Supplementary Information

of

Antibiotic susceptibility of *Escherichia coli* cells during early-stage biofilm formation

Huan Gu[1,2], Sang Won Lee[1,2], Joseph Carnicelli[1,2], Zhaowei Jiang[1,2], and Dacheng Ren[1,2,3,4*]

[1]Department of Biomedical and Chemical Engineering, [2]Syracuse Biomaterials Institute, [3]Department of Civil and Environmental Engineering, [4]Department of Biology, Syracuse University, Syracuse, NY 13244, USA

*Corresponding author:

Dacheng Ren: Phone +1-315-443-4409. Fax +1-315-443-9175. Email : dren@syr.edu

Running title: Bacterial drug-tolerance changes in biofilm formation

Table S1. List of bacterial strains used in this study.

Strain name	References
E. coli RP437	(1)
P. aeruginosa PAO1	(2)
Uropathogenic *E. coli* ATCC 53505	(3)
E. coli ASV (*rrnB*$_{P1}$*::gfp*^{unstable})	(4, 5)
E. coli BW25113	(6)
E. coli JW0452-3 (Δ*acrA*)	(6)
E. coli AR3110 (wild-type)	(7)
E. coli AR182 (Δ*bcsA*)	(7)
E. coli AR282 (Δ*csgB*)	(7)

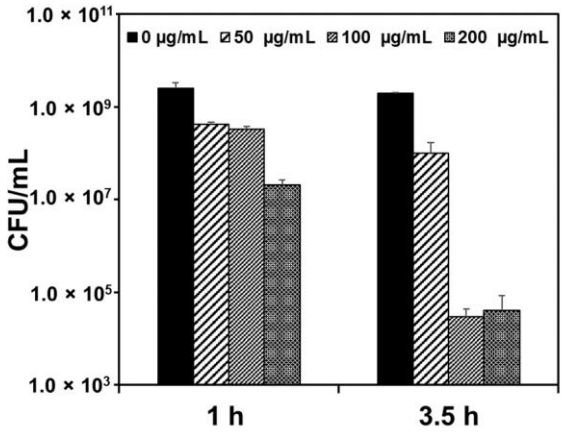

Figure S1. Susceptibility of planktonic *E. coli* RP437 cells to Amp. Planktonic cells were harvested at 2 h after inoculation from flasks with shaking at 200 rpm. Cells were treated in LB medium with Amp (0, 50, 100, or 200 µg/mL) for 1 or 3.5 h. Three biological replicates were tested for each condition.

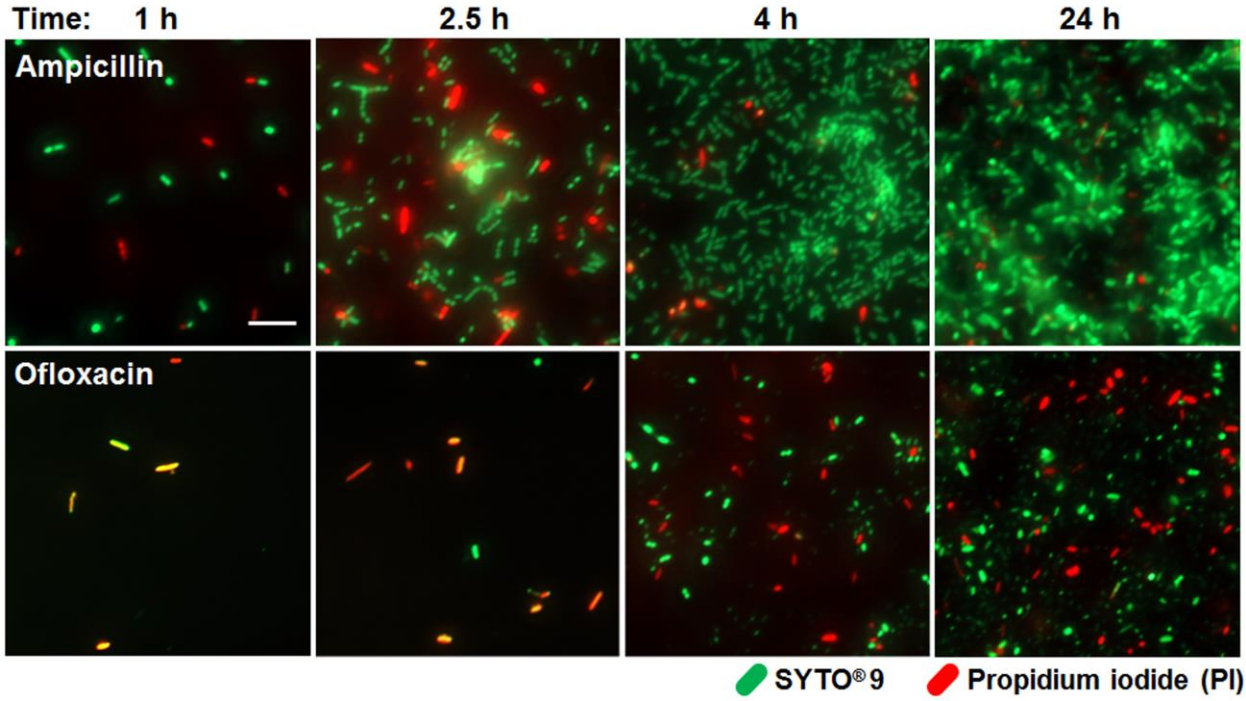

Figure S2. Representative Live/Dead staining images of 1, 2.5, 4, and 24 h *E. coli* RP437 biofilms after 1 h antibiotic treatment (Bar = 10 μm). The antibiotic treatment was conducted in 0.85% NaCl solution. Biomass after 1 h Ofx treatment is lower than that after Amp treatment because Ofx treatment triggered biofilm dispersion. All biofilms were formed on glass surfaces. Three biological replicates were tested and five random images were taken for each sample.

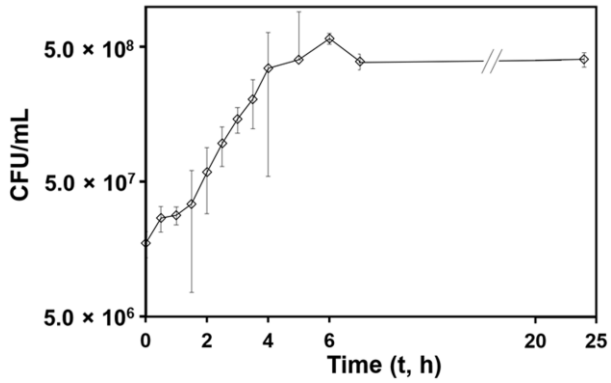

Figure S3. Growth curve of planktonic *E. coli* RP437 cells in static biofilm cultures. Each data point was measured with 14 biological replicates.

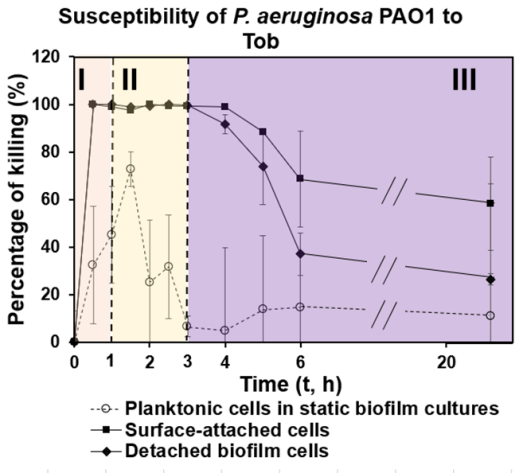

Figure S4. Susceptibility of *P. aeruginosa* PAO1 to 10 μg/mL Tob during early-stage biofilm formation. The antibiotic treatment was conducted in 0.85% NaCl solution. Biofilms were formed on glass surfaces, and each condition was tested with three biological replicates.

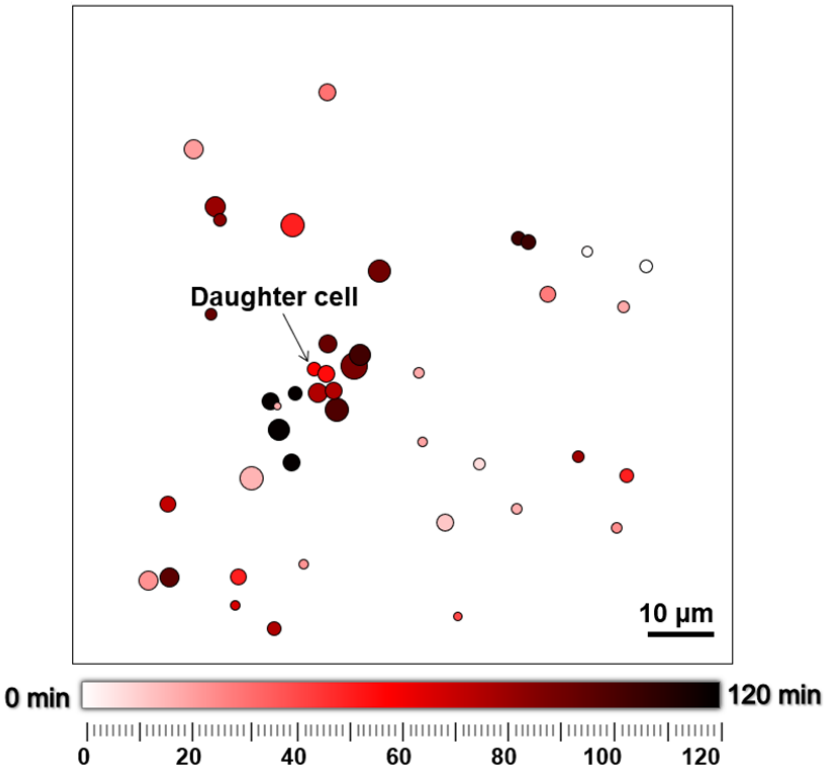

Figure S5. Initial attachment of *E. coli* RP437 cells on glass surfaces. In this figure, each *E. coli* RP437 cell is indicated with a circle. The image shows a 100 µm × 100 µm view of the biofilm. The darkness and diameter of the circle indicate the time point of attachment and cell length at the moment of attachment, respectively. The cell length was scaled down by 1.6 times when determining the circle diameter to fit in the plot. Among all the cells shown in this figure, only one cell was generated due to the division of an attached cell (indicated with an arrow). All the other cells were attached from the planktonic population.

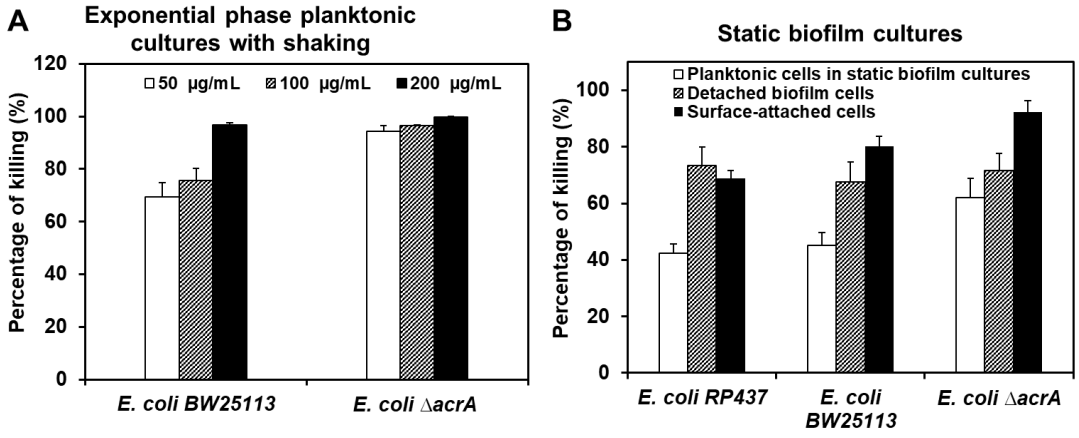

Figure S6. Antibiotic susceptibility of *E. coli* RP437, *E. coli* BW25113, and *E. coli* Δ*acr*A cells. *E. coli* BW25113 is the isogenic wild-type of *E. coli* Δ*acr*A. (A) Susceptibility of 2 h old exponential phase *E. coli* BW25113 and Δ*acr*A cells to Amp (50, 100, or 200 µg/mL) when they were treated in LB medium for 1 h. (B) The susceptibility of 1 h *E. coli* cells to 200 µg/mL Amp. *E. coli* biofilms were formed on glass surfaces, and each condition was tested with three biological replicates.

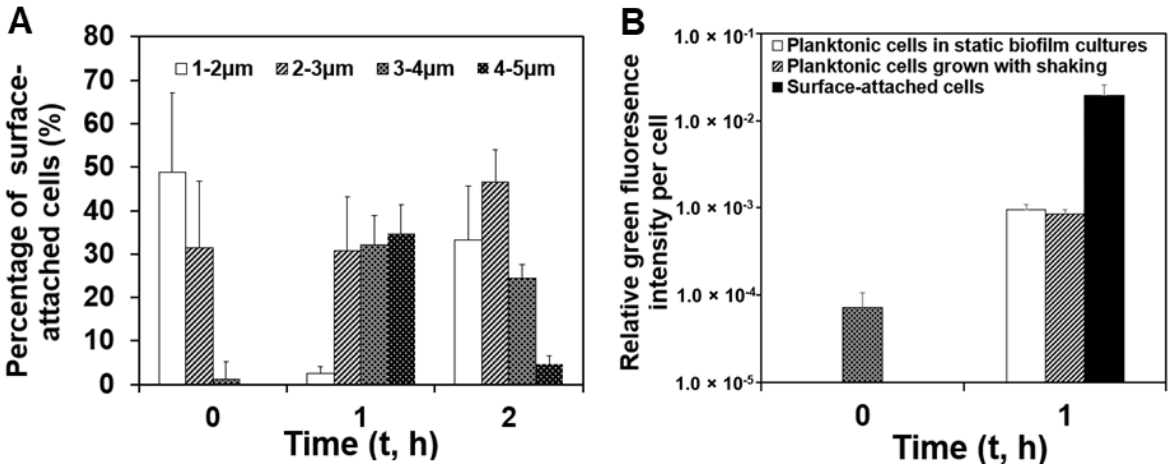

Figure S7. Cellular activities during early-stage biofilm formation. (A) Change of *E. coli* RP437 cell length during biofilm formation. The projected cell length of ~300 cells on the x-y plane was analyzed. (B) Relative green fluorescence of *E. coli* ASV cells before inoculation and 1 h after inoculation. Green fluorescence intensity indicates the expression level of a ribosomal gene *rrnB*. *E. coli* biofilms in Fig. S7 were formed on glass surfaces in LB medium, and each condition was tested with three biological replicates.

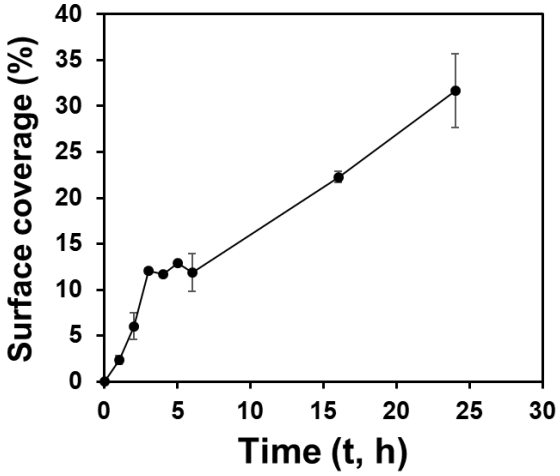

Figure S8. Surface coverage of *E. coli* RP437 biofilms. *E. coli* RP437 was cultured for 24 h to form biofilms on glass surfaces in LB medium. Each condition was tested with three biological replicates.

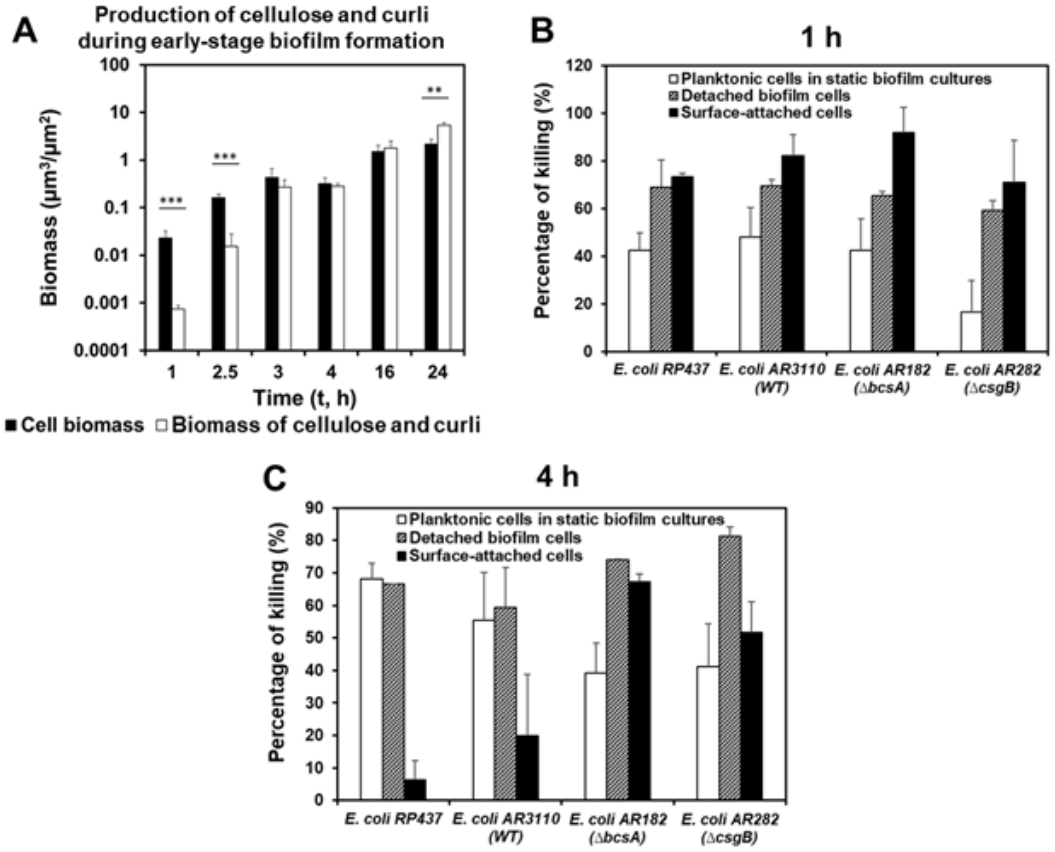

Figure S9. Role of biofilm EPS in the susceptibility of *E. coli* RP437 cells to Amp during early-stage biofilm formation. (A) Quantification of biomass at 1, 2.5, 3, 4, 16, and 24 h after inoculation (*: $p < 0.05$, **: $p < 0.005$, and ***: $p < 0.0005$). The biomass was determined by analyzing the green fluorescent signals from biofilm cells labeled with SYTO®9 and red fluorescence signals from the biofilm matrix labeled with Congo Red. The mass was quantified using COMSTAT(8). (B&C) Susceptibility of surface-attached *E. coli* RP437, *E. coli* AR3110 (WT), *E. coli* AR182 (Δ*bcsA*), and *E. coli* AR282 (Δ*csgB*) cells to 200 μg/mL Amp at 1 (B) or 4 (C) h after inoculation. The antibiotic treatment was conducted in 0.85% NaCl solution. *E. coli* biofilms were formed on glass surfaces in LB medium, and each condition was tested with three biological replicates.

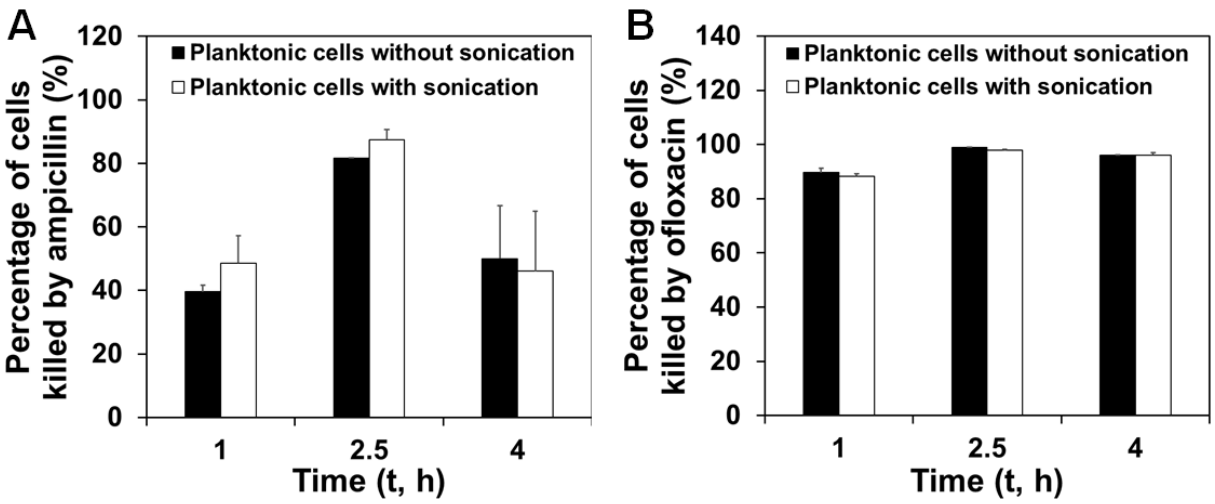

Figure S10. Sonication did not affect the susceptibility of planktonic *E. coli* RP437 cells. The planktonic cells were collected from static biofilm cultures at 1, 2.5, or 4 h after inoculation and treated with Amp (A) and Ofx (B). The antibiotic treatment was conducted in 0.85% NaCl solution. No significant change in antibiotic susceptibility due to sonication was observed. Each condition was tested with three biological replicates.

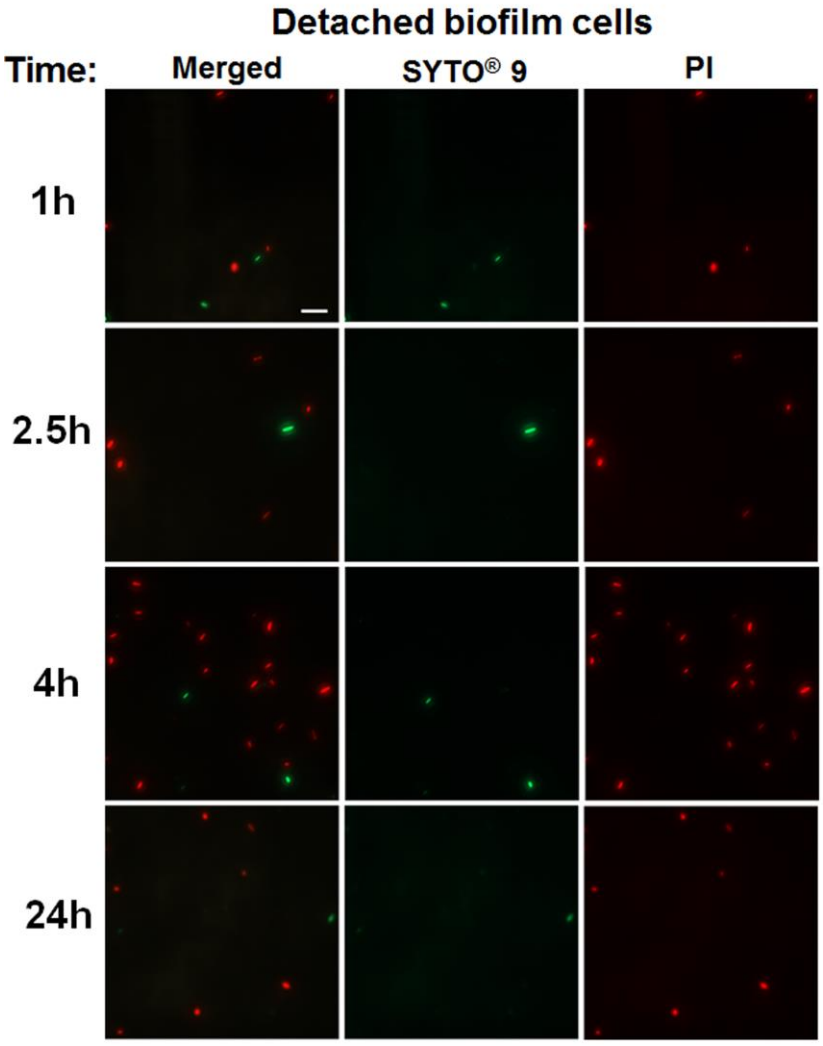

Figure S11. Representative Live/Dead staining images of detached biofilm cells during early-stage biofilm formation (t = 1, 2.5, 4, and 24 h). The antibiotic treatment was conducted in 0.85% NaCl solution. *E. coli* RP437 biofilms were formed on glass surfaces in LB medium, and each condition was tested with three biological replicates. Bar = 10 μm.

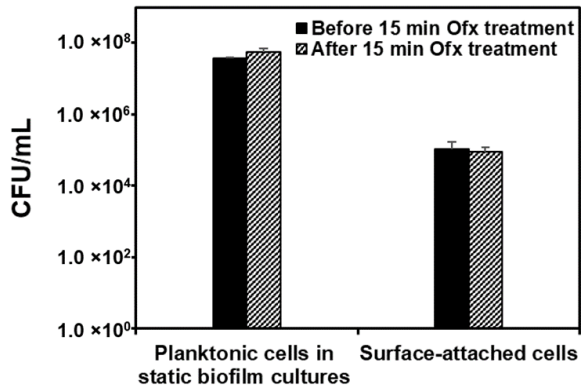

Figure S12. The viability of *E. coli* RP437 cells (1 h after inoculation) before and after 15 min treatment with 10 μg/mL Ofx. The antibiotic treatment was conducted in 0.85% NaCl solution. *E. coli* biofilms were formed on glass surfaces, and each condition was tested with three biological replicates.

References

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