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# PYOCIN S5 ACTIVITY AGAINST *PSEUDOMONAS SYRINGAE* PHYTOPATHOGENIC STRAINS

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**ABSTRACT**

*Pseudomonas syringae* is the most frequently isolated bacterial plant pathogens. Previously we revealed a number of *Pseudomonas aeruginosa* strains – producers of bacteriocins (pyocins) with high activity against *P. syringae*. However, it was unknown, which type of pyocins possesses the activity against *P. syringae* strains. S5 and S1 pyocin genes were observed in the genome of most active strain–producer *P. aeruginosa* UCM B-333. Pyocins were induced by nalidixic acid (100 μg/ml). Using DEAE-cellulose chromatography we isolated two bacteriocin groups from initial lysate. Substances from the 10$^{th}$ fraction had nuclease activity (pyocin S1) and the 27$^{th}$ fraction - pore-forming activity (pyocin S5). It was revealed that S1 pyocin didn't lead to growth inhibition of studied bacterial plant pathogens. However pyocin S5 caused appearance of growth inhibition area with diameter 14-20 mm. So, pyocins of S1 and S5 types are active substances in *P. aeruginosa* UCM B-333 lysates, but only S5 pyocin possess activity against *P. syringae* strains. fpt A receptor gene was revealed in genomes of all analyzed cultures, including used phytopathogenic strains. This fact can explain wide spectrum of pyocin S5 activity. It is suggested that pyocin S5 can be a potential biocontrol agent against phytopathogenic *P. syringae* strains.

**Keywords:** pyocin S5, purification, *Pseudomonas aeruginosa*, antimicrobial activity, *Pseudomonas syringae*

## INTRODUCTION

Bacterial diseases cause the considerable loss of the agriculture crop (**Roberts, 2005; Lian *et al.*, 2008; Suszanowich, 2019**). Among widespread plant pathogens *Pseudomonas syringae* strains are characterized by high harmfulness and a high frequency of isolation (50-80% - on leguminous plants and up to 90% - on cereal crop) (**Gvosdyak *et al.*, 2011; Patyka and Pasichnyk, 2014**). Available chemical pesticides are ineffective for control of these microorganisms spread, and application of such substances has a whole number of disadvantages (**Hassaan and Nemr, 2020**). These substances contaminate, accumulate and have adverse effect on natural system (soil and aquatic), water, plants (growth, metabolism, genotypic and phenotypic changes and impact on plants defense system), human health (genetic alteration, cancer, allergies, and asthma), and preserve food products (**Pathak *et al.*, 2022**). Therefore, one of the ways to solve the problem of crop loss is the search for the new, safe for humans and the environment means for *P. syringae* strains influencing.
Bacteriocins were proposed to prevent plant pathogens dissemination (**Lavermicocca *et al.*, 1999**). Bacteriocins are antimicrobial substances synthesized by majority of microorganisms that are able to inhibit the growth of closely-related strains (**Somsap *et al.*, 2015; Kongkum *et al.*, 2016**). Some researchers consider them to be promising, alternative means to available preparation for influence on plant diseases agents (**Rooney *et al.*, 2020; Balko *et al.*, 2020**). These substances possess high antibacterial activity, not lower than in classic antibiotics, narrow action spectrum, which limits their impact to agents of particular disease only, do not pollute the environment and are safety for human (**Ghequire and De Mot, 2014; Soltani *et al.*, 2021**). In most cases, bacteriocins of Gram-negative bacteria affect strains of the same species to which their producer strain belongs and do not affect microorganisms of other species (**Behrens *et al.*, 2017**). Many different pathovars belong to *P. syringae*, but no bacteriocins have been described that could simultaneously affect most of them.
The production of three type's bacteriocins: pyocins of R, F and S-type is described for *Pseudomonas aeruginosa* (**Ghequire and De Mot, 2014; Behrens *et al.*, 2017**). The first two types are high molecular weight structures, analogues of phage-tails with a molecular weight of 1-10 MDa (**Balko, 2012; Scholl, 2017**). Colicin-like S-type pyocins belong to proteins with a molecular weight of 40-70 κDa (**Behrens *et al.*, 2017**).
In previous studies there were revealed a number of *P. aeruginosa* strains – producers of highly active bacteriocins (**Balko and Avdeeva, 2012**). According to the results of physicochemical and biological analyses, these killer agents were classified as colicin-like S-type pyocins (**Balko *et al.*, 2013**). Bacteriocins, isolated from eleven *P. aeruginosa* strains, were characterized by moderate and high activity against the most of investigated *P. syringae* strains. It was found out, that production of *P. aeruginosa* UCM B-333 bacteriocins depends on the conditions of producer-strain cultivation, whereas synthesis of *P. aeruginosa* UCM B-9 killer agents – on optimization of induction. Both mentioned factors influenced on pyocins activities of other strains. The application of suggested methods gave an opportunity to increase the activity of *P. aeruginosa* UCM B-333 pyocins in more than 40 times against indicator cultures *P. aeruginosa* UCM B-3 and UCM B-10 and extend their influence spectrum to all studied *P. syringae* strains (**Balko *et al.*, 2017**). The ability to affect *P. aeruginosa* strains has been described for all types of pyocins, but the growth inhibition of the bacteria belonging to another species, in particular *P. syringae*, has been revealed by us for the first time. The use of *P. aeruginosa* bacteriocins to inhibit the growth of *P. syringae* may be a new means for controlling the spread of these pathogenic microorganisms.
Each *P. aeruginosa* strain can produce up to five different subtypes of bacteriocins (**Ghequire and De Mot, 2014**). However it remains unknown, which subtype of S pyocins, synthesized by our highly active producer *P. aeruginosa* UCM B-333, is active against *P. syringae*.
So, the objective of our study was to identify subtypes of S-type pyocins in *Pseudomonas aeruginosa* UCM B-333 and to define which of them is capable for inhibition of *Pseudomonas syringae* growth.

## MATERIAL AND METHODS

### Materials

The object of investigation was *Pseudomonas aeruginosa* UCM B-333 from Ukrainian collection of microorganisms (UCM, Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine). We deposited this strain in Depositary of Zabolotny Institute of Microbiology and Virology under number IMV B-7668 as high active producer of bacteriocins.

### Molecular-genetic analysis

Total DNA was isolated from *P. aeruginosa* UCM B-333 cell suspension using Gene Jet Genomic DNA Purification Kit (Thermo Scientific) according to manufacturer's protocol. To detect genes encoding *P. aeruginosa* pyocins S1, S2, S3, S4, S5 a multiplex PCR was carried out. PCR mixture composition, temperature parameters of multiplex PCR were as described (**Dingemans *et al.*,**

**2014**). PCR mixture to amplify AP41 pyocin and pyoM, fptA genes fragments contained 2x Dream Taq PCR Master Mix (Thermo Scientific), 30 pmol of Forward and Reverse primers and 50 ng of DNA. The cycling parameters were as following: one cycle – 95 °C, 2 min; 30 cycles – 95 °C, 10 sec; 57 °C, 15 sec; 72 °C, 25 sec; final extension – 72 °C, 5 min. Amplifications were performed with the thermocycler Mastercycler Personal 5332 (Eppendorf, Germany). For amplification of AP41 and S1-S5 pyocins were used primers according to (**Dingemans *et al.*, 2014**). Primers for the amplification of pyoM and fptA genes were designed using Primer 3 program (http://primer3.ut.ee/):5'-CATCCCTTCAGCAGTTTCGG -3' and 5'-ACGCTCCGAATTGTAGGGAT -3' (pyoM); 5'-ACGAAGGTGATCAAGGGACG-3' and 5'-TTCTCCGCTGATCACCATGT-3' (fptA). Type strain *P. aeruginosa* PAO1 was chosen as a reference control.

RT-qPCR analysis of S1 and S5 gene expression (using SYBRGreen). Samples for the analysis were taken at several points: untreated control (24h culture, 1:10 dilution) and samples incubated for 3, 6 and 18 hours after treatment with nalidixic acid. RNA was isolated from untreated and treated bacteria using TRIzol reagent (ThermoFisher Scientific) with following DNase I treatment. RNA was quantified with «BioPhotometer» ("Eppendorf", Germany). cDNA was synthesized from 1 µg of RNA using RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific). qPCR mix of total volume 20 µl contained 10 µl of Luna Universal qPCRmix (New England Biolabs), 20 pmol of each primer and 3 µl of cDNA. The amplification was performed with QuantStudio ™ 3 Real-Time PCR System (Applied Biosystems) with standard cycling modes recommended by the manufacturer's procedure. 16S rRNA gene expression was chosen as an endogenous control; primer sequences to 16S rRNA gene were given in (**Zelena *et al.*, 2014**). Amplification for each sample and for each gene was carried out in duplicate and each amplification run included no template control. The relative gene expression level was calculated with 2 -ΔΔCt method (**Livak and Schmittgen, 2001**). Statistical significance and analysis were carried out as described in (**Humeniuk *et al.*, 2023**). The primers specificity and absence of dimer formation were confirmed by agarose gel electrophoresis and melting curve analysis.

### Isolation, separation and purification of pyocins

A number of stages of induction, concentration and separation were carried out to obtain purified pyocins. The crude *P. aeruginosa* UCM B-333 lysate was got by induction with nalidixic acid, which was added in final concentration 100 µg/mL to suspension of strain-producers in the logarithmic growth phase (**Balko *et al.*, 2019**). Concentration of bacteriocins was conducted by 70% ammonium sulphate precipitation during 1 day at 4 °C. The sediment was obtained at 30.000 g and 4 °C for 30 min and resuspended in 2 mL of 20 mM Tris-HCl buffer (pH 7.5). The samples were dialyzed through dialysis membrane (MWCO 15 kDa) against 50 mL of 20 mM Tris-HCl buffer for 1 day at 4 °C with a single replacement of dialysis buffer. Purification from insoluble admixture was carried out by low centrifugation at 4.000 g for 30 minutes (**Ito *et al.*, 1970**). Bacteriocins were separated by ion-exchange chromatography. For this purpose column (25 by 130 mm) was filled with DEAE-cellulose and equilibrated with 600 mL of 20 mM Tris-HCl buffer (pH 7.5). 1 ml of bacteriocin sample was applied on this column. Step-by-step elution was conducted with 100 mL of 20 mM Tris-HCl buffer and analogous volume of the same buffer contained 0,1M NaCl. Samples of the eluate (5 mL) were collected into separate sterile tubes. Several authors isolated pyocins with nuclease properties using elution with a buffer containing various concentrations of NaCl - from 0 to 0.25 M. The application of this eluent in the indicated concentrations did not lead to the loss of bacteriocins nuclease activity (**Sano, 1993;Sano *et al.*, 1993**). Protein concentration in the fractions was determined by the measurement of absorbance at 280 nm.

### Pyocin antimicrobial activity

Antimicrobial activity of obtained fractions was tested by "two-layer agar" method (**Ling *et al.*, 2010**) at all stages of bacteriocin separation. Quantitative indices of lysate activities were determined by double serial dilutions method. Substance activity was estimated according to the maximal dilution able to cause formation of lysis zone. The obtained results were counted for 1 ml of studied lysate and expressed in activity units - AU/mL or for convenience in $\times 10^6$ AU/mL (**Saeed *et al.*, 2006**). In our study we included *P. aeruginosa* UCM B-3 and UCM B-10 as indicator cultures. To estimate activities of S1 and S5 pyocin subtypes were used causative agents of plant bacterial diseases: *P. savastanoi* pv. *phaseolicola* UCM B-1026 (reclassified *P. syringae*), *P. syringae* pv. *coronafaciens* UCM B-1154, *P. syringae* pv. *syringae* UCM B-1027, *P. syringae* pv. *atrofaciens* UCM B-1013 and IMV 9290 (Zabolotny Institute of Microbiology and Virology), *P. syringae* pv. *lachrymans* UCM B-1039.

### Pyocin nuclease activity

To check nuclease activity of bacteriocins from the 10th and the 27th fractions, 24 µL of these substances were mixed with the same volume of phage λ DNA (35 ng, «Fermentas»; 0,3 µg/ µL), exposed at 37 °C for 30 min, then frozen at -18 °C. As a control we used untreated DNA of phage λ. Availability of DNA and hydrolysis products was detected by electrophoretic separation in 1% agarose gel (**Sano *et al.*, 1993**). The obtained digital images were processed by TotalLab program.

### Pyocin pore-forming activity

Ability of bacteriocins from the 10th and the 27th fractions to cause pore-formation was tested against *P. aeruginosa* UCM B-10 and *P.savastanoi* pv. *phaseolicola* 4012 (reclassified *P. syringae*). There to 100 µL suspension of indicator culture with titer $10^7$ CFU/mL was blended with the same volumes of substances from the 10th and the 27th fractions. The mixtures were incubated for 1 and 3 hours at 37 °C. Then cells were precipitated by low centrifugation at 10.000 g for 30 minutes, supernatant was taken and frozen at -18 °C. DNA concentration in selected samples was determined with the help of Nanodrop under wavelength 260 nm (**Ling *et al.*, 2010**).

All experiments were carried out at least in two biological and three technical repeats.

## RESULTS

### Typing of pyocin genes

To define *P. aeruginosa* UCM B-333 active substances the typing of S pyocin genes in bacterial genome was performed. As control we chose type strain *P. aeruginosa* PAO1 for which the presence of S2, S4 and S5 pyocin genes was shown (**Elfarash *et al.*, 2012**).

As a result of PCR-analysis, amplification products of 117, 200 and 459 bp, corresponding to S2, S4 and S5 pyocin genes, were obtained in *P. aeruginosa* PAO1 and 459-bp and 646-bp amplicon corresponding to S5 and S1 pyocin genes, respectively, were observed in *P. aeruginosa* UCM B-333 (Figure 1). Thus, among seven analyzed pyocin genes (AP41, S1-S5, M) only two were revealed in *P. aeruginosa* UCM B-333 genome. Other genes weren't detected.

### Relative S1 and S5 pyocin gene activity

It was shown that nalidixic acid can induce synthesis of S-type pyocins (**Balko *et al.*, 2017**), but the dynamic of the induction process has not been studied yet. In the present research bacteria was exposed to nalidixic acid and the relative level of S1 and S5 pyocin gene activity was evaluated during 18 hours after treatment. The expression of both genes was increased up to 6 hours and then decreased on 18th hour (Figure 2).

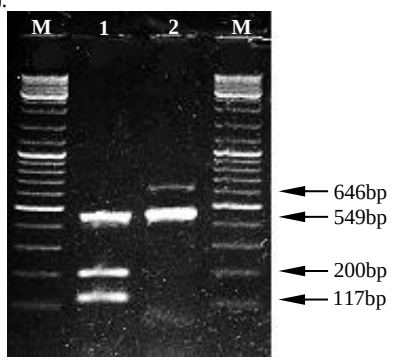


**Figure 1** Electrophoregram of PCR with primers to pyocin genes: 1 - *P. aeruginosa* PAO1, 2 – *P. aeruginosa* UCM B-333, M – GeneRuler DNA Ladder Mix (Thermo Fisher Scientific). The arrows show the fragments of pyocin genes: S1 (646 bp), S2 (117 bp), S4 (200 bp), S5 (459 bp)

The highest expression levels of both genes were observed in 6 hours after treatment when gene activity in comparison with control point increased up to 4,3 (S1) and 2,5 (S5) times. The lowest transcriptional activity was detected in 18 hours after treatment.

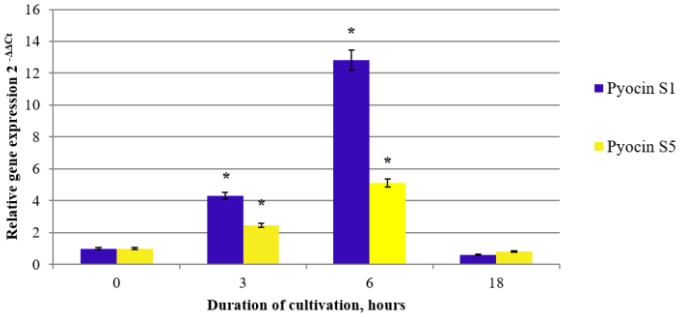


**Figure 2** Relative expression levels of S1 and S5 pyocin genes of *P. aeruginosa* UCM B-333 after treatment with 100 µg/mL nalidixic acid. Significance of differences in comparison with the control values: *p<0.05.

Thus under influence of nalidixic acid the transcription of S1 pyocin gene occurs and the presence of expression products in lysates was shown. Instead, the level of S5 pyocin gene expression was low and most likely depended on the conditions of producer-strain cultivation.

**Pyocin isolation and purification**

To obtain separate fractions of mentioned pyocins, series stages of induction, concentration and separation were conducted. Application of previously described methods of induction optimization (**Balko *et al.*, 2013**) gave us an opportunity to obtain initial *P. aeruginosa* UCM B-333 lysates with high activity indices – $7.68\times10^6$ AU/mL (Table 1).

Bacteriocin concentration was carried out by ammonium sulphate precipitation to 70% saturation. Usage of this method enabled to increase the killer agent activities in 20 times. Precondition for separation of protein substances by ion-exchange chromatography is their purification from insoluble and soluble salt admixture, as well as buffer replacement. For this purpose dialysis with next following low centrifugation was carried out. The obtained bacteriocin sample was characterized by lower parameters of killer activity and protein content then previous one had. But its index of specific activity was rather high – $57.31\times10^6$ AU/mg.

**Table 1** Steps of purification of *P. aeruginosa* UCM B-333 pyocins

| Sample/steps | V, mL | A,×$10^6$AU/mL | $A_{tot}$,×$10^6$AU | C, mg/mL | $A_{sp}$,×$10^6$AU/mg | R, % |
|---|---|---|---|---|---|---|
| Crude lysate | 100 | 7.68 | 768 | 0.20 | 38.40 | 100 |
| 70% $(NH_4)_2SO_4$precipitate | 2 | 153.60 | 307.2 | 2.05 | 74.93 | 40 |
| Dialysate | 1.4 | 38.40 | 53.76 | 0.67 | 57.31 | 7 |
| Fraction 10 | 20 | 0.60 | 12 | 0.43 | 1.39 | 1.6 |
| Fraction 27 | 20 | 0.20 | 4 | 0.26 | 0.77 | 0.5 |

**Legend:** V – total volume, A – pyocin activity, $A_{tot}$ –total activity, C – protein concentration, $A_{sp}$ - specific activity, R - % recovery

The prepared bacteriocin solution was applied to chromatographic column with DEAE-cellulose and step elution was conducted. Two peaks of protein concentration – in the 10th and the 27th fractions were revealed among selected eluants (Figure 3). The 1-20th fractions were eluted with 20 mM Tris-HCl buffer, and the 21-40th fractions – with the same buffer containing 0.1 M NaCl. It should be noted that the highest killer activity indices were detected in the same fractions. They amounted to 0.6 та $0.2\times10^6$ UA/mL accordingly. The obtained peaks coincided in activity and protein concentration, and were also not broad (four fractions by activity and six fractions by protein concentration). Other peaks indicating presence of additional substances in lysate were not revealed. We also detected production of two pyocins using PCR and RT-PCR. Therefore, it was concluded that by means of ion-exchange chromatography we succeeded in separation of two bacteriocin groups from initial lysate. So far as our object was achieved, subsequent elution stages were not conducted.

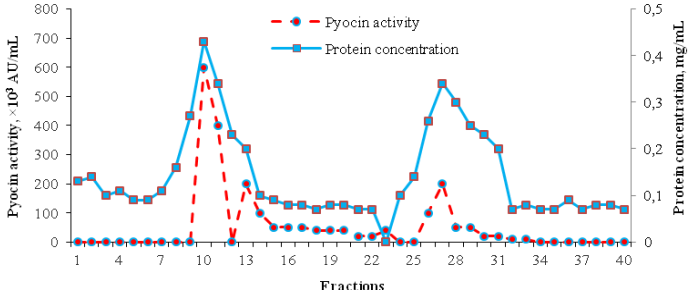


**Figure 3** Elution profile of *P. aeruginosa* UCM B-333 pyocins by ion-exchange chromatography (DEAE-cellulose column). The 1-20th fractions were eluted with 20 mM Tris-HCl buffer, and the 21-40th fractions – with the same buffer containing 0.1 M NaCl. A – pyocin activity, C – protein concentration.

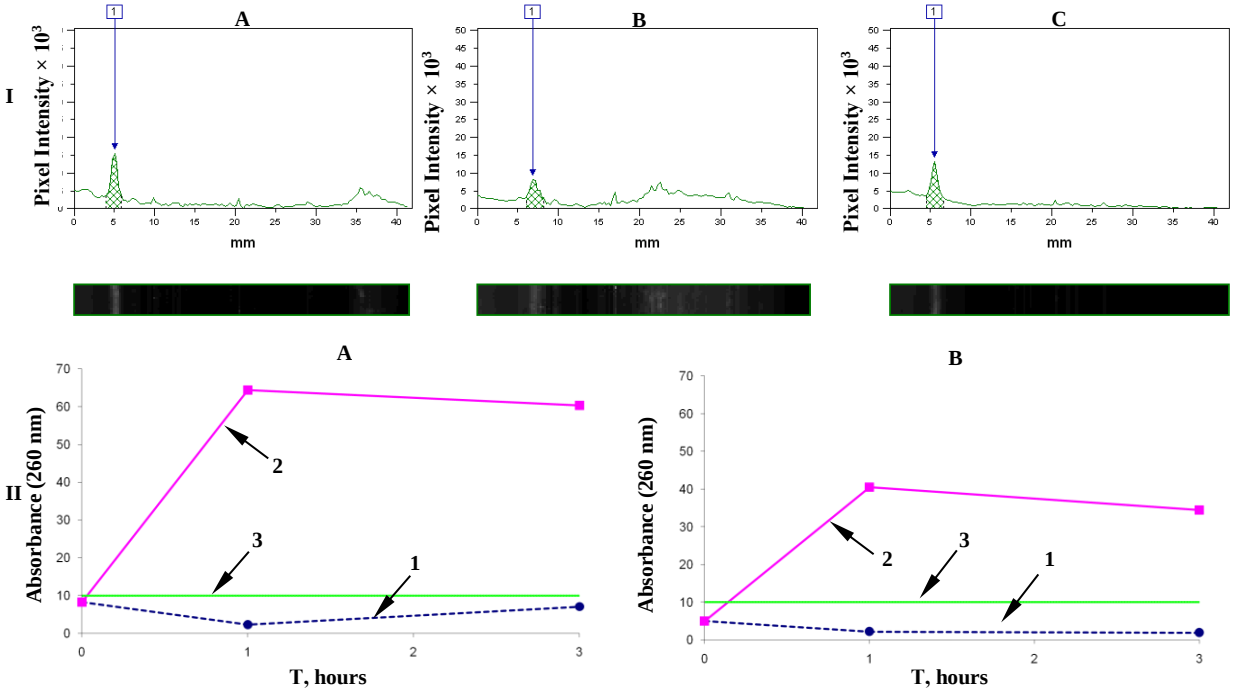


**Figure 4** Pyocin activity of 10th (B) and 27th (C) fractions.
**I.** Densitograms of untreated phage DNA λ (A) and after interaction with the pyocins of 10th (B) and 27th (C) fractions.
**II.** DNA content in the supernatants after interaction of *P. aeruginosa* UCM B-10 (A) and *P. savastanoi* pv. *phaseolicola* 4012 (B) with pyocins of 10th (1) and 27th (2) fractions. 3 - the threshold sensitivity of the device. $p<0.05$

**Pyocin subtypes determination**

The next stage was determination of pyocin subtypes in every selected fraction. It is known that S1 pyocin is characterized by nuclease activity, and S5 pyocin is able to pore-formation (**Elfarash *et al.*, 2014**). Firstly we tested fractions with the highest activity indices (the 10th and the 27th) for their action on phage λ DNA. To improve visualization of obtained results after DNA hydrolysis and its electrophoretic separation in agarous gel digital images of electrophoregrams were processed by TotalLab program. It was established that bacteriocin of the 10th fraction split phage DNA. It became apparent as twofold decrease in altitude of respective peak (Figure 4I)

Additionally appearance of stretched track of undigested DNA fragment was revealed. However similar effect weren't noted for the influence of the 27th fraction bacteriocin. These facts indicated that substance from the 10th fraction had marked nuclease activity, which was a characteristic feature for S1 pyocin. On the other hand, it is obvious that the substances of 27th fraction do not contain bacteriocins with DNase activity.

Impact of the 27th fraction substance on studied indicator cultures was caused by disorder in their cell membrane continuity. It became evident due to more intensive outflow of nucleic acids from *P. aeruginosa* UCM B-10 cells into supernatant after the treatment of indicator culture with killer agents from 27th fraction during 1 and 3 hours. The mentioned parameter increased in 8.1 and 6.9 times as compared with indices before the experiment (Figure 4II A). The analogous influence on *P. savastanoi* pv. *phaseolicola* 4012 caused the rise of cell DNA contents in samples in 7.8 and 7.3 times (Figure 4II B). The noted action mechanism according to Ling H., Saeidi N., Rasouliha B.H. et al (**Ling *et al.*, 2010**) is characteristic for pore-forming S5-subtype pyocins. Other bacteriocins with a similar mechanism of action among the known and predicted *in silico* pyocins for *P. aeruginosa* have not been described (**Ghequire and De Mot, 2014**). The intensity of nucleic acids outflow in supernatant due to influence of substances from the 10th fraction as on *P. aeruginosa* UCM B-10 so as on *P. savastanoi* pv. *phaseolicola* 4012 didn't excel the indices of untreated cultures. Thus it was found that the 27th fraction contained S5 pyocin which caused disorder in cell membrane continuity and led to the death of studied bacterial strains.

**Pyocin killer activity analysis**

To verify the discovered regularities we studied killer activity of 10th and 27th fractions against a number of plant pathogenic bacteria. It was revealed that substance from the 10th fraction, which contains S1 pyocin with nuclease activity, didn't cause growth inhibition of investigated microorganisms. However the application of pore-forming S5 pyocin, found in the 27th fraction, led to appearance of growth inhibition area with diameter 14-20 mm (Figure 5I).

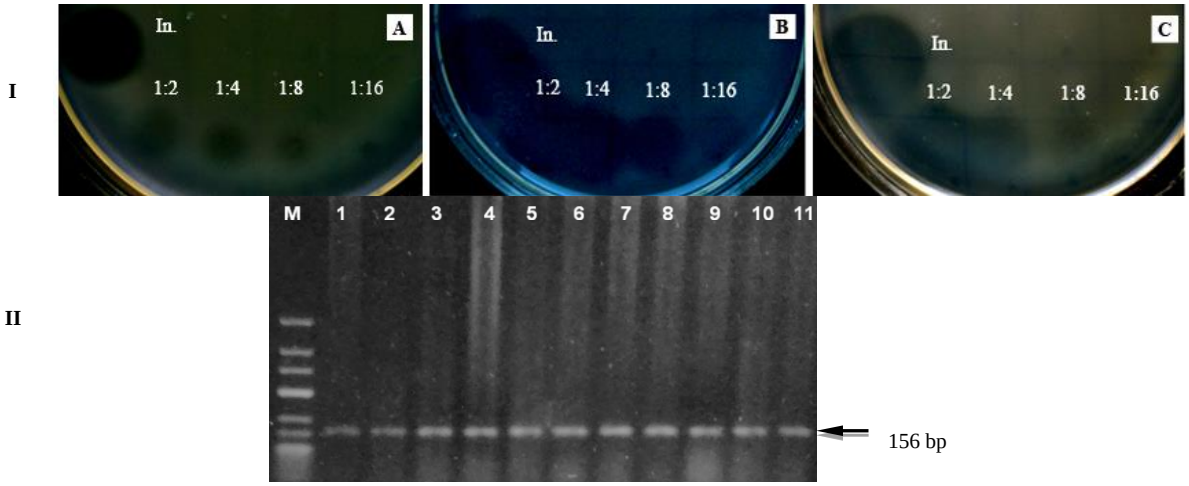


**Figure 5** Influence of pyocin from 27th fraction on investigated microorganisms.
**I.** The effect of pyocin from 27th fraction in the initial concentration (In.) and after dilutions (1:2-1:16) on *P. syringae* pv. *syringae* UCM B-1027 (A), *P. syringae* pv. *coronafaciens* UCM B-1154 (B), *P. syringae* pv. *atrofaciens* UCM B-1013(C).
**II.** Results of PCR with primers to FptA receptor genes in *P. aeruginosa* species: 1- UCM B-1; 2 - UCM B-3; 3 - UCM B-6; 4 - UCM B-7; 5 - UCM B-9; 6 - UCM B-10; 7 - UCM B-13; 8 - UCM B-330; 9 - UCM B-332; 10 - UCM B-335; 11 - UCM B-349. M – GeneRuler DNA Ladder Mix (Thermo Fisher Scientific)

Despite the slight induction and low concentration of this substance in crude *P. aeruginosa* UCM B-333 lysate it remained active even after dilution until 1:8-1:16. At the same time the decrease in diameters of growth inhibition area was observed under every next dilution. Minimal diameters were 6-7 mm.

**3.6. Detection of Fe(III)-pyochelin outer membrane receptor precursor genes**

It is known that the first stage of S5 pyocin action against sensitive cells is their binding to a ferripyochelin FptA receptor (**Elfarash *et al.*, 2014; Behrens *et al.*, 2020**). We have designed primers to TonB-dependent receptor plug domain of the fptA gene. According to PCR-analysis fpt A receptor gene was revealed in genomes of all studied *P. aeruginosa* cultures, including used sensitive strains (Figure 5II).

## DISCUSSION

Nowadays about 25 pyocins of S-type have been described for bacteria of *Pseudomonas* genus (**Rooney *et al.*, 2020**; **Soltani *et al.*, 2021**). The majority of these substances are foreseen *in silico* according to distinctive nucleotide sequences (**Ghequire *et al.*, 2018**). The data about their biological, physicochemical and molecular peculiarities are insufficient. Nucleotide sequences are known only for seven S-type pyocins of *P.aeruginosa* - AP41, S1-S5, M. Therefore amplification and search for pyocin genes in genome of studied strain were conducted on the basis of known sequence data. Obtained results of PCR confirmed presence of S1 and S5 pyocin genes in *P. aeruginosa* UCM B-333 genome (Figure 1), but their expression levels under the influence of used inductor differ considerably (Figure 2). It is known that recA system is responsible for regulation of excretion of majority pyocins including S1 pyocin. The inducing effect of nalidixic acid on microorganisms is caused by nucleic acid damage, which activates RecA-protein. The latter one splits repressor of prtN gene. It leads to the synthesis of PrtN protein, which binds with P-box structure and initiates pyocin transcription (**Sano *et al.*, 1993; Ghequire and De Mot, 2014**). But nucleotide sequence of S5 pyocin gene does not contain P-box structure necessary for binding with transcriptional activator PrtN. However it is supposed that stimulation of S5 pyocin transcription can be triggered by oxidative stress (**Ling *et al.*, 2010**). In our study the induction of pyocins was conducted by recA system activation. Obviously it resulted in more intensive synthesis of S1 pyocin as compared with S5 pyocin (Figure 2).

Application of previously described methods of induction optimization (**Balko *et al.*, 2013**) gave us an opportunity to obtain initial *P.aeruginosa* UCM B-333 lysates with high activity indices – $7.68\times10^6$ AU/mL (Table 1). In turn, according to Sano i Kageyama data (**Sano and Kageyama, 1981**), activity of pyocins AP41 in initial *P. aeruginosa* PAF41 lysates was $10^2$-$1.6\times10^3$AU/mL, pyocin SA189 from *P.aeruginosa* SA189 – $6.4\times10^2$ AU/mL (**Naz *et al.*, 2015**), and pyocin C9 from *P. aeruginosa* C9 – $1.4\times10^4$ AU/mL (**Higerd *et al.*, 1967**). Also positive feature of our lysates was low concentration of protein. It not only heightens specific activity considerably, but also reduces the need to carry out additional stages of purification required for isolation of minimal killer structure. Protein content in our lysates was equal to 0.2 mg/mL (Table 1), whereas according to Higerd et al. this parameter for pyocin C9 was 14.4 mg/mL (**Higerd *et al.*, 1967**), and for pyocin SA189 – 2.9 mg/mL (**Naz *et al.*, 2015**).

The method of ion-exchange chromatography allows to separate protein bacteriocins according to their charges (**Ito *et al.*, 1970**). For separation of bacteriocin mixture and to obtain purified S1 pyocin by DEAE-cellulose chromatography we used elution by 20 mM Tris-HCl buffer without NaCl (Figure 3). The same conditions were applied by Ito et al. (**Ito *et al.*, 1970**), who isolated and characterized this pyocin. These researchers stated that S1 pyocin didn't bind with DEAE-cellulose and passed through the column freely. The isolation of S5 pyocin we conducted by means of elution by Tris-HCl buffer containing 0,1 M NaCl (Figure 3). It should be noted that this method was firstly applied to obtain pyocin of this subtype. Therefore specific activity of our killer agent prevailed over the indices of the substances described above authors (**Higerd *et al.*, 1967; Sano and Kageyama, 1981; Naz *et al.*, 2015**) in 4-5 degrees at majority stages of concentration and purification. But at the stage of purified fractions difference in noted parameters decreased to 10-100 times, and for pyocin C9 - $1\times10^6$ mg/mL (**Higerd *et al.*, 1967**) even predominated a little the activity of the 27nd fraction ($7{,}7\times10^5$ mg/mL; Table 1).

It is known that majority of investigated S-type bacteriocins of *P. aeruginosa* possess nuclease properties (**Sano *et.al.*, 1993; Ghequire and De Mot, 2014**). Among them S2 pyocin is characterized by the highest activity, whereas such indices for S3 and AP41 are slightly lower (**Sano and Kageyama, 1981; Sano *et al.*, 1993; Duport *et al.*, 1995**). Moreover, it was noted that activity of S1 pyocin is twice lower then in S2 pyocin (**Sano *et al.*, 1993**). The minimal duration of bacteriocin contact with phage or plasmid DNA is 30 minutes. During this period

bacteriocins with nuclease activity are able to hydrolyze appropriate substrate. In our study cleavage of phage λ DNA by S1 pyocin from the 10th fraction was incomplete, that was detected as partial residue of linear form and appearance of stretched track of undigested DNA fragment (Figure 4I B). The revealed regularity can be connected not only with lower concentration of S1 pyocin in reacting mixture but with it low ability to hydrolyze DNA too.

Among *P. aeruginosa* S-type pyocins capacity for pore-formation is shown only for S5 pyocin. The influence of this killer agent on bacteria lead to disorder in cell membrane continuity and outflow of cell DNA into extracellular area (**Six *et al.*, 2020**). The substance from the 27th fraction, which contains S5 pyocin, didn't hydrolyze phage DNA (Figure 4I C), but were able to cause pore-formation (Figure 4II). Increase of cell DNA content in samples after influence of bacteriocin from the 27th fraction on *P. aeruginosa* UCM B-10, so as on *P. savastanoi* pv. *phaseolicola* 4012 was similar and exceeded the values of the untreated samples in 6.8-8.1 times. Analogous correlation was observed after bacteriocin-cell interaction during 1 and 3 hours. The threshold sensitivity of analyzer is 10 ng, therefore indices lower then this value can be unreliable. If we equate content of nucleic acids in untreated samples to this minimal index, influence of S5 pyocin on *P. aeruginosa* UCM B-10 during 1 and 3 hours results in increase of DNA content in 6.4 and 6 times (Figure 4II A), and against *P. savastanoi* pv. *phaseolicola* 4012 – in 4 and.3.4 times correspondingly (Figure 4II B). Ling H., Saeidi N., Rasouliha B.H. et al (**Ling *et al.*, 2010**) showed that interaction of S5 pyocin with *P. aeruginosa* DWW3 cells during 1 hour led to increase of nucleic acids outflow approximately in 4 times as compared with reference sample. Our results concerning influence of S5 pyocin on phytopathogenic microorganisms coincide with data of mentioned scientists and activity indices against related strains even exceed. Presented data indicate that our S5 pyocin is more active against closely-related *P. aeruginosa* strains then against phylogeneticaly distant *P. savastanoi* cultures, though its activity concerning last ones was high too (Figure 5I).

It was shown that killer activity spectrum of S5 pyocin is the broadest among all investigated S-type pyocins (**Elfarash *et al.*, 2014**). We found out genes of FptA receptors in genomes of all analyzed cultures (Figure 5II), this fact can explain wide spectrum of S5 pyocin activity. Perhaps this regularity is connected with influence of these substances on all tested *P. syringae* strains.

## CONCLUSION

It was determined that active substances in *P. aeruginosa* UCM B-333 lysates are pyocins of S1 and S5 types, but only S5 pyocin possess activity against phytopathogenic *Pseudomonas syringae* strains. fpt A receptor gene was revealed in genomes of all analyzed cultures, including used phytopathogenic strains. This fact can explain wide spectrum of pyocin S5 activity.

Our results are important for determining the pyocin subtype that inhibit the growth of *P. syringae* strains, as well as for the further development of methods to increase the expression of this particular pyocin by the producer strain. Previously we have shown that separation of pyocins is inexpedient, since it results into considerable decrease in bacteriocin activity indices (**Balko, 2019**). It should also be noted that the use of separation methods in industrial conditions can significantly complicate the process of obtaining and increase the cost of finished preparations of pyocins. Since S1 pyocin does not affect *P. syringae* strains, it is necessary to achieve an increase in the concentration of only S5 pyocin in the initial lysates. Whereafter use of bacteriocins in field is more appropriate in the form of crude lysates. A more technologically complicated way of further practical application of S5 pyocin is also possible. Described pyocin or constructed on their base chimeric or hybrid bacteriocins can be used for development of genetic constructions in biotechnological strains for their further application in national economy.

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