ЕКСПЕРИМЕНТАЛЬНІ ПРАЦІ

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BIOFILM FORMATION BY *PSEUDOMONAS AERUGINOSA* STRAINS WITH DIFFERENT LEVELS OF CYCLIC DI-GUANOSINE MONOPHOSPHATE BIOSYNTHESIS

***Aim**: Swarming motility and biofilm formation abilities determination in P. aeruginosa PA01 pJN2133, with low c-di-GMP level. **Materials and methods.** P. aeruginosa PA01 and P. aeruginosa PA01 pJN2133 were used as a test-organisms. Bacteria were cultivated in 24-walls Nuclon plates in LB medium at 37 °C for 30–60 min for adhesion determination and for 24 hours for biofilm formation. Determination of planktonic cells amount were carried out spectrophotometrically, biofilm formation – by CV-test, and polysaccharide determination – by alcian blue and congo red staining. Swarming motility, hydrophobicity, and z-potential were carried out by common methods. **Results.** It was shown that P. aeruginosa PA01 pJN2133 form biofilm with impaired structure and in 3.7 times less intensive than P. aeruginosa PA01. Even on early stages of the biofilm formation mutant strain shows low ahession ability compare to wild type. P. aeruginosa PA01 pJN2133 planktonic cells amount was higher than in wild type strain at all stages of biofilm formation. P. aeruginosa PA01 pJN2133 swarming motility zones diameter was 62 ± 5 mm and it was in 1.4 times higher than at P. aeruginosa PA01 – 43 ± 3 mm. Swarming motility zones morphology were different. It was shown that cells of each strains change its hydrophobicity from logarithmic to stationary phase, but direction of changes was opposite. P. aeruginosa PA01 pJN2133 cells hydrophobicity increase at stationary phase. P. aeruginosa PA01 cells hydrophobicity were higher in logarithmic phase. Cells of each strains have the same z-potential. **Conclusions**. On the frame of low c-di-GMP amount P. aeruginosa PA01 pJN2133 have low adhesion and biofilm formation abilities. Planktonic cells amount and swarming motility intensity are conversely high.*

Key words: cyclic-di-GMP, Pseudomonas aeruginosa, adhesion, biofilm, swarming motility.

Bis-(3-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is in the spotlight of the scientists as the result of last achievement of microbial genomics and great interests in microbial communities [10, 12, 13]. Cytoplasmic c-di-GMP is a bacterial secondary messenger, that regulates numerous of physiological processes: cell-to-cell communication, biofilm formation, motility, virulence, etc. [1, 2, 4].

Depends on concentration of this regulator bacteria shifts its life-form from motile to sessile (biofilm formation) [12]. It is found that c-di-GMP affects all stages of the biofilm formation process in *Pseudomonas aeruginosa* from the beginning of adhesion to biofilm decay. This compound regulates biosynthesis of matrix components, quorum sensing signal molecules, biosurfactants. [8, 11]. That fact, that many processes change direction in bacterial cells depends on c-di-GMP led to hypo- and hyperproduction strains construction. Their use allows to deepening knowledge in the role of this compound in many bacterial cells processes and the possibility to use this molecule as an instrument for biofilm formation control.

The aim of this work was studding the features of *P. aeruginosa* PA01 pJN2133 swarming motility and biofilm formation in fact that this strain is characterized with low level of c-di-CMP in its cells.

Materials and methods

In this work two strains of *P. aeruginosa* were used. *P. aeruginosa* PA01 – the wild type strain was obtained from the collection of Odessa Mechnikov National University microbiology, virology and biotechnology department. PAO1 pJN2133 with low level of c-di-GMP was kindly provided by Dr. Olena Rzhepishevskaya from University of Umea, Sweden. Strains were cultivated on LB medium (g/l, pepton – 15.0; yeast extract – 10.0; NaCl – 5.0) at 37 °C.

Number of adherent cells, biofilm mass, planktonic cells and matrix exopolysaccharides content were carried out in 24-wells polysteroll Nuclon plates cultivation conditions. Planktonic cells amount was examined spectrophotometrically on wave length 540 nm. Amount of adherent cells was examined after 30 and 60 min of incubation, biofilm mass – after 24 hours of incubation. After washing well content was fixed with 96% ethanol for 10 min and were stained with 1% crystal violet solution. After 15 minutes of incubation each wells were washed and dried. Wells content was lysed by 1.5 lytic solution with 0.1 M NaOH and 1% sodium dodecylsulfate and OD_{592} of each samples were measured [3].

Biofilm matrix exopolysccharides content were studded by staining with congo red (Pel) and alcian blue (alginate). After dissolution in lytic solution OD_{490} and OD_{608} were measured respectively [10].

All spectrophotometrically measurement was carried out with SmartSpec Plus (Bio-Rad, Hungary).

For biofilm morphology discovery 24×24 mm cover glasses was incubated in 35 mm sterile plastic Petri dishes with 2 ml of LB-medium and *P. aeruginosa* cells (10^3 CFU/ml). Plates were incubated for 24 hours at 37 °C. After incubation glasses were fixed with 96% ethanol for 10 min and were stained with 1% crystal violet solution. After drying glasses were analyzed with Primo Star PC, Carl Zeiss microscope and photographed with Olympus DCM (3.0 M pixels) camera.

Swarming motility was studied by the next way. Briefly 2 µl of overnight culture were transferred on the centre of Petri dishes with 0,6% agar medium contain

meat-peptone bullion – 8.0 g/l, glucose – 50.0 g/l, agar-agar – 6.0 g/l. Dishes were incubated for 24 hours at 37 °C. Swarming motility intensity was analyzed by cells spread zones diameter measurement.

Cells surface hydrophobicity was analyzed by MATH-test [14]. Cells from overnight culture were harvested by centrifugation and resuspended in PBS buffer. OD_{600} were adjusted to ~ 0.5. 1 ml of hexodecane was added to 3 ml of cells suspension and mixtures were vortexes vigorously for 1 min. After separation of layers OD_{600} of aqueous phase were measured. Hydrophobicity (%) was calculated by formula: [(A0 – A)/A0] × 100, where A0 – optical density of initial suspension, A – optical density of suspension after hexadecane treatment.

Zeta-potential and cell diameter were measured with Zetasizer Nano ZS, Malvern Instruments.

All experiments were carried out in 3 independent studies with 3–6 repeat.

Statistical analysis was performed by using standard methods of variation analysis. Average values ($\bar{X}$) and their standard error ($S_{\bar{X}}$) were calculated. Reliability of differences was determined by Student's criterion at significance level of not less than 95% ($p \leq 0.05$). All mathematics calculations were performed by using the computer program Excel.

Results and discussion

Biofilm formation process begins with cells attachment to solid surface. Adhesion level is depending on same properties, not only on cell-surface but from abiotic surface as well. Cell properties that affect adhesion are hidrophobicity, z-potential, motility, and exopolysaccharides and biosurfactants secretion. Substrate properties include hidrophobicity, z-potential, and surface architecture [7, 12]. In this study we focused our attention on comparison of these properties in two *P. aeruginosa* strains – PA01 (wild type), and PA01 pJN2133 with low level of c-di-GMP biosynthesis. According to literature data it is known that in *P. aeruginosa* PA01 intracellular content of this messenger equals to 3.5 fmol/mg proteins, and in *P. aeruginosa* PA01 pJN2133 there is an undetectable amount of this compound [9].

Previously, we have shown that the first microcolonies of *P. aeruginosa* PA01 appeared 45 minutes after the start of incubation [6]. Therefore the determination of planktonic and attached cells were carried out after 30 and 60 minutes of incubation (fig. 1). Optical density of inoculums was 0.047 for *P. aeruginosa* PA01 and 0.051 for *P. aeruginosa* PA01 pJN2133.

Obtained results show that from the beginning of cultivation *P. aeruginosa* PA01 pJN2133 shows the lowest than wild type strain ability of attachment to solid surface. After 30 min of incubation attached cells amount of *P. aeruginosa* PA01 was in 1.5 higher. In next 30 min of incubation attached cells amount increased in both cases, but it was on 25% lower in case of *P. aeruginosa* PA01 pJN2133 compare the *P. aeruginosa* PA01. After 60 min of incubation there is a tendency of higher increasing of planktonic cells amount in *P. aeruginosa* PA01.

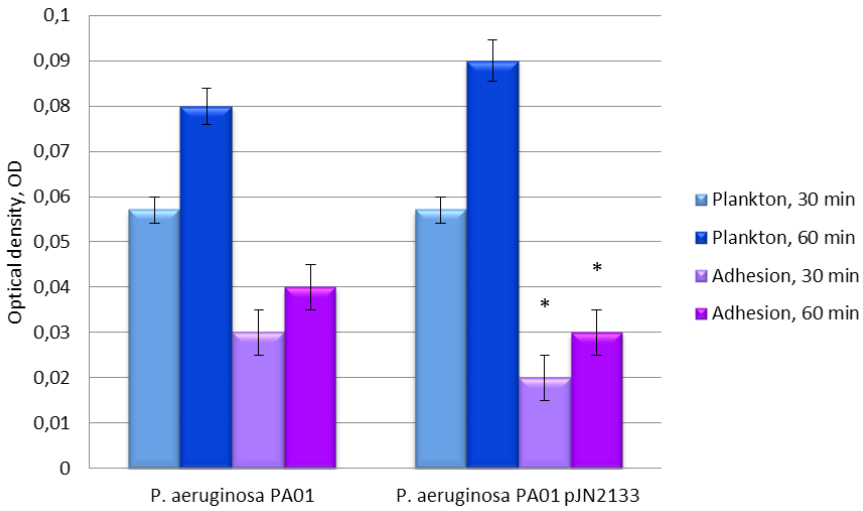

Fig. 1. Attached and free cells amount of *P. aeruginosa* test strains at the first hours of incubation

Note: * – significant difference compared with *P. aeruginosa* PA01

Examination of daily biofilms show that there were significant differences in general form and structure (fig. 2). *P. aeruginosa* PA01 biofilm consists of multicellular 3D-structures. At the same time, *P. aeruginosa* PA01 pJN2133 biofilm was flat and "monolayer" (fig. 2A). The difference from two strains also was noticeable on microcolony level (fig. 2B). *P. aeruginosa* PA01 microcolonies were good formed that consists of matrix enclosed cells. In addition, there are secondary microcolonies formation that enhance biofilm mass. In *P. aeruginosa* PA01 pJN2133 only the small structural units were detected.

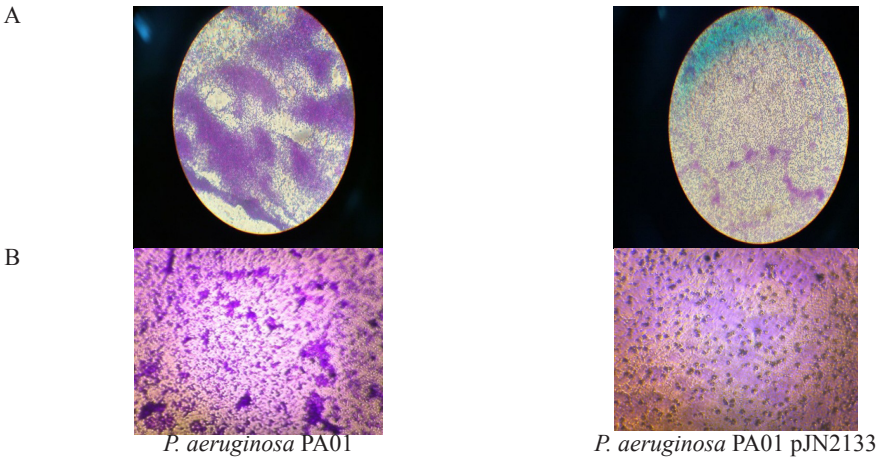

Fig. 2. Images of biofilms, forming by wild and mutant strains of *P. aeruginosa* (Magnification: A – × 200; B – × 400; crystal violet staining)

Quantity examination of the biofilm formation shows (fig. 3) that *P. aeruginosa* PA01 biofilm has in 3.7 times higher mass than in *P. aeruginosa* PA01 pJN2133. ($p<0.001$).

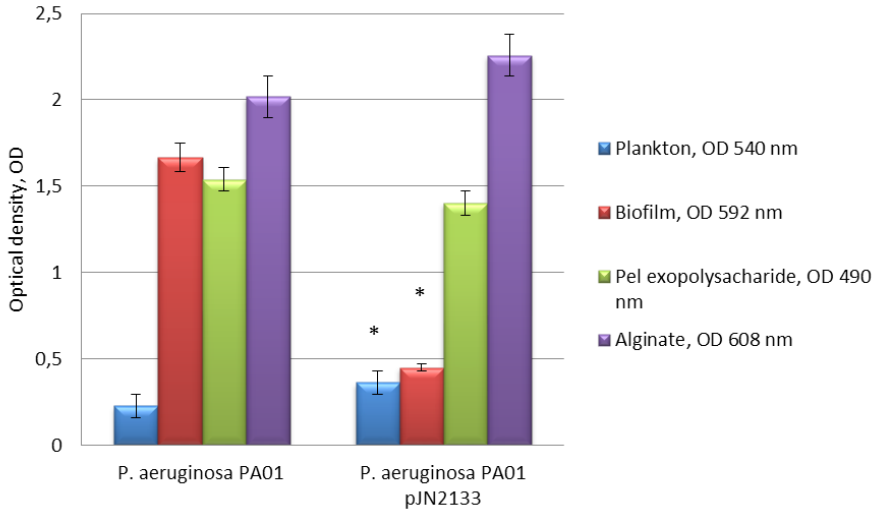

Fig. 3. Biofilm mass and exopolysaccharides ammount

Note: * – significant difference compared with *P. aeruginosa* PA01

However, planktonic cells amount was in 1.6 times higher in the case of *P. aeruginosa* PA01 pJN2133. Pel and alginate exopolysacharides amount was similar in all strains biofilm matrix.

Cell size and physical-chemical properties of the cells surface study show that examined strains have a difference in hydrophobicity. More over, strains hydrophobicity changed during the cultivation (table.).

Table

Cell size and physical-chemical properties of *P. aeruginosa* strains cells surface

Strain	Cell hydrophobicity, %		Cell size, nm	z-potential, - mV
	3 h	24 h		
P. aeruginosa PA01	42.8 ± 2.3	14.2 ± 1.7	666.4 ± 46.4	23.4 ± 1.2
P. aeruginosa PA01 pJN2133	8.3 ± 0.4*	43.1 ± 1.9*	920.8 ± 53.1*	23.6 ± 1.3

Note: * – significant difference compared with *P. aeruginosa* PA01

In logarithmic phase of growth, after 3 h of incubation cell hydrophobicity of *P. aeruginosa* PA01 was higher in 5.2 times then in *P. aeruginosa* PA01 pJN2133. Transition to stationary phase accompanied with opposite changes of hydrophobicity: 3 fold decreasing in wild type and 5 fold increasing in mutant type strain. Cell diameter in *P. aeruginosa* PA01 overnight culture was in 1.4 times lower than in *P. aeruginosa* PA01 pJN2133. Z-potential of the cells was the same.

An important indicator that affects the interaction of bacterial cells with different surfaces and biofilm formation ability is motility, especially swarming. It was shown that *P. aeruginosa* PA01 and *P. aeruginosa* PA01 pJN2133 are distinguish in the swarming motility process characteristics (fig. 4). *P. aeruginosa* PA01 pJN2133 swarming motility zones diameter was 62 ± 5 mm and it was in 1.4 times higher than at *P. aeruginosa* PA01 – 43 ± 3 mm.

P. aeruginosa PA01 *P. aeruginosa* PA01 pJN2133

Fig. 4. Swarming motility of *P. aeruginosa* PA01 and *P. aeruginosa* PA01 pJN2133 cells

P. aeruginosa PA01 swarming zones have not clearly formed central "core" (fig. 4). Branching paths of the cells migration begun from inoculation point is thin in gentle by sight. In the end of each "rays" there is a white thick colony. *P. aeruginosa* PA01 pJN2133 swarming zones looks more rough with 1.5–2.0 cm in diameter centre and thick nonbranching «rays» 2.5 cm in length. Cells in a part of these spread zones more or less evenly along their length and do not form dense colonies. Based on this results, we can conclude that *P. aeruginosa* PA01 pJN2133 cells are more motile than *P. aeruginosa* PA01 cells. Increasing of the motility could be explained by overproduction and secretion of high rhamnolipids amount, because biosurfactants play the main role in swarming motility implementation. If this hypotheses will be confirmed in the next studies, *P. aeruginosa* PA01 pJN2133 could be used as a good rhamnolipids producent.

More over, decreasing in biofilm formation ability on the background of c-di-GMP decreasing make this system an attractive target for novel antimicrobial drugs.

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УТВОРЕННЯ БІОПЛІВКИ ШТАМАМИ *PSEUDOMONAS AERUGINOSA* З РІЗНИМ РІВНЕМ БІОСИНТЕЗУ ЦИКЛІЧНОГО ДИГУАНОЗИНМОНОФОСФАТУ

Реферат

***Мета роботи**: встановлення особливостей утворення біоплівки та переміщення шляхом роїння клітин штаму P. aeruginosa PA01 pJN2133, що характеризується низьким рівнем синтезу цикло-ди-ГМФ. **Матеріали та методи**. Як тест-мікроорганізми використовували штами P. aeruginosa PA01 і P. aeruginosa PA01 pJN2133. Культивування проводили в 24-лункових плоскодонних планшетах Nuclon у середовищі LB при 37 °С впродовж 30–60 хв при визначенні адгезії клітин і впродовж 24 годин при дослідженні утворення біоплівки. Кількість планктонних клітин оцінювали спектрофотометрично, кількість прикріплених клітин і масу біоплівки – за методом забарвлення кристалічним фіолетовим, вміст полісахаридів у матриксі – за методом забарвлення конго червоним або алціановим синім. Роїння, гідрофобність клітин та дзета потенціал оцінювали за загальноприйнятими методами. **Результати**. Встановлено, що P. aeruginosa PA01 pJN2133 у порівнянні з P. aeruginosa PA01 утворює біоплівку з порушеною структурою, маса якої знижена у 3,7 рази. Вже на ранньому етапі формування біоплівки мутантний штам поступається батьківському за кількістю адгезованих клітин. У той же час, на всіх етапах утворення біоплівки P. aeruginosa PA01 pJN2133 кількість планктонних клітин достовірно перевищує рівень, характерний для штаму дикого типу. Діаметр зони розповсюдження клітин P. aeruginosa PA01 pJN2133 шляхом роїння становив 62 ± 5 мм і в 1,4 рази перевищував показник P. aeruginosa PA01 – 43 ± 3 мм. Морфологія зон роїння досліджуваних штамів суттєво різниться за рядом ознак. Показано, що клітини обох штамів при переході з логарифмічної до стаціонарної фази росту змінюють гідрофобність, але спрямованість змін носить протилежний характер: підвищення у разі мутантного штаму, і зниження у разі штаму дикого типу. Клітини обох штамів мають однаковий дзета потенціал. **Висновки.** На тлі низького вмісту у цитоплазмі цикло-ди-ГМФ гальмується адгезія клітин до твердої поверхні і утворення повноцінної біоплівки, що сприяє планктонному способу існування P. aeruginosa PA01 pJN2133. Одержані результати дозволяють припустити, що ці явища обумовлені низькою гідрофобністю клітин мутантного штаму та їх високою здатність до переміщення по поверхні шляхом роїння.*

Ключові слова: цикло-ди-ГМФ, Pseudomonas aeruginosa, адгезія, біоплівка, роїння.

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ОБРАЗОВАНИЯ БИОПЛЕНКИ ШТАММАМИ *PSEUDOMONAS AERUGINOSA* С РАЗНИМ УРОВНЕМ БИОСИНТЕЗА ЦИКЛИЧЕСКОГО ДИГУАНОЗИНМОНОФОСФАТА

Реферат

***Цель работы**: выявление особенностей образования биопленки и передвижения путем роения клеток штамма P. aeruginosa PA01 pJN2133, характеризующегося низким уровнем синтеза цикло-ди-ГМФ. **Материалы и методы.** Как тест-микроорганизмы использовали штаммы P. aeruginosa PA01 и P. aeruginosa PA01 pJN2133. Культивирование проводили в 24-луночных плоскодонных планшетах Nuclon в среде LB при 37 °C в течение 30–60 мин при определении адгезии клеток и в течение 24 часов при исследовании образования биопленки. Количество планктонных клеток оценивали спектрофотометрически, количество прикрепленных клеток и массу биопленки – по методу окраски кристаллическим фиолетовым, содержание полисахаридов в матриксе – по методу окраски конго красным или алцианови синим. Роение, гидрофобность клеток и дзета потенциал оценивали по общепринятым методам. **Результаты.** Установлено, что P. aeruginosa PA01 pJN2133 по сравнению с P. aeruginosa PA01 образует биопленку с нарушенной структурой, масса которой снижена в 3,7 раза. Уже на раннем этапе формирования биопленки мутантный штамм уступает родительскому по количеству адгезированными клеток. В то же время, на всех этапах образования биопленки P. aeruginosa PA01 pJN2133 количество планктонных клеток достоверно превышает уровень, характерный для штамма дикого типа. Диаметр зоны распространения клеток P. aeruginosa PA01 pJN2133 путем роения составил 62 ± 5 мм и в 1,4 раза превышал показатель P. aeruginosa PA01 – 43 ± 3 мм. Морфология зон роения исследуемых штаммов существенно отличается по ряду признаков. Показано, что клетки обоих штаммов при переходе от логарифмической к стационарной фазе роста меняют гидрофобность, но направленность изменений носит противоположный характер: повышение в случае мутантного штамма, и снижение в случае штамма дикого типа. Клетки обоих штаммов имеют одинаковый дзета потенциал. **Выводы**. На фоне низкого содержания в цитоплазме цикло-ди-ГМФ тормозится адгезия клеток к твердой поверхности и образование полноценной биопленки, что способствует планктонных способа существования P. aeruginosa PA01 pJN2133. Полученные результаты позволяют предположить, что эти явления обусловлены низкой гидрофобностью клеток мутантного штамма и их высокой способностью к перемещению по поверхности путем роения.*

Ключевые слова: цикло-ди-ГМФ, Pseudomonas, адгезия, биопленка, роение.

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