Polish Journal of Microbiology
2015, Vol. 64, No 2, 101–106

ORIGINAL PAPER

Characteristics of the *Pseudomonas aeruginosa* PA01 Intercellular Signaling Pathway (Quorum Sensing) Functioning in Presence of Porphyrins Bismuth Complexes

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Submitted 10 December 2012, revised 19 March 2014, accepted 19 March 2015

Abstract

The influence of synthetic and natural porphyrins bismuth complexes on *P. aeruginosa* quorum sensing system was carried out by detection of the pyocyanin, rhamnolipids and autoinducers biosynthesis level. The highest ability to reduce pyocyanin biosynthesis showed Bi(III)-TPP. Rhamnolipids production level also decreased in the presence of studied compounds. This effect was the most expressed in presence of 40 and 80 μM of the synthetic meso-substituted porphyrins. Autoinducers biosynthesis, especially 3-oxo-C_{12}-HSL was suppressed in presence of the bismuth complexes. That suggest that the mechanisms of action of this substances is an inhibition of signaling molecules or/and receptor for them.

Key words: *Pseudomonas aeruginosa* PA01, porphyrins bismuth complexes, *quorum sensing*

Introduction

Today in connection with the high resistance of opportunistic pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *etc.* to traditional antimicrobial drugs, infections that are caused by these bacteria gain high prevalence, especially in patients with various immune deficiencies. Thus, one of the future tasks of modern pharmacology is the search for new antibacterial drugs, which may have inhibitory activity against pathogenic bacteria, especially with non-traditional mechanisms of action. One of the most promising groups of new antimicrobial agents may be compounds that break down bacterial cell-cell signaling pathways (Kociolek, 2009).

Intercellular signaling pathway, known as quorum sensing is a global regulatory mechanism based on the use of small signaling molecules that play a role in gene expression in a bacterial cell population (Bassler, 2002; Brown *et al*., 2001). This mechanism is the basis of many bacterial cell properties such as pathogenicity, and biosynthesis of secondary metabolites. Consequently, studies focused on the regulation of this system, seem to be of promise in biotechnology and medicine.

A quorum sensing system from *P. aeruginosa* (formally an autoinduction system) is based on three families of genes – *las*-, *rhl*- and *pqs*-. Each of these families activates with its own signal molecules: 3-oxo-dodecanoil-homoserine lacton (for *las*- family), butiryl homoserine lacton (for *rhl*- family) and 2-heptyl-3-hydroxy-4-quinolon (for *pqs*- family) (McKnight *et al.*, 2000). *P. aeruginosa* quorum sensing system works based on the binding of signal molecules with specific cytoplasm receptors and "signaling molecules-receptor" complexes formation. These complexes activate the expression of target genes (Winzer and Williams, 2001).

Previously, we demonstrated that synthetic porphyrins and their complexes with metals can possess antimicrobial activity; in particular inhibit bacterial biofilm formation (Galkin *et al.*, 2010). In this study we investigated *P. aeruginosa* PA01 quorum sensing system functions in the presence of synthetic and natural porphyrins bismuth complexes.

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Experimental

Material and Methods

Bacterial strains and growth conditions. *P. aeruginosa* PA01 were obtained from the collection of the microbiology, virology and biotechnology department of Odessa National University named after I.I. Mechnikov.

Bacterial strains were grown on the meat-peptone agar (MPA) and Gis media. For pyocyanin detection, bacterial were strains grown on the PB broth (g/l, peptone – 20; $MgCl_2$ – 1.4; K_2SO_4 – 10).

Chemicals. Synthetic and natural porphyrins bismuth complexes – *meso*-tetra(4-N-methyl-piridyl)porphyrin bismuth complex (Bi(III)-TPP), *meso*-tetra (6-N-methyl-quinolinil)porphyrin bismuth complex (Bi(III)-TQP) and protoporphyrine IX bismuth complex (Bi(III)-PP IX) were synthesized by method (Ishkov *et al.*, 2000) in PLMS-5 of Odessa National University named after I.I. Mechnikov (Fig. 1).

3-oxo-dodecanoyl-homoserine lactone (3-oxo-C_{12}-HSL) and butiryl homoserine lactone (C_4-HSL) standards were obtained from Sigma Aldrich.

2-heptyl-3-hydroxy-4-quinolon (PQS) was synthesized by the method of Somanathan and Smith (1981) in PLMS-5 of Odessa National University named after I.I. Mechnikov.

Cells pre-incubation with discovered compounds. To study the production of pyocyanin and ramnolipids bacterial (2×10^8 CFU/ml) cells were pre-incubated with the test substances in concentrations 0.4; 40 and 80 μM in saline buffer for 1.5 h at 37°C.

Pyocyanin production study. After incubation with porphypins bismuth complexes bacterial cells were washed three times and inoculated to 5 ml of PB broth. Bacterial cells in PB broth were incubated overnight at 37°C. After incubation bacterial cells were removed by centrifugation at 6000 × g for 10 minutes. Pyocyanin from supernatant were extracted and measured by the methods of Essar *et al.* (1990). A 5 ml of culture supernatant were extracted with 3 ml of chloroform. Chloroform layer were transferred to a fresh tubes and re-extracted with 1 ml of 0.2 N HCl. After centrifugation, the top layer was collected and its absorption at 520 nm was measured on μQuant (Bio-Rad) spectrophotometer.

Rhamnolipids production study. For rhamnolipids production study bacterial cells were inoculated to 10 ml of Gis media and were incubated overnight at 37°C. After incubation bacterial cells were removed by centrifugation at 6000 × g for 10 minutes and supernatant were concentrated as follows. The pH of 10 ml of the culture supernatant was adjusted to 6.5, and $ZnCl_2$ was added to a final concentration of 75 mM (Guerra-Santos *et al.*, 1984). The precipitated material was dissolved in 10 ml of 0.1 M sodium phosphate buffer (pH 6.5) and extracted twice with an equal volume of diethyl ether. The pooled organic phases were evaporated to dryness, and the pellets were dissolved in 500 μl of methanol.

The total amount of rhamnolipids was determined using the orcinol assay (Candrasekaran and Bemiller, 1980): 500 μl of the rhamnolipids samples were mixed with 500 μl of an orcinol reagent (0.2 g orcinol, 0.1 g $FeCl_3$ in 100 ml of the 30% HCl). After heating for 20 minutes at 100°C, the samples were cooled for 15 min at room temperature and the OD_{670} was measured on μQuant (Bio-Rad) spectrophotometer.

Autoinducers production study. Level of homoserine lactones synthesis was measured by gas chromatography/mass spectrometry method (Pearson *et al.*, 1995).

Homoserine lactones were extracted from a culture supernatant by ethyl acetate. Organic phase were collected and evaporated to dryness. The pellets were diluted in methanol and purified by HPLC on the C_{18} reverse phases columns in methanol-water gradient.

Gas chromatography/mass spectra were carried out on Hewlett-Packard 5890 with Hewlett-Packard Ultra-1

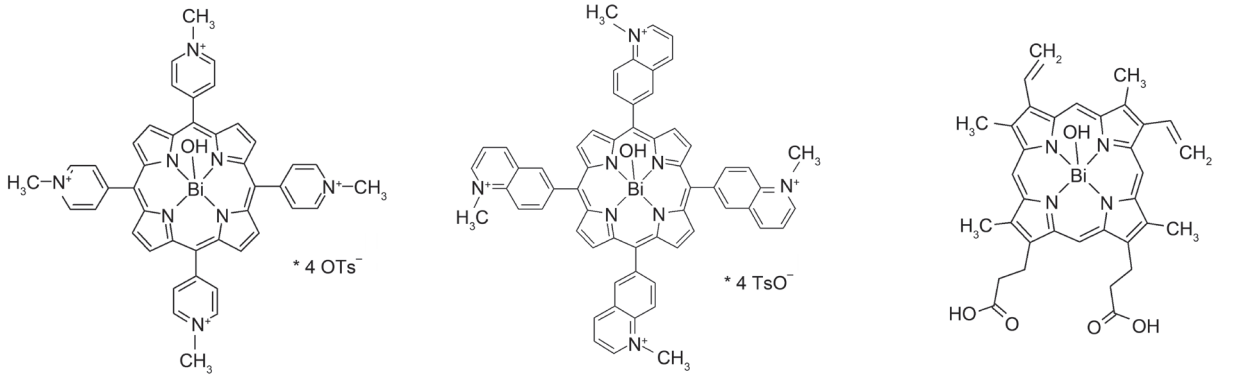

Fig. 1. Porphyrins bismuth complexes, used in the study

capillary column (25 m × 0.2 mm with film thickness of 0.33 μm). Helium as a carrier gas was used. Temperature gradient was at 70 to 240°C with increment by 10°C per minute. Mass spectra were collected by ZAB-HF mass spectrometer (VG Analytical, Manchester, UK) with homoserine lactones standards.

PQS level from culture supernatant was determined by the method of Deziel *et al*. (2004). Ethyl acetate extracts were separated by TLC in dichlormethane-acetonitryl-dioxane mixture (17:2:1) with PQS standards. PQS dotes placement was identified by UV. PQS dots were eluted from TLC plates (ALUGRAM® SIL G/UV_{254}) with ethyl acetate and luminescence of elutes were measured with LUMISTAT at 312 nm.

All experiments were carried out three times.

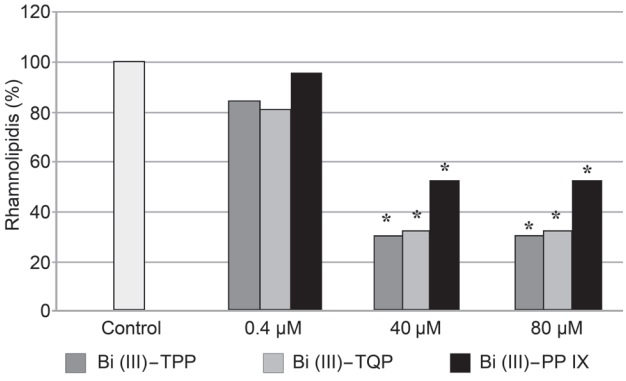

Fig. 2. Rhamnolipids biosynthesis by *P. aeruginos*a PA01 after pre-incubation with porphyrins bismuth complexes
Note: * – significantly different from the control

Results

The influence of synthetic and natural porphyrins bismuth complexes on *P. aeruginosa* quorum sensing system was carried out by detection of the pyocyanin, rhamnolipids and autoinducers biosynthesis level. For these studies bacterial cells were pre-incubated with several concentrations of the synthetic and natural porphyrins bismuth complexes (0.4; 40 and 80 μM). This was done to neutralize the inhibitory activity of used concentrations, which has been shown previously (Galkin *et al.*, 2010).

The study of the biosynthesis of pyocyanin showed that level of this pigment in supernatant of the *P. aeruginosa* PA01 overnight culture decreased in the presence of all concentrations of the compounds studied (Table I).

Determination of the basic pigment level in culture supernatant showed that *P. aeruginosa* PA01 synthesize 6.31 μg per ml of pyocyanin after overnight incubation. After treatment with a 0.4 μM of each compounds the pyocyanin level decreased by a 10%. When higher concentrations were used, the difference in the activity of the studied compounds been observed. After pre-treatment with 40 μM of the Bi(III)-TPP, pyocyanin level decreased by a 38%; Bi(III)-TQP – 30% and Bi(III)-PP IX – 18%. Maximal anti-pyocyanin activity was observed after pre-treatment of *P. aeruginosa* PA01 with an 80 μM of studied compounds. The inhibition of pyocyanin biosynthesis was in case of Bi(III)-TPP for two times, and Bi(III)-TQP and Bi(III)-PP IX – 32% and 25% respectively.

Rhamnolipids production after pre-treatment with synthetic and natural porphyrins bismuth complexes also decreased (Fig. 2). The highest ability to inhibit the synthesis of these metabolites showed Bi(III)-TPP and Bi(III)-TQP. After pre-treatment of the *P. aeruginosa* PA01 cells with 0.4 μM of each compounds the rhamnolipids level in culture supernatant were the same and 80% of the control value. When 40 μM of these compounds were used, rhamnolipids level in the culture supernatant was 32% of the control value, and after pre-treatment with 80 μM – 20 and 23%, respectively. Lowest inhibitory capacity on the rhamnolipids biosynthesis showed Bi(III)-PP IX. Rhamnolipids value after pre-treatment with 0.4; 40 and 80 μM of this compound in overnight culture supernatant was 95, 42 and 52% of the control value, respectively.

The study of the *P. aeruginosa* PA01 quorum sensing autoinducers biosynthesis after pre-treatment with synthetic and natural porphyrins bismuth complexes was conducted in a three time points – after 3, 6 and 24 hours of incubation. Obtained results showed (Table II–IV) that in the control there was a difference in appearance of autoinducers within the investigated time intervals. First the autoinducer, which appeared in the culture medium after three hours of incubation, was 3-oxo-dodecanoyl-homoserine lactone. Butiryl

Table I
Pseudomonas aeruginosa PA01 piocyanin biosynthesis level in presence of the synthetic and natural porphyrins bismuth complexes, μg/ml

Compound	Control	Porphyrins bismuth complexes concentration		
		0.4 μM	40 μM	80 μM
Bi(III)-TPP	6.31 ± 0.42	5.50 ± 0.35	3.86 ± 0.37*	2.91 ± 0.25*
Bi(III)-TQP	6.31 ± 0.42	5.62 ± 0.40	4.36 ± 0.38	4.11 ± 0.28*
Bi(III)-PP IX	6.31 ± 0.42	5.74 ± 0.51	5.17 ± 0.43	4.46 ± 0.37*

homoserine lactone appeared later and reached its maximum concentration after 6 hours of incubation. At time equal to 24 hours from the start of incubation, the levels of homoserine lactones decreased. PQS was detected first time at time point equal 6 hours of incubation and reached its maximum concentration after 24 hours.

Received data showed that after pre-treatment of *P. aeruginosa* PA01 cells with studied substances, autoinducers level in culture supernatant decreased (Tables II–IV). Autoinducers biosynthesis was more sensitive to Bi(III)-TPP. Lowest ability to inhibit an autoinducers biosynthesis showed Bi(III)-PP IX. In the case of Bi(III)-TQP, it was shown that its effects were smaller that the same effects of Bi(III)-TPP, but they were still higher than Bi(III)-PP IX. It was shown that the synthetic porphyrins bismuth complexes posses a higher activity to 3-oxo-C_{12}-HSL and PQS biosynthesis than to C_4-HSL one. In contrast, Bi(III)-PP IX showed the same effect on the biosynthesis of all studied autoinducer.

The results showed that the inhibitory effect of porphyrins bismuth complexes on the biosynthesis of autoinducer was dependent on the concentration of porphyrin. After 6 hours of incubation, *P. aeruginosa* PA01 in culture supernatant that were pre-treated with 0.4 and 40 μM of Bi(III)-TPP concentration of the 3-oxo-C_{12}-HSL was in 2.8 and 4.1 times lower than in control respectively, and after pre-treatment with 80 μM, concentration of this autoinducer in culture supernatant were practically no determinable. After pre-incubation

Table II
Autoinducers biosynthesis of *P. aeruginosa* PA01 after pre-incubation with Bi(III)-TPP

Autoinducer	Bi(III)-TPP concentration, μM	Autoinducers concentration, μM		
		3 hours	6 hours	24 hours
3-oxo-C_{12}-HSL	0	0.65 ± 0.07	1.87 ± 0.23	1.32 ± 0.11
	0.4	traces	0.66 ± 0.14*	0.40 ± 0.15*
	40	0	0.46 ± 0.17*	Traces
	80	0	Traces	0
C_4-HSL	0	traces	12.63 ± 1.07	2.44 ± 0.20
	0.4	traces	7.09 ± 0.87*	1.15 ± 0.18*
	40	0	5.51 ± 1.08*	0.94 ± 0.23*
	80	0	3.76 ± 1.10*	0.73 ± 0.20*
PQS	0	0	2.17 ± 0.16	66.48 ± 4.75
	0.4	0	0.93 ± 0.18*	37.85 ± 4.07*
	40	0	traces	26.74 ± 5.63*
	80	0	traces	15.27 ± 3.81*

Note: * – significant different from control

Table III
Autoinducers biosynthesis of *P. aeruginosa* PA01 after pre-incubation with Bi(III)-TQP

Autoinducer	Bi(III)-TQP concentration, μM	Autoinducers concentration, μM		
		3 hours	6 hours	24 hours
3-oxo-C_{12}-HSL	0	0.65 ± 0.07	1.87 ± 0.23	1.32 ± 0.11
	0.4	0.44 ± 0.13*	1.24 ± 0.23*	1.06 ± 0.14*
	40	0.31 ± 0.12*	0.82 ± 0.12*	0.53 ± 0.10*
	80	0	0.49 ± 0.13*	Traces
C_4-HSL	0	traces	12.63 ± 1.07	2.44 ± 0.20
	0.4	traces	9.33 ± 1.02*	2.15 ± 0.18
	40	traces	6.89 ± 0.76*	1.36 ± 014*
	80	0	4.30 ± 0.50*	1.07 ± 0.09*
PQS	0	0	2.17 ± 0.16	66.48 ± 4.75
	0.4	0	1.35 ± 0.13*	48.67 ± 5.27
	40	0	0.98 ± 0.10*	33.17 ± 4.56*
	80	0	0.71 ± 0.07*	21.83 ± 3.48*

Note: * – significant different from control

Table IV
Autoinducers biosynthesis of *P. aeruginosa* PA01 after pre-incubation with Bi(III)-PP IX

Autoinducer	Bi(III)-PP IX concentration, μM	Autoinducers concentration, μM		
		3 hours	6 hours	24 hours
3-oxo-C_{12}-HSL	0	0.65 ± 0.07	1.87 ± 0.23	1.32 ± 0.11
	0.4	0.61 ± 0.07	1.62 ± 0.21	1.15 ± 0.12
	40	0.53 ± 0.08	1.28 ± 0.14	0.94 ± 0.08
	80	0.40 ± 0.06*	1.23 ± 0.13*	0.90 ± 0.11
C_4-HSL	0	traces	12.63 ± 1.07	2.44 ± 0.20
	0.4	traces	10.32 ± 1.11	2.24 ± 0.25
	40	traces	9.04 ± 1.02*	1.67 ± 0.17
	80	traces	7.85 ± 1.15*	1.36 ± 0.14*
PQS	0	0	2.17 ± 0.16	66.48 ± 4.75
	0.4	0	1.88 ± 0.19	55.18 ± 6.04
	40	0	1.60 ± 0.14*	49.05 ± 5.20
	80	0	1.17 ± 0.09*	40.47 ± 4.33*

Note: * – significant different from control

Table V
Autoinducers biosynthesis of *P. aeruginosa* PA01 after pre-incubation with Bi(III)-PP IX

Autoinducer	Bi(III)-PP IX concentration, μM	Autoinducers concentration, μM		
		3 hours	6 hours	24 hours
3-oxo-C_{12}-HSL	0.4	0.61 ± 0.07	1.62 ± 0.21	1.15 ± 0.12
	40	0.53 ± 0.08	1.28 ± 0.14	0.94 ± 0.08
	80	0.40 ± 0.06*	1.23 ± 0.13*	0.90 ± 0.11
C_4-HSL	0.4	traces	10.32 ± 1.11	2.24 ± 0.25
	40	traces	9.04 ± 1.02*	1.67 ± 0.17
	80	traces	7.85 ± 1.15*	1.36 ± 0.14*
PQS	0.4	0	1.88 ± 0.19	55.18 ± 6.04
	40	0	1.60 ± 0.14*	49.05 ± 5.20
	80	0	1.17 ± 0.09*	40.47 ± 4.33*

with 0.4 and 40 μM Bi(III)-TQP, 3-oxo-C_{12}-HSL a first-detected after 3 hours of incubation, but its concentration were lower than in control in 1.5 and 3.1 times, respectively.

C_4-HSL was not detected up to 6 hours of incubation in all cases (with and without porphyrins pre-treatment). After 6 hours of incubation, concentration of this autoinducer were lower after Bi(III)-TPP and Bi(III)-TQP pre-treatment than in control in 1.8–3.4 and 1.35–2.9 times respectively. After 24 hours of incubation, C_4-HSL concentration was lower in 2–3.3 and 1.1–2.3 times respectively, compared the control.

PQS biosynthesis was completely suppressed during the first 6 hours of incubation after pre-treatment with 40 and 80 μM of Bi(III)-TPP. After pre-treatment with Bi(III)-TQP, PQS were detected in all cases, but its concentration was in 1.6–3.1 times lower than in the control respectively. After 24 hours of incubation PQS concentration in the pre-treated culture were in 1.8, 2.6 and 4.4 times lower respectively in the case of Bi(III)-TPP, and 1.4, 2 and 3 times when Bi(III)-TQP were used.

Bi(III)-PP IX showed no significant effects on the biosynthesis of autoinducers. The highest level of autoinducer biosynthesis inhibition was detected after pre-treatment with 80 μM of this complex – 32–46%.

Discussion

The fact that bacterial quorum sensing system I underlies bacterial pathogenicity, makes it a promising target for novel antimicrobial drugs. Quorum sensing in *P. aeruginosa* controls the production of many virulence factors such as pyocyanin, rhamnolipids, HCN, toxin A, *etc*. Signaling molecules can also act as pathogenicity factors. It was shown that acyl-homoserin

lactones can modulate immune response, induce the death of immune cells, and affect the level of proinflammatory cytokines synthesis (Shiner *et al.*, 2005). Our study showed that synthetic and natural porphyrins bismuth complexes that were studied, could be effective inhibitors of *P. aeruginosa* quorum sensing system. Mechanisms of anti-quorum sensing action of porphyrines bismuth complexes can be linked to its ability block the synthesis of signal molecules. On the other hand, our previous results (Galkin and Ivanitsya, 2011) show that exogenous quorum sensing autoinducers can modify the anti-quorum sensing activity of these compounds. These data suggest that in some cases porphyrins bismuth complexes possibly can compete with autoinducers for binding to their receptors. The discovered ability to inhibit autoinducers biosynthesis and, as a consequence, block pathogenic factors expression (such as pyocyanin and rhamnolipids) and biofilm formation (Galkin *et al.*, 2010) make synthetic and natural porphyrins bismuth complexes very promising for future studies as a new class of antimicrobial drugs.

Acknowledgments

This work was supported by research grants from Ministry of Education and Science of Ukraine.

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