Increased expression of Qnr is sufficient to confer clinical resistance to ciprofloxacin in *Escherichia coli*

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**Background:** Ciprofloxacin, a fluoroquinolone, targets two essential bacterial enzymes, DNA gyrase and topoisomerase IV. Plasmid-borne *qnr* genes, encoding proteins that protect DNA gyrase and topoisomerase IV from inhibition by fluoroquinolones, contribute to resistance development. However, the presence of a plasmid-borne *qnr* gene alone is insufficient to confer clinical resistance.

**Objectives:** We asked whether the level of expression of *qnr* was a limiting factor in its ability to confer clinical resistance and whether expression could be increased without reducing fitness or viability.

**Methods:** *qnrB* and *qnrS* were recombineered onto the chromosome of *Escherichia coli* under the control of constitutive promoters of various strengths. Expression was measured by qPCR, MIC and relative fitness as a function of expression level were determined.

**Results:** For both *qnr* genes there was a positive relationship between the level of *qnr* mRNA and the MIC of ciprofloxacin. The highest MICs achieved with *qnrB* or *qnrS* as the sole resistance determinant were 0.375 and 1 mg/L, respectively, and were reached at expression levels that did not affect growth rate or viability. The *qnrS*-mediated MIC is above the EUCAST clinical breakpoint for resistance to ciprofloxacin. In the absence of Lon protease activity, overexpression of *qnr* genes was associated with high fitness cost, possibly explaining observations of toxicity in other genetic backgrounds.

**Conclusions:** The ability to generate a high MIC without incurring a fitness cost shows that, in an appropriate genetic context, *qnrS* has the potential to generate clinical resistance to ciprofloxacin in one step.

**Introduction**

Ciprofloxacin is a fluoroquinolone with a range of indications, including urinary tract infections frequently caused by *Escherichia coli*.1–3 Fluoroquinolones target DNA gyrase and topoisomerase IV, which cleave and re-ligate DNA and are required for altering supercoiling levels during replication, and for chromosome decatenation.4 When ciprofloxacin binds to either enzyme it traps it in a stable complex with cleaved DNA, leading eventually to cell death.5

Despite being a synthetic and highly potent drug with dual-targeting activity, resistance to ciprofloxacin has reached high frequencies among clinical isolates worldwide.1 In the decade after its introduction all resistance reported in Enterobacteriaceae was associated with chromosomal mutations.8 Recently, plasmid-mediated quinolone resistance (PMQR) has become a frequent component of clinical resistance.6 The first reported PMQR gene reducing susceptibility to ciprofloxacin in Gram-negatives was *qnrA* in *Klebsiella pneumoniae*.6 Subsequently, several classes of *qnr* genes (*qnrB, qnrC, qnrD, qnrS* and *qnrV*) were identified that reduce susceptibility to fluoroquinolones.7,9 Qnr proteins bind to DNA gyrase and topoisomerase IV and protect the enzymes from inhibition by quinolones.7,10

EUCAST sets the clinical breakpoint defining resistance for ciprofloxacin in Enterobacteriaceae at >0.5 mg/L,12 whereas CLSI defines the breakpoint at >4 mg/L.13 No single genetic alteration increases the MIC for susceptible WT *E. coli* above either breakpoint.6,14 Ciprofloxacin-resistant isolates of *E. coli* carry multiple genetic alterations, usually mutations in genes encoding the drug targets, mutations that up-regulate drug efflux and/or a PMQR gene.6,7 In general, the presence of a *qnr* gene in WT *E. coli* leads to an MIC of ciprofloxacin of 0.125–0.25 mg/L.7,9,14,15 A recent study showed that the MIC associated with plasmid-borne *qnr* genes increased from 0.25 to 32 mg/L when bacteria were grown
QnrS is sufficient to cause ciprofloxacin resistance

in urine at pH 5.16. Under the same conditions, the MIC for the susceptible WT increased from 0.015 to 1 mg/L. This shows that MIC can be strongly influenced by environmental conditions though the significance of this very interesting observation for clinical outcomes remains to be determined.

qnr homologues exist on the chromosomes of many bacterial species but their spread into Gram-negative pathogens occurred by carriage on multidrug resistance plasmids. However, the chromosomal location of many qnr genes, and their association with mobile genetic elements in Gram-negatives, suggests there is in principle no barrier to their transfer onto chromosomes in Gram-negatives.

We addressed the potential for chromosomally located qnr in E. coli to confer a clinically relevant resistance level to ciprofloxacin and asked whether the level of resistance would be constrained by associated fitness costs. We report that qnrS can be expressed at levels that increase the MIC above the clinical breakpoint, without incurring significant loss in fitness.

Materials and methods

Media

LB was used as liquid medium for bacterial growth and for solid medium the LB was supplemented with 1.5% agar (LA). Chloramphenicol was used at 30 mg/L, where indicated and counter-selection of sacB was done on LA lacking NaCl, supplemented with 5% sucrose. BBL™ Mueller–Hinton II (Becton, Dickinson & Company, France) was used for MIC determinations.

Strains

A set of isogenic strains was constructed, carrying constitutive promoters of different strengths17 upstream of a cat–sacB cassette at the galK locus in MG1655. Coding sequences for qnrB and qnrS were amplified from clinical isolates of E. coli and transcriptionally fused by recombineering to each of the promoters replacing the cat–sacB cassette.18 A lexA box, located between the native qnrB promoter and the coding sequence,19 was incorporated in the qnr constructs. Strains in which lon was inactivated were constructed by P1-mediated transduction of the lon:kan allele from the Keio collection.20 PCR and DNA sequencing (Macrogen Europe Laboratory, Amsterdam, The Netherlands) were used to confirm the genetic constructions. Genotypes of bacterial strains and oligonucleotide sequences are given in Table S1 and Table S2 (available as Supplementary data at JAC Online).

qPCR

RNA extractions were made in three biological replicates. RNA was isolated at OD260 0.25–0.4 using an RNeasy Mini Kit (Qiagen) and quantified using a Nanodrop NO-1000 spectrophotometer. A DNase Turbo Free (Ambion, Life Technologies) kit was used to remove DNA. cDNA was made using a High Capacity Reverse Transcription kit (Applied Biosystems). qPCRs contained 1 μL of cDNA (diluted 1:10, 1:100, 1:1000), 12.5 μL of PerfeCTa SYBR Green FastMix (Quanta Biosciences), 1.25 μL of 10 μM forward and reverse primers (Table S2) and ddH2O up to a final volume of 20 μL. Eco Real-Time PCR System (ILLUMINA) was used for qPCR. Control housekeeping genes were idhT, cysG and hcaT.21

MIC

A bacterial colony was dispersed in 1 mL of 0.9% NaCl and spread evenly over a Mueller–Hinton II agar plate using a cotton swab; an Oxoid™ M.I.C.Evaluator™ Strip (Thermo Fisher Scientific, Basingstoke, UK) was applied and incubated for 18 h at 37°C.

Growth measurements

Overnight cultures (four biological replicates/strain) were diluted 1:1000 in LB. Growth was measured using Bioscreen C (Qy Growth Curves AB Ltd, Finland) with continuous shaking at 37°C and readings at OD600 every 5 min. The natural logarithm (ln) of each OD600 value was plotted against time. Slopes of linear regression lines for 10 subsequent OD600 readings were computed. Exponential growth rate was calculated by dividing ln(2) with the value of the maximum slope. To evaluate whether qnr overexpression affected bacterial viability, cfu were quantified after plating serial dilutions from stationary-phase cultures onto LA.

Results and discussion

qnr mRNA levels and MIC increase as a function of promoter strength

Isogenic strains were constructed with qnrB or qnrS transcriptionally fused to each of eight constitutively expressed promoters with different strengths (Table S1). The levels of qnrB and qnrS mRNA associated with the different promoters varied over a >100-fold range, with the strongest promoters producing 190-fold (qnrB) and 300-fold (qnrS) the level of the control mRNAs. Differences in mRNA levels for qnrB and qnrS as a function of the different promoters were quantified and related to the MIC of ciprofloxacin (Figure 1). The MIC of ciprofloxacin also increased as a function of the increase in mRNA, up to 0.375 mg/L for qnrB and up to 1 mg/L for qnrS. The maximal MICs remained constant with the four (qnrB) and three (qnrS) strongest promoters (Figure 1). The highest MICs achieved are significantly higher than those associated with the same qnr genes expressed on plasmids in the original clinical isolate, or equivalent genes on plasmids in other genetic backgrounds. For qnrS the MIC achieved with the three strongest promoters is above the level of the clinical resistance breakpoint defined by EUCAST.12

Increased MIC without an incurred fitness cost

In order to assess if the increased expression of the qnr genes incurred a biological fitness cost, exponential growth rates, and bacterial viability counts from the stationary phase, were measured (Table 1). This was done to test whether the metabolic cost of producing Qnr proteins, or an increased level of interactions between Qnr and DNA topoisomerases, would reduce growth rate or bacterial viability. For qnrB, there was no significant variation in either parameter, regardless of promoter strength, mRNA level or MIC value. For qnrS, the two strongest promoters indicated a growth rate reduction of ~5%, but this was not statistically significant (P > 0.05). However, the maximal MIC of 1 mg/L was achieved with the third strongest promoter without reducing the growth rate (Table 1). We conclude that overexpression of these genes, resulting in the case of qnrS in an MIC of up to 1 mg/L, can be achieved without any significant fitness cost in terms of growth rate or viability.

Growth rate in strains overexpressing Qnr is dependent on the activity of Lon protease

Previous in vitro biochemical studies have shown that although qnrB and qnrS protect DNA gyrase at low concentrations, they are inhibitory at higher concentrations.22,23 Although it could be argued that inhibition in vitro might not reflect the complexity of
expression regulation in whole cells, there is a recent publication showing that overexpression in E. coli of different qnr genes found in clinical isolates is toxic, based on observations of strongly reduced viability. 24 We asked what might explain this significant discrepancy with our study in which qnr overexpression was not observed to cause significant toxicity. Whereas we studied qnr expression in MG1655, the Machuca et al. 24 study used E. coli BL21, a strain that is commonly used for protein overexpression studies and lacks two important protease activities, Lon and OmpT. 25 Proteases such as Lon play an important role in E. coli in removing ‘proteins without partners’. 26 We hypothesized that in MG1655, protease activities act to turn over excess Qnr when maximum protection of DNA gyrase has been reached, thus protecting the cell from toxic side effects. Accordingly, the toxicity observed with Qnr overproduction in BL21 might result from the failure to turn over excess proteins. To test this hypothesis, we constructed isogenic strains in which qnrB or qnrS were expressed from the strongest J23-series promoter, J23100, and measured growth rate as a

Figure 1. Ciprofloxacin MIC increases as a function of the level of qnr mRNA. MIC of ciprofloxacin as a function of qnrB (a) and qnrS (b) mRNA levels, relative to the levels of three control housekeeping genes measured by qPCR. Standard deviations in mRNA level are indicated. The sloped line indicates the regression fit.

Table 1. Strain phenotype as a function of qnr expression level

| Strain | Relevant genotype a | mRNA (± SD)b | MIC c | Growth rate (± SD)d | cfu × 10⁸ (± SD)e |
|--------|---------------------|--------------|-------|---------------------|-------------------|
| CH1464 | MG1655              | —            | 0.015 | 1.00 (0.03)         | —                 |
| CH7243 | J23113-qnrB         | 1.62 (0.65)  | 0.03  | 1.01 (0.01)         | 4.61 (0.78)       |
| CH7244 | J23117-qnrB         | 1.96 (0.73)  | 0.12  | 0.98 (0.04)         | 4.22 (0.54)       |
| CH7245 | J23115-qnrB         | 8.31 (1.64)  | 0.25  | 1.04 (0.04)         | 4.12 (0.51)       |
| CH7246 | J23105-qnrB         | 13.59 (3.49) | 0.25  | 1.01 (0.05)         | 3.64 (0.26)       |
| CH7247 | J23110-qnrB         | 39.48 (8.78) | 0.375 | 0.96 (0.07)         | 3.73 (0.61)       |
| CH7248 | J23118-qnrB         | 32.68 (8.89) | 0.375 | 0.99 (0.03)         | 3.91 (0.59)       |
| CH7470 | J23101-qnrB         | 84.54 (21.62) | 0.375 | 1.02 (0.05)         | 4.55 (0.56)       |
| CH7471 | J23100-qnrB         | 193.12 (33.34) | 0.375 | 1.04 (0.03)         | 3.78 (0.98)       |
| CH7249 | J23113-qnrS         | 0.02 (0.01)  | 0.015 | 1.02 (0.06)         | 3.55 (0.32)       |
| CH7250 | J23117-qnrS         | 1.00 (0.23)  | 0.045 | 1.02 (0.02)         | 4.03 (0.63)       |
| CH7251 | J23115-qnrS         | 6.73 (2.42)  | 0.3   | 0.99 (0.05)         | 3.81 (0.37)       |
| CH7252 | J23105-qnrS         | 8.15 (3.63)  | 0.5   | 0.99 (0.07)         | 3.29 (0.36)       |
| CH7253 | J23118-qnrS         | 35.36 (14.2) | 0.75  | 1.06 (0.05)         | 3.82 (0.69)       |
| CH7254 | J23110-qnrS         | 42.61 (11.45) | 1     | 1.02 (0.02)         | 3.46 (0.35)       |
| CH7472 | J23101-qnrS         | 328.30 (62.19) | 1     | 0.94 (0.05)         | 3.37 (0.4)        |
| CH7473 | J23100-qnrS         | 234.37 (57.31) | 1     | 0.96 (0.04)         | 3.33 (0.77)       |

SD, standard deviation.
aStrains are isogenic to MG1655. qnrB and qnrS were placed downstream of J23-series promoters in the galK locus.
bmRNA levels of qnr relative to control genes hcaT, idnT and cysG, with standard deviations.
cMIC of ciprofloxacin in mg/L.
dExponential growth rates relative to CH1464 (MG1655), with standard deviations.
ecfu from serial dilution of overnight cultures, with standard deviations.
Figure 2. lon activity is required to protect strains overexpressing qnr from toxic effects on growth rate. Growth rates of strains overexpressing qnr as a function of lon activity. Control, no qnr gene; QnrB, overexpressed from J23100; QnrS, overexpressed from J23100. Strain genotypes in Table S1.

function of lon activity (Figure 2). The growth rate of the control strain, MG1655, was unaffected by lon activity. However, in each of the strains expressing a qnr gene the growth rate decreased significantly (by 40–50%) in the strains in which lon was inactivated (Figure 2). This shows that Lon plays an important role in preventing potential toxicity associated with Qnr overexpression and explains, at least in part, the discrepancy between our data and the BL21 study.

Conclusions

In a recent study, decreased susceptibility to ciprofloxacin was caused by amplification of plasmid-encoded qnrA during in vitro selection.27 The observation that increased levels of qnr result in an increased MIC is in agreement with our results. However, tandem gene amplifications are inherently unstable and susceptibility returned to normal in the absence of selection, as expected. A point of interest is that qnr genes in natural isolates are known to be located on many different plasmid backbones,7 raising the possibility that there might be significant natural variation in drug susceptibility associated with differences in plasmid copy number or mechanisms controlling gene expression. Our finding that overexpression of qnr can cause significant toxicity in the absence of Lon protease activity shows that the genetic context in which a qnr gene is overexpressed will play an important role in determining the phenotype. Our study suggests that qnr genes, and qnrS in particular, when placed in a genetic context in which they are expressed at high levels, have the potential to generate genetically stable clinical resistance in one step, without incurring significant fitness costs.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at JAC Online.

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