J Antimicrob Chemother 2021; **76**: 606–615
doi:10.1093/jac/dkaa469 Advance Access publication 21 November 2020

Journal of Antimicrobial Chemotherapy

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 (Cip^R) *rpoB* mutations have reduced growth fitness and their impact on clinical resistance development is unclear.

Objectives: To assess the potential for Cip^R *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 Cip^R *rpoB* mutations. To understand how Cip^R *rpoB* mutations reprogramme RNAP.

Methods: *E. coli* strains carrying five different Cip^R *rpoB* alleles were evolved with selection for improved fitness and characterized for acquired mutations, relative fitness and MIC_{Cip}. The effects of *dksA* mutations and a $ppGpp^0$ background on growth and susceptibility phenotypes associated with Cip^R *rpoB* alleles were determined.

Results: The number of distinct Cip^R *rpoB* mutations was estimated to be >100. Mutations in RNAP genes and in *dksA* can compensate for the fitness cost of Cip^R *rpoB* mutations. Deletion of *dksA* reduced the MIC_{Cip} for strains carrying Cip^R *rpoB* alleles. A $ppGpp^0$ phenotype had no effect on drug susceptibility.

Conclusions: Cip^R *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.[1,2] 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*).[6] 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 (Cip^R) 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.[13–16] In the case of costly *rpoB* mutations causing rifampicin resistance (Rif^R) fitness-compensatory mutations are frequently selected and help maintain the resistance clinically, but compensatory mutations have not yet been identified for Cip^R *rpoB* mutations.[17–20]

In this study, we investigated parameters that could influence the potential impact of Cip^R *rpoB* mutations on clinical resistance development in *E. coli*. Our aims were to: (i) estimate the number of distinct Cip^R *rpoB* mutations; (ii) identify and characterize mutations that improve growth fitness of strains carrying Cip^R *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.[14] Deletions of *dksA*, *relA* and *spoT* were introduced into the WT *E. coli* chromosome (*spoT* deletion into a Δ*relA* background) by λ-Red recombineering[21] using the pSIM5 plasmid[22] and a tetracycline resistance cassette (*tetRA*) flanked by FRT sites.[23] 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.[23] *dksA* mutations were moved using the Duplication–Insertion Recombineering method[24] with a pSIM6 recombineering plasmid[22] and a *cat-sacB* selectable/counter-selectable cassette.[25]

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 2×, 4× and 6×, respectively) [Table S1 (available as Supplementary data at *JAC* Online)].[15,26] To simplify calculation, we assumed 22 mutations were isolated once and three mutations isolated twice. The mutational target size N_{RNAP} 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× and the average numbers of single isolations were plotted against the respective mutational target size (Figure S1). N_{RNAP} 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 N_{RNAP} 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 (∼10^6 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 2×10^6 cfu were transferred from an exponentially growing culture into 2 mL of pre-warmed LB (∼10^6 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.[27] 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 mixtures was used to inoculate 2 mL of LB at indicated ciprofloxacin concentrations. Mixed cultures 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 VYB (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=[ln(R(t)/R(0))]/[t].[28] Minimal selective concentrations (MSCs) were determined by calculating the *x*-intercept of a second-order binomial trend curve as previously described.[29,30]

PCR and local sequencing

DNA amplification was performed using 2× 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: TGTGTGTCTGTCATCTCTTT and dksA_rv: TTTACATTCTGGTCGCGT.

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 5VSW[31]) 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.

Isolation of fitness-compensatory mutations

Five RNAP mutations that reduce susceptibility to ciprofloxacin (*rpoB* Δ442–445, S455dup, E1272G, A1277V and E1279G) were previously reconstructed into an isogenic background carrying ciprofloxacin target and efflux mutations (*gyrA* D87G, *gyrB* S464A, *marR* S65fs).[14] These strains were chosen as the starting point for this experimental evolution study. By allelic replacement each of the *rpoB* mutations was shown to reduce growth rate 20%–40% (Table 1 and Table S3), increase MIC_{Cip} from 0.5 to 1–2 mg/L and MIC_{Rif} from 12 to 16–32 mg/L (Table 1) and increase bacterial survival in the presence of ciprofloxacin (Figure S2). WGS of each strain and their isogenic parents was made to determine their genotypes relative to MG1655 prior to experimental evolution (Table S2).

Three independent cultures of each strain carrying Cip^R *rpoB* mutations (15 cultures) were evolved by serial passage selecting for improved fitness. After 100 generations of growth, a single colony per culture was isolated (the largest colony after visual assessment), and growth rate as well as the MIC_{Cip} and MIC_{Rif} were determined for the evolved isolates. Isolates from the majority of cultures (11 of 15) displayed an improved growth rate after 100 generations. The remaining cultures were evolved for 200 generations before isolates with improved growth rate were identified (Table 1). The initial fitness costs imposed by the Cip^R *rpoB* mutations was reduced on average by 52% (23%–100%) compared with the isogenic parental strain without the mutation (CH2133). With one exception (CH8879), the MIC_{Cip} and MIC_{Rif} decreased in concert relative to the unevolved parental strains. Compared with the isogenic strain without *rpoB* mutation (CH2133), the MIC_{Cip} remained elevated ($\geq$1 mg/L) in 9 out of 15 isolates and the MIC_{Rif} remained elevated ($\geq$16 mg/L) in 6 out of the 15 isolates (Table 1 and Table S3). Most notable were two isolates (CH8890 and CH8879) where the fitness cost was reduced by 46% and 39%, respectively, without a change in MIC_{Cip} and two isolates (CH8875 and CH8878) that fully restored fitness while maintaining an elevated MIC_{Cip} (Table 1 and Table S3).

The genomes of the evolved strains were sequenced revealing that all had acquired at least one mutation. Most of the evolved strains (11 of 15) carried only a single significant genetic change (amino acid substitutions or promoter mutations). Seven of these 11 isolates differed only in a single nucleotide from the unevolved parent strains, in each case leading to an amino acid change in a protein-coding sequence. Three of the 11 isolates differed in two nucleotides from the unevolved parents with only one of the mutations causing an amino acid substitution and the other mutation causing a synonymous codon change (2×), or being located in an intergenic region that is not part of any known promoter features (1×). Finally, the last isolate contained a 46 nucleotides-long deletion in the *marR* promoter region accompanied by a synonymous codon change (Table S2).

Overall, only five genes (*rpoA, rpoB, rpoC, dksA* and *marA*) were mutated in multiple strains and each of the 15 strains had a mutation in at least one of these five genes (Table 1). These data indicate these five genes as potential targets for compensatory mutations that reduce the fitness cost of carrying Cip^R *rpoB* mutations. Mutations in four genes (*rpoA, rpoB, rpoC* and *dksA*) were isolated in strains that carried no other genetic change showing these specific mutations are both necessary and sufficient to compensate the cost of the respective Cip^R *rpoB* mutations in these strains (Table S2).

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*.[33] Therefore, the selection of

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

Strain	Relevant genotype[a]			Evolution (generations)	Relative fitness ± SD[d,e]	MIC (mg/L)	
	CIP[b]	*rpoB*[c]	compensatory			CIP	RIF
CH1464	–	–	–	WT	1.00±0.02	0.016	10
CH2133	Cip^R	–	–	isogenic parent	0.91±0.02	0.5	12
CH4959	Cip^R	Δ442–445	–	unevolved	0.63±0.01	2	64
CH8890	Cip^R	Δ442–445	*dksA* D71N	100	0.76±0.00***	2	64
CH8891	Cip^R	Δ442–445	*dksA* L95P	100	0.76±0.00***	1	32
CH8892	Cip^R	Δ442–445	*dksA* D71A	100	0.74±0.00***	1	32
CH3141	Cip^R	S455dup	–	unevolved	0.52±0.01	2	64
CH8953	Cip^R	S455dup	*dksA* nt G-10A, *rpoB* V588L, *marA* I18F	200	0.88±0.03***	0.125	10
CH8954	Cip^R	S455dup	Δ*marA*	200	0.64±0.01***	0.5	32
CH8955	Cip^R	S455dup	*marA* I18T	200	0.61±0.00***	0.5	32
CH3144	Cip^R	E1272G	–	unevolved	0.67±0.02	1	32
CH8956	Cip^R	E1272G	*rpoC* G1136S, Δ*marA*	200	0.81±0.01***	0.125	10
CH8887	Cip^R	E1272G	*rpoC* E1200K	100	0.78±0.00***	0.5	12
CH8889	Cip^R	E1272G	*rpoA* R191P	100	0.79±0.01***	0.5	14
CH2332	Cip^R	A1277V	–	unevolved	0.73±0.00	2	16
CH8875	Cip^R	A1277V	*rpoA* R191C	100	0.91±0.00***	1	12
CH8876	Cip^R	A1277V	*rpoC* H419P	100	0.78±0.00***	1	12
CH8878	Cip^R	A1277V	*rpoB* Q618L	100	0.89±0.01***	1	12
CH3073	Cip^R	E1279G	–	unevolved	0.55±0.00	2	32
CH8879	Cip^R	E1279G	*rpoB* R637C	100	0.69±0.00***	2	16
CH8880	Cip^R	E1279G	*rpoC* G1136V	100	0.72±0.03***	1	12
CH8881	Cip^R	E1279G	*rpoC* L1275Q	100	0.70±0.01***	1	14

CIP, ciprofloxacin; RIF, rifampicin.
[a]Full genotypes are shown in Table S2.
[b]Cip^R: *gyrA* D87G, *gyrB* S464A, *marR* S65fs.
[c]Mutations in *rpoB* that were selected for increased ciprofloxacin resistance.
[d]Fitness ± SD relative to the WT.
[e]Significance compared with the respective unevolved parental strain was calculated using a two-tailed unpaired *t*-test (***$P \leq 0.001$).

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 Cip^R *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.[19,34,35] More than half (6 of 10) of the mutations identified in this study are identical (3×) or alter the same amino acid (3×) as mutations that compensate the fitness cost of Rif^R *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 (Rif^R and Cip^R) rather than the specific cost caused by Cip^R *rpoB* mutations.[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.[36] To ensure that mutations in *dksA* are not generally selected to improve growth even in the absence of Cip^R *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 Cip^R *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×), L95 (1×), A132 (1×) and D137 (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,[37] 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).

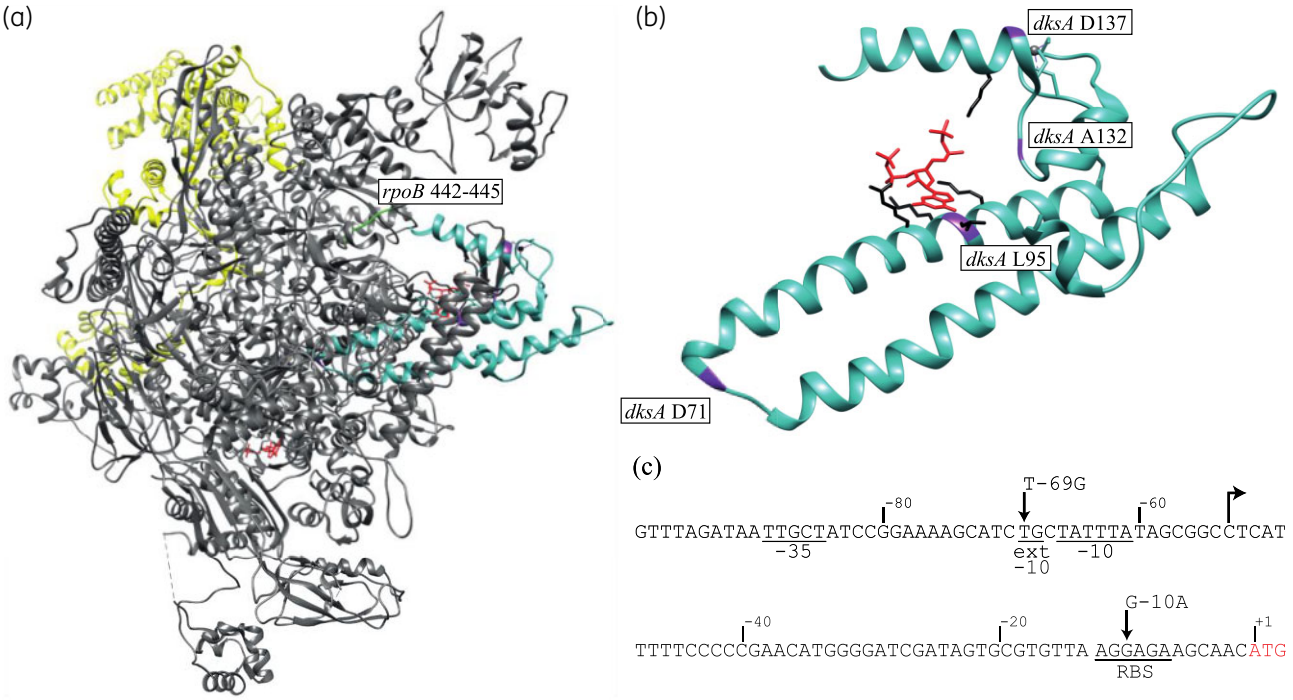

Figure 1. Overview of *dksA* mutations. (a) Structure of RNAP (grey) in complex with RpoD (yellow), DksA (turquoise) and ppGpp (red) (PDB code 5VSW[31]). The RpoB Δ442–445 mutation is indicated in green and DksA mutations are shown in purple. (b) Close-up view of DksA (turquoise) and ppGpp (red). DksA side chains that interact with ppGpp are shown in black and mutated residues in purple. (c) Overview over the *dksA* promoter.[37] The *dksA* start codon is indicated in red, the ribosome- and RNAP-binding sites are shown below the sequence, and the transcriptional start site is indicated by a black arrow over the sequence. Promoter mutations isolated during the evolution experiment are indicated above the sequence.

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 Cip^R *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 Cip^R *rpoB* mutations. The improvement in fitness for strains carrying Cip^R *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 Cip^R *rpoB* mutations can be ameliorated by mutations affecting *dksA*. Accordingly, differences in the frequency of *dksA* mutations selected in the different Cip^R *rpoB* backgrounds do not indicate mechanistic differences between the various Cip^R *rpoB* mutations.

The decrease of ciprofloxacin susceptibility caused by Cip^R 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 Cip^R *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 Cip^R *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 (Δ*dksA*) or ppGpp (ppGpp^0, Δ*relA*/Δ*spoT*) (Table 3). Removal of DksA or ppGpp had no effect on the MIC_{Cip} for either the WT strain or the parental strain (*gyrA* D87G, *gyrB* S464A, *marR* S65fs). Similarly, the MIC_{Cip} for strains carrying each of the

Table 2. Effects of *dksA* mutations in different Cip^R *rpoB* backgrounds

	Relevant genotype				
Strain	CIP[a]	*rpoB*	other	Relative fitness ± SD[b,c]	CIP MIC (mg/L)
CH1464	–	–	–	1.00±0.02	0.016
CH9168	–	–	*dksA* nt T–69G	1.01±0.00$^{n.s.}$	0.016
CH9169	–	–	*dksA* nt G–10A	0.96±0.01*	0.016
CH9285	–	–	*dksA* L95P	1.00±0.02$^{n.s.}$	0.016
CH2133	Cip^R	–	–	0.91±0.02	0.5
CH9171	Cip^R	–	*dksA* nt T–69G	0.92±0.01$^{n.s.}$	0.5
CH9172	Cip^R	–	*dksA* nt G–10A	0.87±0.04$^{n.s.}$	0.5
CH9286	Cip^R	–	*dksA* L95P	0.88±0.01$^{n.s.}$	0.5
CH4959	Cip^R	Δ442–445	–	0.63±0.01	2
CH9174	Cip^R	Δ442–445	*dksA* nt T–69G	0.73±0.00***	1
CH9175	Cip^R	Δ442–445	*dksA* nt G–10A	0.68±0.01**	1
CH9287	Cip^R	Δ442–445	*dksA* L95P	0.76±0.01***	1
CH3141	Cip^R	S455dup	–	0.52±0.01	2
CH9177	Cip^R	S455dup	*dksA* nt T–69G	0.55±0.00**	1
CH9178	Cip^R	S455dup	*dksA* nt G–10A	0.59±0.00***	1
CH9288	Cip^R	S455dup	*dksA* L95P	0.58±0.01***	1
CH3144	Cip^R	E1272G	–	0.67±0.02	1
CH9180	Cip^R	E1272G	*dksA* nt T–69G	0.71±0.01*	1
CH9181	Cip^R	E1272G	*dksA* nt G–10A	0.72±0.00*	1
CH9289	Cip^R	E1272G	*dksA* L95P	0.72±0.01*	1
CH2332	Cip^R	A1277V	–	0.73±0.00	2
CH9183	Cip^R	A1277V	*dksA* nt T–69G	0.76±0.01**	2
CH9184	Cip^R	A1277V	*dksA* nt G–10A	0.77±0.00***	2
CH9290	Cip^R	A1277V	*dksA* L95P	0.78±0.01***	1
CH3073	Cip^R	E1279G	–	0.55±0.00	2
CH9186	Cip^R	E1279G	*dksA* nt T–69G	0.57±0.03$^{n.s.}$	2
CH9187	Cip^R	E1279G	*dksA* nt G–10A	0.58±0.01**	2
CH9291	Cip^R	E1279G	*dksA* L95P	0.56±0.01$^{n.s.}$	2

CIP, ciprofloxacin.
[a]Cip^R: *gyrA* D87G, *gyrB* S464A, *marR* S65fs.
[b]Fitness ± SD relative to the WT.
[c]Significance compared with the respective isogenic parental strain (*dksA*^{WT}) was calculated using a two-tailed unpaired *t*-test ($^{n.s}$, non-significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

five Cip^R *rpoB* alleles was unchanged in a ppGpp^0 background indicating that induction of the stringent-like response by these mutations is fully ppGpp independent. In contrast, deletion of *dksA* reduced the MIC_{Cip} 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^R *rpoB* alleles (MIC_{Cip} for the parental strain is 0.5 mg/L) (Table 3). The only *rpoB* allele unaffected by the *dksA* deletion (*rpoB* E1272G) also caused a smaller increase in MIC_{Cip} (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* E1272G allele. A strain that carries the ciprofloxacin resistance target and efflux mutations (*gyrA* D87G, *gyrB* S464A, *marR* S65fs) was competed against isogenic strains with: (i) the *rpoB* E1272G allele, and (ii) the *rpoB* E1272G allele in combination with the *dksA* deletion (Figure S4). The MSC of ciprofloxacin (MSC_{Cip}) was determined for each competition and displayed a 1.6-fold increase when the *dksA* gene was deleted (*rpoB* E1279G: 0.36 mg/L; *rpoB* E1272G, Δ*dksA*: 0.57 mg/L). A higher MSC generally indicates an increased drug susceptibility. This result indicates that also the *rpoB* E1272G 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_{Cip} associated with the Cip^R *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

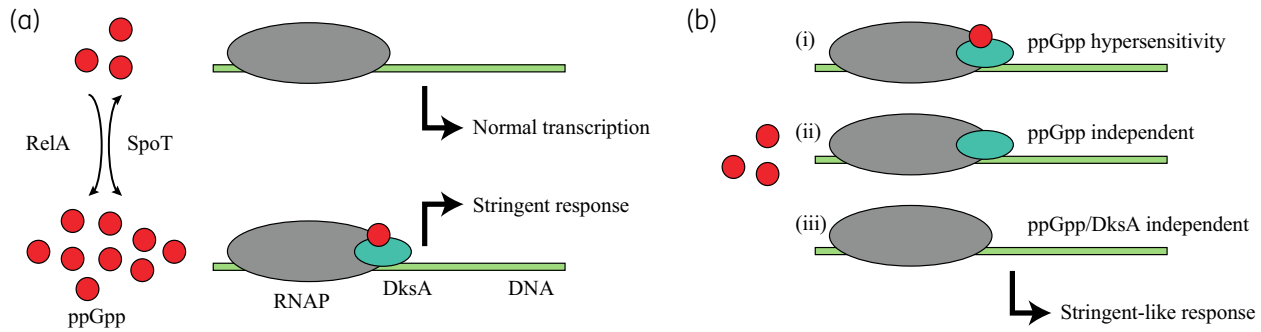

Figure 2. Schematic view of stringent response activation. (a) Upon starvation conditions RelA and/or SpoT cause the accumulation of ppGpp leading to the binding of ppGpp and DksA to the RNAP. The RNAP–DksA–ppGpp complex leads to a shift in the cellular transcriptional pattern. (b) Activation of a stringent-like response at basal ppGpp concentrations. Mutations in *rpoB* could (i) render the RNAP hypersensitive to ppGpp binding, (ii) lead to ppGpp-independent binding of DksA, or (iii) cause ppGpp- and DskA-independent activation of a stringent-like response.

Table 3. Effects of Cip^R *rpoB* alleles in Δ*dksA* and ppGpp^0 backgrounds

	Relevant genotype			
Strain	CIP[a]	*rpoB*	other	CIP MIC (mg/L)
CH1464	–	–	–	0.016
CH9157	–	–	Δ*dksA*	0.016
CH9217	–	–	Δ*relA*, Δ*spoT*	0.016
CH2147	–	Δ442–445	–	0.048
CH9748	–	Δ442–445	Δ*dksA*	0.016
CH2379	–	E1279G	–	0.048
CH9750	–	E1279G	Δ*dksA*	0.032
CH2133	Cip^R	–	–	0.5
CH9201	Cip^R	–	Δ*dksA*	0.5
CH9230	Cip^R	–	Δ*relA*, Δ*spoT*	0.5
CH4959	Cip^R	Δ442–445	–	2
CH9203	Cip^R	Δ442–445	Δ*dksA*	1
CH9231	Cip^R	Δ442–445	Δ*relA*, Δ*spoT*	2
CH3141	Cip^R	S455dup	–	2
CH9205	Cip^R	S455dup	Δ*dksA*	1
CH9232	Cip^R	S455dup	Δ*relA*, Δ*spoT*	2
CH3144	Cip^R	E1272G	–	1
CH9207	Cip^R	E1272G	Δ*dksA*	1
CH9233	Cip^R	E1272G	Δ*relA*, Δ*spoT*	1
CH2332	Cip^R	A1277V	–	2
CH9209	Cip^R	A1277V	Δ*dksA*	1
CH9234	Cip^R	A1277V	Δ*relA*, Δ*spoT*	2
CH3073	Cip^R	E1279G	–	2
CH9211	Cip^R	E1279G	Δ*dksA*	1
CH9235	Cip^R	E1279G	Δ*relA*, Δ*spoT*	2

CIP, ciprofloxacin.
[a]Cip^R: *gyrA* D87G, *gyrB* S464A, *marR* S65fs.
[b]Fitness ± SD relative to the WT.

topology.[40] Therefore, it is possible that the induction of the stringent-like response by the Cip^R *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 Cip^R *rpoB* mutation from each cluster (cluster I: *rpoB* Δ442–445, cluster II: *rpoB* E1279G) and measured the MIC$_{Cip}$ for strains that carry the Cip^R *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 RNAP mutations are selected in combination with many different mutations in *gyrA* (D82N, ΔS83, S83L, D87G, D87Y), *gyrB* (S464A, V467E, ΔC476), *parC* (G78D, S80R) and *parE* (Q428E).[26]

Discussion

Here, we have shown that there is a class of mutations in RNAP 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 RNAP, 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 *ompF*) 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

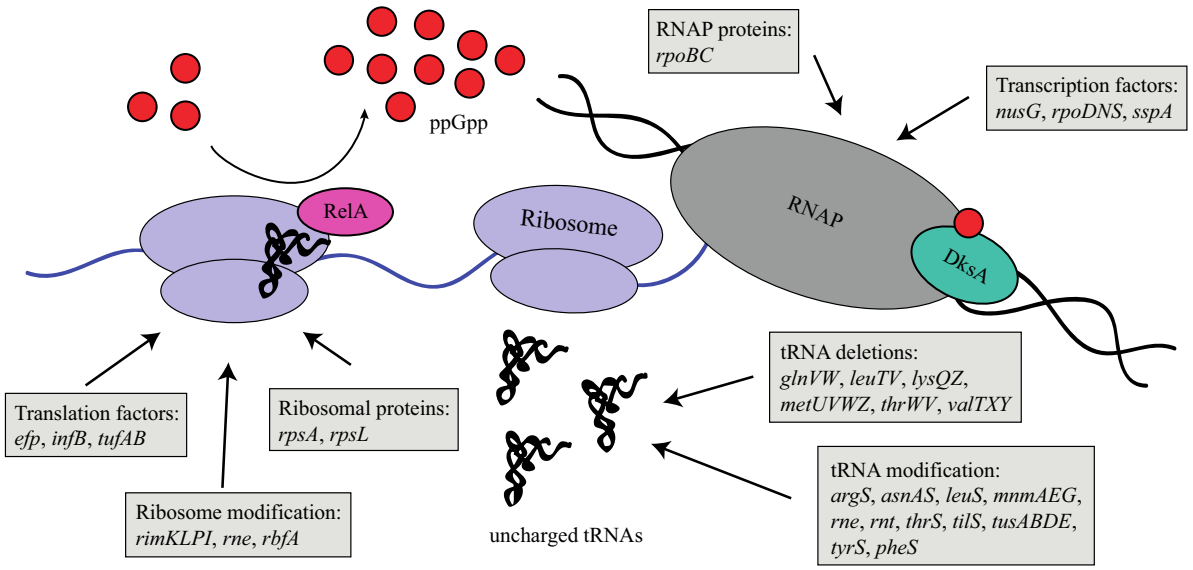

Figure 3. Overview of mutations that could cause an activation of a stringent-like response. Deletions of tRNA genes and mutations in tRNA-modification enzymes could plausibly reduce the flow of correctly charged aminoacyl-tRNAs into the ribosome. Mutated ribosomal proteins, ribosome-modification enzymes and elongation factors could alter the interaction of the ribosomes with uncharged tRNAs and/or RelA. Alterations in RNAP genes and transcription factors could lead to an ppGpp-independent activation of a stringent-like response. A detailed list over all mutations can be found in Tables S1 and S4.

ribosomal proteins, ribosome-modification enzymes and translations elongation factors could also potentially lead to increased binding of uncharged tRNAs to the ribosome and directly or indirectly increase the production of ppGpp. Finally, mutations in RNAP genes or transcription factors could activate a stringent-like response in a ppGpp-independent fashion as shown for the Cip^R *rpoB* mutations in this study (Figure 3, Table S1 and Table S6). We estimate that there are at least 100 distinct mutations in the RNAP genes that could cause this phenotype, which is in the same range as the number of RNAP mutations that can give rise to rifampicin resistance.[20] The mutation rate for rifampicin resistance is around 10^{-8}–10^{-9} per cell per generation,[41,42] indicating that the mutation rate for stringent-like response activating Cip^R *rpoB* mutations probably has a similarly high value. Adding the additional potential mutational targets affecting transcription or translation (discussed above) that could also induce a stringent-like response (Figure 3, Table S1 and Table S6) will most likely increase the mutation rate by one to two orders of magnitude, moving the class into the same order of mutation rate that is observed for mutations that inactivate efflux pump regulators.[13] These data suggest that genes where mutations could modify bacterial gene expression patterns via activation of a stringent-like response constitute a very large and diverse genetic target that could contribute to the evolution of resistance to multiple antibiotics.

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.[14,15] Here, we showed for Cip^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^R *Salmonella*[19,34,35] and *Mycobacterium tuberculosis*[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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