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SHORT COMMUNICATION

Stimulation of Biofilm Formation by Antibiotics

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The biofilms formed by pathogenic microorganisms are responsible for a number of chronic infections resistant to antibiotic treatment [1]. The use of medical devices introduced into a macroorganism, such as catheters, artificial valves, etc. is hindered by biofilm formation [2–4]. Moreover, biofilms are among the major agents responsible for the biocorrosion pipelines and technological equipment. Counteracting resistance of the biofilms to physicochemical treatment, biocides, and antibiotics is of major importance to modern medicine and industry.

It was recently discovered that some compounds of plant origin [5], as well as subbacteriostatic concentrations of certain antibiotics, stimulate biofilm growth, as opposed to preventing it. This phenomenon raises difficulties in preventing biofilm formation.

For example, subinhibitory concentrations of the aminoglycoside antibiotic tobramycin induce biofilm formation by *Pseudomonas aeruginosa* and *Escherichia coli* [6]. Imipinem, a broad-spectrum β-lactam antibiotic, has a similar effect [7].

In our study, antibiotics with different mechanisms of action (azithromycin, rifampicin, and oxacillin) were used as molecular tools for investigating the stages of biofilm formation by nonpathogenic bacteria, namely for detection of the key metabolic processes resulting in formation of the "biofilm phenotype", i.e., in selective expression of the genes involved in biofilm formation. Some of these antimicrobial agents were found to stimulate biofilm formation [8].

The goal of the present work was to compare the sensitivity of planktonic (suspension) cultures and single-species biofilms at different stages of their formation to antibiotics with different mechanisms of action. It was performed for two nonpathogenic microorganisms isolated from the Romashkinskoe oilfield (*Dietzia* sp. and *Kocuria* sp.).

The sensitivity of planktonic cultures to antibiotics was characterized as the antibiotic concentration resulting in 50% growth rate decrease compared to the control without antibiotics (ID_{50}) after 24 h of incubation at 28°C in shaken test tubes (150 rpm) or in the wells of a 96-well polystyrene plate. The sensitivity of biofilms was characterized by the same parameter determined by two methods. The first (classical) approach involved biofilm incubation in 96-well plates, staining with gentian violet or crystal violet, extraction of the dye with 96% ethanol, and measuring OD_{590} of the extract [7]. The second method in our modification involved cell cultivation on a hydrophobic carrier [9] (Teflon blocks, 2 × 2 × 2 mm). While both techniques yielded similar results, the second one was less variable. The biofilms that were incubated during the 24 h prior to addition of antibiotics are henceforth designated as "preformed" ones. Alcian blue, the dye interacting with the matrix polysaccharides and weakly staining the cells, was used to stain the biofilm matrix [10]. Extraction of the dye was carried out with dimethyl sulfoxide, and OD was determined at 540 nm.

The table and figures present the typical experimental results.

The generalized results on the sensitivity of the biofilms and planktonic cultures to three antibiotics are presented in the table. It can be seen that biofilm growth, especially in the case of preformed biofilms, is significantly (ten - or hundredfold) less sensitive to antibiotics than the growth of planktonic cultures.

Since the formation of biofilms results in lower sensitivity to antibiotics and stress factors, introduction of antibiotics in the course of biofilm formation makes it possible to determine the moment of emergence of the "biofilm phenotype", i.e., the time required for the conversion of planktonic cells to the biofilm form (in the presence of a phase interface). For this purpose, antibiotics were added to *Kocuria* sp. suspension in wells or test tubes at inoculation (zero time) and after 2, 4, 6, or 14 h of incubation. The results for azithromycin (assessed after 24 h of incubation) are shown on Fig. 1. In the case of *Dietzia* sp., antibiotics were added at 0, 2, 4, 6, and 24 h. The results for azithromycin (assessed after 48 h of incubation, since the rate of biofilm formation by this organism was lower) are shown on Fig. 2. Similar results were obtained for oxacillin and rifampicin (not shown).

For *Kocuria* sp. biofilms, a significant decrease in the sensitivity to azithromycin was observed from 2 to 4 h (Fig. 1, curves *2* and *3*). In the case of *Dietzia* sp., longer incubation of 4 to 6 h was required (Fig. 2, curves *2* and *3*). No significant increase in antibiotic

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Sensitivity to antibiotics (ID_{50}, μg/mL of the studied bacterial cultures

Bacteria	Culture	Antibiotic		
		Oxacillin	Rifampicin	Azithromycin
Kocuria sp.	Planktonic (test tubes)	0.0025–0.005	0.0015–0.003	0.02–0.025
	Planktonic (plates)	0.005–0.01	0.0015–0.003	0.02–0.05
	Biofilms (forming)	2.5–5.0	0.75–1.0	>1.0
	Biofilms (preformed)	>10	>2.0	>10
Dietzia sp.	Planktonic (test tubes)	0.5–1.0	0.03–0.06	0.5–1.0
	Planktonic (plates)	0.5–1.0	0.05–0.1	0.5–1.0
	Biofilms (forming)	25–50	>2.0	>5.0
	Biofilms (preformed)	>125	>5.0	>50

resistance was observed for appropriate planktonic cultures supplemented with azithromycin during the first 2–6 h of incubation (Figs. 1 and 2, curves *1*). Thus, the density of microbial populations and the degradation of the antibiotic during incubation had no effect on the sensitivity of bacteria.

During this time interval (2–6 h of incubation), transition from reversible adhesion to the irreversible stage probably occurs, with the onset of formation of the extracellular biofilm matrix.

In order to confirm this suggestion, independent staining of biofilms with alcian blue was carried out, along with their staining with crystal violet. Indeed, the increase of antibiotic resistance was accompanied by a sharp increase in matrix staining (Figs. 1 and 2, curves *3*).

We have previously reported that low, sub-bacteriostatic concentrations of some antibiotics stimulated biofilm formation [8]. This phenomenon was further investigated in the present work. A broad range of antibiotic concentrations were added together with the inoculum or after 4–6 h of incubation of the biofilm. Staining with alcian blue and crystal violet was then used to monitor culture growth and matrix formation

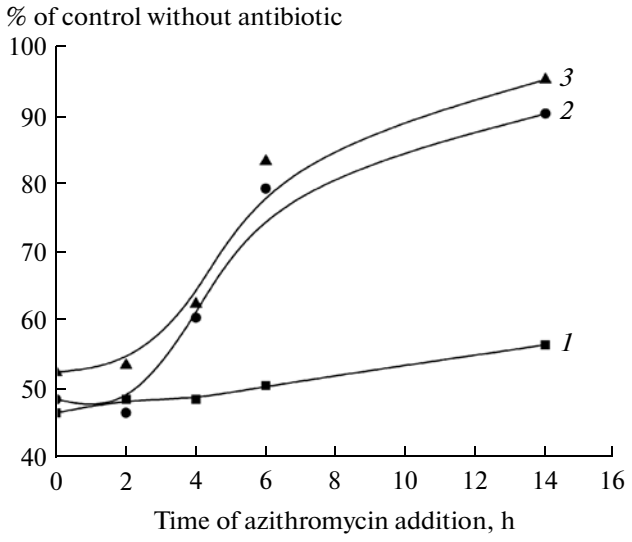

Fig. 1. Sensitivity of *Kocuria* sp. to azithromycin (0.05 μg/mL for the planktonic culture and 1 μg/mL for the forming biofilm) depending on the time of antibiotic introduction: planktonic culture (*1*), biofilm, staining with crystal violet (*2*), and biofilm, staining with alcian blue (*3*).

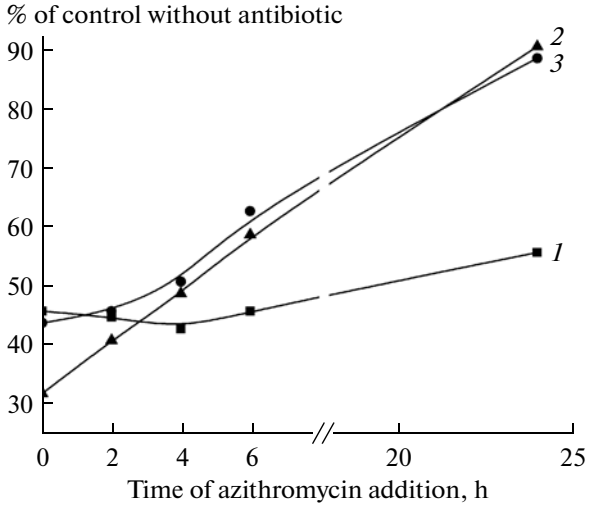

Fig. 2. Sensitivity of *Dietzia* sp. to azithromycin (0.5 μg/mL for the planktonic culture and 5 μg/mL for the forming biofilm) depending on the time of antibiotic introduction: planktonic culture (*1*), biofilm, staining with crystal violet (*2*), and biofilm, staining with alcian blue (*3*).

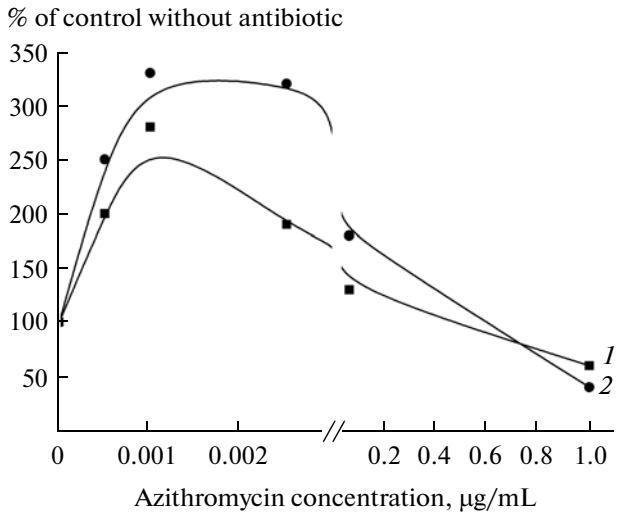

Fig. 3. Stimulation of biofilm formation by *Kocuria* sp. with subbacteriostatic concentrations of azithromycin: staining with crystal violet (*1*) and staining with alcian blue (*2*).

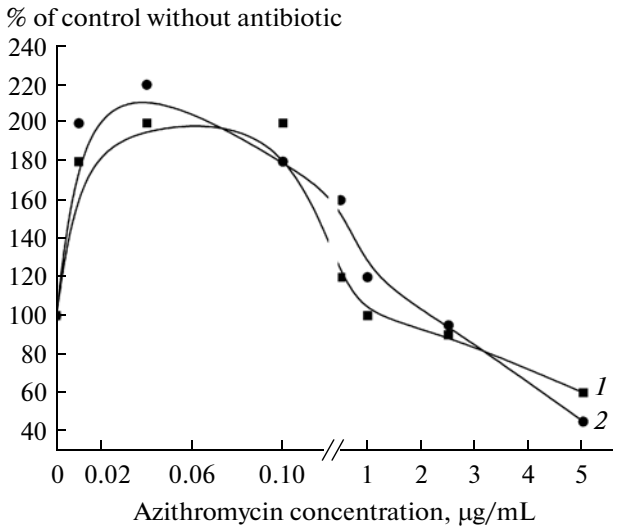

Fig. 4. Stimulation of biofilm formation by *Dietzia* sp. with subbacteriostatis concentrations of azithromycin: staining with crystal violet (*1*) and staining with alcian blue (*2*).

for the subsequent 24 h. The results for azithromycin are presented on Figs. 3 and 4. Low concentrations of the antibiotic, which had little or no effect on the growth of either biofilms or planktonic cultures, were shown to stimulate biofilm formation significantly. The growth rate of the matrix (aclian blue staining, curve *2* on Figs. 3 and 4) exceeded the rate of microbial cell growth (crystal violet staining, curve *1* on Figs. 3 and 4). Rifampicin had a similar effect, while in the case of oxacillin this phenomenon was not observed. Addition of azithromycin or rifampicin after the stage of reversible adhesion (4–6 h) had little or no effect on the stimulation of biofilm formation (not shown).

The mechanism of the stimulation of biofilms formation by antibiotics remains unclear. It was suggested that aminoglycoside antibiotics could have an influence on the level of cyclodiguanosine monophosphate (c-di-GMP), a factor affecting biofilm formation [6]. Stimulation of adhesin synthesis associated with the "ribosomal stress" is another possibility [11].

We suggest that the antibiotics directly or indirectly affecting transcription and translation may imitate the action of stress factors triggering the processes of the "biofilm phenotype" formation involving the RpoS sigma factor and other activators. This suggestion requires further investigation.

In conclusion, it should be mentioned that our results confirm the importance of following the rational regimens of antibiotic treatment, since both the early termination of antibiotics course and their low dosage may stimulate the biofilms formation by pathogenic microorganisms and transition of a disease into the chronic form which is difficult to cure.

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