GENES ENCODING SYNTHESIS OF PHENAZINE-1-CARBOXYLIC ACID IN *Pseudomonas batumici*

V. V. Klochko[1]   [1]Zabolotny Institute of Microbiology and Virology
S. D. Zagorodnya[1]   of the National Academy of Sciences of Ukraine, Kyiv
O. N. Reva[2]   [2]Centre for Bioinformatics and Computational Biology,
                 University of Pretoria, South Africa

E-mail: vvklochko@ukr.net

    The aim of this research was to elucidate the role of fenesin-1-carboxylic acid of *Pseudomonas batumici* and diversity of the genes encoding its synthesis in bacteria of the genus *Pseudomonas*. Phenazine-1-carboxylic acid in the concentration of 10 µg/ml stimulated the biofilm formation by batumin-producing strain. The presence of the corresponding gene in the genome of *P. batumici* was not successfully confirmed by PCR amplification with a set of primers designed for *Pseudomonas*. The complete genome sequencing of *P. batumici* has revealed a homologous gene that could encode synthesis of this compound. Comparative study of sequenced *Pseudomonas* genomes showed presence of at least two genetically diverse groups of phenazine coding orthologous genes. These genes could have distributed among rhizobacteria by the horizontal gene transfer.

    The ability to synthesize the heterocyclic phenazine acids is a distinguishing feature of the metabolism of several species of the genus *Pseudomonas*. Data obtained in the recent years show that the importance of the phenazine for the producers is not limited by its antimicrobial activity. It acts also as an important regulator of gene expression and biofilm formation, participates in the redox reaction and induction of the systemic resistance of plants against pathogens and excites other important effects [1, 2]. Such versatility of the activities of these pigments in bacteria is consistent with an evolutionary hypothesis that the biosynthesis of energetically expensive metabolites in microbial cells is justified only if they are multifunctional [3].

    Pseudomonas batumici, the producer of a medically important polyketide antibiotic batumin, synthesizes also the phenazine-1-carboxylic acid coloring the colonies and endowing the bacteria with a broad antibiotic activity,. Particularly, this compound inhibited the growth of phytopathogenic fungi, agrobacteria, corynebacteria and phytopathogenic *Pseudomonas* at concentrations of 50−400 µg/ml indicating its contribution to the competitiveness of *P. batumici* in the rhizosphere. The ability to synthesize phenazine-1-carboxylic acid (P1CA) is common for several *Pseudomonas* species. Synthesis of this bright yellow pigment was reported for *P. aeruginosa*, *P. putida*, *P. fluorescens* and *P. chlororaphis*. Genetic control of the biosynthesis of P1CA in aforementioned species has been studied quite well. Nucleotide sequences of a fragment of *phzABCDEFG*, *phzI* and *phzR* operons have been determined. A schematic diagram of the biosynthetic pathway of synthesis of this antibiotic was suggested [4].

    Synthesis of P1CA was detected in the strains of *P. batumici,* which produced the polyketide antistaphylococcal antibiotic batumin [5]. Chloroform extracts from the culture liquid comprised the batumin and, depending on the composition of the medium, varying amounts of the phenazine pigment (from trace concentrations up to 250 mg/l).

Genetic encoding of phenazine biosynthesis by *P. batumici* had not been studied previously. The role of this pigment in the biology of *P. batumici* also remained unknown.

Aimes of the present work were to study the role of phenazine-1-carboxylic acid in *P. batumici* and to estimate the diversity of the genes encoding P1CA synthesis in bacteria of the genus *Pseudomonas*.

Materials and Methods

The study object was the batumin-producing type strain *P. batumici* UCM B-321 from the Ukrainian collection of microorganisms (Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine).

Antagonistic activity of *P. batumici* against phytopathogenic fungi and bacteria was studied on the potato agar solid medium (per 1 liter of distilled water: 500 g potato broth; 5 g NaCl; 15 g agar); and on Gauze medium (per 1 liter of distilled water: 30 ml Hottinger bouillon; 5 g peptone; 10 g glucose; 5 g NaCl; 30 g agar-agar) against human opportunistic pathogens, which are listed below.

Phytopathogenic bacteria: *Pseudomonas syringae pv. syringae*, *P. fluorescens*, *Pectobacterium carotovorum*, *Xanthomonas campestris*, *Clavibacter michiganensis*, *Agrobacterium tumefaciens*, *Erwinia aroidea*;

Phytopathogenic fungi: *Mucor plumbeus*, *Fusarium avenaceum*, *Drechslera graminea*, *Rhizopus arrhisus*, *Botrytis cynerea*.

Opportunistic bacterial and fungal pathogens: *Staphylococcus aureus* UCM B-918, *Escherichia coli* UCM B-926, *Pseudomonas aeruginosa* UCM B-900, *Bacillus subtilis* UCM B-901, *Candida albicans* UCM Y-2681.

The strains of phytopathogenic species mentioned above were obtained from the collection of the Department of phytopathogenic bacteria and the Department of physiology and taxonomy of micromycetes of Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine, and the reference strains of the pathogenic microorganisms were obtained from the Ukrainian collection of microorganisms.

Phenazine-1-carboxylic acid was synthesized by *P. batumici* UCM B-321 in Erlenmeyer flasks with 100 ml of the King B medium on a rotary shaker, at 25 °C for 72 hours. The presence of P1CA in the culture liquid was assessed using the liquid chromatography-mass spectrometry (LC/MS), *Agilent* 1200 liquid chromatograph (Agilent Technologies): XDB-C18 column (Zorbax 150 mm×4.6 μm×5 μm) in $ACN:H_2O$ mobile phase (55:45) with 0.5 mmol ammonium acetate at 30 °C with the flow rate 1 ml/min, injection volume of 5 ml under isocratic mode.

P1CA was extracted from the supernatant of the centrifuged cell-less culture liquid by acidifying it with 0.1 N HCl to pH 2−3. The yellow-green precipitate was separated by centrifugation for 10 min at 12.000 g and dissolved in chloroform, which was distilled in a rotary vacuum evaporator at 40 °C. Then the precipitated crystals were dissolved in benzene and extracted with 0.1 N K_2HPO_4 solution (pH 9.0). The resulting extract was again acidified and extracted with benzene. The resulted distilled crystals were re-crystallized from methanol.

Antimicrobial activity of P1CA was studied by the method of serial dilutions on meat peptone agar for bacteria and on beer wort for micromycetes.

Search for the phenazine biosynthesis operon was performed in the complete genome sequence of the strain *Pseudomonas batumici* UCM B-321 (RefSeqNZ_JXDG00000000.1). Genome annotation was processed by RASTServer (http://rast.nmpdr.org/) [6]. Homologous operons in the genomes of other microorganisms were searched for using the program AntiSMASH (http://antismash. secondarymetabolites.org/). Sequence alignment and inference of phylogenetic relationship between the operons of biosynthesis of phenazine-like compounds were carried out by Mauve 2.3.1 [7].

Transmission electron microscopy was performed using JEM-1400 microscope (Jeol, Japan) with an acceleration voltage of 80 kV. Suspension of *P. batumici* UCM B-321 cells was applied to a copper mesh (400 mesh). For contrasting, the samples were stained by 2% uranyl acetate solution. The cell sizes were estimated using JEM-1400 software.

Biofilm formation was studied according to O'Toole [8] by growing *P. batumici* UCM B-321 at 25 °C for 48 hours in 96-well plates on LB medium (per 1 liter of distilled water: 15 g/l peptone, 10 g/l yeast extract, 5 g/l NaCl). Initially, aliquots of 1 and 10 μg/ml P1CA were added to 0.25 ml per well of the cell suspensions of the culture medium ($5 \cdot 10^7$ cell/ml). Biofilms were stained by 0.1% solution of gentian violet 2-F (bioMerieux, France) added in the amount of 0.1 ml per well. Then the microplate photometer Multiskan FC (ThermoFisherScientific, USA) was used to record the opacity at 540 nm.

Significance of the average values was controlled by the $P < 0.05$.

Results and Discussion

Yellow solid crystals were extracted from the culture liquid of *P. batumici* UCM B-321. , Compound's molecular weight determined by LC/MS-analysis (Fig. 1) was 224 (output time 6.9 min). Peaks of the absorption spectrum fell to 250 and 364 nm. The melting temperature was 238 °C. These data allowed to identify the extract as a phenazine-1-carboxylic acid.

A known precursor of phenazines synthesized by *Pseudomonas* is phenazine-1,6-dicarboxylate, which is formed by condensation of two molecules of chorismate. Transformation of 3-oxyanthranilate to phenazine-1,6-dicarboxylate is controlled by a gene with known nucleotide sequence, which is responsible also for the synthesis of isochorismatase [2]. Diagnostic primers developed previously for determination of the phenazine operon in *P. fluorescens* and *P. chlororaphis* were used in this study. PCR amplification with these primers results in synthesis of 620-kb fragments, but the amplification failed with the genomic DNA from *P. batumici* B-321 indicating specificity of the corresponding phenazine genes in this species [9].

The purpose of the further work was to identify an operon involved in the synthesis of the phenazine-like compound by *P. batumici* to carry out a comparative analysis with the known phenazine biosynthesis operons of *Pseudomonas*.

Annotation of the complete genome sequence of *P. batumici* revealed a secondary metabolite biosynthesis operon that showed a significant sequence similarity to the known phenazine operons of other *Pseudomonas*. Homology of these operons was confirmed also by ordering of the respective genes on the chromosomes (Fig. 2).

Other homologous phenazine synthesis operons were discovered in NCBI database by MegaBLAST. In total, ten phenazine synthesis operons including that of *P. batumici* B-321 with an average length of 8000 n.p. were aligned against each other By the program Mauve, which also inferred a phylogenetic tree shown in Fig. 3. Mauve algorithm accounts for both: mutations and genetic rearrangements in long DNA sequences.

It was discovered that the phenazine synthesis operons of *Pseudomonas* bacteria fell into two distinct groups. The sequence of *P. batumici* phenazine operon is more similar to the sequences of the homologous operons in *P. aeruginosa*, although the genome of the type strain *P. aeruginosa* PAO1 comprised two
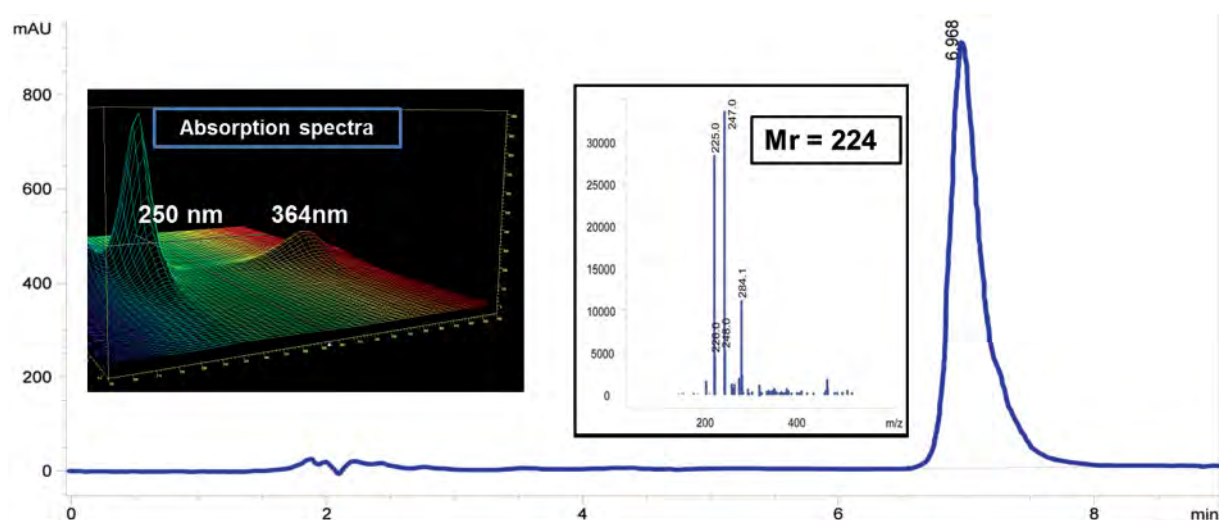

Fig. 1. **LC/MS-analysis and absorption spectra of phenazine-1-carboxylic acid isolated from *P. batumici* B-321**

Fig. 2. **Phenazine biosynthesis operons similar
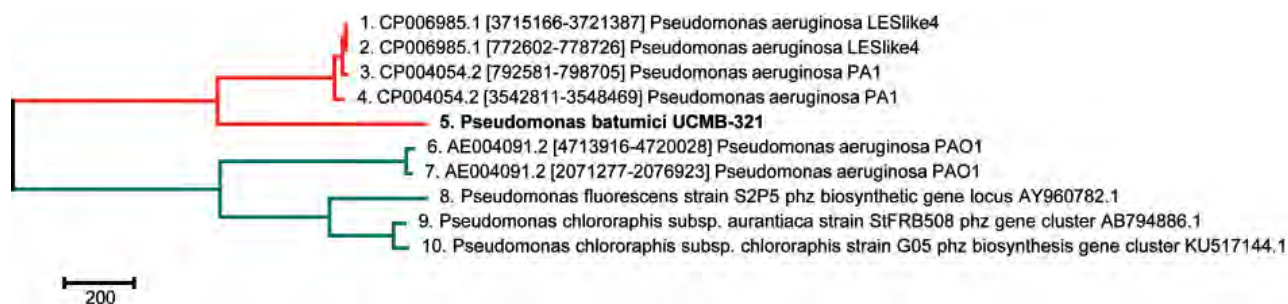to the operon identified in the *P. batumici* B-321 genome**

Fig. 3. **Phylogenetic tree based on the nucleotide sequences
of phenazine biosynthesis operons produced by Mauve 2.3.1**
The branch labels inform about the organism, genome registration numbers
and operon coordinates in the genome

copies of alternative phenazine operon similar to those in *P. fluorescens* and *P. chlororaphis* (Fig. 3). This mosaic distribution of the operons suggests a horizontal exchange of these genes between microorganisms.

The failure with the diagnostic amplification using the standard primers targeting the phenazine biosynthesis operons [9] could be explained by a variability of the target sequences in the two groups of these operons in Pseudomonas as shown in the alignment in Fig. 4.

It was found that the recommended primers were suitable to amplify only from the phenazine operons of *P. fluorescens*, *P. chloraphis* and the type strain *P. aeruginosa* PAO1. DNA segments in the corresponding genes of *P. batumici* and other *P. aeruginosa* strains varied in these regions by insertions and deletions of the targeted nucleotides that made amplification impossible. Analysis of the aligned sequences revealed areas alternative loci of the *phzE* gene (Fig. 5), which would better serve as targets for universal primers.
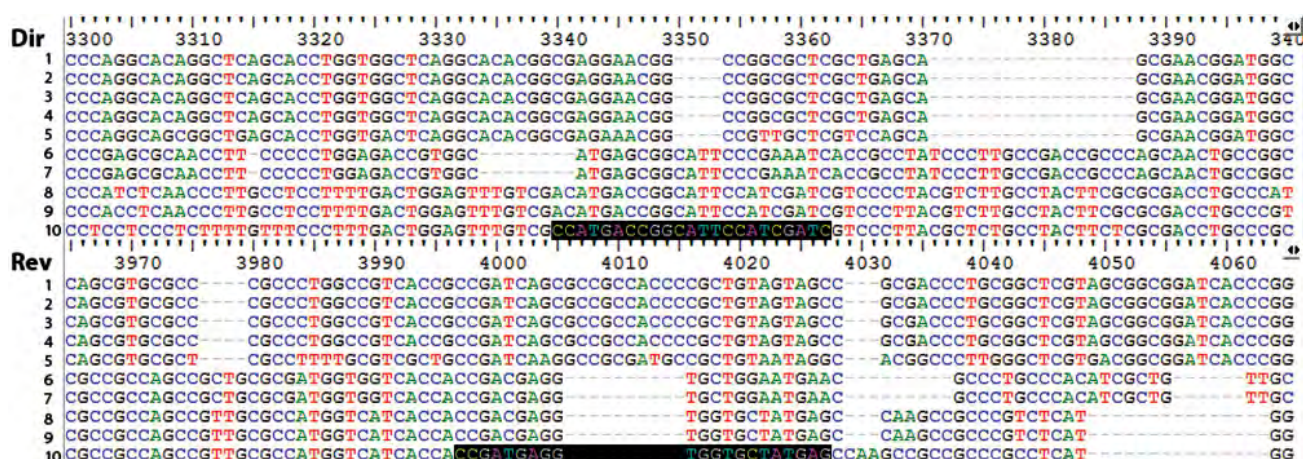

Fig. 4. **Alignment of fragments of phzE targeted by the standard primers:**
Dir — target area of the forward, and Rev — reverse primers; hereinafter: numbers of sequences:
1, 2 — P. aeruginosa LESlike 4; *3, 4 — P. aeruginosa* PA1; *5 — P. batumici* B-321; *6, 7 — P. aeruginosa* PAO1;
8 — P. fluorescens S2P5; *9 — P. chlororaphis* StFRB508; *10 — P. chlororaphis* G05.
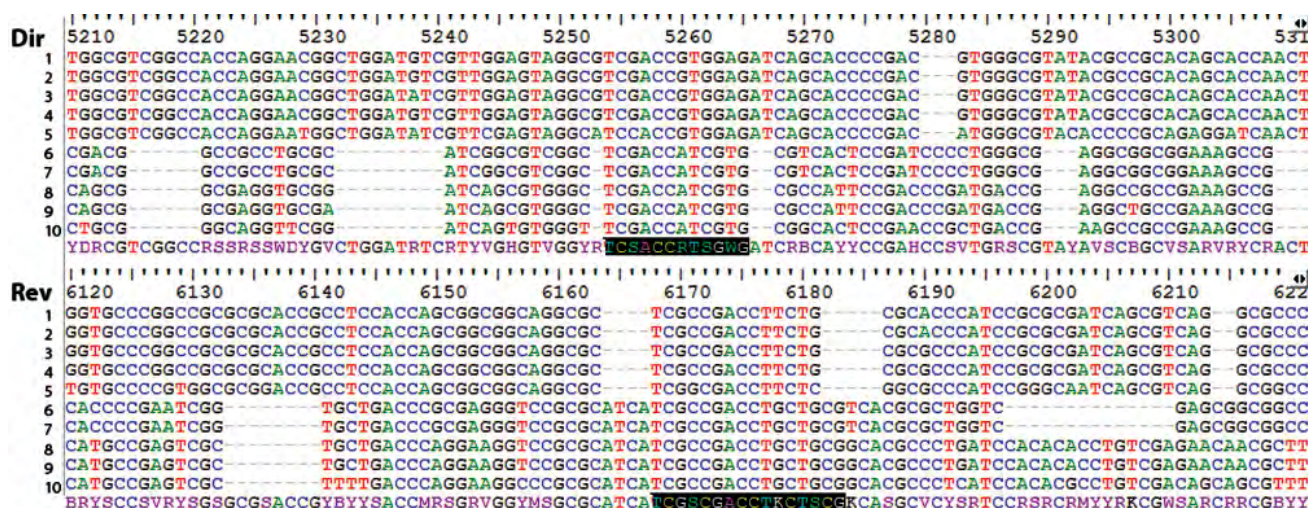Fragments complementary to primers are in black

Fig. 5. **Conserved loci of the *phzE* gene suitable for development of universal primers for a diagnostic amplification of fragments of phenazine synthesis operons in *Pseudomonas***
Sequences were numbered in the same order as in Fig. 4. An unnumbered line corresponds to the consensus sequence

However, the applicability of such primers yet should be confirmed experimentally.

To conclude, the operon of the phenazine biosynthesis of *P. batumici* generally was similar to those of the fluorescent *P. aeruginosa* but the type strain of this species.

The aim of our study was to find out the role of P1CA in ecology of the batumin producer. Considering that *P. batumici* was isolated from the rhizosphere, we made an attempt to evaluate the contribution of the phenazine pigment to the competitiveness of the strain B-321 in soil and rhizosphere. Also we were interested to study the effect of P1CA on opportunistic pathogens to estimate to which extent this activity of *P. batumici* was contributed by batumin and by other secondary metabolites (Table 1).

The highest growth inhibition by *P. batumici* UCM B-321 was exerted against *Staphylococcus* and *Pseudomonas aeruginosa* B-900, which obviously was caused by batumin. Inhibition of *C. albicans* and *B. subtilis*, which were not sensitive to batumin [8−10], may be explained by the activity of P1CA. Inhibition of the phytopathogenic bacteria also may be associated with the synthesis of the phenazine pigment as they are sensitive to this compound even at 50 µg/ml (Table 2).

Antimicrobial activity of P1CA is relatively weak compared to many other antibiotics produced by *Pseudomonas*. However, phenazine was considered as an antifungal agent synthesized by the strains of the genus *Pseudomonas* used for plant protection against fungal diseases. Contrary, batumin does not suppress the growth of fungi at all. Activity of P. batumici against *Agrobacterium*, *Corynebacterium*, and phytopathogenic pseudomonads shown in Table 2 also may be attributed to phenazine.

P1CA may be an important factor of regulation of the processes of biofilm formation by *Pseudomonas* used for plant protection, as it is shown in Fig. 6 on an example with *P. batumici* B-321.

In this experiment, the biofilm formation by *P. batumici* was stimulated by supplementing of P1CA into the medium. Biofilm formation was observed as early as in 24 hours and continued up to 48 hours of cultivation. According to statistical analysis, there was no significant difference between the effects of P1CA The stimulation effect by P1CA on the biofilm formation was equally strong when the compound was applied in concentrations of 1 and 10 µg/ml. In both cases, the rate of biofilm formation uplifted with a statistical reliability ($P < 0.01$) when compared to the biofilm formation rate on the medium without P1CA.

Also it was observed that the cells of *P. batumici* B-321 were 20% longer when grown

Table 1. **Inhibition zones around colonies of *P. batumici* UCM B-321**

Test strain	Growth inhibition zone, mm
Phytopathogenic bacteria	
Pseudomonas syringae pv. syringae	19
Pseudomonas fluorescens	22
Pectobacterium carotovorum	14
Xantomonas campestris	0
Clavibacter michiganensis	0
Agrobacterium tumefaciens	0
Opportunistic microorganisms	
Staphylococcus aureus B-918	Total inhibition
Escherichia coli B-926	17
Pseudomonas aeruginosa B-900	0
Bacillus subtilis B-901	7
*Candida albicans*Y-2681	10

Table 2. **Activity of phenazine-1-carboxylic acid against several phytopathogenic fungi and bacteria**

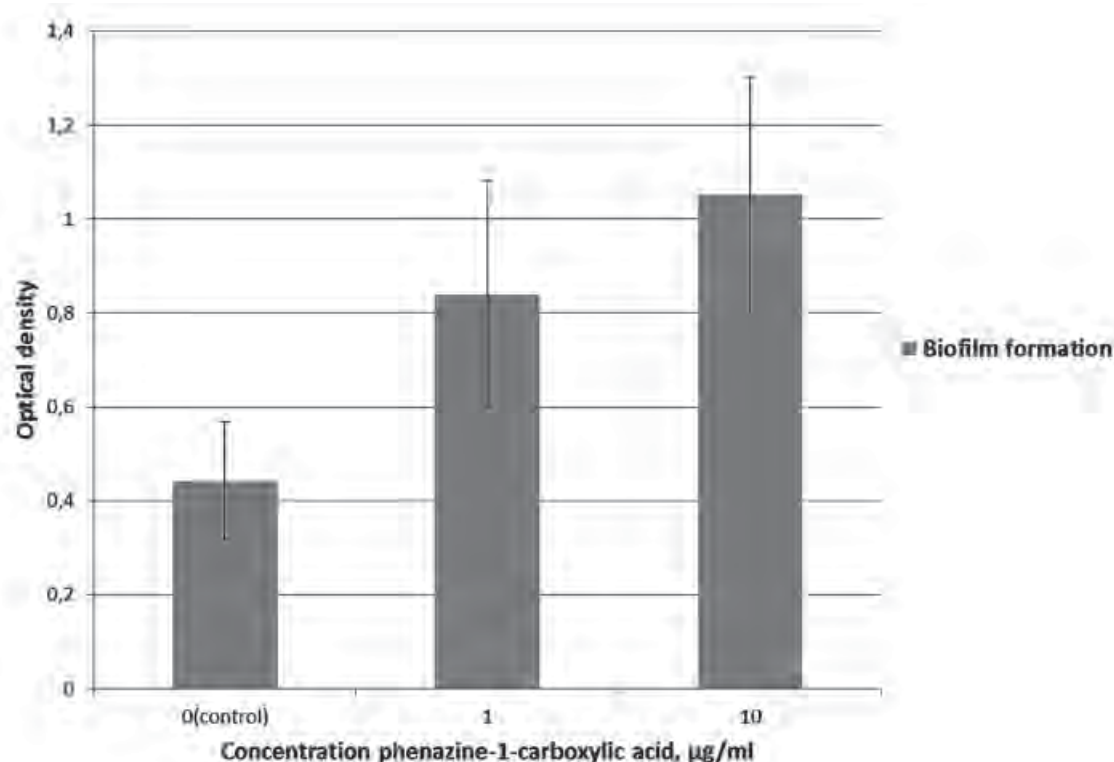| Minimum inhibitory concentration, µg/ml			
Fungi		**Bacteria**	
Muco rplumbeus	200	*Agrobacterium tumefaciens*	200
Fusarium avenaceum	200	*Pseudomonas syringae*	100
Drechslera graminea	50	*Corynebacterium michiganense*	200
Rhizopus arrhisus	100	*Erwinia aroidea*	400

Fig. 6. **Influence of phenazine-1-carboxylic acid on biofilm formation by *P. batumici* UCM B-321 strain**
Statistical significance is given in the text

on the medium with 10 µg/ml P1CA compared to the control growth: $2.81 \pm 0.14 \times 0.72 \pm 0.07$ µm and $2.34 \pm 0.22 \times 0.65 \pm 0.02$ µm, respectively. The observed elongation of the cells may be associated with a down-regulation of the rate of cell division by P1CA [11].

The phenazine biosynthesis was detected in several unrelated taxa of microorganisms, including representatives of Proteobacteria, Actinobacteria and even phylogenetically distant Euryarcheota. Biological properties of these various compounds are poorly studied despite a multitude of publications on practical importance of the phenazine synthesizing fluorescent bacteria of the genus *Pseudomonas* including opportunistic human pathogen *P. aeruginosa* and protecting plants soil saprophyte *P. chlororaphis* subsp. *aureofaciens* [2, 12].

Published earlier comparison of sequences of 16S rRNA [6] showed phylogenetic relatedness of *P. batumici* to *P. chlororaphis*,

which is another profoundly studied P1CA producer. However, in contrast to the latter species, *P. batumici* is unable to synthesize the green fluorescent pigment pyoverdine. Search through the complete genome sequence confirmed absence of the corresponding genes in *P. batumici*. Hence, *P. batumici* is the only non-fluorescent species of *Pseudomonas* capable of producing P1CA. The results signified that the phenazine operons of *P. batumici* was homologous to the phenazine synthesis operons of other representatives of the genus *Pseudomonas*. It resembled to some extent the ancestral variant of this gene linking the two groups of phenazine encoding operons (Fig. 3).

The carried out research demonstrated that the synthesis of P1CA contributed to the competitiveness of *P. batumici* in soil and rhizosphere by supplementing of the antimicrobial activity of batumin. Moreover, P1CA stimulated the biofilm formation by *P. batumici*. According to the literature data, biofilm is the state of the bacterial life cycle, when the microorganisms exert 95–99% of their activities in the natural habitats [13]. Biofilm formation by the fluorescent bacteria of the genus *Pseudomonas* and the role of phenazine in these processes were subjects of numerous studies. Regulated by the quorum-sensing (QS) system, the synthesis of phenazine alters the expression levels of certain genes involved in cell adhesion during the development of biofilms. Phenzine also is involved in the ion reduction from Fe^{3+} to Fe^{2+}. The rhizosphere-dwelling strain *P. chlororaphis* PCL1391 was proved to use phenazine-1-carboxamide to transform Fe^{3+} into easier mobilized Fe^{2+} ions allowing in this way the grow at microaerophilic conditions [14]. In *P. chlororaphis* strain P1CA acts conjointly with 2-oxiphenazine-1-carboxylic acid in regulation of the adhesion of cell in biofilms [15]. Similar effects of P1CA on the stimulation of biofilm formation and iron uptake was reported for the opportunistic pathogen *P. aeruginosa* [16]. It may be supposed that the phenazine plays a similar regulatory role in the life cycle of the non-fluorescent rhizobacterium *P. batumici*.

The data inform the literature and that obtained in the presented research suggests a manifold function of the phenazine substances in Pseudomonas That is still not fully understood and requires further studies.

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ГЕНИ, ЩО КОДУЮТЬ СИНТЕЗ ФЕНАЗИН-1-КАРБОНОВОЇ КИСЛОТИ
У *Pseudomonas batumici*

В. В. Клочко[1], *С. Д. Загородня*[1], *О. М. Рева*[2]

[1]Інститут мікробіології і вірусології
ім. Д. К. Заболотного
НАН України, Київ
[2]Centre for Bioinformatics and Computational Biology, University of Pretoria, South Africa

E-mail: vvklochko@ukr.net

Метою роботи було з'ясування ролі феназин-1-карбонової кислоти *Pseudomonas batumici* та різноманіття генів, що кодують її синтез у бактерій роду *Pseudomonas*. Феназин-1-карбонова кислота в концентрації 10 мкг/мл стимулювала формування біоплівки штамом-продуцентом. Проведення ПЦР з використанням специфічних праймерів, розроблених для *Pseudomonas*, не підтвердило наявність у *P. batumici* гена синтезу феназину. Водночас сиквенування повного геному *P. batumici* виявило гомологічний ген, який, імовірно, кодує синтез цього антибіотика. Порівняльний аналіз геномів бактерій роду *Pseudomonas* показав існування, як мінімум, двох генетично розрізнених груп ортологічних генів, які кодують синтез феназинів. Припускають, що розповсюдження генів синтезу феназинів у ризобактерій пов'язано з горизонтальним перенесенням генів.

Ключові слова: гени *Pseudomonas batumici*, феназин-1- карбонова кислота, біоплівкоутворення.

ГЕНЫ, КОДИРУЮЩИЕ СИНТЕЗ ФЕНАЗИН-1-КАРБОНОВОЙ КИСЛОТЫ
У *Pseudomonas batumici*

В. В. Клочко[1], *С. Д. Загородня*[1], *О. Н. Рева*[2]

[1]Институт микробиологии и вирусологии
им. Д. К. Заболотного НАН Украины, Киев
[2]Centre for Bioinformatics and Computational Biology, University of Pretoria, South Africa

E-mail: vvklochko@ukr.net

Целью работы было выяснение роли феназин-1-карбоновой кислоты *Pseudomonas batumici* и разнообразия генов, кодирующих ее синтез у бактерий рода *Pseudomonas*. Феназин-1-карбоновая кислота в концентрации 10 мкг/мл стимулировала формирование биопленки штаммом-продуцентом. Проведение ПЦР с использованием специфичных праймеров, разработанных для *Pseudomonas*, не подтвердило наличие у *P. batumici* гена синтеза феназина. В то же время секвенирование полного генома *P. batumici* выявило гомологичный ген, который, вероятно, кодирует синтез этого соединения. Сравнительный анализ геномов бактерий рода *Pseudomonas* показал существование, как минимум, двух генетически различимых групп ортологичных генов, кодирующих синтез феназинов. Высказано предположение, что распространение генов синтеза феназинов у ризобактерий связано с горизонтальным переносом генов.

Ключевые слова: гены *Pseudomonas batumici*, феназин-1-карбоновая кислота, биопленкообразование.