Expression of the qepA1 gene is induced under antibiotic exposure

Gerrit Brandis, Jonas Gockel, Linnéa Garoff, Lionel Guy and Diarmaid Hughes

1Department of Medical Biochemistry and Microbiology, Biomedical Center, Uppsala University, Box 582, Uppsala, Sweden

*Corresponding author. E-mail: diarmaid.hughes@imbim.uu.se

Background: The qepA1 gene encodes an efflux pump that reduces susceptibility to ciprofloxacin. Little is known about the regulation of qepA1 expression.

Objectives: To assess the potential role of ciprofloxacin and other antibiotics in the regulation of qepA1 gene expression. To identify the promoter that drives qepA1 expression and other factors involved in expression regulation. To assess whether the identified features are universal among qepA alleles.

Methods: A translational qepA1-yfp fusion under the control of the qepA1 upstream region was cloned into the Escherichia coli chromosome. Expression of the fusion protein was measured in the presence of various antibiotics. Deletions within the upstream region were introduced to identify regions involved in gene expression and regulation. The qepA1 coding sequence and upstream region were compared with all available qepA sequences.

Results: Cellular stress caused by the presence of various antibiotics can induce qepA1 expression. The qepA1 gene is fused to a class I integron and gene expression is driven by the Pc promoter within the integrase gene. A segment within the integron belonging to a truncated dfrB4 gene is essential for the regulation of qepA1 expression. This genetic context is universal among all sequenced qepA alleles.

Conclusions: The fusion of the qepA1 gene to a class I integron has created a novel regulatory unit that enables qepA1 expression to be under the control of antibiotic exposure. This setup mitigates potential negative effects of QepA1 production on bacterial fitness by restricting high-level expression to environmental conditions in which QepA1 is beneficial.

Introduction

Fluoroquinolones, including ciprofloxacin, are a synthetic class of antimicrobial drugs with very good activity against Gram-negative and Gram-positive bacteria. Ciprofloxacin binds to DNA gyrase and topoisomerase IV and inhibits re-ligation of cleaved DNA. The accumulation of DNA breaks is thought to lead to cell death.

Escherichia coli, a major cause of urinary tract infections and invasive septicaemia, is commonly treated using ciprofloxacin. However, many clinical isolates of E. coli have evolved resistance. Evolution of resistance to ciprofloxacin in E. coli is a complex process that almost always includes multiple mutations in genes encoding DNA gyrase (gyrA, gyrB) and topoisomerase IV (parC, parE). Additionally, mutations in genes encoding regulatory proteins of efflux pumps (marR, acrR and soxR) can lead to increased drug efflux and mutations in transcription and translation-related genes (e.g. RNA polymerase and tRNA synthetase genes) can reduce susceptibility to ciprofloxacin by inducing global changes in bacterial protein synthesis.

Horizontally acquired genes also contribute to the development of ciprofloxacin resistance. The qnr genes (qnrA, qnrB, qnrC, qnrD, qnrS and qnrVC) encode proteins that bind to the drug targets and protect the enzymes from ciprofloxacin. A variant of an aminoglycoside acetyltransferase [encoded by aac(6’)-Ib-cr] acetylates ciprofloxacin, thus reducing its activity, and two efflux pumps (encoded by qepA and qepABC) reduce cellular ciprofloxacin concentrations. Clinically observed plasmid-mediated ciprofloxacin resistance genes generally supplement the level of resistance caused by chromosomal mutations in drug target genes.

An exception is an E. coli isolate that acquired resistance by a combination of horizontally acquired genes (qnrS and qepABC) and chromosomal mutations that increase drug efflux. Additionally, it has been shown that overexpression of qnrS in E. coli can result in ciprofloxacin resistance levels above the clinical breakpoint.

Since their discovery, 11 distinct qepA alleles have been identified (qepA1–qepA11). These alleles differ in one to five amino acids from qepA1. The genetic context of the alleles qepA1, qepA2, qepA3, qepA4 and qepA7 shows that the qepA
genes are linked to a truncated class I integron. It is unclear whether the qepA genes are part of the truncated integron and whether the integron plays a role in expression of qepA. Here, we identify two novel qepA alleles, qepA12 and qepA13, and show that 10 of the 13 qepA alleles share an identical upstream region consisting of a truncated integrase gene (intI1) and an ORF containing a partial dfrB4 gene (upstream sequence is unavailable for qepA6, qepA10 and qepA11). Using a translational fusion of the qepA1 gene to a yfp gene (encoding yellow fluorescence protein (YFP)) we show that expression of qepA1 is driven by the PcWTGN--10 promoter within the class I integron. Furthermore, expression of qepA1 was induced by cellular stress caused by several antibiotics, including ciprofloxacin, and we were able to identify a region of around 250 nt that is essential for qepA1 expression.

**Materials and methods**

**Bacterial strains and growth conditions**

All strains were derived from E. coli K12 MG1655 (Table S1, available as Supplementary data at JAC Online). Bacteria were grown at 37°C in LB broth (10 g/L yeast extract, 5 g/L tryptone, 10 g/L NaCl) or on LA plates (LB solidified with 1.5% agar, Oxoid). Strains harbouring temperature-sensitive plasmids were grown at 30°C. Antibiotics were from Sigma–Aldrich (Stockholm, Sweden) and added to induction assays as described. When added to growth medium, concentrations were: chloramphenicol, 25 mg/L; ciprofloxacin, 0.1 mg/L; kanamycin, 50 mg/L; and tetracycline, 15 mg/L. Sucrose counterselection was performed on salt-free LA supplemented with 5% sucrose.

**Strain constructions**

A 1.1 kb segment containing the first 600 nt of the qepA1 gene and 546 nt of the upstream region were amplified from a clinical E. coli isolate (CH459) and inserted into the gatK gene of WT E. coli MG1655 (CH1464) using lambda-red recombineering and sucrose counterselection. The translational fusion of yfp to the qepA1 coding sequence was constructed by inserting a yfp gene that is transcriptionally fused to a kanamycin resistance gene segment. Deletions were constructed by inserting a cat-sacB selectable/counterselectable cassette upstream of qepA1 followed by lambda-red recombineering using ssDNA oligonucleotides to delete various segments. All oligonucleotides are listed in Table S2. The gyrA583L and lexA582S alleles were moved into strains using P1 virA-mediated transduction.

**PCR and local sequencing**

DNA fragments used for recombineering were amplified using Phusion High Fidelity 2× PCR Master Mix (New England Biolabs, Ipswich, USA) and all other DNA amplifications were performed using 2× PCR Mastermix (Thermo Scientific, Waltham, USA) according to the protocols of the manufacturers. Amplification products were purified using SUREClean Plus (Bioline, Germany) and sequencing of purified PCR products was performed by Macrogen (Amsterdam, The Netherlands). Sequences were analysed using CLC Main Workbench 20.0.4 (CLCbio, QIAGEN, Denmark).

**Induction assay**

One millilitre cultures in LB and LB containing subMIC antibiotics were initiated with ~10^6 cfu from overnight cultures and incubated for ~18 h at 37°C with 200 rpm shaking. Growth cultures were diluted 200-fold in PBS and qepA expression measured with a MACSQuant V8 (Miltenyi Biotec) at the average YFP fluorescence level of 30 000 cells. Each strain was grown in at least three biological replicates per antibiotic concentration. Mean fluorescence levels and 95% CIs for each strain were determined using a t-test comparing fluorescence of biological replicates that carry the qepA1-yfp fusion with the fluorescence level of biological replicates lacking the qepA1-yfp fusion and grown in identical conditions.

**Identification and comparison of qepA alleles**

qepA alleles were identified with a BLAST search of the qepA1 nucleotide sequence against the NCBI nucleotide collection database (nr/nt). Coding sequences and upstream regions of all sequences with >90% nucleotide identity were compared using the alignment tool of CLC Main Workbench 20.0.4 (CLCbio, QIAGEN).

**qepA origin analysis**

The protein sequence of qepA (WP_012372821.1) was aligned to three protein databases at NCBI (nr, refseq_protein and refseq_select_prot; alignment performed on 19 November 2020) using blastp. After inspection of the taxonomic distribution of hits, the results of the alignment to refseq protein were selected. Records with less than 50% identity or less than 80% query coverage were discarded, resulting in a dataset of 418 proteins. These sequences and the 22 sequences in Figure 4 of Yamane et al. were aligned with MAFFT v7.471 using the L-INS-i algorithm. Upon inspection of the alignment with SedView v5.0.4, six partial sequences were discarded, resulting in an alignment comprising 434 sequences. The alignment was trimmed with trimAl v1.4rev2, discarding sites with >50% gaps. The trimmed alignment was used to infer a maximum-likelihood tree with IQ-TREE v2.1.2, drawing 1000 ultrafast bootstrap with the UFBoot2 algorithm with the bnni option to reduce the risk of overestimating branch supports. The integrated ModelFinder determined that the best fitting model was LG + F + R10, thus using the LG substitution matrix, empirical state frequency as observed from the data, and determining rate heterogeneity with the FreeRate model with 10 categories. The resulting tree was visualized with FigTree v1.4.4.

**Identification of functional sequence element**

Potential transcription factor binding sites (TFBSs) were identified using the FIMO tool from the MEME suite v5.3.0, using default settings, including a threshold of 10^-5. We used the latest version available for download of PRODORIC (release 8.9) as a TFBS database. The presence of rho-independent terminators was tested using the web tool ARNold, screening both strands. We also used two tools of the RNAstructure package to predict the presence of other secondary structures in the 247 nt segment: FOLD and MaxExpect. All algorithms were run with default settings, activating the DNA option.

**Statistical analysis**

Statistical analysis was performed using R, v3.5.0.

**Results and discussion**

**Expression of qepA1 is induced under cellular stress conditions**

We previously identified a qepA1 gene within an E. coli isolate from a urinary tract infection. To study expression levels of qepA1 in the absence of other resistance factors present within the clinical isolate, we cloned a 1.1 kb segment containing the first 600 nt of the qepA1 gene and 546 nt of the upstream region into the chromosome of WT E. coli MG1655. Subsequently, a yfp gene (encoding YFP) was translationaly fused to the qepA1 coding sequence. This setup enables the use of YFP fluorescence
measurements to indirectly measure qepA1 expression levels (Figure 1a). We initially tested whether qepA1 expression is induced by ciprofloxacin since qepA1 is a quinolone resistance determinant. For this, cultures were grown in the presence of various subMIC concentrations of ciprofloxacin and fluorescence levels were measured. Fluorescence levels increased as a function of ciprofloxacin concentration indicating that qepA1 expression is induced in the presence of ciprofloxacin with a 2.4-fold increase of expression in the presence of 0.008 mg/L ciprofloxacin, corresponding to 0.5 \% MIC of the WT (Figure 1b). We next tested whether qepA1 induction is the direct effect of ciprofloxacin (e.g. by binding to the promoter) or an indirect effect (e.g. due to DNA damage). To test this, we moved a gyrA S83L D87N allele into the strain containing the translational qepA1-yfp fusion and measured expression in various ciprofloxacin concentrations. The gyrA allele reduces susceptibility to ciprofloxacin (MICWT: 0.016 mg/L; gyrA S83L D87N: 0.38 mg/L) without affecting the intracellular ciprofloxacin concentration. If expression of qepA1 is a direct effect of the presence of ciprofloxacin then the gyrA S83L D87N allele should not affect ciprofloxacin-dependent qepA1 expression. Any indirect effects of ciprofloxacin such as DNA damage should be reduced by the gyrA S83L D87N allele so that higher ciprofloxacin concentrations would be required for induction of qepA1 expression. The results of the induction assay showed no increase in qepA1 expression in the presence of 0.008 mg/L ciprofloxacin compared with the 2.4-fold increase in the strain without the gyrA S83L D87N allele. Further increases in ciprofloxacin concentration resulted in increased expression with a 2.5-fold increase in the presence of 0.24 mg/L ciprofloxacin (Figure 1b). This indicates that ciprofloxacin indirectly induces qepA1 expression.

To test whether expression of qepA1 is exclusively induced by quinolones we measured expression levels in the presence of subMIC concentrations of trimethoprim (9.6 mg/L), ampicillin (16 mg/L) and chloramphenicol (8 mg/L). Trimethoprim and ampicillin each caused 4-fold induction of qepA1 expression whereas chloramphenicol did not increase expression (Figure 1c–e). Taken together, the expression analysis indicates that cellular stress caused by the presence of various, but not all, antibiotics can induce qepA1 expression.

qepA1 is fused to an inactive class I integron

The qepA1 gene used in this study is preceded by an ORF that is a fusion of a truncated dfrB4 gene (45 nt) and a segment of unknown origin (144 nt). Both genes (orf and qepA1) are located downstream of the attachment site (attI) of a truncated integrase gene (intI). The integron contains a PcW promoter with a 'TGN' extended −10 motif (PcWTGN−10) that can drive expression
of the integrated genes and an inactive P2 promoter (P2*) \(^{60}\), (Figure 2). A BLAST analysis of the 600 nt that contain the integrase gene and truncated segment of the dfrB4 gene show that it is 99.5% identical to the corresponding segment of an integron found on the Aeromonas caviae pAeac2 plasmid (accession number CP039628). The integron found in A. caviae contains five resistance genes (dfrB4, catB3, aadA, qacEA1 and sul1), which are expressed by a Pch1 promoter. The Pch1 promoter in the A. caviae integron differs in two nucleotides from the PcWTGN\(^{10}\) promoter of the qepA1 integron. These two nucleotide differences are the only differences within the segments containing the integrase and truncated dfrB4 gene. This sequence comparison indicates that the qepA1 region investigated in this study can be divided into three distinct regions: (i) an inactive class I integrase containing the segment from the truncated intI1 gene to the attI1 site (Figure 2, green background); (ii) the 5' segment of a dfrB4 gene including a 56 nt upstream region that was originally integrated into the class I integron (Figure 2, purple background); and (iii) the qepA1 gene including a 146 nt upstream sequence, of which 144 nt form a fused ORF with the truncated dfrB4 gene (Figure 2, red background). This general structure agrees with previously described qepA alleles.\(^{24,27,32-35}\) That dfrB4 is truncated indicates that qepA1 was not inserted by integrase but is rather the result of an integrase-independent recombination event as previously proposed.\(^{32}\)

The qepA gene was probably acquired from Betaproteobacteria

Since the genetic context indicates an unusual fusion of qepA1 to an inactive integron, we decided to determine the evolutionary origin of QepA. The similarity between QepA and MFS-type efflux pumps has previously been described.\(^{24}\) To extend the evolutionary analysis of QepA, we searched the refseq_protein database of NCBI for sequences similar to QepA. We found 418 proteins at least 50% similar to QepA and covering at least 80% of its sequence. A phylogenetic analysis of these and 22 sequences from Yamane et al.\(^{26}\) reveals that all QepA variants cluster in a well-supported clade (Figures 3 and S1). The phylogenetic analyses also show that MFS homologues form clusters with genomes from the same taxon, suggesting these proteins spread mostly by vertical inheritance, or horizontal gene transfer between related species. A notable exception is QepA, exclusively found in Enterobacteriales, which branches inside a large clade constituted of Betaproteobacteria, mostly from Burkholderiales, but also from Nitrosomonadales. The clade most closely related to the QepA proteins contains proteins belonging to six species of Comamonadaceae (one Variorvorax and five Pseudarhodoferax) and to a Pseudomonas asiatica strain MY680.\(^{61}\) Pseudarhodoferax is a recently described genus of the Comamonadaceae.\(^{62}\) The genomes in which the MFS transporter is found were isolated from soil (Pseudarhodoferax soli),\(^{62}\) groundwater (Pseudarhodoferax aquiterra)\(^{63}\) and Arabidopsis thaliana leaves (other Pseudarhodoferax strains). These sequences represent all the available genomes of Pseudarhodoferax, suggesting that the last common ancestor of the genus already harboured the MFS transporter. The phylogenetic placement of the QepA clade as sister to the clade containing all Pseudarhodoferax, within a clade containing mostly Comamonadaceae, is consistent with a scenario where the MFS transporter was horizontally transferred from an ancestor of the Pseudarhodoferax genus to Enterobacteriales.

Expression of qepA1 is driven by the PcWTGN\(^{10}\) promoter

We next focused on the analysis of the region upstream of qepA1. A potential promoter outside the integron has previously been described for the qepA2 allele.\(^{32}\) We noticed that the potential \(-10\) promoter sequence of the qepA2 gene (TGTCGT) differed from the corresponding sequence of the qepA1 gene used in our
study, which contains an additional C nucleotide (TCGTCGT). We asked which of these two potential promoter sequences is more common among qepA variants. A BLAST of qepA1 nucleotide sequences revealed a total of 59 qepA sequences available in the NCBI nucleotide database (Table S3). Interestingly, we identified two qepA alleles not previously described, hereafter referred to as qepA12 (as qepA1 with E394G) and qepA13 (as qepA1 with D627–663) (Table S4). The most frequent qepA allele was qepA1 (39%, 23/59) and most other qepA alleles (9/12) were only represented by 1–3 sequences (Figure 4a). We also identified a synonymous mutation (G496G, GGG to GGA) within qepA9 that was also present in a single qepA4 allele (hereafter named qepA4*). Fourteen of the 59 qepA sequences contained only the qepA coding sequence with no or little (100 nt) of the upstream sequence (Table S3). The upstream regions of the other 45 qepA sequences were compared with the sequence of the qepA1 gene used in our study. We found that 67% (30/45) of the sequences were identical to the upstream region of the qepA1 gene used in our study. Almost all of the remaining sequences differed only within the PcWTGN#10 promoter sequences (10/15) or in the PcWTGN#10 and P2* promoter sequences (2/15) (Table S3). The nucleotide variants found within PcWTGN#10 change the sequence to the PcW or PcH1 promoter (Figure 4b, Table S3). The changes identified within the P2* promoter increase the spacer length, potentially activating it but at
shown for the ciprofloxacin resistance gene by the Pc and potentially the P2 promoters within the integrase.

These data indicate that expression of most of the qepA1 gene is induced by the SOS response.68,69 However, mutation of the qepA1 gene was induced by the three antibiotics that trigger the SOS response but not by chloramphenicol (Figure 1). This suggests that the integration of the qepA1 gene is not dependent on the SOS response.71 If LexA is part of the qepA1 regulation then a strain carrying the lexAInd allele should not exhibit qepA1 induction in the presence of ciprofloxacin. We measured expression of the qepA1-yfp fusion protein in the presence of 0.008 mg/L ciprofloxacin (0.5 x MICCIP) and found that the lexAInd allele did not influence induction of qepA1 relative to the WT lexA allele (P = 0.18, t-test). This indicates that ciprofloxacin-dependent induction of the qepA1 gene is not dependent on the SOS response (Figure 2).

We next asked whether the region between the PcWTGN-10 promoter and the qepA1 gene is involved in the regulation of gene expression. For this, we removed increasingly larger segments of the region downstream of the PcWTGN-10 promoter and measured ciprofloxacin-dependent induction (Figure 2). We found that removal of the segment belonging to the integrase (including P2* and IntI1 promoters, LexA binding site and attI1 site) had no effect on the induction of the qepA1 gene (Figure 2, green background). However, any deletion that included sequence from the truncated dfbB4 segment significantly inhibited qepA1 induction (Figure 2, purple background). We searched the region upstream of qepA1 (247 nt, from the end of the attI1 site, corresponding to position 300 on Figure 2) for the presence of functional elements such as TFBSs and possible hairpin structures, including transcription terminators. No TFBS or rho-independent transcription terminator could be identified in that region. The DNA secondary structure analysis revealed a complex structure comprising eight stems and six loops (Figures S2 and S3). However, the probabilities associated with the stems were, for the most part, lower than 80%. These data indicated that induction of the qepA1 gene is independent of LexA and at least partly dependent on the sequence that was not originally part of the genetic context of the qepA1 gene but we were not able to identify a functional element within this sequence (Figure 2, red background).

### The dfbB4 fragment is essential for qepA1 expression

Previous studies have shown that the bacterial SOS response is triggered by exposure to fluoroquinolones, β-lactams and trimethoprim but not by chloramphenicol.66–68 We found that the qepA1 gene was induced by the three antibiotics that trigger the SOS response but not by chloramphenicol (Figure 1). This suggests that qepA1 might be under the regulation of the SOS response as shown for the ciprofloxacin resistance gene qnrB2.67 The promoter of the intI1 gene contains a LexA binding site and expression of the integrase gene is induced by the SOS response.68,69 However, mutational alteration of the LexA binding site to prevent inhibition of the integrase promoter has shown that this specific LexA binding site has no effect on the transcription from any of the Pc promoter variants.70 To test whether LexA binding to an alternative, so-far-unknown, binding site is involved in regulation of qepA1 expression, we introduced a lexAInd allele into a strain that contains the qepA1-yfp fusion. The lexAInd allele carries a G80D mutation that prevents cleavage of the resulting LexA protein thus inhibiting induction by the SOS response.71 If LexA is part of the qepA1 regulation then a strain carrying the lexAInd allele should not exhibit qepA1 induction in the presence of ciprofloxacin. We measured expression of the qepA1-yfp fusion protein in the presence of 0.008 mg/L ciprofloxacin (0.5 x MICCIP) and found that the lexAInd allele did not influence induction of qepA1 relative to the WT lexA allele (P = 0.18, t-test). This indicates that ciprofloxacin-dependent induction of the qepA1 gene is not dependent on the SOS response (Figure 2).

We next asked whether the region between the PcWTGN-10 promoter and the qepA1 gene is involved in the regulation of gene expression. For this, we removed increasingly larger segments of the region downstream of the PcWTGN-10 promoter and measured ciprofloxacin-dependent induction (Figure 2). We found that removal of the segment belonging to the integrase (including P2* and IntI1 promoters, LexA binding site and attI1 site) had no effect on the induction of the qepA1 gene (Figure 2, green background). However, any deletion that included sequence from the truncated dfbB4 segment significantly inhibited qepA1 induction (Figure 2, purple background). We searched the region upstream of qepA1 (247 nt, from the end of the attI1 site, corresponding to position 300 on Figure 2) for the presence of functional elements such as TFBSs and possible hairpin structures, including transcription terminators. No TFBS or rho-independent transcription terminator could be identified in that region. The DNA secondary structure analysis revealed a complex structure comprising eight stems and six loops (Figures S2 and S3). However, the probabilities associated with the stems were, for the most part, lower than 80%. These data indicated that induction of the qepA1 gene is independent of LexA and at least partly dependent on the sequence that was not originally part of the genetic context of the qepA1 gene but we were not able to identify a functional element within this sequence (Figure 2, red background).
Regulation of qepA expression

Conclusions
Here, we analysed the regulation of qepA1 gene expression. We found that the qepA1 gene is fused to a class I integron and that gene expression is driven by the P2 promoter within the integrase gene. Cellular stress caused by the presence of several, but not all, antibiotics can induce qepA1 expression, and the segment within the integron belonging to a truncated dfrB4 gene is essential for expression regulation. This genetic context is universal among all qepA alleles with available sequence data. Expression of efflux pumps is often tightly regulated since overexpression can be associated with large fitness costs to bacteria. The acquisition of the qepA gene by horizontal gene transfer from Betaproteobacteria and fusion to an inactive class I integron has created a novel regulatory unit that enables qepA expression to be under the control of ciprofloxacin exposure. This setup mitigates potential negative effects of QepA production on bacterial fitness by restricting high-level expression to environmental conditions in which QepA is beneficial.

Funding
This work was supported by grants to Diarmaid Hughes from Vetenskapsrådet (the Swedish Research Council) (grant number 2017-03593) and from the Scandinavian Society for Antimicrobial Chemotherapy (grant numbers SLS-693211, SLS-876451). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Transparency declarations
None to declare.

Supplementary data
Figures S1 to S3 and Tables S1 to S4 are available as Supplementary data at JAC Online.

References
1. Hooper DC, Wolson JS. The fluoroquinolones: pharmacology, clinical uses, and toxicities in humans. Antimicrob Agents Chemother 1985; 28: 716–21.
2. Wolson JS, Hooper DC. The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity in vitro. Antimicrob Agents Chemother 1985; 28: 581–6.
3. Appelbaum PC, Hunter PA. The fluoroquinolone antibacterials: past, present and future perspectives. Int J Antimicrob Agents 2000; 16: 5–15.
4. Dricica K, Malik M, Kerns RJ et al. Quinolone-mediated bacterial death. Antimicrob Agents Chemother 2008; 52: 385–92.
5. Hooper DC, Wolson JS, Ng EY et al. Mechanisms of action of and resistance to ciprofloxacin. Am J Med 1987; 82: 12–20.
6. Khodursky AB, Zechiedrich EL, Cozzarelli NR. Topoisomerase IV is a target of quinolones in Escherichia coli. Proc Natl Acad Sci USA 1995; 92: 11801–5.
7. Kampranis SC, Maxwell A. The DNA gyrase-quinolone complex. ATP hydrolysis and the mechanism of DNA cleavage. J Biol Chem 1998; 273: 22615–26.
8. Dricica K, Hiasa H, Kerns R et al. Quinolones: action and resistance updated. Curr Top Med Chem 2009; 9: 981–98.
9. Croxen MA, Finlay BB. Molecular mechanisms of Escherichia coli pathogenicity. Nat Rev Microbiol 2010; 8: 26–38.
10. Brys A, Hay AD, Lane IF et al. Global prevalence of antibiotic resistance in paediatric urinary tract infections caused by Escherichia coli and association with routine use of antibiotics in primary care: systematic review and meta-analysis. BMJ 2016; 352: i399.
11. Fasugbo O, Gardner A, Mitchell BG et al. Ciprofloxacin resistance in community- and hospital-acquired Escherichia coli urinary tract infections: a systematic review and meta-analysis of observational studies. BMC Infect Dis 2015; 15: 545.
12. Huseby DL, Pietsch F, Brandis G et al. Mutation supply and relative fitness shape the genotypes of ciprofloxacin-resistant Escherichia coli. Mol Biol Evol 2017; 34: 1029–39.
13. Hooper DC, Jacoby GA. Topoisomerase inhibitors: fluoroquinolone mechanisms of action and resistance. Cold Spring Harb Perspect Med 2016; 6: a025320.
14. Pietsch F, Bergman JM, Brandis G et al. Ciprofloxacin selects for RNA polymerase mutations with pleiotropic antibiotic resistance effects. J Antimicrob Chemother 2017; 72: 75–84.
15. Brandis G, Granström S, Leber AT et al. Mutant RNA polymerase can reduce susceptibility to antibiotics via ppGpp-independent induction of a stringent-like response. J Antimicrob Chemother 2021; 76: 606–15.
16. Garoff L, Huseby DL, Praski Alzigirat L et al. Effect of aminoacyl-tRNA synthetase mutations on susceptibility to ciprofloxacin in Escherichia coli. J Antimicrob Chemother 2018; 73: 3285–92.
17. Martínez-Martínez L, Pascual A, Jacoby GA. Quinolone resistance from a transferable plasmid. Lancet 1998; 351: 797–9.
18. Rodríguez-Martínez JM, Machuca J, Cano ME et al. Plasmid-mediated quinolone resistance: two decades on. Drug Resist Updat 2016; 29: 13–29.
19. Hooper DC, Jacoby GA. Mechanisms of drug resistance: quinolone resistance. Ann NY Acad Sci 2015; 1354: 12–31.
20. Tran JH, Jacoby GA, Hooper DC. Interaction of the plasmid-encoded quinolone resistance protein QnrA with Escherichia coli topoisomerase IV. Antimicrob Agents Chemother 2005; 49: 3050–2.
21. Tran JH, Jacoby GA, Hooper DC. Interaction of the plasmid-encoded quinolone resistance protein Qnr with Escherichia coli DNA gyrase. Antimicrob Agents Chemother 2005; 49: 118–25.
22. Tran JH, Jacoby GA. Mechanism of plasmid-mediated quinolone resistance. Proc Natl Acad Sci USA 2002; 99: 5638–42.
23. Robicsek A, Stragliottiz J, Jacoby GA et al. Fluoroquinolone-modifying enzymes: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med 2006; 12: 83–8.
24. Yamane K, Wachina J, Suzuki S et al. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an Escherichia coli clinical isolate. Antimicrob Agents Chemother 2007; 51: 3354–60.
25. Hansen LH, Johannesen E, Burmolle M et al. Plasmid-encoded multidrug efflux pump conferring resistance to ciprofloxacin in Escherichia coli. Antimicrob Agents Chemother 2004; 48: 3332–7.
26. Sorensen AH, Hansen LH, Johannesen E et al. Conjugal plasmid conferring resistance to ciprofloxacin. Antimicrob Agents Chemother 2003; 47: 798–9.
27. Perichon B, Courvalin P, Galimand M. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in Escherichia coli. Antimicrob Agents Chemother 2007; 51: 2464–9.
28. Stragliottiz J, Jacoby GA, Hooper DC et al. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev 2009; 22: 664–89.
29. Sato T, Yokota SL, Uchida I et al. Fluoroquinolone resistance mechanisms in an Escherichia coli isolate, HUE1, without quinolone resistance-determining region mutations. Front Microbiol 2013; 4: 125.
Garloff L, Yadav K, Hughes D. Increased expression of Qnr is sufficient to confer clinical resistance to ciprofloxacin in *Escherichia coli*. *J Antimicrob Chemother* 2018; **73**:348–52.

Ruiz J. In silico analysis of transferable QepA variants and related chromosomal efflux pumps. *Rev Esp Quimioter* 2018; **31**:537–41.

Cattoir V, Poirel L, Nordmann P. Plasmid-mediated quinolone resistance pump QepA2 in an *Escherichia coli* isolate from France. *Antimicrob Agents Chemother* 2008; **52**:3801–4.

Wang D, Huang X, Chen J et al. Characterization of genetic structures of the QepA3 gene in clinical isolates of Enterobacteriaceae. *Front Microbiol* 2015; **6**:1147.

Maneigo V, Felix D, Jones-Dias D et al. Genetic background and expression of the new qepA4 gene variant recovered in clinical TEM-1- and CMY-2-producing *Escherichia coli*. *Front Microbiol* 2017; **8**:1899.

Pork YJ, Yu JK, Kim SI et al. Accumulation of plasmid-mediated fluoroquinolone resistance genes, qepA and qnrS1, in Enterobacter aerogenes co-producing RmtB and class A β-lactamase LAP-1. *Ann Clin Lab Sci* 2009; **39**:55–9.

Portridge SR, Tsafnat G, Coiera E et al. Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiol Rev* 2009; **33**:757–84.

Nazeri H, Cao S, Hasan F et al. Can phylogenetic type predict resistance development? *J Antimicrob Chemother* 2011; **66**:776–87.

Yu D, Ellis HM, Lee EC et al. An efficient recombination system for chromosomе engineering in *Escherichia coli*. *Proc Natl Acad Sci USA* 2000; **97**:5978–83.

Datta S, Costantino N, Court DL. A set of recombineering plasmids for gram-negative bacteria. *Gene* 2006; **379**:109–15.

Goy P, Le Coq D, Steinmetz M et al. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. *J Bacteriol* 1985; **164**:918–21.

Ellis HM, Yu D, DelIttio T et al. High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. *Proc Natl Acad Sci USA* 2001; **98**:6742–6.

Thomason LC, Costantino N, Court DL. E. coli genome manipulation by P1 transduction. *Curr Protoc Mol Biol* 2007; Chapter 1: Unit 1.17.

Altschul SF, Gish W, Miller W et al. Basic local alignment search tool. *J Mol Biol* 1990; **215**:403–10.

Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013; **30**:772–80.

Gouy M, Guindon S, Gascuel O. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 2010; **27**:221–4.

Capella-Gutierrez S, Silio-Martinez JM, Gabaldon T. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 2011; **27**:519–20.

Nguyen LT, Schmidt HA, von Haeseler A. FastTree2: approximately maximum-likelihood trees for large alignments. *Bioinformatics* 2014; **30**:196–203.

Hoang DT, Chenomor O, von Hoeseaer A et al. UFBoot2: improving the ultrafast bootstrap approximation. *Mol Biol Evol* 2018; **35**:518–22.

Kalyaanamoorthy S, Minh BQ, Wong THK et al. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods* 2017; **14**:587–9.

Rambaut A, FigTree. https://github.com/rambaut/figtree.

Grant CE, Bailey TL, Noble WS. FIMO: scanning for occurrences of a given motif. *Bioinformatics* 2011; **27**:1017–8.

Bailey TL, Boden M, Buske FA et al. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res* 2009; **37**:W202–8.

Munch R, Hiller K, Barg H et al. PRODORIC: prokaryotic database of gene regulation. *Nucleic Acids Res* 2003; **31**:266–9.

Naville N, Guilloit-Gaudreffroy A, Marchais A et al. ARNold: a web tool for the prediction of rho-independent transcription terminators. *RNA Biol* 2011; **8**:11–3.

Reuter JS, Mathews DH. RNAstructure: software for RNA secondary structure prediction and analysis. *BMC Bioinformatics* 2010; **11**:129.

Mathews DH, Disney MD, Childs JL et al. Incorporating chemical modification constraints into a dynamic programming algorithm for prediction of RNA secondary structure. *Proc Natl Acad Sci USA* 2004; **101**:7287–92.

Wu Y, Shi B, Ding X et al. Improved prediction of RNA secondary structure by integrating the free energy model with restraints derived from experimental probing data. *Nucleic Acids Res* 2015; **43**:7247–59.

Datta S, Costantino N, Court DL. A set of recombineering plasmids for gram-negative bacteria. *Gene* 2006; **379**:109–15.

Gay P, Le Coq D, Steinmetz M et al. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. *J Bacteriol* 1985; **164**:918–21.

Ellis HM, Yu D, DiTizio T et al. High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. *Proc Natl Acad Sci USA* 2001; **98**:6742–6.

Thomason LC, Costantino N, Court DL. E. coli genome manipulation by P1 transduction. *Curr Protoc Mol Biol* 2007; Chapter 1: Unit 1.17.

Altschul SF, Gish W, Miller W et al. Basic local alignment search tool. *J Mol Biol* 1990; **215**:403–10.

Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013; **30**:772–80.

Gouy M, Guindon S, Gascuel O. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 2010; **27**:221–4.

Capella-Gutierrez S, Silio-Martinez JM, Gabaldon T. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 2011; **27**:519–20.

Nguyen LT, Schmidt HA, von Hoeseaer A et al. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* 2015; **32**:268–74.

Hoang DT, Chenomor O, von Hoeseaer A et al. UFBoot2: improving the ultrafast bootstrap approximation. *Mol Biol Evol* 2018; **35**:518–22.

Kalyaanamoorthy S, Minh BQ, Wong THK et al. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods* 2017; **14**:587–9.

Rambaut A, FigTree. https://github.com/rambaut/figtree.

Grant CE, Bailey TL, Noble WS. FIMO: scanning for occurrences of a given motif. *Bioinformatics* 2011; **27**:1017–8.

Bailey TL, Boden M, Buske FA et al. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res* 2009; **37**:W202–8.