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INFLUENCE OF THE EXOGENOUS QUORUM SENSING AUTOINDUCERS ON *PSEUDOMONAS AERUGINOSA* RHAMNOLIPIDS BIOSYNTHESIS

The aim *of this investigation was to discover the effect of Pseudomonas aeruginosa exogenic QS autoinducers: N-(3-oxo-dodecanoyl)homoserinlacton (3-oxo-C_{12}-HSL), N-butiryl-homoserinlacton (C_4-HSL), and 2-heptyl-3-hydroxy-4-quinolon (PQS) on P. aeruginosa ATCC 15692 mono- and di-rhamnolipids biosynthesis.* ***Methods.*** *Pseudomonas aeruginosa ATCC 15692 were cultured on Giss medium with 2% glucose at 37 °C for 24 h. The investigations were performed in «plancton-biofilm» system with using of «Nunclon» 48-well plates. Di- and monorhamnolipids separation was conducted by TLC methods on Alugram Sil G /UV 254 TLC plates. Di- and monorhamnolipids were eluted separately and their content was determined by the orcinic test. Dirhamnolipids /monorhamnolipids ratio was calculated taking a monorhamnolipids content as 1 unit. There were used in this work homoserinlactones (Sigma Aldrich) and 2-heptyl-3-hydroxy-4-quinolon synthesized in ONU Biotechnological scientific-educational center.* ***Results****. It was determined that exogenous 3-oxo-C_{12}-HSL showed no effects on rhamnolipids biosynthesis. In presence of the two others autoinducers: C_4-HSL and PQS, rhamnolipids content increases. C_4-HSL at the concentrations of 5 and 10 μM caused increasing of the biosurfactants biosynthesis in 3.4 and 4.1 times. In presence of PQS in 40-80 μM concentration range a proportional increase in the synthesis was observed. Its level increased in 1.9; 3.3 and 5.2 times in presence of 40, 60 and 80 μM signaling quinolon concentration respectively. After 24 hours of incubation di- and monorhamnolipids ratio was 2,2:1. In presence of C_4-HSL it was 2:1 at the concentration of this autoinducer 5 μM and 2,4:1 at the concentration of 10 μM N-butiryl-homoserinlacton.* ***Conclusions.*** *PQS greatly increased dirhamnolipids biosynthesis, especially Rha-Rha-C_{10}-C_{10}. At PQS concentration of 40, 60 and 80 μM Rha-Rha-C_{10}-C_{10} /Rha-C_{10}-C_{10} ratio was 3:1, 3,6:1 and 4,5:1, respectively. It was shown that supernatants of bacterial cultures, contained increased amounts of dirhamnolipids, showed the highest emulsifying activity.*

K e y w o r d s: Pseudomonas aeruginosa, di- and monorhamnolids, autoinducers, quorum sensing.

Pseudomonas aeruginosa rhamnolipids have a wide spectra of biological activity, especially antimicrobial and antitumor mode of action [9, 10]. Due to their high emulsifying capacity they can be used in bioremediation of the polluted soil [7] and for oil recovery enhancement [12]. *P. aeruginosa* biosurfactants are the rhamnolipids mixture with different molecular structure that mainly consists of di- and monorhamnolipids, contained two fatty acid residues in their structure, mostly β-hydroxydodecanoyl-β-hydroxydodecanoat (C_{10}-C_{10}). Dirhamnolipids are more soluble in water and posses the highest emulsifying activity [8].

It has been previously shown that in presence of the exogenous signal quinolon (PQS) — *P. aeruginosa* quorum sensing autoinducer, rhamnolipids biosynthesis increases [2]. In this connection it is interesting to ascertain the effect of other signal molecules, especially, homoserinlactons on this process, common rhamnolipids content and dirhamnolipids /monorhalipids ratio.

The aim of this work was to evaluate the effect of *Pseudomonas aeruginosa* exogenic QS autoinducers: N-(3-oxo-dodecanoyl)homoserinlacton (3-oxo-C_{12}-HSL), N-butiryl-homoserinlacton (C_4-HSL), and 2-heptyl-3-hydroxy-4-quinolon (PQS) on *P. aeruginosa* ATCC 15692 mono- and di-rhamnolipids biosynthesis.

The investigations were performed in «plancton-biofilm» system with using of the «Nunclon» 48-well plates. *P. aeruginosa* ATCC 15692 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 for 24 h at 37 °C. Rhamnolipids extraction and detection methods have been described earlier [2].

Rhamnolipids separation was performed with TLC method on Alugram Sil G /UV 254 TLC plates (Germany) in chlorophorm-methanol-water (65:12:2) mixture [11]. Rhamnolipids spots placement was determined by color reaction with rhamnose and acetic acid— sulphuric acid—anis aldehyde solution (50:1:0.05) and color reaction with fatty acid and 10% phosphomolybdic acid—ethanol. In both cases TLC plates treated with the specific reagents were heated at 80 °C till pink-orange or blue staining appearance, in the first and second cases, respectively.

Di- and monorhamnolipids were eluted separately with chlorophorm. The samples were centrifuged at 1500 g for 30 minutes for silica-gel removal. After centrifugation a chloroform layer was taken took away and evaporated. Residue was diluted at 100 μM of methanol and rhamnolipids concentration was determined using orcinol-assay [5]. Dirhamnolipids / monorhamnolipids ratio was calculated taking a monorhamnolipids content as 1 unit.

Culture supernatants emulsifying activity was determined using method [6]. 5 ml of supernatants and vegetable oil were placed in graduated tubes

of 1 cm in diameter. The samples were shaken vigorously for 5 minutes to obtain homogeneous emulsion and incubated at room temperature for 24 hours. After incubation the height of the column of the emulsion and its density were measured.

There were used in this work homoserinlactones (Sigma Aldrich) and 2-heptyl-3-hydroxy-4-quinolon synthesized in ONU Biotechnological scientific-educational center. Homoserinlactons were used at the concentrations of 5 and 10 μM, PQS — 40, 60 and 80 μM. The data concerning a physiological concentration of autoinducers were used while concentrations choosing.

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

Statistical analysis was performed using standard methods of variational analysis. Average values ($\bar{X}$) and their standard error ($S_{\bar{X}}$) were calculated. Reliability of differences was determined by Student's criterion at a significance level of not less than 95% ($p \leqslant 0.05$). All mathematics calculations were performed using the computer program Excel [1].

Results and discussions

It was shown that rhamnolipids total content in control cultures were 3.75±0.28 μg /мl. The results showed that exogenous 3-oxo-C_{12}-HSL exhibited no effects on rhamnolipids biosynthesis (fig. 1). At the same time in presence of the two others autoinducers: C_4-HSL and PQS, rhamnolipids content increases. C_4-HSL at concentrations of 5 and 10 μM caused increase of the biosurfactants biosynthesis in 3.4 and 4.1 times.

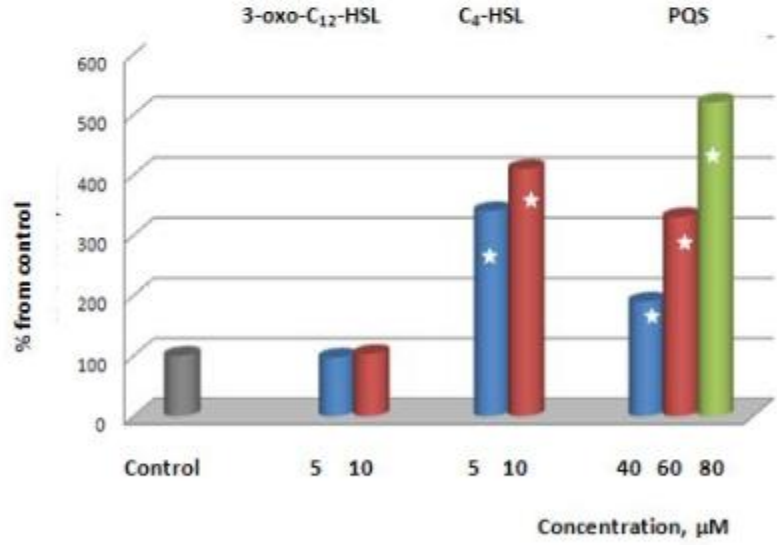

Fig. 1. Rhamnolipids biosynthesis in presence of *Pseudomonas aeruginosa* quorum sensing autoinducers

Note: — the differences were significant in comparison with control

In presence of PQS in 40-80 μM concentration range proportional increasing in the synthesis was observed. Its level increased in 1.9; 3.3 and 5.2 times in presence of 40, 60 and 80 μM signaling quinolon concentration, respectively.

In addition to assessing the total rhamnolipids content, there were discovered di- and monorhamnolipids content in test samples separately (table). The obtained results show that C_4-HSL and PQS increase Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10} level. C_4-HSL used in 5 μM concentration lead to proportional increase of the both rhamnolipids forms. In presence of 10 μM of this autoinducer dirhamnolipid concentration was in 4,6 times and monorhamnolipides in 3,7 times higher compare the control. PQS in all concentrations led to increase of dirhamnolipid level in 4,6-6,8 times. Monorhamnolipd level increased in 1.5-3 times. Presumably, PQS lead to rhamosyltransferase 2 activation, that catalyze dirhamnolipids biosynthesis from monorhamnolipids.

Based on the results shown in Table there were calculated dirhamnolipid /monoramnolipid ratio. After 24 hours of incubation this ratio was 2.2:1. In presence of C_4-HSL it was 2:1 in concentration of this autoinducer 5 μM and 2.4:1 in concentration 10 μM N-butiryl- homoserinlacton. PQS greatly increased dirhamnolipids biosynthesis, especially Rha-Rha-C_{10}-C_{10}. At PQS concentration 40, 60 and 80 μM Rha-Rha-C_{10}-C_{10} /Rha-C_{10}-C_{10} ratios were 3:1, 3.6:1 and 4.5:1, respectively.

Table

P. aeruginosa **quorum sensing autoinducers action on di- and monorhamnolipids content**

Variant	Dirhamnolipid, mg /ml	Monorhamnolipid, mg /ml
Control	1.92 ± 0.15	0.96 ± 0.11
3-oxo-C12-HSL 5 μM	1.85 ± 0.20	0.97 ± 0.14
3-oxo-C12-HSL 10 μM	1.94 ± 0,18	1.03 ± 0.09
C4-HSL 5 μM	6.98 ± 0.47*	3.49 ± 0.24*
C4-HSL 10 μM	8.93 ± 0.73*	3.72 ± 0.41*
PQS 40 μM	4.38 ± 0.36*	1.46 ± 0.15*
PQS 60 μM	7.96 ± 0.67*	2.21 ± 0.17*
PQS 80 μM	13.10 ± 0.89*	2.91 ± 025*

Note: * — distinctions are reliable as compared to control

P. aeruginosa culture supernatants emulsifying activity in presence of the various PQS concentrations are shown on Fig. 2.

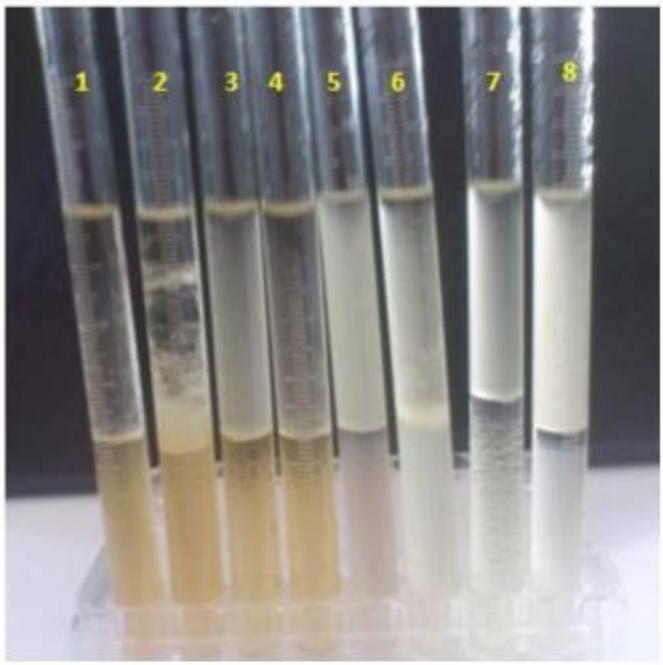

Fig. 2. Effect of PQS on the emulsifying activity of daily culture supernatant of *P. aeruginosa*

Note: 1 — medium control; 2 — supernatant of control culture; 3 — medium and Tween 20; 4 — medium and Tween 60; 5 — medium and Tween 80; 6 — culture supernatant and 40 μM PQS; 7 — culture supernatant and 60 μM PQS; 8 — culture supernatant and 80 μM PQS

After 24 hours of incubation emulsifying layer height was about a half from total oil column. Supernatants of cultures that grew in presence of PQS completely emulsified all oil in the sample. By emulsifying ability they did not concede standard emulsifiers such as Tween 20 and Tween 80. Supernatant of test-strain culture grown in presence of PQS in concentration 80 μM exceeded the variant with Tween 80 in emulsion density. It was interesting that increased content of rhamnolipids provided high emulsion stability. After 72 h emulsifying layer height in control culture decreased to 1 cm, but in the test samples emulsifying layer height was 4–4.5 cm.

Thus, there were shown in the research that two *P. aeruginosa* quorum sensing autoinducers — C_4-HSL and PQS could stimulate biosurfactants biosynthesis added in culture medium together with bacterial cells. Signaling quinolon enriches the rhamnolipids mixture with dirhamnose form. Dirhamnolipid part increasing leads to increase of the emulsifying activity.

The obtained results allow to advance the hypothesis that signal quinolon can posses activation of rhamnosyltranspherase 2 due to *rhlC*, expression enhancement. Moreover the possibility that PQS can directly activate rhamnosyltranspherase 2 is not excluded.

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ВПЛИВ ЕКЗОГЕННИХ АУТОІНДУКТОРІВ QUORUM SENSING НА СИНТЕЗ РАМНОЛІПІДІВ *PSEUDOMONAS AERUGINOSA*

Реферат

Метою даної роботи була оцінка впливу аутоіндукторів QS *Pseudomonas aeruginosa* — N-(3-оксо-додеканоїл)гомосеринлактону (3-оксо-C_{12}-АГЛ), N-бутирил-гомосеринлактону (C_4-АГЛ), и 2-гептил-3-гідрокси-4-хінолону (PQS) на синтез ди- і монорамноліпідів штамом *P. aeruginosa* ATCC 15692. **Методи.** *Pseudomonas aeruginosa* ATCC 15692 культивували на середовищі Гиса с 2% глюкози при 37 °С 24 години. Дослідження проводили в системі планктон—біоплівка в 48-лункових полістиролових плоскодонних планшетах «Nunclon». Розділення ди- і монорамноліпідів здійснювали за допомогою ТШХ на пластинках Alugram Sil G /UV 254. Ди- і монорамноліпіди роздільно елюювали з пластинок і визначали їх вміст в орциновому тесті. Співвідношення дирамноліпід / монорамноліпід розраховували, приймаючи за 1 одиницю вміст монорамноліпіда. У роботі були використані аутоіндуктори quorum sensing *P. aeruginosa*: гомосеринлактони (Sigma Aldrich) и 2-гептил-3-гідрокси-4-хінолон, синтезований у Біотехнологічному науково-навчальному центрі ОНУ імені І.І. Мечникова. **Результати.** Екзогенний 3-оксо-C_{12}-АГЛ не впливає на синтез рамноліпідів. В присутності двох інших аутоіндукторів: C_4-АГЛ и PQS, вміст рамноліпідів суттєво збільшується. C_4-АГЛ у концентраціях 5 і 10 мкМ викликав підвищення синтезу біосурфактантів *P. aeruginosa* в 3,4 і 4,1 рази, відповідно. В присутності PQS в діапазоні концентрацій 40-80 мкМ спостерігалося пропорційне підвищення синтезу рамноліпідів. Їх рівень зростав в 1,9; 3,3 і 5,2 рази при 40, 60 и 80 мкМ сигнального хінолону, відповідно. Крім оцінки загального вмісту рамноліпідів у дослідних зразках визначали співвідношення ди- и монорамноліпідів. Через добу у контролі це співвідношення становило 2,2:1. За присутності C_4-АГЛ воно майже не змінювалося і дорівнювало 2:1 при концентрації аутоіндуктора 5 мкМ та 2,4:1 при 10 мкМ N-бутирил-гомосеринлактону. Сигнальний хінолон суттєво підвищував синтез дирамноліпідів і, перш за все, Rha-Rha-C_{10}-C_{10}. За концентрацій PQS 40, 60 і 80 мкМ співвідношення Rha-Rha-C_{10}-C_{10} /Rha-C_{10}-C_{10} становило 3:1, 3,6:1 и 4,5:1, відповідно. Показано, що супернатанти культур з підвищеним вмістом дирамноліпідів мають більш високу емульгувальну активність.

Ключові слова: *Pseudomonas aeruginosa*, ді- и монорамноліпіди, аутоіндуктори *quorum sensing*.

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ВЛИЯНИЕ ЭКЗОГЕННЫХ АУТОИНДУКТОРОВ QUORUM SENSING НА СИНТЕЗ РАМНОЛИПИДОВ *PSEUDOMONAS AERUGINOSA*

Реферат

Целью данной работы была оценка влияния экзогенных аутоиндукторов QS Pseudomonas aeruginosa — N-(3-оксо-додеканоил)гомосеринлактона (3-оксо-C_{12}-АГЛ), N-бутирил-гомосеринлактона (C_4-АГЛ), и 2-гептил-3-гидрокси-4-хинолона (PQS) на синтез ди- и монорамнолипидов штаммом *P. aeruginosa* ATCC 15692. **Методы.** Pseudomonas aeruginosa ATCC 15692 культивировали на среде Гисса с 2% глюкозы при 37 °С 24 часа. Исследования проводили в системе планктон—биоплёнка в 48-луночных полистироловых плоскодонных планшетах «Nunclon». Разделение ди- и монорамнолипидов проводили с помощью ТСХ на пластинках Alugram Sil G /UV 254. Ди- и монорамнолипиды раздельно элюировали с пластинок и определяли их количественное содержание с помощью орцинового теста. Соотношение дирамнолипид /монорамнолипид рассчитывали, принимая з 1 единицу содержание монорамнолипида. В работе были использованы аутоиндукторы quorum sensing *P. aeruginosa*: гомосеринлактоны (Sigma Aldrich) и 2-гептил-3-гидрокси-4-хинолон, синтезированный в Биотехнологическом научно-учебном центре ОНУ имени И.И. Мечникова. **Результаты.** Экзогенный 3-оксо-C_{12}-АГЛ не влияет на синтез рамнолипидов. В присутствии двух других аутоиндукторов: C_4-АГЛ и PQS, содержание рамнолипидов значительно возрастает. C_4-АГЛ в концентрациях 5 и 10 мкМ вызывал повышение синтеза биосурфактантов *P. aeruginosa* в 3,4 и 4,1 раза, соответственно. В присутствии PQS в диапазоне концентраций 40—80 мкМ наблюдалось пропорциональное увеличение синтеза рамнолипидов. Их уровень возрастал в 1,9; 3,3 и 5,2 раза при 40, 60 и 80 мкМ сигнального хинолона, соответственно. Кроме оценки общего содержания рамнолипидов в исследуемых пробах определяли соотношении ди- и монорамнолипидов. Через сутки в контроле это соотношение равнялось 2,2:1. В присутствии C_4-АГЛ оно почти не менялось и составляло 2:1 при концентрации аутоиндуктора 5 мкМ и 2,4:1 в присутствии 10 мкМ N-бутирил-гомосеринлактона. Сигнальный хинолон значительно увеличивал синтез дирамнолипидов и, прежде всего, Rha-Rha-C_{10}-C_{10}. При концентрация PQS 40, 60 и 80 мкМ соотношение Rha-Rha-C_{10}-C_{10} / Rha-C_{10}-C_{10} составляло 3:1, 3,6:1 и 4,5:1, соответственно. Показано, что супернатанты культур с повышенным содержанием дирамнолипидов обладают более высокой эмульгирующей активностью.

Ключевые слова: *Pseudomonas aeruginosa*, ди- и монорамнолипиды, аутоиндукторы *quorum sensing*.

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