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Fitness cost constrains the spectrum of *marR* mutations in ciprofloxacin-resistant *Escherichia coli*

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Objectives: To determine whether the spectrum of mutations in *marR* in ciprofloxacin-resistant clinical isolates of *Escherichia coli* shows evidence of selection bias, either to reduce fitness costs, or to increase drug resistance. MarR is a repressor protein that regulates, via MarA, expression of the Mar regulon, including the multidrug efflux pump AcrAB-TolC.

Methods: Isogenic strains carrying 36 different *marR* alleles identified in resistant clinical isolates, or selected for resistance *in vitro*, were constructed. Drug susceptibility and relative fitness in growth competition assays were measured for all strains. The expression level of *marA*, and of various efflux pump components, as a function of specific mutations in *marR*, was measured by qPCR.

Results: The spectrum of genetic alterations in *marR* in clinical isolates is strongly biased against inactivating mutations. In general, the alleles found in clinical isolates conferred a lower level of resistance and imposed a lower growth fitness cost than mutations selected *in vitro*. The level of expression of MarA correlated well with the MIC of ciprofloxacin. This supports the functional connection between mutations in *marR* and reduced susceptibility to ciprofloxacin.

Conclusions: Mutations in *marR* selected in ciprofloxacin-resistant clinical isolates are strongly biased against inactivating mutations. Selection favours mutant alleles that have the lowest fitness costs, even though these cause only modest reductions in drug susceptibility. This suggests that selection for high relative fitness is more important than selection for increased resistance in determining which alleles of *marR* will be selected in resistant clinical isolates.

Introduction

Ciprofloxacin is a second-generation fluoroquinolone antibiotic widely used in everyday clinical practice. Due to its potent bactericidal activity against a broad range of clinically relevant Gram-negative and Gram-positive bacteria, it is one of the first-line treatments for many infections.[1,2] Ciprofloxacin, and other fluoroquinolones, target the two essential bacterial enzymes, DNA gyrase and topoisomerase IV, involved in regulating supercoiling of DNA during DNA replication and mRNA transcription. The drug binds to the enzymes as they are in complex with DNA, thereby preventing them from resealing the double-stranded DNA breaks created while handling supercoiling. This blocks the progression of the replication fork, leading to an accumulation of double-stranded DNA breaks, and eventually cell death.[3,4]

In *Escherichia coli*, individual resistance mutations usually confer only small increases in resistance to ciprofloxacin and in order to reach clinically relevant levels of resistance (the European breakpoint for clinical resistance is 0.5 mg/L)[5] the bacteria need to acquire several genetic alterations in a stepwise fashion.[6,7] Resistance to ciprofloxacin in clinical isolates of *E. coli* is strongly associated with combinations of mutations that alter the drug targets DNA gyrase (encoded by *gyrA* and *gyrB*) and topoisomerase IV (encoded by *parC* and *parE*) and mutations that upregulate drug efflux.[8–12]

Bacterial cells have membrane transport systems that regulate vital processes such as influx of essential nutrients and efflux of toxic compounds (including antibiotics) to ensure cellular homeostasis. Mutations that upregulate the expression of efflux pumps result in increased expulsion of toxic compounds and reduced susceptibility of the toxic compounds. Bacteria commonly carry genes encoding several different efflux systems, which have been categorized into five major families of transporters: (i) the ATP-binding cassette (ABC) family; (ii) the major facilitator (MFS) family; (iii) the

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multidrug and toxic compound extrusion (MATE) family; (iv) the small multidrug resistance (SMR) family; and (v) the resistance–nodulation–division (RND) family.[13,14] Some efflux pumps are specific for one substrate, while others can expel a wide variety of structurally very different compounds.

The major efflux system in *E. coli* is the AcrAB-TolC pump, belonging to the RND family of transporters.[15,16] This pump is composed of an inner membrane transporter protein (AcrB), an outer membrane protein (TolC) and a periplasmic adapter protein (AcrA) that couples the transporter and the exit channel to form a continuous channel out of the cell. AcrAB-TolC is a non-specific pump and can pump out a large number of compounds with little chemical similarity, including antibiotics (e.g. fluoroquinolones, tetracyclines, penicillins, chloramphenicol, rifampicin, novobiocin, fusidic acid), detergents (SDS), Triton X-100, oxidative agents, disinfectants and bile salts.[17] If the expression of the AcrAB-TolC pump is upregulated, the consequence is reduced susceptibility to multiple antimicrobial agents.

The expression of the AcrAB-TolC efflux pump is regulated at several levels.[13,18,19] The expression of AcrAB, encoded by the *acrAB* operon, is negatively regulated by the local transcriptional repressor protein AcrR and positively regulated by the global transcriptional regulator protein MarA. MarA is part of the *marRAB* operon, whose expression is regulated by the local transcriptional repressor protein MarR. Two dimers of MarR bind to two direct repeat sequences within the operator region of the *marRAB* operon, preventing binding of the RNA polymerase and thereby blocking transcription.[20] The MarA regulon has been identified by microarray studies to include around at least 60 genes spread out through the chromosome and involved in a variety of functions, and among these genes are, for example, *acrAB* and *tolC* of the efflux pump.[21,22]

Mutations that lead to relief of transcriptional repression by MarR result in increased transcription of the *marRAB* operon, consequent upregulation of the AcrAB-TolC efflux pump and reduced susceptibility to effluxed antibiotics. The phenotypes of mutations affecting expression of the MarA regulon extend beyond antibiotic susceptibility. MarA has been shown to be a virulence factor in *E. coli*, important for persistence in a murine urinary tract infection (UTI) model.[23] In addition, substrates of AcrAB include bile salts and mammalian hormones, indicating that expression of the pump plays a role in bacterial survival in their ecological niche.[24] Such mutations include inactivating mutations (deletions, insertions, frameshifts, nonsense mutations) in *marR*, single amino acid substitutions in *marR* residues involved in target DNA binding or dimerization of MarR sub-units, or mutations in the operator region of the *marRAB* operon to which MarR dimers bind to repress.[12,25–27]

In a recent study,[7] we combined sequence analysis of ciprofloxacin-resistant clinical isolates of *E. coli* and experimental measurements (mutation rate, MIC, relative fitness) with *in silico* modelling to explain the prevalence of dominant genotypes and the order in which resistance mutations accumulate. The study found that the most frequent genotype in ciprofloxacin-resistant clinical isolates of *E. coli* is *gyrA* S83L D87N *parC* S80I due to its relative fitness advantage over other mutations. Upon selective pressure of ciprofloxacin, the most common initial mutation was found to be *gyrA* S83L, followed by *parC* S80I and then *gyrA* D87N. All studied clinical isolates with a ciprofloxacin MIC >16 mg/L (17 strains) were found to have a fourth mutation or more, either another target mutation (*parC* or *parE*) and/or efflux regulator mutations (*marR*, *acrR* or *soxR*).

In this study, we screened the literature on fluoroquinolone-resistant clinical isolates of *E. coli* to identify the spectrum of mutations that appears in the efflux regulator MarR. The aims of the study are: (i) to characterize the mutations in terms of resistance-related parameters (MIC and relative fitness); (ii) to ask whether the spectrum of mutations in *marR* in ciprofloxacin-resistant clinical isolates is dominated by inactivating mutations of the MarR repressor, as would be expected by selection for relief of repression; and (iii) if the spectrum of mutations in MarR is non-random, to ask whether this can be explained by differential effects on drug susceptibility and/or on the relative fitness of the mutants, associated with different types of mutations.

Materials and methods

Media and growth conditions

Bacteria were routinely grown in LB broth (Oxoid Ltd, Basingstoke, UK) or on Luria agar (LA; LB solidified with 1.5% agar, Oxoid Ltd, Basingstoke, UK). Growth competition experiments were performed in Mueller–Hinton (MH) broth (Difco Becton Dickson, MD, USA). MH agar plates (Difco Becton Dickinson, MD, USA) were used for MIC tests, according to EUCAST recommendations. When not specified otherwise, bacteria were grown at 37 °C overnight (16–18 h) and liquid cultures were aerated by shaking at 180–200 rpm.

Bacterial strains

Genetic constructions were made in the *E. coli* K12 MG1655[28] genetic background. Putative efflux mutations in *marR* were identified after a literature search of fluoroquinolone-resistant clinical isolates of *E. coli* (Table S1, available as Supplementary data at *JAC* Online) and were introduced into MG1655 (LM179), or into an isogenic resistant strain, LM693, carrying three drug-target mutations by Lambda Red recombineering.[29] Information on the clinical origins of the isolates (most are UTI or bacteraemia isolates), and their independence, is given in Table S1. The resistance marker *kan-sacB* was first inserted into different locations in *marR* using double-stranded recombineering with selection on LA plates containing kanamycin (50 mg/L). In a second Lambda Red recombineering step the *kan-sacB* cassette was replaced by the desired mutation in *marR* using single-stranded oligonucleotide recombineering[30] with counter-selection for sucrose resistance.[31] Each single-stranded oligonucleotide carried homology to the sequences flanking the inserted cassette and contained the mutation to be introduced. Mutations in *marR* from *in vitro* selections (Table S2) were moved from the strains in which they were originally selected into the WT *E. coli* background by duplication–insertion recombineering.[32] All *marR* mutations introduced into the reconstructed mutants were confirmed by local DNA sequencing.

Competitive fitness measurements

The mutant strains' competitive fitness relative to the fitness of the WT was determined in growth competition assays. Competitor strains were tagged by inserting a yellow (YFP) or dTomato (dT) fluorescent marker into the chromosome by P1-mediated transduction using standard methods.[33] Four to six independent cultures of a mutant strain with the YFP marker were competed against the same number of independent cultures of the WT with the dT marker. Another four to six cultures of each competitor with the dye-swapped combination were also competed. Competing cultures were mixed in a 1:1 ratio, 128-fold diluted in MH in a 96-well plate and grown overnight at 37 °C and 900 rpm in a PHMP-4 Thermoshaker

(Grant-bio, Cambridge) allowing for seven generations of growth per overnight cycle. The next day, the ratios of the competitors were determined using a magnetic-activated cell sorter (MACSQuant® VYB, Miltenyi Biotech, Germany). The cultures of competing strains were again 128-fold diluted in a 96-well plate with fresh MH and allowed to grow overnight once more, with competitor ratios being measured again the next day. Competitions were performed in MH without ciprofloxacin (0 mg/L) and in MH with different concentrations of ciprofloxacin (0.002, 0.004, 0.006, 0.008 and 0.01 mg/L). Fitness differences between the competitors were calculated as the change in the ratio of the competitor strains between the two measurement timepoints according to previously described methods.[34]

Antibiotics

Kanamycin (Sigma–Aldrich, Stockholm, Sweden) was used at a final concentration of 50 mg/L. A 1 mg/mL stock solution of ciprofloxacin (Sigma–Aldrich, Stockholm, Sweden) was further diluted upon use to the final concentrations stated in the text.

MIC determination

The MIC of ciprofloxacin was determined using Oxiod™ M.I.C.Evaluator™ strips (Thermo Fisher Scientific, Basingstoke, UK) on MH agar plates according to the manufacturer's instructions. MIC was read after 18–20 h incubation at 37 °C.

PCR amplification and local DNA sequencing

DNA regions to be sequenced were PCR amplified and sent to Macrogen Europe (Amsterdam, the Netherlands) for sequencing. Routine PCR reactions were run in a S1000™ Thermal Cycler (Bio-Rad Laboratories, Berkeley, CA, USA) using Taq PCR Master Mix (Thermo Scientific, Waltham, MA, USA) according to the following protocol: 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and ending with 72 °C for 5 min before cooling to 4 °C. For amplification of recombineering cassettes, Phusion® High-Fidelity PCR Master Mix with HF buffer (New England Biolabs, Ipswich, MA, USA) was used according to the following PCR conditions: 98 °C for 30 s followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 2.5 min and ending with 72 °C for 7 min before cooling to 4 °C. For reverse transcription of RNA into cDNA a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used with the following protocol: 25 °C for 10 min, 37 °C for 2 h and 85 °C for 5 min. The oligonucleotide primers used are listed in Table S3.

mRNA preparation and real-time PCR (qPCR) measurements

qPCR was used to determine the effect of individual *marR* mutations on the expression of the genes *marA*, *acrAB* and *tolC*. Total RNA was extracted from cultures grown in LB with no antibiotics added, during exponential growth (OD_{600} 0.25–0.3), using an RNeasy Mini Kit (Qiagen). Cellular DNA was removed from the RNA preparations using a Turbo DNA-free™ kit (Ambion). RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA was diluted 1:10, 1:100 and 1:1000 in ddH_2O. qPCR was performed with Fast SYBR™ green master mix (Applied Biosystems) using Eco Real-Time PCR Systems (Illumina) with the following thermal steps: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All qPCR measurements were done with three biological replicates. Two housekeeping genes, *hcaT* and *cysG*, were used as reference genes to quantify the transcriptional responses.[35] The oligonucleotide primers used are listed in Table S3.

Results

Different spectrum of marR mutations in resistant clinical isolates and in in vitro-selected resistant mutants

MarR is a non-essential repressor protein that regulates expression of *marA*, the regulator of the Mar regulon that includes the AcrAB-TolC efflux pump.[13,18] Mutations in *marR* can cause upregulation of the AcrAB-TolC pump and reduce susceptibility to ciprofloxacin and other antimicrobial drugs.[12,27,36] We investigated whether the spectrum of mutations associated with fluoroquinolone-resistant isolates was dominated by inactivating mutations as would be expected for a non-essential gene.[37] DNA sequences of ciprofloxacin-resistant *E. coli* clinical isolates (Table S1), and mutants selected *in vitro* (Table S2), were screened for putative *marR* resistance mutations. Among the resistant clinical isolates we identified 26 different mutations associated with 49 different isolates (Table S1). Among the mutants selected *in vitro* we identified 72 different mutations among 78 isolates (Table S2). As MarR is a non-essential protein, inactivating mutations (deletions, insertions, frameshifts, nonsense mutations and amino acid substitutions) could in theory appear at any position throughout the gene. Mapping the mutations onto *marR* (Figure 1) showed that while mutations from both clinical and *in vitro* selections are widely distributed, there is also evidence for a locational bias. For example, among the clinical isolates single amino acid substitutions are preferentially located within the DNA-binding region of the gene. However, even more striking was the strong bias in the type of mutation associated with clinical isolates versus *in vitro*-selected mutants. There was a significant bias in the relative proportions of single amino acid substitutions and predicted inactivating mutations, when comparing the clinically selected isolates and the *in vitro*-selected mutants (Figure 2). Among clinical isolates approximately 78% were single amino acids substitutions (Table S1), whereas this class accounted for only 13% of the *in vitro*-selected mutants (Table S2). The relative frequency of different mutational classes among the *in vitro*-selected mutants (dominated by frameshift mutations, Figure 1) is in accordance with the type of distribution expected for the occurrence of spontaneous mutations that inactivate a non-essential gene in the absence of strong purifying selection.[37] In contrast, the relative frequencies of mutation types among the clinical isolates suggest a strong selection bias against gene-inactivating mutations (Table S1). This selection bias might be caused by the fitness consequences of overexpressing the AcrAB efflux pump, but it could also be associated with other phenotypes of a misregulated MarA regulon that reduce bacterial persistence or virulence.[23,24,38] To test the hypothesis that the relative frequency of *marR*-inactivating mutations in clinical isolates is limited by counter-selection, we constructed a set of strains carrying individual *marR* mutations. Isogenic strains were constructed in MG1655, carrying 21 mutations found in clinical isolates (14 single amino acid substitutions, 5 predicted inactivating mutations and 2 small in-frame deletions, Table S1) and 15 mutations selected *in vitro* (1 in the non-coding sequence upstream of *marR*, 3 single amino acid substitutions and 11 predicted inactivating mutations, Table S2). The mutations chosen for reconstruction included all of the mutations identified in the coding sequence of

marR in clinical isolates (with the exception one large duplication and one large deletion), and a representative subset of the *in vitro*-selected mutants (based on their locations throughout the coding sequence on *marR*).

Ciprofloxacin susceptibility and relative fitness of isogenic mutant strains

The MIC of ciprofloxacin and the relative fitness in competition assays (in the absence of ciprofloxacin) were measured for each of the isogenic mutant strains (clinical, Table S4; *in vitro*-selected, Table S5). The MIC for each of the mutants was greater than that for the WT MG1655 strain (0.015 mg/L), with values that ranged from 0.023 up to 0.06 mg/L. The values for relative competitive growth fitness of the individual mutants in the absence of ciprofloxacin differed by up to 10% per generation. One striking feature of the data is that the mutants with the highest relative fitness (clustering around a value of 1) are also those with the lowest MIC. In contrast, mutants with the highest MIC values tend to have the lowest fitness values (Figure 3). A second striking feature of the data is that all of the alleles with the highest relative fitness values were originally found in clinical isolates, whereas all of the *in vitro*-selected mutants cluster in a group of alleles with high MIC and low fitness (Figure 3). Finally, mutations that are predicted to inactivate MarR form a cluster with high MIC and low relative fitness (Figure 3). These distinct groupings (low MIC being associated with high fitness, and high MIC being associated with lower fitness) were maintained when 13 of the *marR* mutations were placed in a ciprofloxacin-resistant genetic background carrying multiple target mutations (Table S6). We concluded that among the clinical isolates with mutations in *marR* there is a strong selection for high relative fitness in the absence of ciprofloxacin that is associated with relatively small increases in MIC. An implication of this conclusion is that if a low-cost *marR* mutation arose as a first-step mutation during the evolution of resistance, it could persist and act as a stepping stone for the selection of subsequent low-cost *gyrA* and *parC* mutations.

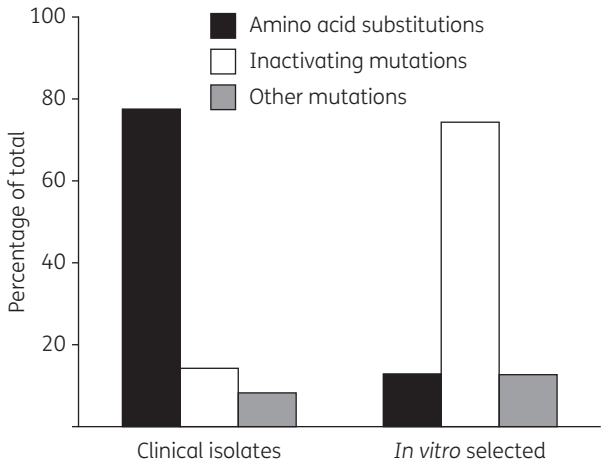

Figure 2. Distribution of different mutation types in *marR*. Mutations are defined as sequence differences relative to *marR* in *E. coli* MG1655. Amino acid substitution mutations refer to single substitutions relative to the protein sequence of MarR. Inactivating mutations are defined as: nonsense mutations, frameshift mutations, out-of-frame deletions and insertion/deletion mutations >9 nt. Other mutations as defined as: mutations upstream of the coding sequence, and 3–9 nt in-frame duplications/insertions/deletions.

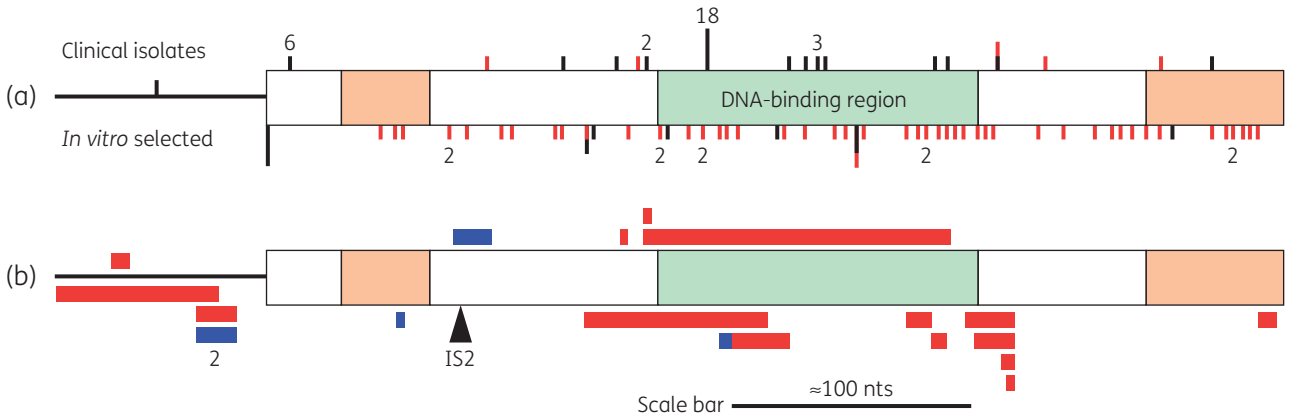

Figure 1. Location of genetic differences in *marR* in resistant mutants. Linear maps of *marR*, drawn approximately to scale. The boxed area represents the coding sequence (144 codons) and the black line to the left represents the upstream 90 nt containing putative regulatory sequences: Shine–Dalgarno sequence (nt – 9 to – 12);[44] RNA polymerase −10 and −35 promoter sequences (nt – 35 to – 63);[44,45] MarR binding sites (nt – 1 to – 23 and −35 to – 58);[44,45] Possible AcrR binding site (nt – 63 to – 72);[46] Mar-box sequence (nt – 80 to – 99).[47,48] Green shading represents sequence coding for the DNA-binding region of MarR (aa 55–100). Brown shading represents sequence coding for the regions involved in dimerization of MarR (aa 10–22 and 123–144).[49] Sequence differences relative to *marR* from MG1655 are indicated: differences found in ciprofloxacin-resistant clinical isolates are shown above each map; mutations selected for ciprofloxacin resistance *in vitro* are shown below each map. Each genetic change is unique unless indicated otherwise by an associated number on the map (e.g. 18 ciprofloxacin-resistant clinical isolates had an amino acid change at codon K62 in the DNA-binding region). (a) Single-nucleotide substitutions relative to *marR* in MG1655 are shown as black bars, single-nucleotide additions/deletions (frameshift-causing) are shown as red bars. Longer bars represent multiple different changes at the same location. (b) Deletions are shown in red, duplications are shown in blue, and the location of an IS element insertion is shown with a black triangle. The shortest deletions/duplications indicated are 3 nt.

Relative fitness of marR mutations increases as a function of ciprofloxacin concentration

Because many of the *marR* alleles found in clinical isolates confer very modest increases in ciprofloxacin MIC, it could be asked whether these increases have any selective significance in the presence of ciprofloxacin. To address this we competed isogenic strains carrying clinically selected *marR* alleles against the isogenic WT strain, at different concentrations of ciprofloxacin (Figure 4).

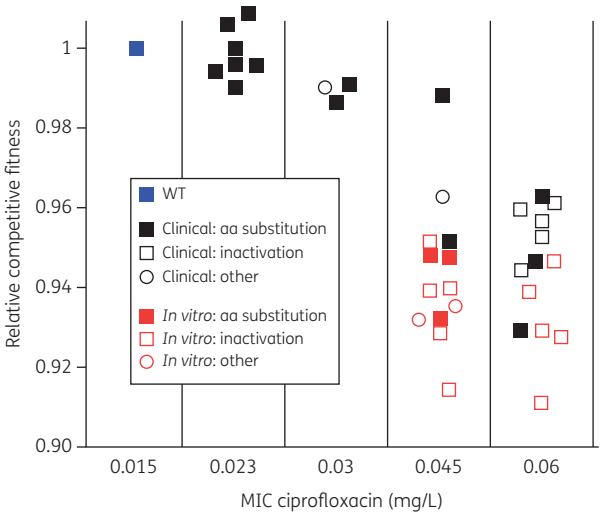

Figure 3. Relationship between MIC and relative fitness of *marR* mutations. All strains are isogenic in the MG1655 WT background. Relative fitness was measured in growth competition experiments in the absence of drug. Numerical data with standard deviations are shown in Table S4 (clinical isolates) and Table S5 (isolates selected *in vitro*).

The clinical alleles chosen were four single amino acid substitutions and two predicted inactivating mutations. The competition assays showed that all six mutations conferred a competitive fitness advantage that increased as a function of ciprofloxacin concentration. The single amino acid substitutions in *marR* with the lowest MICs (D76G and V79I, MIC 0.023 mg/L) each had a relative fitness similar to that of the WT in the absence of ciprofloxacin (1.01 ± 0.01 and 1.00 ± 0.01), but showed a selective advantage over WT as drug concentrations approached the MIC for the WT (Figure 4). Mutations with higher MIC values (L78M, MIC 0.03 mg/L and R73G, A52fs and Q110*, MIC 0.06 mg/L) had a lower fitness than the WT in the absence of drug (in agreement with the growth competition measurements made in the absence of ciprofloxacin and shown in Figure 3), but a significantly greater relative fitness than the susceptible WT as the ciprofloxacin concentration approached the MIC for the WT (Figure 4). We concluded that all of the mutations found in clinical isolates conferred a selective advantage in the presence of ciprofloxacin approaching the MIC for the WT.

Mutations in marR affect the level of the positive regulator marA and efflux pump components

We investigated whether the differences in MIC and relative fitness associated with the different mutations in *marR* correlate with the relative level of expression of *marA*, the regulator of the Mar regulon. We used qPCR to measure the level of *marA* mRNA in isogenic strains carrying 15 different mutations in *marR*, representing the full range of variation in MIC (Table S7). The strains tested included several with mutations in *marR* that cause a 4-fold increase in MIC of ciprofloxacin, from 0.015 to 0.06 mg/mL, corresponding to the maximum fold increase in MIC previously measured for mutations

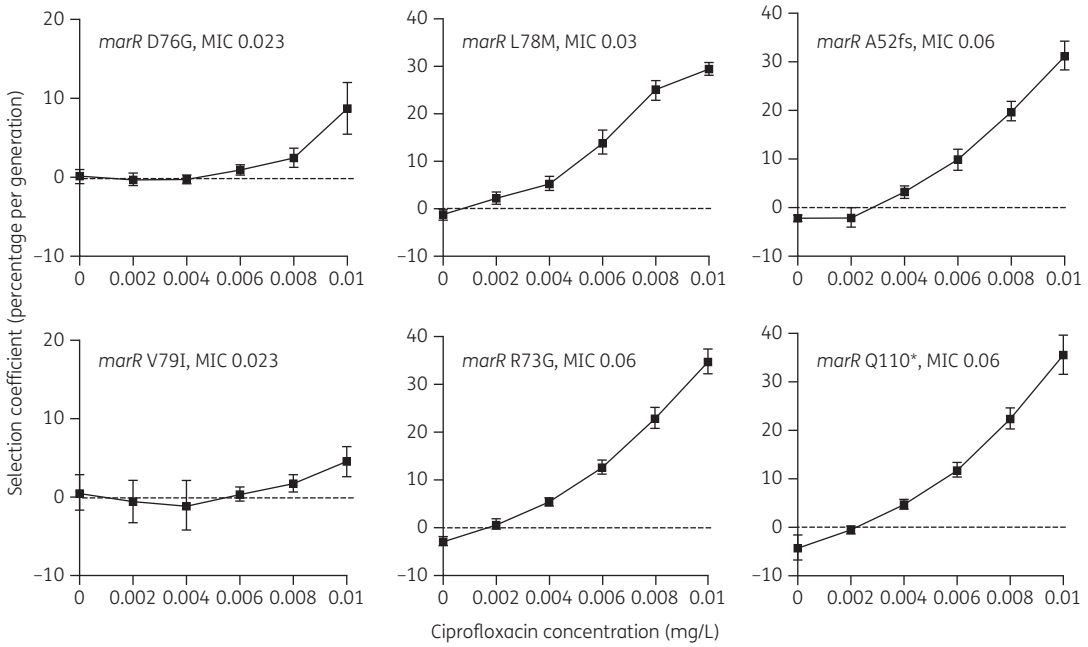

Figure 4. Selection of *marR* mutants as a function of ciprofloxacin concentration. Growth competition assays between the susceptible WT and isogenic mutant strains carrying the *marR* allele indicated. Data are the average of 8–12 independent experiments.

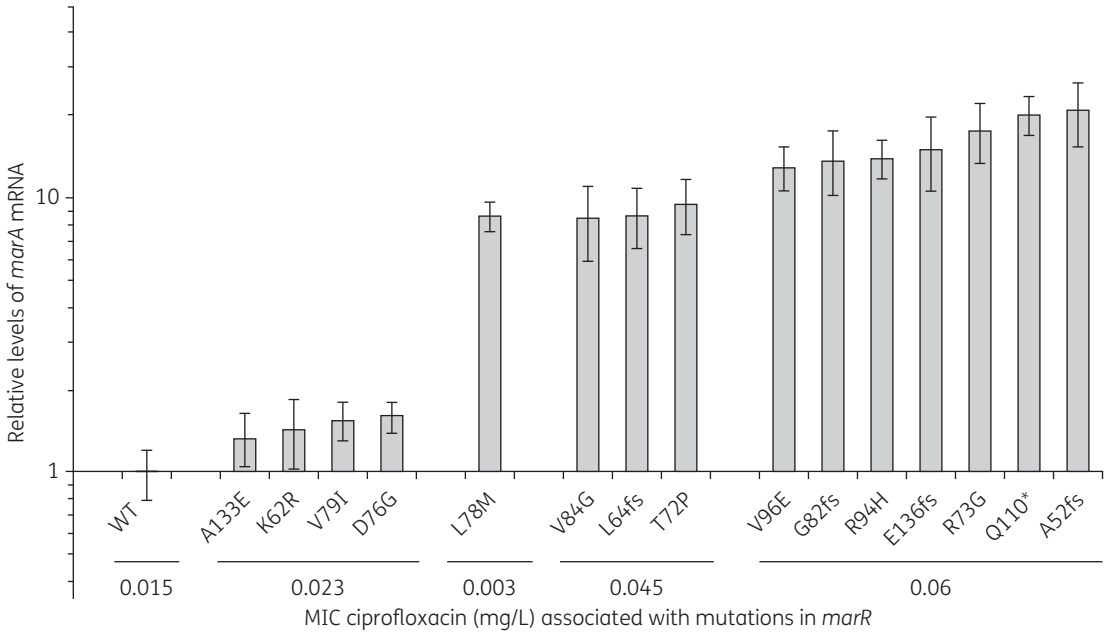

Figure 5. Level of *marA* mRNA as a function of *marR* mutation. *marA* expression as a function of *marR* mutations associated with different MIC values of ciprofloxacin. The difference in *marA* expression relative to the WT is statistically significant for all *marR* mutants (*P* values, Table S7). Data are an average of three independent biological experiments, each with three technical replicates.

that inactivate the MarR repressor.[6,39,40] While the normal physiological regulation of the Mar regulon is associated with inducers that interact with the MarR repressor to modulate its interaction with the operator sequence, in the case of mutations that reduce the activity of the MarR repressor the expectation is that induction should occur in the absence of any chemical inducer. The results showed a strong correlation between the MIC associated with a particular mutation and the relative level of *marA* mRNA measured by qPCR in the absence of any added chemical inducer (Figure 5). We concluded that mutations in *marR* that increase the MIC of ciprofloxacin relieve the repression of *marA*, and that the degree of de-repression can explain the increase in MIC. We next investigated whether these increases in the level of *marA* caused an associated increase in the level of components of the AcrAB-TolC efflux pump. Using qPCR we measured the levels of mRNA for *acrA*, *acrB* and *tolC*, in each of the 15 *marR* mutants (Table S7). The data showed a positive correlation between increases in the level of *marA* mRNA and increases in the levels of each of the mRNA's encoding efflux pump components (Figure 6). Although the error bars are somewhat wider for *acrA* (R^2 value 0.78) than for *acrB* (R^2 value 0.93), the slopes of the correlations for *acrA* and *acrB* are almost identical, increasing from approximately 1 up to 3 over the same range of *marA* mRNA concentrations (Figure 6). We concluded that the degree of reduced susceptibility to ciprofloxacin associated with individual mutations in *marR* correlates with increased expression of the positive regulator *marA*, and of the components of the AcrAB-TolC efflux pump.

Discussion

One of the factors contributing to reduced susceptibility to ciprofloxacin in *E. coli* is overexpression of the major multidrug efflux pump AcrAB-TolC.[12,13,41,42] Expression of this pump is transcriptionally activated by MarA, which in turn is negatively regulated by the repressor protein MarR.[13,19] Mutants of *E. coli* in which the AcrAB-TolC pump is constitutively overexpressed frequently have mutations in *marR*.[7,12] Because *marR* is a non-essential gene, a variety of different mutational types could be expected to reduce repressor activity and lead to overexpression of the efflux pump. The expectation, based on mutational target size,[37] is that the most frequently occurring class of mutations in *marR* should be gene-inactivating mutations. These inactivating mutations would include partial or complete deletions, insertions of IS elements, nonsense mutations and frameshift mutations, all of which are predicted to lift repression by inactivating MarR, and consequently induce overexpression of the efflux pump. Repressor inactivation would be expected to induce the greatest increase in pump expression and thus the greatest increase in MIC. If, however, de-repression of the Mar regulon incurred a fitness cost, then there might be a counter-selection against gene-inactivating mutations. The factors contributing to the fitness costs *in vivo* could include the costs directly associated with upregulating the AcrAB efflux pump, but they could also include other factors regulated by MarA, including resistance to bile salts,[38] or impaired virulence.[23] Accordingly, if fitness cost were a significant constraining factor in clinical strains, the hypothesis predicts that the mutations that would be preferentially selected in resistant isolates would be those that reduced susceptibility without incurring significant fitness costs.

In this study, we tested the hypothesis that there is a trade-off between reduced susceptibility and increased fitness costs that acts as a selective constraint on the particular *marR* mutations that can accumulate in resistant clinical isolates. To address the question we surveyed the frequencies of different mutational

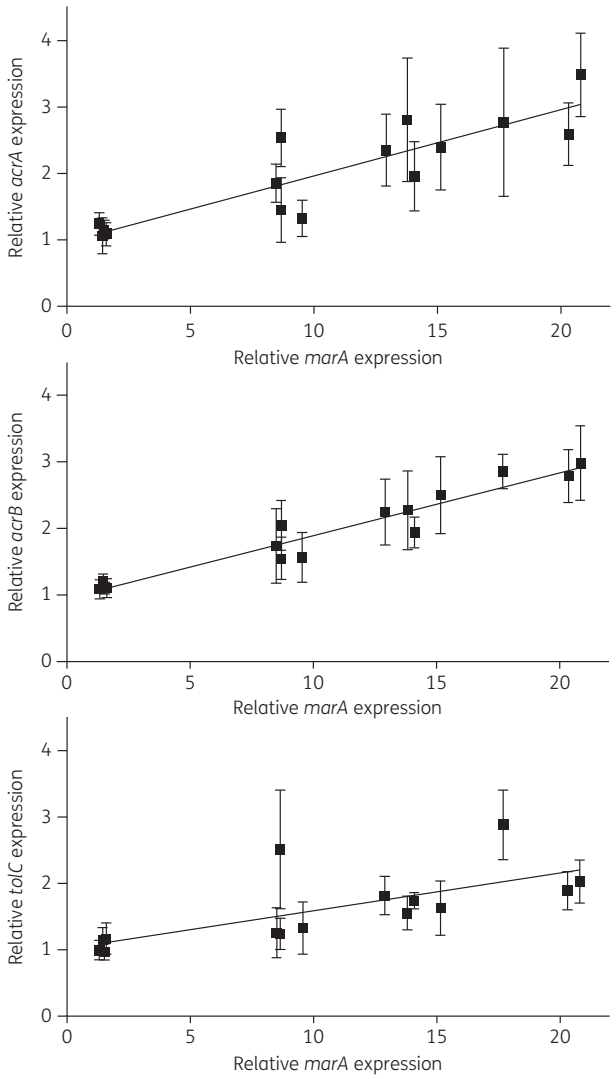

Figure 6. The mRNA level of *acrAB-tolC* as a function of *marA* level. Correlation between the mRNA levels of *acrAB* and *tolC* as a function of *marA* expression level caused by different mutations in *marR*. The correlation values are: *acrA* $R^2 = 0.78$, *acrB* $R^2 = 0.93$ and *tolC* $R^2 = 0.49$. Detailed data and analysis of statistical significance are given in Table S7.

types that are selected for reduced susceptibility *in vitro*, relative to the mutational types that are found in resistant clinical isolates (Figure 1). The two groups of strains had very different distributions of the different mutational types, with a very strong bias among the clinical isolates against gene-inactivating mutations (Figure 2). This difference in mutational types also correlated with a significant difference in the phenotypes associated with the mutations (Figure 3). In general, the mutations selected in clinical isolates were strongly biased in favour of those that incurred very small fitness costs, and conferred small reductions in susceptibility to ciprofloxacin. The mutations selected *in vitro* were heavily biased in favour of inactivating mutations and generally caused greater reductions in susceptibility.

Our data suggest that in balancing the fitness costs of constitutive overexpression of the Mar regulon, versus the benefit of reduced susceptibility, clinical isolates favour mutations in *marR* that confer only small reductions in susceptibility to ciprofloxacin. This conclusion is probably not limited to mutations in *marR* but may have more a general application. Expression of the AcrAB pump is also regulated by other non-essential regulator proteins, AcrR and SoxR.[13] Mutations in each of these regulators have been identified in clinical isolates, and when selected *in vitro* have been shown to cause upregulation of pump expression and reduced susceptibility.[13,43] However, a recent analysis of the genome sequences of several hundred ciprofloxacin-resistant clinical isolates of *E. coli* showed that the frequency of mutations in any of the pump regulators was very small relative to the frequency of target mutations.[7] The low frequency of mutations in these non-essential genes is indicative of a strong purifying selection and suggests that constitutive upregulation of the AcrAB efflux pump is not a favoured strategy for developing clinical resistance. This strong selection against incurring fitness costs could also explain why those clinical isolates with the highest levels of resistance often carry additional mutations in drug target genes such as *gyrA*, *gyrB*, *parC* and *parE*,[7] even though such specific amino acid substitution mutations are expected to occur much less frequently in target genes than inactivating mutations in non-essential repressor genes.

We conclude that in the clinical strains the selection pressure to reduce fitness costs severely constrains the spectrum of mutations that can be accommodated in *marR*. Although selection favours mutations in *marR* that cause only modest reductions in susceptibility to ciprofloxacin, our measurements of competitive fitness (Figure 4) show that these selected mutations can confer a growth advantage in the presence of increasing concentrations of ciprofloxacin.

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Transparency declarations

None to declare.

Supplementary data

Tables S1–S7 are available as Supplementary data at *JAC* Online.

References

1 Castro W, Navarro M, Biot C. Medicinal potential of ciprofloxacin and its derivatives. *Future Med Chem* 2013; **5**: 81–96.

2 Pitout JD, Chan WW, Church DL. Tackling antimicrobial resistance in lower urinary tract infections: treatment options. *Expert Rev Anti Infect Ther* 2016; **14**: 621–32.

3 Chen CR, Malik M, Snyder M *et al*. DNA gyrase and topoisomerase IV on the bacterial chromosome: quinolone-induced DNA cleavage. *J Mol Biol* 1996; **258**: 627–37.

4 Fabrega A, Madurga S, Giralt E *et al*. Mechanism of action of and resistance to quinolones. *Microb Biotechnol* 2009; **2**: 40–61.

5 EUCAST. MIC distributions and ECOFFs. http://www.eucast.org/mic_distributions_and_ecoffs/.

6 Marcusson LL, Frimodt-Moller N, Hughes D. Interplay in the selection of fluoroquinolone resistance and bacterial fitness. *PLoS Pathog* 2009; **5**: e1000541.

7 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; doi:10.1093/molbev/msx052.

8 Nazir H, Cao S, Hasan F *et al*. Can phylogenetic type predict resistance development? *J Antimicrob Chemother* 2011; **66**: 778–87.

9 Strahilevitz J, Jacoby GA, Hooper DC *et al*. Plasmid-mediated quinolone resistance: a multifaceted threat. *Clin Microbiol Rev* 2009; **22**: 664–89.

10 Heisig P. Genetic evidence for a role of parC mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. *Antimicrob Agents Chemother* 1996; **40**: 879–85.

11 Webber M, Piddock LJV. Quinolone resistance in *Escherichia coli*. *Vet Res* 2001; **32**: 275–84.

12 Komp Lindgren P, Karlsson Å, Hughes D. Mutation rate and evolution of fluoroquinolone resistance in *Escherichia coli* isolates from patients with urinary tract infections. *Antimicrob Agents Chemother* 2003; **47**: 3222–32.

13 Li XZ, Plesiat P, Nikaido H. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin Microbiol Rev* 2015; **28**: 337–418.

14 Nikaido H, Pages JM. Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiol Rev* 2012; **36**: 340–63.

15 Eicher T, Brandstatter L, Pos KM. Structural and functional aspects of the multidrug efflux pump AcrB. *Biol Chem* 2009; **390**: 693–9.

16 Muller RT, Pos KM. The assembly and disassembly of the AcrAB-TolC three-component multidrug efflux pump. *Biol Chem* 2015; **396**: 1083–9.

17 Koronakis V, Eswaran J, Hughes C. Structure and function of TolC: the bacterial exit duct for proteins and drugs. *Annu Rev Biochem* 2004; **73**: 467–89.

18 Blair JM, Richmond GE, Piddock LJ. Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. *Future Microbiol* 2014; **9**: 1165–77.

19 Grkovic S, Brown MH, Skurray RA. Regulation of bacterial drug export systems. *Microbiol Mol Biol Rev* 2002; **66**: 671–701.

20 Martin RG, Rosner JL. Binding of purified multiple antibiotic-resistance repressor protein (MarR) to mar operator sequences. *Proc Natl Acad Sci U S A* 1995; **92**: 5456–60.

21 Barbosa TM, Levy SB. Differential expression of over 60 chromosomal genes in *Escherichia coli* by constitutive expression of MarA. *J Bacteriol* 2000; **182**: 3467–74.

22 Pomposiello PJ, Bennik MH, Demple B. Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. *J Bacteriol* 2001; **183**: 3890–902.

23 Casaz P, Garrity-Ryan LK, McKenney D *et al*. MarA, SoxS and Rob function as virulence factors in an *Escherichia coli* murine model of ascending pyelonephritis. *Microbiology* 2006; **152**: 3643–50.

24 Piddock LJ. Multidrug-resistance efflux pumps—not just for resistance. *Nat Rev Microbiol* 2006; **4**: 629–36.

25 Piddock LJ. Mechanisms of fluoroquinolone resistance: an update 1994-1998. *Drugs* 1999; **58** Suppl 2: 11–8.

26 Oethinger M, Podglajen I, Kern WV *et al*. Overexpression of the marA or soxS regulatory gene in clinical topoisomerase mutants of *Escherichia coli*. *Antimicrob Agents Chemother* 1998; **42**: 2089–94.

27 Alekshun MN, Kim YS, Levy SB. Mutational analysis of MarR, the negative regulator of marRAB expression in *Escherichia coli*, suggests the presence of two regions required for DNA binding. *Mol Microbiol* 2000; **35**: 1394–404.

28 Blattner FR, Plunkett G 3rd, Bloch CA *et al*. The complete genome sequence of *Escherichia coli* K-12. *Science* 1997; **277**: 1453–74.

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

30 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.

31 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.

32 Näsvall J, Knöppel A, Andersson DI. Duplication-insertion recombineering: a fast and scar-free method for efficient transfer of multiple mutations in bacteria. *Nucleic Acids Res* 2017; **45**: e33.

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

34 Dykhuizen DE. Experimental studies of natural selection in bacteria. *Annu Rev Ecol Syst* 1990; **21**: 373–98.

35 Zhou K, Zhou L, Lim Q *et al*. Novel reference genes for quantifying transcriptional responses of *Escherichia coli* to protein overexpression by quantitative PCR. *BMC Mol Biol* 2011; **12**: 18.

36 Sato T, Yokota SI, Uchida I *et al*. Fluoroquinolone resistance mechanisms in an *Escherichia coli* isolate, HUE1, without quinolone resistance-determining region mutations. *Front Microbiol* 2013; **4**: 125.

37 Andersson DI, Hughes D, Roth JR. The origin of mutants under selection: interactions of mutation, growth and selection. In: A Böck, JB Kaper, PD Karp, FC Neidhardt, T Nyström, JM Slauch, CL Squires, D Ussery, eds. *EcoSal-Escherichia coli and Salmonella: Cellular and Molecular Biology*. Washington, DC: ASM Press, 2011. doi: 10.1128/ecosalplus.5.6.6.

38 Rosenberg EY, Bertenthal D, Nilles ML *et al*. Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. *Mol Microbiol* 2003; **48**: 1609–19.

39 Machuca J, Briales A, Diaz-de-Alba P *et al*. Effect of the efflux pump QepA2 combined with chromosomally mediated mechanisms on quinolone resistance and bacterial fitness in *Escherichia coli*. *J Antimicrob Chemother* 2015; **70**: 2524–7.

40 Kern WV, Oethinger M, Jellen-Ritter AS *et al*. Non-target gene mutations in the development of fluoroquinolone resistance in *Escherichia coli*. *Antimicrob Agents Chemother* 2000; **44**: 814–20.

41 Blair JM, Webber MA, Baylay AJ *et al*. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 2015; **13**: 42–51.

42 Swick MC, Morgan-Linnell SK, Carlson KM *et al*. Expression of multidrug efflux pump genes acrAB-tolC, mdfA, and norE in *Escherichia coli* clinical isolates as a function of fluoroquinolone and multidrug resistance. *Antimicrob Agents Chemother* 2011; **55**: 921–4.

43 Wang H, Dzink-Fox JL, Chen M *et al*. Genetic characterization of highly fluoroquinolone-resistant clinical *Escherichia coli* strains from China: role of acrR mutations. *Antimicrob Agents Chemother* 2001; **45**: 1515–21.

44 Martin RG, Rosner JL. Transcriptional and translational regulation of the marRAB multiple antibiotic resistance operon in *Escherichia coli*. *Mol Microbiol* 2004; **53**: 183–91.

45 Cohen SP, Hachler H, Levy SB. Genetic and functional analysis of the multiple antibiotic resistance (mar) locus in *Escherichia coli*. *J Bacteriol* 1993; **175**: 1484–92.

46 Lee JO, Cho KS, Kim OB. Overproduction of AcrR increases organic solvent tolerance mediated by modulation of SoxS regulon in *Escherichia coli*. *Appl Microbiol Biotechnol* 2014; **98**: 8763–73.

47 Martin RG, Jair KW, Wolf RE Jr *et al*. Autoactivation of the marRAB multiple antibiotic resistance operon by the MarA transcriptional activator in *Escherichia coli*. *J Bacteriol* 1996; **178**: 2216–23.

48 Martin RG, Gillette WK, Rhee S *et al*. Structural requirements for marbox function in transcriptional activation of mar/sox/rob regulon promoters in *Escherichia coli*: sequence, orientation and spatial relationship to the core promoter. *Mol Microbiol* 1999; **34**: 431–41.

49 Alekshun MN, Levy SB, Mealy TR *et al*. The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 Å resolution. *Nat Struct Biol* 2001; **8**: 710–4.