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KINETICS OF DIRHAMNOLIPIDS BIOSYNTHESIS AND RHAMNOSYLTRANSFERASE 2 ACTIVITY IN THE PRESENCE OF *PSEUDOMONAS AERUGINOSA* SIGNAL QUINOLONE

Aim: *Discovery of P. aeruginosa ATCC 15692 dirhamnolipids biosynthesis and rhamnosyltransferase 2 activity in the presence of Pseudomonas aeruginosa exogenous quorum sensing signal molecule 2-heptyl-3-hydroxy-4-quinolon (PQS).* ***Methods.*** *Pseudomonas aeruginosa ATCC 15692 were cultured in the Giss medium with 2% glucose at 37 °C for 24 h. All the discoveries were performed in «plancton-biofilm» system with using of the «Nunclon» 48-well plates. Dirhamnolipids separation conducted by TLC methods on Alugram Sil G/UV 254 TLC plates. Dirhamnolipids were eluted separately and its content was determined by the orcinol test. Rhamnosyltransferase 2 (RhlC) activity was analysed in P. aeruginosa cell extracts using a rhamnosyltransferase assay specific for the addition of L-rhamnose to monorhamnolipid. 2-heptyl-3-hydroxy-4-quinolon synthesized in ONU Biotechnological scientific-educational center.* ***Results****. The synthesis of dirhamnolipids in control culture is activated from the early stationary phase and the content of the biosurfactants is increased fivefold up 10 to 24 hour – up 0.83 to 4.3 mg/ml. Addition of increasing concentrations of PQS did not affect the growth of P. aeruginosa but increased dirhamnolipids content. After 10 h of growth, there were approximately 4.6 times more biosurfactant in the cultures supplemented with PQS compared with the control. After 24 hours its level in culture medium was 20.68 mg/ml in the presence of 80 μM PQS and 4.3 mg/ml in the absence of PQS. The additions of PQS at the time of inoculation are sufficient to induce RhlC activity during the transition to stationary phase. So, after eight hours in the presence of 40, 60 or 80 μM PQS rhamnosyltransferase 2 activity was higher at 40%, 75% and 93%, respectively. After 24 hours this enzymatic activity was 1.6, 1.8 and 2.1 times higher as compared with the control.*

Key words: Pseudomonas aeruginosa, dirhamnolids, PQS, rhamnosyltransferase 2.

Pseudomonas aeruginosa rhamnolipids have a wide spectra of biological activity, especially antimicrobial and antitumor mode of action [10, 13]. Due to its high emulsifying capacity they can be used in bioremediation of the polluted soil [8] and for oil recovery enhancement [15]. *P. aeruginosa* biosurfactants are the rhamnolipids mixture with different molecular structure that mainly consists of di- and monorhamnolipids, that have two fatty acid residues in their structure, mostly

β-hydroxydodecanoyl-β-hydroxydodecanoat. Dirhamnolipids are more soluble in water and posses the highest emulsifying and antitumor activity [9].

We have previously shown that the exogenous signal quinolon (PQS) increased rhamnolipids biosynthesis and dirhamnolipid/monorhamnolipid ratio, and hypothesized that it was able to activate rhamnosyltransferase 2 [7].

Three enzymatic reactions are required in the final steps of rhamnolipids biosynthesis in *P. aeruginosa* [12]: 1) RhlA is involved in the synthesis of the HAAs, the fatty acid dimers, from two 3-hydroxyfatty acid precursors; 2) the membrane-bound RhlB rhamnosyltransferase 1 uses dTDP-L-rhamnose and an HAA molecule as precursors, yielding monorhamnolipids; 3) these monorhamnolipids are in turn the substrates, together with dTDP-L-rhamnose, of the RhlC rhamnosyltransferase 2 to produce dirhamnolipids. Unfortunately, few works have characterized these three enzymes.

The aim of this study was discovering of *Pseudomonas aeruginosa* ATCC 15692 dirhamnolipids biosynthesis and rhamnosyltransferase 2 activity in presence of the exogenous quorum sensing signal molecule 2-heptyl-3-hydroxy-4-quinolon (PQS).

Materials and Methods

All investigations were performed in «plancton-biofilm» system with using of the «Nunclon» 48-well plates. *P. aeruginosa* ATCC 15692 overnight cultures were diluted with sterile saline buffer and were added in the plate wells containing 1 ml of Giss media to final cell concentration equal 10^3 CFU. The plates were incubated from 2 h to 24 h at 37 °C. Optical density of cultures (λ 540 nm) and dirhamnolipids content were determined each 2 hours during the day.

Rhamnolipids separation was performed with TLC method on Alugram Sil G/UV 254 TLC plates (Germany) in chloroform-methanol-water (65:12:2) mixture [14]. Rhamnolipids spots placement was determined by color reaction with rhamnose and acetic acid–sulphuric acid–anis aldehyde solution (50:1:0.05) and TLC plates were heated at 80 °C till appearance of pink-orange staining.

Dirhamnolipids were eluted with chlorophorm. The samples were centrifugated at 1500 g for 30 minutes for silica-gel removal. After centrifugation chloroform layer was taken away and evaporated. Residue was diluted at 100 μM and rhamnolipids concentration was determined by using orcinol-assay [5].

Rhamnosyltransferase 2 activity was analysed in *P. aeruginosa* cell extracts using a rhamnosyltransferase assay specific for the addition of L-rhamnose to monorhamnolipid [11]. Cells from stationary phase cultures were washed with 100 mM Tris-100 mM NaCl buffer, pH 7, and ruptured by sonication. Whole-cell extracts were incubated with 0.5 mg of dTDP-L-rhamnose and 1.5 mg of monorhamnolipid for 4 h at 37 °C. Monorhamnolipid used in the assay was purified from *P. aeruginosa* strain ATCC 9027, which lacks the ability to produce dirhamnolipid [16]. Dirhamnolipid were separated by TLC and determined using orcinol-assay [5]. One transferase unit corresponds to the incorporation of one nmol of rhamnose from TDP-rhamnose into monorhamnolipid per hour.

We have used in this work 2-heptyl-3-hydroxy-4-quinolon that was being synthesized in ONU Biotechnological scientific-educational center, TDP-rhamnose was obtained from PhD V. Osetrov. PQS was used at the concentrations of 40, 60 and 80 μM. While choosing the concentrations there were used the data about physiological concentrations of autoinducers.

All the experiments were carried out triple with 6 repeats in each case.

Data are reported as the mean ± standard deviation. Reliability of differences was determined by Student's criterion at a significance level of not less than 95% ($p \leq 0.05$). All the mathematical calculations were performed using the computer program Excel [1].

Results and Discussion

The study of kinetics of planktonic cells growth and diramnolipids biosynthesis of control cultures of *P. aeruginosa* show that dirhamnolipids appear in the culture medium in the late logarithmic growth phase – between 6 and 8 hour of cultivation (Fig. 1).

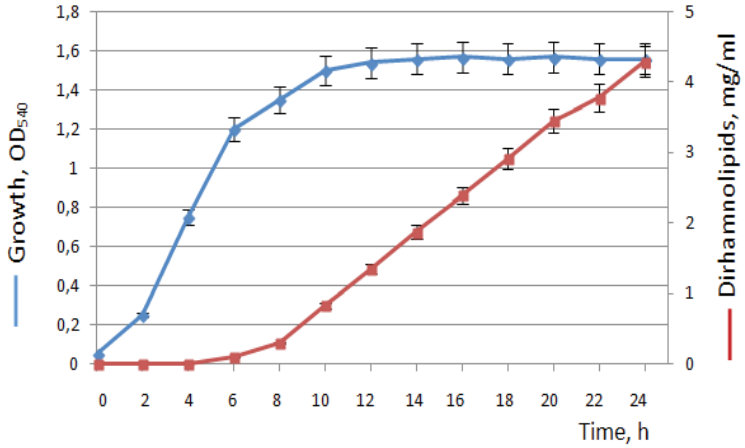

Fig. 1. Kinetics of planktonic bacteria growth and diramnolipids biosynthesis of control cultures of *P. aeruginosa*

The synthesis of dirhamnolipids is activated from the early stationary phase and the content of the biosurfactants is increased fivefold up 10 to 24 hour – up 0.83 to 4.3 mg/ml. McKnight S. et al. have demonstrated that PQS production is also initiated in early stationary phase [6] and production of rhamnolipids are reduced in PQS-deficient mutants [4]. All these data suggest an important role of *P. aeruginosa* quinolone signal in the synthesis of biosurfactants. Therefore, we studied the effect of exogenous PQS on the planktonic cells growth and diramnolipid biosynthesis.

Fig. 2 shows that addition of increasing concentrations of PQS did not affect the growth of *P. aeruginosa* PAO1. These results are consistent with data [4] showed that of exogenously added PQS at concentrations from 10 to 100 μM did not affect the growth of *P. aeruginosa* PAO1 *lecA::lux*.

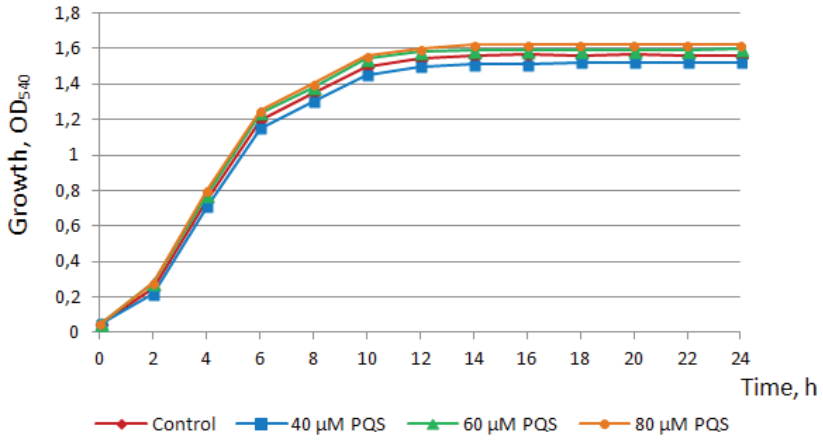

Fig. 2. Kinetics of *P. aeruginosa* planktonic bacteria growth in presence of quinolone signal molecule

Addition of increasing concentrations of PQS enhanced dirhamnolipids content in a concentration-dependent manner (Fig. 3)

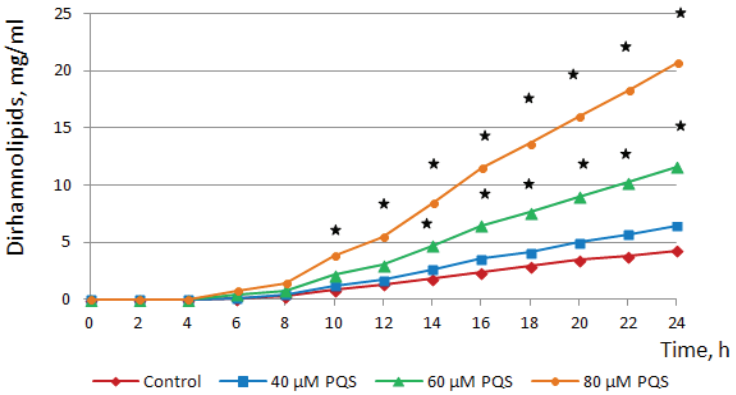

Fig. 3. Kinetics of *P. aeruginosa* diramnolipids biosynthesis in presence of quinolone signal molecule

Note: – distinctions are reliable as compared to control

The results presented in Fig. 3 show that the addition of PQS at concentration 80 μM had the greatest effect on the dirhamnolipids biosynthesis. After 10 h of growth, there is approximately 4.6 times more biosurfactant in cultures supplemented with PQS compared with the control. After 24 hours its level in the culture medium was 20.68 mg/ml in the presence of 80 μM PQS and 4.3 mg/ml in the absence of PQS.

In the presence of 60 μM PQS dirhamnolipids content was 11.6 mg/ml – 2.7 times greater than the control but less than two from the result obtained at adding of 80 μM PQS.

Further there were performed the analysis of activity of rhamnosyltransferase 2 (RhlC), which catalyses the addition of dTDP-L-rhamnose to the monorhamnolipid-accepting molecule [3]. The study was conducted via 8, 16 and 24 hours (Table).

Table

Effect of PQS on rhamnosyltransferase 2 activity in *Pseudomonas aeruginosa* (units/mg protein)

Control	8 hour	16 hour	24 hour
	4.2 ± 1.5	**7.8 ± 1.6**	**10.3 ± 2.8**
PQS 40 μM	5.9 ± 2.0	10.3 ± 2.3	16.6 ± 2.6
PQS 60 μM	7.4 ± 1.8	13.8 ± 2.7	18.7 ± 3.7*
PQS 80 μM	8.1 ± 2.6*	17.2 ± 3.5*	21.5 ± 4.1*

Note: * – distinctions are reliable as compared to control

The results indicate that the activity of RhlC increases in control cells 2.5 times during cultivation from 8 to 24 hours. This increase in activity was not associated with increased cell contents (Fig. 2) and due to enhanced expression of *rhlC* gene that encodes rhamnosyltransferase 2. The additions of PQS at the time of inoculation are sufficient to induce RhlC activity during the transition to stationary phase. So, after eight hours in the presence of 40, 60 or 80 μM PQS rhamnosyltransferase 2 activity was higher at 40%, 75% and 93%, respectively. After 24 hours this enzymatic activity was 1.6, 1.8 and 2.1 times higher as compared with the control.

As it is known *P. aeruginosa* regulates the transcription of an array of genes by quorum sensing [11]. In the case of rhamnolipids biosynthesis, the product of RhlI is the signal butanoyl-homoserine lactone, C4-HSL, which acts as the activating ligand of the transcriptional regulator RhlR. The RhlR/C4-HSL complex then binds to a specific sequence in the *rhlAB* regulatory region to activate the transcription. The level of expression of *rhlAB* is thus dependent on the local environmental concentration of this signal. The expression of the second rhamnosyltransferase, encoded by *rhlC*, is coordinately regulated with *rhlAB* by the same quorum sensing regulatory pathway. As it has been shown previously, addition of increasing concentrations of exogenous PQS enhanced C4-HSL levels (3.5 times more C4-HSL in presence of 60 mM PQS compared with the control) and the transcription of certain genes in a concentration-dependent manner [4].

Therefore, PQS controls production of rhamnolipids by stimulating the RhlR/C4-HSL quorum sensing system. Additionally, PQS acts as inducing ligands of PqsR regulator and PqsE, which upregulates *rhlAB* transcription [2].

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КІНЕТИКА БІОСИНТЕЗУ ДИРАМНОЛІПІДІВ ТА АКТИВНІСТЬ РАМНОЗІЛТРАНСФЕРАЗИ 2 ЗА ПРИСУТНОСТІ СИГНАЛЬНОГО ХІНОЛОНУ *PSEUDOMONAS AERUGINOSA*

Реферат

Мета: *дослідження біосинтезу дірамноліпідів P. aeruginosa ATCC 15692 та активності рамнозілтрансферази 2 за присутності екзогенної сигнальної молекули – 2-гептил-3-гідроксі-4-хінолону (PQS).* **Методи.** *Pseudomonas aeruginosa ATCC 15692 культивували у середовищі Гісса с 2% глюкози при 37°С 24 год. Дослідження проводили в системі планктон–біоплівка у 48-лункових планшетах «Nunclon». Виділення дірамноліпідів проводили за використання ТШХ на пластинах Alugram Sil G/UV 254. Дірамноліпіди елюювали з пластин і визначали їх кількісний вміст за допомоги орцинового тесту. Активність рамнозілтрансферази 2 (RhlC) аналізували у безклітинному екстракті за реакцією приєднання L-рамнози до монорамноліпіду. 2-гептил-3-гідроксі-4-хінолон був синтезований у Біотехнологічному науково-навчальному центрі ОНУ імені І.І. Мечникова.* **Результати.** *Синтез дірамноліпідів активується в контрольній культурі в ранню стаціонарну фазу і вміст дірамноліпідів підвищується п'ятикратно між 10 і 24 годинами – з 0,83 до 4,3 мг/мл. Внесення зростаючих концентрацій PQS не впливало на ріст P. aeruginosa, але підвищувало вміст дірамноліпідів. Через 10 годин він перевищував рівень контролю приблизно у 4,6 рази. Через 24 години вміст біосурфактанту в присутності 80 мкМ PQS становив 20,68 мг/мл проти 4,3 мг/мл за відсутності PQS. Внесення PQS одночасно з інокуляцією суттєво індукувало активність RhlC у порівнянні з контролем. Так, через вісім годин за присутності 40, 60 або 80 мкМ PQS активність рамнозілтрансферазы 2 була вище на 40%, 75% і 93%, відповідно. Через 24 години ферментативна активність перевищувала контроль у 1,6, 1,8 та 2,1 рази, відповідно.*

Ключові слова: Pseudomonas aeruginosa дірамноліпіди, PQS, рамнозілтрансфераза 2.

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КИНЕТИКА БИОСИНТЕЗА ДИРАМНОЛИПИДОВ И АКТИВНОСТЬ РАМНОЗИЛТРАНСФЕРАЗЫ 2 В ПРИСУТСТВИИ СИГНАЛЬНОГО ХИНОЛОНА *PSEUDOMONAS AERUGINOSA*

Реферат

Цель: *изучение биосинтеза дирамнолипидов P. aeruginosa ATCC 15692 и активности рамнозилтрансферазы 2 в присутствии экзогенной сигнальной молекулы –*

2-гептил-3-гидрокси-4-хинолона (PQS). **Методы.** *Pseudomonas aeruginosa ATCC 15692 культивировали в среде Гисса с 2% глюкозы при 37 °С 24 часа. Исследования проводили в системе планктон–биоплёнка в 48-луночных планшетах «Nunclon». Выделение дирамнолипидов осуществляли с помощью ТСХ на пластинах Alugram Sil G/UV 254. Дирамнолипиды элюировали с пластин и определяли их количественное содержание с помощью орцинового теста. Активность рамнозилтрансферазы 2 (RhlC) анализировали в бесклеточном экстракте по реакции присоединения L-рамнозы к монорамнолипиду. 2-гептил-3-гидрокси-4-хинолон был синтезирован в Биотехнологическом научно-учебном центре ОНУ имени И.И. Мечникова.* **Результаты.** *Синтез дирамнолипидов активируется в контрольной культуре в раннюю стационарную фазу и содержание дирамнолипидов повышается пятикратно между 10 и 24 часами – с 0,83 до 4,3 мг/мл. Внесение увеличивающихся концентраций PQS не влияло на рост P. aeruginosa, но повышало содержание дирамнолипидов. Через 10 часов оно превышало уровень контроля примерно в 4,6 раза. Через 24 часа содержание биосурфактанта в присутствии 80 мкМ PQS составляло 20,68 мг/мл против 4,3 мг/мл в отсутствии PQS. Внесение PQS одновременно с инокуляцией существенно индуцировало активность RhlC по сравнению с контролем. Так, через восемь часов в присутствии 40, 60 или 80 мкМ PQS активность рамнозилтрансферазы 2 была выше на 40%, 75% и 93%, соответственно. Через 24 часа ферментативная активность превышала контроль в 1,6, 1,8 и 2,1 раза, соответственно.*

Ключевые слова: Pseudomonas aeruginosa, дирамнолипиды, PQS, рамнозилтрансфераза 2.

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