ISSN 0026-2617, Microbiology, 2015, Vol. 84, No. 3, pp. 319–327. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © A.V. Gannesen, M.V. Zhurina, M.A. Veselova, I.A. Khmel', V.K. Plakunov, 2015, published in Mikrobiologiya, 2015, Vol. 84, No. 3, pp. 281–290.

EXPERIMENTAL ARTICLES

Regulation of Biofilm Formation by *Pseudomonas chlororaphis* in an in vitro System

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Received October 21, 2014

Abstract—The mutants of *Pseudomonas chlororaphis* 449 with completely or partially suppressed accumulation of *N*-acyl homoserine lactones exhibited the absence or a pronounced decrease of their capacity for stimulation of biofilm growth in the presence of azithromycin. Biofilms of the wild type strain preformed in the presence of the stimulatory azithromycin concentrations exhibited more intense staining with a polysaccharide-specific dye 1,9-dimethyl methylene blue (DMMB) and were more resistant to heat shock. These findings indicate accumulation of the structural matrix polysaccharides, which play a protective role under conditions of thermal shock. Extremely low azithromycin concentrations (0.001–0.01 μg/mL) inhibit biofilm formation by *P. chlororaphis* 449 and *P. chlororaphis* 66 with suppression of the synthesis of DMMB-staining polysaccharides.

Keywords: biofilms, antibiotics, activation of biofilm formation, quorum sensing
DOI: 10.1134/S0026261715030042

A number of regulatory systems participating in formation of bacterial biofilms rely on such components as cyclic diguanosine monophosphate (c-di-GMP), "small" RNA (sRNA), indole derivates, and other substances [1]. The system of "quorum sensing" (QS) is one of the most important systems of global metabolic regulation, involved in biofilm formation.

The functioning of QS system in gram-negative bacteria is mediated by two types of proteins. One type is responsible for the synthesis of signal molecules commonly referred as autoinducers, usually *N*-acyl homoserine lactones (AHL). The second type of the proteins interacts with autoinducers, launching transcription of specific genes [2–4]. Over 70 genera of the α-, β-, and γ-proteobacteria are known to use AHL-involving QS [5]. In *Vibrio fischeri*, AHL synthesis is controlled by the *luxI* gene encoding AHL-synthase LuxI. When a critical concentration threshold is reached, AHL binds to the LuxR transcription activator and transcription of QS-dependent genes, including *luxI*, is launched. Thus, the concentration of signal molecules in the medium has an avalanche-like increase. Since AHL are able to diffuse inside and outside of cells, the AHL pool correlates with cell density, providing control of gene expression depending on the population density. At least three QS systems operate in pseudomonads. The most studied systems, LasIR and RhlIR (LuxIR system homologues), use AHL as signal molecules, while the third system, AQ, is alkil quinolone-dependent [6, 7].

Although involvement of AHL-dependent QS-system in biofilm formation was demonstrated in several studies, molecular mechanisms of these processes remain incompletely understood. It was shown recently that inactivation of the AHL synthase gene *cviI* in *Chromobacterium violaceum* leads to impaired synthesis of the components of the polysaccharide extracellular polymer matrix (EPM), accompanied by increased sensitivity of the biofilms to extreme environmental conditions and antibiotics [8, 9].

The goal of the present work was to study the effect of low (sub-bacteriostatic) concentrations of azithromycin on *P. chlororaphis* wild type strains, modified strains, and mutants with defects in some global regulatory systems, in order to reveal the role of those systems in the regulation of biofilm formation by azithromycin.

MATERIALS AND METHODS

Subjects of study. The subjects of study were pure cultures of gram-negative saprotrophic bacteria *P. chlororaphis* (strains 66 and 449), modified strains, and mutants of the latter strain, obtained in the laboratory of Expression regulation of genes in microorganisms, Institute of Molecular Genetics, Russian Academy of Sciences (IMG RAS) and stored in collection of IMG RAS. The basic characteristics of those microorganisms are summarized in the table.

Cultivation and storage of microorganisms. The microorganisms were stored on semiliquid (0.3%

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Features of bacteria used in the study

Bacterial strains	Characteristics of the strains	Source
P. chlororaphis 449	Prototroph; able to synthesize AHL	Collection of the Institute of Molecular Genetics, Russ. Acad. Sci.
P. chlororaphis 66	Prototroph; able to synthesize AHL	Collection of the Institute of Molecular Genetics, Russ. Acad. Sci.
P. chlororaphis 449, mutant 2	Mutation in the *rpoS* gene; able to synthesize AHL	[10]
P. chlororaphis 449, mutant 3	Mutation in the *gacS* gene; ability to synthesize AHL is decreased	[11]
P. chlororaphis 449, mutant 4	Mutation in the *phzB* gene; able to synthesize AHL	[11]
P. chlororaphis 449, mutant 5	Mutation in the *phzA* gene; able to synthesize AHL	[11]
P. chlororaphis 449/pME6000, strain 6e	Contains a vector plasmid pME6000, Tc-r; able to synthesize AHL	[11]
P. chlororaphis 449/pME6000, strain 7e	Contains plasmid pME6863, including the cloned gene *aiiA*, Tc-r; is unable to accumulate AHL	[11]

agar) LB medium in agar columns under paraffin oil at 4–6°C. In case the of the mutants 2, 3, 4, and 5, sterile kanamycin solution (final concentration 100 μg/mL) was added to the medium for storage. In the case of strains 6e and 7e, sterile tetracycline solution was used (final concentration up to 40 μg/mL). Bacteria were grown in liquid LB medium at 29–30°C on a shaker (150 rpm) for 20–24 h. These cultures were used as inocula for biofilm experiments.

The biofilms were obtained and their sensitivity to inhibitors was studied as described previously [9].

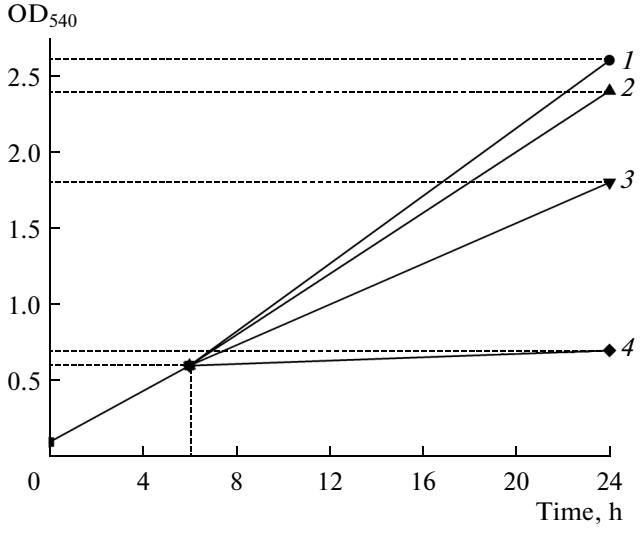

Fig. 1. Graph illustrating a method of calculating of *P. chlororaphis* 449 planktonic culture sensitivity to heat shock: 29°C (*1*), 34°C (*2*), 37°C (*3*), and 45°C (*4*).

Effect of heat stress on growth of biofilms of *P. chlororaphis* 449 in the presence of azithromycin. The sensitivity of planktonic cultures and biofilms to heat stress was characterized as the ratio of increase of a parameter proportional to the biomass of a studied object (optical density or light scattering) in the presence and absence of azithromycin at studied temperatures to its increase at the optimal temperature (29°C). The calculation method is shown on Fig. 1 for *P. chlororaphis* 449 planktonic culture without the antibiotic. The culture was incubated for 6 h, the time required for development of the "biofilm" phenotype and beginning of matrix formation [8]. In this case, the optical density (OD_{540}) determined as light scattering by suspensions of 6-h planktonic cells reached the value of 0.6. After transfer of the culture to experimental temperature conditions, the incubation continued for another 24 h. Increase of the optical density of planktonic cell suspension at 29°C was 2.6 – 0.6 = 2.0 unit OD. This value was accepted as 100%. Culture growth at other temperatures was assessed similarly. For example, at 37°C, it was 1.6 – 0.6 = 1.0 units OD. Thus, the relative culture growth at this temperature was 1.0/2.0 × 100 = 50%. This value was plotted on the graphs (Figs. 2–9). The relative growth of biofilms was calculated similarly (based on optical density of the dyes extracted from preliminary stained biofilms). The measurements were conducted at 590 and 670 nm for crystal violet (CV) and 1,9-dimethyl methylene blue (DMMB), respectively [9].

Microscopy. For phase contrast microscopy (PCM) studies and epifluorescence microscopy (EFM) Axio Imager, D1 Carl Zeiss, objective lens ×40 microscope was used. Biofilms were formed on microscopic slides. For this purpose, standard slides were cut into three parts and were cleaned by incubation in

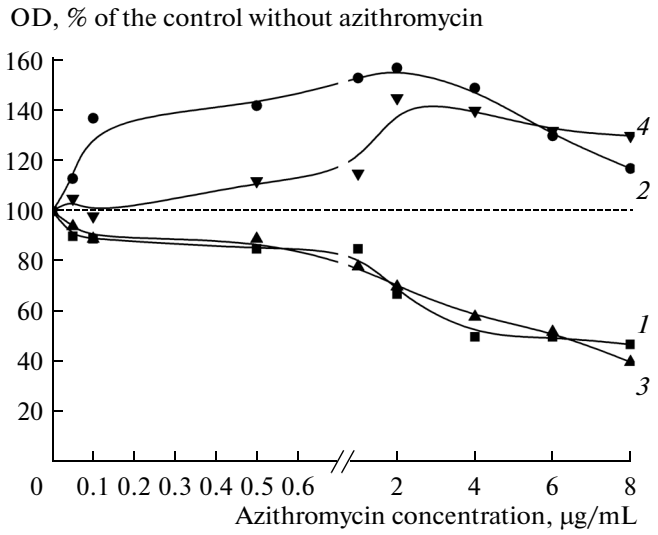

Fig. 2. Growth of planktonic cultures (*1*, *3*) and biofilms (*2*, *4*) of *P. chlororaphis* 449 (*1*, *2*) and mutant 2 (*3*, *4*) at different azithromycin concentrations. Biofilms stained with CV. On this and following figures a dashed line indicates the control level without addition of the antibiotic.

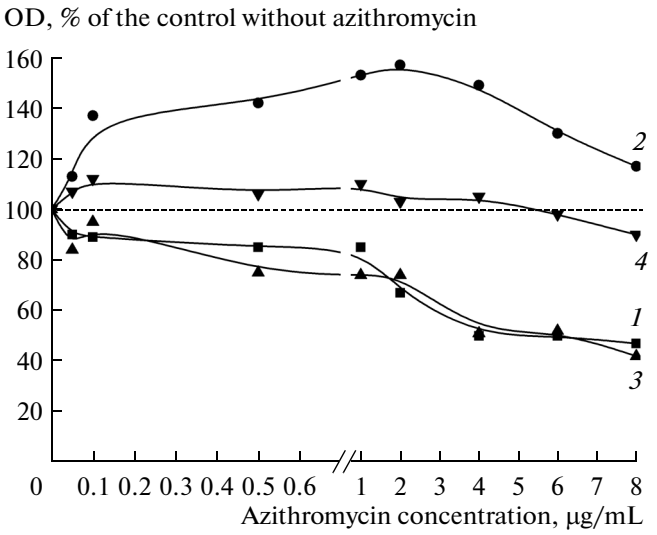

Fig. 3. Growth of planktonic cultures (*1*, *3*) and biofilms (*2*, *4*) of *P. chlororaphis* 449 (*1*, *2*) and mutant 3 (*3*, *4*) at different azithromycin concentrations. Biofilms stained with CV.

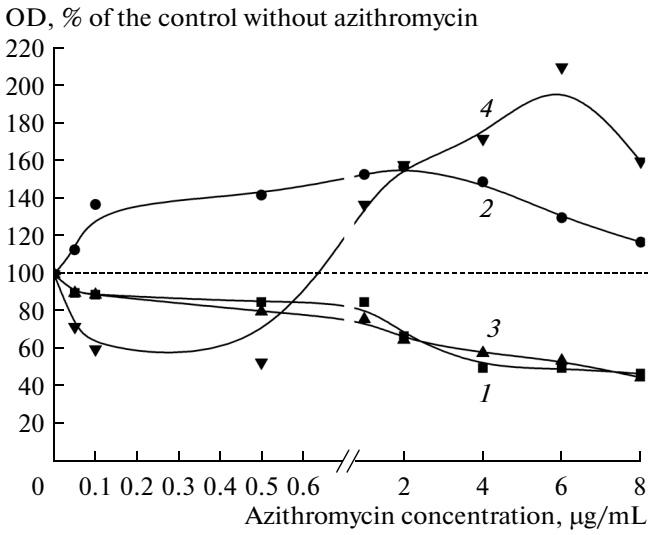

Fig. 4. Growth of planktonic cultures (*1*, *3*) and biofilms (*2*, *4*) of *P. chlororaphis* 449 (*1*, *2*) and mutant 4 (*3*, *4*) at different azithromycin concentrations. Biofilms stained with CV.

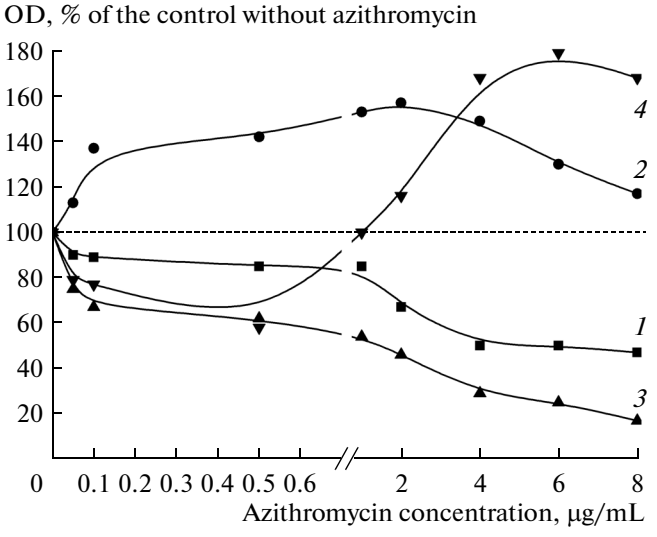

Fig. 5. Growth of planktonic cultures (*1*, *3*) and biofilms (*2*, *4*) of *P. chlororaphis* 449 (*1*, *2*) and mutant 5 (*3*, *4*) at different azithromycin concentrations. Biofilms stained with CV.

chromic mixture for a day. After washing, the slides were sterilized in tubes with 5 mL LB medium (1 atm), which were then inoculated with 50 μL of the culture. After 24 h of cultivation, the planktonic cell suspension was decanted, the slides were dried, and the biofilms were fixed by heating gently in a gas burner flame. For PCM, the biofilms were stained with DMMB according to [12], with some modifications. To obtain stock solution of the dye, 20 mg DMMB (double salt with zinc chloride) was dissolved in 5 mL 96% ethanol. The stock solution of 0.2 g sodium formate (anhydrous) and 0.2 mL 99% formic acid in 95 mL distilled water (pH 3.3) were prepared separately. After mixing, the solution, which is stable for several months at room temperature, was stored in the dark. For EFM, DAPI [13] and the fluorescent stain FilmTracer™ SYPRO Ruby biofilm matrix stain (according to the manufacturer's instructions) [14] were used.

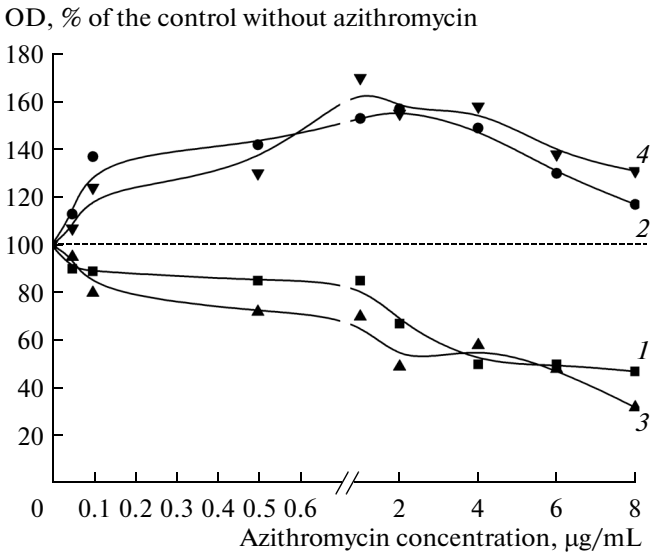

Fig. 6. Growth of planktonic cultures (*1, 3*) and biofilms (*2, 4*) of *P. chlororaphis* 449 (*1, 2*) and strain 6e (*3, 4*) at different azithromycin concentrations. Biofilms stained with CV.

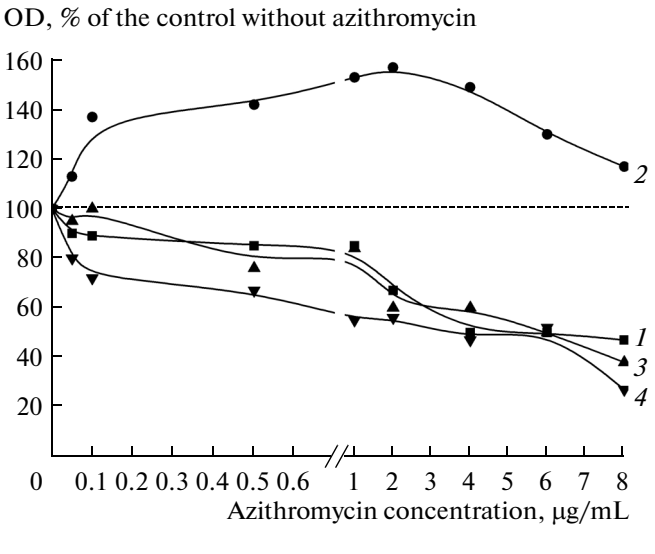

Fig. 7. Growth of planktonic cultures (*1, 3*) and biofilms (*2, 4*) of *P. chlororaphis* 449 (*1, 2*) and strain 7e (*3, 4*) at different azithromycin concentrations. Biofilms stained with CV.

Statistical reliability of the results is based on selection of a typical experiment by the nonparametric method of comparing paired data, taking into account "the sign test" [15]. As a result of four or more statistical variants for each sample, the median of relative values (in %) was chosen as an average value. Graphical data were processed in the Origin 8.6 software package using B-spline function.

RESULTS AND DISCUSSION

To study the regulatory patterns of biofilm formation regulation in *P.chlororaphis* 449, we chose the mutants with an interruption in several systems of global metabolic regulation that take part in biofilm formation regulation [16]: (a) in the gene *rpoS* encoding the sigma S subunit of RNA polymerase, RpoS (mutant 2); (b) in the two-component global regulation system GacA-GacS (mutant 3); (c) in the *phz* operon system, encoding the synthesis of phenazine antibiotics (mutants 4 and 5). We also used the strain *P. chlororaphis* 449, transformed by the plasmid pME6863, which contained the cloned heterological gene *aiiA* encoding homoserine lactonase. This enzyme breaks down AHL, the signal molecules of the QS systems [17]. As a result, it disturbs the functioning of QS regulation (strain 7e). For comparison, strain 6e was used, containing the vector plasmid pME6000 which does not influence AHL synthesis and bacterial cell migration on solid surfaces (see table).

Our experiments were aimed at investigation of the effect of these mutations on the patterns of biofilm formation by *P. chlororaphis* 449. The approach chosen for the purpose was based on the use of a macrolide antibiotic azithromycin as a biochemical tool. Azithromycin is a macrolide antibiotic widely used for clinical treatment of biofilm-related infections [18].

Previously, we used azithromycin to study the dynamics of biofilm formation. It was found that low, sub-bacteriostatic concentrations of this antibiotic could significantly activate the growth of biofilms of several gram-positive bacteria [19]. It was important to determine whether azithromycin had the same effect on gram-negative bacteria *P. chlororaphis* 449 and whether the disorders in the regulatory systems mentioned above affected this effect.

***Azithromycin* effect on *P. chlororaphis 449* wild type and mutant strains**. In mutant 2, the *rpoS* gene, which encodes the sigma S subunit of RNA polymerase, is inactivated, resulting in suppression of the synthesis of phenazine antibiotics with no significant effect on the synthesis of four type of *N*-acyl homoserine lactones (*N*-butanoyl-L-homoserine lactone, *N*-hexanoyl-L-homoserine lactone, *N*-(3-oxo-hexanoyl)-L-homoserine lactone and a minor AHL) or on bacterial ability to move over the surface of a dense medium (swarming) [10]. The effect of azithromycin on planktonic culture and biofilm growth of this mutant (in comparison with the same parameters for the wild type) are presented on Fig. 2. It can be seen that growth of planktonic cultures of both strains is almost equally sensitive to azithromycin. Low concentrations of azithromycin (0.1–1 μg/mL), which do not significantly influence the growth of planktonic cultures, significantly stimulate (1.4 to 1.6 times) the growth of biofilms of both strains, similarly to what was shown previously for gram-positive bacteria [19]. Thus, mutation of the *rpoS* gene does not significantly influence (qualitatively) the nature of the action of azithromycin.

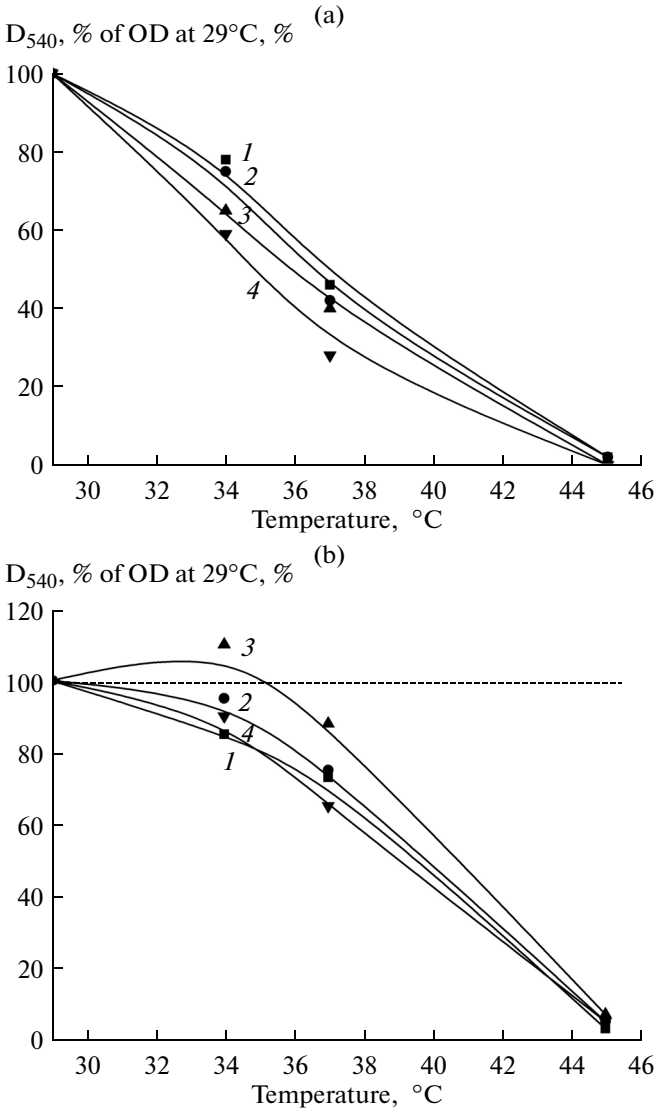

Fig. 8. Growth of planktonic culture (a) and biofilm (b) of *P. chlororaphis* 449 at different temperatures. Without antibiotic (*1*); in the presence of azithromycin: 6 μg/mL (*2*), 8 μg/mL (*3*), and 15 μg/mL (*4*). Biofilms stained with CV.

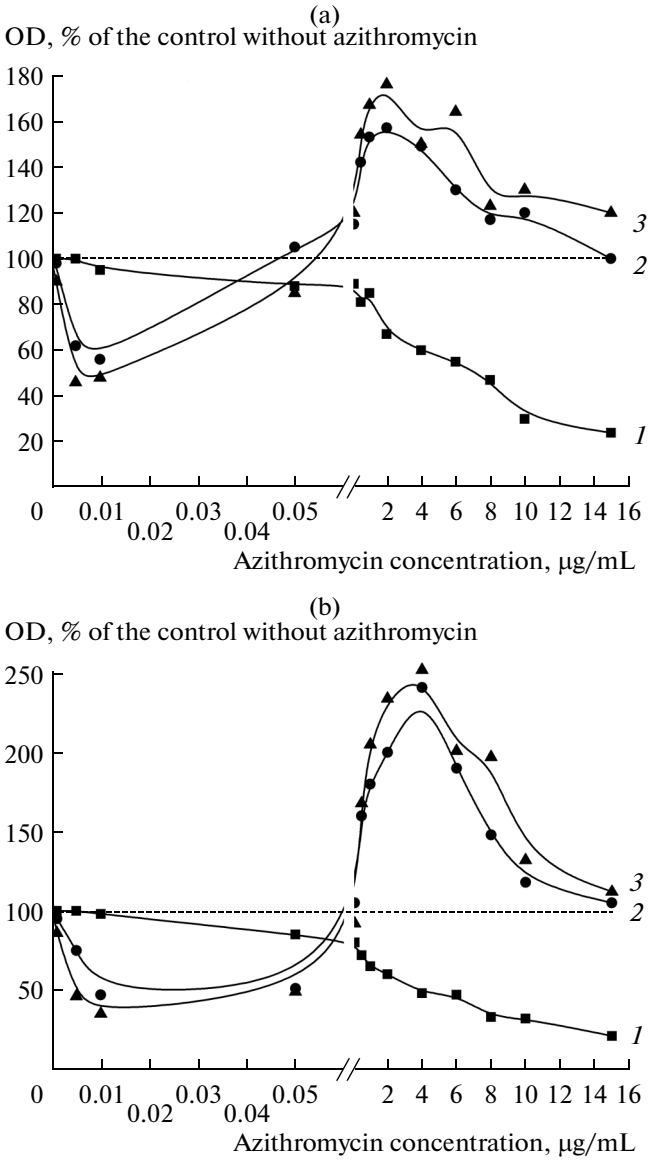

Fig. 9. Effect of low concentrations of azithromycin on planktonic culture (*1*) and biofilm (*2, 3*) growth of *P. chlororaphis* 449 (a) and *P. chlororaphis* 66 (b). Biofilms stained with CV (*2*) or DMMB (*3*).

In mutant 3, the two-component system GacA-GacS, which regulates the synthesis of all types of AHL, phenazine antibiotics, exoproteases, polygalacturonase, pectin methyl estherase, and antagonistic activity over phytopathogenic fungi, is impaired. The mutation is localized in the *gacS* gene encoding sensor kinase GacS. This mutant retains the capacity for synthesis of only some AHL types, and the level of synthesis is lower than in the wild type [11]. As can be seen from Fig. 3, planktonic cultures of these strains do not differ in their sensitivity to azithromycin. This mutant retained the tendency for stimulation of biofilm formation by low concentrations of azithromycin, though it is much less pronounced than in the wild type strain. It is obvious that the set (or level) of AHL serves as an essential factor for development of this effect.

The mutants 4 and 5 were obtained by transposon mutagenesis (using mini-Tn5Km) and contain inactivated genes of the phenazine operone: *phzB* (mutan 4) and *phzA* (mutant 5). As a result of the absence of phenazine synthesis, the mutants do not have the orange color typical of the wild type strain. However, synthesis of the complete set of AHL is preserved in this mutant [11].

Figs. 4 and 5 demonstrate the effects of azithromycin on the growth of planktonic cultures and biofilms of mutants 4 (Fig. 4) and 5 (Fig. 5). The sensitivity to azithromycin in planktonic cultures of mutants 4 and 5 was close to that in planktonic cultures of the wild type strains (mutant 5 was slightly more sensitive). While the stimulation of biofilm growth by sub-inhibitory concentrations of the antibiotic was observed for both mutants, it developed at higher concentrations (1–8 μg/mL). In the lower concentration region (0.05–0.5 μg/mL), considerable inhibition of this process was observed. The role of this phenomenon will be discussed further.

Strain 7e contains the plasmid pME6863, which includes the cloned gene of *N*-acyl homoserine lactonase (AiiA) responsible for the degradation of all types of AHL produced by this strain; degradation is accompanied by a drastic decrease in phenazine synthesis and impaired ability of bacterial cells to swarm. As we have already mentioned, strain 6e bears a vector plasmide pME6000, which does not affect AHL synthesis [20].

Figures 6 and 7 illustrate the influence of azithromycin on growth of planktonic cultures and biofilms of strains 6e (Fig. 6) and 7e (Fig. 7). It is obvious that introduction of the plasmid pME6000 (strain 6e) which does not lead to AHL synthesis decrease, affected significantly neither the azithromycin sensitivity of the planktonic culture nor the stimulating effect of this antibiotic on biofilms (Fig. 6). On the contrary, strain 7e, which contains the plasmid pME6860 inducing AHL degradation, fully lost its capacity for the stimulation of biofilm formation in the presence of low azithromycin concentrations. Moreover, biofilm growth became slightly more sensitive to effects of this antibiotic than the growth of planktonic culture (Fig. 7). Thus, according to the results of our experiments, the stimulatory effect of low concentrations of azithromycin on biofilm formation positively correlates with capacity for AHL synthesis. This correlation will be discussed below.

Sensitivity to heat stress. There is evidence that azithromycin and other macrolide antibiotics in sub-inhibitory concentrations stimulate the synthesis of biofilm matrix polysaccharides in clinical strains of *Staphylococcus epidermidis* [21]. On the other hand, our previous studies confirmed an important role of the polysaccharide matrix of the biofilms of several gram-positive and gram-negative saprotrophic bacteria in resistance not only to biocides, but also to such environmental stress factors as heat and acidic or osmotic shock [8].

If stimulation of *P. chlororaphis* 449 biofilm formation by azithromycin implied enhanced synthesis of the polysaccharide matrix, then such "stimulated" biofilms might be more resistant to heat. To verify this assumption, we studied the sensitivity of *P. chlororaphis* 449 biofilms to heat stress in the presence of stimulatory concentrations of azithromycin.

According to the results shown on Fig. 8, sensitivity of planktonic cultures to heat shock in the presence of azithromycin slightly increased (Fig. 8a). On the contrary, the sensitivity of biofilms to heat stress in the presence of azithromycin in growth-stimulating concentrations (6–8 μg/mL) slightly decreased (Fig. 8b).

This fact correlates with our hypothesis concerning biofilm growth stimulation in the presence of azithromycin, accompanied by increased synthesis of the DMMB-stained polysaccharide matrix, which, according to the literature [8], plays an important role in biofilm resistance to biocides and physicochemical stress factors. Additional evidence for this conclusion will be provided in the section of this study dedicated to microscopic research.

What is the probable biochemical mechanism of biofilm growth stimulation by azithromycin? Based on the literature, the following possible reasons for the stimulation of biofilm formation in pathogenic bacteria may be suggested.

First of all, enhanced biosynthesis of adhesion factors may occur, for example in the case of action of sub-inhibitory concentrations of the antibiotic imipenem on *Acinetobacter baumannii* [22]. Secondly, antibiotics may have a positive effect on the synthesis of QS regulation system factors required for biofilm formation, as was shown for macrolides in case of *C. violaceum* [23]. Additional evidence for importance of QS system in bacterial biofilm formation is provided by the work, in which another stress factor (hydrogen peroxide) was shown to stimulate growth of *P. aeruginosa* PAO1 biofilms. Moreover, this effect (similar to our experiments with azithromycin) was not observed in the presence of the plasmid pME6860, which contains the heterologous gene *aiiA* encoding *N*-acyl homoserine lactonase [24]. Finally, antibiotics may affect the regulatory systems involving cyclic diguanosine monophosphate (c-di-GMP), which participates in the biosynthesis of components of the polysaccaride biofilm matrix, as was shown for aminoglycosides in case of *E. coli* [25].

Thus, antibiotics not only induce death and growth inhibition of microorganisms, but are able to act as stress signatory molecules in sub-inhibitory concentrations [26].

Effect of ultralow concentrations of azithromycin on biofilm formation by pseudomonads. Investigation of the effect of a broad range of azithromycin concentrations on *P. chlororaphis* 449 revealed an unusual pattern: in regions of ultralow concentrations of the antibiotic which did not affect planktonic culture growth (0.001–0.01 μg/mL), biofilm formation was significantly inhibited (Fig. 9a).

Experiments with another strain, *P. chlororaphis* 66 confirmed this effect (Fig. 9b).

A similar effect was described in a recent work dedicated to detailed study of the inhibitory effect of macrolide antibiotics (erythromycin and azithromycin) on

stationary biofilms of *Porphyromonas gingivalis*, typical for dental plaque. Inhibitory effect on biofilms appeared at lower concentrations than the inhibitory concentrations for growth of planktonic cultures and for protein synthesis. Investigation of azithromycin-exposed biofilms revealed impaired matrix synthesis, while the amount of living cells remained the same [27].

It is still unclear how the same antibiotic can have different effects on the same bacterial biofilm, acting as an inhibitor at very low concentrations and as a stimulator at higher ones. Normally, a reverse pattern is common for toxic substances, i.e., stimulation at low concentrations and inhibition at high ones.

A possible explanation of this phenomenon is as follows. Biofilm matrix is a complex multi-component structure of biopolymers (DNA, proteins and polysaccharides). Since ultralow concentrations of azithromycin do not affect DNA and protein synthesis, we can suggest that in this case it targets mainly polysaccharide synthesis. Several polysaccharides take part in biofilm formation in pseudomonads. If synthesis of one of them is repressed by ultralow concentrations of azithromycin and synthesis of the second one is activated by higher concentrations of azithromycin, and if both processes are simultaneous, biofilms may be formed according to the variant shown on Fig. 9. At ultralow concentrations of azithromycin, biofilm growth is inhibited due to repressed synthesis of the first component, while synthesis of the second component has not been activated yet. At higher concentrations of azithromycin, activated synthesis of the second component compensates for the inhibition of the first component.

Undoubtedly, this hypothesis is speculative and lacks experimental proof.

Phase contrast microscopy (PCM) and epifluorescent microscopy (EFM) application. PCM. According to the literature, synthesis of the polysaccharides of the polymeric matrix is the most likely the target for azithromycin at both biofilm growth repression and biofilm stimulation. One of the methods making it possible to trace accumulation of the biofilm matrix polysaccharides is staining with DMMB, a specific stain for polysaccarides. In our previous work, we demonstrated that repression of the matrix polysaccharide synthesis (due to azithromycin activity or as a result of mutation) in some gram-negative bacteria correlates with decreased biofilm staining by DMMB [8].

Results shown on Figs. 10a–10c prove that matrix polysaccharides are the main target for repression or stimulation of biofilm growth by azithromycin, since the changes were more pronounced in DMMB-stained biofilms than in those stained with nonspecific CV.

The possible correlation of these conclusions was tested by microscopy of the biofilms exposed to various concentrations of azithromycin during their formation and stained with the polysaccharide-specific stain DMMB .

The results of phase contrast microscopy of *P. chlororaphis* 449 stained with DMMB after 6-h incubation (time required for matrix formation) in the presence of 0.03 (inhibitory concentration) and 8 μg/mL azithromycin (stimulating concentration) are shown on Fig. 10a. Azithromycin was not added to the control. Preliminary experiments demonstrated azithromycin not reacting with the stain and not influencing the staining process if added immediately before biofilm staining.

It can be seen that synthesis of the polysaccharide matrix components (stained with DMMB) decreased considerably in the presence of ultralow, "inhibiting" concentration of azythromycin (0.03 μg/mL), with a remarkable amount of cells becoming "naked." On the contrary, in the presence of higher, "stimulating" concentrations of azithromycin (8 μg/mL) synthesis of the polysaccharide matrix components increased remarkably. These results confirm our suggestion of a dose-dependent regulatory effect of azithromycin on the synthesis of the polysaccharide matrix components.

EFM. The same incubation time (6 h) was used for biofilm incubation, which was sufficient for formation of a mature matrix. Since DAPI, apart from staining DNA, also exhibits nonspecific proper fluorescence, it is suitable for assessment of the total matrix content in biofilms. DAPI is accumulated in the matrix and visualizes it due to proper fluorescence of the unbound stain. Under these conditions, the fluorescence of matrix-embedded cells is masked [13]. Comparison of DAPI-stained biofilms in the control without azithromycin with the biofilms formed in the presence of "inhibitory" (0.03 μg/mL) and "stimulating" (8 μg/mL) concentrations of azithromycin demonstrated the total content of the matrix to decrease in the first case and to increase in the second case (Fig. 10b).

Since low concentrations of azithromycin affect neither growth of microbial cells nor protein and DNA synthesis, it may be assumed that decreased matrix content probably resulted from repression of polysaccharide synthesis.

The SYPRO Ruby dye stains proteins of the matrix associated with the cells and loosely located in the matrix. Comparison of the same variants of biofilms (as in Figs. 10a, 10b) stained with this stain (Fig. 10c) revealed the biofilm becomes looser in the presence of "inhibitory" azithromycin concentrations, with decreased protein content. According to previously expressed opinions, this decrease is probably due to impaired structure of the polysaccharide matrix, resulting in a loss of some proteins released into environment from the matrix. No significant changes in protein content in the biofilm were observed at "stimulating" concentrations of the antibiotic.

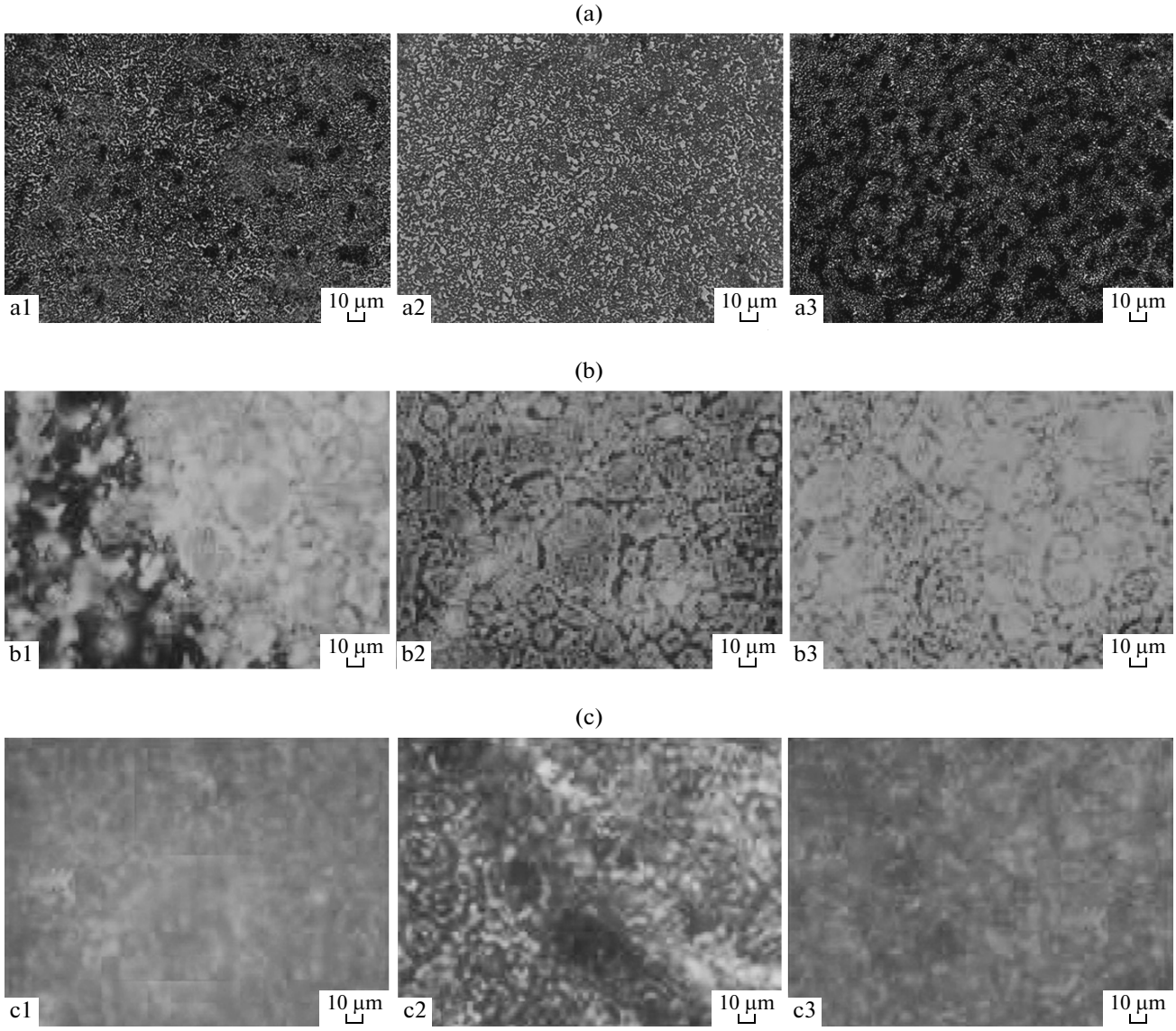

Fig. 10. Light microscopy of *P. chlororaphis* 449 biofilms grown in the presence of different concentrations of azithromycin: control without antibiotic (*1*), 0.03 μg/mL azithromycin (*2*), and 8.0 μg/mL azithromycin (*3*). PCM, staining with DMMB, dark areas correspond to the matrix (a); EFM, staining with DAPI, light areas correspond to the matrix (b); and EFM, staining with RUBY, light areas correspond to the matrix (c).

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, project no. 13-04-01145.

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Translated by E. Botchkova