*J Antimicrob Chemother* 2021; **76**: 77–83
doi:10.1093/jac/dkaa417 Advance Access publication 22 October 2020

Journal of Antimicrobial Chemotherapy

# Resistance/fitness trade-off is a barrier to the evolution of MarR inactivation mutants in *Escherichia coli*

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*Received 25 May 2020; accepted 9 September 2020*

**Background:** Mutations that inactivate MarR reduce susceptibility to ciprofloxacin and competitive growth fitness in *Escherichia coli*. Both phenotypes are caused by overexpression of the MarA regulon, which includes the AcrAB-TolC drug efflux pump.

**Objectives:** We asked whether compensatory evolution could reduce the fitness cost of MarR-inactivating mutations without affecting resistance to ciprofloxacin.

**Methods:** The cost of overexpressing the AcrAB-TolC efflux pump was measured independently of MarA overexpression. Experimental evolution of MarR-inactive strains was used to select mutants with increased fitness. The acquired mutations were identified and their effects on drug susceptibility were measured.

**Results:** Overexpression of the AcrAB-TolC efflux pump was found not to contribute to the fitness cost of MarA regulon overexpression. Fitness-compensatory mutations were selected in *marA* and *lon*. The mutations reduced the level of MarA protein thus reducing expression of the MarA regulon. They restored growth fitness but also reduced resistance to ciprofloxacin.

**Conclusions:** The fitness cost caused by overexpression of the MarA regulon has multiple contributing factors. Experimental evolution did not identify any single pump-independent cost factor. Instead, efficient fitness compensation occurred only by mechanisms that reduce MarA concentration, which simultaneously reduce the drug resistance phenotype. This resistance/fitness trade-off is a barrier to the successful spread of MarR inactivation mutations in clinical isolates where growth fitness is essential.

## Introduction

Fluoroquinolones are antimicrobial drugs that inhibit the activity of two essential enzymes, DNA gyrase and topoisomerase IV.[1,2] Ciprofloxacin is a clinically important fluoroquinolone with very good activity against several Gram-negative species,[3] including *Escherichia coli*, which is one of the major causes of urinary tract infections and invasive septicaemia.[4] However, many clinical isolates of *E. coli* have evolved resistance to ciprofloxacin, restricting therapeutic options.[5,6] The evolution of resistance to ciprofloxacin in *E. coli* is a complex multistep process.[7] Chromosomal mutations and horizontally acquired plasmid-borne genes can each contribute to building up the level of resistance in clinical isolates.[8] Clinical resistance almost always involves the selection of multiple target mutations affecting DNA gyrase and topoisomerase IV[9] although an exception has been identified.[10]

In addition to target mutations many resistant clinical isolates also have mutations in transcriptional regulators such as *marR*.[11,12] MarR is a transcriptional repressor protein that locally regulates the expression of the *marRAB* operon. MarR repression can be relieved upon exposure to different inducing agents or by mutations.[13] Upon relief of repression, *marRAB* transcription is activated. MarA is a global transcriptional regulator of more than 100 genes[14,15] and MarB has been reported to repress the expression of the *marRAB* operon by reducing the rate of transcription of *marA*.[16] Mutations that inactivate the non-essential repressor protein MarR result in a decreased susceptibility to multiple antibiotics.[17] The MDR phenotype is mediated by a MarA-dependent increase in expression of the AcrAB-TolC efflux pump, which is the major efflux pump in *E. coli* and it can expel a wide variety of compounds. The resistance phenotype mediated by inactivation mutations in the *marR* gene is, however, often associated with a significant fitness cost.[18]

In a recent study we observed a strong bias against mutations that inactivate MarR in ciprofloxacin-resistant clinical isolates of *E. coli*.[18] We studied the relationship between drug susceptibility

and relative fitness using isogenic strains carrying 36 different *marR* mutations identified in clinical isolates or selected for resistance *in vitro* and found that single amino acid substitutions in general confer a low level of resistance combined with high fitness. In contrast, inactivating mutations in *marR* cause higher resistance but at the cost of significantly reduced relative fitness. In spite of this, high fitness-cost *marR* mutations are sometimes observed in ciprofloxacin-resistant clinical isolates.[18] This suggests the possibility that some clinical isolates may have mechanisms to reduce the fitness cost of inactivating mutations in *marR*, for example by the acquisition of additional mutations that are fitness-compensatory. In order to study whether the high fitness cost associated with high ciprofloxacin MIC ($MIC_{Cip}$) *marR* mutations can be genetically compensated for, we chose two characterized frameshift-deletion mutations in *marR* (I18fs and C51fs) that are each associated with a relatively large reduction in susceptibility to ciprofloxacin and a relatively high fitness cost.[18] Using these two *marR* mutants as starting points, we performed experimental evolution to select for improved growth fitness. The aims of the study were to determine whether there is an evolutionary pathway to acquire mutations that reduce the fitness costs of *marR* mutations, and whether such improved fitness can be achieved without reversing the resistance phenotype.

## Materials and methods

### Bacterial strains and growth conditions

All strains were derivatives of *E. coli* K12 MG1655.[19] Construction of the *marR* I18fs and *marR* C51fs strains has been previously described.[18] Genotypes of the strains used in this study are shown in Table S1 (available as Supplementary data at *JAC* Online). Bacteria were grown in LB broth (Oxoid Ltd, Basingstoke, UK) or on Luria agar (LA; LB solidified with 1.5% agar; Oxoid) at 37°C overnight (liquid cultures were aerated by shaking at 180–200 rpm). Growth competition experiments were performed in LB.

### Selection and construction of acrR mutations

Putative efflux mutations in *acrR* were identified from ciprofloxacin-resistant mutants selected for resistance *in vitro* and from a literature search of fluoroquinolone-resistant clinical isolates of *E. coli* (Table S2).[9,12,20–24] Mutant *acrR* alleles were constructed into an isogenic background using Lambda-Red recombineering.[25] The selectable marker *kan-sacB* was first inserted into different locations in *acrR* by double-stranded recombineering. In a second step, the *kan-sacB* cassette was replaced by the desired *acrR* allele using single-stranded oligonucleotide recombineering,[26] counterselecting for sucrose resistance.[27] All *acrR* mutations were confirmed by local DNA sequencing.

### Experimental evolution

Ten independent lineages of each of the non-revertible out-of-frame deletion mutants CH6295 (*marR* I18fs) and CH6300 (*marR* C51fs) (Table S1) were grown for 500 generations in LB. After each cycle of 10 generations, the lineages were serially passaged by transferring 2 μL of overnight culture into 2 mL of fresh medium. After 500 generations, dilutions were plated on LA and incubated at 37°C overnight. The largest colony (based on visual assessment) was isolated from each lineage for further analysis.

### MIC determination

The MIC of ciprofloxacin was determined using Oxoid™ M.I.C.Evaluator™ strips (Thermo Fisher Scientific, Basingstoke, UK) on Mueller–Hinton agar plates (Difco Becton Dickinson, MD, USA).

### Competitive-fitness measurements

The competitive fitness of isolates relative to the parental strains was determined in growth competition assays. Each competitor strain was labelled with fluorescent markers, either yellow fluorescence protein (YFP) or dTomato (dT), by P1-mediated transduction.[28] Four independent cultures of a mutant strain labelled with the YFP marker were competed against the same number of independent cultures of the parental strain labelled with the dT marker. The same was done with the dye-swapped combination, resulting in a total of eight independent experiments for each competition pair. Overnight cultures were mixed in a 1:1 ratio, diluted 128-fold in LB and transferred to a 96-well plate. Cultures were allowed to grow overnight at 37°C and 900 rpm in a PHMP-4 Thermoshaker (Grant Bio, Cambridge, UK). After the first growth cycle, cultures were diluted 128-fold in fresh LB, allowing for an additional seven generations of growth. Competitor ratios were determined using a magnetic-activated cell sorter (MACSQuant® VYB, Miltenyi Biotech, Germany) after each growth cycle. The change in the ratio of competitor strains between the two MACSQuant measurements were used to calculate relative fitness as previously described.[29]

### WGS

Genomic DNA was prepared from evolved clones using a Masterpure DNA Purification Kit (Epicentre, Illumina Inc., Madison, WI, USA). Genomic DNA was resuspended in elution buffer (EB) and then diluted and quantified using a Qubit device (Invitrogen via Thermo Fisher Scientific). Libraries for WGS were prepared using Nextera XT Library Preparation and Index kits (Illumina) according to manufacturer instructions. Library quality was assessed using a TapeStation device (Agilent Technologies, Santa Clara, CA, USA) using High Sensitivity D5000 ScreenTape and reagents. The libraries were sequenced on a MiSeq device using a 600 cycle V3 reagent kit (Illumina). Reads were processed, aligned and analysed using CLC Genomics Workbench V9 (CLCbio, QIAGEN, Denmark).

### mRNA preparation and real-time quantitative PCR (qPCR) measurements

Bacterial cultures were grown in LB at 37°C until exponential growth ($OD_{600}$ 0.25–0.3) and total RNA was extracted using the RNeasy Mini Kit (QIAGEN). Chromosomal DNA was removed by treating the RNA preparations with DNase using the Turbo DNA-free™ Kit (Ambion) and RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). After diluting the cDNA 1:10, 1:100 and 1:1000 in double-distilled $H_2O$, qPCR was performed with Fast SYBR™ Green Master Mix (Applied Biosystems) using the Eco Real-Time PCR System (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. Housekeeping genes *hcaT* and *cysG* were used as reference genes.[30]

## Results

### Fitness cost caused by inactivation of MarR is not associated with overexpression of AcrAB-TolC

*E. coli* strains that carry the inactivation mutants of MarR used in this study display a reduced growth fitness of 0.78–0.81 relative to the isogenic WT and an increased $MIC_{Cip}$ of 0.045–0.06 mg/L ($MIC_{Cip}$ of isogenic WT: 0.015 mg/L) (Table 1). The resistance phenotype is thought to be mainly caused by overexpression of

**Table 1.** Ciprofloxacin susceptibility and relative fitness of relevant strains

| Strain | Relevant genotype[a] | $MIC_{Cip}$ (mg/L) | Fitness ± SD[b] |
|---|---|---|---|
| CH1464 | WT | 0.015 | 1.00±0.01 |
| CH6295 | *marR* I18fs | 0.06 | 0.81±0.02 |
| CH6300 | *marR* C51fs | 0.045 | 0.78±0.02 |
| CH6971 | *marR* I18fs, Δ*marA* (4.8 kb) | 0.015 | 0.94±0.03 |
| CH7278 | *marR* I18fs, *lon* ntC-26T | 0.03 | 0.93±0.01 |
| CH6970 | *marR* I18fs, *arcA* P58R | 0.06 | 0.96±0.02 |

[a]See Table S1 for details on Δ*marA* deletions. Number in parentheses shows total size of deletion. All isolates with the *marR* I18fs mutation also carry an *mngA* duplication (nt −49 to −44) mutation in the chromosome.
[b]Competitive fitness ± SD relative to the WT (CH1464).

the AcrAB-TolC efflux pump.[31] We initially tested whether overexpression of the efflux pump is also responsible for the fitness cost of a MarR inactivation mutation. The expression of AcrAB is modulated by the local regulator AcrR. Mutations that reduce AcrR activity lead to overexpression of the AcrAB-TolC efflux pump that is independent of the MarA regulon.[32] A previous study on the fitness effect of *marR* mutations showed that fitness costs varied between different *marR* alleles.[18] Therefore, we constructed a set of 30 *acrR* alleles found in clinical ($n = 17$)[9,12,20,21,24] and *in vitro* selected ($n = 13$)[33] isolates in an isogenic background to account for possible phenotypic variations among *acrR* alleles. The levels of ciprofloxacin susceptibility and bacterial fitness were measured for all 30 isolates. The results showed that the *acrR* alleles increased $MIC_{Cip}$ to 0.023–0.045 mg/L, which is as high as in the *marR* C51fs allele-carrying strain (Tables 1 and S2). This agrees with the notion that overexpression of the AcrAB-TolC efflux pump is the main contributing factor to reduced ciprofloxacin susceptibility of MarR inactivation mutations. Contrarily, the relative fitness of isolates that carry the *acrR* alleles was 0.99 with no significant difference between clinically selected *acrR* alleles (0.988±0.003) and *in vitro* selected alleles (0.990±0.001) (Table S2). This shows that only a small fraction of the fitness cost associated with the MarR inactivation mutations is caused by overexpression of the AcrAB-TolC efflux pump. These data indicate that it should be theoretically possible to select fitness-compensatory mutations that reduce the fitness cost of a MarR inactivation mutation without losing the associated reduction in ciprofloxacin susceptibility.

### *Fitness-compensatory evolution of isolates with MarR inactivation mutations*

The MarA regulon consists of more than 100 genes and it is not known which part of the regulon is responsible for the decreased competitive fitness observed in isolates that carry MarR inactivation mutations.[14,15] The fitness cost could be the cumulative result of many small costs caused by the changes in expression of multiple genes, or the majority of the fitness cost could be caused by a single gene/operon within the MarA regulon. In the latter case, it should be possible to mutationally alter expression of the specific gene/operon thus restoring cellular fitness without loss of the resistance phenotype. This would be unlikely to be the case if the fitness cost is spread over multiple genes because each individual gene would require to be mutated. To determine whether the fitness costs associated with *marR* mutations could be compensated for by the acquisition of secondary mutations we experimentally evolved two mutant strains in the absence of drug selective pressure. The chosen mutants carry non-revertible out-of-frame deletions in the coding sequence of *marR* (I18fs and C51fs) to reduce the probability of compensation by reversion, or intragenic mutation. The two mutants were previously characterized[18] and have an increased $MIC_{Cip}$ (0.06 and 0.045 mg/L, respectively, relative to 0.015 mg/L for the isogenic WT) and a reduced competitive fitness (approximately 0.8 in LB) relative to WT (Table 1). Ten independent lineages of each mutant strain were evolved by serial passage in LB for 500 generations (50 serial passages). At the endpoint of the evolution, cultures were diluted and plated on LA for single colonies. A single colony from each lineage was isolated and analysed by WGS. All 20 sequenced strains had acquired at least one additional mutation during the evolution (Table 2). Six of the strains had acquired a single additional mutation, while the remaining 14 strains had acquired between two and six additional mutations (Table 2). Mutations in four different genes (*marA*, *lon*, *arcA* and *gltP*) were identified in multiple isolates. Almost half of the evolved strains (9/20) had acquired a mutation in *marA* and in three of the evolved strains the *marA* mutation was the only mutation acquired during the evolution (Figure 1a). Another four mutants had acquired a mutation in *lon*, a gene encoding a protease that degrades MarA.[34] In one of these four mutants the *lon* mutation was the only mutation acquired during the evolution (Figure 1b). Six of the evolved strains had mutations in *arcA* (one additional strain had a mutation in *arcB*), encoding a two-component transcriptional regulation system involving gene expression under microaerobic and anaerobic conditions.[35,36] In two strains a mutation in *arcA* was the only mutation acquired during the evolution (Figure 1c). Finally, nine isolates carried mutations affecting the *gltP* gene, which encodes a glutamate and aspartate transporter,[37] but all of these strains had acquired additional mutations (Figure 1d). It has previously been shown that mutations in *arcA* and *gltP* are frequently selected in *E. coli* during adaption to growth in LB,[38] indicating that these mutations were not selected to improve the fitness cost caused by the inactivation of MarR mutation but represent a general adaptation to the growth medium. Thus, further work was focused on mutations affecting *marA* and *lon*.

### *Mutations in marA and lon restore fitness but also increase drug susceptibility*

The $MIC_{Cip}$ for each of the 20 whole-genome sequenced mutants was measured (Table 2). Of the 10 mutants that were evolved from CH6295 (*marR* I18fs), 8 had an $MIC_{Cip}$ that was lower than the parental strain (reduced to between 0.015 and 0.03 mg/L). The two remaining mutants showed little or no change relative to the parental $MIC_{Cip}$ (0.045–0.06 mg/L). Of the 10 mutants that were evolved from CH6300 (*marR* C51fs), 6 had $MIC_{Cip}$ values that were reduced (0.03 and 0.015 mg/L) while the remaining 4 mutants had unchanged $MIC_{Cip}$ values of 0.045 mg/L. Overall, all isolates that carried a mutation in *marA* or *lon* displayed a significantly decreased $MIC_{Cip}$ while isolates with unchanged $MIC_{Cip}$ values were associated with mutations in *acrA* and *gltP* (Table 2).

**Table 2.** Genotypes and $MIC_{Cip}$ of evolved isolates

| Strain | Parental genotype[a] | Acquired mutations[b] | $MIC_{Cip}$ (mg/L) |
|---|---|---|---|
| CH1464 | WT | WT | 0.015 |
| CH6295 | *marR* I18fs | parental strain | 0.06 |
| CH6881 | *marR* I18fs | *marA* T125<>IS1 | 0.023 |
| | | *citC* ntA-171T | |
| CH6882 | *marR* I18fs | *gltP* ntA-115T | 0.045 |
| | | *yqeG* I42L | |
| | | *rpoC* H419P | |
| | | *dosC* ntT-259<>IS5 | |
| | | *nanM* I10<>IS2 | |
| CH6883 | *marR* I18fs | *lon* E4E (GAG>GAA) | 0.03 |
| | | *rhaD* ntA-143C | |
| | | *rhaD* ntC-217T | |
| CH6884 | *marR* I18fs | *marA* M63K | 0.015 |
| | | *arcA* P58R | |
| | | *sapD* A195P | |
| | | *gcvP* V885M | |
| | | *phnN* L20L (CTG>CTA) | |
| | | *yjdA* A726P | |
| CH6885 | *marR* I18fs | *lon* ntC-26T | 0.03 |
| | | *ydeN* D508A | |
| | | *ygiF* A294A (GCG>GCT) | |
| CH6886 | *marR* I18fs | *lon* E4E (GAG>GAA) | 0.03 |
| CH6969 | *marR* I18fs | Δ*marA* (1.9 kb) | 0.023 |
| | | *rbsR* G165fs | |
| CH6970 | *marR* I18fs | *arcA* P58R | 0.06 |
| CH6971 | *marR* I18fs | Δ*marA* (4.8 kb) | 0.015 |
| CH6972 | *marR* I18fs | *marA* Y37H | 0.023 |
| | | *gltP* ntA-115T | |
| CH6300 | *marR* C51fs | parental strain | 0.045 |
| CH6921 | *marR* C51fs | Δ*marA* (1.1 kb) | 0.015 |
| CH6922 | *marR* C51fs | *arcA* R16H | 0.03 |
| | | *gltP* ntA-115T | |
| | | *malE* W184* | |
| CH6923 | *marR* C51fs | *arcA* L65F | 0.045 |
| CH6924 | *marR* C51fs | *lon* E4E (GAG>GAA) | 0.023 |
| | | *gltP* ntA-115T | |
| CH6925 | *marR* C51fs | *marA* S32* | 0.015 |
| | | *gltP* ntA-115T | |
| CH6926 | *marR* C51fs | *marA* I18F | 0.015 |
| | | *gltP* ntA-115T | |
| | | Δ*ppsA* (3 kb) | |
| CH6973 | *marR* C51fs | *gltP* ntA-115T | 0.045 |
| | | *gltB* R117H | |
| | | *arcB* S83fs | |
| CH6974 | *marR* C51fs | *arcA* G59A | 0.045 |
| | | *gltP* ntA-115T | |
| CH6975 | *marR* C51fs | Δ*marA* (5.0 kb) | 0.015 |
| CH6976 | *marR* C51fs | *arcA* I205L | 0.045 |
| | | *gltP* ntA-115T | |
| | | *leuX* ntG-22T | |

An asterisk denotes a stop codon.
[a]All isolates with the *marR* I18fs mutation also carry an *mngA* duplication (nt −49 to −44) mutation in the chromosome.
[b]See Table S1 for details on Δ*marA* deletions. Numbers in parentheses show total size of deletions.

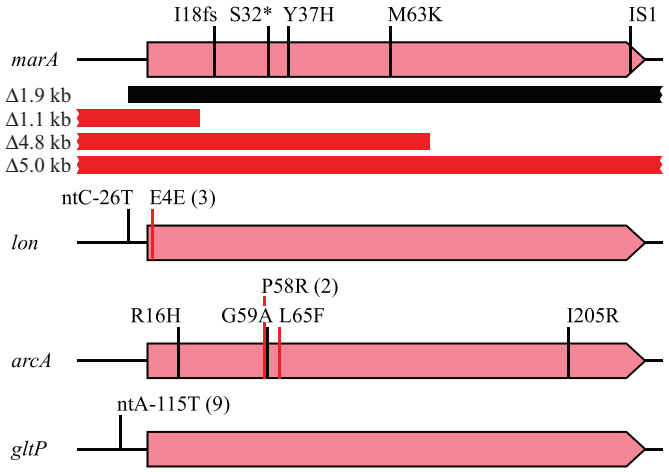


**Figure 1.** Distribution of mutations in *marA*, *lon*, *arcA* and *gltP* selected during the evolution experiment. Deleted segments of *marA* are indicated below and extend outside the displayed region as indicated by jagged ends. Mutations and deletions marked in red were the only additional mutations acquired in an evolved strain, indicating that each alone is sufficient to account for the mutant phenotype. Numbers in parentheses indicate independent isolation events of the marked mutation.

To confirm that mutations in *marA* and *lon* actually improve growth fitness, and to quantify the improvement, we constructed fluorescently labelled strains that carried a single mutation in *marA* [Δ*marA* (4.8 kb)] or *lon* (*lon* ntC-26T) in the *marR* I18fs background. Growth competition experiments showed that both mutations increased the competitive fitness from 0.81 to 0.93 (*lon*) and 0.94 (*marA*) relative to the WT (Table 1). We conclude that mutations in *marA* or *lon* are sufficient to improve the growth defect caused by *marR* inactivation mutations and that this improved fitness is always accompanied by a reduction of the resistance phenotype.

### *Compensatory mutations reduce cellular MarA concentration*

We asked how the compensatory mutations restore cellular fitness. Expression of *lon* and *marA* were measured to identify how the compensatory mutations affected mRNA levels of these two genes. The expression levels of the *acrA*, *acrB* and *tolC* efflux pump genes were used as an indirect measure of MarA protein concentration as it determines the expression of the MarA regulon, which is dependent on MarA concentrations. Inactivation of *marR* (*marR* I18fs) led to a 13-fold increase of *marA* mRNA level and 3-fold increased expression of the AcrAB-TolC efflux pump components relative to the isogenic WT (Table 3). Unsurprisingly, the deletion of the *marA* gene abolishes *marA* expression and restores expression of *acrAB* and *tolC* to WT levels. Expression levels of *lon* remained unchanged in both mutants (Table 3). Next, we measured expression levels of the five genes in strains that carried the *marR* I18fs mutation in combination with either of the two *lon* mutations identified in this study (*lon* ntC-26T and *lon* E4E, GAG to GAA). Expression of *lon* increased in both mutants approximately 3-fold, showing that the mutations led to up-regulation of *lon* expression.

**Table 3.** Relative mRNA expression levels

| Strain | Relevant genotype[a] | mRNA level ± SD[b] | | | | |
|---|---|---|---|---|---|---|
| | | *lon* | *marA* | *acrA* | *acrB* | *tolC* |
| CH1464 | WT | 1.00 ± 0.40 | 1.00 ± 0.11 | 1.00 ± 0.17 | 1.00 ± 0.14 | 1.00 ± 0.21 |
| CH6295 | *marR* I18fs | 1.09 ± 0.22 | 12.79 ± 1.92 | 2.82 ± 0.53 | 2.69 ± 0.29 | 3.67 ± 0.70 |
| CH6971 | *marR* I18fs, Δ*marA* (4.8 kb) | 1.11 ± 0.34 | 0.01 ± 0.01 | 0.54 ± 0.12 | 0.69 ± 0.08 | 0.68 ± 0.12 |
| CH7278 | *marR* I18fs, *lon* ntC-26T | 3.71 ± 0.37 | 12.16 ± 2.38 | 1.52 ± 0.25 | 1.70 ± 0.23 | 1.75 ± 0.20 |
| CH6886 | *marR* I18fs, *lon* E4E | 2.79 ± 0.56 | 12.10 ± 1.90 | 1.44 ± 0.22 | 1.53 ± 0.27 | 2.14 ± 0.52 |

[a]See Table S1 for details on the Δ*marA* deletion. Number in parentheses shows total size of deletion.
[b]mRNA level ± standard deviation relative to the WT (CH1464).

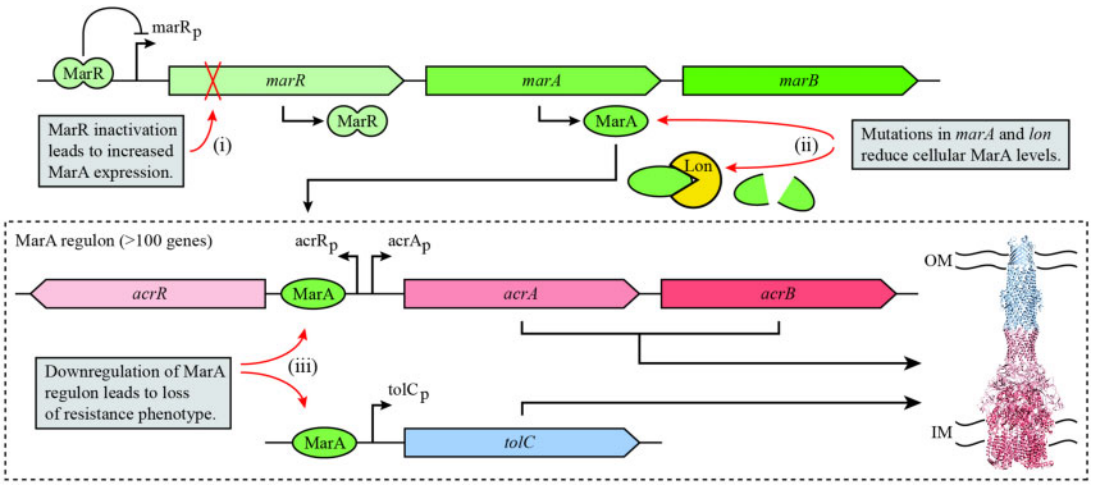


**Figure 2.** Mechanism of action of fitness-compensatory mutations in *marA* and *lon*. Inactivation mutations in MarR lead to increased expression of MarA. The MarA regulon contains >100 genes, including genes encoding AcrAB-TolC, which is responsible for the resistance phenotype. Compensatory mutations in *marA* abolish MarA activity while mutation in *lon* increases expression of Lon protease thus reducing MarA concentration. The resulting reduced expression of the MarA regulon increases cellular fitness but leads to loss of the resistance phenotype. OM, outer membrane; IM, inner membrane.

The expression of *marA* mRNA remained unchanged in both *lon* mutant strains but the mRNA levels of the pump components (*acrAB*, *tolC*) were down-regulated to a 1.7-fold increase relative to the WT compared with a 3-fold increase in the parental *marR* I18fs strain. This indicates that elevated Lon concentrations led to increased degradation of MarA protein in these mutants (Table 3). Overall, the data show that both types of compensatory mutation act via the reduction of cellular MarA concentration, either by deletion of the *marA* gene or by increased MarA degradation. In each case the reduction of MarA concentration results in decreased expression of the AcrAB-TolC efflux pump and a loss of the resistance phenotype (Figure 2).

## Discussion

Gene-inactivating mutations in *marR* can arise in *E. coli* under selection for reduced susceptibility to ciprofloxacin, but they carry a large relative fitness cost.[18] We asked whether such mutants could subsequently evolve by acquisition of fitness-compensatory mutations and whether such mutations would affect susceptibility to ciprofloxacin. To address this question, we measured the fitness cost of overexpression of the AcrAB-TolC efflux pump and experimentally evolved two strains carrying non-revertible mutations in *marR*. Our main findings were that (i) overexpression of the AcrAB-TolC efflux pump does not significantly contribute to the fitness cost caused by a MarR inactivation mutation, (ii) mutation in *marA* and *lon* can compensate for the fitness cost by reducing the cellular concentration of MarA and (iii) fitness compensation occurs with loss of the antibiotic-resistance phenotype associated with the *marR* mutations. Mutations that were identified in *arcA* and *gltP* are not specific to adaptation of MarR inactivation mutations and most likely represent adaptation to the growth medium.[38] Mutations in several other genes were acquired in several of the sequenced strains, but none of these genes were mutated more than once, they were only found in combination with additional mutations and none of the genes was associated with the MarA regulon (Table 2).[14,15] While some or all of these mutations might, in principle, contribute to the growth compensation, in this study we have decided to focus only on the two genes *marA* and *lon*, where the data show that the compensation is directly linked to the fitness cost caused by the parental *marR* mutation.

Notably, 7 of the 20 strains did not carry mutations in *marA* or *lon*. Each of these isolates carried a mutation in *arcA* and/or *gltP* (Table 2), indicating that these mutations by themselves lead to a significant increase in cellular fitness. We tested this hypothesis by measuring the relative fitness of an isolate that had acquired only a single *arcA* mutation during the evolution experiment (CH6970: *marR* I18fs, *arcA* P58R). The relative fitness of this strain was indistinguishable from the strains that carried mutations in *marA* or *lon* (Table 1). The seven isolates without *marA* or *lon* mutations most likely represent instances where the mutations in *arcA* and/or *gltP* happened to appear first. Extending the time of the evolution experiment would probably result in additional *marA* or *lon* mutations within these lineages.

Among the 20 whole-genome-sequenced evolved strains, 9 had acquired mutations within the *marA* coding sequence (or acquired large deletions including *marA*) and 4 had acquired mutations within or upstream of *lon* (Table 2). Growth competition experiments showed that single mutations in either *marA* or *lon* are sufficient to restore cellular fitness (Table 1). Overproduction of Lon has previously been shown to inhibit translation in *E. coli* and reduce cellular fitness.[39] The positive effect caused by Lon overproduction in this study is most likely due to the relatively low level of overproduction (3-fold compared with ∼40-fold[39]) so that the cost of overproduction is balanced by the positive effect due to increased MarA degradation. The strains with mutations in *marA* had $MIC_{Cip}$ values that were changed back to the WT level (Table 2). The strains that acquired mutations within or upstream of *lon* also had increased susceptibility to ciprofloxacin, although not fully back to the WT susceptibility level (Table 2). Measurements of mRNA levels of *acrA*, *acrB* and *tolC* showed that these compensatory mutants had a reduced level of each of the efflux pump components (Table 3). Taken together these data show that mutations in *marA* or *lon* compensate for the fitness costs of the *marR* mutation by reducing or abolishing transcriptional regulation of the MarA regulon (Figure 2). The predicted effects of the selected mutations in *marA* or *lon* agree with the known activities of these genes.[14,15,34] Thus, *marA*, the direct transcriptional regulator of the *mar* regulon, was genetically inactivated by several of the selected compensatory mutations.

Our data show that overexpression of the AcrAB-TolC efflux pump is only a minor contributing factor to the fitness cost imposed by inactivation of MarR (Table S2). Therefore, it is theoretically possible to reduce the fitness cost without losing the resistance phenotype. The MarA regulon includes more than 100 genes and it is not known which of these genes are responsible for the observed fitness cost.[14,15] If the majority of the fitness cost were caused by altered expression of a single gene or operon within the MarA regulon then it should be possible to mutationally alter expression of this specific gene/operon thus restoring cellular fitness without loss of the resistance phenotype. A fitness cost that is caused by the complex interaction of expression changes of multiple genes cannot be easily compensated for without altering the expression of the whole MarA regulon. Our experiments showed that fitness-compensatory evolution of strains with an inactivated *marR* gene acts via reduction of cellular MarA concentration. These data indicate that the fitness cost imposed by MarR inactivation is the result of multiple contributing factors and compensatory evolution is closely associated with reduced expression of the regulon and concomitant loss of the resistance phenotype.

In conclusion, we have shown that high fitness-cost *marR* mutations can be compensated for by the acquisition of mutation in *marA* or *lon* but the increase in cellular fitness is accompanied by the loss of the resistance phenotype. This fitness/resistance trade-off poses a barrier to the evolution of MarR inactivation mutants in clinical isolates where both high fitness and high resistance levels are required for successful propagation.[7]

## Funding

This work was supported by grants to Diarmaid Hughes from Vetenskapsrådet (the Swedish Research Council) (grant number 2017-03593) and 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

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

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