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# A marked Copy of the Manuscriptinfluence of Oxyr on Susceptibility of Planktonic and Sessile *Escherichia Coli* Cultures to Ciprofloxacin and Cefotaxime

**Zoya Samoilova*, Nadezhda Muzyka, Galina Smirnova, Oleg Oktyabrsky**
Laboratory of Physiology and Genetics of Microorganisms, Institute of Ecology and Genetics of Microorganisms, Russian Academy of Sciences, Perm Federal Research Center, Perm, Russia

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**Abstract**

Alternation of bacterial antioxidant defense pathways might affect susceptibility to antibiotics in dual ways. Using a relatively simple model based on wild-type and *oxyR Escherichia coli* mature biofilms, their counterpart planktonic cultures and exponentially growing planktonic cultures, we explored the role of OxyR-mediated metabolism alternations in modulation of susceptibility to antibiotics ciprofloxacin and cefotaxime. All three types of cultures were placed in fresh medium,1 h after antibiotics were added and incubation continued further for 2 h. Killing rates of antibiotics were determined, biofilm eradication using crystal violet assay was estimated, expression of *rpoS, katG, sulA* genes as well as HPI and HPII catalase activity were measured. Biofilms of both strains were more recalcitrant to ciprofloxacin and cefotaxime at all tested concentrations compared to exponentially growing planktonic cultures. In *oxyR* biofilms killing rate of ciprofloxacin was lower, and killing rate of cefotaxime was higher compared to the parental strain. Compared to biofilms, wild-type biofilm counterpart planktonic cultures showed higher tolerance to low doses of ciprofloxacin, while *oxyR* plankton demonstrated higher tolerance to cefotaxime. Higher recalcitrance of *oxyR* biofilms to ciprofloxacin may be caused by an increase in persister cells under conditions of enhanced oxidative stress and activated SOS response.

**Keywords**: biofilms; antibiotics; RpoS, OxyR and SOS regulons; *Escherichia coli*

## Introduction

Ciprofloxacin- and cefotaxime-treated urinary tract infections are often associated with biofilm-producing bacteria such as *Escherichia coli* which demonstrated resistance to both antibiotics in 49.9 and 28% of described cases, respectively [1]. Promising strategies to combat multidrug-resistant and antibiotic tolerant microorganisms include alternation of central bacterial metabolism [2,3]. Since formation of reactive oxygen species (ROS) is a key trigger of stress response, modulation of antioxidant defense pathways might affect development of antibiotic tolerance/susceptibility. ROS including $H_2O_2$, have been proposed to explain antibiotic-induced bacterial cell death, regardless of primary antibiotic targets.[4] This subject remains a matter of intense debate, as other scientists published conflicting results, including those about the role of OxyR-controlled defenses in lethal activity of antibiotics [5-7].

OxyR factor stimulates transcription of genes involved in bacterial defense against peroxide stress, decreasing the levels of $H_2O_2$ and unincorporated iron, thereby reducing DNA damage caused by Fenton chemistry and preventing cell death [8].

OxyR role in biofilm formation varied from stimulation [9-11] to inhibition [12, 13].

*Email: samoilova@iegm.ru

Here, we used an experimental model based on mature biofilms, their counterpart plankton and exponentially growing planktonic cultures of *Escherichia coli* strains to study effect of OxyR deficiency on susceptibility of *E. coli* biofilm and planktonic cultures to bactericidal antibiotics of different classes: fluoroquinolone ciprofloxacin and β-lactam cefotaxime.

## Materials and methods

### Bacterial strains and growth conditions

*E. coli* strains BW25113 (wt) and JW3933 (*oxyR*) were from Keio collection [14]. The strains carrying transcriptional gene fusions *katG*::*lacZ* and *rpoS*::*lacZ* were constructed by transformation of the parental strain and the *oxyR* mutant with plasmids pKT1033 [15] and pRS415 *katF5*, respectively. The strains with *sulA(sfiA)*::*lacZ* fusions were created by P1 transduction from the strain DM4000 [16].

Bacteria were grown overnight in M9 minimal glucose (2 g/l) medium [17]. After centrifugation the cells were resuspended in fresh M9 medium (4 g/l glucose) supplemented with 0.2% casamino acids and 10 μg/ml thiamine to an initial optical density at 600 nm ($OD_{600}$) of 0.1. Wells of 96-well polystyrene microtitre plates were inoculated with 200 μl of culture prepared as described above and incubated at 37°C during 22 h to produce biofilms.

All reagents for determination β-galactosidase and catalase activity, antibiotics ciprofloxacin and cefotaxime, thiamine, casamino acids, agar, Luria-Bertani broth, were from Sigma-Aldrich Chemical Co (St Lous, MO, USA). Other reagents were of analytical grade (Reachim, Russia).

### Antibiotic treatments

Mature biofilms were washed twice with 0.9% NaCl, 200 μl aliquots of fresh M9 (4 g/l glucose) medium supplemented with 0.2% casamino acids and 10 μg/ml thiamine were added in each well. The plates were incubated at 37°C with shaking (340 rpm) in Shaker Thermostat Sky Line (ELMI, USA) during 1 h, then antibiotics 0.03 μg/ml (2 MIC) and 0.3 μg/ml (20 MIC) ciprofloxacin or 1 μg/ml (12.5 MIC) and 10 μg/ml (125 MIC) cefotaxime were added and incubation continued further for 2 h. Mass biofilm formation and colony forming units (CFU) were determined at time zero and every hour of cultivation.

Two types of planktonic cultures were used. One (wt-16, *oxyR*-16) was derived from 16 h night culture prepared as described above for biofilm production, another (wt-22, *oxyR*-22) was derived from 22 h planktonic counterparts of the biofilms by its dilution with the fresh medium to $OD_{600}$ of 0.1. 200 μl aliquots of these cultures were placed in wells of polysterene microtiter plates, incubated for 1 h, treated with antibiotics and further incubated for 2 h. $OD_{600}$ was measured every hour of incubation using xMark™ spectrophotometer (Bio-Rad, USA) and samples for CFU determination were taken.

### Biofilm formation assay

Mass biofilm formation (BF) was monitored using the modified crystal violet microplate biofilm assay [18]. Wells of 96-well polysterene microtiter plates containing mature biofilms were prepared as described above. Control wells contained bacteria-free medium only. At time zero and every hour of cultivation $OD_{600}$ of each well was measured, broth was removed and wells were rinsed twice with 200 μl of sterile saline. The wells were air-dried and 150 μl per well of 0.1% crystal violet solution was added for 30 min. Then, the colourant was discarded and the wells were rinsed five times with distilled water. The plates were air-dried for 1 h. To quantify biofilms, 200 μl of 96% ethanol was pipetted into each well. After 5 min, 125 μl of the solution was transferred to a separate plate where the $OD_{540}$ was measured using xMark™ spectrophotometer. BF was calculated as follows: BF = AB – CW, where AB is the $OD_{540}$ of stained attached bacteria and CW is the $OD_{540}$ of stained control wells.

### Determination of killing rates of antibiotics

To compare bactericidal activity of antibiotics in different cultures, killing rates after 2 h exposure with antibiotics were calculated from the CFU data for each culture. The rate of antibiotic-induced bacterial killing (ψ*)* was calculated based on density decline of viable bacteria (CFU/ml) over a defined period [19]. Colony-forming ability (CFU/ml) was estimated as previously described [18]. Preliminary experiments revealed a decrease in plating efficiency of *oxyR* mutant on LB agar plates under aerobic conditions. Colony-forming ability of this strain was restored in the presence of catalase (500 U/ml) during serial dilutions and in plates with LB agar. Therefore, all colony-forming assays were performed with the addition of catalase.

**Determination of β-galactosidase and catalase activity**

We explored the role of OxyR, RpoS and SOS regulons in susceptibility to antibiotics measuring β-galactosidase activity in reporter strains carrying fusions of the genes *katG*, *rpoS* and *sulA* with the gene *lacZ* [17] using a SmartSpec Plus Spectrophotometer (Bio-Rad, USA). Aliquots of 340 µl of similar planktonic culture samples were collected from two similar wells and $OD_{600}$ was measured. Then 170 µl of these aliquots were transferred into two tubes each containing 690 µl of reducing buffer [17]. Then, 5 µl of sodium deoxycholate and 5 µl of toluene were added to each tube and incubated for 30 min at 37°C. Then 170 µl of 2-nitrophenyl-β-D-galactopyranoside (ONPG) were added to each test tube and incubated for 5 min. "Blank" tube was supplied with 170 µl of reducing buffer instead. The reaction was stopped by addition of 170 µl of $K_2CO_3$ and $OD_{420}$ and $OD_{550}$ were measured.

β-galactosidase activity was expressed in Miller units, calculated using the formula:

$(OD_{420} - 1.75 \times OD_{550} / \Delta OD_{600} \times t) \times 6000$,

where $OD_{420}$ and $OD_{550}$ – optical density of the samples, $\Delta OD_{600}$ is the difference between $OD_{600}$ of bacterial culture and $OD_{600}$ of bacteria-free medium, t – duration of exposition with ONPG, 6000 – coefficient taking into account dilution of the culture.

In biofilms, broth was removed and the plate wells were rinsed to remove nonadherent bacteria. Fresh M9 was further added to each well and biofilms were sonicated by two 1 min pulses (37 kHz, 30W) with 1 min pause time between them in a water bath sonicator (Ultrasonic cleaning unit Elmasonic S10 H, Elma, Germany). $OD_{600}$ and β-galactosidase activity were measured as described above.

HPI and HPII catalase activity in planktonic cultures was determined by the spectrophotometric method [20].

**Statistical analysis of the data**

Each result is indicated as the mean value of at least five 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 Statistica 6 (ver. 6, 2001; StatSoft Inc.).

**Results**

**Effect of *oxyR* deficiency on biofilm eradication in the absence and presence of antibiotics**

Similarly to previously published data [9], *oxyR* mutant demonstrated an increase in biofilm formation after 22 h of static incubation compared to the wild-type strain. Biofilm formation, calculated as crystal-violet staining ($OD_{540}$), normalised to total bacterial growth ($OD_{600}$), was 0.160 ± 0.016 and 0.258 ± 0.016 for the wild-type strain (wt-bf) and the *oxyR* mutant (*oxyR*-bf), respectively. Subsequent incubation in fresh M9 without antibiotics resulted in significant biofilm eradication, although wt-bf retained 60% of the initial biofilm compared to only 25% in *oxyR*-bf (Figure-1), apparently due to changes in the structure of biofilm and/or the signal transduction pathways associated with OxyR.

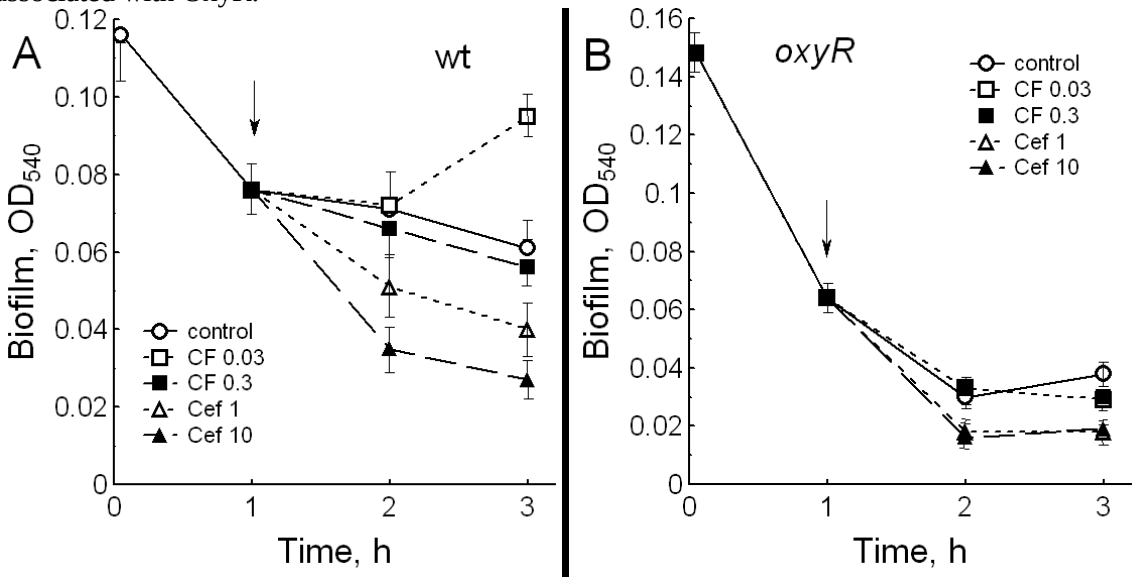


**Figure 1**-Influence of antibiotics and *oxyR* deletion on biofilm eradication in fresh medium.

**A.** Effect of ciprofloxacin (0.03 and 0.3 μg/ml) or cefotaxime (1 and 10 μg/ml) on BW25113 (wt) biofilms. **B**. Effect of ciprofloxacin (0.03 and 0.3 μg/ml) or cefotaxime (1 and 10 μg/ml) on JW3933 (Δ*oxyR*) biofilms. Antibiotics were added at the moment designated by the arrow. Values are the means and standard error (vertical bars) from at least five independent experiments.

Addition of 0.03 μg/ml of ciprofloxacin (CF) after 1 h incubation in fresh medium even stimulated biofilm formation by wt-bf, whereas a higher dose (0.3 μg/ml) had no significant effect. In contrast, cefotaxime (Cef) accelerated biofilm eradication in a dose-dependent manner (Fig. 1A). In *oxyR* strain, both doses of CF did not affect biofilm eradication, while both concentrations of Cef reduced $OD_{540}$ to the same extent (Figure-1B).

**Comparison of bactericidal activity of ciprofloxacin and cefotaxime in planktonic cultures and biofilms**

In preliminary experiments, planktonic cultures obtained from 22 h biofilm counterpart plankton (wt-22, *oxyR*-22) grew about 2 times more slowly than planktonic cultures derived from a normal night culture (wt-16, *oxyR*-16). After 1 h incubation in the fresh medium without antibiotics specific growth rates calculated from $OD_{600}$ data were 1.10 ± 0.02 $h^{-1}$ and 0.47 ± 0.03 $h^{-1}$ for wt-16 and wt-22, respectively, and 0.94 ± 0.02 and 0.51 ± 0.03 $h^{-1}$ for *oxyR*-16 and *oxyR*-22, respectively.

Biofilms of both strains were more recalcitrant to CF and Cef at all concentrations tested compared to the wt-16 and *oxyR*-16 planktonic cultures (Fig. 2 A,B). A distinctive feature of wt-22 was a higher tolerance to low doses of both antibiotics compared to wt-16 (Fig. 2 C,D), which was in agreement with our previous findings that regardless of reasons causing changes of growth rate, an increase in the growth rate led to an increase in the ciprofloxacin-induced killing rate [21]. The functional OxyR protein was necessary for the development of this tolerance to low doses of CF, while the presence of the *oxyR* mutation was not significant in the case of Cef.

Notably, killing rate of low CF doses in wt-22 (ψ = -0.08) was even lower than that in wt-bf (ψ = -1.31). Similarly, *oxyR*-22 was less susceptible to low doses of Cef (ψ = 0.47) than *oxyR*-bf (ψ = -1.57).

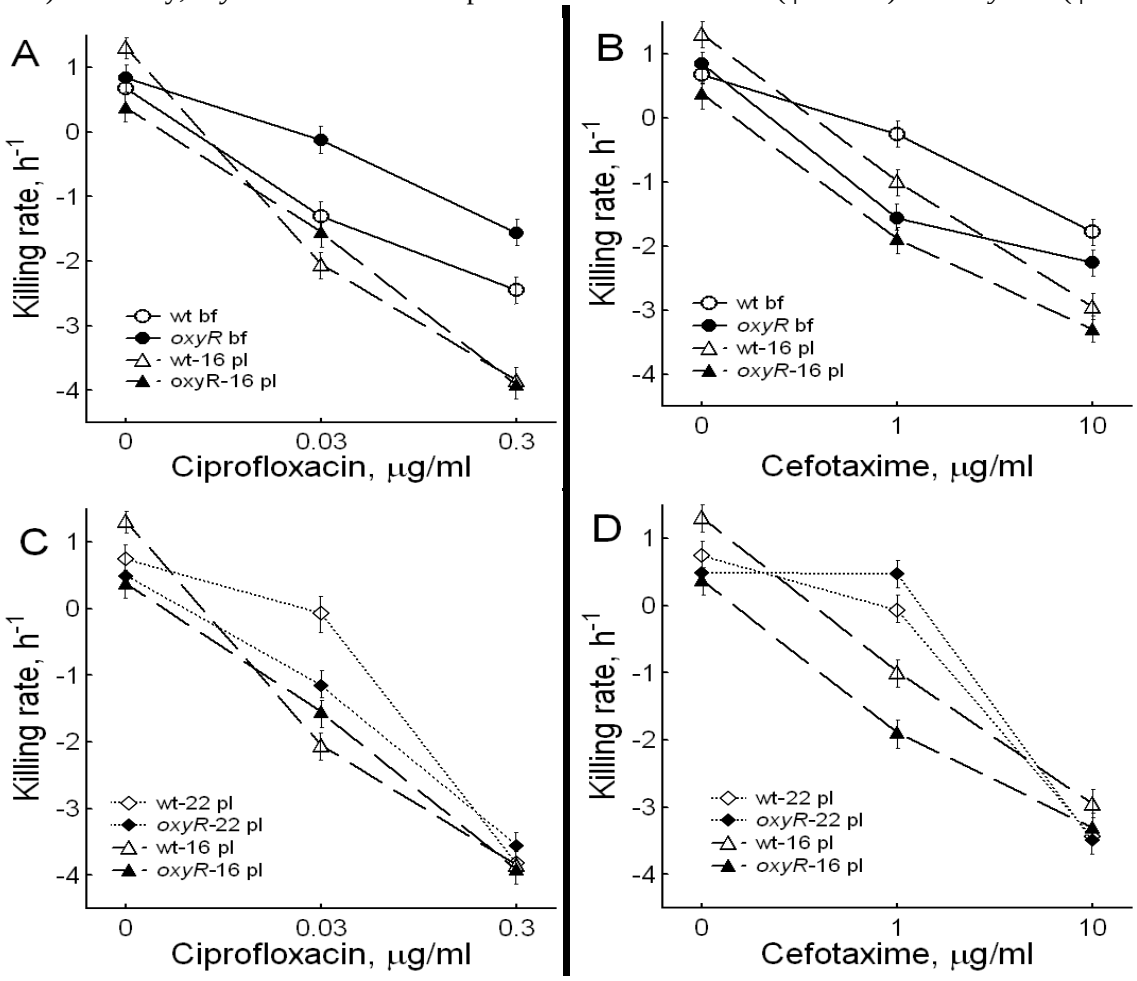


**Figure 2**-Killing rates of ciprofloxacin (**A**, **C**) and cefotaxime (**B**, **D**).

**A, B.** Comparison of wild-type and *oxyR* biofilms with wt-16 and *oxyR*-16 planktonic cultures.**C, D.** Comparison of two types of planktonic cultures.

**Determination of β-galactosidase activity in *rpoS::lacZ, katG::lacZ, sulA::lacZ* gene fusions and catalase activity in planktonic and biofilm cultures**

Expression of *rpoS* gene was minimal in wt-16 and *oxyR*-16 planktonic cells (Figure-3A), which corresponded to high growth rates of these cultures. The level of expression of the *rpoS* gene increased 1.5-2 times in slow-growing wt-22 and *oxyR*-22 and more than 4-fold in wt-bf and *oxyR*-bf. Biofilms also demonstrated a higher level of *sulA* expression, indicating an increased expression of SOS regulon compared to planktonic cultures (Figure-3B). Earlier, *recA* and other SOS response genes were significantly induced in mature biofilms compared to exponentially grown planktonic cells [22]. Absence of *oxyR* increased *sulA* gene expression by 2.5 times in both planktonic cultures compared to the wild type strain (Figure-3B), revealing an increased oxidative stress and DNA damage in the *oxyR* mutant under these conditions.

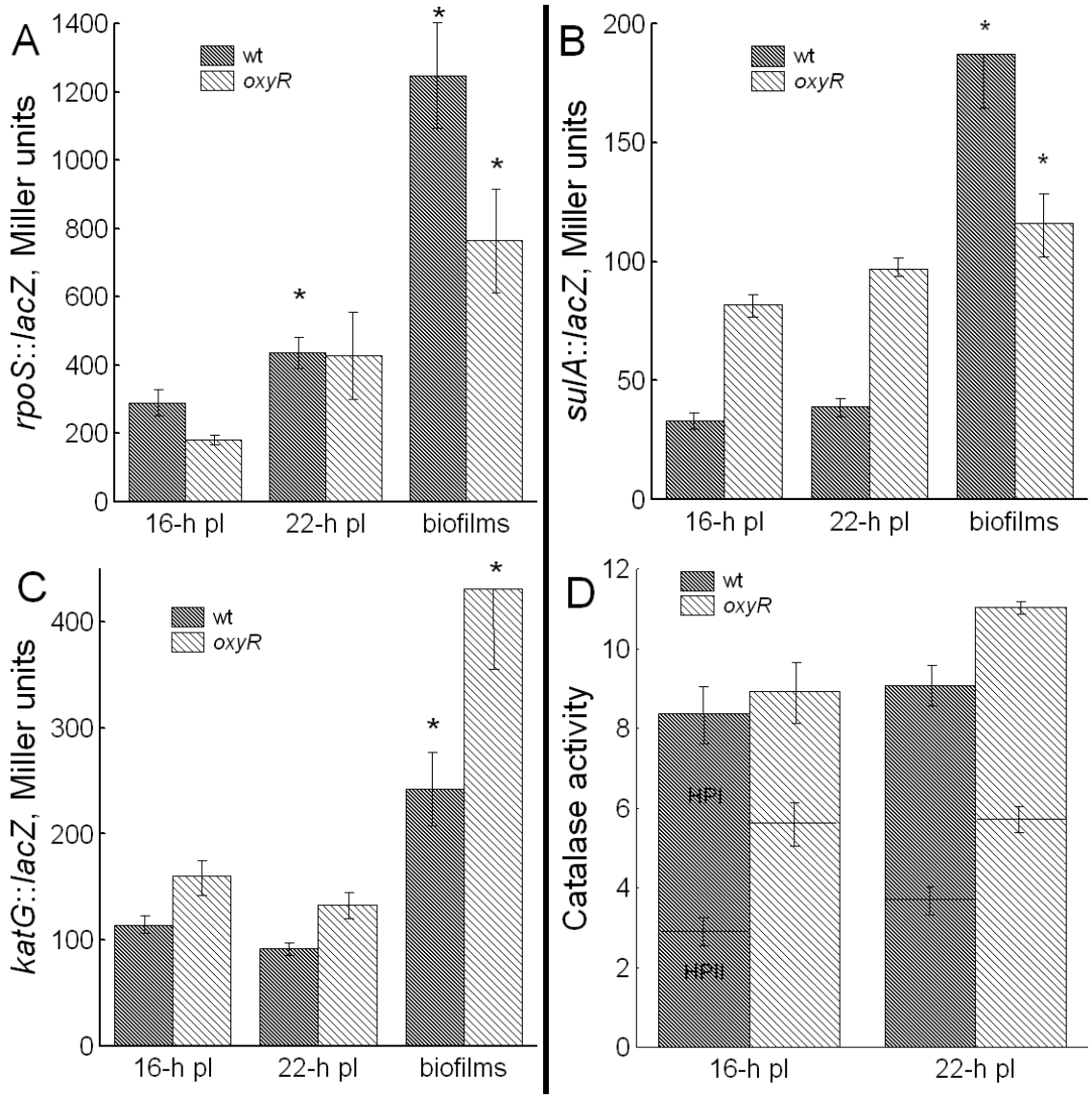


**Figure 3**-Expression of the *rpoS*, *katG*, *sulA* genes and catalase activity in planktonic cells and biofilms. **A**, **B**, **C.** The β-galactosidase activity of *rpoS*::*lacZ*, *sulA*::*lacZ*, *katG*::*lacZ*.

**D.** Catalase activity (μmol/min · mg protein). The presented data were obtained in an hour after the start of incubation in fresh medium. Values are the means and standard error (vertical bars) from at least five independent experiments. Statistical differences compared to the 16-h planktonic cultures ($P = 0.05$) are noted with an asterisk.

The expression of *katG* gene of the OxyR regulon, was not significantly different in both planktonic cultures, but it increased 2.1-fold and 2.7-fold in wt-bf and *oxyR*-bf compared to planktonic cells (Fig. 3C) which was consistent with the findings that cells in biofilms undergo endogenous oxidative stress [23]. The expression of *katG* can be controlled by both OxyR and RpoS transcriptional regulators [24]. Therefore, enhanced expression of *katG* gene in *oxyR* mutant was presumably caused by RpoS-controlled expression. Determination of catalase activity in planktonic cultures of the wild-type and *oxyR* strains confirmed the role of RpoS in induction of catalase HPI (encoded by *katG*), especially in *oxyR*-22 planktonic cells (Fig. 3D). Total catalase activity in these cells was higher than in wt-22 planktonic culture due to the increased activity of both HPI and HPII (encoded by RpoS-regulated *katE* gene) catalases. Unfortunately, we were unable to measure catalase activity in biofilms because of the small number of cells.

**Discussion**

Deletion of *oxyR* was reported to increase the susceptibility of *Klebsiella pneumoniae* to antibiotics due to a decrease in expression of *acrB*, encoding a component of multiple drug resistance system AcrAB [25]. Here we showed that OxyR deficiency significantly influenced susceptibility to CF in biofilms and *oxyR*-22. The effect was opposite: *oxyR*-bf became more recalcitrant than wild-type biofilms, whereas *oxyR*-22 lost ability to withstand low doses of CF better than *oxyR*-16. Alternatively, Cef demonstrated increased killing efficiency in *oxyR* biofilms compared to wild-type biofilms, while *oxyR*-22 retained higher tolerance to low doses of antibiotic than *oxyR*-16. Specific growth rate of bacterial cultures contributes to susceptibility of *E. coli* to killing by antimicrobials [21] and is inversely proportional to intracellular concentrations of global regulator of gene expression guanosine tetraphosphate (ppGpp) and general stress response regulator RpoS. Typically associated with starvation and stress, RpoS controls many defensive genes, which overlap partially with those that are under OxyR control (*katG*, *ahpCF*, *dps*) [24]. ppGpp-mediated stringent response and RpoS can be involved in generation of highly tolerant bacterial persisters to antibiotics [26,27]. Here, both wt-22 and *oxyR*-22 grew 2 times slower and showed 1.5 and 2.4 times higher levels of *rpoS* expression than wt-16 and *oxyR*-16. Observed low growth rate and RpoS regulon induction might explain decreased lethal effect of low Cef dose in wt-22 and *oxyR*-22 and low CF dose in wt-22. Under these conditions, tolerance of wt-22 and *oxyR*-22 planktonic cultures was the same or even higher than those of the corresponding biofilms. Similarly, earlier studies showed that stationary cultures of *Pseudomonas aeruginosa* had the same or even higher tolerance to antibiotics than biofilms [28]. In case of CF, *oxyR* deficiency in *oxyR*-22 prevented an increase in tolerance, probably because of lack of some vital function controlled by OxyR. It does not seem to be *katG* gene coding for catalase HPI, since *katG* expression and catalase activity in *oxyR*-22 were even slightly higher than in wt-22.

Significant increase in *rpoS*, *sulA* and *katG* expression in both wild-type and *oxyR* biofilms compared to wt-16 and *oxyR*-16 may indicate an increase in persister cells which led to a decrease of CF and Cef killing efficiency in wild-type biofilms. *oxyR* deletion resulted in a strong biofilm eradication that was accelerated by Cef, which could be the reason for the decrease in *oxyR* biofilm recalcitrance toward Cef compared to wild-type biofilms. By contrast, lack of *oxyR* may enhance endogenously induced oxidative stress in biofilms, thereby promoting formation of persister cells with participation of toxin-antitoxin modules and the SOS response [23,27]. Eventually, an increase in tolerance to CF in *oxyR* biofilms might occur as we have observed.

Data presented here highlight the need of in-depth exploration of involvement of bacterial antioxidant pathways in antibiotic tolerance.

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