antimicrobial resistance genes and norfloxacin targets expression. Instead, multiple ribosomal protein genes were downregulated.which could beattributed to a decrease in the ribosomal protein promoter activity, modulated by ppGpp. Under sublethal norfloxacin stress and high ppGpp levels, the cells underwent filamentation suggesting that ppGpp regulates the response of *S. sonnei* to norfloxacinWe speculate that high ppGpp levels play roles in modulating the developmentof antibiotic resistance in the sensitive strain. Indeed, the adaptive evolution of UKMCC1015 in the presence of norfloxacin revealed that the evolved strain 5c developed a mutation in *gyrA* (Asp87Tyr) gene conferring reduced norfloxacin susceptibility and reduced filamentation. These results collectively suggest that ppGpp may not be involved in regulating the mutant's response to norfloxacin exposure.

Keywords

Shigella sonnei UKMCC1015, whole genome sequencing, transcriptome analysis, sub-lethal conditions

Data Summary

The complete genome of *Shigella sonnei* UKMCC1015 and its plasmids have been deposited at GenBank under accession numbers CP060117, CP060118 and CP060119. RNA-sequencing data are accessible through BioSample accession number SAMN16871737.

Introduction

Shigella species is a common causal agent of episodic global diarrhoea, affecting mainly children younger than 5 years (Baker & The 2018). Among the four species of *Shigella, Shigella dysenteriae* and *Shigella boydii* are infrequently isolated, while *Shigella flexneri* and *Shigella sonnei* are more commonly encountered in clinical settings. *S. flexneri* is commonly associated in resource-poor countries, while *S. sonnei* is usually associated with the disease in high-income countries. Due to the improving economic prosperity of many countries, *S. sonnei* has recently replaced *S. flexneri* as the dominant cause of bacillary dysentery globally (Thompson, Duy & Baker 2015). The current population of *S. sonnei* descended from a common ancestor that led to distinct lineages and spread to other parts of the world, with strains in Asia that are mostly descendants of one lineage from Europe (Holt *et al.* 2012).

The etiological shift of shigellosis to *S. sonnei* has also been reported in Malaysia (Banga Singh *et al.* 2011; Koh *et al.* 2012). Most studies on *Shigella* in Malaysia have focused on its antimicrobial susceptibility profiles and there is lack of data on its genetics and phylogeny. Additionally, among all 36 *S. sonnei* complete genomes provided in the NCBI database (last accessed on 09/2020), there are no genomes of *S. sonnei* strains from Southeast Asia.

The misuse and overuse of antibiotics, has resulted in an increased concentration of antibiotics in the environment, exacerbating the antimicrobial resistance crisis (Gonzalez-Plaza *et al.* 2019). Among all antimicrobial-resistant bacteria, fluoroquinolone-resistant *Shigella spp.* is the priority 3 (medium) category for new antibiotics research and development. Studies have shown that low antibiotic concentrations might contribute to antimicrobial resistance (Bhattacharya *et al.* 2017; Lee *et al.* 2017; Gumus *et al.* 2018). Antibiotic concentrations higher than the minimum inhibitory concentration (MIC) leads to antimicrobial effects on susceptible cells. Concentrations lower than the MIC (also known as sub-MIC) can act as a signal for diverse responses that ultimately induce tolerance (Bernier

& Surette 2013). As antibiotic pollution leads to sub-MIC levels of antibiotics in the environment, this condition can enrich pre-existing resistant variants and cause a selective pressure for the development of *de novo* resistance (Li *et al.* 2018). Thus, the effect of sub-MIC on antibiotic tolerance in sensitive bacterial strains needs to be well understood to counteract the inevitable presence of resistant strains.

Norfloxacin is one of the first fluoroquinolones synthesised. Although it is not the preferred drug for treating shigellosis, its mechanism of action is similar to the recommended ciprofloxacin. All fluoroquinolones act by inhibiting DNA gyrase and topoisomerase IV. Fluoroquinolones bind at the enzyme-DNA interface, blocking ligation of the DNA and leading to lethal chromosomal breaks (Aldred, Kerns & Osheroff 2014). By inhibiting bacterial DNA synthesis, it will ultimately cause bacterial cell death. Exposure to sub-MIC fluoroquinolone can promote resistance via mutations in the DNA gyrase gene (Ranjbar & Farahani 2019). However, the transcriptional responses of a sensitive bacterium to low levels of fluoroquinolone before developing antimicrobial resistance are not well characterised. This study presents the antibiotic susceptibility profile and characterisation of the *S. sonnei* strain UKMCC1015 from Malaysia. We present the first complete reference genome for *S. sonnei* from South East Asia. Next, RNA-sequencing (RNA-Seq) was used to investigate how this fluoroquinolone-sensitive bacterium responds to a sub-lethal level of a second-generation fluoroquinolone, norfloxacin. The results show that multiple ribosomal protein operons are differentially expressed and novel pathways are involved on exposure to sub-lethal levels of norfloxacin.

Methods and Materials

Strain growth and culture conditions

Bacterial strains and plasmids used in this study are listed in Table S4. *Shigella sonnei* UKMCC1015 strain is a clinical isolate purchased from the University Kebangsaan Malaysia Culture Collection (UKMCC). *E. coli* DH10β was used for general cloning purposes. The strain was cultured in Mueller-Hinton broth (Oxoid, UK) and incubated at 37°C with shaking at 200 rpm overnight. When necessary, 50 µg/mL of kanamycin (KAN) and 100 µg/mL of ampicillin (AMP) were added to the culture media for selection. Total genomic DNA was extracted from the mid-log culture using the Wizard® Genomic DNA Purification Kit from Promega following the manufacturer's protocol.

Antimicrobial susceptibility testing

The antimicrobial susceptibility profile of the *S. sonnei* UKMCC1015 strain was determined by agar disk-diffusion method using different antibiotics (Figure 1b). The results were interpreted according to the CLSI guidelines (CLSI 2020). Muller Hinton Agar was inoculated with standardised bacterial

suspension of 0.5 MacFarland. Antibiotic disks were placed on the agar surface and the plates were incubated at 37˚C. After 16 to 18 hours of incubation, the zone of inhibition (diameter of the halo) was measured. The minimum inhibitory concentration (MIC) for norfloxacin was determined using the broth-microdilution method. *E. coli* ATCC25922 was used as the quality control.

Genome sequencing, assembly and annotation

We used a hybrid approach to obtain the complete genome, employing long-read Oxford Nanopore Technology and short-read Illumina MiSeq sequencing. Briefly, for long-read sequencing, DNA libraries were prepared using the Ligation Sequencing Kit (SQK-LSK109) according to the manufacturer's protocol and sequenced on a MinION FLOW-MIN106 flowcell and MinION MK1B sequencing device (Oxford Nanopore Technologies). Base-calling was conducted with Guppy v3.2.10 on MinKnow 3.6.17 using a fast base calling configuration. For Illumina MiSeq sequencing, DNA libraries were prepared using the Nextera XT library preparation kit and sequenced using a 2 X 250 bp paired-end configuration. The Illumina reads were trimmed using Trimmomatic v0.39 with parameters ILLUMINACLIP:adapters.fa:2:30:10:1:true, SLIDINGWINDOW:5:20, LEADING:20, TRAILING:20 (Bolger, Lohse & Usadel 2014) and used for error correction using Pilon v1.23 (Walker *et al.* 2014). For the genome assembly, multiple long-read assemblers were used: Flye v2.6 (Kolmogorov *et al.* 2019), Raven v1.1.10 (https://github.com/lbcb-sci/raven) and Miniasm-Minipolish v0.3 (https://github.com/rrwick/Minipolish). Then, Trycycler (https://github.com/rrwick/Trycycler) was used to obtain the consensus long-read assemblies from different long-read assemblers. The long reads were also used for error correction using Medaka v1.0.3 (https://github.com/nanoporetech/medaka). The genome was annotated using PGAP (Tatusova *et al.* 2016). Additionally, antimicrobial resistance (AMR) genes were annotated using the Comprehensive Antibiotic Resistance Database (CARD) (Alcock *et al.* 2020). The genome and plasmids in UKMCC1015 were constructed using BRIG (Alikhan *et al.* 2011).

Growth curve under different antibiotic concentrations

To determine the antibiotic concentration used for transcriptome analysis, the growth curve of *S. sonnei* UKMCC1015 was determined using 0X MIC, 0.2X MIC (0.006 µg/ml), 0.5X MIC (0.015 µg/ml) and 1.0X MIC (0.03 µg/ml) of norfloxacin. Briefly, the overnight bacterial culture was aliquoted to 50 ml of broth containing the different antibiotic concentrations. The OD at 600 nm was obtained every 15 minutes for 5.5 hours. The growth curve was performed in triplicates. Since the bacterial growth was not strongly inhibited at 0.2X MIC norfloxacin, further analysis was performed at 0.2X MIC to study the effect of a low dose of norfloxacin on the transcriptome of *S. sonnei*.

Total RNA extraction

RNA isolation of the samples was performed in duplicates. *S. sonnei* UKMCC1015 were grown overnight at 37°C with shaking at 200 rpm. Four 250 ml conical flasks containing MH broth were inoculated with the overnight culture to an initial optical density of 0.05 at 600nm. 0.2X MIC of norfloxacin was added to two of the experimental cultures. The remaining two cultures were used as controls that were not exposed to norfloxacin. All cultures were incubated at 37°C with shaking at 200 rpm until it reached the mid-log phase (OD 600 nm of 0.5). After adding stop mix (95% absolute ethanol and 5% TRIzol™ reagent (Invitrogen, US) to the bacterial culture, they were immediately frozen and kept at -80°C until RNA extraction. Total RNA extraction was done using TRIzol reagent according to the manufacturer's instructions. Briefly, the frozen bacterial culture were defrosted on the ice at room temperature, and the cells were collected by centrifuging at 4°C, 4000 rpm for 10 minutes. The obtained pellet was resuspended with TRIzol and incubated at room temperature for 5 minutes for complete dissociation of nucleoprotein complexes. After adding chloroform and incubating at room temperature for 5 minutes, the mixture was centrifuged at 13,000 rpm at 4°C for 15 minutes. The colourless aqueous phase was obtained, and an equal amount of isopropanol was added. The mixture was incubated on ice for 10 minutes and then centrifuged at 13,000 rpm for 10 minutes. 75% ethanol was added to the pellet to wash the RNA from the salt residue. After centrifuging at 13000 rpm at 4°C for 5 minutes, the supernatant was completely removed. The pellet was air-dried for 10 minutes and resuspended in sterile Milli-Q water.

cDNA library preparation and sequencing

rRNA depletion was performed using the MICROB*Express* Bacterial mRNA Enrichment kit (Thermo Fisher Scientific, Massachusetts, USA) following the manufacturer's protocol and cDNA library preparation using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Massachusetts, USA). DNA library quality control was done by measuring concentration using the Qubit 2.0 fluorometer (Invitrogen, California, USA) and assessing the size distribution using TapeStation 2200 (Agilent Technologies, California, USA). All libraries were pooled equimolar, and 15 pM was loaded into the MiSeq cartridge. Sequencing was performed on the MiSeq system using a 150 bp run configuration.

RNA bioinformatics analyses

The *S. sonnei* UKMCC genome (GenBank assembly accession: GCA_014217935.1) was used as the reference genome to map the RNA sequencing data. Genome annotation was obtained from the NCBI PGAP annotation. *S. sonnei* Ss046 ncRNA sequences from the BSRD database (Li *et al.* 2013) were used to BLAST against the *S. sonnei* genome to find additional ncRNA genes in the genome. BLAST hits were then appended to the annotation. To properly merge the two annotation sources, if

annotations of the two sources overlapped, the PGAP annotation took precedence over the BLAST BSRD hits.

Illumina adapter sequences and low-quality bases were trimmed off using Trimmomatic v0.39 with similar parameters as the genome assembly above. The trimmed, quality-filtered reads were mapped to the reference genome using Bowtie2 v2.3.5 and SAMtools v1.9 (Langmead & Salzberg 2012; Li *et al.* 2009). The alignments on annotated genes were counted using htseq-count v0.11.2 using parameters mode = union, order = pos, and minaqual = 0 (Anders, Pyl & Huber 2015). Features included in the htseq counting were CDS, rRNA, ncRNA, tRNA, antisense RNA and riboswitch. Differential abundance analyses were conducted using DESeq2 (Love, Huber & Anders 2014) on the Galaxy platform, while the visualisation through volcano plot was done using the web-server VolcaNoseR (Goedhart & Luijsterburg 2020). The Clusters of Orthologous Genes (COGs) were determined using eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) web-based server (Huerta-Cepas *et al.* 2018). Heatmaps were made using Python package seaborn (Waskom 2021) and matplotlib (Hunter 2007).

Recombinant DNA techniques and oligonucleotides

Plasmid DNA and PCR products were extracted and purified using the FavorPrep™ Plasmid Extraction Mini Kit and the GEL/PCR Purification Kit according to the manufacturer's instructions. For PCR, Q5® High-Fidelity, *Taq* DNA polymerases (New England Biolabs) and 2x*Taq* PCR MasterMix (SolarBio) were used according to the manufacturer's recommendations. Cloning was performed using restriction enzymes (New England Biolabs & Vivantis Technologies), Quick CIP and T4 DNA ligase (New England Biolabs) described by the manufacturer. All primers used in this study were synthesised by Integrated DNA Technologies (Table S4).

Construction of ribosomal promoter-reporter plasmids

Plasmids were constructed using the restriction enzyme-based cloning approach. The promoter replacement approach was applied to clone different ribosomal protein (r-protein) promoters into the pUltra-RFP (Figure 4b). R-protein promoter sequences of 151 nucleotides directly upstream of the transcriptional start site (TSS) were used in this analysis. The TSS was identified based on the TSS in *E. coli* (Lemke *et al.* 2011). The r-protein promoters rpsJp, rpsLp, rpsMp, rpsPp, rplNp, and rpsFp were amplified from *S. sonnei* UKMCC1015 gDNA using primers HTO-0203/HTO-0204, HTO-0205/HTO-0206, HTO-0207/HTO-0208, HTO-0209/HTO-0210, HTO-0199/HTO-0200, and, HTO-0201/HTO-0202 respectively, and ligated to pUltra-RFP by *Eco*RI and *Pst*I restriction sites. The resulting constructs were pUltra-RFP_rpsJp, pUltra-RFP_rpsLp, pUltra-RFP_rpsMp, pUltra-RFP_rpsPp, pUltra-RFP_rplNp, and, pUltra-RFP_rpsFp, respectively. The ppGpp synthetase gene, *relA* was amplified from the *S. sonnei* UKMCC1015 gDNA using primers HTO-0267/HTO-0268 and ligated to pBAD24 vector, an arabinose-inducible plasmid by NdeI and

XbaI restriction sites, resulting in the construction of pBAD24_*relA*. Plasmids constructed were transformed into *E. coli* DH10β via the heat-shock/calcium chloride procedure (Sambrook & Russell 2006). The constructs were verified by colony PCR and Sanger sequencing (Integrated DNA Technologies, IDT). The plasmids were extracted and inserted into the *S. sonnei* strain.

Fluorescence assay

To analyse the effect of norfloxacin on the promoter activity of various r-protein promoters, overnight cultures of the *S. sonnei* mutants were diluted to a starting OD of 600 nm of 0.05 and norfloxacin, at a final concentration of 0.2X MIC, was added. Cultures without norfloxacin stress served as controls. The cultures were incubated at 37˚C with shaking at 250 rpm. When the cells reached an OD 600 nm of 0.5, the cells were spun down by centrifugation (13,000 rpm, 5 minutes). The cell pellets were resuspended in an equal volume of 1X phosphate buffered saline (1X PBS) and vortexed. Then, 200 µL of the cells were added to a transparent, flat 96-well plate with a clear bottom. Relative fluorescence unit (RFU) was measured using the Infinite M200 (Tecan) microplate reader. Absorbance was measured at OD 600 nm and the fluorescence signals were detected using 535 nm/620 nm (excitation/emission) and collected as top readings. Absorbance and fluorescence readings were corrected for background absorbance and fluorescence from the medium. The RFU value was normalised against the bacterial cell density (RFU/OD 600 nm) using the formula:

$$RFU/OD\ 600\ nm = \frac{RFU_{raw} - RFU_{medium}}{OD_{raw} - OD_{medium}}$$

Promoter motif prediction

MEME (Multiple EM for Motif Elucidation) analysis was performed to identify regulatory motifs around the six r-protein promoter regions of UKMCC1015 (Bailey & Elkan 1994). The nucleotide sequences (30 bp upstream and an additional 4 bp downstream of the predicted TSS (-30 to +4) were selected as the primary input, and the motifs can only be predicted on the given strand.

Observation of cellular morphology by microscopy

Bacterial cells were streaked on nutrient agar plates supplemented with 0.5X MIC norfloxacin and incubated overnight at 37˚C. Cells were stained with Gram stain and visualised using the Leica microscope at 1000X magnification. 0.2% L-arabinose was added to the media for *relA* overexpression. Bacteria cultured on plain nutrient agar serve as controls. All the *S. sonnei* cells used in this assay contain the pUltra-RFP_rpsJp plasmid.

In vitro evolution of *S. sonnei* UKMCC1015 under norfloxacin stress

An overnight culture of the sensitive *S. sonnei* UKMCC1015 was grown in MHB broth at 37˚C with 200 rpm shaking. A subculture of the bacteria was inoculated on to a fresh MHB culture with 1X MIC norfloxacin, and this was repeated for a total of 14 days. The culture was then plated on MH agar plates containing different concentrations of norfloxacin. One colony, designated strain 5c, was selected from the plate containing 3.0X MIC of norfloxacin. The new MIC of this strain was determined using the broth microdilution method. Genomic DNA extraction was performed, sent for short read Illumina sequencing and the reads were processed similarly to the UKMCC1015 wild type. Genome assembly was carried out using SPAdes v3.13.0 (Nurk *et al.* 2013), and annotated using Prokka (Seemann 2014). SNPs were detected using Snippy, with the sensitive wild type UKMCC1015 serving as the reference.

Results

In this study, we characterised the antimicrobial susceptibility profile and the complete genome of *S. sonnei* UKMCC1015 from Malaysia and investigated the transcriptomic changes of this bacterium to a sublethal dose of norfloxacin.

Presence of antimicrobial resistance (AMR) genes in the ***S. sonnei* UKMCC1015** strain

The antimicrobial susceptibility testing revealed that this *S. sonnei* UKMCC1015 clinical strain was resistant to erythromycin, but susceptible to other antibiotics such as ciprofloxacin sulfamethoxazole-trimethoprim, ampicillin-sulbactam, cefotaxime, nalidixic acid (Figure 1a). This strain was also sensitive to norfloxacin as the MIC was determined to be 0.03 µg/ml using the broth dilution method. As this *S. sonnei* strain is sensitive toward two fluoroquinolones (ciprofloxacin and norfloxacin), we investigated the presence of any antimicrobial resistance (AMR) genes within the genome through whole genome sequencing. A closed chromosome and two plasmids were obtained for *S. sonnei* strain UKMCC1015 using hybrid assembly (Figure 1b, assembly information in Table S5). The chromosome has a size of 4,926,332 bp, while the two plasmids, UKMCC1015_2 and UKMCC1015_3, have sizes of 7,231 and 2,101 bp, respectively, with an average GC content of 51.03% for the complete genome. UKMCC1015 strain annotation identified 45 AMR genes involved in the resistance of fluoroquinolone, macrolides, tetracycline among many other antibiotics (Figure 1b, Table S1). The AMR genes detected in the genome are mostly efflux pumps (82%- 37 genes out of 45), while 4 genes are responsible for antibiotic inactivation, and 4 genes are responsible for drug target alteration. Nineteen of the genes coding for efflux pumps can potentially confer resistance to fluoroquinolones. However, we observed no mutations in any of the 4 genes that are commonly associated with fluoroquinolone resistance -DNA Gyrase A and B (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*). No AMR genes were detected on the two plasmids.

Identification of differentially expressed genes (DEGs) during exposure to 0.2X MIC norfloxacin

In order to identify genes that might be involved in response to sublethal norfloxacin exposure, RNA-sequencing was performed on the fluoroquinolone-sensitive *S. sonnei.* The growth curve of *S. sonnei* UKMCC1015 under different concentrations of norfloxacin indicated that 0.2X MIC norfloxacin had the least effect on bacterial growth (Figure 2a) and, therefore, this concentration was selected for RNA-Seq analysis. The total RNA of *S. sonnei* UKMCC1015 grown on normal broth (control) and under 0.2X MIC norfloxacin (experimental) was extracted, sequenced and analysed (Table S6). From the PCA plot (Figure S1), the control groups were distinct from the experimental group. The total read counts of all the genes were used in the DESeq2 analysis to determine the differentially expressed genes (DEGs) (adjusted p-value ≤ 0.05, $\log_2$ (fold-change) ≥ 2). A total of 112 DEGs were identified in response to norfloxacin challenge. 31 genes show >2-fold upregulation, while 81 genes show >2-fold downregulation (Figure 2b, Table S3). Among these 112 genes, 1 is a non-coding RNA, *gcvB*. The remaining 111 genes were categorised using the COG database to further elucidate their role in *S. sonnei* response toward 0.2X MIC norfloxacin (Figure 2c). The top five categories were J (translation, ribosomal structure and biogenesis) with 49 genes, K (transcription) with 12 genes, S (function unknown) with 9 genes, F (nucleotide transport and metabolism) with 9 genes, and C (energy production and conversion) with 9 genes. No AMR genes and norfloxacin target genes were among the DEGs. None of the 45 AMR genes annotated in the *S. sonnei* UKMCC1015 genome were differentially expressed during 0.2X MIC norfloxacin exposure (Figure 2d) and no changes in the expression of norfloxacin target genes (*gyrA, gyrB, parC,* and *parE*) were observed.

Downregulation of ribosomal protein genes in response to sub-lethal norfloxacin exposure

RNA-Seq analysis revealed that among the 49 DEGs in the COG category J (translation, ribosomal structure and biogenesis) (Figure 2d), two genes, *iraP* and *raiA,* were upregulated. The remaining genes (n = 47) in category J were downregulated, and included 37 genes encoding ribosomal proteins (r-proteins), which are involved in the assembly of the ribosomal subunits (Shajani et al. 2011). Given that r-proteins are commonly expressed in operons, in which multiple genes are co-transcribed (Osbourn & Field 2009), we postulated that the downregulation of the genes in category J could be due to promoter regulation at the operon level. Interestingly, 38/47 downregulated genes are expressed in 8 operons (Figure 3a), supporting our idea that any regulation of these genes must be through the promoters of these operons.

To further validate our hypothesis, promoter-reporter fusions were constructed (Figure 3b) to study the activity of the r-protein promoters upon 0.2X MIC norfloxacin exposure. Multiple promoters regulate the expression of operons 1 and 2, therefore, these two operons are excluded from our analysis. Promoters from five out of the remaining 6 operons i.e. operons 3 - 8 (rplNp, rpsFp, rpsJp, rpsLp, rpsMp, and rpsPp) were selected for analysis because these operons are under the control of only a

single promoter (Figure 3a), allowing direct measurements of the promoter activity on the operon transcription. The *rpsF* was not differentially expressed, hence its promoter rpsFp was used as a control in this analysis. The fluorescence intensity was measured and compared between 0X MIC (control) and 0.2X MIC norfloxacin. The normalised RFU/OD 600 nm changes in response to 0.2X MIC norfloxacin for each selected promoter are shown in Figure 3c. The rpsJp, rpsPp, rpsMp, and rplNp promoters exhibited a significant decrease in the normalised RFU/OD 600 nm upon exposure to 0.2X MIC norfloxacin with a percentage reduction of 5.81% (p-value = 0.0018), 7.28% (p-value = 0.004), 7.50% (p-value = 0.036), and 2.17% (p-value = 0.044), respectively, consistent with the RNA-seq findings, indicating that sub-lethal levels of norfloxacin reduced the activities of the r-protein promoters. The activity of the native promoter of the pUltra-RFP reporter plasmid that served as the negative control was not significantly affected (Figure S2).

Identification of GC-rich motifs in the r-protein promoters

Several r-protein promoters are commonly regulated by the guanosine tetraphosphate and guanosine pentaphosphate alarmone, collectively referred to as (p)ppGpp (ppGpp from here on). (Lemke et al. 2011). Inhibition of transcription by ppGpp may require the presence of a GC-rich sequence downstream of the -10 promoter region (Sanchez-Vazquez *et al.,* 2019). To determine whether this ppGpp discriminator sequence is present in the r-protein promoters, we analysed the promoter sequence of the selected r-protein promoters (rpsPp, rpsLp, rpsFp, rpsMp, rplNp, and rpsJp) using MEME. Besides a typical prokaryotic -10 consensus (TATAAT), we also identified a GC-rich motif downstream from the -10 consensus in all the six r-protein promoters from the MEME analysis (Figure 4). This motif may serve as the ppGpp discriminator sequence, inhibiting multiple r-protein promoters in response to 0.2X MIC norfloxacin.

ppGpp modulates *S. sonnei* UKMCC1015 response upon sublethal norfloxacin challenge

High ppGpp levels lead to the downregulation of multiple r-protein operons (Lemke *et al.* 2011). We postulated that sublethal norfloxacin exposure triggers the production of ppGpp and subsequently inhibits the expression of the r-proteins. It was reported that fluoroquinolone antibiotics (enrofloxacin, ciprofloxacin, and norfloxacin) at sublethal concentrations induced filamentation in bacteria (Bai et al. 2010; Bos et al. 2014; Herold et al. 2005). Therefore, a filamentation assay was conducted to verify the effect of norfloxacin on the morphology of *S. sonnei* and to study the relationship between norfloxacin exposure and ppGpp production. The filamentation process is aided by the SulA cell division inhibitor (Justice et al. 2002). In accordance with previous studies, the expression of *sulA* was upregulated by a log2 fold-change of 1.4 (Table S2), and *S. sonnei* UKMCC1015 formed long filaments after being challenged with sublethal level of norfloxacin (Figure 5a).

To investigate whether filamentation also occurs in response to elevated ppGpp levels, we constructed a *relA* overexpression strain (*S. sonnei relA*$^+$) under the control of an arabinose-inducible promoter and artificially raise the ppGpp levels via L-arabinose induction. In response to 0.2% L-arabinose induction, filamentation was observed in *S. sonnei relA*$^+$ (Figure 7b). The number of filamentous bacteria increased upon exposure to 0.2% arabinose induction and 0.5X MIC norfloxacin (Figure 7b). No filamentation was observed in *S. sonnei* UKMCC1015 (control strain) in response to 0.2% L-arabinose (Figure 7b). Both sublethal norfloxacin exposure and high ppGpp levels induce filamentation and support the notion that elevated ppGpp levels modulate the *S. sonnei* response to sublethal norfloxacin challenge.

Norfloxacin-adapted strain 5c does not depend on ppGpp to cope with sublethal norfloxacin challenge

It has been shown that filamentation and elevated ppGpp levels could increase the mutability of bacteria and promote antibiotic resistance (Bos et al. 2014; Das & Bhadra 2020; Hobbs & Boraston 2019). Therefore, we hypothesised that the formation of filament and the accumulation of ppGpp during norfloxacin exposure induce mutagenesis, leading to the development of antibiotic resistance in *S. sonnei*. To verify our speculation, the sensitive *S. sonnei* UKMCC1015 was propagated in the presence of 1.0X MIC norfloxacin for 14 days. This *in vitro* evolution process produced an evolved *S. sonnei* strain 5c with norfloxacin MIC of 0.30 µg/mL, which is 10 times higher than the parental UKMCC1015 strain (0.03 µg/mL). To identify the genetic basis of the reduced norfloxacin susceptibility, whole genome sequencing was performed on strain 5c. Overall, we observed single nucleotide polymorphisms (SNPs) in *gyrA, narG, galU, ymfD*, deletion in *ompC* and duplication in *ompR* of strain 5c (Figure 6a).

Through the evolutionary process, strain 5c developed higher norfloxacin resistance compared to UKMCC1015. To determine how this evolved strain 5c responds to sublethal norfloxacin stress, we indirectly measure the ppGpp levels using the filamentation assay. In the absence of norfloxacin stress, strain 5c adopted rod-shaped morphology, similar to UKMCC1015 (Figure 5a, 6b). Strain 5c formed fewer filaments than the parental UKMCC1015 when exposed to 0.5X MIC norfloxacin (Figure 5a, 6b), suggesting that ppGpp levels in strain 5c are lower than in UKMCC1015. To support our postulation that ppGpp production is not triggered in strain 5c, we measured the changes in the rpsJp promoter activity in response to 0.2X MIC norfloxacin. Indeed, no significant reduction in the fluorescence intensity was observed (Figure 6c, Figure S2h), indicating that ppGpp production is not induced in strain 5c during sublethal norfloxacin exposure and that the activity of the r-protein promoter remained unchanged.

Discussion

S. sonnei UKMCC1015 contains AMR genes but is susceptible to numerous antibiotics

This study investigated the effects of sub-lethal exposure to fluoroquinolone antibiotics on *Shigella sonnei*, a pathogen responsible for shigellosis. The *S. sonnei* strain UKMCC1015 was found to be sensitive to the majority of the antibiotics tested, with the exception of erythromycin, a macrolide antibiotic. CARD identified 45 AMR genes, mostly (82%) efflux pumps in this strain and the presence of *mphB* could explain the strains resistance to erythromycin (Noguchi et al. 1996). Although the over-expression of efflux pumps is associated with antibiotic resistance (Webber & Piddock 2003), the mere presence of these efflux pump genes may not be sufficient to confer any resistance towards specific antibiotics, explaining the susceptibility of this strain to other antibiotics. *S. sonnei* UKMCC1015 also contains multiple AMR genes that are linked to fluoroquinolone resistance as annotated by CARD. Most fluoroquinolone resistance is developed through chromosomal mutations in the genes encoding the target proteins, or plasmid-located genes that encode for plasmid-mediated quinolone resistance (PMQR) genes, *qnr* (Hooper & Jacoby 2016). This fluoroquinolone-sensitive strain does not have any chromosomal mutations, nor are there any AMR genes present in the plasmids of *S. sonnei* UKMCC1015.

The influence of sublethal norfloxacin on the transcriptomic profile of *S. sonnei*

We used transcriptomics to understand the effects of sub-lethal norfloxacin on the gene expression of the antibiotic-sensitive *S. sonnei* UKMCC1015 after 14 days of exposure. In response to 0.2X MIC of norfloxacin, *S. sonnei* differentially expressed 112 genes. The results show that the exposure to sub-MIC of norfloxacin causes *S. sonnei* to 1) maintain the expressions of AMR genes and the targets of the norfloxacin, DNA gyrase and topoisomerase IV; and 2) downregulate the expression of the r-proteins leading to downregulation of translation, transcription, energy production and nucleotide production to cope with the sub-lethal norfloxacin.

Sublethal norfloxacin exposure has no effect on the expression of AMR genes or antibiotic targets

Sub-lethal norfloxacin exposure of *S. sonnei* did not differentially regulate the expression of AMR genes such as those responsible for antibiotic efflux. Several past studies on fluoroquinolone-resistant *Shigella* indicate that the overexpression of genes encoding efflux pumps contributes to their resistance to fluoroquinolones (Kim *et al.,* 2008; Yang *et al.,* 2008; Taneja *et al.,* 2015). Other responses towards fluoroquinolones include downregulation of membrane porins to reduce membrane permeability and prevent antibiotic accumulation within the cell (Fabrega *et al.* 2009). This suggests that the initial transcriptional response of fluoroquinolone-sensitive bacteria is different from resistant

ones. Instead of overexpressing the efflux pumps available, the sensitive strain perhaps uses other pathways to respond to 0.2X MIC norfloxacin.

Additionally, the expressions of the norfloxacin targets (DNA gyrase and topoisomerase IV) did not change significantly during exposure to sub-lethal norfloxacin. Machuca *et al.* (2017) reported that low-level quinolone-resistant *E. coli* downregulates the genes of DNA gyrase subunits. In contrast, exposure to norfloxacin induced the expression of *gyrA* and *gyrB* in *Acinetobacter baumannii* (Heo *et al.,* 2014). Similarly *E. coli* upregulates *gyrA* expression as a response toward DNA gyrase inhibitors such as norfloxacin and aminocoumarin (Neumann & Quinones 1997; Anderle *et al.* 2008). Even though the main fluoroquinolone resistance mechanism is linked to chromosomal mutations of *gyrA* and *parC* (Fabrega *et al.* 2009) and not their regulation, the upregulation of the drug target may be an attempt to oversaturate the antibiotics. This increases the levels of DNA gyrase not bound to the drug, thus, reducing the amount of double-stranded DNA breaks that the antibiotics can cause.

Downregulation of r-protein expression in response to sublethal norfloxacin challenge

From the transcriptomic analysis, it was shown that exposure to sub-lethal concentration of norfloxacin resulted in the under-expression of multiple r-protein operons, even though r-proteins are not the target of this antibiotic. Five of the six r-protein promoters (rpsJp, rpsPp, rpsMp, rplNp, and rpsFp) yielded results consistent with the RNA-seq findings. There was no significant change in the rpsFp promoter activity (control) in response to norfloxacin stress, supporting the RNA-seq results that *rpsF* is not a DEG (Figure S2f). Besides, the activity of the pUltra-RFP native promoter (negative control) was not affected. These results show that the effects of norfloxacin are specific to r-protein promoters and suggest that a reduction in r-protein promoter activities drives the repression of multiple r-protein gene transcripts during 0.2X MIC norfloxacin exposure. However, we observed a smaller decrease in the r-protein promoter activity compared to a larger reduction from the RNA-Seq analysis. Besides, the rpsLp promoter activity was significantly upregulated by 2.08% (Figure 3c; Figure S2d). This discrepancy could be due to the delay between transcription and maturation of the fluorescent protein. The synthesis of fluorescent protein involves transcription and translation. Once synthesised, the fluorescent proteins undergo maturation in order to fluoresce (Balleza *et al.* 2018).

ppGpp inhibits the expression of r-proteins during sublethal norfloxacin exposure

The downregulation of r-proteins may be associated with the alarmone ppGpp. ppGpp is involved in the stringent response triggered during amino acid or fatty acid starvation or other environmental stress such as osmotic shock, heat shock and oxygen variation (Pacios *et al.* 2020). Some roles of ppGpp include growth rate control by inhibiting the production of ribosomal RNA, and the regulation of general metabolism through the expression of genes in amino acid biosynthesis (Hauryliuk *et al.,* 2015). Besides, ppGpp may mediate the downregulation of nucleotide synthesis and subsequently a

decrease in DNA replication. As a result, the bacteria decrease the effect of norfloxacin on the DNA gyrase and topoisomerase IV, enzymes involved in DNA replication. This is also supported by how *gyrA* and *parC,* the targets of norfloxacin, are not being upregulated. Instead of increasing the number of targets and DNA replication to overwhelm the concentration of antibiotics (Heo et al. 2014), this fluoroquinolone-sensitive *S. sonnei* decreases its activities and becomes semi-dormant. As reported for *E. coli,* it is possible that the elevated ppGpp levels may lead to the inhibition of transcription initiation for multiple r-proteins operons (Lemke *et al.* 2011).

Our RNA-Seq and fluorescence assay results suggest that *S. sonnei* increases the levels of intracellular ppGpp and subsequently downregulates the r-protein operon expression as a response to low levels of norfloxacin. This hypothesis is further supported by the presence of a GC-rich motif downstream of the -10 promoter consensus (Figure 4). This sequence could be the ppGpp discriminator region that facilitates the inhibition of r-protein promoters. Therefore, the repression of the r-protein promoter should downregulate the transcription of the downstream r-protein genes. However, this does not apply to the operon controlled by rpsFp (Figure 3a), one of the promoters that contain the GC-rich ppGpp discriminator sequence. Two genes within that operon (*rpsF* and *rpsR*) were not identified as DEGs (Figure 3a). This observation suggests that aside from ppGpp inhibition, other regulatory mechanisms may be involved in response to 0.2X MIC norfloxacin exposure. Our analysis shows that the fluoroquinolone-sensitive *S. sonnei* copes with sub-lethal norfloxacin stress, possibly through an unknown pathway that includes increasing ppGpp levels in the cell.

Norfloxacin stress triggers the production of ppGpp and induces filamentation

We also investigated the possible association between norfloxacin exposure and stringent response using filamentation assay. Filamentation is considered one of the bacterial survival strategies under stressful conditions such as antibiotic exposures (Justice et al. 2008). The cells continue to grow without dividing during filamentation resulting in the formation of elongated cells (Justice et al. 2008). *E. coli*, for example, has been shown to form filaments after exposure to different fluoroquinolone antibiotics (Bai et al. 2010; Bos et al. 2014). As a result, these changes in cellular morphology serve as an indicator of cell division inhibition (Bai et al. 2010). Long filaments were observed in this study when the *S. sonnei* UKMCC1015 was stressed with a sublethal concentration of norfloxacin. The filamentation process is aided by the SulA cell division inhibitor, which interacts with FtsZ, inhibiting the formation of Z-ring and cell division in *E. coli* (Justice et al. 2002). In our RNA-Seq analysis, *sulA* expression was upregulated in response to a sublethal norfloxacin exposure (Table S2) consistent with previous research (Bai et al. 2010; Amir et al. 2014).

We discovered that *S. sonnei* formed filaments when *relA*, which encodes for the ppGpp synthetase enzyme (Steinchen et al. 2020), was induced with 0.2% L-arabinose. This observation indicates that

elevated levels of ppGpp levels also lead to filamentous growth. Yamaguchi et al. (2015) hypothesised that extremely high and low levels of ppGpp interfere with FtsZ assembly (Yamaguchi et al. 2015).

The ppGpp alarmone affects a plethora of bacterial physiology (Spira & Ospino 2020). As a result, the downregulation of multiple r-protein operons observed in our study could be a consequence of an increase in ppGpp production in response to sublethal norfloxacin stress. Overall, both exposures to a sublethal dose of norfloxacin and high levels of ppGpp induce filamentation. Therefore, we propose that sublethal norfloxacin exposure triggers ppGpp production, causing *S. sonnei* to form filaments. The activation of ppGpp-dependent stringent response maybe a stop-gap arrangement that allows time for the fluoroquinolone-sensitive *S. sonnei* UKMCC1015 to develop adaptive mutations that can enable it to thrive under sublethal norfloxacin stress.

Norfloxacin exposure induces mutagenesis and promote antibiotic resistance via an accumulation of ppGpp and filament formation

Since norfloxacin induces DNA damage, the absence of cell division during filamentation allows DNA repair to occur, reducing the risk of transmitting damaged DNA to the daughter cells (Justice et al. 2008). Filamentous bacteria contain multiple copies of chromosomes (Justice et al. 2008). Recombination between chromosomes aids in DNA repair and may facilitate the evolution of antibiotic resistance via an increase in the rate of mutagenesis (Bos et al. 2014). An increase in bacterial mutability is also linked with the stringent response (Hobbs & Boraston 2019) and studies have shown that ppGpp can lead to antibiotic tolerance and the formation of persister cells, thus, contributing to antibiotic resistance (Das & Bhadra 2020).

To study the link between antibiotic exposure, stringent response and the promotion of antibiotic resistance, we conducted adaptive laboratory evolution on *S. sonnei* UKMCC1015 under 1.0X MIC norfloxacin stress. Through the evolution process, we obtained an evolved strain 5c with a norfloxacin MIC of 0.3 µg/mL, which is 10-fold higher than the parental strain. Despite the fact that strain 5c is still considered norfloxacin-sensitive (norfloxacin MIC sensitive breakpoint ≤ 4 µg/mL) (CLSI 2020), this evolved bacterium is less susceptible to norfloxacin after only 14 days of evolution under 1.0X MIC norfloxacin condition. Whole genome sequencing revealed this evolved strain accrued SNP mutations in *gyrA, narG, galU, ymfD*, and a deletion in *ompC* and a duplication of *ompR* of strain 5c. The SNP detected in the fluoroquinolone target gene, *gyrA*, changes the amino acid at position 87 from aspartic acid to tyrosine (Asp87Tyr) (Figure 6a). Asp87 and Ser83 are the major residues for the binding of quinolones to GyrA and the most commonly mutated sites observed in quinolone-resistant bacteria (Hooper & Jacoby 2016). Therefore, the substitution at location Asp87 might explain the increase in norfloxacin MIC of strain 5c. This suggests that antibiotic exposure can induce mutations in the genome.

ppGpp does not modulates the response of norfloxacin-adapted strain 5c upon sublehthal norfloxacin exposure

Since strain 5c showed reduced susceptibility to norfloxacin, we next examined the effects of sublethal norfloxacin exposure on this evolved strain. Under stressful conditions, bacteria develop a filamentous phenotype. We observed that 5c formed fewer filaments than the unevolved UKMCC1015 when exposed to 0.5X MIC of norfloxacin. A reduction in filamentous growth suggests that strain 5c could better resist a sublethal norfloxacin challenge. The *gyrA* mutation in strain 5c may, at least in part, confer protection against a low dose of norfloxacin exposure. A mutated gyrase A may prevent norfloxacin binding, making it less likely to cause DNA damage and prevent filamentation in strain 5c. Given that ppGpp triggers filamentation in UKMCC1015, it is possible that strain 5c may respond to sublethal norfloxacin exposure through an adaptive mechanism distinct from the ppGpp-dependent response seen in the parental strain. To test our hypothesis that ppGpp production is not induced in strain 5c, we conducted the fluorescence assay to examine changes in the rpsJp promoter activity in response to 0.2X MIC norfloxacin. We discovered that, unlike UKMCC1015, strain 5c did not downregulate the rpsJp promoter activity upon sublethal norfloxacin stress, implying that ppGpp is not induced in strain 5c to repress the expression of r-protein operons. As a result, the evolved strain could use a ppGpp-independent regulatory mechanism to cope with norfloxacin exposure.

Proposed adaptive response of *S. sonnei* UKMCC1015 to a sublethal norfloxacin exposure

Sublethal norfloxacin exposure may trigger the accumulation of ppGpp and activate the stringent response in *S. sonnei* UKMCC1015, which is responsible for the downregulation of several r-protein operons. Besides, the bacteria develop filamentous morphology to cope with antibiotic stress. Filamentation enables the bacteria to repair the DNA damage caused by norfloxacin before dividing. The process of chromosomal recombination may increase the rate of bacterial mutagenesis, driving the evolution of antibiotic resistance. An evolved strain 5c was generated after 14 days of propagating the sensitive UKMCC1015 under norfloxacin stress. Strain 5c developed a mutation in one of the norfloxacin targets (GyrA) and showed reduced norfloxacin susceptibility as a result of this adaptive evolutionary process (Figure 7).

Conclusions

Overall, our whole genome and the transcriptomic studies provide new insights into how a fluoroquinolone-sensitive *S. sonnei* UKMCC1015 responds to a sublethal norfloxacin challenge. The commonly reported norfloxacin targets (DNA gyrase and topoisomerase IV) were not differentially expressed. However, many r-protein genes were downregulated, which may represent a consequence

of the activation of ppGpp-associated stringent response. This study provides new insights into the multitude of responses *S. sonnei* can have to a low concentration of antibiotics, including the involvement of the stringent response and the alarmone ppGpp. Finally, our study highlights the connection between ppGpp and the promotion of antibiotic resistance during prolonged antibiotic stress and establishes the idea that the antimicrobial response of a sensitive strain to a sublethal antibiotic challenge differs from that of a resistant strain. Future studies should focus on the exact pathways involving the stringent response that can aid in the discovery of potential targets for novel antibiotics or anti-resistance agents.

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Conflict of Interest

The authors have no conflict of interests to declare.

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Figure legends

Figure 1: Phenotypic and genotypic characteristics of *Shigella sonnei* UKMCC1015. a. Antibiotic susceptibility profile of *S. sonnei* UKMCC1015. **b.** Closed chromosome of *S. sonnei* UKMCC1015, with UKMCC1015_1 (chromosome) and two plasmids, UKMCC1015_2 (bottom left) and UKMCC1015_3 (bottom right). UKMCC1015_1 chromosome, from inner ring outwards: GC skew, Genome Islands (Grey, obtained from IslandViewer predicted with at least 2 prediction methods), Prophage regions (Blue, underlined, obtained from Phaster), AMR genes (Black, obtained from Abricate using databases CARD). Plasmids of UKMCC1015, from the inner ring outwards: GC content, GC skew, genes in orange.

Figure 2: Effects of sub-MIC of norfloxacin towards *S. sonnei* UKMCC1015. a. Growth curve of *S. sonnei* UKMCC1015 under different sub-MIC of norfloxacin (0X, 0.2X, 0.5X and 1.0X MIC). **b.** Volcano plot of transcriptome analysis. **c.** COG categorisation of the 111 genes annotated by the non-supervised Orthologous Group (eggNOG). **d.** Expressions of all AMR genes and norfloxacin targets (*gyrA, gyrB, parC* and *parE*) which are not differentially expressed; and the expressions of the differentially expressed genes (DEGs) in Category J (translation, ribosomal structure and biogenesis).

Figure 3: Schematic illustration of r-protein operons, promoter exchange approach and the effects of 0.2X MIC norfloxacin (NOR) on the promoter activity a. Ribosomal protein operons of *S. sonnei* UKMCC1015. Green indicates differentially expressed genes (DEGs), and grey indicates non-differentially expressed genes. **b.** Construction of r-protein reporter plasmid via promoter exchange approach **c.** Normalised RFU/OD 600 nm changes for rpsJp, rpsMp, rpsPp, and rpsLp, rplNp, rpsFp r-protein promoters and pUltra-RFP promoter in response to 0.2X MIC norfloxacin stress. Three biological replicates were used for each promoter. Statistical significance (P-value < 0.05) was determined using Paired t-test (one-tailed, n=3); one asterisk (*): P-value < 0.05, two asterisks (**): P-value < 0.01, and ns: non-significant.

Figure 4: Promoter motif prediction of the ribosomal proteins. Motif predicted by MEME within the -30 to +4 region of the rpsJp, rpsPp, rpsFp, rpsMp, rplNp, and rpsLp r-protein promoter sequences of *S. sonnei* UKMCC1015.

Figure 5: *S. sonnei* filamentation assay. Cellular morphology of *S. sonnei* UKMCC1015 strains in response to **a.** 0.5X MIC norfloxacin (NOR), **b.** 0.2% L-arabinose induction along with 0.5X MIC NOR. Cells were observed under 1000X magnification using the Leica light microscope.

Figure 6: Characterisation of evolved strain 5c and the effects of 0.2X MIC norfloxacin on the r-protein promoter activity. a. SNPs identified in strain 5c after evolution. **b.** Cellular morphology of strain 5c in response to 0.5X MIC norfloxacin (NOR). Cells were observed under 1000X magnification using the Leica light microscope. **c.** Normalised RFU/OD 600 nm for rpsJp promoter in

strain 5c in response to 0.2X MIC norfloxacin (NOR) challenge. Three biological replicates were used and statistical significance was determined using paired t-test (one-tailed, n = 3); ns: non-significant.

Figure 7: Overview of the adaptation of *S. sonnei* UKMCC1015 to sublethal norfloxacin (NOR) exposures. Yellow cells are the sensitive cells (UKMCC1015), and blue cells are the evolved strain 5c. Red and blue arrows indicate activation and inhibition, respectively.

a.

Antibiotics	Mass (µg)	Class	CLSI sensitive breakpoint (diameter, mm)	Zone of inhibition (diameter, mm)					Interpretation
				Rep1	Rep2	Rep3	Mean	SE	
Ciprofloxacin (CIP)	5	Fluoroquinolone	≥ 26	32	36	34	34.0	1.15	**Sensitive**
Sulfamethoxazole-trimethoprim (SXT)	25	Folate pathway antagonist	≥ 16	27	30	31	29.3	1.20	**Sensitive**
Cefotaxime (CTX)	30	Cephems (b-lactam)	≥ 26	35	38	36	36.3	0.88	**Sensitive**
Nalidixic acid (NAL)	30	Quinolones	≥ 19	24	27	27	26.0	1.00	**Sensitive**
Chloramphenicol (CHL)	30	Phenicols	≥ 18	29	28	28	28.3	0.33	**Sensitive**
Ampicillin-sulbactam (SAM)	20	b-lactam combination	≥ 15	25	26	26	25.7	0.33	**Sensitive**
Erythromycin (E)	15	Macrolide	-	0	0	0	0	-	**Resistant**

b.

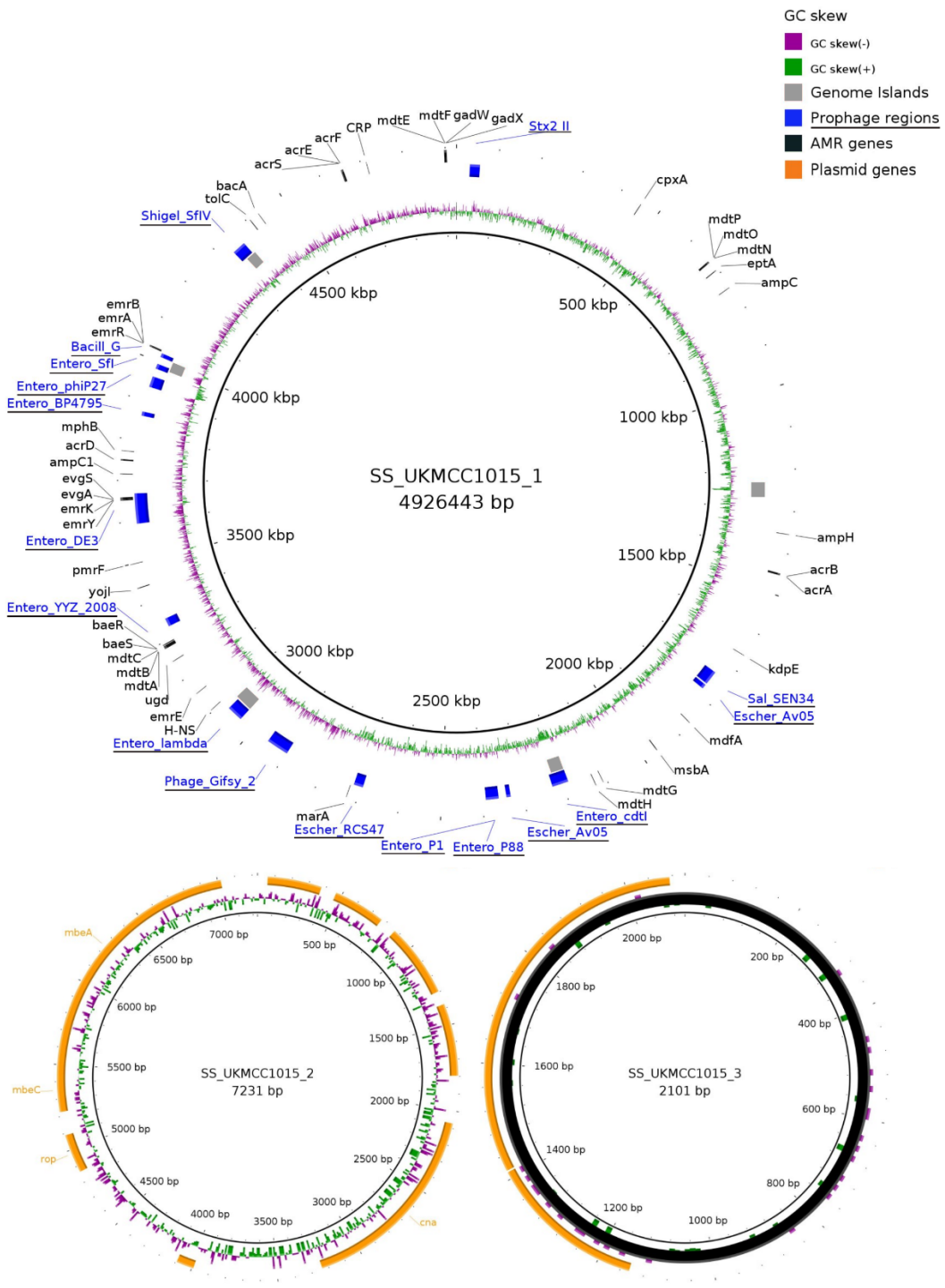


Figure 1

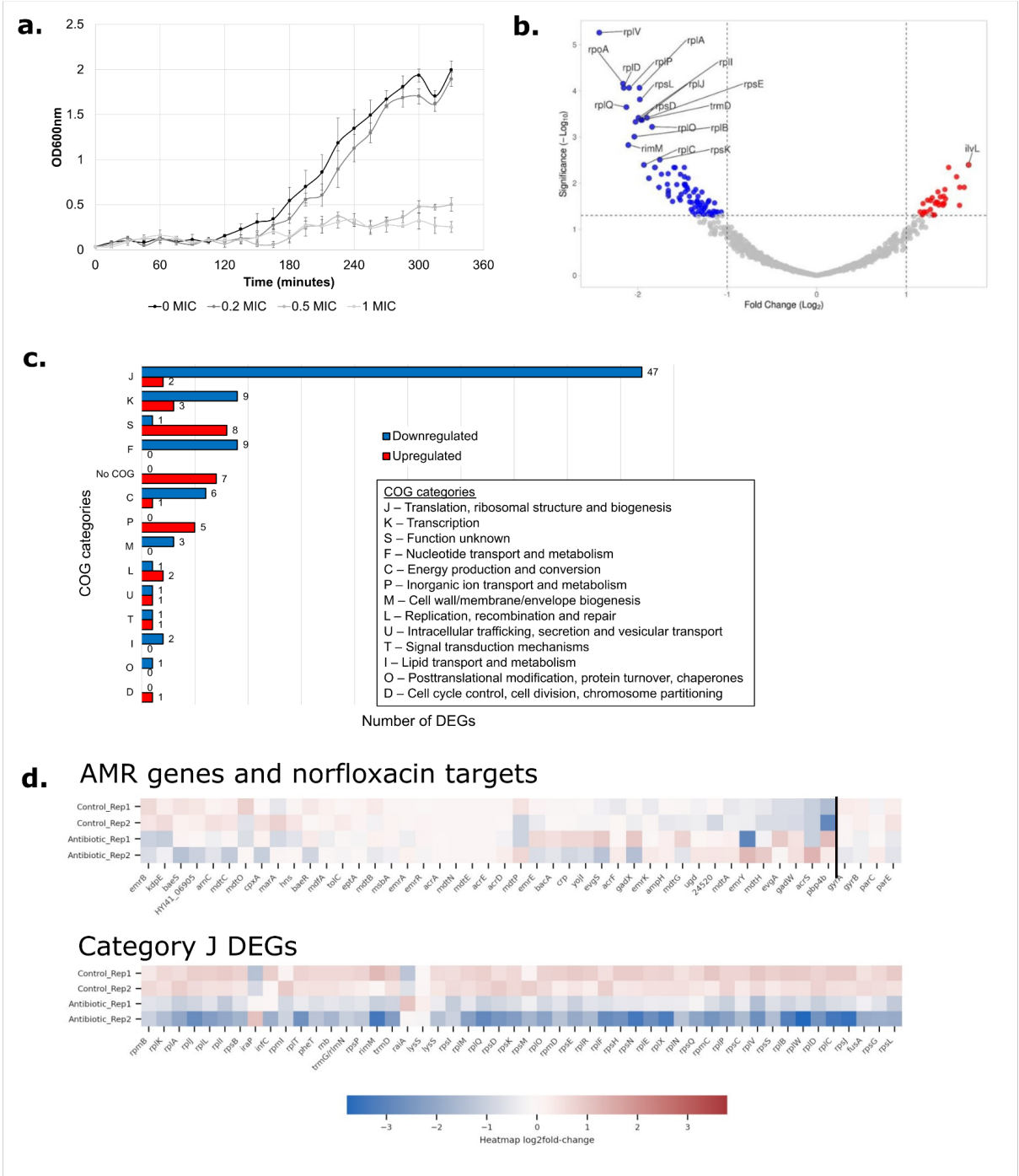

a.
OD600nm
Time (minutes)
0 MIC
0.2 MIC
0.5 MIC
1 MIC
b.
Significance (−Log10)
Fold Change (Log2)
rplV
rpoA
rplA
rplD
rplP
rplI
rpsL
rplJ
rpsE
rplQ
rpsD
trmD
rplO
rplB
rimM
rplC
rpsK
ilvL
c.
COG categories
Number of DEGs
Downregulated
Upregulated
COG categories
J – Translation, ribosomal structure and biogenesis
K – Transcription
S – Function unknown
F – Nucleotide transport and metabolism
C – Energy production and conversion
P – Inorganic ion transport and metabolism
M – Cell wall/membrane/envelope biogenesis
L – Replication, recombination and repair
U – Intracellular trafficking, secretion and vesicular transport
T – Signal transduction mechanisms
I – Lipid transport and metabolism
O – Posttranslational modification, protein turnover, chaperones
D – Cell cycle control, cell division, chromosome partitioning
d.
AMR genes and norfloxacin targets
Control_Rep1
Control_Rep2
Antibiotic_Rep1
Antibiotic_Rep2
Category J DEGs
Heatmap log2fold-change

Figure 2

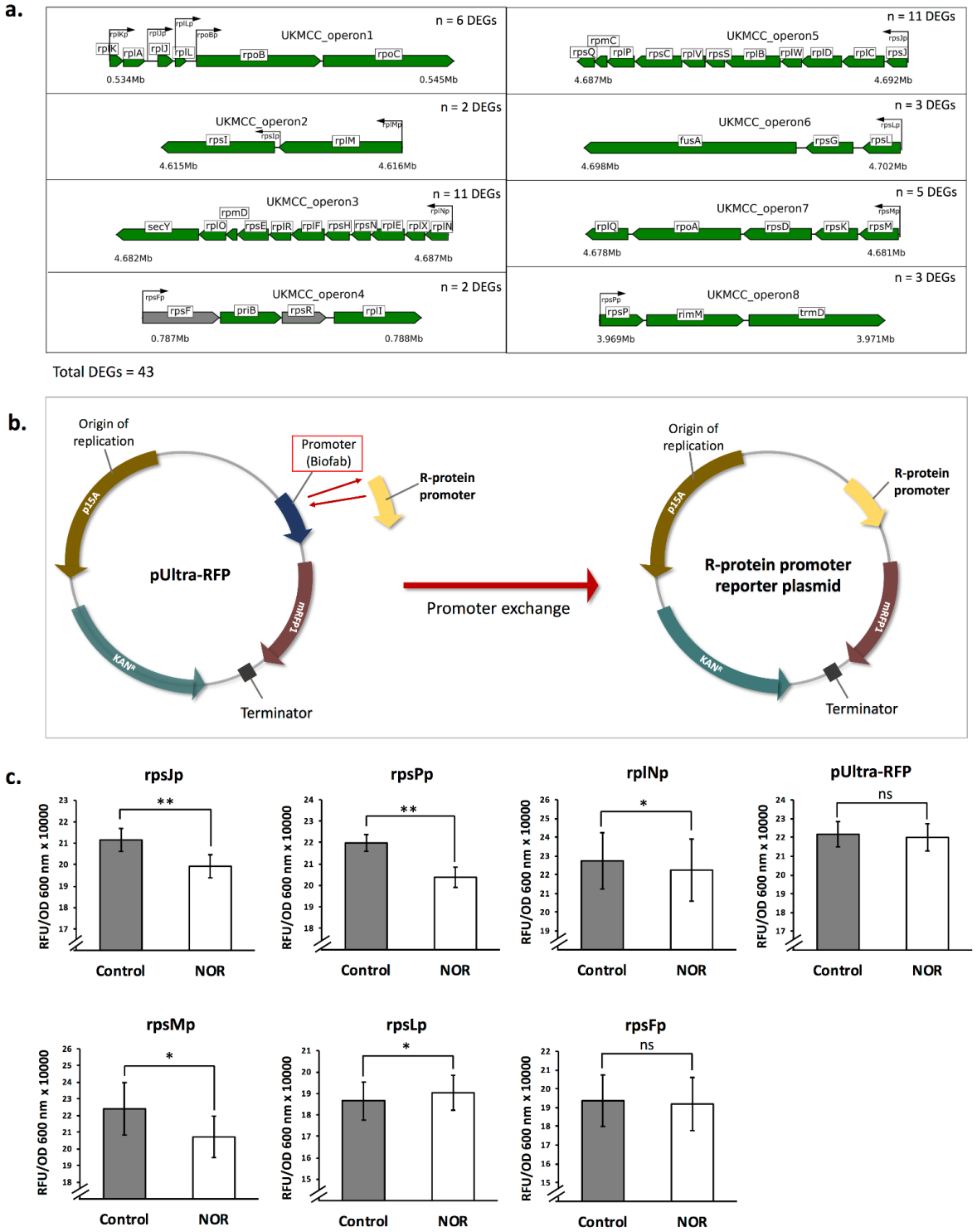

a.
n = 6 DEGs
UKMCC_operon1
0.534Mb
0.545Mb
n = 11 DEGs
UKMCC_operon5
4.687Mb
4.692Mb
n = 2 DEGs
UKMCC_operon2
4.615Mb
4.616Mb
n = 3 DEGs
UKMCC_operon6
4.698Mb
4.702Mb
n = 11 DEGs
UKMCC_operon3
4.682Mb
4.687Mb
n = 5 DEGs
UKMCC_operon7
4.678Mb
4.681Mb
n = 2 DEGs
UKMCC_operon4
0.787Mb
0.788Mb
n = 3 DEGs
UKMCC_operon8
3.969Mb
3.971Mb
Total DEGs = 43
b.
Origin of replication
Promoter (Biofab)
R-protein promoter
pUltra-RFP
Terminator
Promoter exchange
R-protein promoter reporter plasmid
c.
rpsJp
rpsPp
rplNp
pUltra-RFP
rpsMp
rpsLp
rpsFp
RFU/OD 600 nm x 10000
Control
NOR
ns

Figure 3

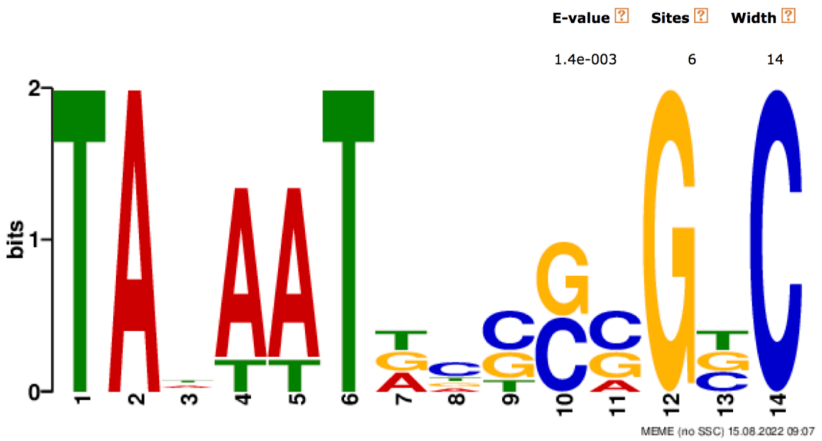


Figure 4

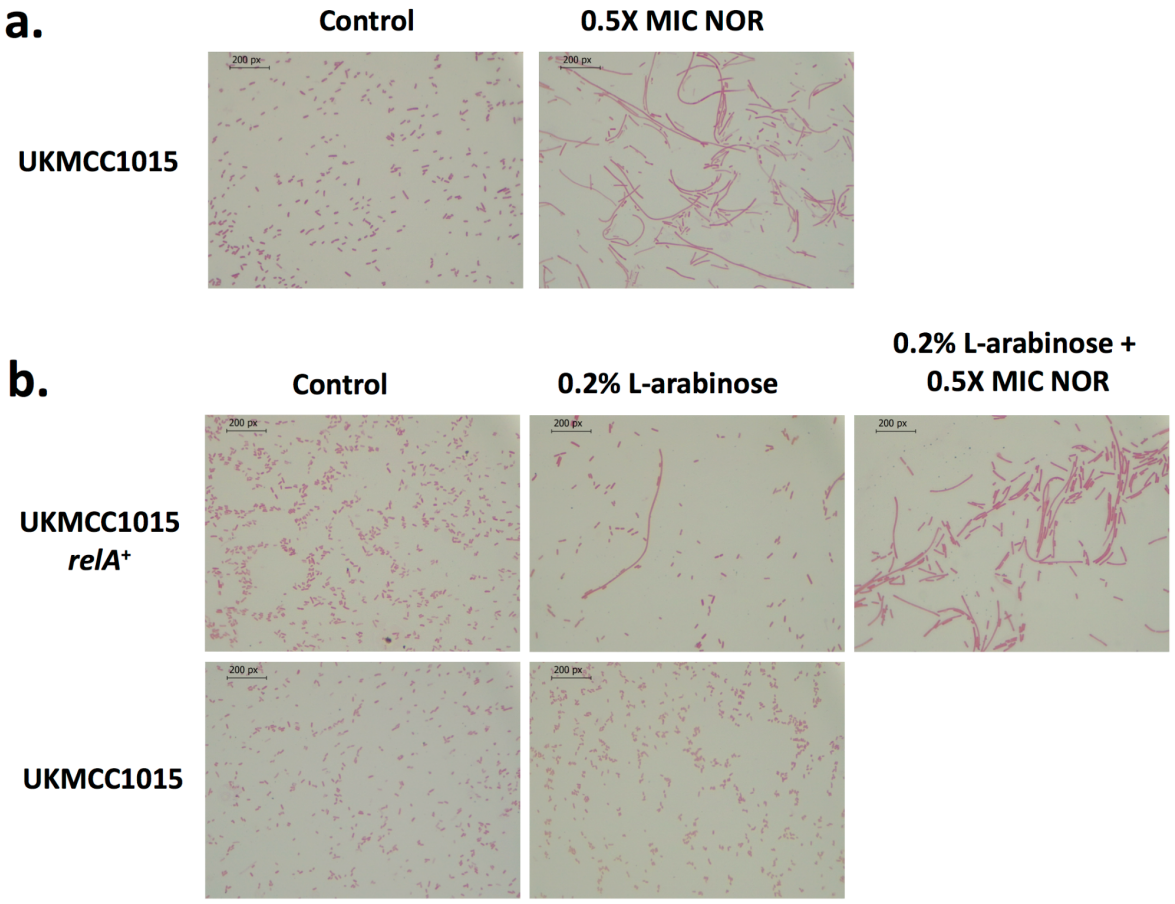


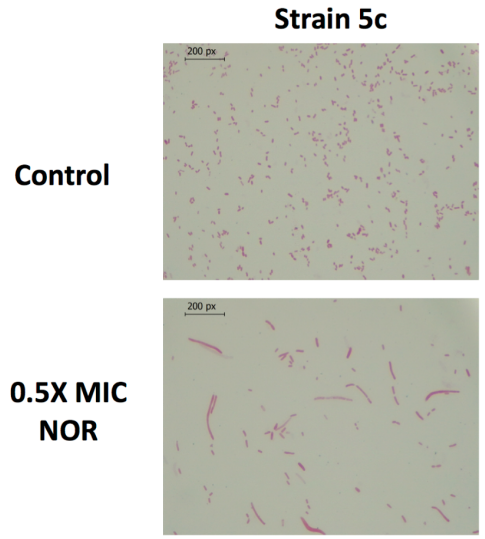
Figure 5

a.

Gene	Amino acid change
gyrA	Asp87Tyr
narG	Asn705Lys
ompC	Phe104del[a]
ompR	Arg210dup[b]
galU	Glu238Gly
ymfD	Thr104Pro

[a] del: deletion; [b] dup: duplication

b.



c.

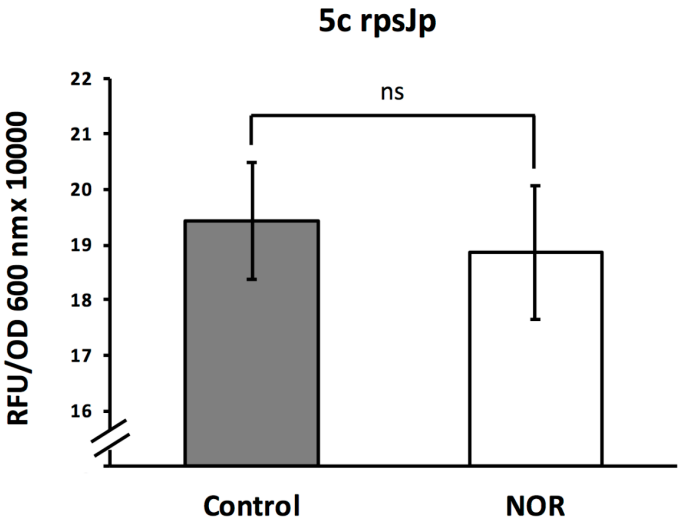


Figure 6

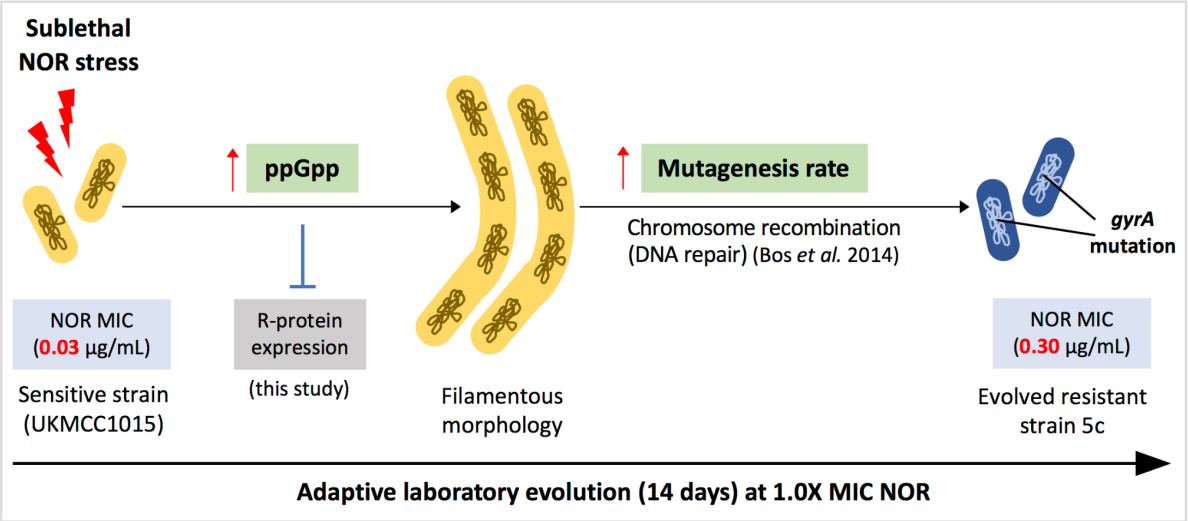

Sublethal
NOR stress
ppGpp
Mutagenesis rate
Chromosome recombination
(DNA repair) (Bos et al. 2014)
gyrA
mutation
NOR MIC
(0.03 µg/mL)
R-protein
expression
(this study)
NOR MIC
(0.30 µg/mL)
Sensitive strain
(UKMCC1015)
Filamentous
morphology
Evolved resistant
strain 5c
Adaptive laboratory evolution (14 days) at 1.0X MIC NOR

Figure 7

Supplementary Data

Table S1: List of antimicrobial resistance (AMR) genes in the genome of UKMCC1015, identified by Abricate using the CARD database.

Table S2: Total list of genes and non-coding RNAs annotated in the transcriptomic study. Bold indicates the differentially expressed genes (DEGs) in *Shigella sonnei* UKMCC1015 in response to 0.2X MIC of norfloxacin.

Table S3: Bacterial strains and plasmids used in this study.

Table S4: Primers used in this study. Restriction sites used for cloning are underlined.

Table S5: Genome assembly information of *S. sonnei* UKMCC1015 and strain 5c.

Table S6: RNA sequencing results of all 4 experimental sample groups.

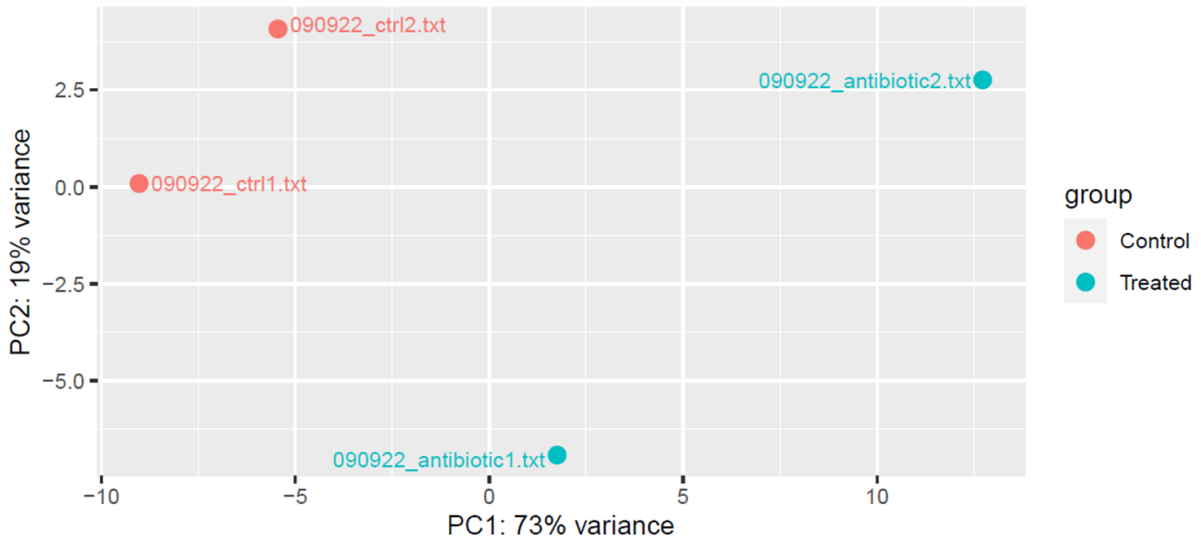


Figure S1: Principle component analysis (PCA) plot of RNA sequencing data. Gene expression changes towards 0.2X MIC of norfloxacin were investigated between the control and experimental group.

a.

rpsJp BioRep	RFU/OD 600 nm	
	Control	0.2X MIC NOR
1	200986	188463
2	215002	204124
3	218522	205089
Average	211503	199226
Standard error	5356	5388
Percentage change (%)	-5.81	
Paired t-test (one-tailed)	0.0018	

b.

rpsPp BioRep	RFU/OD 600 nm	
	Control	0.2X MIC NOR
1	224427	211359
2	212007	195108
3	222990	204922
Average	219808	203796
Standard error	3922	4725
Percentage change (%)	-7.28	
Paired t-test (one-tailed)	0.004	

c.

rpsMp BioRep	RFU/OD 600 nm	
	Control	0.2X MIC NOR
1	202154	194806
2	254523	232011
3	215327	194778
Average	224001	207198
Standard error	15727	12406
Percentage change (%)	-7.50	
Paired t-test (one-tailed)	0.036	

d.

rpsLp BioRep	RFU/OD 600 nm	
	Control	0.2X MIC NOR
1	186530	191047
2	171021	175873
3	201763	204047
Average	186438	190322
Standard error	8875	8141
Percentage change (%)	+2.08	
Paired t-test (one-tailed)	0.020	

e.

rplNp BioRep	RFU/OD 600 nm	
	Control	0.2X MIC NOR
1	198701	190712
2	249384	246477
3	233993	230116
Average	227359	222435
Standard error	15002	16550
Percentage change (%)	-2.17	
Paired t-test (one-tailed)	0.044	

f.

rpsFp BioRep	RFU/OD 600 nm	
	Control	0.2X MIC NOR
1	180643	181397
2	179265	174385
3	221087	219931
Average	193665	191904
Standard error	13717	14159
Percentage change (%)	-0.91	
Paired t-test (one-tailed)	0.199	

g.

pUltra-RFP BioRep	RFU/OD 600 nm	
	Control	0.2X MIC NOR
1	208891	205656
2	224294	228795
3	231923	225654
Average	221703	220035
Standard error	6774	7246
Percentage change (%)	-0.75	
Paired t-test (one-tailed)	0.327	

h.

5c rpsJp Biorep	RFU/OD 600 nm	
	Control	0.2X MIC NOR
1	214011	209032
2	190976	189457
3	177882	167227
Average	194289	188572
Standard error	10560	12076
Percentage change (%)	-2.94	
Paired t-test (one-tailed)	0.0825	

Figure S2: Average normalised RFU/OD 600 nm, standard error, percentage changes and p-value for **a.** rpsJp, **b.** rpsPp, **c.** rpsMp, **d.** rpsLp, **e.** rplNp, **f.** rpsFp r-protein promoters, **g.** pUltra-RFP promoter for control and 0.2X MIC norfloxacin (NOR) exposure, and **h**. strain 5c rpsJp during 0.2X MIC norfloxacin stress. Experiments were conducted in triplicates.