Study of the contribution of active defense mechanisms to ciprofloxacin tolerance in Escherichia coli growing at different rates

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Abstract Using rpoS, tolC, ompF, and recA knockouts, we investigated their effect on the physiological response and lethality of ciprofloxacin in E. coli growing at different rates on glucose, succinate or acetate. We have shown that, regardless of the strain, the degree of changes in respiration, membrane potential, NAD+/NADH ratio, ATP and glutathione (GSH) strongly depends on the initial growth rate and the degree of its inhibition. The deletion of the regulator of the general stress response RpoS, although it influenced the expression of antioxidant genes, did not significantly affect the tolerance to ciprofloxacin at all growth rates. The mutant lacking TolC, which is a component of many E. coli efflux pumps, showed the same sensitivity to ciprofloxacin as the parent. The absence of porin OmpF slowed down the entry of ciprofloxacin into cells, prolonged growth and shifted the optimal bactericidal concentration towards higher values. Deficiency of RecA, a regulator of the SOS response, dramatically altered the late phase of the SOS response (SOS-dependent cell death), preventing respiratory inhibition and a drop in membrane potential. The recA mutation inverted GSH fluxes across the membrane and abolished ciprofloxacin-induced H2S production. All studied mutants showed an inverse linear relationship between logCFU ml-1 and the specific growth rate. Mutations shifted the plot of this dependence relative to the parental strain according to their significance for ciprofloxacin tolerance. The crucial role of the SOS system is confirmed by dramatic shift down of this plot in the recA mutant.

Keywords Ciprofloxacin · Glutathione · Hydrogen sulfide · Membrane potential · ompF, rpoS, tolC and recA knockouts · Respiration · Specific growth rate

Introduction

An in-depth understanding of the physiological response of microorganisms to antibiotic stress and the mechanisms for killing bacteria can be the basis for finding ways to increase the efficiency of existing and...
new antibiotics and prevent the emergence of antibiotic-resistant strains of microorganisms. It is well known that a susceptibility of bacteria to antibiotic-mediated killing is strongly affected by their growth rate. Drug tolerance increases with decreasing growth rates and reduces to a minimum in fast growing cultures (Tuomanen et al. 1986; Eng et al. 1991; Sufya et al. 2003; Greulich et al. 2015; Lee et al. 2018; Smirnova and Oktyabrsky 2018; Pontes and Groisman 2019). We have recently shown that an increase in the specific growth rate (μ) by 0.1 h⁻¹ leads to an increase in the ciprofloxacin-induced killing rate by about 1 h⁻¹, regardless of the reasons affecting the growth rate (medium composition, temperature, pretreatment with various reagents, or mutations) (Smirnova and Oktyabrsky 2018). The specific growth rate can be considered as an integral parameter reflecting the activity of interrelated core cellular processes (chromosome replication, transcription, and translation) and, accordingly, the number of active targets for antibiotics with different types of action (Pontes and Groisman 2020). Target inactivity could block antibiotic action. However, simultaneously with a change in the number of targets, a change in the growth rate is accompanied by metabolic reprogramming under complex regulatory control by signal transduction factors and regulators, including guanosine tetraphosphate (p)ppGpp and the regulator of the general stress response RpoS (σS) (Steinchen and Bange 2016). The levels of both (p)ppGpp and RpoS are inversely proportional to the specific growth rate, increasing linearly with its decrease (Ihsen and Egli 2004; Potrykus et al. 2011). RpoS controls the expression of many proteins involved in the protection of vital biomolecules, i.e., proteins, DNA, and the cell envelope, which provides a high level of resistance of stationary-phase cells to various stresses, including osmotic shock, oxidative stress, heat shock, and acid and base shock (Hengge 2008; Navarro Llorens et al. 2010). In addition, it has been shown that the composition of the medium and the growth rate of bacteria can affect the activity of multidrug efflux pumps, in particular AcrAB-TolC (Rand et al. 2002; Bailey et al. 2006). An increase in the (p)ppGpp level may be accompanied by the induction of the SOS response (Maslowska et al. 2019). Therefore, there is a possibility that differences in the activity of bacterial defense systems associated with a change in the growth rate will make some contribution to the development of antibiotic tolerance in addition to a change in the activity of specific targets. This is especially interesting in connection with the radical-based hypothesis of antibiotic killing proposed by the Collins group (Kohanski et al. 2007). According to this hypothesis, metabolic changes induced by different bacterial antibiotics downstream of their target-specific interactions lead to an increase in the production of reactive oxygen species (ROS), in particular hydroxyl radicals, which contribute to cell damage and death (Belenky et al. 2015; Lobritz et al. 2015; Yang et al. 2017). From this point of view, factors that alter the production of radicals and the activity of antioxidant systems should significantly affect the efficacy of antibiotics. However, radical-based mechanism of antibiotic killing was challenged by several research groups (Ezraty et al. 2013; Liu and Imlay 2013; Keren et al. 2013) and was actively debated in the scientific community (Imlay 2015; Van Acker and Coenye 2017).

The aim of this work was to study how the growth rate of bacteria affects the physiological response to ciprofloxacin and how defense systems, the activity of which is modulated by growth rate, contribute to the development of antibiotic tolerance. Variation in specific growth rates was achieved by growing bacteria on different sources of carbon and energy (glucose, sodium succinate, and sodium acetate).

Fluoroquinolone ciprofloxacin kills bacteria by damaging their DNA through interaction with gyrase and topoisomerase IV, eventually leading to chromosome fragmentation and cell death (Drlica et al. 2008; Bush et al. 2020). It is assumed that ROS are involved in the mechanism of quinolone-mediated rapid killing (Goswami et al. 2006; Dwyer et al. 2007; Hong et al. 2020). However, the exact mechanisms that lead to bacterial death, especially the role of ROS in lethality, require further study. Quinolone-induced DNA damage activates the RecA/LexA-controlled SOS gene network, resulting in the production of various repair proteins and contributing significantly to ciprofloxacin tolerance (Theodore et al. 2013). The concentration of ciprofloxacin in E. coli cells depends on the activity of its entry through the OmpF porin (Hirai et al. 1986) and its pumping out with the participation of bacterial efflux pumps (mainly AcrAB), the central component of which is the outer membrane efflux protein TolC (Goswami et al. 2016). To study the role of the SOS response and other stress responses in tolerance to...
ciprofloxacin at different growth rates, mutants with deletions of the recA, rpoS, ompF, and tolC genes were used.

**Materials and methods**

**Materials**

Ciprofloxacin, LB-agar, DMSO, DiBAC 4(3) and all reagents for determination of H₂O₂, superoxide, glutathione, β-galactosidase activity and NAD⁺/NADH ratio were from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents, including components of M9 medium were of analytical grade (Reachim, Moscow, Russia).

**Strains and growth conditions**

A parental strain of *Escherichia coli* BW25113 (wt) and single-knockout mutants JW0912 (ΔompF), JW2669 (ΔrecA), JW5503 (ΔrpoS), JW5503 (ΔtolC), JW3901 (ΔoxyR) and JW2414 (ΔcysM) were from the Keio collection (Baba et al. 2006). All the strains were not resistant to ciprofloxacin. The strains carrying transcriptional gene fusions katG::lacZ and katE::lacZ were constructed by transformation of the parental strain and the rpoS mutant with plasmids pKT1033 and pRS katE16, respectively (Tao et al. 1989; Mulvey et al. 1990). The strains with sodA::lacZ and sulA(sfiA)::lacZ fusions were created by P1 transduction from the strains QC772 and DM4000, respectively (Carlioz and Touati 1986; Volkert et al. 1989).

Overnight cultures (100 ml) were grown with shaking (150 rpm) in M9 minimal medium (15.13 g Na₂HPO₄·12 H₂O, 3 g KH₂PO₄, 1 g NH₄Cl, 0.5 g NaCl, 0.246 g MgSO₄·7 H₂O, 0.011 g CaCl₂ per liter of distilled water) (Miller 1972) with 10 mM of glucose, sodium succinate or sodium acetate at 37 °C. After centrifugation, these cells were resuspended in 100 ml of fresh medium to OD₆₀₀ of 0.1 and then grown as described above. In mid-log phase (OD₆₀₀ of 0.4) bacteria were treated with 0.3 (20 × MIC) or 3 μg ciprofloxacin (CF) ml⁻¹ and growth was monitored for 2–2.5 h. The specific growth rate (μ) was calculated by equation μ = Δln OD₆₀₀ /Δt, where t is the time in hours.

Real-time monitoring of dissolved oxygen (dO₂), pH, and extracellular sulfide (S²⁻)

Dissolved oxygen and pH in *E. coli* cultures were continuously measured directly in the flasks using a Clarke oxygen electrode InPro 6800 (Mettler Toledo) and a pH electrode ESC-10601/7 (“IT” Russia), respectively. The dO₂/pH controller of BioFlo 110 fermentor (New Brunswick Scientific Co., USA) was used for data recording. Ciprofloxacin was added without stop of rotation of flasks.

 Extracellular sulfide was also detected directly in the flasks using the system of sulfide-specific ion-selective chalcogenide XC-S²⁻-001 (Sensor Systems Company, Russia) and reference electrodes and a computer pH/ion meter cpX-2 (IBI Pushchino, Russia). All parameters were continuously and synchronously processed in real time using a single hardware-software complex, including several registration units.

**Determination of ATP and NAD⁺/NADH ratio**

ATP concentration was measured using the luciferin-luciferase ATP determination kit (Molecular Probes). For ATP extraction, 50 μl of the cell suspension was mixed with 450 μl of cell disruption reagent (DMSO). The extraction was completed within 5 min and the ATP concentration was determined according to the manufacturer’s protocol.

NAD⁺-NADH pool was determined as described previously (Leonardo et al. 1996). Duplicate 1.0-ml samples were placed in Eppendorf tubes and centrifuged at 15,000 g for 1 min. After removal of the supernatant, the pellets were immediately frozen and then 250 μl of either 0.2 M HCl (for NAD⁺ extraction) or 0.2 M NaOH (for NADH extraction) were added to the frozen pellets. Specific dinucleotides were extracted for 10 min at 100 °C in a ThermoShaker TS-100C (BioSan, EU) and then the tubes were centrifuged at 5000 g for 5 min to remove cellular debris. Assay of the extracts containing specific dinucleotide species were performed in triplicate by the recycling assay as previously described (Leonardo et al. 1996). Coenzyme standards from 1.5 to 0.05 μM were used to calibrate the assay.
Study of cell viability and membrane potential

For colony-forming studies, 2-ml culture samples were washed (centrifuged for 2 min at 15,000 g and resuspended in an equal volume of 0.9% NaCl), serially diluted in 0.9% NaCl, mixed with molten soft LB-agar (0.8%) at 42 °C and poured onto plates with solid LB-agar (1.5%). Colonies were counted over 24-h incubation at 37 °C.

Changes in the membrane potential ($\Delta \psi$) were evaluated using $\Delta \psi$-sensitive fluorescent dye DiBAC$_4$(3) (Wickens et al. 2000) as described previously (Smirnova et al. 2015). Samples of log-phase cells treated with the protonophore carbonylcyanide $m$-chlorophenylhydrasone (CCCP, 20 lM) were used as positive control. Fluorescent cells were counted using Leica DM2000 microscope as earlier described (Smirnova et al. 2015). Total cell number was counted in transmitted light. About 1000 cells were counted for every sample and all experiments were conducted 3–6 times on separate days.

Measurement of extracellular superoxide and H$_2$O$_2$

Extracellular superoxide was monitored by the method (Korshunov and Imlay 2006) using the ability of superoxide to reduce ferricytochrome c. Cells were grown in M9 medium with glucose, succinate or acetate to OD$_{600}$ of 0.5, centrifuged and resuspended to OD$_{600}$ of 0.2 in three 50-ml flasks containing 12 ml of prewarmed medium. One of these flasks was used for growth monitoring. Two other flasks additionally contained 20 lM cytochrome c. Superoxide dismutase (SOD) (30 U ml$^{-1}$) was added to one of two flasks, and they were incubated at 37 °C with shaking at 150 rpm for 2.5 h. At 30-min intervals, samples (1.5 ml) were withdrawn, passed through membrane filters, and the reduced cytochrome c was immediately determined as described in the method (Korshunov and Imlay 2006). The difference between the concentrations of reduced cytochrome c in the flasks with and without SOD is equivalent to the concentration of extracellular superoxide.

For H$_2$O$_2$ determination, cells were grown as described in growth conditions. At intervals, 2-ml aliquots of culture were removed, passed through membrane filters and H$_2$O$_2$ was measured by Amplex Red-horseradish peroxidase detecting system (AR/HRP) (Seaver and Imlay 2001) using spectrofluorimeter Shimadzu RF-1501 (Shimadzu, Japan) ($\lambda_{\text{ex}}$ 563 nm and $\lambda_{\text{em}}$ 587 nm). The concentration of H$_2$O$_2$ in the samples was calculated from the calibration curve.

Determination of glutathione and β-galactosidase activity

Extracellular glutathione (GSH$_{\text{out}}$) was determined in samples, which were removed at 15-min intervals by rapid filtration through 0.45 μm-pore-size filters. For the determination of intracellular glutathione (GSH$_{\text{in}}$), 5 ml samples of cell culture was harvested by centrifugation (8000 × g for 5 min) at different time points and prepared as described previously (Smirnova et al. 2012). GSH was measured using the DTNB-glutathione reductase recycling method (Tietze 1969) modified as described previously (Smirnova et al. 2012).

Changes in the expression of the tested genes were estimated by determination of β-galactosidase activity (Miller 1972) in E. coli strains carrying the appropriate gene fusions.

Determination of ciprofloxacin concentration in the growth medium

Changes in ciprofloxacin concentration in growing cultures were determined by its fluorescence ($\lambda_{\text{ex}}$ 271 nm and $\lambda_{\text{em}}$ 416 nm) in samples taken through a membrane filter using a Shimadzu RF-1501 spectrofluorimeter (Shimadzu, Japan). Since substances absorbing at 270 nm accumulate in the medium with increasing culture density, a series of calibration curves were created for different OD$_{600}$ values. For this purpose, ciprofloxacin at concentrations of 0, 0.3, 0.6, 1.2, 1.5, and 3 μg ml$^{-1}$ was added to the filtrates obtained at different OD$_{600}$ from control cultures, and fluorescence was determined. The concentration of ciprofloxacin in the test samples was determined by calibrations performed for the corresponding value of OD$_{600}$.

Statistical analysis of the data

Each result is indicated as the mean value of three to six independent experiments ± the standard error of the mean (SEM). Significant difference was analyzed...
by Student’s t-test. A P-value of 0.05 was used as the cut-off for statistical significance. Results were analyzed by means of the program packet Statistica 6 (StatSoft Inc. 2001).

Results

Influence of the studied mutations and ciprofloxacin on the growth rate and cellular energetics

Before studying the effect of any mutation on tolerance to a particular antibiotic, it is necessary to determine how this mutation affects the growth rate of bacteria, that is, the number of active targets for the antibiotic. Mutants lacking the rpoS, recA, ompF and tolC genes grew at the same rate, approximately 0.68 h⁻¹, as the parent when glucose was used as a substrate (Fig. 1a). Growth on less energy-efficient succinate and acetate reduced the specific growth rate in all strains approximately to the same extent (by 1.5 and 3.6 times, respectively). Therefore, the effect of these mutations on the tolerance to ciprofloxacin is determined by their functional significance, and not by the effect on the growth rate.

Interacting with DNA gyrase, ciprofloxacin inhibits replication and transcription, which leads to a decrease in the growth rate (Drlica et al. 2008; Bush et al. 2020). In all strains studied, the inhibitory effect of ciprofloxacin on the specific growth rate (μ) was less pronounced at a dose of 0.3 μg CF ml⁻¹, when a 30-min delay in the fall of μ was observed (Fig. 1b, c). The degree of growth inhibition decreased with a decrease in the initial growth rate and was minimal in cultures grown on acetate. The slower decline in μ in the ompF mutant (Fig. 1d) is apparently caused by the decreased penetration of ciprofloxacin into cells lacking OmpF porin, which is confirmed by measurements of the ciprofloxacin concentration in the

Fig. 1 Effect of mutations and energy substrates on E. coli growth rate in the absence (a) and in the presence of 0.3 μg CF ml⁻¹ (b) or 3 μg CF ml⁻¹ (c, d). E. coli wild-type and mutant cells were grown in M9 minimal medium with glucose, succinate or acetate. Ciprofloxacin was added at OD₆₀₀ of 0.4 at the time indicated by the arrow. Values are the means and standard error (vertical bars) from at least three independent experiments.
medium (Fig. 1S). Other mutations, including *recA*, did not significantly affect ciprofloxacin-induced growth inhibition, except that in the *recA* mutant, unlike the parent, μ did not drop below zero after 90 min of exposure to 3 μg CF ml⁻¹ (Fig. 1c).

We also studied how ciprofloxacin affects other indicators of cellular metabolic activity. One of the most important physiological parameters under aerobic conditions is the rate of respiration. Respiratory acceleration is considered a source of ROS in the radical-based hypothesis of antibiotic lethality (Kohanski et al. 2007; Lobritz et al. 2015). To monitor oxygen consumption during treatment with ciprofloxacin, we used the method of continuous registration of dO₂ with a Clark electrode directly in flasks with bacterial cultures. Despite the constant rotation, the concentration of dissolved oxygen in untreated cultures gradually decreased with an increase in biomass (control curves in Fig. 2a, b). The rate of this decrease, expressed as dO₂/OD₆₀₀ · min, was 1.12 ± 0.04, 0.66 ± 0.01 and 0.16 ± 0.01 for glucose, succinate and acetate, respectively. The lower basal level of dO₂ was SOS-dependent (Fig. 2a, b). The oxygen consumption mode in the first phase depended on the type of energy substrate and antibiotic concentration and was expressed as dO₂/OD₆₀₀.

The consumption of the substrate was accompanied by acidification of the medium due to the accumulation of acid by-products during growth on glucose, while alkalinisation of the medium occurred during growth on succinate and acetate (Fig. 2S-a, b). Ciprofloxacin dose-dependently suppressed glucose consumption, but the consumption of acetate and succinate continued at a rate that did not differ significantly from the control. Interestingly, despite the same growth rate as the wild type, the *recA* mutant showed lower substrate consumption (Fig. 2S-b) as well as a slower decrease in dO₂ during growth on all studied substrates (Fig. 2b).

The respiratory chain of *E. coli* conserves energy via the generation of a proton motive force (PMF), which is the sum of the transmembrane pH gradient (ΔpH) and the membrane potential (ΔΨ). Dissipation of ΔΨ is considered as one of the markers of programmed cell death (Dwyer et al. 2012; Erental et al. 2014). To determine the ability of cells to maintain membrane potential, we used the fluorescent dye DiBAC₄ (3), which stains only depolarized cells (Wickens et al. 2000). The percentage of depolarized cells in untreated cultures was higher during growth on succinate and acetate, 2.7 ± 0.1 and 2.2 ± 0.1, respectively, compared to 0.9 ± 0.1 during growth on glucose. Cultures exposed to 3 μg CF ml⁻¹ maintained the level of depolarized cells below 10% for an hour, and then a rapid decrease in membrane potential was observed (Fig. 2c). In accordance with our previous work (Smirnova et al. 2017), this phase was SOS-dependent and coincided with the SOS-dependent phase of respiratory inhibition. The percentage of depolarized cells was 5 times lower in the *recA* mutant compared to the parent (Fig. 2c). During growth on succinate and acetate, the ciprofloxacin-induced decrease in membrane potential was less pronounced than on glucose, which indicates less cell damage under these conditions. The SOS-dependent phase of a decrease in membrane potential was also observed when *E. coli* was treated with a bactericidal dose of H₂O₂ (10 mM), but was absent when exposed to chloramphenicol (25 μg ml⁻¹) or a bacteriostatic dose of H₂O₂ (2 mM) (Fig. 3S-c).

The growth rate of bacteria is largely determined by the rate of production of NADH, which is a source of reducing equivalents in the respiratory chain for creating an electrochemical proton gradient and ATP synthesis with the participation of ATP synthase. Kohanski et al. (2007) reported that bactericidal
Antibiotics cause a decrease in the NADH pool as a consequence of accelerated respiration. Under our conditions, the steady-state level of NADH, which results from its production and consumption, was even lower and, accordingly, the NAD$^+/NADH$ ratio was higher in cells growing on glucose than in cells growing on succinate and acetate (Fig. 2d). 20 min after the addition of 3 l gC Fm l$^{-1}$, there was a decrease in NADH and a twofold increase in the NAD$^+/NADH$ ratio in comparison with the untreated culture on glucose, while the level of NADH on succinate increased 1.7 times (Fig. 2d, e). No changes in these parameters were observed on acetate. Intracellular ATP increased after ciprofloxacin treatment in a dose-dependent manner (Fig. 2S-c). For all substrates, this increase was gradual at 0.3 l gC ml$^{-1}$ and, conversely, quickly peaked at 3 l gC ml$^{-1}$ (Fig. 2S-d), which coincided with the growth inhibition mode at various concentrations of ciprofloxacin (Fig. 1b, c). The recA mutant showed the same mode of ATP changes as the parental strain (Fig. 2S-d).

Changes in the redox state of cells growing with various sources of carbon and energy

An increase in ROS production and a shift in the redox state of glutathione towards its oxidised form are usually regarded as evidence of oxidative stress. Both
processes were observed under the action of bactericidal antibiotics (Dwyer et al. 2014; Belenky et al. 2015). However, we have previously shown that in *E. coli* only superoxide generator menadione was able to reduce the GSH/GSSG ratio, decreasing GSH and increasing GSSG, while H$_2$O$_2$ did not cause a drop in the GSH/GSSG ratio in a wide range of concentration (1–10 mM) (Smirnova et al. 2000). We also showed that after a 20-min exposure, ciprofloxacin reduced both the production of extracellular superoxide and the level of extracellular H$_2$O$_2$, but significantly increased the level of intracellular GSH$_{in}$ and extracellular GSH$_{out}$ glutathione in cells growing on glucose (Smirnova et al. 2017).

Here we showed that growing on succinate had no effect on ROS generation compared to glucose, while the use of acetate reduced superoxide production and H$_2$O$_2$ accumulation by 1.5 and 2.2 times, respectively (Fig. 3S-a, b). At OD$_{600}$ of 0.4, the level of GSH$_{in}$ was 1.3 times lower for succinate and 1.4 times higher for acetate as compared to cells grown on glucose (Fig. 3a). With all substrates, the intracellular glutathione level gradually increased during the observation period. In untreated cells, about 10% of the total synthesized glutathione was excreted into the medium, where its concentration per biomass unit was maintained at an approximately constant level (Fig. 3b). Treatment with 3 µg CF ml$^{-1}$ caused a transient increase in GSH$_{in}$ that was proportional to the degree of growth inhibition and had significantly lower amplitude in cells growing on acetate and succinate compared to glucose (Fig. 3a). The increase in the level of GSH$_{in}$ was accompanied by an increase in its extracellular concentration, which was higher for glucose and succinate than for acetate (Fig. 3b). The release of GSH was especially accelerated after 60 min of exposure to ciprofloxacin, which corresponds to the phases of SOS-dependent inhibition of respiration and a rapid drop in membrane potential (Fig. 2a, c). To clarify the role of the SOS-response in altering glutathione levels, we measured intracellular and extracellular glutathione in the recA mutant. Unexpectedly, an inversion of GSH levels inside and outside the cells was observed: GSH$_{in}$ was low and stable during observation, while GSH$_{out}$ was 4.5 times higher and gradually increased with increasing biomass (Fig. 3c). That is, most of the glutathione in this mutant left the cells as it was synthesized. The addition of 3 µg CF ml$^{-1}$ did not change the level of GSH$_{in}$ in this mutant, but accelerated its release into the medium. Further studies are needed to clarify the relationship between RecA and transmembrane glutathione fluxes and the possible involvement of GSH in the regulation of RecA, which contains thiol groups, whose mutations can affect its activity (Weisemann and Weinstock 1988).

We have previously shown that, during growth on glucose, a high dose of ciprofloxacin (3 µg ml$^{-1}$) provokes a reversible release of sulfide from *E. coli* cells into the medium (Tyulenev et al., 2018). However, this dose of ciprofloxacin did not induce H$_2$S release when cells were grown on succinate or acetate (Fig. 3d). As in the case of valine and chloramphenicol (Smirnova et al. 2019), H$_2$S generation under exposure of *E. coli* to ciprofloxacin depended on the activity of cysteine synthase B (CysM) and was absent in the cysM mutant (Fig. 3e). H$_2$S production was also absent in the recA mutant (Fig. 3e). It was previously reported that H$_2$S can protect cells against antibiotic killing (Shatalin et al. 2011). To clarify this possibility under our conditions, we studied the effect of ciprofloxacin on the growth rate and the number of CFU in the cysM mutant with abolished H$_2$S production. No significant differences were found between the wild-type strain and the cysM mutant (Fig. 4S).

Expression of antioxidant genes in *E. coli* growing with various carbon and energy sources

When ROS accumulate in the cell, bacteria respond through the OxyR, SoxRS and RpoS regulons, which control the transcription of genes encoding ROS-scavenging enzymes (Imlay 2013). The expression of genes *katG* and *katE*, encoding catalases HPI (KatG) and HPII (KatE), is also positively regulated by RpoS (Ivanova et al. 1994), the level of which is inversely proportional to the specific growth rate (Ihssen and Egli 2004). According to these data, the slower growth of *E. coli* on less energy-efficient succinate and acetate was accompanied by an RpoS-dependent increase in the expression of the *katG* and *katE* genes as compared with the growth on glucose (Fig. 4a, b). The expression of the sulA gene, which is controlled by SOS-regulon, also increased with a decrease in the growth rate (Fig. 4c). This growth rate dependence of sulA expression may be caused by an increase in the level of (p)ppGpp in slower growing cells, which leads to the
induction of genes involved in the SOS response, as shown for the *E. coli* stringent response (Durfee et al. 2008). The *rpoS* mutation did not significantly affect the expression of *sulA*::*lacZ* during growth on glucose, but additionally increased it by 1.7 and 2.4 times on succinate and acetate, respectively. RpoS is known to positively regulate DNA polymerases II (*polB*) and IV (*DinB*) independently of LexA (Maslowska et al. 2019); therefore, additional induction of the SOS response in the *rpoS* mutant may be a compensatory mechanism for maintaining their required level.

The data on the induction of antioxidant regulons under the action of bactericidal antibiotics are contradictory (Kohanski et al. 2007; Liu and Imlay 2013; Dwyer et al. 2014). We have previously shown that the expression of the genes *sodA* and *katG*, as well as the level of total catalase activity, significantly decreased during the action of ciprofloxacin on *E. coli* growing in a minimal medium with glucose (Smirnova et al. 2017). Ciprofloxacin treatment of cells growing on succinate or acetate also led to a decrease in the expression of *katG*::*lacZ* (Fig. 4d), while in response to *H*₂*O*₂, its expression increased on all these substrates (Fig. 3S-d). In untreated cultures, growth on acetate led to an increase in *sodA*::*lacZ* expression (Fig. 4e), which was probably associated with the removal of repression of the *sodA* gene by the ArcAB regulatory system at high levels of *dO*₂ (Compan and...
Treatment of cells with 3 \( \mu \text{g} \text{CF ml}^{-1} \) reduced the expression of \( \text{sodA}::\text{lacZ} \) during growth on all tested substrates. The effect of ciprofloxacin on the expression of the \( \text{katE} \) gene was proportional to the degree of growth inhibition by the antibiotic: it increased with an increase in the concentration of ciprofloxacin and with the transition to a more energy-efficient substrate. The addition of 3 \( \mu \text{g} \text{CF ml}^{-1} \) increased \( \text{katE} \) expression 1.5-fold when cells grew on glucose, 1.2-fold if growth was on succinate and had no significant effect during growth on acetate (not shown).

Consistent with previous data, ciprofloxacin treatment caused an immediate activation of the SOS response (Theodore et al. 2013). Regardless of the substrate used, the increase in \( \text{sulA}::\text{lacZ} \) expression was more pronounced with a lower antibiotic dose (Fig. 4f). Perhaps the lower level of \( \text{sulA} \) induction with 3 \( \mu \text{g} \text{CF ml}^{-1} \) (3 times) compared to 0.3 \( \mu \text{g} \text{CF ml}^{-1} \) (10 times) was associated with a sharper inhibition of transcription and translation at a high concentration of the antibiotic.

**Effect of ompF, tolC, rpoS, and recA mutations on ciprofloxacin lethality**

Among all tested mutations, only mutations in the \( \text{recA} \) and \( \text{ompF} \) genes could significantly affect the lethal activity of ciprofloxacin, as follows from the analysis of killing curves (Fig. 5). The effect of \( \text{ompF} \) deficiency strongly depended on the concentration of ciprofloxacin. At 0.3 \( \mu \text{g} \text{CF ml}^{-1} \), its bactericidal activity decreased by 2 times in cells growing on glucose or succinate (Fig. 5a, b), while at 3 \( \mu \text{g} \text{CF ml}^{-1} \), it increased 9, 3, and 2 times during growth on glucose, succinate, and acetate, respectively.
compared to the parental strain (Fig. 5d, e, f). RecA deficiency reduced the number of CFU ml\(^{-1}\) by 2–4 orders of magnitude compared to the parental strain, depending on the concentration of ciprofloxacin and the substrate used. The absence of RpoS only slightly (1.7 times) increased the lethal activity of ciprofloxacin when cells were grown on succinate at both antibiotic concentrations or on glucose at 0.3 \(\mu\)g CF ml\(^{-1}\) (Fig. 5a, b, e). The \textit{tolC} mutant showed the same tolerance to ciprofloxacin as the parent.

The bactericidal activity of quinolones, including ciprofloxacin, is biphasic: the lethality of the drugs increases to a concentration known as the optimal bactericidal concentration (OBC), after which the bactericidal activity then declines (Lewin et al. 1991). In wild type \textit{E. coli}, the OBC is about 0.3 \(\mu\)g CF ml\(^{-1}\). Accordingly, 0.3 \(\mu\)g CF ml\(^{-1}\) showed a higher bactericidal activity (up to one order of magnitude) than 3 \(\mu\)g CF ml\(^{-1}\) during the growth of wild-type cells and \textit{rpoS} and \textit{tolC} mutants on all tested substrates. In contrast, the dose 3 \(\mu\)g CF ml\(^{-1}\) was more lethal for the \textit{recA} mutant during growth on glucose (Fig. 5a, d). The opposite effect of the \textit{ompF} mutation on the sensitivity of \textit{E. coli} to low and high doses of ciprofloxacin can be explained by the shift of the OBC for this mutant towards a higher dose of ciprofloxacin compared to the parental strain due to a decrease in the penetration of the antibiotic into cells. In the \textit{ompF} mutant, the dose of 3 \(\mu\)g CF ml\(^{-1}\) corresponded to the OBC, causing a maximum decrease in CFU; a further increase in the concentration to 10 \(\mu\)g CF ml\(^{-1}\) was accompanied by a decrease in the lethality of the antibiotic (not shown).

Depending on the substrate used, the bactericidal activity of ciprofloxacin decreased in the sequence: glucose, succinate, acetate, regardless of the \textit{E. coli} strain and the concentration of the antibiotic (Fig. 5). Earlier, we showed for a wild-type strain that there is
an inverse correlation between logCFU ml$^{-1}$ and the specific growth rate of bacteria growing on different energy sources (Smirnova and Oktyabrsky 2018). Our current work has revealed the existence of such a relationship for all studied mutants (Fig. 6a, b). The degree of shift of the graphical dependence of logCFU ml$^{-1}$ on the specific growth rate along the ordinate in the mutant relative to the wild-type strain reflects the contribution of the corresponding gene to the tolerance to ciprofloxacin.

Oxidative stress is thought to be involved in the lethal effects of ciprofloxacin and other quinolones (Goswami et al. 2006; Dwyer et al. 2007; Hong et al. 2020). However, the absence of RpoS, which controls the induction of H$_2$O$_2$ scavengers KatG and KatE and the ferrous sequestering protein Dps (Navarro Llorens et al. 2010), only slightly affected the bactericidal activity (Fig. 5). To test the role of RpoS in protection against oxidative stress under our conditions, we studied the bactericidal activity of H$_2$O$_2$ in E. coli cells growing with different energy sources. During growth on glucose, the rpoS mutant was 3 orders of magnitude more sensitive to 10 mM H$_2$O$_2$ than the parent, but this dose did not kill both strains in the case of succinate and acetate (Fig. 3S-e). Apparently, a decrease in the number of targets for oxidative damage and their more efficient repair during slow growth were more important for survival than the induction of RpoS-controlled defense systems.

![Fig. 6](image)

**Fig. 6** All studied mutants showed an inverse linear relationship between logCFU ml$^{-1}$ and the specific growth rate (a, b). Mutations shifted the plot of this dependence relative to the parental strain according to their significance for ciprofloxacin tolerance. Data are taken from the killing curves (Fig. 5) one hour after antibiotic treatment. Effect of additives on survival of ciprofloxacin-treated E. coli wt (c) and oxyR (d) during growth recovery on antibiotic-free agar. Bacteria were grown in M9 minimal medium with glucose up to OD$_{600}$ of 0.4, and then part of the culture was treated with 0.3 µg CF ml$^{-1}$. Samples of untreated (control) and ciprofloxacin treated cells were removed and, after serial dilutions, plated on LB agar without additives or with the addition of catalase (500 U ml$^{-1}$) or DMSO (5%). Values are the means and standard error (vertical bars) from at least three independent experiments. The time of CF addition is indicated by an arrow.
It was recently reported that ROS may be involved in quinolone-mediated killing not directly during antibiotic exposure, but after plating bacteria on antibiotic-free agar (Hong et al. 2020). To test this possibility under our conditions, we plated bacteria treated with ciprofloxacin in liquid culture on antibiotic-free LB agar containing catalase or DMSO. The presence of catalase did not affect the number of surviving cells in the wild-type strain treated with 0.3 μg CF ml⁻¹ (Fig. 6c). However, in line with previous data (Hong et al. 2020), the addition of DMSO to LB agar increased logCFU ml⁻¹ by an order of magnitude. It is known that DMSO traps hydroxyl radicals; therefore, it is possible that oxidative stress is involved in the killing of this part of cells. At the same time, DMSO can inhibit bacterial growth (Fig. 5S), and its protective effect may, at least in part, be caused by growth delay and more efficient DNA repair, since a similar effect was observed when using M9 agar without DMSO instead of LB agar with DMSO (Fig. 6c). The procedure for determining the colony forming ability (washing, serial dilutions, and inoculation on the agar surface) involves a change in the composition of the medium, temperature and oxygen concentration and itself represents a series of stresses for the cells. Apparently, defense mechanisms in wild-type cells make it possible to avoid the damaging effect of these stresses. In particular, the addition of catalase and DMSO to agar has no effect on the change in logCFU ml⁻¹ in the control culture (Fig. 6c). In contrast, the plating efficiency of the oxyR mutant was increased by two orders of magnitude (to the level of the wild-type) with the addition of catalase and by one order of magnitude when the LB agar contained DMSO (Fig. 6d, control curves). These data indicate that the plating bacteria itself induces oxidative stress if the antioxidant system is compromised. In the presence of catalase and DMSO, ciprofloxacin-induced killing increased in parallel with the increase in the plating efficiency of the oxyR mutant (Fig. 6d). However, even in the presence of catalase, the survival of the oxyR mutant was 2 orders of magnitude higher than that of the parent (Fig. 2c, d), which may be due to the lower growth rate of the mutant during antibiotic treatment (μoxyR = 0.22 h⁻¹; μwt = 0.68 h⁻¹).

In general, although the possibility of a contribution of oxidative stress to DNA damage during post-antibiotic growth recovery is not ruled out, the magnitude of this contribution is difficult to distinguish using anti-ROS additives that inhibit bacterial growth.

Discussion

The specific growth rate of bacteria is an integral parameter reflecting the activity of interrelated core processes of cellular metabolism (replication, transcription, and translation), and through global regulators, primarily (p)ppGpp and RpoS, can affect the degree of induction of various defense systems. The activity of core metabolic processes determines the number of active targets for antibiotics of different classes and, accordingly, their antimicrobial, including lethal, efficacy (Pontes and Groisman 2020). At the same time, the concept developed in the last decade by Collins’ group and their followers suggests that, unlike bacteriostatic drugs, the effect of bactericidal antibiotics on their targets leads to toxic changes in metabolism, which are accompanied by an increase in the production of reactive oxygen species that contribute to antibiotic lethality (Kohanski et al. 2007). In this work, we tested how the growth rate of E. coli affects the nature of metabolic changes under the action of the fluoroquinolone ciprofloxacin and how protective systems, the activity of which depends on the growth rate, modify its lethal efficacy. One of the central propositions of the hypothesis of the lethal activity of antibiotics with the participation of ROS is the acceleration of respiration under the action of bactericidal antibiotics, in contrast to bacteriostatic drugs, which inhibit oxygen consumption (Kohanski et al. 2007; Lobritz et al. 2015). The fluoroquinolone norfloxacin has previously been shown to stimulate the rate of oxygen consumption by E. coli cells (Dwyer et al. 2014; Lobritz et al. 2015), but another study noted that norfloxacin has a weak effect on respiration (Liu and Imlay 2013). In the present work, we have established that the effect of ciprofloxacin on oxygen and substrate consumption can vary from moderate stimulation to sharp inhibition, depending on the previous bacterial growth rate and the degree of its inhibition by the antibiotic. In general, the degree of respiratory inhibition increases with the number of active sites with which ciprofloxacin interacts. Regardless of the growth substrate used, we recorded an increase in the level of intracellular ATP upon treatment of cells with ciprofloxacin, which may be a
consequence of inhibition of energy-dependent processes and was previously noted as characteristic of bacteriostatic antibiotics (Lobritz et al. 2015). In the hypothesis of Kohanski et al. (2007) depletion of the NADH pool and a reversible increase in the NAD+/NADH ratio under the action of bactericidal antibiotics is considered as an indicator of the acceleration of electron transport along the respiratory chain with the formation of ROS. We observed these changes in the NAD+/NADH ratio 20 min after ciprofloxacin treatment of *E. coli* growing on glucose. However, ciprofloxacin treatment of *E. coli* growing on succinate was accompanied by an increase in NADH level, which was previously noted under the action of bacteriostatic drugs (Lobritz et al. 2015). Unlike Dwyer et al. (2014), we were unable to register the induction of OxyR and SoxRS regulons under our conditions using the fusions *katG*:::lacZ and *sodA*:::lacZ, which is consistent with the results previously obtained by Liu and Imlay (2013). Belenky et al. (2015) showed that bactericidal antibiotics stimulate the synthesis of glutathione, causing modest increases in the levels of GSH, coupled with much larger increases in GSSG, which may be indicative of an ongoing antioxidant response to antibiotic-dependent ROS induction and protein damage. In the present work and earlier (Smirnova et al. 2017), we also observed an increase in the level of intracellular GSH and a proportional increase in GSSG upon treatment of *E. coli* with ciprofloxacin. However, at the same time, acceleration of glutathione synthesis and an increase in its intracellular pool was a characteristic feature of the stress response under the action of bacteriostatic chloramphenicol (Smirnova et al. 2019) and cell starvation caused by depletion of glucose (Tyulenev et al. 2018), phosphate (Smirnova et al. 2012) or isoleucine (when treated with valine) (Smirnova et al. 2019). We suggest that this acceleration of GSH synthesis is a response to a temporary increase in the pool of free cysteine with a sharp inhibition of protein synthesis (Smirnova et al. 2019). Glutathione acts as a buffer for cysteine, maintaining its homeostasis, which prevents the possible negative consequences of an increase in its level and its participation as a reductant of Fe$^{3+}$ in the Fenton reaction (Park and Imlay 2003). In addition, glutathione can perform many other functions, including maintaining redox balance and regulating enzyme activity and ion fluxes (Smirnova and Oktyabrsky 2005). In the absence of the multidrug efflux pump TolC-AcrAB, exogenous glutathione can stimulate the release of ciprofloxacin from *E. coli* cells (Goswami et al. 2016). H$_2$S production, which was observed under the action of ciprofloxacin, is also involved in the maintenance of cysteine homeostasis under stresses associated with inhibition of protein synthesis (Tyulenev et al. 2018; Smirnova et al. 2019). H$_2$S, as a gaseous signaling molecule, may have pleiotropic effects, including modulating antibiotic sensitivity and oxidative stress (Shatalin et al. 2011; Mironov et al. 2017). Thus, interacting with its targets, ciprofloxacin caused significant metabolic perturbations in the cellular energetics and the content of redox-active thiols. However, we did not reveal a strict specificity of the effects of bactericidal ciprofloxacin in comparison with changes in metabolism, which were recorded under stresses of a bacteriostatic nature. The degree of changes strongly depended on the growth rate of bacteria (the number of active targets) and the concentration of the antibiotic, which apparently determines their variability and the discrepancy between the results of researchers using different experimental models.

Under aerobic conditions, growing *E. coli* cells generate a steady flux of superoxide anion radicals and H$_2$O$_2$ as a result of the inadvertent transfer of either a single electron or two consecutive electrons from the redox moieties of flavoenzymes to oxygen (Imlay 2013). Metabolic changes occurring under various stresses, including antibiotic action, might be expected to cause a temporary imbalance in ROS production, moderate doses of which can participate in intracellular signaling (Sies 2017), while large doses can damage cellular macromolecules (Imlay 2013). The question is whether these doses of ROS are sufficient to kill bacteria and whether the action of bactericidal antibiotics creates conditions under which the regulatory capabilities of the protective systems that maintain low intracellular levels of ROS, free iron and cysteine are exceeded. We have previously shown that the absence of glutathione in the *gshA* mutant does not significantly contribute to the tolerance of *E. coli* to ciprofloxacin, and any exogenous additives affecting the intracellular redox state modulate antibiotic sensitivity in full accordance with their ability to alter bacterial growth rate (Smirnova et al. 2016; 2017). In this work, the absence of H$_2$S production in the *cysM* mutant did not affect its sensitivity to ciprofloxacin. Deletion of *rpoS*, which significantly reduced the
expression of the \textit{katG} and \textit{katE} genes encoding HPI and HPII catalases, did not cause a shift of the plot of the dependence of log CFU ml\textsuperscript{-1} on the specific growth rate relative to the parent strain, which indicates the absence of a significant contribution of RpoS-controlled defense systems to ciprofloxacin tolerance in our conditions. Previously, it was shown that the presence of the \textit{rpoS} mutation does not affect the development of tolerance to the fluoroquinolone ofloxacin (Fung et al. 2010). We also did not register a significant effect on the tolerance to ciprofloxacin of the \textit{tolC} gene deletion. It is possible that with prolonged bacterial growth in the presence of low (comparable to MIC) doses of the antibiotic, the contribution of efflux pumps and RpoS-controlled mechanisms to the development of tolerance may be higher. Under our conditions (a minimal medium without exogenous cysteine and with low iron), the main factors determining the lethal activity of ciprofloxacin were the rate of its entry into the cell with the participation of OmpF and the repair of damaged targets with the participation of the SOS system. \textit{ompF} and especially \textit{recA} mutations caused a significant shift in the plot of the dependence of log CFU ml\textsuperscript{-1} on the specific growth rate relative to the parental strain, which indicates the importance of these genes.

Our experimental data fit well into the scenario of ciprofloxacin action described by Theodore et al. (2013). Quinolones inhibit DNA supercoiling and relaxation by binding to both gyrase and DNA and stabilising the gyrase-DNA-cleaved complex (Drlica et al. 2008; Bush et al. 2020). This stage, apparently, is not rapidly lethal, but inhibits replication and transcription and induces the SOS response. However, after the gyrase removing from DNA, double-strand DNA breaks (DSBs) are formed, which can lead to chromosome fragmentation and cell death. Quinolone-induced damage can be repaired by the SOS system, but since \textit{E. coli} can successfully repair only up to four simultaneous DSBs, slowly growing cells with fewer replication forks and reduced transcriptional activity experience fewer breaks and are able to repair them and survive. Fast-growing cells receive more damage and die, which explains the inverse linear dependence of log CFU ml\textsuperscript{-1} on the specific growth rate for all studied \textit{E. coli} strains. The SOS response is precisely timed and synchronised according to the size of the damage and the time elapsed since the discovery of the damage (Maslowska et al. 2019); with severe DNA damage and a constant inducing signal, the induction of SOS-dependent toxins TisB and DinQ, which form pores in the cytoplasmic membrane, occurs (Dörr et al. 2010; Brantl and Jahn 2015). The characteristic physiological changes at this stage of the SOS response, which began after about 60 min of exposure to the antibiotic, were a complete cessation of growth and respiration, a drop in the membrane potential and ATP concentration, and the release of glutathione from cells into the medium. The described events, which can be considered as markers of an extreme SOS response or programmed cell death (Dwyer et al. 2012; Erental et al. 2014), were absent in the \textit{recA} mutant. The complete cessation of metabolic processes and the transition to a dormant state at this phase is an extreme way to avoid further DNA damage.

The most commonly used test for bactericidal activity is the determination of CFU after plating diluted cultures treated with antibiotics on antibiotic-free agar plates to create killing curves. It has recently been reported that a significant proportion of cells may not die directly during treatment with quinolones, but during the post-stress period, when growth is recovered on plates without antibiotics. The authors suggest that the main cause of cell death during this period is a self-amplifying burst of ROS, which are the dominant factor in all types of quinolone-mediated lethality (Hong et al. 2020). Our experiments to determine changes in the physiological parameters of culture, including the growth rate, respiration, ATP and membrane potential, showed that during the first hour of exposure to ciprofloxacin, the cells retain high metabolic activity, that is, they remain alive. On the killing curve, this period corresponds to the maximum rate of bacterial death. This paradox indicates that the death of a large proportion of the population may indeed occur during growth recovery after removal of the antibiotic. It has been shown that upon treatment with quinolones, there are two phases of induction of the SOS response: immediately after antibiotic addition and during growth recovery of persisters after antibiotic removal (Goormaghtigh and Van Melderen 2019). In order to resume growth, cells need to clear the stalled transcription complexes, restore replication forks, and repair DSBs (Theodore et al. 2013). Our study has shown that the plating on agar itself can provoke oxidative stress in the \textit{oxyR} mutant with the
damaged antioxidant system. It can be assumed that ciprofloxacin-mediated DNA damage may disturb the normal induction of antioxidant systems and cause additional oxidative damage to DNA during plating of bacteria and resumption of respiration and growth, thereby complicating the repair process. However, the contribution of ROS to post-stress quinolone lethality requires further research, since many of the anti-ROS agents used by Hong et al. (2020), in particular DMSO, slow down bacterial growth. It has been reported that (p)ppGpp, which is elevated in slowly growing cells, can facilitate DNA repair by promoting UvrD-mediated RNAP backtracking (Kamarthapu et al. 2016), and is also required to promote new origin formation (Myka et al. 2019). Additional research on the processes occurring during the post-stress period will help to improve understanding of the mechanisms of quinolone-mediated lethality.

In general, the results obtained allow us to conclude that the degree of metabolic perturbations induced by ciprofloxacin directly depends on the concentration of the antibiotic and the specific growth rate preceding stress, while the survival is inversely proportional to the growth rate. Growth rate, being an integral indicator of metabolic activity, determines the number of targets that can be damaged at a given intracellular antibiotic concentration. In this regard, the main factors influencing the tolerance to ciprofloxacin were the presence of OmpF porin and an active SOS response. The crucial role of the SOS system is confirmed by dramatic shift down of the plot of the relationship between log CFU ml−1 and specific growth rate in the recA mutant relative to the parent. The RpoS-mediated increase in the activity of antioxidant systems in slowly growing cells did not significantly contribute to ciprofloxacin tolerance.

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References

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M et al (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:1–11. https://doi.org/10.1038/msb4100050

Bailey AM, Webber MA, Piddock LJV (2006) Medium plays a role in determining expression of *acrB*, *marA*, and *soxS* in *Escherichia coli*. Antimicrob Agents Chemother 50:1071–1074. https://doi.org/10.1128/AAC.50.3.1071-1074.2006

Belenky P, Ye JD, Porter CBM, Cohen NR, Lobritz MA, Ferrante T, Jain S, Korry BJ, Schwarz EG, Walker GC, Collins JJ (2015) Bactericidal antibiotics induce toxic metabolic perturbations that lead to cellular damage. Cell Rep 13:968–980. https://doi.org/10.1016/j.celrep.2015.09.059

Brantl S, Jahn N (2015) sRNAs in bacterial type I and type III toxin-antitoxin systems. FEMS Microbiol 39:413–442. https://doi.org/10.1093/femsre/fuv003

Bush NG, Diez-Santos I, Abbott LR, Maxwell A (2020) Quinolones: mechanism, lethality and their contributions to antibiotic resistance. Molecules 25:5662. https://doi.org/10.3390/molecules25255662

Carlioz A, Touati D (1986) Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? EMBO 5:623–630

Compan I, Touati D (1993) Interaction of six global transcriptional regulators in expression of manganese superoxide dismutases in *Escherichia coli* K-12. J Bacteriol 175:1687–1696. https://doi.org/10.1128/jb.175.6.1687-1696.1993

Dörr T, Vulić M, Lewis K (2010) Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. PLoS Biol 8:e1000317. https://doi.org/10.1371/journal.pbio.1000317
Drlica K, Malik M, Kerns RJ, Zhao X (2008) Quinolone-mediated bacterial death. Antimicrob Agents Chemother 52:385–392. https://doi.org/10.1128/AAC.01617-06
Durfee T, Hansen AM, Zhi H, Blattner FR, Ding JJ (2008) Transcription profiling of the stringent response in Escherichia coli. J Bacteriol 190:1084–1096. https://doi.org/10.1128/JB.01092-07
Dwyer DJ, Kohanski MA, Hayete B, Collins JJ (2007) Gyrase inhibitors induce an oxidative damage cellular death pathway in Escherichia coli. Mol Sys Biol 3:91. https://doi.org/10.1038/msb4100135
Dwyer DJ, Camacho DM, Kohanski MA, Callura JM, Collins JJ (2012) Antibiotic-induced bacterial cell death exhibits physiological and biochemical hallmarks of apoptosis. Mol Cell 46:561–572. https://doi.org/10.1016/j.molcel.2012.04.027
Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, Chan CTY, Lobritz MA, Braff D, Schwarz EG, Ye JD, Pati M, Vercruysse M, Ratillo PS, Allison KR, Khalil AS, Ting AY, Walker GC, Collins JJ (2014) Antibiotics induce redox-related physiological alterations as part of their lethality. Proc Natl Acad Sci USA 111:E2100–E2109. https://doi.org/10.1073/pnas.1401876111
Eng RHK, Padberg FT, Smith SM, Tan EN, Cherubin CE (1991) Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. Antimicrob Agents Chemother 35:1824–1828. https://doi.org/10.1128/aac.35.9.1824
Erental A, Kalderon Z, Saada A, Smith Y, Engelberg-Kulka H, Eng RHK, Padberg FT, Smith SM, Tan EN, Cherubin CE (1991) Killing by bactericidal antibiotics. Cell 46:561–572. https://doi.org/10.1016/j.molcel.2007.06.049
Erental A, Kalderon Z, Saada A, Smith Y, Engelberg-Kulka H (2014) Apoptosis-like death, an extreme SOS response in Escherichia coli. Mbio. https://doi.org/10.1128/mBio.01426-14
Ezraty B, Vergnes A, Banzhaf M, Duverger Y, Huguenot A, Brochado AR, Su SY, Espinosa L, Loiseau L, Py B, Typas A, Barras F (2013) Fe-S cluster biosynthesis controls uptake of aminoglycosides in a ROS-less death pathway. Science 340:1583–1587. https://doi.org/10.1126/science.1238328
Fung DKC, Chan EWC, Chin ML, Chan RCY (2010) Deletion of a bacterial starvation stress response network which can mediate antibiotic tolerance development. Antimicrob Agents Chemother 54:1082–1093. https://doi.org/10.1128/AAC.01218-09
Goormaghtigh F, Van Melderen L (2019) Single-cell imaging and characterization of Escherichia coli persisters cells to ofloxacin in exponential cultures. Sci Adv. https://doi.org/10.1126/sciadv.aav9462
Goswami M, Mangoli SH, Jawali N (2006) Involvement of reactive oxygen species in the action of ciprofloxacin against Escherichia coli. Antimicrob Agents Chemother 50:949–954. https://doi.org/10.1128/AAC.50.3.949-954.2006
Goswami M, Subramanian M, Kumar R, Jass J, Jawali N (2016) Involvement of antibiotic efflux machinery in glutathione-mediated decreased ciprofloxacin activity in Escherichia coli. Antimicrob Agents Chemother 60:4369–4374. https://doi.org/10.1128/AAC.00414-16
Greulich P, Scott M, Evans MR, Allen RJ (2015) Growth-dependent bacterial susceptibility to ribosome-targeting antibiotics. Mol Syst Biol 11:796. https://doi.org/10.1525/msb.20145949
Hengge R (2008) The two-component network and the general stress sigma factor RpoS (σ^5) in Escherichia coli. Adv Exp Med Biol 631:40–53. https://doi.org/10.1007/978-0-387-78885-2_4
Hirai K, Aoyama H, Irikura T, Iyobe S, Mitsuhashi S (1986) Differences in susceptibility to quinolones of outer membrane mutants of Salmonella typhimurium and Escherichia coli. Antimicrob Agents Chemother 29:535–538. https://doi.org/10.1128/aac.29.3.535
Hong Y, Li Q, Gao Q, Xie J, Huang H, Drlica K, Zhao X (2020) Reactive oxygen species play a dominant role in all pathways of rapid quinolone-mediated killing. J Antimicrob Chemother 75:576–585. https://doi.org/10.1093/jac/dkz485
Ilhens J, Egli T (2004) Specific growth rate and not cell density controls the general stress response in Escherichia coli. Microbiology 150:1637–1648. https://doi.org/10.1099/mic.0.26849-0
Imlay JA (2013) The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nature Rev Microbiol 11:443–454. https://doi.org/10.1038/nrmicro3032
Imlay JA (2015) Diagnosing oxidative stress in bacteria: not as easy as you might think. Curr Opin Microbiol 24:124–131. https://doi.org/10.1016/j.mib.2015.01.004
Ivanova A, Miller C, Glinsky G, Eisenstark A (1994) Role of the rpoS(katF) in oxyR independent regulation of hydroperoxidase I in Escherichia coli. Mol Microbiol 12:571–578. https://doi.org/10.1111/j.1365-2958.1994.tb01043.x
Kamarthapu V, Epshtein V, Benjamin B, Proshkin S, Mironov A, Cashel M, Nucler E (2016) ppGpp couples transcription to DNA repair in E. coli. Science 352:993–996. https://doi.org/10.1126/science.aad6945
Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K (2013) Killing by bactericidal antibiotics does not depend on reactive oxygen species. Science 339:1213–1216. https://doi.org/10.1126/science.1232688
Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ (2007) A common mechanism of cellular death induced by bactericidal antibiotics. Cell 130:797–810. https://doi.org/10.1016/j.cell.2007.06.049
Korshunov S, Imlay JA (2006) Detection and quantification of superoxide formed within the periplasm of Escherichia coli. J Bacteriol 188:6326–6334. https://doi.org/10.1128/jb.00554-06
Lee AJ, Wang S, Meredith HR, Zhuang B, Dai Z, You L (2018) Robust, linear correlations between growth rates and β-lactam-mediated lysis rates. Proc Natl Acad Sci USA 115:4069–4074. https://doi.org/10.1073/pnas.1719504115
Leonardo MR, Dailly Y, Clark DP (1996) Role of NAD in killing by bactericidal antibiotics. Cell 130:797–810. https://doi.org/10.1016/j.cell.2007.06.049
Lewin CS, Morrissey I, Smith JT (1991) The mode of action of quinolones: the paradox in activity of low and high concentrations and activity in the anaerobic environment. Eur J Clin Microbiol Infect Dis 10:240–248
Liu Y, Imlay JA (2013) Cell death from antibiotics without the involvement of reactive oxygen species. Science 339:1210–1213. https://doi.org/10.1126/science.1237251
Tyulenev A, Smirnova G, Muzyka N, Ushakov V, Oktyabrsky O (2018) The role of sulfides in stress-induced changes of Eh in Escherichia coli cultures. Bioelectrochemistry 121:11–17. https://doi.org/10.1016/j.bioelechem.2017.12.012

Van Acker H, Coenye T (2017) The role of reactive oxygen species in antibiotic-mediated killing of bacteria. Trends Microbiol 25:456–466. https://doi.org/10.1016/j.tim.2016.12.008

Volkert MR, Gately FH, Hajec LI (1989) Expression of DNA damage-inducible genes of Escherichia coli upon treatment with methylating, ethylating and propylating agents. Mutation Res 217:109–115

Weisemann JM, Weinstock GM (1988) Mutations at the cysteine codons of the recA gene of Escherichia coli. DNA 7:389–398

Wickens HJ, Pinney RJ, Mason DJ, Gant VA (2000) Flow cytometric investigation of filamentation, membrane patency and membrane potential in Escherichia coli following ciprofloxacin exposure. Antimicrob Agents Chemother 44:682–687. https://doi.org/10.1128/AAC.44.3.682-687.2000

Yang JH, Bening SC, Collins JJ (2017) Antibiotic efficacy – context matters. Curr Opin Microbiol 39:73–80. https://doi.org/10.1016/j.mib.2017.09.002

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