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Fitness Costs and Stability of a High-Level Ciprofloxacin Resistance Phenotype in *Salmonella enterica* Serotype Enteritidis: Reduced Infectivity Associated with Decreased Expression of *Salmonella* Pathogenicity Island 1 Genes▿†

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The fitness costs associated with high-level fluoroquinolone resistance were examined for phenotypically and genotypically characterized ciprofloxacin-resistant *Salmonella enterica* serotype Enteritidis mutants (104-cip and 5408-cip; MIC, >32 μg/ml). The stability of the fluoroquinolone resistance phenotype in both mutants was investigated to assess whether clones with better fitness could emerge in the absence of antibiotic selective pressure. Mutants 104-cip and 5408-cip displayed altered morphology on agar and by electron microscopy, reduced growth rates, motility and invasiveness in Caco-2 cells, and increased sensitivity to environmental stresses. Microarray data revealed decreased expression of virulence and motility genes in both mutants. Two clones, 104-revert and 1A-revertC2, with ciprofloxacin MICs of 3 and 2 μg/ml, respectively, were recovered from separate lineages of 104-cip after 20 and 70 passages, respectively, on antibiotic-free agar. All fitness costs, except motility, were reversed in 104-revert. Potential mechanisms associated with reversal of the resistance phenotype were examined. Compared to 104-cip, both 104-revert and 1A-revertC2 showed decreased expression of *acrB* and *soxS* but still overexpressed *marA*. Both acquired additional mutations in SoxR and ParC, and 1A-revertC2 acquired two mutations in MarA. The altered porin and lipopolysaccharide (LPS) profiles observed in 104-cip were reversed. In contrast, 5408-cip showed no reversal in fitness costs and maintained its high-level ciprofloxacin resistance for 200 passages on antibiotic-free agar. In conclusion, high-level ciprofloxacin resistance in *S*. Enteritidis is associated with fitness costs. In the absence of antibiotic selection pressure, isolates may acquire mutations enabling reversion to an intermediate-level ciprofloxacin resistance phenotype associated with less significant fitness costs.

Salmonella enterica serotype Enteritidis is one of the most common causes of food-borne salmonellosis worldwide. Historically, *S.* Enteritidis has remained susceptible to most antibiotics, unlike more-common serotypes, such as *Salmonella enterica* serotypes Typhimurium, Virchow, Newport, and Hadar, in which resistance to a wide range of antimicrobial agents is common (16, 29). A number of reports have documented an increasing prevalence of nalidixic acid resistance among nontyphoidal *Salmonella* isolates, in particular *S.* Enteritidis (5, 26, 42). Such isolates typically show decreased susceptibility to ciprofloxacin (MIC, 0.12 to 1.0 μg/ml), although the MICs are within the susceptible range of the interpretive criteria of the Clinical and Laboratory Standards Institute (CLSI) (12). There is increasing evidence that fluoroquinolone therapy for infections caused by *Salmonella* strains with reduced susceptibility to fluoroquinolones may result in treatment failure (28, 34, 44). To date, high-level fluoroquinolone resistance remains relatively uncommon in *Salmonella* isolates, compared with that in other *Enterobacteriaceae*. However, the emergence and clonal spread of fluoroquinolone-resistant *Salmonella enterica* serotype Typhimurium DT204 strains were observed in the 1990s, and these strains presently reoccur in serotypes such as *S.* Typhimurium (22), *S.* Choleraesuis (9), and *S.* Schwarzengrund (31).

Well-documented mechanisms associated with the development of high-level fluoroquinolone resistance include mutations that reduce the affinity for the antibiotic targets DNA gyrase and/or DNA topoisomerase IV, active efflux due to overproduction of the AcrAB-TolC efflux pump, and plasmid-mediated protection of target topoisomerases (17, 20). The contribution of the decreased membrane permeability resulting from altered porin expression and lipopolysaccharide (LPS) profiles to quinolone resistance is currently unclear (19, 27, 35).

Mutations in antibiotic target genes and overexpression of multidrug resistance (MDR) efflux pumps have been associated with fitness costs, including reduced growth rates and

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TABLE 1. Phenotypic and genotypic characteristics of the *S.* Enteritidis strains used in this study

Strain	MIC (μg/ml)[a]		Mutation(s) in target structure[b]				Mutation(s) in regulator of AcrAB-TolC[b]			
	NAL	CIP	GyrA	GyrB	ParC	ParE	SoxR	SoxS	MarA	RamR
104	>256	0.25	D87Y	*	*	*	*	*	*	*
104-cip	>256	>32	D87Y, S83F	*	*	*	R20H	E52K	*	*
104-revert	>256	4	D87Y, S83F	*	D79N	*	E16G, R20H	E52K	*	*
1A-revertC2	>256	2	D87Y, S83F	*	D79N	*	R20H, R133C	E52K	I111N, H115Q	*
5408	>256	0.25	D87Y	*	*	*	*	*	*	*
5408-cip	>256	>32	D87Y	E466D	*	V461G	*	*	*	G25A
NCTC 13349	8	0.032	*	*	*	*	*	*	*	*

[a] Values represent means of results from 3 separate determinations. NAL, nalidixic acid; CIP, ciprofloxacin.
[b] D, aspartic acid; Y, tyrosine; S, serine; F, phenylalanine; E, glutamic acid; G, glycine; R, arginine; H, histidine; K, lysine; N, asparagine; I, isoleucine; C, cysteine; Q, glutamine; A, alanine; *, wild-type allele (no mutation).

virulence, which may limit the survival of resistant strains in the absence of antibiotic selective pressure (1, 3, 23, 39, 47). However, stabilization of resistance can occur through the development of compensatory mutations that restore fitness without loss of the original level of resistance (2). In contrast to the wealth of information available on the mechanisms leading to high-level fluoroquinolone resistance in *Salmonella*, few studies to date have investigated the fitness costs associated with this phenotype (18, 45). Data from these studies suggest that mechanisms that confer high-level ciprofloxacin resistance in *Salmonella* have a prohibitive fitness cost and may thus limit the emergence and spread of highly resistant clones in the absence of antibiotic selection.

In a previous study, we genotypically and phenotypically characterized two *in vitro*-selected ciprofloxacin-resistant *S.* Enteritidis mutants (32). The aim of this study was to assess their growth characteristics, colony morphology, motility, invasiveness, and acid and osmotic tolerance and to investigate whether clones with better fitness could emerge following serial transfer in the absence of antibiotic selective pressure. We examined the global gene expression in both mutants to investigate potential molecular mechanisms associated with the observed fitness costs. Mechanisms associated with the reversal of one of the mutants to a low-level ciprofloxacin resistance phenotype associated with lesser fitness costs were also investigated.

MATERIALS AND METHODS

Bacterial strains used. The parent *S.* Enteritidis strains 104 and 5408, displaying high-level nalidixic acid resistance and reduced susceptibility to ciprofloxacin and harboring a single *gyrA* mutation (D87Y), were isolated from poultry and a human, respectively. Quinolone-resistant mutants 104-cip and 5408-cip were obtained from their isogenic parents after seven selection steps on tryptone soya agar (TSA; Oxoid, New Hampshire, United Kingdom) with increasing concentrations of ciprofloxacin (0.25 to 16 μg/ml; Sigma-Aldrich, Ireland), as previously described (32). Two clones, 104-revert and 1A-revertC2, were derived from separate lineages of 104-cip after 20 and 70 passages, respectively, on antibiotic-free TSA (Table 1). The reference strain *S.* Enteritidis PT4 NCTC 13349 was also included in this study.

Stability of the resistance phenotype and molecular subtyping of isolates. Ciprofloxacin-resistant mutants were serially passaged on TSA in the absence of ciprofloxacin for 200 generations. Cultures were tested for their quinolone resistance every 3 days. Individual isolates were subtyped by pulsed-field gel electrophoresis (PFGE). PFGE was performed following XbaI digestion of genomic DNA according to the standard 1-day PulseNet protocol of the Centers for Disease Control and Prevention (CDC) (http://pulsenetinternational.org/protocols/protocols.asp) (6).

Bacterial growth curves. Bacterial growth was monitored by measuring optical density at 600 nm (OD_{600}) in tryptone soya broth (TSB; Oxoid, New Hampshire, United Kingdom). Briefly, all bacteria were initially cultured on TSA at 37°C for 18 h, and an isolated colony was then inoculated into 10 ml TSB. A 1-ml aliquot of the overnight culture was inoculated into a conical flask containing 100 ml TSB and incubated in a shaking incubator aerobically at 37°C. Absorbance readings were taken every hour for 8 h and then after 24 h with a spectrophotometer (Biomate 5; Thermospectronic, Cambridge, United Kingdom).

Electron microscopy. Bacterial cells were grown to stationary phase in Luria-Bertani (LB) broth (Difco, NJ), harvested by centrifugation at $1,968 \times g$ for 10 min, and resuspended in phosphate-buffered saline (PBS; Sigma-Aldrich, Ireland) and 2.5% glutaraldehyde (fixative) at a concentration of approximately 10^8 cells per ml. Five microliters of the cell suspension was pipetted onto Formvar carbon-stabilized copper grids and incubated for 2 min. Negative staining was performed with 5 μl 2% phosphotungstic acid for 1 min. The grids were examined using a JEOL JEM-2000 FX microscope under standard operating conditions.

Antimicrobial susceptibility testing. MICs for nalidixic acid, ciprofloxacin, ampicillin, chloramphenicol, tetracycline, and sulfamethoxazole-trimethoprim were determined by an Etest with Mueller-Hinton agar (Difco) by following the manufacturer's instructions (AB-Biodisk, Solna, Sweden). The MICs were evaluated by using the breakpoints of the CLSI (11). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms. All antibiotics were supplied by Oxoid.

Phenotype microarray. The ability of the bacterial strains to respond to environmental stresses (pH and osmotic) were examined by the use of OmniLog Biolog phenotype microarrays (Biolog, Inc., Hayward, CA). Briefly, bacteria were grown on blood agar overnight at 37°C. Colonies were picked with a sterile cotton swab and suspended in 10 ml IF-0a (Biolog), and the cell density was adjusted to an OD_{600} of 0.035 with a spectrophotometer (Biomate 5; Thermospectronic, Cambridge, United Kingdom). A 750-μl aliquot of this cell suspension was added to 150 ml IF-10 (Biolog). Microtiter plates (PM-9 and PM-10) were inoculated with 100 μl of cell suspension per well and then incubated at 37°C for 48 h in an OmniLog incubator and monitored continuously for color changes in the wells. Kinetic data were analyzed with OmniLog PM software.

Swim motility assay. One microliter of an overnight culture was spotted in the middle of a swim plate (LB broth and 0.1% gellan gum [Sigma]) and allowed to dry for 1 h at room temperature. Plates were incubated at 30°C overnight. The region of visible colony spread on the agar was measured with a ruler (mm) (14, 37).

Swarm motility assay. Bacterial cells were grown overnight in LB broth, harvested by centrifugation, washed once, and resuspended at a 50-fold concentration. Two microliters of this cell suspension was spotted onto a motility plate (LB broth and 0.2% gellan gum) and incubated at 30°C overnight. The region of visible colony spread on the agar was measured as described above (14, 37).

Adherence and invasion assays. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (vol/vol) fetal bovine serum (FBS), 1% penicillin-streptomycin solution, 1% (vol/vol) minimal nonessential amino acids in Triton X-100, and 1% fungizone (250 μg/ml amphotericin B) in a humidified atmosphere of 5% CO_2 at 37°C. (All reagents were supplied by Biosciences, Dublin, Ireland.) Caco-2 cells (5×10^5 cells/0.5 ml) were seeded into 24-well plates and cultured for approximately 15 days. Cell medium was changed three times a week. An overnight culture of bacteria was diluted 1 in 100 in LB broth and allowed to grow to mid-log phase. The bacterial cells were

collected by centrifugation (1,968 × *g* for 10 min) and resuspended in DMEM at a concentration of approximately 10^8 bacteria per ml. The Caco-2 cells in the 24-well plates were washed twice in PBS, serum-free medium was then added, and cells were allowed to equilibrate for 2 h in 5% CO_2 at 37°C. The serum-free medium was removed, and the Caco-2 cells were incubated with the bacterial inoculum for 1 h in 5% CO_2 at 37°C. For the adhesion assay, after infection, the Caco-2 cells were washed four times with PBS before disruption with a 1-ml volume of PBS containing 1% Triton X-100 (Sigma-Aldrich) at room temperature for 5 min. The number of bacteria per well was determined by plating out appropriate dilutions of this final suspension onto TSA. The adhesion level is reported as the number of adhered bacteria minus the number of invaded bacteria. For the invasion assay, after the bacteria were allowed to adhere to the monolayers, wells were washed twice with serum-free medium, replaced with serum-free medium containing gentamicin sulfate (50 μg/ml; Sigma-Aldrich), and incubated for 30 min to kill all external bacteria. The medium was then removed, and the cells were washed twice with PBS to remove any residual gentamicin sulfate before lysis with 1% Triton X-100. The number of bacteria invaded was estimated by plating serial dilutions. Adhesion and invasion assays were performed on three separate occasions, with four wells per assay used for each isolate.

PCR amplification and sequencing of the QRDRs of quinolone target genes and the local and global regulators of *acrAB-tolC*. Genomic DNA was extracted from overnight cultures in TSB (Oxoid) at 37°C by using a Wizard genomic DNA purification kit (Promega, Madison, WI). The quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* and the local (*acrR*) and global regulators of *acrAB-tolC* (*ramA*, *ramR*, *marORAB*, and *soxRS*) were amplified and sequenced as previously described (32).

Expression analysis of efflux transporter gene *acrB* and global regulators *marA*, *soxS*, *ramA*, and *rob*. RNA extraction and real-time quantification of RNA templates by real-time one-step RT-PCR were carried out as previously described (32). Relative gene expression was calculated using the $\Delta\Delta C_T$ method (25).

LPS and porin analyses. LPS and porin analyses were carried out as previously described (32). Briefly, LPS was prepared from whole bacteria by proteolytic digestion of cellular proteins with proteinase K and separated by SDS-PAGE, and the bands were visualized by silver staining. For porin analysis, total cell proteins were separated by SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with polyclonal antibodies (1:2,000 dilution) directed against denatured OmpF porin or with F4 polyclonal antibody directed against the L3 internal loop of *E. coli* porins. These antibodies directed against denatured OmpF and the L3 internal porin loop recognize the denatured enterobacterial porins, including *Salmonella* F and D porins (40). The detection of antigen-antibody complexes was performed with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit IgG antibodies.

Gene expression microarray. RNA was extracted from six separate cultures (biological replicates) of each parent and mutant strain. A separate microarray analysis was performed with each biological replicate.

Microarray design and manufacture. The nonredundant multiserotype microarray contained 5,660 PCR products that currently cover 95% of all genes in the genomes of *Salmonella* serovar Typhimurium strain LT2, *S.* Typhimurium strain SL1344, *S.* Typhi strain CT18, *S.* Typhi strain Ty2, *S.* Paratyphi A strain SARB42, and *S.* Enteritidis strain PT4. The array represents each gene in a separate spot, deposited in 50% dimethyl sulfoxide (DMSO) onto the amino silane-modified surfaces of bar-coded Corning Ultra-GAPS glass slides (catalogue no. 40015; Corning). Each glass slide contained triplicate identical arrays.

RNA labeling and hybridization. cDNA probes were labeled with Cy3 and Cy5 dye-linked dUTP by direct incorporation during reverse transcription from total RNA to cDNA, as described in the protocol available at http://cmgm.stanford.edu/pbrown/protocols/4_Ecoli_RNA.txt, with minor modifications. Fifty micrograms of total RNA and 2.4 μg of random hexamers were resuspended in 30 ml of water, and subsequently, the amounts and volumes of all components were doubled compared to those used in the Brown protocol. Furthermore, 2 μl of RNasin (F. Hoffmann, La Roche, Ltd., Basel, Switzerland) was added to the reverse transcription mixture, and the reaction mixture was incubated at 42°C for 2 h. After the first hour of incubation, a further 2 μl of Superscript II reverse transcriptase was added. Probes were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and eluted in 1 mM Tris-HCl, pH 8.0. Subsequently, all probes were dried down and resuspended in 10 μl sterile water.

Hybridization and data acquisition. Probes were hybridized to the *Salmonella* array overnight in 25% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% (vol/vol) SDS at 42°C by using a hybridization chamber (Corning, New York, NY) submerged in a water bath. Protocols suggested by the manufacturer for hybridizations in formamide buffer were applied for prehybridization, hybridization, and posthybridization processing. Microarrays were scanned with a ScanArray Lite Laser scanner (PerkinElmer Life and Analytical Sciences, Waltham, MA), using ScanArray Express 3.0 software, or with a GenePix 4100A scanner (Molecular Devices, Sunnyvale, CA) and GenePix Pro software.

Data analysis. Signal intensities were quantified using the QuantArray 3.0 software package (Packard BioChip Technologies, Billerica, MA). Spots were analyzed by adaptive quantitation and subsequently statistically analyzed using WebArray (46). The following parameters were used: background subtraction was performed using the "half" method, print-tip Loess normalization was employed within arrays, and scale normalization was used between arrays.

Real-time RT-PCR versus microarray analysis. Gene expression analyses of five differentially transcribed regulators of *S.* Enteritidis were assessed by real-time reverse transcriptase PCR (RT-PCR) (as described above) and compared to gene expression microarray results.

Statistical analysis. Data are presented as means ± standard deviations (SD). Statistical comparisons were made using the two-tailed Student *t* test. A *P* value less than 0.05 was considered significant.

Microarray data accession number. The *Salmonella* data from this study have been deposited in NCBI's Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE15024.

RESULTS

Stability of the high-level ciprofloxacin resistance phenotype and subtyping of isolates. The separate lineages of 104-cip reverted to intermediate levels of ciprofloxacin resistance (MICs of 4 μg/ml [104-revert] and 2 μg/ml [1A-revert C2]) after 20 and 70 passages on antibiotic-free agar, respectively, and maintained this phenotype throughout the rest of this study (Table 1). In contrast, 5408-cip maintained its high-level ciprofloxacin resistance phenotype (MIC, ≥32 μg/ml) for 200 passages in the absence of antibiotic. All isolates showed identical PFGE restriction profiles (data not shown).

Growth and morphology of ciprofloxacin-resistant mutants. Mutant strains 104-cip and 5408-cip demonstrated lower growth rates and formed smaller colonies than their respective parent strains (Fig. 1). The growth profiles of both parent strains were identical to that of the reference strain (data not shown). The growth profiles of 104-revert and 1A-revertC2 were the same as that of their parent strain, 104. The two reverted mutants formed colonies similar in size to those formed by their isogenic parents. Electron microscopy analysis (Fig. 2) revealed that 104-cip rods were significantly longer (1.16 ± 0.3 μm versus 1.02 ± 0.27 μm; $P < 0.0005$) and wider (0.45 ± 0.07 μm versus 0.43 ± 0.07 μm; $P < 0.005$) than those of the parent strain. Rods of the mutant 5408-cip were significantly shorter (1.00 ± 0.28 μm versus 1.10 ± 0.31 μm; $P < 0.0005$) and wider (0.51 ± 0.08 μm versus 0.46 ± 0.08 μm; $P < 0.0005$) than those of its parent strain. After 200 passages on agar in the absence of antibiotic, 5408-cip continued to form small colonies and had a reduced growth rate in broth (data not shown).

Susceptibility of mutants to environmental stresses and antibiotics. Both 104-cip and 5408-cip displayed increased pH and osmotic susceptibilities compared to their isogenic parents in the phenotype microarrays. The mutant 104-cip had increased susceptibility to 5 to 6.5% sodium chloride; 3% sodium formate; 5 to 11% sodium lactate; 5% urea; 100 mM sodium nitrite; and pHs 4.5, 9.5, and 10.0. The 5408-cip mutant showed increased susceptibility to 3 to 6.5% sodium chloride; 5 to 6% potassium chloride; 3 to 6% sodium formate; 2 to 6% urea; 4 to 10% sodium lactate; 20 to 50 mM sodium benzoate, pH 5.2; 10 to 100 mM sodium nitrite; and pHs 4.5, 9.5, and 10.0. Both

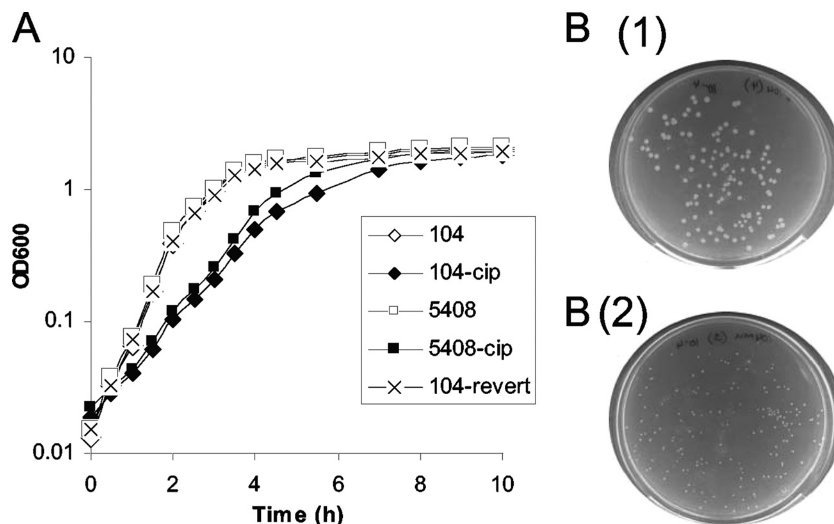

FIG. 1. Growth profiles of *S.* Enteritidis wild-type strains (104 and 5408), their isogenic ciprofloxacin-resistant mutants (104-cip and 5408-cip), and the reverted mutant (104-revert) (A) and colony morphology of parents [B (1)] and isogenic mutants [B (2)] (B). Both 104-cip and 5408-cip formed small colonies on agar. 104-revert formed colonies similar to those of the parent strains. For purposes of clarity, 1A-revertC2 and the *S.* Enteritidis reference strain (S100) are not included in the growth curve below. Both showed growth profiles similar to those of 104, 5408, and 104-revert.

104-cip and 5408-cip displayed a lack of acid and alkali habituation through production of decarboxylases and deaminases and a lack of ability to accumulate osmolytes. Both reverted mutants showed pH and osmotic susceptibilities similar to those of the parent strain. We previously reported that 104-cip showed decreased susceptibility to tetracycline, ampicillin, and chloramphenicol and that 5408-cip showed decreased susceptibility to sulfamethoxazole-trimethoprim, ampicillin, and chloramphenicol (32). Compared to 104-cip, 104-revert and 1A-revertC2 displayed increased susceptibility to tetracycline, ampicillin, and chloramphenicol (Table 2).

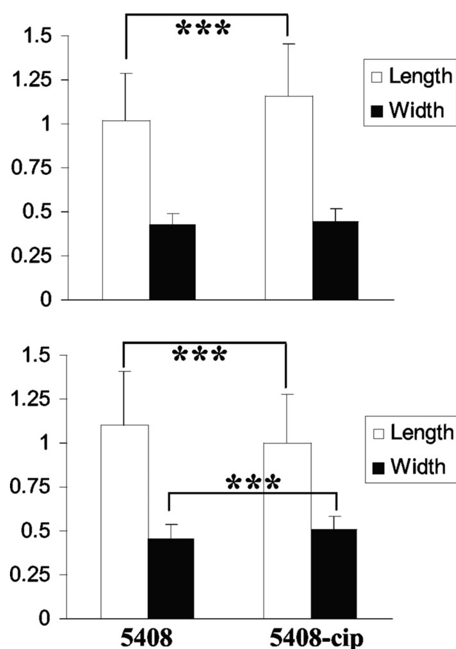

FIG. 2. Comparison of the widths and lengths of wild-type *S.* Enteritidis strains (104 and 5408) and their isogenic ciprofloxacin-resistant mutants (104-cip and 5408-cip) as determined by electron microscopy. Results are expressed as means ± SD for 400 measurements for individual *Salmonella* rods from 6 different fields. Statistical comparisons were made with unpaired Student *t* tests. **, $P < 0.005$; ***, $P < 0.0005$.

Mutant motility, adherence, and invasion. The mutated 104-cip, 104-revert, and 5408-cip strains showed 66, 90, and 93% reductions in swim motility and 36, 36, and 46% reductions in swarm motility, respectively, compared to their parent strains. The parent strains showed swim and swarm motilities identical to those of the reference strain. The mutant 104-cip showed significantly lower levels of adherence to Caco-2 monolayers than its isogenic parent (104) (6.47 ± 0.08 versus 6.84 ± 0.22 log CFU adhered; $P < 0.0005$). The 104-revert strain showed adherence levels similar to those observed for 104. Strains 5408 and 5408-cip adhered in similar numbers (Fig. 3). Mutants 104-cip and 5408-cip were significantly ($P < 0.0005$) less invasive in Caco-2 cells than their parent strains. The log numbers of CFU invaded in 104-cip and 5408-cip were 4.84 ± 0.05 and

TABLE 2. Antimicrobial susceptibility phenotypes of *S.* Enteritidis strains

Strain	MIC (μg/ml)[a]			
	TC	TS	AM	CL
104	1	0.125	1	2
104-cip	16	0.25	16	128
104-revert	2	0.064	4	8
1A-revertC2	0.50	0.032	0.50	2
5408	1	0.064	1	4
5408-cip	2	0.25	4	16

[a] Values represent means of results from 3 separate determinations. AM, ampicillin; CL, chloramphenicol; TC, tetracycline; TS, trimethoprim-sulfamethoxazole.

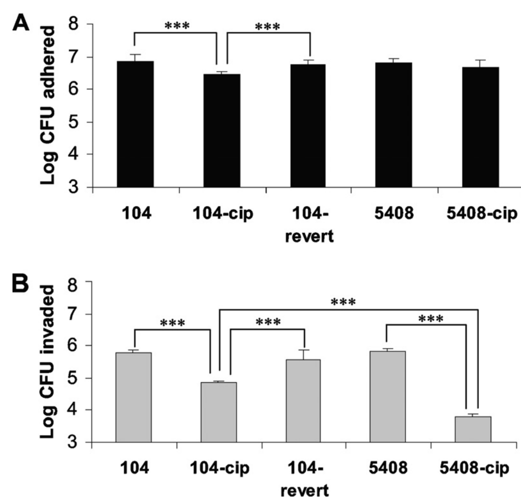

FIG. 3. Adherence (A) and invasion (B) of Caco-2 monolayers by *S.* Enteritidis wild-type strains (104 and 5408), their isogenic ciprofloxacin-resistant mutants (104-cip and 5408-cip), and the reverted mutant (104-revert). Results are expressed as mean ± SD log numbers of CFU invaded in 12 wells from three separate experiments. Statistical comparisons were made with unpaired Student *t* tests. ***, $P < 0.0005$.

3.78 ± 0.09, respectively, compared to the log numbers of CFU invaded by their respective parents (5.76 ± 0.1 and 5.8 ± 0.11, respectively). Strain 104-revert showed levels of invasion in Caco-2 cells similar (5.58 ± 0.3 log CFU) to those observed for its parent strain (Fig. 3). Both 104 and 5408 showed levels of adherence and invasion similar to those observed for the *S.* Enteritidis reference strain (data not shown).

Genotypic characteristics of the mutants. In our previous study, we genotypically characterized mutants 104-cip and 5408-cip (32). The mutant 104-cip harbored two GyrA (D87Y and S83F) mutations, and 5408-cip harbored single GyrA (D87Y), GyrB (E466D), and ParE (V461G) mutations. Both mutants overexpressed *acrB*. The global regulator genes *soxS* and *marA* were overexpressed in 104-cip, and *ramA* was overexpressed in 5408-cip. Mutations were found in SoxR (R20H) and in SoxS (E52K) in 104-cip and in RamR (G25A) in 5408-cip (Tables 1 and 3). In this study, both 104-revert and 1A-revertC2 showed decreased expression of *acrB* and *soxS* compared to 104-cip but retained increased expression of *marA*, albeit at a lower level of expression than that in 104-cip. Expression of *ramA* and *rob* in both reverted mutants was similar to that in 104-cip (Table 3). Both reverted isolates maintained the previously identified double GyrA mutations (S83F and D87Y), the single SoxS mutation (E52K), and the single SoxR mutation (R20H). An additional mutation was found in SoxR in 104-revert (E16G) and in 1A-revertC2 (R113C). A mutation was observed in ParC (D79N) in both reverted isolates. 1A-revertC2 acquired two mutations in MarA (I111N and H115Q) (Table 1).

LPS and porin profiles. We previously examined the LPS and porin profiles of 104-cip and 5408-cip (32). The LPS profile of 104-cip showed significant loss of short and intermediate O-chain LPSs compared to the levels for its parent strain (data not shown). The 104-cip mutant showed decreased production of OmpF (Fig. 4). No changes were observed in the LPS or OmpF profile of 5408-cip. Both 104-revert and 1A-revertC2 gained short and intermediate O-chain LPSs (data not shown), and 104-revert showed increased OmpF production relative to 104-cip (Fig. 4).

Gene expression microarray. Microarray data revealed that 64 genes were differentially regulated in 104-cip and 97 genes in 5408-cip compared to what was observed for their respective parent strains. Two detailed tables representing the differentially regulated genes in both mutants can be found in the supplemental material. Activated and repressed genes were classified into clusters of orthologous groups (COGs) as defined at http://www.ncbi.nlm.nih.gov/COG/grace/fiew.cgi (Table 4). Several genes involved in energy production and conservation (31 genes), carbohydrate transport and metabolism (24 genes), and amino acid transport and metabolism (19 genes) were differentially regulated in both mutants (Table 4). Decreased expression of outer membrane porins was observed in *ompF* and *ompW* in 5408-cip and in *ompC* in 104-cip, along with an increased expression level of *ompX* in 5408-cip. A total of 26 genes involved in cell motility (including *fimA*, *fliCHGTZ*, *motAB*, *cheAWMRBYZ*, and *flgLKFEDCN*) were downregulated in 5408-cip. In contrast, only 3 genes (*flgED* and *orgA*) were decreased in 104-cip. Both mutants showed downregulation of *Salmonella* pathogenicity island 1 (SPI-1) genes involved in invasion (*invJIGF*, *hilCD*, and *prgKIH*) (included in the no-COG category). Among the genes that were upregulated in 104-cip, a number were SoxS regulated, including *acnA*, *fpr*, *ribA*, *sodA*, *lpxC*, and *soxS* itself. Increased expression of the efflux genes *acrB* and *acrA* was detected in 104-cip and 5408-cip. Comparisons of real-time RT-PCR and microarray results (Table 5) showed that there were similar trends occurring between the two methods for *acrB* and *rob* in the two mutants and for *soxS* in 104-cip.

DISCUSSION

S. Enteritidis is the predominant serotype associated with egg-borne salmonellosis in humans. This serotype is invasive in poultry and therefore has the potential to contaminate eggs by trans-ovarian transmission following colonization of the intestinal tract (43). In this study, the fitness costs associated with high-level ciprofloxacin resistance in *S.* Enteritidis isolates, including reduced growth rates, altered morphology, decreased motility, and invasiveness in Caco-2 cells and increased susceptibility to environmental stresses, such as pH and osmotic stimuli, all could negatively affect intestinal colonization, thereby limiting the spread of resistant clones.

TABLE 3. Gene expression analyses of *S.* Enteritidis isolates by real-time RT-PCR

Strain	Fold change in gene expression[a]				
	acrB	*soxS*	*marA*	*ramA*	*rob*
104-cip	6.1 ± 1.5	26.1 ± 4.0	8.9 ± 0.6	1.2 ± 0.2	−4.6 ± 0.2
104-revert	−1.9 ± 0.4	−5.0 ± 0.9	3.0 ± 0.4	−1.45 ± 0.1	−1.75 ± 0.5
1A-revertC2	1.5 ± 0.3	1.7 ± 0.1	6.6 ± 1.1	1.6 ± 0.2	−2.2 ± 0.2
5408-cip	5.4 ± 1.6	−3.4 ± 0.5	1.3 ± 0.2	33.7 ± 4.0	−2.3 ± 1.0

[a] RNA was extracted from cultures growing at log phase in LB broth. Gene expression data represent means ± SD of results from 3 independent total RNA extractions. Changes in gene expression are relative to the levels for the respective parental strains.

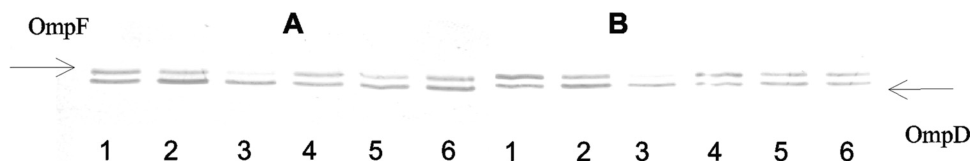

FIG. 4. The detection of porins was carried out using the polyclonal antibodies directed against denatured OmpF porin (A) or the F4 polyclonal antibody directed against the L3 internal loop of *E. coli* porins (B). Lanes 1, 104; lanes 2, 104-revert; lanes 3, 104-cip; lanes 4, 5408; lanes 5, 5408-cip; lanes 6, *S.* Enteritidis NCTC 13349. Arrows indicate the migration of F and D porins, respectively.

Microarray data revealed consistent downregulation both in mutants of invasion genes found within SPI-1, which encodes a type III secretion system (TTSS), and in flagellar biosynthesis genes, which correlated with the observed phenotypes of decreased motility and epithelial cell invasiveness. Invasion, preceded by adherence, is a highly coordinated activity, involving fimbriae, flagella, chemotaxis, LPS, and the TTSS (4, 21, 24). The greater reductions in motility and invasiveness of 5408-cip than in those of 104-cip correlated with the significantly decreased expression of a larger number of genes involved in flagellar biosynthesis and chemotaxis in this isolate. The possibility that the altered LPS profile in 104-cip may also have influenced its adherence and invasiveness cannot be excluded. Microarray data from this study showed a shift in metabolic activity to support the energy requirements associated with the overexpression of AcrAB in both ciprofloxacin-resistant mutants. In particular, the expression levels of a number of enzymes of the Krebs cycle were upregulated, consistent with the channeling of reducing power to the terminal electron transport system.

TABLE 4. Classification of regulated genes in ciprofloxacin-resistant mutants according to COGs

Category[a]	No. of genes regulated			
	104-cip activated	104-cip repressed	5408-cip activated	5408-cip repressed
Energy production and conversion	11	8	5	7
Carbohydrate transport and metabolism	3	7	0	14
Amino acid transport and metabolism	4	3	2	10
Coenzyme transport and metabolism	2	0	0	0
Inorganic ion transport and metabolism	1	0	1	0
Translation, ribosomal structure, and biogenesis	0	0	3	0
Transcription	1	0	2	3
Replication, recombination, and repair	1	0	0	0
Defense mechanisms	2	0	2	0
Signal transduction mechanisms	0	1	0	1
Cell wall/membrane/envelope biogenesis	1	1	0	3
Cell motility	0	3	0	26
Posttranslational modification, protein turnover, and chaperones	3	1	0	1
General function prediction only	1	0	1	4
Function unknown	2	0	3	1
No COG[b]	0	8	1	7
Total	32	32	20	77

[a] Genes were classified according to COGs as defined at http://www.ncbi.nlm.nih.gov/COG/grace/fiew.cgi.
[b] Repressed genes in 104-cip and 5408-cip were all SPI-1 genes. The activated gene in 5408-cip is the outer membrane protease gene *ompX*.

Mechanisms widely associated with high-level fluoroquinolone resistance, such as multiple topoisomerase mutations and overexpression of MDR efflux pumps, have been associated with fitness costs (1, 3, 23, 39). However, the mechanisms by which these resistance traits cause fitness burdens, such as reduced growth rates and virulence, have not been elucidated. Decreased growth in high-level fluoroquinolone resistance isolates harboring multiple topoisomerase mutations has been associated with decreased DNA supercoiling (3, 23). Decreased supercoiling indicates the presence of a less efficient DNA gyrase (15) which could in turn slow replication and thereby affect growth. Growth defects could also result from the inopportune efflux of nutrients and metabolic intermediates by overexpressed MDR efflux pumps (30). However, evidence to support this hypothesis is currently lacking. Expression of genes associated with bacterial virulence, such as invasion and flagellar genes, is influenced by changes in DNA supercoiling (13) and is activated by quorum sensing (10, 41). As efflux pumps have been shown to expel quorum-sensing signals (7, 33), it is reasonable to speculate that altered quorum-sensing signal homeostasis, resulting from increased AcrAB activity and/or altered DNA supercoiling, may have contributed to the decreased virulence of isolates in this study. Currently, we are investigating the effects of the topoisomerase mutations on supercoiling.

No restoration of growth rates or decrease in the level of resistance was observed in 5408-cip after 200 passages in the absence of antibiotic. In contrast, 104-cip reverted to an intermediate-level ciprofloxacin resistance phenotype (104-revert) after 20 passages, with reversal of all fitness costs except motility. The ability of 104-cip to revert to a lower-level resistance phenotype was confirmed by the recovery of another reverted clone (1A-revertC2) 70 generations after a separate lineage of 104-cip was put into serial transfer. Expression of *acrB* was decreased in both 104-revert and 1A-revertC2, providing convincing evidence that overexpression of this pump makes a significant contribution to the fitness burden of fluoroquinolone resistance. The decreased expression of *acrB* in both reverted isolates was associated with decreased expression of the global regulator *soxS*. It would be interesting to speculate that the additional SoxR mutations acquired by the reverted mutants (E16G in 104-revert and R113C in 1A-revertC2) were responsible for the observed decrease in expression of *soxS* and

TABLE 5. Gene expression analyses of select transcriptional regulators of *S.* Enteritidis[a]

Strain	Change in gene expression (*P*)[b]									
	acrB		*soxS*		*marA*		*ramA*		*rob*	
	RT-PCR	Array	RT-PCR	Array	RT-PCR	Array	RT-PCR	Array	RT-PCR	Array
104-cip	6.1 ± 1.5	2.22 (2.4E−11)	26.1 ± 4.0	1.19 (3.5E−05)	8.9 ± 0.6	*0.15 (0.07)*	1.2 ± 0.2	*0.16 (0.5)*	−4.6 ± 0.2	−0.8 (3.2E−06)
5408-cip	5.4 ± 1.6	1.53 (1.8E−06)	−3.4 ± 0.5	*−0.15 (0.25)*	1.3 ± 0.2	*0.17 (0.03)*	33.7 ± 4.0	0.9 (1.1E−03)	−2.3 ± 1.0	−0.34 (2.2E−03)

[a] RNA was extracted from cultures growing at log phase in LB. RT-PCR data represent means ± SD of results from 3 independent total RNA extractions. Changes in gene expression are relative to the levels for the respective parental strains.

[b] Array data were gathered from six biological replicates. Analysis was done using WebArray (46), employing the print-tip Loess and scale methods for within- and between-array normalizations, respectively. The cutoff values for genes downregulated and upregulated in the ciprofloxacin-resistant mutants ($\log_2$ ciprofloxacin-resistant-mutant/wild-type ratio values) were <1 ($P < 0.01$) and >1.0 ($P < 0.01$), respectively. Log_2 ciprofloxacin-resistant-mutant/wild-type ratio values with *P* values greater than 0.01 are marked in italics to indicate lower confidence levels.

the associated decrease in *acrB* expression, even in the genetic background of elevated *marA* expression. A mutation at position E16 in the helix-turn-helix region of the SoxR protein in *E. coli* has previously been reported, and it is thought that alterations in this region of the protein could disrupt DNA binding (8).

Reversal to an intermediate-level ciprofloxacin resistance phenotype was also associated with restoration of normal OmpF expression and LPS profiles. Although we have shown that these membrane alterations do not make a significant contribution to the resistance phenotype in 104-cip (32), the possibility that they may have contributed to the fitness burden of ciprofloxacin resistance in this isolate cannot be excluded. OmpF is controlled by the *mar* and *sox* regulons (36, 38). The normal expression of OmpF in 104-revert, despite elevated expression of *marA*, may be a consequence of the decreased expression of *soxS* and the greater role played by this regulator in antibiotic resistance in 104-cip (32). The significance of the acquired *marA* mutations in 1A-revertC2 is currently unclear.

Interestingly, both reverted mutants acquired the same mutation in *parC* (D79N). Kugelberg et al. reported that after serial passage in laboratory medium, the fitness of slow-growing fluoroquinolone-resistant *P. aeruginosa* isolates with a *gyrA* mutation and decreased DNA supercoiling was increased by a compensatory mutation(s) that restored supercoiling to normal levels. However, no mutations were found in any of the genes expected to affect supercoiling (23). The possibility that this *parC* mutation may contribute to fitness restoration by compensating for the potential negative effects of resistance-associated topoisomerase mutations on global supercoiling remains to be explored.

In conclusion, this study demonstrates that high-level ciprofloxacin resistance in *S.* Enteritidis *in vitro*-derived mutants is associated with fitness costs. Evolution in the absence of antibiotic selective pressure may result in mutational events favoring a reversion to a lower-level resistance phenotype associated with lesser fitness costs, rather than the acquisition of compensatory mutations that would maintain resistance while ameliorating the fitness burden.

The fitness costs of high-level ciprofloxacin resistance, in the absence of evidence of compensatory evolution, may account for the lack of emergence and spread of highly resistant *S.* Enteritidis clones along the farm-to-fork continuum to date. The lack of fitness costs observed in isolates showing high-level nalidixic acid resistance and decreased susceptibility to ciprofloxacin may be a contributory factor in the widespread dissemination of this phenotype. Infection with these first-step mutants could lead to generation of second-step mutants rapidly after fluoroquinolone therapy, which could ascend to dominance during the course of treatment and potentially result in treatment failure.

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