THE EFFECT OF PSEUDOMONAS AERUGINOSA SIGNAL QUINOLONE ON THE RHAMNOLIPIDS BIOSYNTHESIS AND RHAMNOSYLTRANSFERASE 2ACTIVITY

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Abstract

Aim: Discovery of the *P. aeruginosa* ONU 302rhamnolipids biosynthesis andrhamnosyltransferase 2 activity in presence of the *Pseudomonas aeruginosa*exogenous quorum sensing signal molecule2-heptyl-3-hydroxy-4-quinolon (PQS). **Methods.** *Pseudomonas aeruginosa* ONU 302were cultured in the Giss medium with 2% glucose at 37^{o}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 arhamnosyltransferase assayspecific for the addition of L-rhamnose to monorhamnolipid. **Results**. The synthesis of rhamnolipids in control culture is activated from the early stationary phase and the content of the biosurfactants is increasedfivefold up 10 to 24 hour – up 0.66 to 3.44 mg/ml. Addition of increasing concentrations of PQS did not affect the growth of*P.aeruginosa* but were enhanced rhamnolipids content and dirhamnolipids proportion in the biosurfactants mixture. After 24 hours total biosurfactant level in culture medium in the presence of 80 µM PQS was 3.7 times higher compared with the control. The dirhamnolipid/monorhamnolipid ratio was 2.2 times higher. The additions of PQS at the time of inoculation are sufficient to induce RhlC activity during the transition to stationary phase.So, after 24 hours in the presence of 40, 60 or 80 µM PQS rhamnosyltransferase 2 activity was higher at 40%, 70% and 120%, respectively, as compared with the control.

Keywords: Pseudomonas aeruginosa, rhamnolipids, PQS, rhamnosyltransferase 2.

Introduction

Pseudomonas aeruginosa rhamnolipids have a wide spectra of biological activity, especially antimicrobial and antitumor mode of action (Piljac and Piljac, 1995; Vatsa et al., 2010). Due to its high emulsifying capacity they can be used in bioremediation of the polluted soil (Nguyen et al., 2008) and for oil recovery enhancement (Wang et al., 2007). *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 highest emulsifying and antitumor activity (Peker et al., 2003).

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 can activaterhamnosyltransferase 2 (Mukhlis Abedalabas et al., 2013).

Three enzymatic reactions are required in the final steps of rhamnolipids biosynthesis in *P. aeruginosa* (Soberon-Chavez et al., 2005): 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 the *Pseudomonas aeruginosa*ONU 302rhamnolipids biosynthesis andrhamnosyltransferase 2 activity in presence of the exogenous quorum sensing signal molecule2-heptyl-3-hydroxy-4-quinolon (PQS).

Materials and Methods

Bacterial strain *P. aeruginosa* ONU 302 used in this study are obtained from cultures collections of Department of Microbiology, Virology and Biothechnology of OdessaNational Mechnikov University.

All researchwere performed in "plancton-biofilm" system with using of the «Nunclon» 48-well plates. *P. aeruginosa* ONU 302overnight 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.Plates were incubated from 2 h to 24 h at 37^{o}C. Optical density of cultures (λ 540 nm) and rhamnolipids content were determined each 2 hour during the day.

Rhamnolipidsseparation were performed with TLC method on Alugram Sil G/UV 254 TLC plates (Germany) in chloroform-methanol-water (65:12:2) mixture(Wadekaret al., 2012). Rhamnolipidsspotsplacementwasdeterminedbycolorreaction with rhamnoseandacetic acid–sulphuric acid–anis aldehyde solution (50:1:0.05) and TLC plates were heated at 80 ^{o}C till pink-orange staining appearance.

Di- and monorhamnolipids were eluted with chlorophorm. Samples were vortex at 1500 g for 30 minutes for silica-gel removal. After centrifugation chloroform layer were took away and evaporated. Residue was diluted at 100 μM and rhamnolipids concentration were determined using orcinol-assay (Koch et al., 1991). Dirhamnolipids/monorhamnolipids ratio was calculated taking a monorhamnolipids content as a 1 unit.

Rhamnosyltransferase 2 activity was analysed in *P.aeruginosa* cell extracts using arhamnosyltransferase assayspecific for the addition of L-rhamnose to mono-rhamnolipid (Rahim et al., 2001). Cells from stationary phase cultures were washed with 100 mM Tris-100 mM NaClbuffer, pH 7, and ruptured by sonication. Whole-cell extractswere incubated with 0.5 mg of dTDP-L-rhamnose and1.5 mg of monorhamnolipid for 4 h at 37 ^{o}C.Monorhamnolipid used in the assay was purified from *P.aeruginosa* strain ATCC9027, which lacks the ability toproduce dirhamnolipid (Wild et al., 1997). Dirhamnolipid were separated by TLC and determined using orcinol-assay (Koch et al., 1991). 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 extractswas determined by using the Lowry method (Lowry et al., 1951).

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 a concentrations of 40, 60 and 80 μM.Data about a 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

The study of kinetics of planktonic cells growth and ramnolipids biosynthesis of control cultures of *P.aeruginosa*ONU 302show that rhamnolipids appears in the culture medium in the late logarithmic growth phase – between 6 and 8 hour of cultivation (Figure 1).

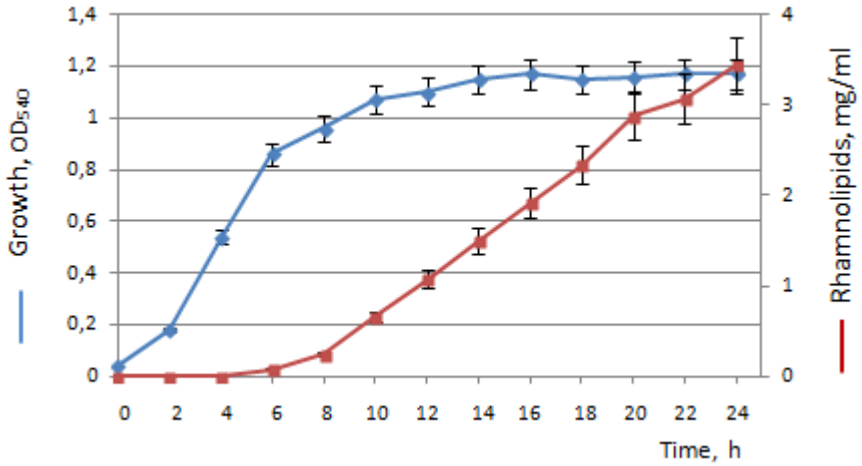

Figure 1. Kinetics of planktonic bacteria growth and rhamnolipids biosynthesis of control cultures of *P.aeruginosa* ONU 302

The synthesis of rhamnolipids is activated from the early stationary phase and the content of the biosurfactants is increasedfivefold up 10 to 24 hour – up 0.66 to 3.44 mg/ml. McKnight S. et al. have demonstrated that PQS production is also initiated in early stationary phase (McKnight et al., 2000) and production of rhamnolipids are reduced in PQS-deficient mutants (Diggle et al., 2003).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.

Figure 2 shows that addition of increasing concentrations of PQS did not affect the growth of *P.aeruginosa* ONU 302. These results are consistent with data (Diggle et al., 2003) which showed thatof exogenously added PQS at concentrations from 10 to 100 µM did not affect the growth of *P. aeruginosa* PAO1 *lecA::lux*.

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

The results presented in Fig. 3 show that the addition of PQS at concentration 80 µM had the greatest effect on the rhamnolipids biosynthesis. After 10 h of growth, there is approximately 4.5 times more biosurfactant in cultures supplemented with PQS compared with the control.After 24 hours its level in culture medium was 12.7 mg/ml in the presence of 80 µM PQS and 3.44 mg/ml in the absence of PQS.

In the presence of 60 µM PQS rhamnolipids content was 8.9 mg/ml – 2.6 times greater than the control but less than 1.4 times from result obtained at adding of 80 µM PQS.

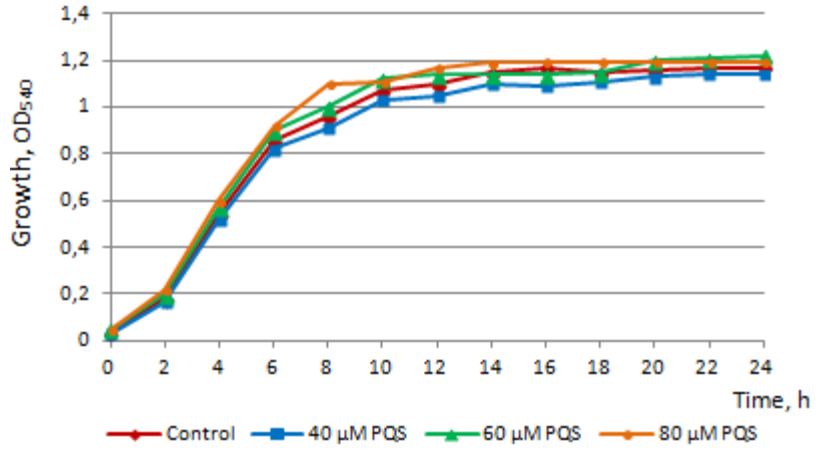

Figure 2. Kinetics of *P.aeruginosa* planktonic bacteria growth in presence of quinolone signal molecule

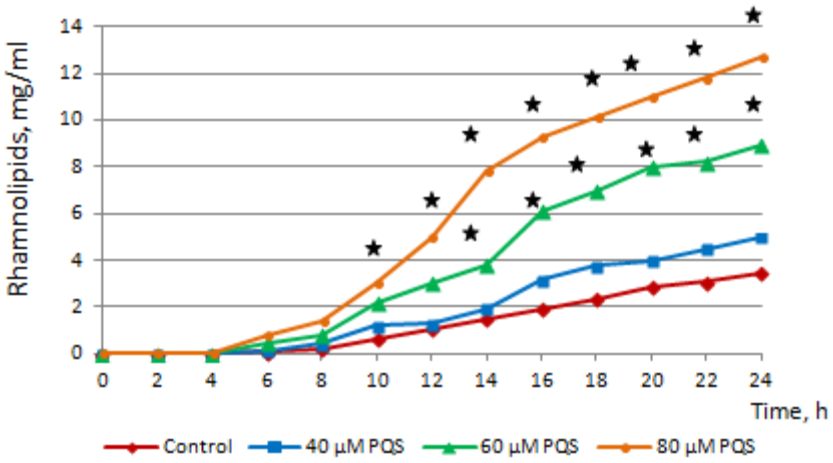

Figure 3. Kinetics of P.aeruginosa ONU 302 rhamnolipids biosynthesis in presence of quinolone signal molecule

★ Note: – distinctions are reliable as compared to control

The exogenous signal quinolon not only increased total rhamnolipids biosynthesis, but also dirhamnolipid/monorhamnolipid ratio in *P. aeruginosa* ONU 302. The data presented in table 1 show that dirhamnolipids fraction increases with the time of cultivation and that the PQS show greatlyinfluences on the dirhamnolipids biosynthesis at concentration 80 µM.

Table 1. Effect of PQS on dirhamnolipids/monorhamnolipids ratio

	8 hour	16 hour	24 hour
Control	0.8 : 1	1.2 : 1	1.9 : 1
PQS 40 µM	1.3 : 1	2.0 : 1	2.7 : 1
PQS 60 µM	1.5 : 1	2.2 : 1*	3.3 : 1*
PQS 80 µM	1.6 : 1*	2.7 : 1*	4.2 : 1*

Note: Dirhamnolipids/monorhamnolipids ratio was calculated taking a monorhamnolipids content as a 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 2.4 time higher. The dirhamnolipid/monorhamnolipid ratio increased 24hours later in 1.4; 1.7 and 2.2 times in 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 302.

Further analysis was performed activity of rhamnosyltransferase 2 (RhlC), which catalyses the addition of dTDP-L-rhamnose to the monorhamnolipid-accepting molecule (Burger et al., 1963).The study was conducted via 8, 16 and 24 hours (Table 2).

Table 2. Effect of PQS on rhamnosyltransferase 2 activity in *Pseudomonas aeruginosa* ONU 302 (units/mg protein)

	8 hour	16 hour	24 hour
Control	3.6 ± 1.5	6.6 ± 1.6	8.7 ± 2.8
PQS 40 μM	5.0 ± 2.0	8.8 ± 2.3	14.1 ± 2.6
PQS 60 μM	6.3 ± 1.8	11.7 ± 2.7	15.9 ± 3.7*
PQS 80 μM	6.9 ± 2.6*	14.6 ± 3.5*	18.3 ± 4.1*

Note: * – distinctions are reliable as compared to control

The results indicate that the activity of RhlC increases in control cells 2.4 times during cultivation from 8 to 24 hours.This increase in activity was not associated with increased cell contents (Figure 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 92%, respectively. After 24 hours this enzymatic activity was 1.6, 1.8 and 2.1 times higher as compared with the control.

As is known *P. aeruginosa* regulates the transcription of an array of genes by quorum sensing(Rahim et al., 2001). 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 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 (Diggle et al., 2003).

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 PqsEwhich upregulates *rhlAB* transcription (Abdel-Mawgoud et al., 2011).

Conclusion

It is concluded that, *Pseudomonas aeruginosa*quorum sensing signal molecule2-heptyl-3-hydroxy-4-quinolon (PQS)can be usedin biothechnology to increase the yield of biosurfactantsand enrichthem with dirhamnolipids

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