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Muchlis Abedalabas, M.B. Galkin, T.O. Filipova

Odesa National Mechnykov University,
2, Dvoryanska str., Odesa, 65082, Ukraine, tel.: +38 (048) 765 33 61,
e-mail: tphilippova@onu.edu.ua

RHAMNOLIPIDS BIOSYNTHESIS IN *PSEUDOMONAS AERUGINOSA* ONU 301 IN THE PRESENCE OF EXOGENOUS SIGNALING QUINOLONE

Aim: *Discovery of the P. aeruginosa ONU 301* ***rhamnolipids biosynthesis and rhamnosyltransferase 2 activity*** *in presence of Pseudomonas aeruginosa exogenous quorum sensing signal molecule 2-heptyl-3-hydroxy-4-quinolon (PQS).* ***Methods.*** *Pseudomonas aeruginosa ONU 302 were cultured in the Giss medium with 2% glucose at 37 °C 24 h. All discoveries were performed in «plancton-biofilm» system with using of the «Nunclon» 48-well plates. Di- and monorhamnolipids separation conducted by TLC methods and its content was determined by 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.* ***Results****. After 16 h of growth, there is approximately 4.2 times more biosurfactant in cultures supplemented with PQS compared with the control. After 24 hours its level in culture medium was 16.0 mg/ml in the presence of 80 μM PQS and 3.7 mg/ml in the absence of PQS. The dirhamnolipids content in control culture after 8 hours was less than the monorhamnolipids. But after 24 hours its level was in 2.86 time higher. The dirhamnolipid/monorhamnolipid ratio increased 24 hours later in 1.25; 1.55 and 2.25 times in presence of 40, 60 and 80 μM signaling quinolon concentration, respectively. 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 1.5, 1.9 and 2.2 times higher as compared with the control. After 24 hours this enzymatic activity was higher at 45%, 85% and 110%, respectively.* ***Conclusion.*** *It is concluded that, Pseudomonas aeruginosa quorum sensing signal molecule 2-heptyl-3-hydroxy-4-quinolon (PQS) can be used in biothechnology to increase the yield of biosurfactants and enrich them with dirhamnolipids*

Key words: Pseudomonas aeruginosa, rhamnolipids, 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 in *P. aeruginosa* PA01, and hypothesized that it could 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 [2, 11].

The aim of this study was discovering of *Pseudomonas aeruginosa* ONU 301 rhamnolipids biosynthesis and rhamnosyltransferase 2 activity in presence of the exogenous quorum sensing signal molecule 2-heptyl-3-hydroxy-4-quinolon (PQS).

Materials and Methods

Bacterial strain *P. aeruginosa* ONU 301 used in this study was obtained from cultures collections of Department of Microbiology, Virology and Biothechnology of Odesa National Mechnykov University.

All researches were performed in «plancton-biofilm» system with using of the «Nunclon» 48-well plates. *P. aeruginosa* ONU 301 overnight cultures diluted with sterile saline buffer were added in the plate wells containing 1 ml of Giss media to final cell concentration equal 10^3 CFU. The plates were incubated at 37 °C. Optical density of cultures (λ 540 nm) and rhamnolipids content were determined after 8, 16 and 24 hours.

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 pink-orange staining appearance.

Di- and monorhamnolipids were eluted with chlorophorm. The samples were vortexed 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 were determined using orcinol-assay [4]. Dirhamnolipids/monorhamnolipids ratio was calculated taking monorhamnolipids content as 1 unit.

Rhamnosyltransferase 2 activity was analysed in *P. aeruginosa* cell extracts using 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]. Dirhamnolipids were separated by TLC and determined using orcinol-assay [4]. One transferase unit

corresponds to the incorporation of one nmol of rhamnose from TDP-rhamnose into monorhamnolipid per hour. The total protein content of the whole-cell extracts was determined by using the Lowry method [5].

We used in this work 2-heptyl-3-hydroxy-4-quinolon that was synthesizing in ONU Biotechnological scientific-educational center, TDP-rhamnose was obtained from PhD V. Osetrov. PQS was used at concentrations of 40, 60 and 80 μM. Data about physiological concentration of autoinducers were used while concentrations choosing.

All 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 mathematics calculations were performed using the computer program Excel.

Results and Discussion

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 [3]. 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 planctonic cells growth and ramnolipids biosynthesis.

The results show that addition of increasing concentrations of PQS does not affect the growth of *P. aeruginosa* ONU 301. These results coincide with data [3] showed that of exogenously added PQS at concentrations from 10 to 100 μM do not affect the growth of *P. aeruginosa* PAO1 *lecA::lux*.

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

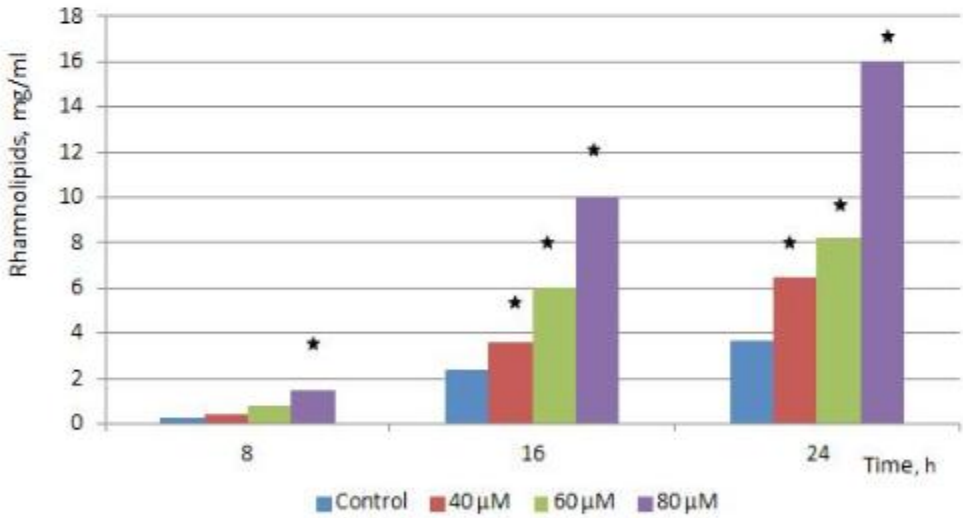

Fig. Kinetics of *P. aeruginosa* ONU 301 rhamnolipids biosynthesis in presence of quinolone signal molecule

Note: – distinctions are reliable as compared to control

The results presented in Fig. show that the addition of PQS at concentration 80 μM had the greatest effect on the rhamnolipids biosynthesis. After 16 h of growth, there is approximately 4.2 times more biosurfactant in cultures supplemented with PQS compared with the control. After 24 hours its level in culture medium was 16.0 mg/ml in the presence of 80 μM PQS and 3.7 mg/ml in the absence of PQS.

In the presence of 60 μM PQS rhamnolipids content was 8.2 mg/ml – 2.2 times greater than the control but less than 2 times from result obtained at adding of 80 μM PQS.

The exogenous signal quinolon not only increased total rhamnolipids biosynthesis, but also dirhamnolipid/monorhamnolipid ratio in *P. aeruginosa* ONU 301. The data presented in Table 1 show that dirhamnolipids fraction increases with the time of cultivation and that *P. aeruginosa* ONU 301 show greatly influences on the dirhamnolipids biosynthesis at concentration 80 μM.

Table 1

Effect of PQS on dirhamnolipids/monorhamnolipids ratio

	8 hour	16 hour	24 hour
Control	0.7 : 1	1.4 : 1	2.0 : 1
PQS 40 μM	1.4 : 1	2.0 : 1	2.5 : 1
PQS 60 μM	1.4 : 1	2.1 : 1*	3.1 : 1*
PQS 80 μM	1.7 : 1*	3.2 : 1*	4.5 : 1*

Note: Dirhamnolipids/monorhamnolipids ratio was calculated taking monorhamnolipids content as 1 unit;
* – distinctions are reliable as compared to control

The dirhamnolipids content in control culture after 8 hours was less than the monorhamnolipids. But after 24 hours its level was in 2.86 time higher. The dirhamnolipid/monorhamnolipid ratio increased 24 hours later in 1.25; 1.55 and 2.25 times in the presence of 40, 60 and 80 μM signaling quinolon concentration, respectively. Thus, the PQS increases the proportion of dirhamnolipids in the total biosurfactants mixture which is synthesized by *P. aeruginosa* ONU 301.

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 [2]. The study was conducted via 8, 16 and 24 hours of cultivation (Table 2).

The results indicate that the activity of RhlC increases in control cells 2.7 times during cultivation from 8 to 24 hours. This increase in activity is not associated with increased cell contents but 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 1.5, 1.9 and 2.2 times higher as compared with the control. After 24 hours this enzymatic activity was higher at 45%, 85% and 110%, respectively.

Table 2

Effect of PQS on rhamnosyltransferase 2 activity in *Pseudomonas aeruginosa* ONU 301 (units/mg protein)

Variant \ Time	8 hour	16 hour	24 hour
Control	2.9 ± 0.7	5.6 ± 0.9	7.8 ± 2.3
PQS 40 μM	4.3 ± 1.2	7.8 ± 1.3	11.3 ± 2.4
PQS 60 μM	5.6 ± 1.1	10.1 ± 1.8	14.5 ± 2.9*
PQS 80 μM	6.5 ± 2.0*	12.6 ± 2.2*	16.4 ± 3.1*

Note: * – distinctions are reliable as compared to control

We have previously shown that exogenous PQS has the same effect on the rhamnolipids biosynthesis of other strains of *P. aeruginosa* [7]. 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 the presence of 60 μM PQS compared with the control) and the transcription of certain genes in a concentration-dependent manner [3].

Therefore, PQS controls production of rhamnolipids by stimulating the RhlR/C4-HSL quorum sensing system. Additionally, PQS act as inducing ligands of PqsR regulator and PqsE which upregulates *rhlAB* transcription [1].

It is concluded that, *Pseudomonas aeruginosa* quorum sensing signal molecule 2-heptyl-3-hydroxy-4-quinolon (PQS) can be used in biothechnology to increase the yield of biosurfactants and enrich them with dirhamnolipids.

Мухліс Абедалабас, М.Б. Галкін, Т.О. Філіпова

Одеський національний університет імені І.І. Мечникова,
вул. Дворянська, 2, Одеса, 65082, Україна, тел.: +38 (048) 765 33 61,
e-mail: tphilippova@onu.edu.ua

БІОСИНТЕЗ РАМНОЛІПІДІВ *PSEUDOMONAS AERUGINOSA* ONU 301 ЗА ПРИСУТНОСТІ ЕКЗОГЕННОГО СИГНАЛЬНОГО ХІНОЛОНУ

Реферат

Мета: *дослідження біосинтезу рамнолiпідів P. aeruginosa ONU 301 та активності рамнозилтрансферази 2 за присутності екзогенної сигнальної молекули – 2-гептил-3-гідрокси-4-хінолону (PQS).* **Методи.** *Pseudomonas aeruginosa ONU 301 культивували у середовищі Гісса с 2% глюкози при 37 °С 24 год. Дослідження проводили в системі планктон–біоплівка у 48-лункових планшетах «Nunclon». Виділення дирамноліпідів проводили за використання ТШХ на пластинах Alugram Sil G/UV 254. Дирамноліпіди елюювали з пластин і визначали їх кількісний вміст за допомоги орцинового тесту. Активність рамнозилтрансферази 2 (RhlC) аналізували у безклітинному екстракті за реакцією приєднання L-рамнози до монорамноліпіду. 2-гептил-3-гідрокси-4-хінолон був синтезований у Біотехнологічному науково-навчальному центрі ОНУ імені І.І. Мечникова.* **Результати.** *Через 16 годин вміст рамнолiпідів перевищував рівень контролю приблизно у 4,2 рази. Через 24 години вміст біосурфактантів в присутності 80 мкМ PQS становив 16,0 мг/мл проти 3,7 мг/мл за відсутності PQS. Вміст дирамноліпідів у контрольній культурі через 8 год був меншим ніж монорамноліпідів, у той же час, через добу його рівень був більшим у 2,86 разу. Співвідношення дирамноліпід/монорамноліпід через 24 год підвищувалося у 1,25; 1,55 та 2,25 разу за присутності 40, 60 і 80 мкМ сигнального хінолону, відповідно. 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 рамнозилтрансферазы 2 зростала у 1,5; 1,9 і 2,2 рази у порівнянні з контролем. Через 24 години ферментативна активність перевищувала контроль на 45%, 85% та 110%, відповідно.* **Висновки**. *Сигнальний хінолон Pseudomonas aeruginosa (PQS) може бути використаний в біотехнології для підвищення виходу біосурфактантів та збагачення їх суміші дирамноліпідами.*

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

Мухлис Абедалабас, Н.Б. Галкин, Т.О. Филиппова

Одесский национальный университет имени И.И. Мечникова,
ул. Дворянская, 2, Одесса, 65082, Украина
e-mail: tphilippova@onu.edu.ua

БИОСИНТЕЗ РАМНОЛИПИДОВ *PSEUDOMONAS AERUGINOSA* ONU 301 В ПРИСУТСТВИИ ЭКЗОГЕННОГО СИГНАЛЬНОГО ХИНОЛОНА

Реферат

Цель: *изучение биосинтеза рамнолипидов P. aeruginosa ONU 301 и активности рамнозилтрансферазы 2 в присутствии экзогенной сигнальной молекулы – 2-гептил-3-гидрокси-4-хинолона (PQS).* **Методы.** *Pseudomonas aeruginosa ONU 301 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.** *Через 16 часов содержание рамнолипидов превышало уровень контроля примерно в 4,2 раза. Через 24 часа содержание биосурфактантов в присутствии 80 мкМ PQS составляло 16,0 мг/мл против 3,7 мг/мл в отсутствии PQS.*

Содержание дирамнолипидов в контрольной культуре через 8 часов было меньшим чем монорамнолипидов, в то же время, через сутки его уровень был выше в 2,86 раза. Соотношение дирамнолипид/монорамнолипид через 24 часа увеличивалось в ***1,25; 1,55 и 2,25 раза в присутствии 40, 60 и 80 мкМ сигнального хинолона****, соответственно. Внесение PQS одновременно с инокуляцией существенно индуцировало активность RhlC по сравнению с контролем. Так, через восемь часов в присутствии 40, 60 и 80 мкМ PQS активность рамнозилтрансферазы 2 повышалась в 1,5; 1,9 и 2,2 раза. Через 24 часа ферментативная активность превышала контроль на 45%, 85% и 110%, соответственно.* **Выводы.** *Сигнальный хинолон Pseudomonas aeruginosa (PQS) может быть использован в биотехнологии для повышения выхода биосурфактантов и обогащения их смеси дирамнолипидами.*

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

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