Mutant RNA polymerase can reduce susceptibility to antibiotics via ppGpp-independent induction of a stringent-like response

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Received 29 April 2020; accepted 12 October 2020

Background: Mutations in RNA polymerase (RNAP) can reduce susceptibility to ciprofloxacin in Escherichia coli, but the mechanism of transcriptional reprogramming responsible is unknown. Strains carrying ciprofloxacin-resistant (CipR) rpoB mutations have reduced growth fitness and their impact on clinical resistance development is unclear.

Objectives: To assess the potential for CipR rpoB mutations to contribute to resistance development by estimating the number of distinct alleles. To identify fitness-compensatory mutations that ameliorate the fitness costs of CipR rpoB mutations. To understand how CipR rpoB mutations reprogramme RNAP.

Methods: E. coli strains carrying five different CipR rpoB alleles were evolved with selection for improved fitness and characterized for acquired mutations, relative fitness and MICCip. The effects of dksA mutations and a ppGpp0 background on growth and susceptibility phenotypes associated with CipR rpoB alleles were determined.

Results: The number of distinct CipR rpoB mutations was estimated to be >100. Mutations in RNAP genes and in dksA can compensate for the fitness cost of CipR rpoB mutations. Deletion of dksA reduced the MICCip for strains carrying CipR rpoB alleles. A ppGpp0 phenotype had no effect on drug susceptibility.

Conclusions: CipR rpoB mutations induce an ppGpp-independent stringent-like response. Approximately half of the reduction in ciprofloxacin susceptibility is caused by an increased affinity of RNAP to DksA while the other half is independent of DksA. Stringent-like response activating mutations might be the most diverse class of mutations reducing susceptibility to antibiotics.

Introduction

Ciprofloxacin is an important antibiotic with activity against Gram-negative and Gram-positive bacteria.1 It binds to DNA gyrase and topoisomerase IV and inhibits the re-ligation of cleaved DNA.3,4 The accumulation of DNA breaks leads to bacterial chromosome fragmentation and ultimately to cell death.5 Ciprofloxacin resistance mutations are commonly located in genes encoding the drug targets DNA gyrase (gyrA, gyrB) and topoisomerase IV (parC, parE).5 Additionally, mutations in genes encoding regulatory proteins of efflux pumps (marR, acrR and soxR) can lead to increased drug efflux.6 There are also horizontally acquired genes that reduce susceptibility to ciprofloxacin by protecting the drug target, modifying the drug, or encoding a novel efflux pump.7–11 In Gram-negatives such as Escherichia coli no individual mutation or acquired gene is sufficient to increase resistance above the clinical breakpoint.12,13 Recent research has revealed that mutations in transcription- and translation-related genes, including RNA polymerase (RNAP) and tRNA synthetase genes, can reduce susceptibility to ciprofloxacin.14,15 These mutations lead to global changes in bacterial protein synthesis with a net benefit under ciprofloxacin-selection conditions. Mutations in tRNA synthetase genes were shown to induce the bacterial stringent response by reducing the supply of aminoacylated-tRNA to the ribosome, but the underlying mechanism by which mutations in the RNAP itself reduce susceptibility has not been elucidated. Although their clinical impact is not yet clear, polymorphisms within the RNAP genes are found in about 6% of ciprofloxacin-resistant (CipR) clinical E. coli.14 Some of these mutations are similar to those selected in vitro, but sequencing data alone cannot determine whether they were selected to reduce ciprofloxacin susceptibility. A factor that could potentially limit the role of transcription/translation-related resistance mutations in the clinical setting is that they generally cause a substantial fitness...
cost. The mutations described so far reduce bacterial fitness by 20%–50% and might be counter-selected. In the case of costly rpoB mutations causing rifampicin resistance (RifR) fitness-compensatory mutations are frequently selected and help maintain the resistance clinically, but compensatory mutations have not yet been identified for CipR rpoB mutations.

In this study, we investigated parameters that could influence the potential impact of CipR rpoB mutations on clinical resistance development in E. coli. Our aims were to: (i) estimate the number of distinct CipR rpoB mutations; (ii) identify and characterize mutations that improve growth fitness of strains carrying CipR rpoB mutations; and (iii) use the compensatory mutations to identify the underlying mechanism by which the transcriptional pattern of the RNAP is altered to reduce susceptibility to ciprofloxacin.

Materials and methods

Bacterial strains and growth conditions

Strains were derived from the E. coli K12 strain MG1655. Bacteria were grown at 37°C in LB broth or on Luria agar (LA) plates (LB solidified with 1.5% agar, Oxoid). Antibiotics were purchased from Sigma–Aldrich (Stockholm, Sweden). Final concentrations of antibiotics were: ciprofloxacin, 0.008–4 mg/L (see MIC test); rifampicin, 1–32 mg/L (see MIC test); tetracycline, 15 mg/L; chloramphenicol, 60 mg/L; ampicillin, 100 mg/L.

Strain constructions

The construction of strains containing rpoB mutations was described previously. Deletions of dksA, relA and spoT were introduced into the WT E. coli chromosome (spoT deletion into a ΔrelA background) by λ-Red recombineering using the pSIMS plasmid and a tetracycline resistance cassette (tetRA) flanked by FRT sites.

Deletions and fluorescence markers were moved between strains using P1 virA-mediated transduction. Excision of tetRA was done by expression of Flp recombinase from a pCP20 plasmid. dksA mutations were moved using the Duplication-Insertion Recombineering method with a pSIM6 recombinase plasmid and a cat-sacB selectable/counter-selectable cassette.

Estimation of RNAP mutational target size

Independent mutations selected for reduced susceptibility to ciprofloxacin were isolated in rpoB or rpoC (22 once and three mutations 2X, 4X and 6X, respectively) (Table S1 available as Supplementary data at JAC Online). To simplify calculation, we assumed 22 mutations were isolated once and three mutations isolated twice. The mutational target size NNRAP was defined as the total number of distinct mutations that will most likely result in the observed distribution (22 singles, three doubles) when 28 random mutations are randomly selected. We simulated evolution experiments by selecting 28 random mutations for various mutational target sizes (60, 70, ... , 170). Selection of random mutations was performed using Excel for Mac 15.40 (Microsoft). Each simulation was repeated 500 times and the average numbers of single isolations were plotted against the respective mutational target size (Figure S1). NNRAP was calculated based on a second-order polynomial trend line. This calculation assumes all mutations have an equal chance of being selected, which is most likely not the case. Mutational target size NNRAP is therefore an underestimate because mutations selected more frequently will skew the distribution to more multiple isolations.

Evolution by serial passage

Independent lineages of each strain were grown overnight with shaking at 37°C in 2 mL of LB. After each cycle of growth, 2 μL of culture was transferred into 2 mL of fresh LB medium to initiate the next cycle. Every 10 cycles (100 generations) cultures were diluted in 0.9% NaCl and approximately 100 cfu were spread onto LA plates and grown overnight at 37°C to visually assess growth improvement. For each lineage, the largest colony was isolated and exponential growth rates of the isolated clones were measured. After 20 cycles (200 generations) isolates from all lineages displayed improved growth rates.

Growth rate measurements

Exponential growth rates were measured using a Bioscreen C machine (Oy Growth curves Ab Ltd). Cultures were grown overnight in LB, diluted 3000-fold in fresh LB (~10⁶ cfu/mL), then 300 μL incubated at 37°C with continuous shaking in honeycomb microtitre plates. OD (600 nm) was measured at 5 min intervals. Doubling times were calculated from the increase in OD over a sliding window of 10 measurement points. Maximum exponential growth rates were defined as the measurement window with the minimal doubling time. All measurements were performed on three independent cultures.

MIC determination

MICs were determined using broth microdilution in 96-well round-bottomed microtiter plates. Bacteria were suspended in 0.9% NaCl to 0.5 McFarland and 100-fold diluted in 100 μL of LB medium containing rifampicin (1, 2, 4, 8, 10, 12, 14, 16 and 32 mg/L) or ciprofloxacin (0.008, 0.016, 0.032, 0.048, 0.064, 0.125, 0.25, 0.5, 1, 2 and 4 mg/L). MICs were assessed visually after incubation for 18 h at 37°C. All measurements were performed on three independent cultures.

Time–kill assay

Approximately 2×10⁸ cfu were transferred from an exponentially growing culture to 2 mL of pre-warmed LB (~10⁶ cfu/mL) containing ciprofloxacin at 0, 0.25, 0.5, 2 and 4 mg/L. Cultures were grown at 37°C in a shaking water bath and bacterial survival was assessed after 0, 2 and 4 h by plating dilutions on LA plates.

Growth competition experiments

Growth competition experiments were performed as previously described. Briefly, strains for competition experiments were labelled with a galK::SYFP2–kanR cassettes (SYFP2 encodes a yellow fluorescence protein) and competed against the isogenic parental strain without rpoB mutation (CH2133) labelled with a galK::mTagBFP2–kanR cassettes (mTagBFP2 encodes a blue fluorescence protein). For each competition, three independent cultures of each strain were grown for 18 h at 37°C in LB. The fluorescence marker strains to be competed were mixed 1:1 and then 2 μL of the mixes was used to inoculate 2 mL of LB at indicated ciprofloxacin concentrations. Mixtures were grown for 24 h at 37°C. Populations were analysed in the initial 1:1 mixture and following the growth cycle (representing 10 generations of growth difference) with a MACSQuant VIB (Miltenyi Biotec). For each population, 10000 cells were counted and the ratio YFP:BFP was determined. The ratios were used to calculate the selective coefficients for each culture using the equation s=[In(R(t)/R(0))]/t. Minimal selective concentrations (MSCs) were determined by calculating the x-intercept of a second-order binomial trend curve as previously described.

PCR and local sequencing

DNA amplification was performed using 2X PCR Mastermix (Thermo Scientific, Waltham, MA, USA), according to the protocol of the manufacturer.
Amplification products were purified using SureClean Plus (BioLine, Germany), according to the protocol of the manufacturer, and sequencing of purified PCR products was performed by Macrogen (Amsterdam, the Netherlands). Sequences were analysed with the CLC Main Workbench 8.0.1 (CLCbio, Qiagen, Denmark). Primers to amplify and sequence the dksA gene were dksA_fw: TGTGTGTCGTATCATCTTTT and dksA_rv: TTATACATTCTGGTCCGTT.

**WGS**

Genomic DNA was prepared using the MasterPure DNA Purification Kit (Epicentre, Illumina Inc., Madison, WI, USA), according to the manufacturer’s instructions. Samples were prepared for sequencing according to Nextera® XT DNA Library Preparation Guide (Rev. D) (Illumina Inc.). Sequencing was performed using a MiSeq™ desktop sequencer, according to the manufacturer’s instructions (Illumina Inc.). Sequences were analysed with the CLC Genomic Workbench 11.0.1 (CLCbio, Qiagen, Denmark). Full genotypes of sequenced strains are shown in Table S2.

**Structural analysis**

Molecular graphics and analyses on an X-ray crystal structure of E. coli RNAP and DksA/ppGpp complex (PDB code 5VSW3)1 were performed with chimera version 1.13.1.32

**Statistical analysis**

Statistical analysis of the relative growth rate measurements was performed with the R software version 3.5.0 using two-tailed unpaired t-tests.

**Results**

**Many RNAP mutations reduce susceptibility to ciprofloxacin**

Previous work identified six rpoB mutations that reduce susceptibility to ciprofloxacin.14 Additional RNAP mutations affecting rpoB or rpoC were subsequently identified during selections for ciprofloxacin resistance.15,26 Currently, we know of 25 distinct RNAP mutations, with 15 mutations in rpoB and 10 in rpoC (Table S1). Their locations in the RNAP structure fall within two clusters previously identified.14 Surprisingly, most mutations (22 of 25) were isolated only once, indicating that a large number of distinct RNAP mutations is able to reduce susceptibility to ciprofloxacin. Based on the observed distribution of mutations, the total mutational target size was estimated to include more than 100 distinct mutations (see the Materials and methods section). This large mutational target size suggests RNAP mutations are a common class among mutations selected to reduce susceptibility to ciprofloxacin.

**Evaluation of putative fitness-compensatory mutations**

Four mutations affected marA of which one deleted the entire marA gene and another deleted the marA promoter (Table 1). Inactivation mutations of MarA have recently been shown to be selected to reduce the fitness cost imposed by mutations in marR, which cause overexpression of marA.13 Therefore, the selection of
marA mutations in this study is most likely due to the presence of a marR S65fs mutation in genetic background rather than the specific cost imposed by the CipR rpoB mutations. Second-site mutations in RNAP genes (rpoA, rpoB and rpoC) were previously shown to reduce the fitness costs imposed by rpoB mutations that increase resistance to rifampicin.\textsuperscript{19,34,35} More than half (6 of 10) of the mutations identified in this study are identical (3\% \times 3) or alter the same amino acid (3\% \times 3) as mutations that compensate the fitness cost of RifR rpoB mutations. These mutations have previously been characterized and are likely to represent a general mechanism to compensate the fitness cost imposed by rpoB mutations (RifR and CipR) rather than the specific cost caused by CipR rpoB mutations.\textsuperscript{19,34,35} Therefore, further analysis was focused on the novel class of mutations selected in dksA. Faster growing isolates can be selected even for WT E. coli MG1655, usually as a result of increased uptake of amino acids or sugars from the growth medium.\textsuperscript{16} To ensure that mutations in dksA are not generally selected to improve growth even in the absence of CipR rpoB mutations, we evolved three cultures of the isogenic parental strain (CH2133) for 200 generations and sequenced the dksA genes of the resulting evolved isolates. None of the three isolates carried a mutation in dksA, indicating that the identified mutations are specific for strains with CipR rpoB alleles. Twenty additional lineages of the strain containing rpoB Δ442–445 (the strain where three of the four dksA mutations were selected during the evolution experiment) were evolved for 100 generations and the dksA gene of a single isolate per lineage was sequenced to identify additional independent compensatory dksA mutations. Mutations in dksA were identified in 18 of the 20 isolates, increasing the number of independent dksA mutations to 22 (Figure 1 and Table S4). The majority (20 of 22) of mutations were amino acid substitutions affecting four different residues in DksA: D71 (15\% \times 3), L95 (1\% \times 3), A132 (1\% \times 3) and D137 (3\% \times 3). These mutations in dksA are localized in the coiled-coil tip (D71) that interacts with the catalytic site of the RNAP, or in amino acids that are part of the ppGpp-binding pocket (L95, A132 and D137) (Figure 1b). The remaining two mutations were located upstream of the coding sequence in the dksA regulatory region,\textsuperscript{27} one mutation (dksA nt G–10A) in the ribosomal-binding site (RBS) and one (dksA nt T–69G) in the extended −10 element (ext −10) (Figure 1c).

### Table 1. Genotypes and phenotypes of evolved isolates and parental strains

| Strain       | Relevant genotype\textsuperscript{a} | Evolution (generations) | Relative fitness ± SD\textsuperscript{c} | MIC (mg/L) |
|--------------|--------------------------------------|-------------------------|------------------------------------------|------------|
| CH1464       | CipR                                 | WT                      | 1.00±0.02                                | 0.016      |
| CH2133       | CipR                                 | isogenic parent         | 0.91±0.02                                | 0.5        |
| CH4959       | CipR Δ442–445                        | 100                     | 0.76±0.00**                               | 2          |
| CH8890       | CipR Δ442–445 dksA D71N              | 100                     | 0.76±0.00**                               | 2          |
| CH8891       | CipR Δ442–445 dksA L95P              | 100                     | 0.76±0.00**                               | 1          |
| CH8892       | CipR Δ442–445 dksA D71A              | 100                     | 0.74±0.00**                               | 1          |
| CH1314       | CipR S455dup                         | 200                     | 0.88±0.03**                               | 0.125      |
| CH8953       | CipR S455dup dksA nt G–10A, rpoB     | 200                     | 0.64±0.01***                              | 0.5        |
| CH8954       | CipR S455dup ΔmarA                   | 200                     | 0.61±0.00**                               | 0.5        |
| CH8955       | CipR S455dup marA I18T               | 200                     | 0.61±0.00**                               | 0.5        |
| CH3144       | CipR E1272G                          | unevolved               | 0.67±0.02                                | 1          |
| CH8956       | CipR rpoC G1136S, ΔmarA              | 200                     | 0.81±0.01***                              | 0.125      |
| CH8887       | CipR rpoC E1200K                     | 100                     | 0.78±0.00**                               | 0.5        |
| CH8889       | CipR rpoA R191P                      | 100                     | 0.79±0.01**                               | 0.5        |
| CH2332       | CipR A1277V                          | unevolved               | 0.73±0.00                                | 2          |
| CH8875       | CipR rpoA R191C                      | 100                     | 0.91±0.00**                               | 1          |
| CH8876       | CipR rpoA H419P                      | 100                     | 0.78±0.00**                               | 1          |
| CH8878       | CipR rpoB Q618L                      | 100                     | 0.89±0.01**                               | 1          |
| CH3073       | CipR E1279G                          | unevolved               | 0.55±0.00                                | 2          |
| CH8879       | CipR rpoB R637C                      | 100                     | 0.69±0.00**                               | 2          |
| CH8880       | CipR rpoB G1136V                     | 100                     | 0.72±0.03**                               | 1          |
| CH8881       | CipR rpoB L1275Q                     | 100                     | 0.70±0.01**                               | 1          |

CIP, ciprofloxacin; RIF, rifampicin.

\textsuperscript{a}Full genotypes are shown in Table S2.

\textsuperscript{b}CipR: gyrA D87G, gyrB S464A, marR S65fs.

\textsuperscript{c}Mutations in rpoB that were selected for increased ciprofloxacin resistance.

\textsuperscript{d}Fitness ± SD relative to the WT.

\textsuperscript{e}Significance compared with the respective unevolved parental strain was calculated using a two-tailed unpaired t-test (**P < 0.001).
Mutations in dksA increase fitness of strains carrying each of the five rpoB mutations

During the evolution experiment, dksA mutations were only isolated in strains carrying the rpoB mutations located in cluster I (rpoB Δ442–445 and S455dup). The absence of dksA mutations selected in isolates with rpoB mutations in cluster II (rpoB E1272G, A1277V and E1279G) could be due to chance (e.g. compensatory mutations in RNAP genes are more frequent or have a larger effect than dksA mutations), or indicate that the underlying mechanism of the rpoB mutations in cluster I differs from those in cluster II. In the latter hypothesis, dksA mutations might not have an effect on the rpoB mutations in cluster II. Three dksA mutations (dksA nt T–69G, nt G–10A and L95P) representing the three different mutation types (ext –10, RBS and protein alteration) were moved into each of the five unevolved parental strains carrying CipR rpoB mutations, the isogenic parental strain with WT rpoB, and a ciprofloxacin-susceptible WT strain (Table 2). The dksA mutations were either neutral or imposed a small fitness cost in the parental strain and the WT. In contrast, all three dksA mutations had a positive effect on the fitness of strains carrying each of the five different CipR rpoB mutations. The improvement in fitness for strains carrying CipR rpoB mutations in cluster I (mean improvement 24%) was slightly larger than for cluster II (mean improvement 16%) (Table 2, Table S5 and Figure S3). These data show that the fitness cost imposed by each of five CipR rpoB mutations can be ameliorated by mutations affecting dksA. Accordingly, differences in the frequency of dksA mutations selected in the different CipR rpoB backgrounds do not indicate mechanistic differences between the various CipR rpoB mutations.

The decrease of ciprofloxacin susceptibility caused by CipR rpoB mutations is partly dependent on DksA but independent of ppGpp

The identification of fitness-compensatory mutations in dksA indicates that the mechanism responsible for transcriptional reprogramming is related to the stringent response. Under starvation conditions, the cellular concentration of the alarmone ppGpp is increased by RelA and/or SpoT. Binding of ppGpp to the RNAP increases its affinity for the transcription factor DksA and the resulting formation of an RNAP–DksA–ppGpp complex leads to a shift in the cellular transcriptional pattern referred to as the stringent response (Figure 2a).38 The significance of selecting compensatory mutations in dksA is that it indicates that the CipR rpoB mutations induce a stringent-like response in the absence of a starvation signal, as has been described for similar mutations in the RNAP genes.39 Theoretically, the CipR rpoB mutations could affect transcriptional reprogramming in different ways: (i) render RNAP hypersensitive to ppGpp, leading to RNAP–DksA–ppGpp complex formation at basal ppGpp concentrations; (ii) create a conformational change in RNAP causing ppGpp-independent binding of DksA; or (iii) change the conformation of the RNAP such that a stringent-like response is activated independently of ppGpp and DskA (Figure 2b). To distinguish between these possibilities, we measured in each strain the effects on ciprofloxacin susceptibility of the absence of DksA (ΔdksA) or ppGpp (ppGpp0, ΔrelA/ΔspoT) (Table 3). Removal of DksA or ppGpp had no effect on the MICCip for either the WT strain or the parental strain (gyrA D87G, gyrB S464A, marR S65fs). Similarly, the MICCip for strains carrying each of the

Figure 1. Overview of dksA mutations. (a) Structure of RNAP (grey) in complex with RpoD (yellow), DksA (turquoise) and ppGpp (red) (PDB code 5VSW31). The RpoB Δ442–445 mutation is indicated in green and DksA mutations are shown in purple. DksA side chains that interact with ppGpp are shown in black and mutated residues in purple. (b) Close-up view of DksA (turquoise) and ppGpp (red). DksA side chains that interact with ppGpp are shown in black and mutated residues in purple. (c) Overview over the dksA promoter.37
five Cip<sup>R</sup> rpoB alleles was unchanged in a ppGpp<sup>0</sup> background indicating that induction of the stringent-like response by these mutations is fully ppGpp independent. In contrast, deletion of dksA reduced the MIC<sub>Cip</sub> for strains carrying four of the five rpoB alleles (from 2 to 1 mg/L) corresponding to the loss of half of the resistance caused by the Cip<sup>R</sup> rpoB alleles (MIC<sub>Cip</sub> for the parental strain is 0.5 mg/L) (Table 3). The only rpoB allele unaffected by the dksA deletion (rpoB<sup>E1272G</sup>) also caused a smaller increase in MIC<sub>Cip</sub> (from 0.5 to 1 mg/L) than the other alleles. A MIC measurement may be too crude to test the effect of the dksA deletion on this particular rpoB allele. Therefore, we performed competition experiments in the presence of various concentrations of ciprofloxacin to test whether deletion of the dksA gene also affects the rpoB<sup>E1272G</sup> allele. A strain that carries the ciprofloxacin resistance target and efflux mutations (gyrA<sup>D87G</sup>, gyrB<sup>S464A</sup>, marR<sup>S65fs</sup>) was competed against isogenic strains with: (i) the rpoB<sup>E1272G</sup> allele, and (ii) the rpoB<sup>E1272G</sup> allele in combination with the dksA deletion (Figure S4). The MSC of ciprofloxacin (MSC<sub>Cip</sub>) was determined for each competition and displayed a 1.6-fold increase when the dksA gene was deleted (rpoB<sup>E1279G</sup>: 0.36 mg/L; rpoB<sup>E1272G</sup>, dksA<sup>nt</sup>: 0.57 mg/L). A higher MSC generally indicates an increased drug susceptibility. This result indicates that also the rpoB<sup>E1272G</sup> allele displays an increased ciprofloxacin susceptibility when the dksA gene is deleted. Taken together, these results show that approximately half of the increase in MIC<sub>Cip</sub> associated with the Cip<sup>R</sup> rpoB alleles is caused by binding of DksA to RNAP (ppGpp-independent activation of a stringent-like response) while the other half is caused by a change in the RNAP transcription pattern (ppGpp- and DksA-independent activation of a stringent-like response).

**Activation of the stringent-like response is independent from the strain background**

It has previously been shown that DNA gyrase mutations can alter the global patterns of gene expression due to changes in the DNA

### Table 2. Effects of dksA mutations in different Cip<sup>R</sup> rpoB backgrounds

| Strain       | Relevant genotype | Relative fitness ± SD<sup>b,c</sup> | CIP MIC (mg/L) |
|--------------|-------------------|--------------------------------------|----------------|
| CH1464       | –                 | 1.00±0.02                            | 0.016          |
| CH9168       | –                 | 1.01±0.00 n.s.                        | 0.016          |
| CH9169       | –                 | 0.96±0.01*                           | 0.016          |
| CH9285       | –                 | 1.00±0.02 n.s.                        | 0.016          |
| CH2133       | Cip<sup>R</sup>   | 0.91±0.02                            | 0.5            |
| CH9171       | Cip<sup>R</sup>   | 0.92±0.01 n.s.                        | 0.5            |
| CH9172       | Cip<sup>R</sup>   | 0.87±0.04 n.s.                        | 0.5            |
| CH9286       | Cip<sup>R</sup>   | 0.88±0.01 n.s.                        | 0.5            |
| CH9495       | Cip<sup>R</sup> Δ442-445 | 0.63±0.01                           | 2              |
| CH9174       | Cip<sup>R</sup> Δ442-445 | 0.73±0.00 ***                      | 1              |
| CH9175       | Cip<sup>R</sup> Δ442-445 | 0.68±0.01 ***                      | 1              |
| CH9287       | Cip<sup>R</sup> Δ442-445 | 0.76±0.01 ***                      | 1              |
| CH3141       | Cip<sup>R</sup> S455dup | 0.52±0.01                           | 2              |
| CH9177       | Cip<sup>R</sup> S455dup | 0.55±0.00 ***                      | 1              |
| CH9178       | Cip<sup>R</sup> S455dup | 0.59±0.00 ***                      | 1              |
| CH9288       | Cip<sup>R</sup> S455dup | 0.58±0.01 ***                      | 1              |
| CH3144       | Cip<sup>R</sup> E1272G | 0.67±0.02                           | 1              |
| CH9180       | Cip<sup>R</sup> E1272G | 0.71±0.01                           | 1              |
| CH9181       | Cip<sup>R</sup> E1272G | 0.72±0.00*                          | 1              |
| CH9289       | Cip<sup>R</sup> E1272G | 0.72±0.01*                          | 1              |
| CH2332       | Cip<sup>R</sup> A1277V | 0.73±0.00                           | 2              |
| CH9183       | Cip<sup>R</sup> A1277V | 0.76±0.01 ***                      | 2              |
| CH9184       | Cip<sup>R</sup> A1277V | 0.77±0.00 ***                      | 2              |
| CH9290       | Cip<sup>R</sup> A1277V | 0.78±0.01 ***                      | 1              |
| CH3073       | Cip<sup>R</sup> E1279G | 0.55±0.00                           | 2              |
| CH9186       | Cip<sup>R</sup> E1279G | 0.57±0.03 n.s.                        | 2              |
| CH9187       | Cip<sup>R</sup> E1279G | 0.58±0.01 ***                      | 2              |
| CH9291       | Cip<sup>R</sup> E1279G | 0.56±0.01 n.s.                        | 2              |

CIP, ciprofloxacin.

<sup>a</sup>Cip<sup>R</sup>: gyrA<sup>D87G</sup>, gyrB<sup>S464A</sup>, marR<sup>S65fs</sup>.

<sup>b</sup>Fitness ± SD relative to the WT.

<sup>c</sup>Significance compared with the respective isogenic parental strain (dksA<sup>WT</sup>) was calculated using a two-tailed unpaired t-test (n.s., non-significant; *, P<0.05; **, P<0.01; ***, P<0.001).
Thus, we selected one CipR mutation from each cluster (cluster I: rpoB Δ442–445, cluster II: rpoB E1279G) and measured the MIC_{CIP} for strains that carry the CipR rpoB mutations but no other ciprofloxacin resistance mutations. The presence of either rpoB mutations led to a 3-fold increase of the MIC_{CIP} (from 0.016 to 0.048 mg/L), which is comparable to the 4-fold MIC_{CIP} increase seen in strains with the ciprofloxacin resistance mutations in the strain background (Table 3). As expected, deletion of the dksA gene increased susceptibility to ciprofloxacin to 0.016–0.032 mg/L (Table 3). We conclude that the activation of the stringent-like response by the rpoB mutations is independent of ciprofloxacin resistance mutations in the strain background. This agrees with the finding that RNA polymerase mutations are selected in combination with many different mutations in gyrA (D82N, S83, S83L, D87G, D87Y), gyrB (S464A, V467E, AC476), parC (G78D, S80R) and parE (Q428E).14,15

**Table 3. Effects of CipR rpoB alleles in ΔdksA and ppGpp\(^{b}\) backgrounds**

| Strain | Relevant genotype | CIP MIC (mg/L) |
|--------|------------------|----------------|
| CH1464 | – – ΔksA         | 0.016          |
| CH9157 | – – ΔrelA, AspoT | 0.016          |
| CH9217 | – Δ442–445       | 0.048          |
| CH9748 | – Δ442–445 ΔksA  | 0.016          |
| CH2379 | – E1279G         | 0.048          |
| CH9750 | – E1279G ΔksA    | 0.032          |
| CH2133 | Cip\(^{b}\) – –  | 0.5            |
| CH9201 | Cip\(^{b}\) ΔksA | 0.5            |
| CH9230 | Cip\(^{b}\) ΔrelA, AspoT | 0.5 |
| CH4959 | Cip\(^{b}\) Δ442–445 | 2              |
| CH9203 | Cip\(^{b}\) Δ442–445 ΔksA | 1              |
| CH9231 | Cip\(^{b}\) Δ442–445 ΔrelA, AspoT | 2              |
| CH3141 | Cip\(^{b}\) S455dup | 2              |
| CH9205 | Cip\(^{b}\) S455dup ΔksA | 1              |
| CH9232 | Cip\(^{b}\) S455dup ΔrelA, AspoT | 2              |
| CH3144 | Cip\(^{b}\) E1272G | –              |
| CH9207 | Cip\(^{b}\) E1272G ΔksA | 1              |
| CH9233 | Cip\(^{b}\) E1272G ΔrelA, AspoT | 1              |
| CH2332 | Cip\(^{b}\) A1277V | –              |
| CH9209 | Cip\(^{b}\) A1277V ΔksA | 1              |
| CH9234 | Cip\(^{b}\) A1277V ΔrelA, AspoT | 2              |
| CH3073 | Cip\(^{b}\) E1279G | –              |
| CH9211 | Cip\(^{b}\) E1279G ΔksA | 1              |
| CH9235 | Cip\(^{b}\) E1279G ΔrelA, AspoT | 2              |

CIP, ciprofloxacin.

\(^{a}\)Cip\(^{b}\): gyrA D87G, gyrB S464A, marR S65fs.

\(^{b}\)Fitness ± SD relative to the WT.

**Discussion**

Here, we have shown that there is a class of mutations in RNA polymerase that reduce susceptibility to ciprofloxacin by ppGpp-independent activation of a stringent-like response. Approximately half of the effect involves ppGpp-independent binding of DksA to the RNA polymerase, but the other half is independent of both ppGpp and DksA. These results concur with those from a previous study showing that mutations in aminoacyl-tRNA synthetases could decrease antibiotic susceptibility by activating the stringent response.15 Activation of the stringent response was shown to alter the expression of multiple genes related to antibiotic resistance (e.g. mdtK, acrZ and ampF) thus leading to a net benefit in the presence of multiple antibiotics including fluoroquinolones, rifampicin, chloramphenicol, β-lactams and trimethoprim.14,15 Recent selections for reduced susceptibility to ciprofloxacin that we have performed have revealed a large number of genes where mutations could potentially exert their effects by activating a stringent-like response in E. coli (Figure 3, Table S1 and Table S6).14,15 Examples include mutations that delete tRNA genes and mutations affecting tRNA-modification enzymes or aminoacyl-tRNA synthetases that could plausibly reduce the flow of correctly charged aminoacyl-tRNAs into the ribosome, leading to induction of the stringent response. This mechanism has been shown for mutations in tRNA synthetase genes encoding LeuS and AspS.15 Similarly, mutations identified in topology.40 Therefore, it is possible that the induction of the stringent-like response by the CipR rpoB mutations is dependent on the ciprofloxacin resistance mutations present in the strain background (gyrA D87G, gyrB S464A, marR S65fs). To address this possibility, we selected one CipR rpoB mutation from each cluster
The clinical impact of this new class of resistance mutations is hard to estimate. Due to its diversity it is difficult to determine if specific mutations in clinical isolates were selected to increase antibiotic resistance. Additionally, mutations in RNAP and aminoacyl-tRNA synthetase genes have been associated with a reduction of bacterial fitness in the range of 20%-50%, which could potentially reduce their clinical impact. Here, we showed for Cip\textsuperscript{r} rpoB mutations that bacterial fitness can be restored by compensatory mutations while maintaining an elevated resistance level (Table 1) as has previously been shown for rpoB mutations in Rif\textsuperscript{r} Salmonella\textsuperscript{19,34,35} and Mycobacterium tuberculosis\textsuperscript{17,18,43–48} isolates. These results indicate that it is possible to activate a stringent-like response that decreases bacterial susceptibility to various antibiotics without incurring the fitness cost associated with a full activation of the stringent response.

Funding
This work was supported by grants to D.H. 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 S4 and Tables S1 to S6 are available as Supplementary data at JAC Online.

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