ISSN 0026-2617, Microbiology, 2014, Vol. 83, No. 6, pp. 723–731. © Pleiades Publishing, Ltd., 2014.

EXPERIMENTAL ARTICLES

Activation of Formation of Bacterial Biofilms by Azithromycin and Prevention of This Effect

S. V. Mart'yanov, M. V. Zhurina, G. I. El'-Registan, and V. K. Plakunov[1]

Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia

Received May 30, 2014

Abstract—Growth of members of most of the studied genera of gram-positive (*Dietzia*, *Kocuria*, and *Rhodococcus*) and gram-negative bacteria (*Pseudomonas* and *Chromobacterium*) in biofilms exhibited higher resistance to a translation inhibitor, azithromycin compared to the growth of planktonic cultures of the same strains. Low concentrations of azithromycin were found to stimulate biofilm formation by the studied saprotrophic strains. The rate of synthesis of the polysaccharide matrix component exceeded the rate of cell growth, indicating implementation of the biofilm phenotype under these conditions. It was found that an alkylhydroxybenzene (AHB) compound 4-hexylresorcinol was capable of almost uniform suppression of growth of both planktonic cultures and biofilms of the saprotrophic strains under study. In some cases, combined action of azithromycin and AHB resulted in an additive inhibitory effect and prevented the stimulation of biofilm growth by subinhibitory azithromycin concentrations. Thus, AHB may be considered a promising antibiofilm agent.

Keywords: biofilms, antibiotics, stimulation of biofilm formation, prevention of biofilm formation, alkylhydroxybenzenes, 4-hexylresorcinol

DOI: 10.1134/S0026261714060113

The stimulatory effect of low antibiotic concentrations on biofilm formation by pathogenic microorganisms is receiving increasing attention in literature [1–3].

In our earlier work, we described the stimulatory effect of subinhibitory antibiotic concentrations on formation of biofilms by a number of gram-positive oil-oxidizing bacteria [4]. It is, however, not self-evident that subinhibitory antibiotic concentrations should exert the same effects on pathogenic and saprotrophic bacteria; this issue was to be addressed in special studies. The evolution of pathogenic microorganisms occurs in intense interaction with the macroorganism they infect. A prerequisite for their reproductive success is the development of strategies for resistance to protective mechanisms of the macroorganism, as well as to antibiotics used for the chemotherapy of infectious diseases. It is not surprising, therefore, that the biofilms formed by pathogens are highly resistant to antibiotics. If antibiotic therapy is prematurely discontinued and the antibiotic concentrations in the macroorganism decrease to subinhibitory levels, this may result in activating biofilm formation.

Saprotrophic microorganisms that inhabit the soil, aquatic, or subterranean ecosystems usually do not have to deal with antibiotics, at least not at the concentrations used for chemotherapy. Therefore, the resistance of their biofilms to antibiotics is not essential for their survival. Accordingly, this kind of antibiotic resistance seems to involve a general biological phenomenon that is based upon the biofilm properties, rather than the environmental conditions. Even if we suggest that saprotrophic microorganisms have acquired antibiotic resistance from pathogenic microorganisms via horizontal gene transfer, the manifestation of this capacity outside an infected macroorganism provides additional evidence that it represents a general biological phenomenon.

As for the mechanism of stimulation of biofilm formation by antibiotics, no consensus has yet been reached in the relevant literature. In a number of works, the influence of low antibiotic concentrations on bacterial adherence to a solid surface was detected. For instance, it was established [2] that subinhibitory concentrations of imipenem, a bacterial cell wall synthesis-suppressing antibiotic of the carbapenem group, increased adherence of *Acinetobacter baumanii* cells to polystyrene.

It was revealed that subinhibitory concentrations of the antibiotic tobramycin enhanced biofilm formation by *Pseudomonas aeruginosa* strain PAO1 [5]. However, other researchers established that subinhibitory tobramycin concentrations suppressed the N-acetyl homoserine lactone (AHL)-dependent quorum sensing (QS) system in the strain *P. aeruginosa* PUPa3, resulting in a decrease in biofilm formation [6].

[1] Corresponding author; e-mail: plakunov@inmi.host.ru

It was suggested that the activating effect of tobramycin on biofilm formation is related to the level of cyclic diguanosine monophosphate (c-di-GMP) [7]. The synthesis and degradation of this compound is catalyzed by the enzymes diguanylate cyclase (DGC) and phosphodiesterase (PDE), respectively. Both the positive and negative regulation of a number of biological processes depends on the binding of c-di-GMP to regulatory proteins. This results in changes in their conformation which exert an influence on intracellular processes. It was established that transition of bacteria from the planktonic to the biofilm phenotype is subject to regulation by the proteins with DGC and PDE activities. For instance, the biofilms formed by a *P. aeruginosa* mutant with an increased c-di-GMP content maturate poorly and do not disperse. Presumably, the c-di-GMP level in the cell gradually decreases during biofilm maturation. In a number of pathogenic bacteria, exopolysaccharide synthesis is also regulated via c-di-GMP. The regulatory pathways that depend on the QS system and c-di-GMP are not directly interlinked; nonetheless, there may be indirect links between them. For example, the QS-regulated transcription factor AphA of *Vibrio cholerae* influences expression of the DGC- and PDE-encoding genes.

Other researchers also revealed that in *P. aeruginosa* induction of biofilm formation in the presence of subinhibitory aminoglycoside concentrations involves c-di-GMP. The *arr* gene (the aminoglycoside-dependent regulator) codes for PDE. Accordingly, this enzyme activity is decreased in the cells with a mutant *arr* gene; this is accompanied by an increase in the cells' sensitivity to tobramycin. It was established that addition of guanosine triphosphate (GTP), which inhibits the activity of this phosphodiesterase, results in suppressing biofilm formation [1].

The aforementioned data suggest that it is not currently feasible to single out a general pattern underlying the effect of low antibiotic concentrations on biofilm formation. Therefore, this phenomenon should be addressed in further studies.

The goal of the present work was to find out whether low concentrations of the antibiotic azithromycin stimulate biofilm formation in a number of gram-positive and gram-negative bacteria not investigated previously in this respect and to test the suggestion that biofilm formation can be selectively suppressed.

MATERIALS AND METHODS

Subjects of research. In this work, we used pure cultures of the gram-positive petroleum-degrading bacteria *Kocuria rhizophila* and *Dietzia natronolimnaea* isolated from the stratal water of the Romashkinskoe oil deposit, and *Rhodococcus equi* isolated from the soil of the Hoa Lak settlement (Vietnam). For comparison, we also used gram-negative bacteria *Pseudomonas chlororaphis* 66, *Chromobacterium violaceum* WT (the type strain ATCC 31552), and its mutant CV026. These strains were a generous gift of Prof. I.A. Khmel' (Institute of Molecular Genetics, Russian Academy of Sciences). In the mutant strain, the *cviI* gene of synthase responsible for AHL synthesis was inactivated by inserting the mini-Tn5 transposon.

Cultivation and storage of the microorganisms. The microorganisms were stored in stabs of LB medium under paraffin oil at 4–6°C, except for the *Chromobacterium* cultures that were stored at room temperature; 100 μg/mL kanamycin was added to the medium for the CV026 mutant. To prepare the inoculum, bacteria were grown in LB medium at 29°C on a shaker (150 rpm) for 20–24 h. These cultures were used as inoculum in our studies with biofilms.

Obtaining biofilms and assessing their sensitivity to inhibitors. To compare the sensitivity of planktonic cultures and biofilms to antibacterial agents and physicochemical factors, we used the Teflon blocks technique that was developed in our laboratory [4]. For biofilm formation, 3 g of Teflon blocks (4 × 4 × 4 mm) were placed in test tubes with 3 mL of LB medium, sterilized at 1 atm, and inoculated (typically, with 50–60 μL). Biofilms formed on the Teflon blocks, while planktonic (suspension) cultures developed in the same test tube in liquid medium. Upon incubation, the planktonic culture was separated and its growth was assayed from the level of relative optical density (light absorption + light scattering) at 540 nm. Planktonic cells were removed from the blocks by washing with 1% NaCl. The biofilms were fixed with 96% ethanol. After removing the alcohol, the biofilms were stained with 0.1% aqueous solution of crystal violet (CV) (3 mL per test tube). In an additional series of experiments, the biofilm matrix was stained with 1,9-dimethylmethylene blue (DMMB) [8]; this method was modified by us as follows: a weighed amount of DMMB was used to prepare a 160 μg/mL solution in the buffer containing 200 mg of sodium formate and 200 μL of concentrated formic acid per 100 mL of water (pH 3.0); 3 mL of the solution was added to each test tube. CV was extracted with 96% ethanol and DMMB was extracted with the decomplexating solution (1.64 g sodium acetate, 152.85 g guanidine chloride, and 40 mL isopropanol per 400 mL of water, pH 6.8). Optical density of the solutions was measured at 590 and 670 nm for CV and DMMB, respectively.

Sensitivity to antibiotics and 4-hexylresorcinol. The sensitivity of a planktonic culture to an inhibitor was determined from the inhibitor concentration decreasing the growth rate by 50% (IC_{50}) after 24 h of incubation at 28°C in test tubes on a shaker (150 rpm). The

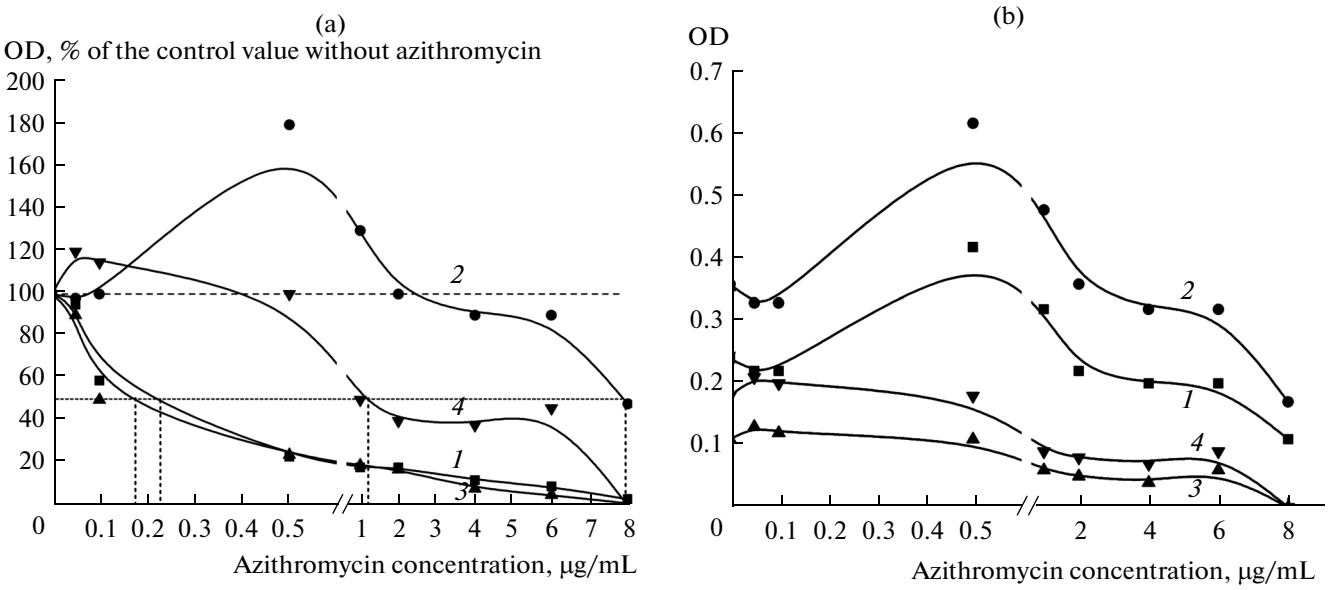

Fig. 1. Effect of azithromycin on the growth of planktonic cultures and biofilms of *C. violaceum*.: planktonic cultures (*1*, *3*) and biofilms (*2*, *4*) of the wild-type strain WT (*1*, *2*) and the CV026 mutant (*3*, *4*); the biofilms were stained with DMMB; growth, % of that in the control without the antibiotic (a); biofilms of the wild-type strain (*1*, *2*) and the CV026 mutant (*3*, *4*); the biofilms were stained with DMMB (*1*, *3*) or CV (*2*, *4*); the optical density of the extracts of the dyes were measured (b). Dashed line (here and in the following figures) indicates the control level; dotted line indicates the 50% growth suppression level.

azithromycin stock solution (25 mg/mL) was prepared in 96% ethanol, and the 4-hexylresorcinol stock solution (50 mg/mL), in dimethyl sulfoxide; the solutions were diluted with liquid LB medium. With slowly growing bacteria (*Dietzia natronolimnaea* and *R. equi*), the incubation was carried out for 36–48 h. The sensitivity of biofilms was characterized using an analogous parameter, which was obtained after staining the biofilms on the blocks and extracting the dye (as described in the preceding subsection of Materials and Methods).

Microscopy. Phase contrast microscopy (PCM) was performed using an Axio Imager D1 microscope (Carl Zeiss, Germany) with a ×40 objective lens. The biofilms were formed on slides. For this purpose, slides were longitudinally cut into three parts with a glass cutter and cleaned by incubating in chromic–sulfuric acid mixture for one day. The cleaned slides were sterilized at 1 atm in test tubes with 5 mL of LB medium. After growing the culture for 24 h, the planktonic cell suspension was discarded, the slides were dried, and the biofilms were fixed by cautiously heating them in a gas burner flame. Samples for PCM were stained according to the aforementioned method with DMMB.

Statistical treatment of the results was based on choosing a typical experiment by the non-parametrical method of comparing data pairs using the sign test [9]. Graphical data were processed with Origin 8.6 software using the β-spline function. At least 4 statistically independent repeats of each experiment were conducted.

RESULTS AND DISCUSSION

As mentioned above, biofilm formation in some systems is enhanced by low antibiotic concentrations. This phenomenon is of paramount importance, particularly in terms of infection chemotherapy; however, it has been investigated only for a limited number of bacterial species. Therefore, we conducted detailed studies of the effects of a wide range of concentrations of the antibiotic azithromycin on planktonic cultures and biofilms of a number of saprotrophic microorganisms. Special attention was given to *C. violaceum*, since both a wild-type strain (WT) and a mutant (CV026) with impaired synthesis of AHLs are essential components of the QS regulatory system, were available. Although the specifics of the molecular mechanisms involved in the interaction between the QS system and biofilm formation remain unknown, preliminary data obtained in our laboratory indicate that synthesis of the polysaccharide components of the biofilm extracellular matrix is disrupted in the *C. violaceum* CV026 mutant [10]. As a result, the sensitivity of biofilm formation to extreme environmental factors is increased [11]. Higher sensitivity of the mutant to azithromycin compared to the wild-type strain, WT, should be expected.

The results of determining the sensitivity of *C. violaceum* to azithromycin are presented in Fig. 1. It can be seen that the biofilms of this microorganism were

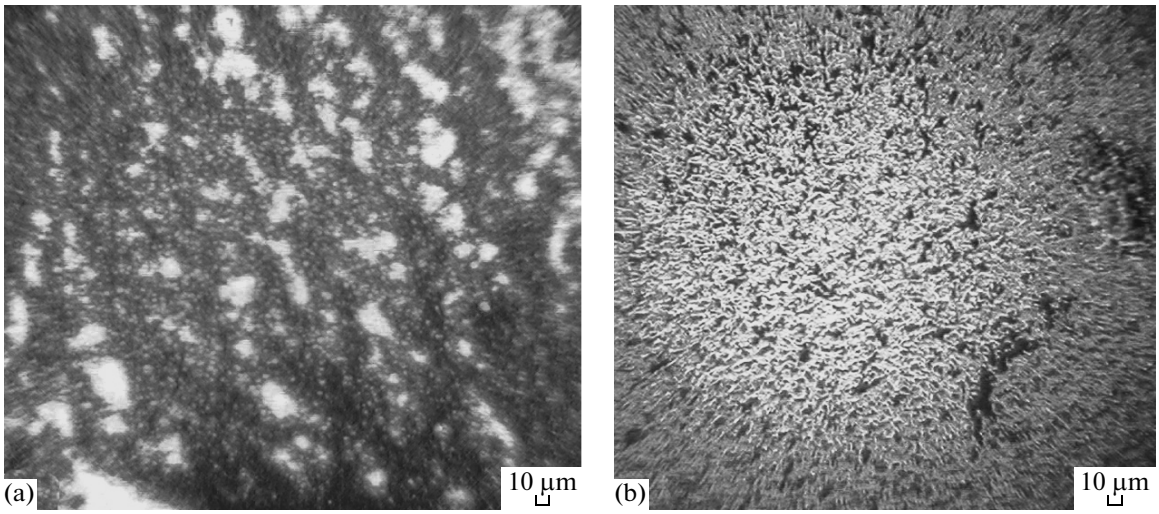

Fig. 2. PCM of *C. violaceum* biofilms grown on slides and stained with DMMB. The wild-type strain WT (a) and the CV026 mutant (b). The polysaccharide matrix stains dark (bluish-violet) and microcolonies do not stain.

more resistant to the effect of azithromycin than planktonic cultures (similar to other bacteria investigated by us earlier). However, the resistance of the CV026 mutant biofilm was almost an order of magnitude lower than that of the biofilm of the wild-type strain (WT). Pronounced stimulation of biofilm formation (up to 160% of biofilm formation in the control system without the antibiotic) was observed within the antibiotic concentration range (0.1–1.0 μg/mL) that is subinhibitory for the planktonic culture. As for the mutant, such stimulation was much less significant and peaked at lower antibiotic concentrations.

The data on the stimulation and suppression of biofilm formation, as well as on the quantitative differences in biofilm accumulation between the wild type and the mutant of *C. violaceum*, were confirmed by us in studies with CV, which stains all biofilm components, not only acidic polysaccharides. Importantly, the data on biofilm formation presented on Fig. 1b are expressed not in relative units (i.e., percentage of the control value without the antibiotic, as shown in Fig. 1a) but in absolute values of the OD of the extracts of the dyes used in the studies. This enabled us to conduct quantitative comparative studies on biofilm accumulation in different systems. These studies revealed that the total biofilm accumulation level under standard conditions was 2–2.5 times lower in the mutant (Fig. 1b, curves *3* and *4*) than in the wild-type strain (Fig. 1b, curves *1* and *2*).

Comparing the biofilms of these strains by means of PCM lent support to the earlier data obtained in our laboratory using PCM, epifluorescence, atomic force, and laser interference microscopy [10]. These data indicated a drastic decrease in the content of polysaccharide matrix in the mutant biofilm (Fig. 2). While most cells of the wild-type strain (Fig. 2a) were enclosed in the polysaccharide matrix that stained and became bluish-violet (dark on a photograph), a majority of mutant microcolonies did not stain (Fig. 2b). Only solitary "islands" of the polysaccharide matrix occurred, which plausibly resulted from reversion of a small number of the mutant cells to the wild-type phenotype.

Therefore, we confirmed our earlier suggestion [10] that impairing the QS system by inhibiting AHL synthesis results in decreased capacity for biofilm formation, which is chiefly due to suppression of the synthesis of the polysaccharide component of the matrix.

As mentioned in the Introduction, developing strategies for controlling biofilms and particularly the activation of their formation in the presence of low antibiotic concentrations is one of the most important issues in terms of medicine and with respect to technological processes.

We investigated the influence on biofilm formation of 4-hexylresorcinol, a representative of alkylhydroxybenzenes (termed AHBs hereafter). The choice of this compound was due to the fact that AHBs are natural autoregulatory molecules ("adaptogens") that can interfere with a wide spectrum of biochemical processes [12, 13]. Importantly, 4-hexylresrcinol has already been used as a food additive (E586) [14], i.e., it is not toxic for humans, which is of particular interest in terms of medical application of our results. There is evidence in the literature that this compound may suppress the formation of some bacterial biofilms [15]. 4-Hexylresorcinol exhibits relatively low antibacterial activity (see below). However, synergistic effects should be expected if 4-hexylresorcinol is combined with antibiotics, e.g., azithromycin, in systems in which 4-hexylresorcinol suppresses biofilm formation.

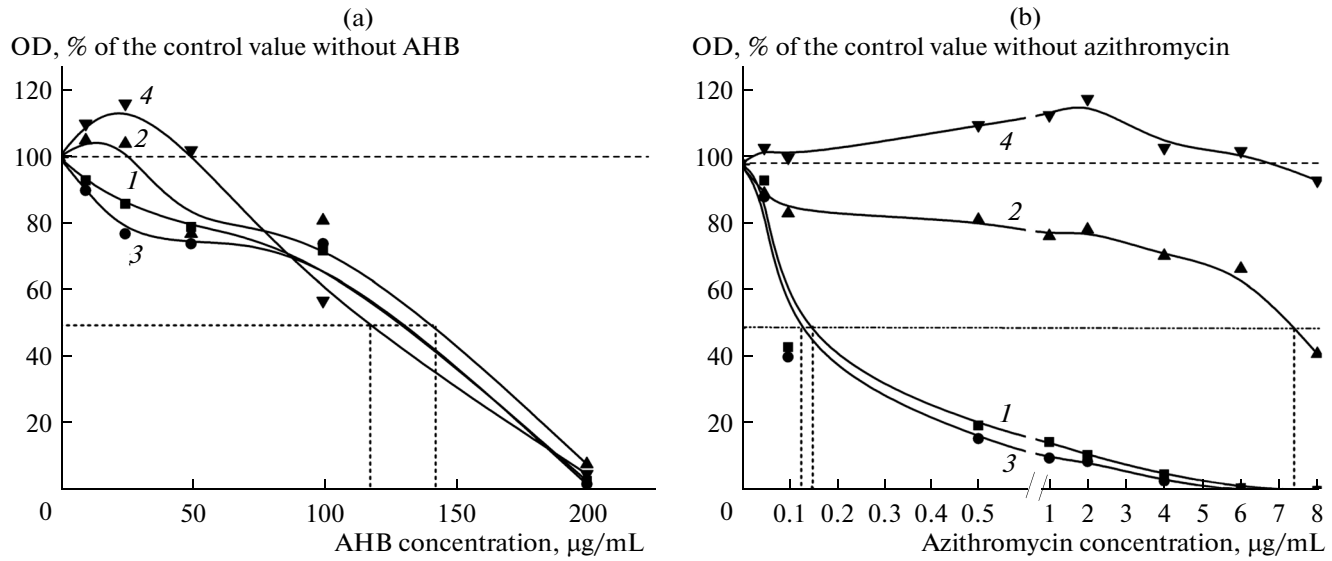

Fig. 3. Effect of AHB and azithromycin on the growth of planktonic cultures and biofilms of *C. violaceum*. Effect of AHB: The wild-type strain WT (*1*, *2*), the CV026 mutant (*3*, *4*); planktonic cultures (*1*, *3*), and biofilms (*2*, *4*) (a); and combined effect of azithromycin and AHB (25 μg/mL). The wild-type strain WT (*1*, *2*), the CV026 mutant (*3*, *4*); planktonic cultures (*1*, *3*), and biofilms (*2*, *4*); the biofilm was stained with DMMB (b).

Conducting such studies is of paramount importance for the following two reasons. First, biofilm formation in technological systems, e.g., oil pipelines, very frequently occurs during oil recovery and oil product transportation. It results in pipeline obstruction and equipment corrosion. Therefore, controlling this process is of special technological importance. Second, selecting nontoxic compounds that enhance the effect of antibiotics on oil-degrading and model microorganisms can have other medically important applications: it can be used to fight bacterial infections caused by biofilm-forming microorganisms.

The effects of AHB and its combination with azithromycin were investigated on gram-negative bacteria *C. violaceum* and *P. chlororaphis* 66 and gram-positive bacteria *D. natronolimnaea*, *K. rhizophila*, and *R. equi*. The combined effect of azithromycin and AHB was examined at AHB concentrations that only exerted a weak inhibitory effect on culture growth per se (suppressing growth by 5–10%).

***C. violaceum*.** Some strains of this saprotrophic microorganism used as a model may occasionally cause infections [16]. Importantly, the stimulatory effect of azithromycin on the growth of the biofilms of the CV026 mutant was less significant; it was shifted towards lower antibiotic concentrations in comparison to the wild-type strain WT. The planktonic cultures and biofilms of these strains were characterized by similar, relatively low AHB sensitivity levels. The IC_{50} value varied between 120 and 140 μg/mL (Fig. 3a). Low AHB concentrations even stimulated growth of the biofilm of the CV026 mutant to a certain degree.

When azithromycin and AHB (25 μg/mL) were used in combination, the sensitivity of the growth of planktonic cultures of both strains and of the biofilms of the wild-type strain did not change significantly (Fig. 3b). However, similar to the data given above, the biofilm growth-stimulating effect was completely removed in the wild-type strain. Conversely, in the mutant strain, in the presence of AHB, azithromycin caused a marked stimulation of biofilm growth. This probably indicated a regulatory role of AHB in this process. Plausibly, AHB can "imitate" the role of AHL. The mechanism of action of AHB awaits further research.

***P. chlororaphis* 66.** This microorganism is a saprotroph; it has been isolated from the soil. However, it can oxidize paraffins and is able to grow in M9 mineral medium with hexadecane as the sole carbon source. A large number of representatives of this genus are opportunistic or obligate pathogens that form biofilms in the infected macroorganism, causing chronic obstinate diseases, e.g., complicated cystic fibrosis [17].

Our studies revealed that *P. chlororaphis* 66, similar to *C. violaceum*, exhibited low sensitivity to the inhibitory effect of AHB (Fig. 4a). It may be seen that the IC_{50} values for the planktonic culture and the biofilm were 120 and ~200 μg/mL, respectively. However, if azithromycin and AHB acted in combination, the sensitivity of the planktonic culture to the antibiotic increased somewhat. The IC_{50} value decreased from 5 to 3 μg/mL (Fig. 4b). Most importantly, the biofilm synthesis-stimulating effect at subinhibitory azithromycin concentrations (0.05–5 μg/mL) was completely removed.

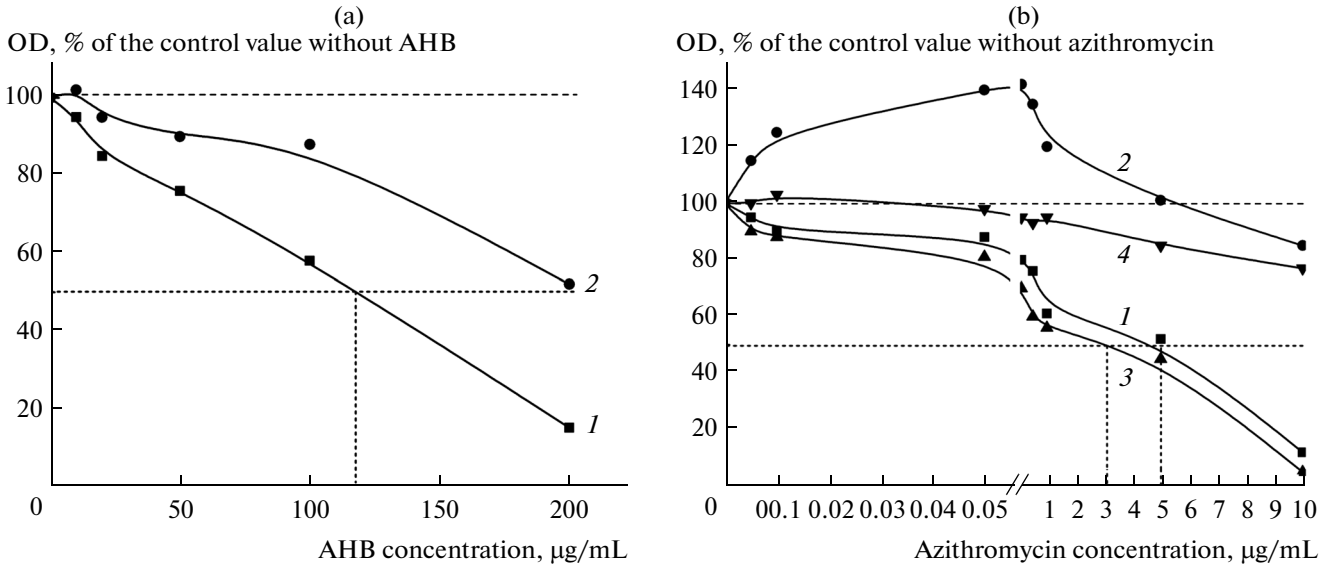

Fig. 4. Effect of AHB and azithromycin on the growth of planktonic cultures and biofilms of *P. chlororaphis* 66. Effect of AHB: planktonic culture (*1*) and biofilm (*2*) (a); and effect of azithromycin (*1*, *2*) and the azithromycin and AHB (20 μg/mL) mixture (*3*, *4*); planktonic cultures (*1*, *3*) and biofilms (*2*, *4*); the biofilm was stained with DMMB (b).

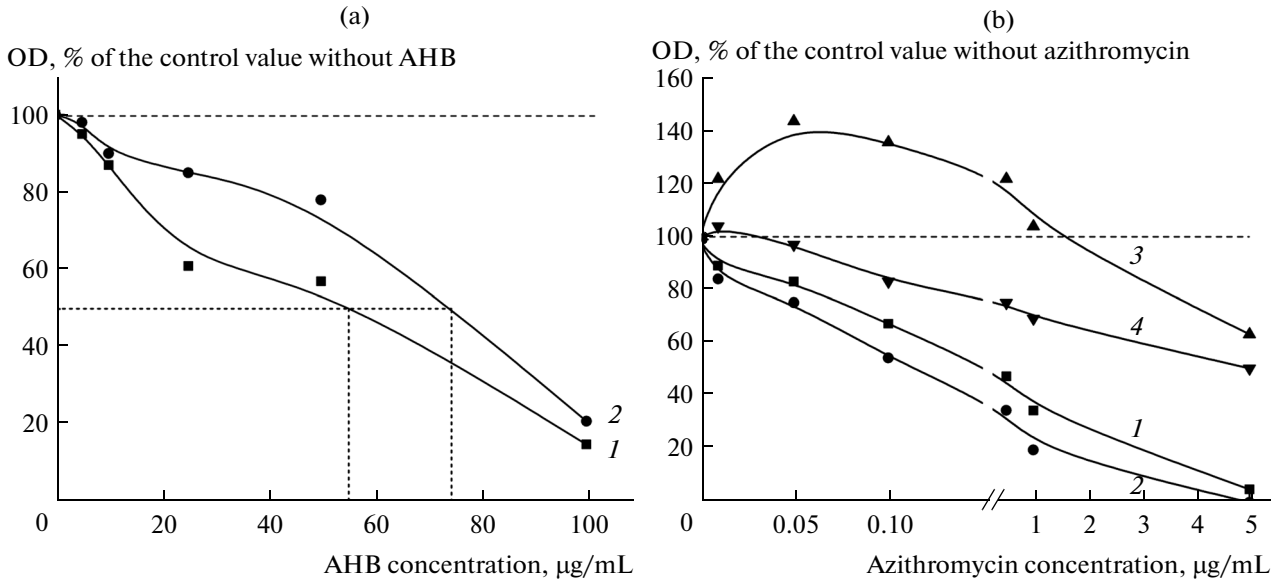

Fig. 5. Effect of AHB and azithromycin on the growth of planktonic cultures and biofilms of *D. natronolimnaea*. Effect of AHB: planktonic culture (*1*) and biofilm (*2*) (a); and effect of azithromycin (*1*, *3*) and the azithromycin and AHB (10 μg/mL) mixture (*2*, *4*); planktonic cultures (*1*, *2*) and biofilms (*3*, *4*); the biofilm was stained with DMMB.

D. natronolimnaea. Although the typical representatives of this genus are saprotrophic oil-degrading bacteria, the genus also includes opportunistic pathogens [18]. Therefore, this bacterium may be regarded as a model that is of not only technological but also medical importance.

Testing the influence of AHB on the growth of *D. natronolimnaea* revealed that the activity of this inhibitor on the planktonic culture and in the biofilm did not differ significantly, unlike the activity of azithromycin. The IC_{50} values were 55 and 75 μg/mL for the planktonic culture and the biofilm, respectively (Fig. 5a).

A much more important fact was that, apart from enhancing the inhibitory effect of azithromycin, AHB at a concentration of 10 μg/mL (which only inhibited the growth of both planktonic culture and the biofilm by 10–12%) completely removed the biofilm forma-

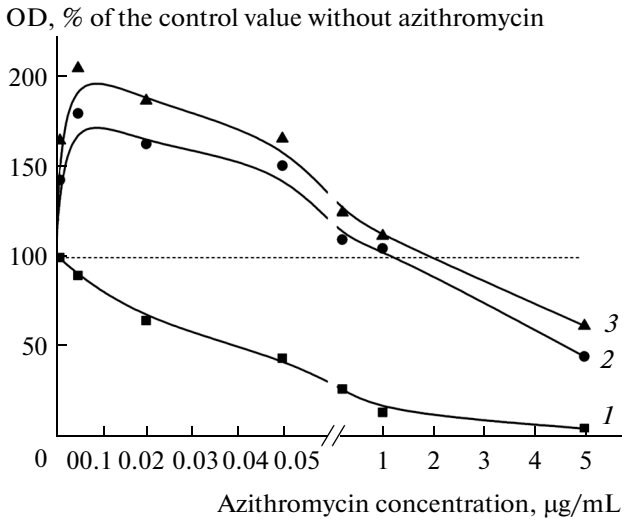

Fig. 6. Effect of azithromycin on the growth of planktonic cultures (*1*) and biofilms (*2*, *3*) of *K. rhizophila* stained with CV (*2*) or DMMB (*3*).

tion-stimulating effect that was produced by low concentrations of this antibiotic (Fig. 5b).

K. rhizophila. The typical representatives of this genus are saprotrophs. For instance, the culture isolated in our laboratory via the transitional stage of biofilm formation from stratal water of the Romashkinskoe oil deposit was capable of growth on the M9 mineral medium with hexadecane as the sole carbon source. However, cases of bacteraemia, including those caused by a *K. kristinae* strain that forms biofilms on catheters [19], have been described.

The planktonic culture of the isolated strain of *K. rhizophila* was highly sensitive to azithromycin [4]. However, at subinhibitory concentrations, the antibiotic caused an appreciable stimulation of biofilm growth (Fig. 6) and especially of the synthesis of the polysaccharide component of the matrix (determined by DMMB staining), which indicated formation of mature biofilms.

Among all the tested bacteria, the *K. rhizophila* culture was the most sensitive to the inhibitory effect of AHB (Fig. 7a).

With respect to sensitivity to AHB, no significant differences between the planktonic culture and the biofilm of *K. rhizophila* were revealed (similar to *D. natronolimnaea*). The IC_{50} value of the planktonic culture and the biofilm were 7.5 and 16 μg/mL, respectively (DMMB staining), and 24 μg/mL (CV staining). The higher sensitivity revealed by DMMB staining probably indicated preferential suppression of the synthesis of the polysaccharide components of the biofilm matrix in the presence of AHB.

When added in combination, azithromycin and AHB (5 μg/mL) produced an additive effect on the planktonic culture of *K. rhizophila*. However, the most important point was that, as in the case of *D. natronolimnaea*, no biofilm synthesis-stimulating effect occurred within the subinhibitory concentration range (shown in Fig. 7b). This effect clearly manifested itself with azithromycin alone (Fig. 6).

R. equi. This culture is also saprotrophic, although some strains of this species can be classified as opportunistic pathogens [20].

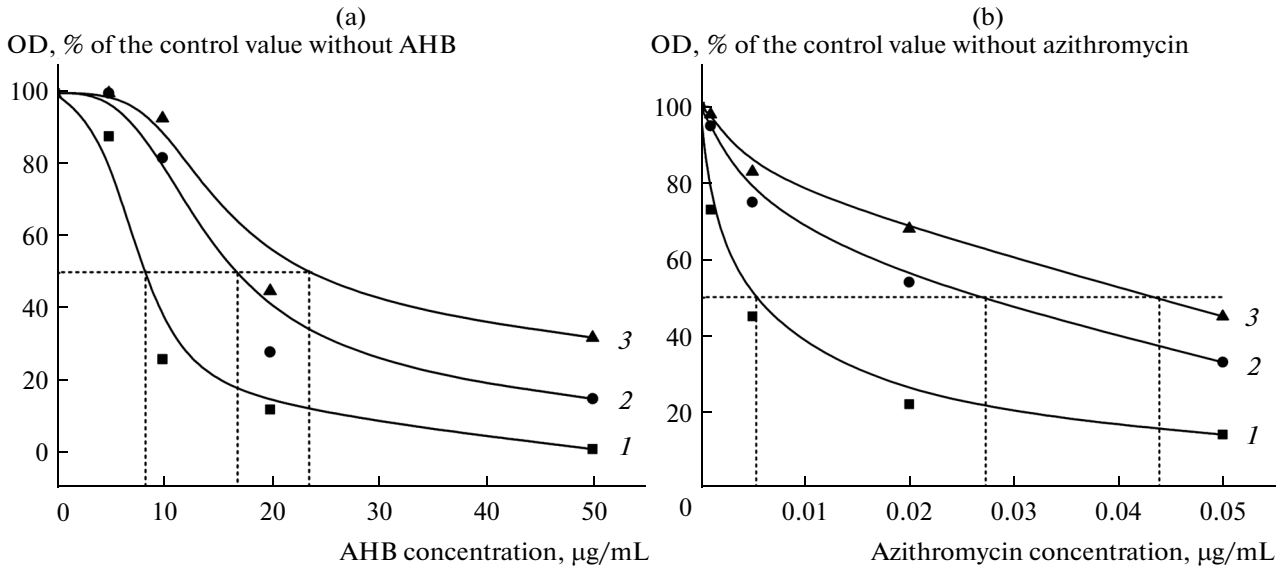

Fig. 7. Effect of AHB and azithromycin on the growth of planktonic cultures and biofilms of *K. rhizophila*. Effect of AHB: planktonic culture (*1*) and biofilm (*2*, *3*) stained with DMMB (*2*) or CV (*3*) (a); and combined effect of azithromycin and AHB (5 μg/mL); planktonic culture (*1*) and biofilm (*2*, *3*) stained with DMMB (*2*) or CV (*3*).

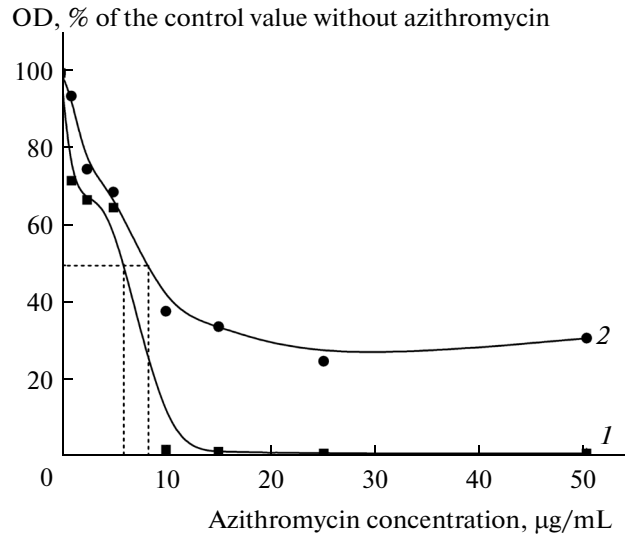

Fig. 8. Effect of azithromycin on the growth of planktonic culture (*1*) and biofilm (*2*) of *R. equi* stained with DMMB.

Studies concerning the effect of azithromycin on this microorganism revealed an extraordinary pattern (Fig. 8). For the sensitivity determined as the IC_{50} value, the differences between the planktonic culture and the biofilm were insignificant: 6 and 8 μg/mL for the planktonic culture and the biofilm, respectively. However, if the azithromycin concentration was over 15 μg/mL, growth of the planktonic culture was completely suppressed, whereas that of the biofilm persisted, at least up to azithromycin concentrations of 50 μg/mL. The sensitivity of the planktonic culture and of the biofilm to the AHB effect also revealed a peculiar pattern: the biofilm was more sensitive at low AHB concentrations (up to 30 μg/mL). However, the growth of planktonic culture was completely suppressed by 50 μg/mL AHB, whereas the biofilm continued to grow (Fig. 9a).

When added in combination, azithromycin and AHB (30 μg/mL) exhibited a synergistic inhibitory effect on the growth of the planktonic culture (IC_{50} decreased from 6 to 0.2 μg/mL) and of the biofilm (IC_{50} = 4 μg/mL), and biofilm growth was almost completely suppressed by 10 μg/mL azithromycin (Fig. 9b).

The results obtained indicate that the combination of AHB with an antibiotic holds much promise in terms of controlling biofilm formation in both gram-positive and gram-negative bacteria. The most important fact is that, apart from enhancing the effect of the antibiotic on planktonic cultures, it prevents the stimulation of biofilm formation by low, subinhibitory antibiotic concentrations that can be established in a macroorganism if the regimen of antibiotic chemotherapy is not maintained. The conclusion can be drawn that the mechanism of action of AHB involves suppressing the synthesis of matrix polysaccharides because its inhibitory effect is more prominent if biofilms are stained with DMMB, a polysaccharide-specific dye.

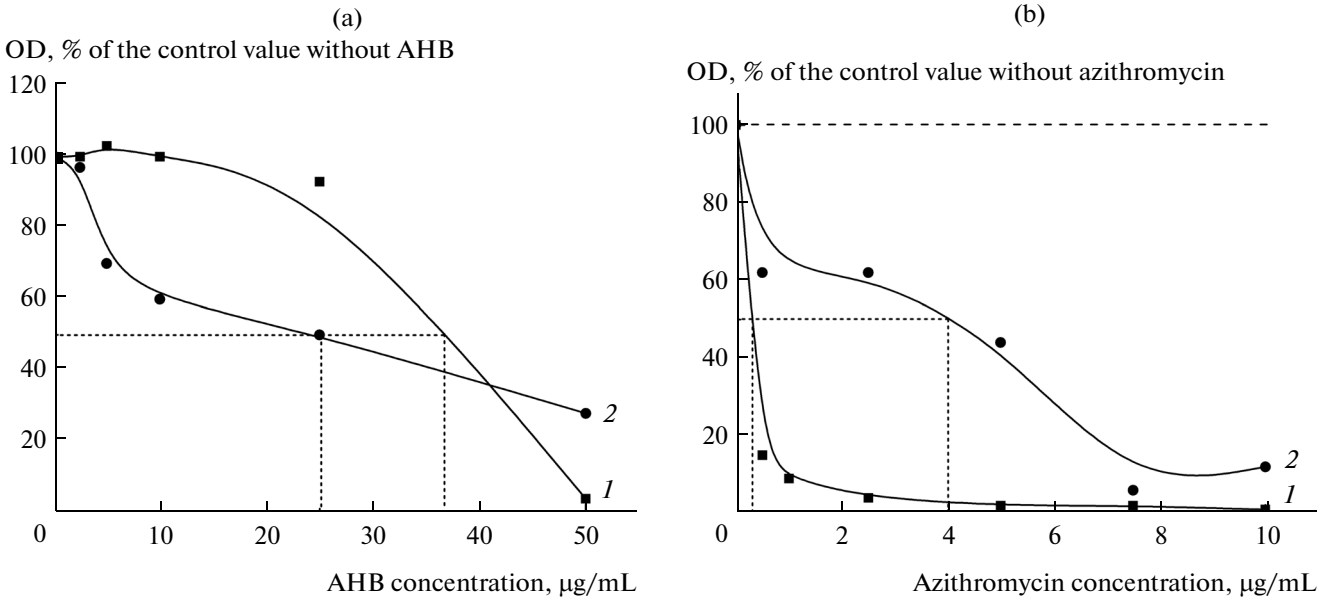

Fig. 9. Effect of AHB and azithromycin on the growth of planktonic cultures (*1*) and biofilms (*2*) of *R. equi*. Effect of AHB: planktonic culture (*1*) and biofilm (*2*); and combined effect of azithromycin and AHB (30 μg/mL): planktonic culture (*1*) and biofilm (*2*) stained with DMMB (b).

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic research (project no. 13-04-01145).

REFERENCES

1. Hoffman, L., D'Argenio, D., MacCoss, M., Zhang, Z., Jones, R., and Miller, S., Amynoglycoside antibiotics induce bacterial biofilm formation, *Nature*, 2005, vol. 436, pp. 1171–1755.
2. Nucleo, L., Steffanoni, G., Fugazza, R., Migliavacca, R., Pagani, L., and Landini, P., Growth in glucose-based medium and exposure to subinhibitory concentrations of imipenem induce biofilm formation in a multidrug-resistant clinical isolate of *Acinetobacter baumannii, BMC Microbiol.*, 2009, vol. 9, p. 270. http://www.biomedcentral.com/1471-2180/9/270
3. Zaitseva, Yu.V., Plyuta, V.A., and Khmel', I.A., Effect of phenolic compounds of plant origin on formation of microbial biofilms and quorum sensing regulatory systems, in *XXIII Mezhdunarodnaya zimnyaya molodezhnaya nauchnaya shkola "Perspektivnye napravleniya fiziko-khimicheskoi biologii i biotekhnologii"* (23rd Int. Winter Youth Sci. School "Prospects of Physicochemical Biology and Biotechnology"), Moscow, 2011, p. 144.
4. Strelkova, E. A., Zhurina, M. V., Plakunov, V. K., and Belyaev, S. S., Stimulation of biofilm formation by antibiotics, *Microbiology* (Moscow), 2012, vol. 81, no. 2, pp. 259–262.
5. Linares, J.F., Gustafsson, I., Baquero, F., and Martinez, J.L., Antibiotics as intermicrobial signaling agents instead of weapons, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, vol. 103, pp. 19484–19489.
6. Babić, F., Venturi, V., and Maravić-Vlahoviček, G., Tobramycin at subinhibitory concentrations inhibits the RhlI/R quorum sensing system in a *Pseudomonas aeruginosa* environmental isolate, *BMC Infect. Dis.*, 2010, vol. 10, p. 148.
7. Worthington, R.J., Richards, J.J., and Melander, C., Small molecule control of bacterial biofilms, *Org. Biomol. Chem.*, 2012, vol. 10, pp. 7457–7474.
8. Peeters, E., Nelis, H.J., and Coenye, T., Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates, *J. Microbiol. Methods*, 2008, vol., 72, pp. 157–165.
9. Paulson, D.S., *Biostatics and Microbiology*, Springer Science + Business Media, 2008.
10. Zhurina, M.V., Kostrikina, N.A., Parshina, E.Yu., Strelkova, E.A., Yusipovich, A.I., Maksimov, G.V., and Plakunov, V.K., Visualization of the extracellular polymeric matrix of *Chromobacterium violaceum* biofilms by microscopic methods, *Microbiology* (Moscow), 2013, vol., 82, no. 4, P. 517–524.
11. Strelkova, E.A., Pozdnyakova, N.V., Zhurina, M.V., Plakunov, V.K., and Belyaev, S.S., Role of the extracellular polymer matrix in resistance of bacterial biofilms to extreme environmental factors, *Microbiology* (Moscow), 2013, vol., 82, no. 2, pp. 119–125.
12. Bukharin, O.V., Gintsburg, A.L., Romanova, Yu.M., and El'-Registan, G.I., *Mekhanizmy vyzhivaniya bakterii* (Mechanisms of Bacterial Survival), Moscow: Meditsina, 2005.
13. Nikolaev, Yu.A., Borzenkov, I.A., Tarasov, A.L., Loiko, N.G., Kozlova, A.N., Gal'chenko, V.F., and El'-Registan, G.I., Role of alkylhydroxybenzenes in Bacterial Adaptation to Unfavorable Growth Conditions, *Microbiology* (Moscow), 2010, vol., 79, no. 6, pp. 747–752.
14. Provision of the Chief State Healh Officer "On Establishment of SanPin 2.3.2.2364-08", http://zakonbase.ru/content/part/572439?print=1.
15. Pozdnyakova, N.V., Zhurina, M.V., Plakunov, V.K., and El'-Registan, G.I., Suppression of formation of microbial biofilms. Biomedical aspect, "*Biologiya – nauka XXI veka". Materialy Mezhdunarodnoi konferentsii* (Proc. Int. Conf. "Biology—The Science of the 21st Century), Moscow: MAKS, 2012, pp. 720–721.
16. Hagiya, H., Murase, T., Suzuki, M., Shibayama, K., Kokumai, Y., Watanabe, N., Maki, M., and Otsuka, F., *Chromobacterium violaceum* nosocomial pneumonia in two Japanese patients at an intensive care unit, *J. Infect. Chemother.* 2014, vol. 20, pp. 139–142.
17. Haley, C.L, Colmer-Hamood, J.A, and Hamood, A.N., Characterization of biofilm-like structures formed by *Pseudomonas aeruginosa* in a synthetic mucus medium, *BMC Microbiol.*, 2012, vol. 12, pp. 181–190.
18. Koerner, R.J., Goodfellow, M., and Jones, A.L., The genus *Dietzia*: a new home for some known and emerging opportunist pathogens, *FEMS Immunol. Med. Microbiol.*, 2009, vol. 55, pp. 296–305.
19. Ma, E.S.K., Wong, C.L.P., Lai, K.T.W., Chan, E.S.H., Yam, W.C, and Chan, A.C.W., *Kocuria kristinae* infection associated with acute cholecystitis, *BMC Infect. Dis.*, 2005, vol. 5, p. 60. http://www.biomedcentral.com/1471-2334/5/60
20. Yamshchikov, A.V., Schuetz, A., and Lyon, G.M., *Rhodococcus equi* infection, *Lancet Infect. Dis.*, 2010, vol. 10, pp. 350–359.

Translated by A. Oleskin