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Effect of the efflux pump QepA2 combined with chromosomally mediated mechanisms on quinolone resistance and bacterial fitness in *Escherichia coli*

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Objectives: The aim of the study was to determine the interplay between the plasmid-mediated *qepA2* gene and multiple chromosomally mediated fluoroquinolone resistance determinants in the development of fluoroquinolone resistance in *Escherichia coli* and its influence on bacterial fitness.

Methods: *E. coli* ATCC 25922 and derived isogenic strains harbouring different chromosomally mediated fluoroquinolone resistance determinants were electroporated with pBK-CMV vector encoding QepA2. The MICs of fluoroquinolones were determined by standardized microdilution. The mutant prevention concentration (MPC) was evaluated. Bacterial fitness was analysed using Δ*lacZ* system competition assays.

Results: The ciprofloxacin MIC for strains harbouring the *qepA2* gene was 4- to 8-fold higher compared with strains without the *qepA2* gene. The *qepA2* gene also increased the MPC of ciprofloxacin 4- to 16-fold. Combination of the *qepA2* gene plus two to three additional mechanisms conferred a clinically relevant resistance level. The presence of the *qepA2* gene was associated with fitness costs in strains with mutations in the *gyrA* and/or *parC* genes, although the presence of an additional deletion of the *marR* gene compensated for this fitness cost by increasing bacterial fitness by 5%–23%.

Conclusions: The additive effect of chromosomally mediated fluoroquinolone resistance mechanisms and the *qepA2* gene led to clinical levels of fluoroquinolone resistance. Under competitive conditions, the *qepA2* gene had a biological cost in *E. coli* that was compensated for by the presence of an additional deletion in the *marR* gene.

Keywords: *E. coli*, *qepA*, resistance mechanisms

Introduction

Resistance to fluoroquinolones in Enterobacteriaceae mainly involves mutations in chromosomal genes encoding DNA gyrase and topoisomerase IV and decreased accumulation of fluoroquinolones due to changes in permeability.[1] Three different types of plasmid-mediated quinolone resistance (PMQR) or transferable mechanism of quinolone resistance have been described in Gram-negative microorganisms: Qnr proteins, an acetyltransferase [AAC(6′)-Ib-cr] and efflux pumps (QepA and OqxAB).[2]

The QepA determinant was described in *Escherichia coli* clinical isolates from Japan and Belgium in 2007. The *qepA1* gene encodes an efflux pump protein [belonging to the major facilitator superfamily (MFS)] and confers decreased susceptibility to hydrophilic fluoroquinolones.[3,4] In 2008, a new variant of this determinant, QepA2, was reported in France.[5]

The aim of this study was to investigate the effect of the *qepA2* gene and chromosomally mediated quinolone resistance mechanisms on quinolone susceptibility and biological cost. To evaluate this interplay, we constructed a collection of 21 isogenic *E. coli* strains carrying combinations of four chromosomally mediated quinolone resistance mechanisms that produced and did not produce QepA2 proteins.

Materials and methods

Bacterial strains and cloning assays

A set of 10 isogenic strains derived from *E. coli* ATCC 25922 (wild-type, WT) and harbouring Ser83Leu mutation and/or Asp87Asn mutation in GyrA and/or Ser80Arg mutation in ParC and/or a *marR* gene deletion[6] were used as background strains for combination with the *qepA2* gene (Table 1).

E. coli BicA was used for *qepA2* gene amplification.[5] The *qepA2* gene, including the promoter sequences, was amplified using primers PreQepA2 (5′-ggaagatcttccGGTCGATGTTTGATGTTATGGAG-3′) and PostQepA2 (5′-ggaagatcttccAGAGCCGCCTGAGGCCATGCGAC-3′) and cloned into the pBK-CMV vector (underlined nucleotides correspond to the BglII site). This construct was electroporated into the 10 isogenic strains (Table 1).

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by microdilution according to CLSI guidelines.[7] The quinolones used were nalidixic acid, ciprofloxacin, levofloxacin, moxifloxacin, norfloxacin and ofloxacin (Sigma–Aldrich, Madrid, Spain). Mutant prevention concentrations (MPCs) of ciprofloxacin and levofloxacin were determined as previously described.[8]

Growth competition assays

E. coli ATCC 25922 Δ*lacZ* was used as the standard strain against mutants constructed in the growth competition assays.[6] This strain was also tested against its parent, showing that the Δ*lacZ* mutation was neutral. Isogenic strains with *qepA2* were tested against *E. coli* ATCC 25922 Δ*lacZ* pBK-CMV. Each competition was tested in at least six independent experiments (Table 1). Growth competition assays were performed as described previously.[6] Relative fitness was calculated as the ratio of the $\log_{10}$ cfu density of any strain harbouring some quinolone resistance mechanism to that of the control strain.[6,9] Statistical significance of difference in fitness relative to the WT was measured using Student's *t*-test (two-tailed $P<0.05$).

Results and discussion

The interplay between chromosomally mediated quinolone resistance and *qnr* genes in susceptibility to fluoroquinolones and bacterial fitness has recently been described.[6] However, the effect of the combination of other PMQR genes, such as *qepA*, with chromosomally mediated mechanisms is unknown. In the present study, a set of 21 isogenic *E. coli* strains was used to evaluate the relationship between chromosomally encoded fluoroquinolone resistance mutations and *qepA2*, drug susceptibility and bacterial fitness.

For microdilution results, the margin of error of MIC values was ±1 half-doubling step, so that any change of at least two dilutions was considered significant. The presence of the *qepA2* gene increased ciprofloxacin and norfloxacin (hydrophilic fluoroquinolones) MICs for all *E. coli* genotypes (Table 1). In any combination, the ciprofloxacin MIC for strains harbouring the *qepA2* gene increased 4- to 8-fold compared with strains without the *qepA2* gene. The additional presence of *qepA2* increased the norfloxacin MIC 4- to 16-fold. The MICs of the more hydrophobic quinolones evaluated were not significantly modified (Table S1, available as Supplementary data at *JAC* Online). Together, a combination of the *qepA2* gene plus two to three chromosomal quinolone resistance mechanisms was enough to modify the clinical category for hydrophilic fluoroquinolones, supporting the idea that the PMQR determinants have a relevant role in producing clinical resistance to quinolones.[6]

EUCAST breakpoints are more restrictive than those defined by CLSI, including those for fluoroquinolones in Enterobacteriaceae.[7,10] We observed frequent discrepancies between CLSI and EUCAST in strains containing the *qepA2* gene combined with *gyrA* and/or *parC* modifications. These discrepancies were observed in 20% (10% susceptible/intermediate plus 10% intermediate/resistant) and 50% (40% susceptible/resistant plus 10% intermediate/resistant) of the full collection for ciprofloxacin and norfloxacin, respectively.

Taking EUCAST criteria into account, the epidemiological cut-off for ciprofloxacin in *E. coli* is established as 0.032 mg/L (www.eucast.org), which is considered to correspond to strains without known quinolone resistance mechanisms.[10] Herein, we showed evidence of the existence of strains carrying the *qepA2* gene (EC60) or a deletion of the *marR* gene (EC01)[6] whose ciprofloxacin MICs were lower than the epidemiological cut-off (Table 1), making it necessary to revise this epidemiological cut-off.

MPC levels were evaluated in three isogenic pairs harbouring one or two resistance mechanisms with or without *qepA2* (EC60, EC62, EC64 compared with WT, EC02, EC04) (Table 1).[6] Ciprofloxacin MIC values ranged between 0.016 and 1 mg/L. Ciprofloxacin MPC values for strains harbouring the *qepA2* gene were 4- to 16-fold (4–128 mg/L) higher than for strains without *qepA2* and, in most cases, greatly exceeded breakpoint concentrations for resistance according to CLSI and EUCAST criteria and the C_{max} reached for this quinolone (Table S2).[7,10,11]

In growth competition assays, the *qepA2* gene alone had no effect on growth fitness. In general, this PMQR gene in combination with mutations in *gyrA* or *parC* had a moderate fitness cost (Figure S1). Unexpectedly, the deletion in the *marR* gene compensated for the fitness cost associated with QepA2. Strains carrying *qepA2* and a deletion in the *marR* gene compensated for the fitness values, which ranged between 1.05 and 1.23 (Table 1 and Figure S1). The mechanism that caused this phenomenon is unknown; although, in accordance with previous data, certain chromosomal mutations could modify the fitness cost associated with PMQR.[12] Analysis of the promoter regions of *qepA2* using the BPROM informatics tool (www.softberry.com) found a *cpxR* binding site, but we did not find any *marR* boxes that might explain the interaction between marR and QepA2.

Comparison of EC64 versus EC65 and EC68 versus EC69 showed that the presence of a deletion of the *marR* gene increased the MIC of ciprofloxacin 2- to 4-fold. In both cases, a 23% increase in bacterial fitness was also observed (Table 1). At least *in vitro*, adding a single resistance mechanism may significantly modify bacterial fitness (~20%) and simultaneously increase MICs. Accordingly, particular combinations of resistance mechanisms (e.g. QepA2 and Δ*marR*) could be conserved in the absence of antimicrobial pressure, because they improve the relative fitness of the mutant strain and reduce drug susceptibility according to recent literature.[6,9]

Altogether, these results contrast with the central dogma of evolution, which states that resistance mechanisms confer a fitness cost and constitute an adaptive disadvantage in the absence of antimicrobials. Thus, the development of increasing rates of fluoroquinolone resistance could occur even in the absence of these drugs. Accordingly, strategies designed to reduce antimicrobial resistance rates should take the bacterial

Table 1. Genotypes, ciprofloxacin susceptibility and fitness of isogenic strains

Strain[a]	*gyrA*1	*gyrA*2	*parC*	*marR*	*qepA2*	MIC of ciprofloxacin (mg/L)	Fitness (standard deviation)[b]	*P*[c]	*n*[d]	Source or reference
ATCC 25922	—	—	—	—	—	0.004	1.00 (0.08)	—	18	
ATCC 25922 pBK-CMV	—	—	—	—	—	0.004	1.00 (0.07)	—	10	
EC01	—	—	—	Δ	—	0.015	1.08 (0.06)	0.01	10	6
EC02	S83L	—	—	—	—	0.06	1.03 (0.02)	0.14	6	13
EC03	S83L	—	—	Δ	—	0.125	0.99 (0.11)	0.90	6	6
EC04	S83L	—	S80R	—	—	0.25	1.20 (0.05)	<0.0001	10	13
EC05	S83L	—	S80R	Δ	—	0.5	1.07 (0.03)	0.005	10	6
EC06	S83L	D87N	—	—	—	0.06	1.14 (0.11)	0.006	10	6
EC07	S83L	D87N	—	Δ	—	0.125	1.04 (0.04)	0.15	10	6
EC08	S83L	D87N	S80R	—	—	1	1.04 (0.05)	0.20	6	6
EC09	S83L	D87N	S80R	Δ	—	2	1.02 (0.04)	0.53	6	6
EC60	—	—	—	—	pBK-qepA2	0.015	1.02 (0.13)	0.71	10	this study
EC61	—	—	—	Δ	pBK-qepA2	0.06	1.14 (0.06)	<0.001	10	this study
EC62	S83L	—	—	—	pBK-qepA2	0.25	0.89 (0.13)	0.06	10	this study
EC63	S83L	—	—	Δ	pBK-qepA2	0.5	1.05 (0.12)	0.28	10	this study
EC64	S83L	—	S80R	—	pBK-qepA2	1	1.00 (0.01)	0.97	6	this study
EC65	S83L	—	S80R	Δ	pBK-qepA2	2	1.23 (0.11)	<0.001	10	this study
EC66	S83L	D87N	—	—	pBK-qepA2	0.25	0.92 (0.06)	0.02	10	this study
EC67	S83L	D87N	—	Δ	pBK-qepA2	0.5	1.11 (0.03)	<0.001	10	this study
EC68	S83L	D87N	S80R	—	pBK-qepA2	4	0.93 (0.05)	0.01	10	this study
EC69	S83L	D87N	S80R	Δ	pBK-qepA2	16	1.16 (0.02)	<0.001	10	this study

[a]Genotype. Strains are isogenic to *E. coli* ATCC 25922 and carry only the chromosomal modifications and/or the *qepA2* gene shown. Resistance-associated mutations located in the GyrA and ParC proteins have been defined as resistance mechanisms that alter the target site.
[b]Mean fitness per generation relative to WT. Standard deviation (in parentheses) measured in pairwise competition against the isogenic WT (Δ*lacZ*).
[c]Statistical significance of difference in fitness relative to WT (Student's *t*-test, two-tailed *P* value).
[d]Number of independent competition experiments on which the fitness value was based.

fitness of resistant strains into account and, in specific situations, reduced use of antimicrobials may not be enough.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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