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QUORUM SENSING AUTOINDUCERS BIOSYNTHESIS BY BIOFILM CULTURES OF *PSEUDOMONAS AERUGINOSA* STRAINS WITH DIFFERENT LEVELS OF THE CYCLIC DIGUANOZINMONOPHOSPHATE

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Мета роботи – встановити залежність між вмістом цикло-ди-ГМФ і здатністю *P. aeruginosa* до утворення біоплівки і синтезу аутоіндукторів системи кворуму.

Матеріали та методи дослідження. В роботі були використані штам дикого типу *P. aeruginosa* PAO1 і штами *P. aeruginosa* з низьким (PAO1pJN2133) та підвищеним (PAO1 ΔwspF) рівнями циклічного дигуанозинмонофосфату. Культивування проводили у 24-луноквих плоскодонних планшетах Nuclon при 37 °C у середовищі LB. Масу біоплівки визначали у CV-тесті. Вимірювання здійснювали на спектрофотометрі SmartSpecPlus (Bio-Rad, Hungary) при довжині хвилі 592 нм. Ацил-гомсеринлактони екстрагували підкисленим етилацетатом та визначали їх кількість методом ГХ/МС. Визначення вмісту цикло-ди-ГМФ проводили з використанням репортерної плазмиди Seattle шляхом вимірювання інтенсивності флуоресценції клітин у біоплівках.

Результати дослідження. Встановлено, що штам *P. aeruginosa* PAO1 pJN2133, внутрішньоклітинний вміст цикло-ди-ГМФ якого є у 4 рази меншим ніж у *P. aeruginosa*, утворює біоплівку, маса якої у 3,5 рази нижча ніж у батьківського штаму. Штам *P. aeruginosa* PAO1 ΔwspF перевищує *P. aeruginosa* PAO1 за цими показниками у 1,5 рази та 33 %, відповідно. Ще більш виражені відмінності виявляються при співставленні між собою мутантних штамів. За рівнем цикло-ди-ГМФ *P. aeruginosa* PAO1 ΔwspF переважає штам PAO1 pJN2133 у 5,9 рази, а за масою біоплівки – у п'ять разів. Найбільшу кількість сигнальних молекул системи кворум сенсінг синтезує штам з низьким рівнем вторинного месенджера.

Висновки. Між внутрішньоклітинним вмістом цикло-ди-ГМФ і здатністю до утворення біоплівки існує прямо-пропорційна залежність: чим вище вміст вторинного месенджера, тим більша маса біоплівки. Концентрація аутоіндукторів QS у середовищі зворотнім чином зв'язана з внутрішньоклітинним вмістом цикло-ди-ГМФ: у порівнянні з батьківським штамом підвищена у штаму з низьким вмістом вторинного месенджера і знижена у штаму з його підвищеним рівнем

Ключові слова: *Pseudomonas aeruginosa*, quorum sensing, біоплівки, цикло-ди-ГМФ, аутоіндуктори QS

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1. Introduction

Pseudomonas aeruginosa is a widespread microorganism, can colonize numerous ecological niche and cause opportunistic infections in humans. Such properties are related to its ability to form biofilms and produce various pathogenic factors [1, 2]. The process of biofilm development consists of three key stages: adhesion to any surface and the transition from a mobile to a sedentary lifestyle; growth of microcolonies, maturation of a biofilm and differentiation of its cells; destruction of the biofilm and resettlement of cells for colonization of new areas. The life cycle of biofilms is controlled by two signalling systems – the intercellular communication system (quorum sensing, QS) and cyclic diguanosine monophosphate (cyclic-di-GMP). Separately, both systems have been studied in depth, but studies of their interaction have begun recently. Therefore, there are many unresolved issues that need further study [3].

2. Literary review

Cyclic-di-GMP is an intracellular secondary messenger that coordinates the transition of bacteria from a

mobile to an attached state [4, 5]. It was found that in many species of bacteria the concentration of cyclic-di-GMP in biofilm cells is much higher than in freely existing ones [6]. Thus, it is shown that *P. aeruginosa* in the biofilm contains an average of 75–110 pmol cyclic-di-GMP per mg of total cell extract, while planktonic cells contain < 30 pmol/mg. It is assumed that bacterial cells use cyclic-di-GMP to control the development of the biofilm and the change in its concentration is a signal for the transition of the biofilm from one stage to another [7]. The level of the secondary messenger in the cell depends on the rates of its synthesis and decay. The cyclic-di-GMP molecule is synthesized from two GTP molecules by enzymes called diganylate cyclase (DGC) and degrades to 5'-phosphoguanlyl-(3'-5')phosphoguanosine (pGpG) and / or GMP by phosphodiesterase (PDE). These enzymes come in multiple forms and are usually associated with a variety of PerArnt-Sim (PAS) sensory domains, allowing the cell to receive numerous internal and external signals. The genome of *P. aeruginosa* PAO1 encodes 41 proteins involved in cyclic-di-GMP metabolism [8]. Due to this, this sec-

ondary messenger is involved in numerous cellular functions, including cell cycle regulation, differentiation, biofilm formation and dispersion, motility and virulence [9].

Quorum sensing (QS) is a mechanism that is activated in response to cell density and used by many bacteria to coordinate gene transcription. In QS, bacteria constitutively express signalling molecules called autoinducers. When the concentrations of QS molecules reach the threshold, they bind to certain bacterial receptors and trigger a coordinated program of increased transcription of bacterial virulence genes. This mechanism is particularly important for the expression of pathogenicity in *P. aeruginosa*. Two interconnected units of QS *P. aeruginosa* use acyl-homoserine lactone signalling molecules: Las and Rhl systems [10, 11]. LasI synthase is responsible for synthesis N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), which is recognized by the LasR receptor. The Rhl system uses N-butanoyl-homoserine lactone (C4-HSL) produced by RhlI synthase and bound to the RhlR receptor [12]. The third, the Pqs system, uses the signal quinolone specific to *P. aeruginosa*: 2-heptyl-3-hydroxy-4-quinolone (PQS), and interacts closely with the Las and Rhl units [13]. The most important functional effect of QS activation is the formation of a biofilm, which provides benefits for the survival of bacteria in any ecosystem. A study of the Las-deficient strain of *P. aeruginosa* showed that it forms an undeveloped biofilm that does not contain classical multicellular fungal-like structures. These data suggest that the QS system is not absolutely necessary for the formation of biofilms, but it is responsible for the development of mature biofilms. This is supported by the presence of fungal-like structures of eDNA and rhamnolipids, the synthesis of which is under the control of the QS system [14, 15].

Thus, according to the literature, we can conclude that both signalling systems are necessary for bacteria to form a full-fledged biofilm, which indicates the relevance of in-depth study of their interaction.

3. The purpose and objectives of the study

The aim of the work is to establish the relationship between the content of cyclic-di-GMP and the ability of *P. aeruginosa* to form a biofilm and the synthesis of autoinducers of the quorum system.

Research objectives:

1. To establish the relationship between the content of cyclic-di-GMP and the mass of the formed biofilm of the *P. aeruginosa* studied strains.
2. To investigate the level of biosynthesis of autoinducers of QS by biofilm cultures of the *P. aeruginosa* studied strains.

4. Materials and methods of the research

The wild-type strain of *P. aeruginosa* PA01 from the collection of cultures of the Department of Microbiology, Virology and Biotechnology of ONU named after I.I. Mechnikov and strains of *P. aeruginosa* with low (PA01 pJN2133) and elevated (PA01 $\Delta wspF$) levels of cyclic diguanosine monophosphate were used provided by O. Rzhepishevskaya from the University of Umeå, Sweden. The work was performed in 2018–2019.

Cultivation was performed in 24-well flat-bottomed plates Nuclon at 37 °C LB medium with the following composition (g/l): peptone – 15.0, yeast extract – 10.0, sodium chloride – 5.0.

Each well was assigned 8 wells: four to determine the mass of the biofilm and four – the number of cells in the biofilm. The mass of the biofilm was determined using the CV test [16]. Measurements were performed on a spectrophotometer Smart Spec Plus (Bio-Rad, Hungary) at a wavelength of 592 nm.

To determine the number of cells, the biofilms were removed from the bottom of the wells with a soft plastic spatula, saline (PS) was added, and the cells were homogenized and precipitate by centrifugation. The pellet was suspended in 1 ml of PS and the number of cells was determined spectrophotometrically.

The liquid taken from the wells was centrifuged to remove planktonic cells and the autoinducers were extracted three times from the supernatant with acidified ethyl acetate. The content of acyl-homoserinlactones was determined by GC/MS using chromatographic mass spectrometric system Agilent 6890N / 5973 inert [17], signal quinolone – by HPLC on a liquid chromatograph Agilent 1260 Infinity II [18]. Commercial samples of SIGMA-ALDRICH autoinducers were used as standards.

Determination of cyclic-di-GMP content was performed using the Seattle reporter plasmid (pMH487 (pCdrA:gfpS)) according to the method [19]. Before introducing the plasmid into the cells, they were electroporated on a Bio-Rad MicroPulser, 1.6–2.5 kV, 25 μ F, 200 ω . After two hours of incubation with the plasmid, the cells were washed and transferred to 96-well black Costar plates. After 24 h of cultivation at 37 °C, the culture fluid was removed, the biofilms were washed with phosphate buffer and the fluorescence was measured in relative units (extinction λ 485 nm, emission λ 535 nm, λ_{OD} 595 nm) on a Tecan GENios plate reader. After measuring the fluorescence intensity, the number of cells in the biofilms was determined and the relative values for each strain were calculated.

All experiments were performed in three independent experiments with 3-4 replicates in each.

Statistical processing of research results was carried out using conventional methods of variation analysis. The average values of the indicators (\bar{X}) and their standard error (SX S) were calculated. Significance of differences between means was determined by Student's criterion, assessing the reliability of the results at a significance level of at least 95 % ($p \leq 0.05$). Mathematical calculations were performed using the computer program Excel [20].

5. The results of the study

Used in the work of *P. aeruginosa* strains with low (PA01 pJN2133) and high (PA01 $\Delta wspF$) relative to the parent strain content of cyclic-di-GMP were constructed in the laboratory of Caroline S. Harwood [9]. The level of cyclic-di-GMP in cells depends on the activity of two specific enzymes – diguanylate synthase (WspR) and cyclo-diguanylate phosphodiesterase (protein PA2133). Depletion of the secondary messenger level in *P. aeruginosa* pJN2133 is achieved by additional copies of the PA2133 gene introduced into

cells as part of plasmid pJN105 (empty vector). Mutation in the *wspF* gene, the product of which is a negative regulator of diguanylate synthase, contributes to the increase in cyclic-di-GMP levels in *P. aeruginosa* Δ *wspF*.

Given that the increase in cyclic-di-GMP levels is a signal for the transition of bacterial cells from free to attached [4], we have previously studied the architecture of biofilms formed by the studied strains of *P. aeruginosa* [21]. Significant differences in their general appearance and structure were found between strains of *P. aeruginosa* PA01 and *P. aeruginosa* PA01 Δ *wspF*, on the

one hand, and strain *P. aeruginosa* PA01 pJN2133, on the other. According to light and laser confocal microscopy, multicellular three-dimensional structures were present in *P. aeruginosa* PA01 and PA01 Δ *wspF* biofilms, while *P. aeruginosa* PA01 pJN2133 biofilm did not contain such formations and appeared "monolayer". In addition, in the biofilm of strain PA01 pJN2133 its components: cells and extracellular matrix, were evenly distributed on the surface.

Quantitative characteristics of biofilms of the studied strains and their content of cyclic-di-GMP are shown in Fig. 1.

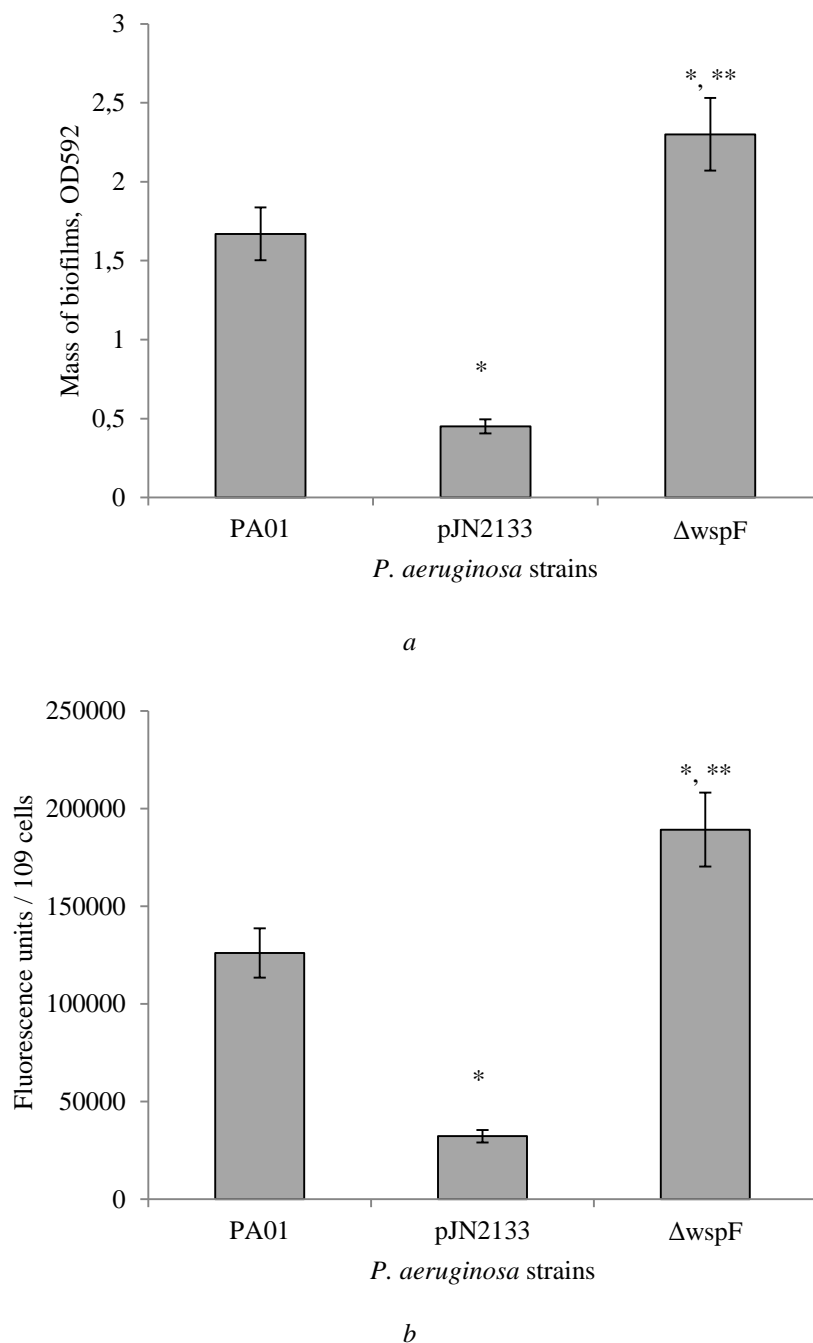


Fig. 1. The relationship between the content of cyclic-di-GMP and the mass of biofilms of the studied strains of *P. aeruginosa*:

- a* – mass of biofilms; *b* – the content of cyclic-di-GMP in biofilm cells;
 * – the difference is significant in comparison with strain PA01 ($p \leq 0.01$);
 ** – the difference is significant compared to strain pJN2133 ($p \leq 0.001$)

The obtained results revealed a direct dependence of the mass of biofilms on the level of the secondary messenger in its cells. Thus, the strain of *P. aeruginosa* PA01 pJN2133, the intracellular content of cyclic-di-GMP which is 4 times less than that of *P. aeruginosa*, forms a biofilm, the mass of which is 3.5 times lower than that of the parent strain. The strain of *P. aeruginosa* PA01 $\Delta wspF$ exceeds *P. aeruginosa* PA01 by 1.5 times

and 33 %, respectively. Even more pronounced differences are found when comparing mutant strains. The cyclic-di-GMP level of *P. aeruginosa* PA01 $\Delta wspF$ is 5.9 times higher than the PA01 pJN2133 strain, and the biofilm weight is five times higher.

Presented in Fig. 2 data show opposite to the content of the secondary messenger changes in the levels of autoinducers of the quorum sensing system.

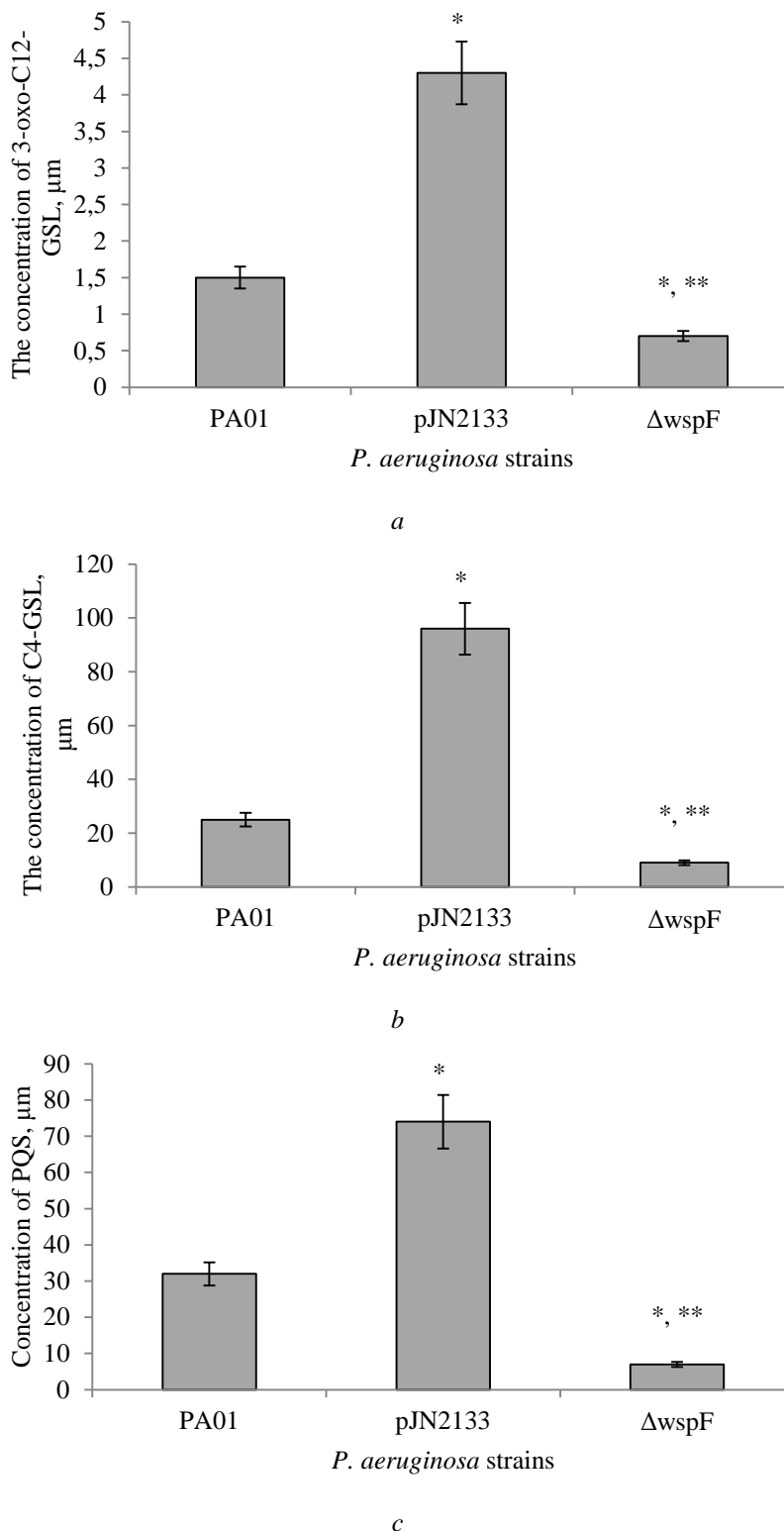


Fig. 2. The level of QS autoinducers in the supernatants of biofilm cultures of the studied strains of *P. aeruginosa*: a – the concentration of 3-oxo-C12-HSL; b – concentration of C4-HSL; c – PQS concentration;

* – the difference is significant in comparison with strain PA01 ($p \leq 0.01$);

** – the difference is significant compared to strain pJN2133 ($p \leq 0.001$)

Against the background of low levels of cyclic-di-GMP strain *P. aeruginosa* PA01 pJN2133 synthesizes in biofilm culture an increased number of major autoinducers QS compared to *P. aeruginosa* PA01: 2.9 times 3-oxo-C12-HSL, 3.8 times C4-HSL and 2.3 times PQS. Under the same conditions, the cultivation of *P. aeruginosa* PA01 $\Delta wspF$, characterized by an increased content of cyclic-di-GMP, produces significantly fewer of these signalling molecules: 2.1 times 3-oxo-C12-HSL, 2.8 times C4-HSL and 4.6 times PQS. Comparison of mutant strains indicates that *P. aeruginosa* PA01 pJN2133 in the synthesis of autoinducers QS: 3-oxo-C12-HSL, C4-HSL and PQS is dominated by *P. aeruginosa* PA01 $\Delta wspF$ in 6.1; 5.4 and 11.4 times, respectively.

6. Discussion of research results

Thus, when cultured in biofilm cultures, *P. aeruginosa* cells with low cyclic-di-GMP content produce more autoinducers of the three main links of the intercellular communication system than cells of the parent strain and, in particular, the strain with elevated levels of the secondary messenger. These results coincide with the data of other authors who, using transcriptome analysis, showed an increase in the expression of *rhl* and *pqs* operons in another mutant *P. aeruginosa* with low levels of cyclic-di-GMP [22]. Given that mutants with a low content of the secondary messenger form a biofilm with a broken architecture, it is possible to propose a strategy to combat biofilm infections aimed at reducing the level of the secondary messenger. If such substances can be found, they will become an important alternative to modern antimicrobial agents, to which bacteria in biofilms are virtually insensitive [23, 24]. In the early stages of the study of the QS in *Pseudomonas*, it was believed that the activation of its *rhl* and *pqs* systems, which is accompanied by an increase in the synthesis of the corresponding autoinducers, promotes the formation of biofilms [12, 13]. Our results do not fully agree with this opinion. It can be assumed that cyclic-di-GMP makes a more important contribution to the regulation of biofilm formation. According to a number of authors, the role of QS is to regulate the synthesis of exoDNA, which stabilizes biofilms (*rhl*), and polysaccharides of their matrix. In addition, this system is responsible for the synthesis of numerous exoproducts, in particular, rhamnolipids, which are effective biosurfactants and are involved in maintaining the architecture of biofilms [25, 26]. In general, it should be noted that the mechanisms of interaction of the two regulatory systems: cyclic-di-GMP and QS, have not been definitively resolved. The relevance of further research is due to two facts. On the one hand, some secondary metabolites of the QS system, the synthesis of

which is affected by the secondary messenger, are factors in the pathogenicity of *P. aeruginosa*. On the other hand, a number of these secondary metabolites are potentially useful biotechnological products, in particular, exoenzymes, pyocyanin, rhamnolipids. Earlier we found that the introduction of exogenous synthetic PQS in the culture of *P. aeruginosa* PA01 increases the synthesis of biosurfactants and changes the ratio of mono- and dirhamnolipids [27]. Therefore, in the future it is planned to determine the ability of the studied strains to biosynthesis of such products.

Study limitations. The limitation of the study is the use of only three strains of *P. aeruginosa*, which makes it impossible to consider the established relationships between the studied parameters inherent in all members of this species. It is necessary to expand the range of strains due to other collection, clinical and selected from natural ecological niche samples.

Prospect for further research. The study will help deepen knowledge about the relationship between the two signalling systems of bacterial cells. Determining the dependence of the biosynthesis of secondary metabolites controlled by the QS system on the content of cyclic-di-GMP will make it possible to develop ways to regulate these processes – to inhibit in case of infections and stimulate in case of biotechnologically useful products.

7. Conclusions

There is a directly proportional relationship between the intracellular content of cyclic-di-GMP and the ability to form a biofilm. The *P. aeruginosa* strain with an increased level of secondary messenger forms the strongest biofilm, the weight of which is 1.33 and 5 times greater than the wild-type strain and the strain with a low content of cyclic-di-GMP.

The concentration of QS autoinducers in the medium is inversely related to the intracellular content of cyclic-di-GMP: compared to the parent strain, it is increased in the strain with a low content of the secondary messenger and decreased in the strain with its increased level.

Conflict of interests

Authors declare no conflict of interests.

Gratitude

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