Emergence of ciprofloxacin heteroresistance in foodborne *Salmonella enterica* serovar Agona

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Abstract

Bacterial heteroresistance has been increasingly identified as an important phenomenon for many combinations of antibiotic and bacteria, including vancomycin in *Staphylococcus aureus*, carbapenems in *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, and penicillin in *Streptococcus pneumonia*. We identified an isolate of *Salmonella* from food which yielded a population that demonstrated heteroresistance to ciprofloxacin. Population analyses profile curves identified a resistant subpopulation at a low frequency (10^{-9} to 10^{-7}). Whole Genome Sequencing and pulsed-field gel electrophoresis analyses confirmed that these two subpopulations are isogenic with 6 single-nucleotide polymorphisms and two small deletions distinguishing the resistant subpopulation from the susceptible. Both the resistant subpopulation and the parental population possessed a T57S substitution in ParC and carried *qnrS*. The resistant subpopulation was distinguished by over-expression of *acrB* and *acrF* and a deletion within *rsxC* and associated alteration in expression of *soxS*. The resistant population had a competitive advantage against the parental population when grown in the presence of bile salts but was attenuated in the adhesion and invasion of human intestinal cells. The presence of ciprofloxacin heteroresistant populations in food animals may represent a threat as traditional susceptibility testing cannot easily identify this phenomenon.

Importance

We report the identification of ciprofloxacin heteroresistance in *Salmonella* isolated from chicken meat. Heteroresistance is a concern as the presence of resistant sub populations is often missed by susceptibility testing and ciprofloxacin remains an important therapy for salmonellosis. We determined that heteroresistance resulted from a combination of mutations in fluoroquinolone target genes and overexpression of efflux pumps associated with a deletion in *rsxC*. This study alerts that heteroresistance to ciprofloxacin exists in *Salmonella* in the food chain and that careful interpretation of antibiotic susceptibility is necessary.

Introduction

Non-typhoidal *Salmonella* are one of the most important foodborne pathogens and a leading cause of diarrhea worldwide. Non-typhoidal *Salmonella* infections are usually self-limiting, but severe infections can occur (1, 2). Fluoroquinolones (FQs), especially ciprofloxacin, are one of the drugs of choice for the treatment of salmonellosis although resistance to FQs has emerged (3). In gram-negative bacteria, FQ resistance is primarily due to mutations in target genes encoding for DNA gyrase and topoisomerase IV(4). Other mechanisms of FQ resistance include over-production of chromosomal multidrug resistance (MDR) efflux pumps (5) and acquisition of plasmid-mediated quinolone resistance (PMQR) determinants. Amongst the multiple MDR efflux pumps, AcrAB, which belongs to the resistance-nodulation-cell division (RND) family, is the most important determinant of FQ efflux (6). In *Salmonella*, AcrAB is regulated by the local repressor AcrR and global regulators RamA, MarA and SoxS (7). PMQRs include the 'Qnr' proteins, a variant of the common aminoglycoside acetyltransferase, AAC(6')-Ib-cr (8), and efflux pumps QepA (9, 10) and OqxAB (11). Both MDR efflux and PMQRs confer relatively modest changes in susceptibility which do not result in clinical 'breakpoint' resistance without a synergistic mutation within *gyrA*.

Heteroresistance is a phenomenon whereas a seemingly isogenic population contains a subpopulation with different susceptibility to a given antibiotic than the majority. Studies in recent years have reported the presence of heteroresistance in clinical settings which have been associated with treatment failure (12). Upon antibiotic exposure, the proportion of resistant mutants can increase leading to selection of a resistant population and therapy failure (13). Heteroresistance is thought to be an important intermediate stage before the development of full resistance (14). A population with a heterogeneous level of drug susceptibility gains robustness in terms of ability to survive antibiotic exposure without committing all members of the population to become resistant if there is an associated fitness cost in drug-free conditions (14-17). The first description of heteroresistance was by Alexander and Leidy in 1947 (18, 19) who noticed subpopulations with altered

streptomycin susceptibility in *Haemophilus influenzae*. Jevons (in 1960) reported two *Staphylococcus aureus* subpopulations with quite different methicillin susceptibilities (20). More recently there are increasing reports of heteroresistance including cephalosporins and penicillins heteroresistance in *Acinetobacter baumannii* (21), penicillin heteroresistance in *Streptococcus pneumoniae* (14) and commonly imipenem and meropenem in *Pseudomonas aeruginosa* (22). Heteroresistance to colistin, which is often the only effective antibiotic against widely resistant isolates and used a last resort therapy has been reported from multidrug-resistant *Acinetobacter baumannii* (13, 23).

Heteroresistance to ciprofloxacin has been reported but not in *Salmonella* (17), given the importance of *Salmonella* infections and use of FQs in treatment development of heteroresistance would be a concern. In this study, we isolated a ciprofloxacin heteroresistant *Salmonella* from animals and investigated mechanisms responsible for the heteroresistance phenotype.

Results

Identification of ciprofloxacin heteroresistance amongst *Salmonella* isolates

A total of 61 *Salmonella* isolates were obtained from 36 chicken meat and 47 pork specimens. Among the 61 *Salmonella* isolates, ciprofloxacin resistance (MIC_{CIP} range: 1-32 mg/L) and susceptibility (MIC_{CIP} range: 0.008-0.5 mg/L) rates were 11.48% and 88.52% respectively. PAP of the ciprofloxacin susceptible isolates identified the presence of one *Salmonella* Agona strain which was isolated from chicken meat displaying heteroresistance to CIP (Fig. 1). The PAP curve indicated that the frequencies of the presence of resistant subpopulations were between 10^{-9} to 10^{-7}. PFGE profiles of *Xbal*-digested genomic DNA indicated identical banding profiles between the parent and heteroresistant population (Fig. 1). Antimicrobial susceptibility testing of the resistant subpopulation showed a 16-fold increases in MIC_{CIP} compared to its parent strain (Table 1). The heteroresistant strain was of multilocus sequence type ST13 (Table 1).

Resistance mechanisms contributing to ciprofloxacin heteroresistance in *Salmonella*

We analyzed known mechanisms of FQ resistance in this pair of strains to identify any differences between the susceptible and resistant isolates. The MIC of ciprofloxacin of HZC9-R (4 mg/L) increased 16-fold relative to native strain HZC9 (0.25 mg/L) which is considered intermediate by CLSI breakpoints, and resistant by EUCAST rules. A T57S substitution in ParC and the presence of the PMQR gene *qnrS* were found in both strains. Expression of both *acrB* and *acrF* in HZC9-R were raised (2.43-fold and 1.57-fold, respectively) compared to HZC9 (Table1) and expression of *qnrS* in HZC9-R was significantly higher than in HZC9 (Fig. 2), suggesting the involvement of efflux pump expression and *qnrS* expression in ciprofloxacin resistance in HZC9-R. Supporting the idea that efflux expression was important in dictating the phenotype of HZC9-R, accumulation assays revealed significantly less accumulation of resazurin in this strain compared to the parent (Fig 2).

Growth and growth competition between resistant strains and their native

strains

Growth curves showed both subpopulations in LB broth required similar time to reach stationary phase but HZC9-R achieved a slightly lower cell density than the susceptible subpopulations (Data not shown). Competitive growth assays of the sub populations against a common wild-type strain (marked by the presence of the *lac* operon allowing blue-white screening) showed that subpopulation HZC9-R was more competitive than the parent population (Fig. 3).

We also assessed the competitive ability between the resistant and susceptible subpopulations directly in 0%, 0.1%, and 1% bile by pyrosequencing in antibiotic-free LB broth in three independent experiments. The mean competition coefficients between HZC9-R and HZC9 were 2.03, 3.00 and 3.57 (Fig. 3). This result indicated that resistant *Salmonella* HZC9-R had a competitive growth advantage in the presence of bile.

Association and invasion ability of resistant strain and its parent strains

To determine if there were any differences in the association and invasion ability of this pair of strains, we measured the ability of each to associate and invade INT 407 cells *in vitro* (Fig. 4). Results are expressed as the percentage of the inoculum that effectively associated and invaded the INT 407 cells. HZC9-R (MIC_{CIP}=4 mg/L) strain had lower virulence than HZC9 (MIC_{CIP}=0.25 mg/L).

The virulence of each subpopulation was also investigated by using Galleria mellonella larvae infection model. The result was consistent with the association and invasion assay in that HZC9 was more virulent than HZC9-R (Fig. 4).

Genomic analysis

Whilst the initial investigations suggested efflux over-expression as a mechanism determining the phenotype of HZC9-R there was no immediate reason for this. Hybrid genome assemblies based on nanopore and illumina data were prepared for both strains. Each had a single chromosome (4.9Mb) and three distinct plasmids of (92kb, 49kb and 10kb) as shown in table S1. There were a small number of differences present between the strains including 6 SNPs as well as a small number of deletions, including a 250bp deletion within *rsxC* present in HZC9-R (Table S2 and

Fig. S1).

Paraquat induces immediate *soxS* expression in HZC9-R

RsxC has been shown in *E. coli* to constitute a reducing system to reset expression of *soxS* once induced by reducing SoxR but has not been identified as having a role in ciprofloxacin resistance in *Salmonella*. We measured expression and sensitivity to induction of *soxS* in HZC9-R and tested the impact of inactivating *rsxC* in HZC9. The results showed that expression of *soxS* in HZC9-R was higher than in HCZ9 and responded to induction much more quickly (Fig. 8). Furthermore, inactivation of *rsxC* in HZC9 led to decreased susceptibilities (2-4 fold) to various drugs and dyes (Table 2).

Discussion

Heteroresistance has been recognized as an important step in the development of highly resistant populations but often cannot be detected by traditional antibiotic susceptibility testing which can therefore give misleading sensitivity results that can lead to treatment failures (17). Heteroresistant phenomena, which render compromising activity of clinical relevant antimicrobials, are increasingly reported in a variety bacteria, including *Haemophilus influenzae, Staphylococcus aureus, Acinetobacter baumannii, Streptococcus pneumonia,* and *Pseudomonas aeruginosa* (13, 14, 20-23). In the present study, we for the first time identified one ciprofloxacin heteroresistant *Salmonella* strain HZC9 with a subpopulation of resistant cells with an elevated MIC_{CIP} 8-fold higher than its parent strain. This indicates that this resistant subpopulation is likely to expand and confer full resistance if ciprofloxacin is chosen for therapy. This raises the likelihood that traditional testing cannot easily identify the presence of ciprofloxacin heteroresistance and the necessity of careful interpretation of antibiotic susceptibility. To explore whether these resistant subpopulations have any fitness cost we compared their ability to grow in media alone and in the presence of bile, a proxy for conditions in the gut. The resistant strain grew more slowly than the sensitive counterpart in all conditions in isolation (data not shown) but was able to outcompete the resistant strain when mixed together suggesting some interplay between the subpopulations.

To clarify the underlying mechanisms contributing to the heteroresistant phenotypes, we analyzed possible mechanisms among resistant and susceptible subpopulations. Both resistant and susceptible strains (HZC9-R and HZC9) harbored a single amino acid change of T57S in ParC. This substitution has been suggested to be seen in some serovars without a role in FQ resistance, one study speculated that *Salmonella enterica* serovar Typhimurium*,* serovar Schwarzengrund*, and* serovar Hadar each have a different role from *parC* T57S mutation in quinolone resistance (24), however data from another study suggested that a T57S mutation in ParC increased ciprofloxacin susceptibility of isolates (25). Overexpression of *acrB* (2.43-fold) and *acrF* (1.57-fold) and significantly higher expression of *qnrS* were detected in

HZC9-R relative to HZC9 which could help explain the ciprofloxacin resistance. Several studies have shown that the acquisition of mutations that increase efflux, mainly due to the overexpression of AcrAB, are key steps along with mutations in QRDRs in the development of quinolone resistance in *Salmonella* (25, 26). Of the 6 SNPs present between the strains, none were in known regulators of *acrAB* or *acrEF*. Comparison of the genomes of the two strains revealed a ~250 bp deletion of gene *rsxC* on the chromosome in HZC9-R. Both expression of *soxS* in HZC9-R and the 2-4 fold decreased susceptibilities to drugs and dyes in HZC9 Δ*rsxC*::*tet*^{R} mutant suggested that increased efflux in HZC9-R was activated by increased *soxS* activity which resulted from mutation of *rsxC*. Therefore, inactivation of *rsxC* is a relevant mechanism of fluoroquinolone resistance in *Salmonella* which should be considered when searching for the genetic basis for resistance.

In conclusion, to the best of our knowledge, this is the first study to report *Salmonella* ciprofloxacin heteroresistance. Ciprofloxacin heteroresistance in *Salmonella* strains can be explained by a combination of target gene mutations, overexpression of efflux pumps caused by *rsxC* mutation and carriage of a PMQR. The present study alerts that ciprofloxacin heteroresistance exists in isolates from the food chain and will not be identified by traditional antibiotic susceptibility testing.

Materials and methods

Salmonella isolation, Serotyping, and Antimicrobial susceptibility testing

61 *Salmonella* strains were isolated from 36 chicken meat and 47 pork specimens obtained in 2016 from four supermarkets, in Guangzhou, Guangdong, China. Approximately 25 g samples cut into pieces were put into 200 mL buffered peptone water (BPW) and then incubated at 37°C for 12 h. 1 mL aliquots of BPW cultures were then transferred to 10 mL of selenite cysteine broth and incubated at 37°C for 24 h. The enriched culture was streaked onto chromogenic medium selective for *Salmonella* (CHROM agar Microbiology, France) and incubated for another 24 h at 37°C. Single purple colonies were selected from each plate and then confirmed as *Salmonella* using the API20E system (bioMérieux, Marcy l'Étoile, France) and identified by MALDI-TOF MS (Axima-Assurance-Shimadzu). *Salmonella* isolates were serotyped using *Salmonella* specific O and H antigens (Statens Serum Institut, Denmark) by the slide agglutination test according to the Kauffmann-White scheme. MICs of ciprofloxacin were determined in triplicate for each bacterial strain using the broth microdilution method according to the CLSI reference methods**.** *Escherichia coli* ATCC 25922 was used as a quality control strain. The genetic relatedness of heteroresistant strains was initially assessed by PFGE after *Xba*I-digestion of genomic DNA using a CHEF-MAPPER System (Bio-Rad Laboratories, HercμLes, CA), as previously described (27).

Population analyses profiling (PAP)

PAP was conducted for resistance to ciprofloxacin as described previously with some modifications (21). Briefly, bacteria were streaked out on Muller-Hinton agar plates and incubated at 37°C for 24 h. Single colonies were sub-cultured in 5 mL of Muller-Hinton broth and grown overnight. Cultures were serially diluted and plated onto Muller-Hinton agar plates containing 2-fold dilutions of ciprofloxacin at concentrations ranging from 0.5 to 16-fold MIC. A 10^{-6} dilution of the culture was plated onto Muller-Hinton agar plates containing no drug for determination of colony numbers which were counted after incubation at 37°C for 48 h. Three independent assays were performed, and mean colony numbers were calculated and plotted on a

semi-logarithmic graph with colony numbers on the vertical axis and drug concentration on the horizontal axis. Colonies grown on drug-containing plates (4 to 16-fold MIC) were sub-cultured in drug-free plates for 1 week and MICs were determined using the broth micro-dilution method.

Detection of mutations within QRDRs, presence of PMQR genes, and expression of *acrB*, *acrF* and *qnrS*

Mutations in *gyrA*, *gyrB*, *parC*, and *parE* were detected by PCR (28) and sequencing, results were compared against with the genome of *Salmonella* Typhimurium LT2. The presence of PMQR determinants *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr, qepA,* and *oqxAB*) was analyzed by PCR using primers and conditions described previously (29).

For gene expression analyses, bacterial strains were grown in minimal media to an OD_{600} of 0.6, and total RNA was isolated according to Fàbrega et al. (30). The expression of *acrB*, *acrF* genes was analyzed by RT-qPCR (31). The 16S rRNA was used as an internal control gene for normalization. The relative expression level of each gene was calculated using the ΔΔCt method. Data is presented as mean ± SD. Three independent assays were performed, and each RNA sample was tested in triplicate.

Fluorescence reporter plasmids for measuring expression of target genes

The promoter regions of *acrA*, *ramA*, *marA*, *soxS* were cloned into plasmid pMW82 to control *gfp* expression individually. Each resulting promoter fusion plasmid was transformed into strains by electroporation. Transformants were inoculated into LB broth containing 50 µg/mL carbenicillin (to maintain the plasmid) incubated at 37 °C with shaking for around 12 hours and cultures were normalized at OD_{600} 0.1. Three biological and three technical replicates were included in each assay. 200 µL of each normalized culture was added into wells of a 96-well plate and simultaneous measurement of fluorescence reader at excitation and emission of 485 nm and 520 nm and absorbance at a wavelength of 600 nm were conducted at 37 °C in a BMG FLUOstar Omega plate reader every 10 minutes for 9 hours.

Accumulation assays

Resazurin accumulation assays were used to assess differences in accumulation and efflux ability. Strains of interest were grown to log phase by 1:100 inoculation from overnight cultures. Cells were harvested, washed by PBS, and normalized at OD600 0.1. 180 μL of normalized cultures were mixed with 10 μL of resazurin (to give a final concentration of 10 μg/mL) and 10 μL of pump inhibitor PAβN (to give a final concentration of 0.125 μg/mL) or only resazurin and transferred into a 96-well plate. Simultaneous measurement of fluorescence at excitation and emission of 544nm and 590nm and absorbance at a wavelength of 600 nm were conducted in Omega FLUOstar plate reader every 5-10 minutes for few hours. *Salmonella* 14028s Δ *tolC*::*cat* was used as a control strain. Three technical replicates and at least three biological replicates were conducted. Cell-only and resazurin-only reactions were set as controls.

Bacterial growth kinetic and competition assays

To determine growth kinetics, strains were incubated overnight in Luria-Bertani (LB) broth at 37°C with shaking, and diluted to OD_{600} = 0.1, then 30 μL was added into 15 mL fresh LB broth and incubated with shaking (200 rpm). Samples were serially diluted and plated onto LB agar plates to estimate growth every two hours. The plates were incubated at 37°C for 24 h before counting colonies. Three independent assays were performed for each strain.

We tested competition between strains in 0%, 0.1%, and 1% bile in antibiotic-free LB broth. Each test was run with nine replicates. The relative abundance of each strain in populations was determined using pyrosequencing to identify the single nucleotide variations in the strains according to the method of Baker et al (32). The DNA from the competitive growth assays was amplified by PCR in triplicate using biotinylated primer pairs targeting the region containing the single nucleotide polymorphism to distinguish the two organisms in the assay by mutations in the chromosome according to Single Nucleotide Polymorphisms (SNP) analyses based WGS (position: 1347348). All PCR amplifications were visualized on 1% agarose gels prior to pyrosequencing. The purified PCR products were pyrosequenced at the BGI Company. Competition coefficients were calculated by measuring the

percentage yield of the single nucleotide in the genome. A competition coefficient >1 means that the resistant strain was the more abundant strain; meanwhile, a competition coefficient <1 means that the resistant strain was the less abundant strain.

Competition assays between each subpopulation and *Salmonella* 14028s/*lac* (a *S.* Typhimurium14028s mutant carrying the *lacIZ* genes downstream of *glmS*) were also carried out to determine the competitive ability of the two subpopulations. Overnight cultures were normalized at an OD_{600} nm of 1. Each strain was (1:100) added into universals containing 5 mL LB broth. CFU of each strain were determined by plating appropriate dilutions on LB agar plates containing X-gal and IPTG (blue-white screening) both at the beginning and after 24 hours of co-culturing at 37 °C. Competitive coefficients of each subpopulation against the 14028s/*lac* mutant strain were calculated between 0 hour and 24 hour time points. Two biological and three technical replicates were used.

Adhesion, invasion assays and *Galleria mellonella* infection model

The ability of strains to adhere to and invade INT 407 cells (human embryonic intestine cell line stored in our laboratory) was assayed using a previously described method (33). Each assay was performed at least three times with three technical replicates for each strain.

Galleria mellonella larvae infection model was used to assay the virulence of different strains. Wax worm larvaes were purchased from livefoods.co.uk. Healthy larvae (no pupation or melanisation) of similar size were selected for bacteria injection. A preliminary which was conducted to determine the infectious dose of *Salmonella* (*S.* Typhimurium 14028s was used) found that an inoculation of $2*10^4$ CFU resulted in 50% lethality after 72 hours under 37 ℃. Overnight cultures of each strain were diluted and normalized in PBS and a certain volume of dilution were injected into the third hindmost left proleg of ten larvae. Inoculation were also confirmed by plating different dilutions onto LB agar plates and counting CFUs on the next day. PBS injection and no injections were included as controls. Survival rates were checked over time. 3 biological replicates were included in this experiment.

Genome sequencing

For Illumina sequencing, DNA was prepared from 500 µL of overnight cultures using a standard alkaline lysis procedure. 50 µL of DNA- binding magnetic beads (KAPA Pure beads, Roche diagnostics) were used to purify DNA before beads were washed by fresh 80% ethanol three times and left to air dry for 1-2 minutes. 200 µL of 5 mM Tris-Cl was added into each eppendorf and mixed by vortexing and pipetting for 5 minutes. The tubes were centrifuged for 10 minutes at 11,000 g and the isolated DNA was transferred into a new eppendorf. DNA was quantified using Qubit dsDNA HS Assay kit (Q32851) on a Qubit 3.0 instrument (Invitrogen, MA, USA) according to the manufacturer's instructions. Genome sequencing was carried out on the Illumina NextSeq 500 platform (Illumina, San Diego, CA). Libraries were prepared using a Nextera XT DNA protocol from genomic DNA normalized to 0.5 ng/µL with 10 mM Tris-HCl. The final pool was quantified using Qubit dsDNA HS Assay kit (Q32851) on a Qubit 3.0 instrument (Invitrogen, MA, USA) and run on a high sensitivity D1000 ScreenTape (Agilent Catalogue No. 5067-5579) using the Agilent Tapestation 4200 to calculate the final library pool molarity. The pool was run at a final concentration of 1.8 pM on an Illumina NextSeq500 instrument using a mid-output.

For Nanopore sequencing, strains were incubated overnight in LB broth at 37°C with shaking and the genomic DNA was extracted using the RevoluGen PuriSpin Fire Monkey Kit. DNA was quantified and qualified by Qubit dsDNA HS Assay kit on a Qubit 3.0 instrument (Invitrogen, MA, USA) and Genomic DNA screen Tape according to the manufacturer's instructions.

Libraries were prepared using the rapid barcoding kit (Oxford Nanopore catalogue number SQK-RBK004) according the manufacturer's instructions. 400 ng DNA was used for library preparation and 75 µL of the library was loaded on an R9.4 MinION flowcell. Size of the DNA fragments was assessed using the Agilent 2200 Tape Station (Agilent Catalogue No. 5067-5579) before sequencing and after final library pool preparation.

Hybrid assembly, annotations and SNP analyses

Sequencing reads were uploaded to virtual machines provided by the MRC

CLIMB (the Cloud Infrastructure for Microbial Bioinformatics) project using BaseMount (34). Analyses of sequencing data used 'NanoStat v 0.1.1' to calculate statistics of long reads. Adapters from Oxford Nanopore reads were trimmed using 'Porechop v 0.2.3'. 'NanoFilt v 0.1.0' was used for filtering and trimming of long reads after Porechop bioinformatics analyses. 'fastp v 0.19.5' was used to provide all-in-one preprocessing for all FASTQ files. 'MultiQC v 1.7' was used to take results of fastp bioinformatics analyses and create a single report. Hybrid assemblies of Illumina and Nanopore reads after bioinformatics analyses were then performed using the Unicycler assembly pipeline for bacteria genomes and finally assemblies were then annotated with 'Prokka v 1.13'. Annotations were visualized in 'Artemis'. SNPs in isolates were identified using 'Snippy v 4.2.3'. Comparison of the genomes of HZC9 and HZC9-R was done by using BRIG (BLAST Ring Image Generator).

Strains and genetic manipulation

An *rsxC* gene deletion mutant was created using the λ-red recombineering system-based, gene doctoring technique as previously described(35). Two homologous regions which include 330 bp upstream and downstream of the part which would be deleted were amplified by PCR. Colony PCR and Sanger sequencing were carried out to check part deletion of *rsxC* with primers annealing outside the region to be modified.

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Disclosure declaration

No authors have any conflicts to declare.

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Author Contributions

Conceived and designed the experiments: C-ZZ, ET, NMT, MAW and H-XJ.

Performed the experiments: C-ZZ, YZ, X-MD, and X-LL. Analyzed the data: C-ZZ, X-LL, NMT, H-ZD, MAW and H-XJ. Contributed reagents/materials/analyses tools: C-ZZ, YZ, X-MD, and X-LL. Wrote the paper: C-ZZ, MAW and H-XJ.

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549 **Table 1. Susceptibility, genotypes and gene expression of strains used in the**

550 **study.**

Strain	MIC_{CIP} (mg/L)	MLST	Serotype	Target gene mutations				PMQR	Gene expression values (+ SEM)	
				gyrA	*gyrB*	*parC*	*parE*		*acrB*	*acrF*
HZC9	0.25	ST13	Agona	-	-	T57S	-	*qnrS*	1	1
HZC9-R	4	ST13	Agona	-	-	T57S	-	*qnrS*	2.43(1.23)	1.57(0.94)

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552 **Table 2. Susceptibilities of subpopulations and *rxsC* deletion mutant**

Strain	MIC_{ACR} (mg/L)	MIC_{KAN} (mg/L)	MIC_{AMP} (mg/L)	MIC_{CEF} (mg/L)	MIC_{CIP} (mg/L)	MIC_{AZI} (mg/L)	MIC_{CHL} (mg/L)
HZC9	64	4	16	<0.125	0.5	4	128
HZC9 Δ*rsxC*::*tet*^{R}	128	4	64	<0.125	1	8	256
HZC9-R	128	2	128	0.5	4	32	512

553 ACR, Acriflavine; KAN, Kanamycin; AMP, Ampicillin; CEF, Cefotaxime; CIP,

554 Ciprofloxacin; AZI, Azithromycin; CHL, Chloramphenicol; *tet*^{R}, tetracycline resistance

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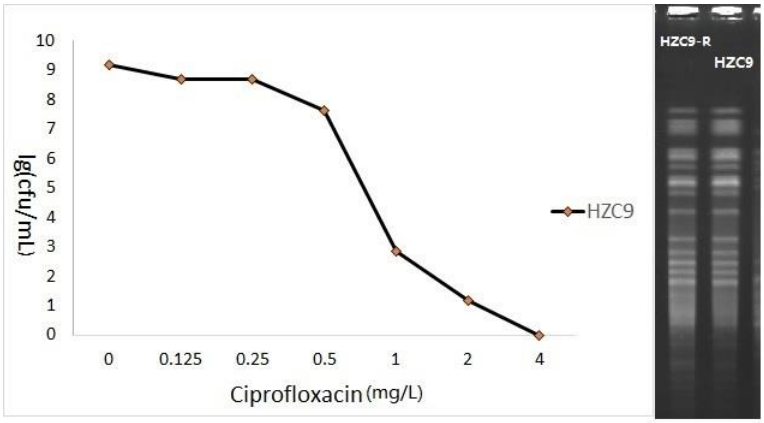


Figure 1. Susceptibility of HZC9 to ciprofloxacin as demonstrated by population analyses and PFGE profiles of *Xbal*-digested genomic DNA of HZC9 and HZC9-R.

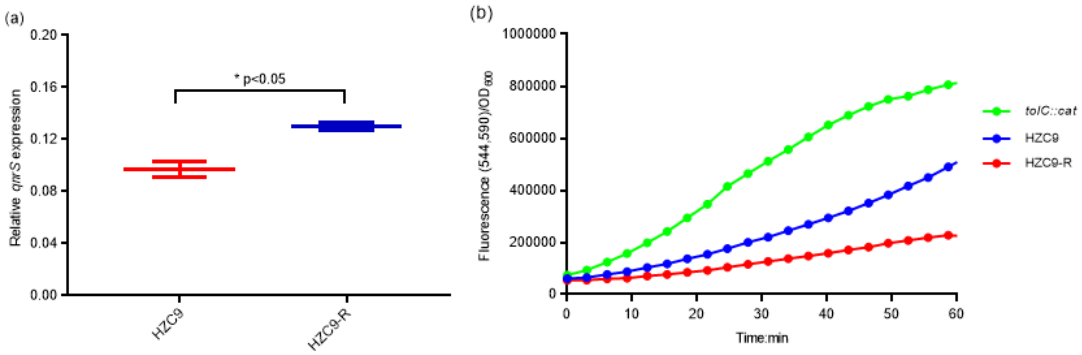


Figure 2. (a) Relative expression of *qnrS* in HZC9 and HZC9-R. Values show mean ± SD from three independent assays with three technical replicates in each. (b) Resazurin accumulation by HZC9, HZC9-R (and reference strain *tolC*::*cat*). Values shown mean data from three experiments, each with three technical replicates.

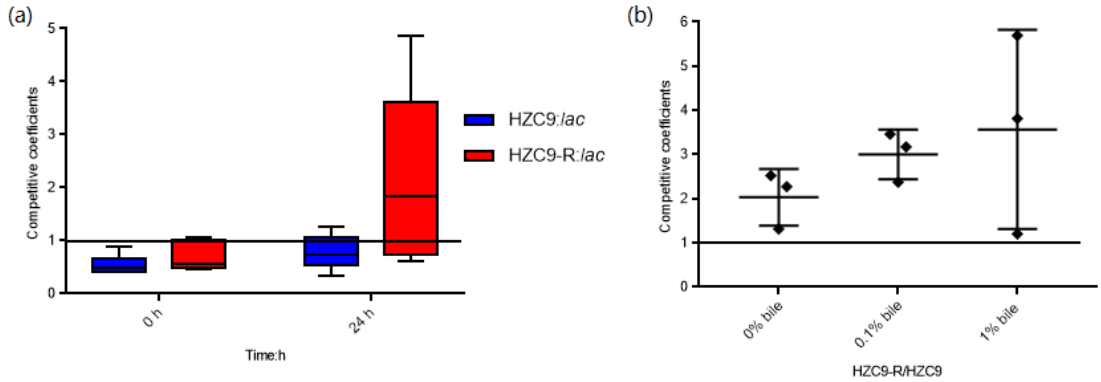


Figure 3. (a) *In vitro* bacterial competition assays by blue-white screening. Competition coefficient values obtained from each independent experiment are plotted; a competition coefficient of 1 is noted by a horizontal line. (b) *In vitro* bacterial competition assays by pyrosequencing. Competition coefficient values obtained from each independent experiment are plotted; means are noted by short continuous horizontal lines with error bars indicating SD.

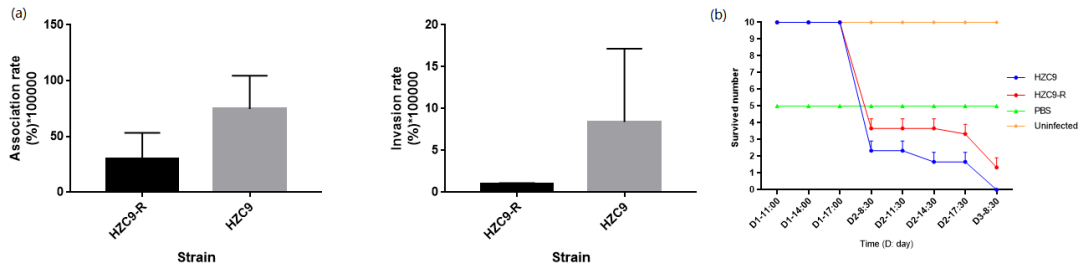
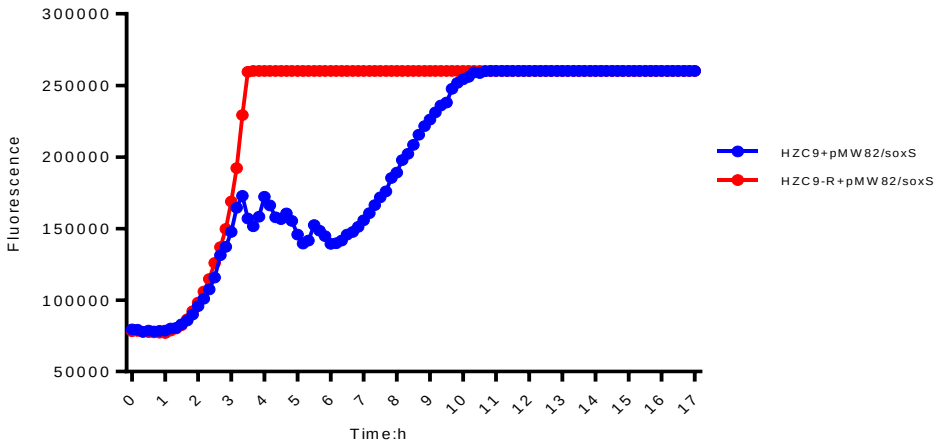


Figure 4. (a) Ability of strains to associate and invade INT 407 epithelial cells. Results are the mean of three independent experiments ±standard error. (b) Virulence of strains in a Galleria mellonella larvae infection model. Survival numbers were counted over time.



586

587 **Figure 5**. Expression of *soxS* in HZC9 and HZC9-R measured by *gfp*-reporter

588 assays. Fluorescence was measured in arbitrary units and data show averages from

589 three biological and three technical replicates.

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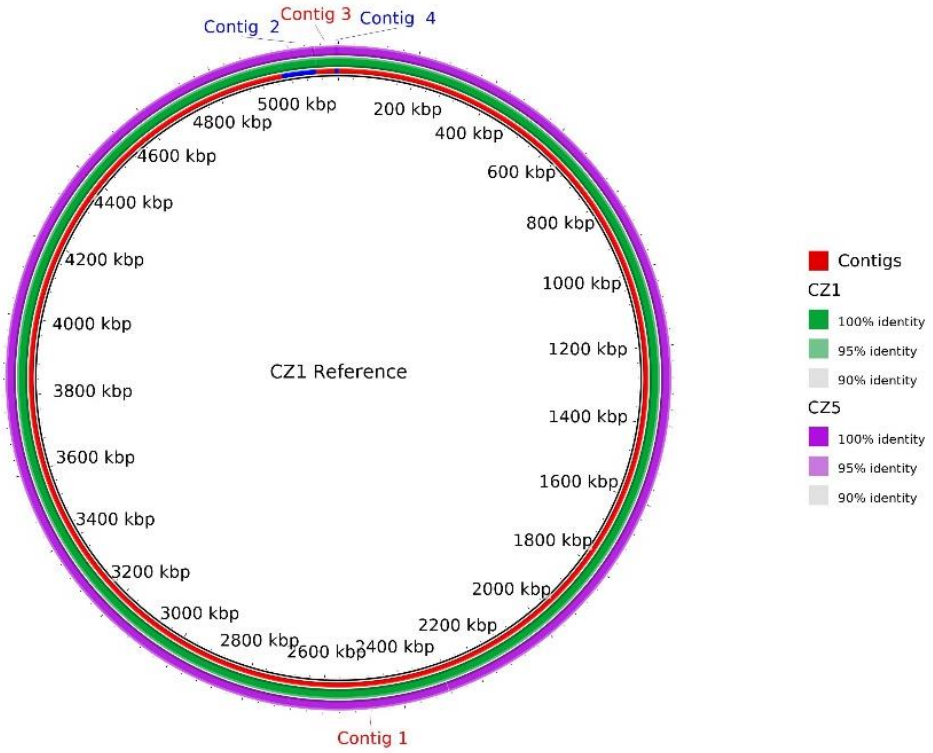
Supplemental Material

Figure legends

Figure S1. Map of genomic comparison of HZC9 (CZ1) and HZC9-R (CZ5). HZC9 (CZ1) was used as the reference. Four contigs on the map represent chromosome, plasmid 1, plasmid 2 and plasmid 3 individually.



607 **Table S1. Genetic differences between HZC9 and HZC9-R.**

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	HZC9 (CZ1)	HZC9-R (CZ5)	Difference
Chromosome (bp)	4,972,924	4,972,563	-361
Plasmid 1 (bp)	92,109	92,110	+1
Plasmid 2 (bp)	49,297	48,872	-425
Plasmid 3 (bp)	10,047	10,047	=

609

610 **Table S2. Single-Nucleotide Polymorphisms (SNPs) found between two strains**

	Position and SNPs					
Gene	1183585	1347348	1902973	2330556	2337500	2633210
	motB	hypothetical	*sifA*	hypothetical	intergenic	hypothetical
HZC9	C	C	G	A	C	G
HZC9-R	A	T	A	T	T	A

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