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PHENOTYPIC AND GENOTYPIC CRITERIA FOR THE SCREENING OF HIGHLY ACTIVE S-TYPE PYOCINS *PSEUDOMONAS AERUGINOSA* PRODUCERS

Bacteriocins of Pseudomonas aeruginosa, especially S-type pyocins, show high efficiency as analogs of antimicrobial drugs. Various screening methods can be used to identify producers of highly active pyocins, but there are no clear criteria for selecting perspective strains. The ***aim*** *of this work was to determine criteria that can be used during phenotypic and genotypic screening for the selection of perspective highly active S-type pyocin P. aeruginosa producers.* ***Methods.*** *The objects of investigation were 40 P. aeruginosa strains. Pyocins were obtained from each culture, relative coefficients of activity spectrum and sensitivity were determined for all the strains used. The obtained results of the phenotypic screening were compared with the data of the genotypic screening.* ***Results.*** *The use of the proposed method of activity assessment according to the lysis intensity made it possible to phenotypically assess the expression of pyocin genes. It was established that according to the new criteria, only one strain — P. aeruginosa UCM B-333 — can be included in the group of the most active pyocin producers that inhibit the growth of more than 75% of indicator cultures. The majority of representatives of maximally and highly active producers were characterized by high resistance to the action of other pyocins, which can be considered as an additional criterion for the selection of perspective strains. During genotypic screening, it was established that the quantity of pyocin genes in the genome cannot be interpreted as a clear criterion of the producer's perspective. However, 50% of representatives of maximally and highly active pyocin producers were characterized by the presence of two pyocin genes, while in 47.7% of moderately active and 54.5% of low active producers, one pyocin gene was detected more often. It was established that with widening the bacteriocin activity spectrum, the detection frequency of pyocin S1 and S5 genes increases, and for pyocin S2 and S3 genes — decreases. Thus, among the producers of maximally and highly active bacteriocins, pyocin S1 and S5 genes were identified with the highest frequency — 42.8% and 78.6%, and pyocin S2 and S3 genes — with the lowest one — 28.6% and 7.1%, respectively. Genes*

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of pyocin S4 with tRNase activity were detected with equally high frequency in all groups of producers. ***Conclusions.*** *The method of activity assessment by the lysis intensity allows not only to determine the presence of pyocins, but also to phenotypically evaluate the level of their expression, which is an important criterion for the selection of perspective producers. Bacteriocins with a wider activity spectrum are synthesized by P. aeruginosa strains with higher resistance to the action of pyocins from other cultures. The most optimal genotypic criterion for the selection of a highly perspective pyocin producer, detection of genes combination of bacteriocins with different mechanisms of action — with DNase activity (pyocin S1) and the ability to pore formation (pyocin S5) — can be considered.*

Keywords: *S-type pyocins, Pseudomonas aeruginosa, producers of highly active bacteriocins, phenotypic and genotypic screening.*

Increasing frequency of isolation of antibiotic-resistant strains of microorganisms, including *Pseudomonas aeruginosa*, a representative of the ESKAPE group of pathogens, poses a global threat to human health (De Oliveira et al., 2020). One of the variants for solving this problem is the necessity to introduce new antimicrobial substances into clinical practice (Wu et al., 2015). It is known that *P. aeruginosa* is able to produce bacteriocins (pyocins) — antibiotic-like substances that affect only closely related strains and do not inhibit the growth of normal human microbiota (Ghequire et al., 2018; Behrens et al., 2017). Among pyocins, there are distinguished high-molecular R- and F-type bacteriocins with a molecular weight of about 1—10 MDa, which resemble the phage tails, as well as colicin-like S-type bacteriocins, which include low-molecular proteins with a molecular weight of 20 to 100 kDa (Ghequire & De Mot, 2014). We also showed that *P. aeruginosa* can synthesize microcin-II-like bacteriocins — substances of peptide nature (Balko, 2019; Balko et al., 2019). The high efficiency of pyocins, especially the S type, as analogu**es** of antimicrobial drugs has been shown in a number of studies *in vitro* and *in vivo* (Behrens et al., 2017; Soltani et al., 2021; McCaughey et al., 2016). Seven bacteriocins of this type — S1-S5, AP41, and M — have been previously described in the literature (Michel-Briand & Baysse, 2002); the existence of eight more is predicted *in silico* (Ghequire & De Mot, 2014).

Phenotypic screening methods are widely used to detect producers of biologically active substances, including bacteriocins (Gudzenko et al., 2019; Dankevich, 2017; Balko & Avdeeva, 2012). However, these classical approaches have some disadvantages: the long duration of the study, significant resource consumption, the need to optimize cultivation conditions, etc. (De Oliveira et al., 2020). Molecular genetic screening for the presence of pyocin genes in *P. aeruginosa* strains can be regarded as a faster and more sensitive method of detecting bacteriocin producers. However, the criteria that must be followed when choosing perspective producers of pyocins with the maximum spectrum of activity remain unclear — the number of pyocin genes in the genome, the subtype of the identified bacteriocins, which determines the nature of their antimicrobial activity, and the ratio of different pyocin subtypes.

Therefore, the **aim** of this work was to determine criteria that can be used during phenotypic and genotypic screening for the selection of perspective highly active S-type pyocin *P. aeruginosa* producers.

Materials and Methods. The objects of investigation were 40 *Pseudomonas aeruginosa* strains: UCM B-1 — UCM B-3, UCM B-5 — UCM B-10, UCM B-12 — UCM B-16, UCM B-329 — UCM B-349, UCM B-351 — UCM B-353, UCM B-900, UCM B-907, from Ukrainian Collection of Microorganisms (UCM, Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine).

Isolation of bacteriocins (pyocins) from 40 *P. aeruginosa* strains were carried out after induction with nalidixic acid at a final concentration of 100 μg/mL, as previously described (Balko et al., 2013).

Antimicrobial activity of pyocins was tested by the «two-layer agar» method (Balko & Avdeeva, 2012). All 40 *P. aeruginosa* strains noted above were used as indicator cultures.

The activity spectrum of *P. aeruginosa* bacteriocins against the used indicator cultures was assessed by the transparency of the formed lysis zones (Tovkach, 1998). In the case of the appearance of a completely transparent zone of lysis in the area of bacteriocin application, the index of pyocin activity was interpreted as 1. When non-absolute transparency of the lysis zone was observed, the index of activity was assessed as 0.75, moderately expressed transparency — as 0.67, partial transparency — as 0.5, light transparency — as 0.33, and insignificant transparency — as 0.25. The absence of growth inhibition zone was considered as the absence of antimicrobial activity, and in this case, the activity index was interpreted as 0. Subsequently, all activity indices of the certain pyocin were summed up, divided by the number of used indicator cultures and multiplied by 100 to evaluate as a percentage. The obtained coefficient was designated as A — the relative coefficient of the pyocin activity spectrum. The studied producers were placed in order of decreasing coefficient A of bacteriocins synthesized by them. The number of the certain strain in order of decreasing coefficient of activity spectrum of pyocins synthesized by it was marked as NA and was considered as a perspective of the producer.

The resistance of *P. aeruginosa* indicator cultures to the action of pyocins was determined similarly to the approach described above (Tovkach, 1998). For this, the activity indices of all pyocins on the corresponding culture were summed up, divided by the number of applied pyocins and multiplied by 100 to express as a percentage. The obtained coefficient was denoted as S — the relative coefficient of the sensitivity spectrum. Indicator strains were placed in order of increasing S. The number of the certain culture in order of increasing sensitivity spectrum was designated as NS. The resistance of the *P. aeruginosa* strain to the action of pyocins was evaluated according to this coefficient.

Molecular genetic screening for the presence of pyocin genes in 40 studied *P. aeruginosa* strains was performed using PCR. Total DNA was isolated from *P. aeruginosa* cell suspension using GeneJetGenomic DNA Purification Kit (ThermoScientific) according to manufacturer's

Table 1. **List of primers used in the study**

Primer	Sequence (5'→3')	Amplicon size, bp	Reference
S1_F	CGACCCATTGCTGACCTTAT	646	(Dingemans et al., 2014)
S1_R	CCGCCTCAATACTTGCTTTG		
S2_F	ATGCTTTGCCTCAACTGACC	117	(Dingemans et al., 2014)
S2_F	TCAAGGCATTGTTTGCAGTC		
S3_F	CCGCTGGAAGTGGACATTTA	312	(Dingemans et al., 2014)
S3_R	CCCCTCCTCTAGCAATCCTT		
S4_F	GAGGGTGGACTGAGGTTGAA	200	(Dingemans et al., 2014)
S4_R	ATCGTTCCGATCTGCAATTT		
S5_F	ACGCAGGACAGAAGCAGAAC	459	(Dingemans et al., 2014)
S5_R	GCAATACCCACAAGCCAACT		
AP41_F	GGCGTTGCTACCGGTAATG	304	(Dingemans et al., 2014)
AP41_R	CGCTTTCCAGAGGGACTACA		
M_F	CATCCCTTCAGCAGTTTCGG	132	This study
M_R	ACGCTCCGAATTGTAGGGAT		

protocol. To detect genes encoding *P. aeruginosa* pyocins S1, S2, S3, S4, and S5, a multiplex PCR was carried out. PCR mixture composition, temperature parameters of multiplex PCR were as described elsewhere (Dingemans et al., 2014). The PCR mixture to amplify pyocin AP41 and M gene fragments contained 2x DreamTaq PCR Master Mix (ThermoScientific), 30 pmol of Forward and Reverse primers, and 50 ng of DNA. The cycling parameters were as follows: 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). Primers for the amplification of the pyocin M gene were designed using GenBank, KEGG, MEGA 6, BLAST, and Primer 3 program (http://primer3.ut.ee/). Primers used for amplification are listed in Table 1. Type strain *P. aeruginosa* PAO1 containing genes of pyocin S2, S4, and S5 (Elfarash et al., 2012) was chosen as a reference control.

Results. Conducting an extended evaluation of the pyocin activity spectrum made it possible to divide 40 studied *Pseudomonas aeruginosa* strains into five groups of producers. Strains were classified as maximally active, if their bacteriocins inhibited the growth of more than 75 % of *P. aeruginosa* strains. When bacteriocins influenced 50—75 % of cultures, producer-strains were regarded as highly active. In the case of affecting more than 25 % but less than 50 % of the *P. aeruginosa* strains, their producers were classified as moderately active. When bacteriocins were active against less than 25 % of the studied cultures, these strains were classified as low-active producers. In case of the absence of detectable inhibition after cultures treatment with supernatants, producers were classified as inactive. It should be noted that only *P. aeruginosa* UCM B-333 was included in the group of maximally active producers (Table 2).

Bacteriocins of this strain were characterized by the widest spectrum of activity and affected

Table 2. **Arrangement of *Pseudomonas aeruginosa* strains according to the pyocin activity spectrum**

Maximally and highly active			Moderately active			Low active and inactive		
NA	Strain	A, %	NA	Strain	A, %	NA	Strain	A, %
1	B-333	75.7	15	B-10	47.2	30	B-16	22.2
2	B-335	71.9	16	B-15	44.4	31	B-329	19.4
3	B-13	71.1	17	B-334	44.4	32	B-348	19.4
4	B-7	69.2	18	B-338	44.4	33	B-347	19.4
5	B-9	68.2	19	B-900	41.7	34	B-331	13.9
6	B-6	64.9	20	B-345	41.7	35	B-339	5.6
7	B-353	64.4	21	B-336	38.9	36	B-3	0
8	B-349	62.7	22	B-352	38.9	37	B-2	0
9	B-330	61.1	23	B-337	36.1	38	B-5	0
10	B-332	59.6	24	B-12	33.3	39	B-8	0
11	B-340	55.6	25	B-14	30.6	40	B-351	0
12	B-907	52.1	26	B-344	30.6			
13	B-1	50.7	27	B-343	27.8			
14	B-342	50.0	28	B-341	27.8			
			29	B-346	25.0			

NA — number in order of decreasing pyocin activity spectrum; A — relative coefficient of the pyocin activity spectrum.

75.7 % of the studied cultures. Since the group of maximally active producers contained only one representative with activity indicators that slightly differed from 75%, we decided to combine this group with the highly active producers. The newly formed group of maximally and highly active producers included 14 strains. 15 cultures were referred to the group of moderately active producers. The group of low-active producers was represented by 6 cultures, among them *P. aeruginosa* UCM B-339 has the narrowest activity spectrum. Its activity index was about 5.6 %, i.e., slightly higher than 0 %. Therefore, we decided to combine representatives of this group with cultures whose pyocins didn't inhibit growth of the used strains. As a result, a new group was formed — low and inactive producers, to which 11 cultures belonged.

The majority of colicin-like S-type pyocins are made of two proteins — the bacteriocin itself and the immunity protein (Soltani et al., 2021). The function of the latter is to protect the producer cells from the lytic action of their own bacteriocins (Elfarash et al., 2012). Quantity of pyocin subtypes will obviously correlate with quantity of immunity protein varieties on the surface of producer cells. So, we assumed, that the activity spectrum of pyocins can be evaluated by checking the resistance of the producer culture to the action of bacteriocins synthesized by other strains. For this, each of the *P. aeruginosa* strains — producers of pyocins was exposed to the action of bacteriocins obtained from other *P. aeruginosa* cultures. To emphasize the applied methodology, in this experiment, the used microorganisms were designated not as producers but as indicator strains. So, we determined the sensitivity indices (S) and assessed their correlation with the activity indices (A) obtained at the previous stage of this study. According to results of this study, all 40 *P. aeruginosa* strains were divided into four groups. Cultures that were sensitive to less than 25 % of used bacteriocins were evaluated as maximally resistant (Table 3). In the case of sensitivity from 25 to 50 % of applied pyocins, the indicator strains were marked as highly resistant, and in the range from 50 to 75 % — as moderately resistant. Cultures whose growth was inhibited by more than 75 % of bacteriocins were classified as low-resistant. Comparing the results of these two research methods, it was established that producers that synthesized pyocins with an extended spectrum of activity are

Table 3. **Arrangement of *Pseudomonas aeruginosa* strains by the sensitivity to pyocins synthesized by other cultures**

Maximally resistant			Highly resistant			Moderately resistant			Low resistant		
NA	Strain	S,%	NA	Strain	S,%	NA	Strain	S,%	NA	Strain	S,%
1	B-330	7.3	10	B-337	25.7	20	B-8	50.4	31	B-351	76.6
2	B-333	10.4	11	B-338	27.1	21	B-345	52.4	32	B-352	78.8
3	B-9	13.4	12	B-343	27.8	22	B-2	53.7	33	B-346	80.8
4	B-6	13.4	13	B-353	31.9	23	B-16	56.1	34	B-334	84.9
5	B-900	13.9	14	B-336	32.8	24	B-329	56.1	35	B-331	86.0
6	B-7	18.8	15	B-15	37.8	25	B-3	58.5	36	B-349	86.2
7	B-1	19.5	16	B-12	38.3	26	B-5	62.2	37	B-348	94.0
8	B-13	22.0	17	B-14	43.9	27	B-339	65.5	38	B-342	94.5
9	B-335	24.3	18	B-907	46.3	28	B-332	65.9	39	B-341	96.0
			19	B-10	46.3	29	B-340	66.9	40	B-344	97.5
						30	B-347	68.3			

NA — number in order of increasing sensitivity spectrum; S — relative coefficient of the sensitivity spectrum.

characterized by lower indices of sensitivity to the action of bacteriocins of other strains.

P. aeruginosa is characterized by the multiplicity of bacteriocin production (Ghequire et al., 2018; Riley & Chavan, 2007). This means that any strain can simultaneously synthesize one or several different subtypes of S-type pyocins. However, it is impossible to estimate the quantity and subtypes of pyocins synthesized by the certain culture using the described phenotypic methods. For this, for each group of bacteriocin producers, we determined the presence of pyocin genes in their genomes by PCR.

It was determined that strains from the group of maximally and highly active producers contained determinants of pyocin S1-S5 subtypes (Table 4). Among 14 representatives of this group, pyocin S5 genes were detected most often (11 strains), pyocin S4 genes were revealed less frequently (8 strains), and only one culture contained pyocin S3 genes.

Table 4. **Distribution of S-type pyocin genes among the group of maximally and highly active *Pseudomonas aeruginosa* producers**

NA	Strain-pyocin producer, *P. aeruginosa* (UCM)	Pyocin genes				
		S1	S2	S3	S4	S5
1	B-333	+	—	—	—	+
2	B-335	—	—	—	+	+
3	B-13	—	+	—	+	+
4	B-7	+	—	—	—	+
5	B-9	+	—	—	—	+
6	B-6	+	—	—	—	+
7	B-353	—	—	—	+	+
8	B-349	—	—	—	+	—
9	B-330	—	+	—	+	+
10	B-332	—	+	—	—	+
11	B-340	—	—	—	+	—
12	B-907	+	—	—	—	—
13	B-1	—	+	—	+	+
14	B-342	+	—	+	+	+

NA — number in order of decreasing pyocin activity spectrum, «+» — the presence of the pyocin gene, «—» — the absence of the pyocin gene.

In the group of moderately active producers, the ratio of genes of different pyocin S subtypes significantly differed from similar parameters in the previous group (Table 5). It should be notedthat in this and the following tables, cultures are arranged and numbered in the same way as at the stage of determining their activity, i.e, in order of decreasing pyocin activity. This presentation of PCR results allows to compare the data on the genes detection with the indices of pyocin's activity. Among 15 representatives of this group, pyocin S4 genes (9 strains) and pyocin S2 genes (8 strains) were detected most often. The frequency of genes detection for the other pyocin subtypes was approximately the same.

11 strains formed the group of low active and inactive producers (Table 6). They were combined into one group according to the activity spectrum of pyocins. However, the results of genetic screen-

Table 5. **Distribution of S-type pyocin genes among the group of moderately active *Pseudomonas aeruginosa* producers**

NA	Strain-pyocin producer, *P. aeruginosa* (UCM)	Pyocin genes				
		S1	S2	S3	S4	S5
15	B-10	+	—	—	—	—
16	B-15	—	+	—	—	—
17	B-334	—	+	—	—	—
18	B-338	—	—	+	+	—
19	B-900	—	—	—	+	+
20	B-345	+	—	—	+	+
21	B-336	—	+	—	—	—
22	B-352	—	—	—	+	—
23	B-337	—	+	—	+	—
24	B-12	—	+	—	—	—
25	B-14	—	+	—	—	—
26	B-344	+	—	+	+	+
27	B-343	—	+	—	+	+
28	B-341	—	—	+	+	—
29	B-346	—	+	—	+	+

NA — number in the order of decreasing pyocin activity spectrum, «+» — the presence of the pyocin gene, «—» — the absence of the pyocin gene.
Coefficients NA are given according to their values in Table 2.

ing established some differences between representatives of this group. Thus, in the genomes of 6 low active producers (cultures numbered 30—35), pyocin S4 genes were most often localized (5 strains), while the genes of other pyocins were detected half as often. Instead, the representatives of inactive producers (cultures numbered 36—40) contained only pyocin S2 genes among all tested bacteriocin genes. It should be noted that during molecular genetic screening, pyocin AP41 and M amplicons were not detected in any of the *P. aeruginosa* strains studied.

When analyzing the dependence of the producer's perspective on the presence of different quantity of pyocin genes in the genome, it was established that 16 of the 40 investigated strains (40 %) contained one gene, 14 (35 %) — two genes, 8 (20 %) — three genes, and only 2 (5 %) — four genes. It should be noted that in each group of bacteriocin producers there were strains that contained one, two, and three genes (Tables 4—6). However, 50 % of the representatives of the group of maximally and highly active pyocin producers were characterized by the presence of two genes (Table 4), while in moderately active and low-active producers, one gene was detected more often — 47.7 % (Table 5) and 54.5 % (Table 6), respectively. Thus, the quantity of identified pyocin genes in the genome cannot be interpreted as a clear criterion of a producer's perspective.

Among the studied cultures, 33 strains (82.5%) contained pyocins with DNase properties, 23 strains (57.5%) — with tRNase properties, and 18 strains (45.0%) — with the ability to form pores. Analyzing the dependence of the producer's perspectives on the mechanism of its pyocins action, it was established that among the representatives of maximally and highly active producers, 10 cultures (71.4%) synthesized pyocins with DNase activity, 8 strains (57.1%) — with tRNase activity, 11 cultures (78.6%) — with pore-forming activity (Table 4).

For moderately active producers, the detection frequencies of pyocins with DNase and tRNase activity did not differ significantly from the characteristics of the previous group — 86.7% and 60%, respectively. However, bacteriocins with pore-forming activity were observed half as often — 33.3% (Table 5). Similar tendency was typical for the group of low and inactive producers. In the genomes of these microorganisms, bacteriocin genes with DNase and tRNase activity were detected in 10 (90.9%) and 6 cultures (54.5%), respectively, but only 2 strains (18.2%) contained pore-forming pyocin genes (Table 6). So, bacteriocin genes with DNase (pyocin S1, S2, and S3) and tRNase (pyocin S4) activities were detected with the same frequency in all groups of producers. Subsequently, the distribution of certain genes with DNase activity among the studied cultures was analyzed. Thus, pyocin S1 genes were not detected among the representatives of the group of low and inactive producers. Instead, 63.6% of the cultures contained pyocin S2 genes, and 27.2% had pyocin S3 genes (Table 6). For the group of moderately active producers, the ratio was somewhat different.

Table 6. **Distribution of S-type pyocin genes among the group of low and inactive *Pseudomonas aeruginosa* producers**

NA	Strain-pyocin producer, *P. aeruginosa* (UCM)	Pyocin genes				
		S1	S2	S3	S4	S5
30	B-16	—	—	+	+	—
31	B-329	—	+	—	—	—
32	B-348	—	—	+	+	—
33	B-347	—	—	+	+	+
34	B-331	—	—	—	+	—
35	B-339	—	+	—	+	+
36	B-3	—	+	—	—	—
37	B-2	—	+	—	—	—
38	B-5	—	+	—	—	—
39	B-8	—	+	—	—	—
40	B-351	—	+	—	+	—

No. — number in the order of decreasing pyocin activity spectrum, «+» — the presence of the pyocin gene, «—» — the absence of the pyocin gene.
Coefficients NA are given according to their values in Table 2.

Pyocin S1 genes contained a significantly higher percentage of strains — 20%, and the frequency of isolation of pyocin S2 and S3 genes slightly decreased — 53.3% and 20%, respectively (Table 5). In the group of maximally and highly active producers, pyocin S1 genes were identified with the highest frequency — 42,8%, and pyocin S2 and S3 ones — with the lowest frequency — 28.6% and 7.1%, respectively (Table 4). The above shows that with widening the bacteriocin activity spectrum, the detection frequency of pyocin S1 genes increases, while the frequency of isolation of pyocin S2 and S3 genes decreases, and bacteriocin genes with pore-forming activity (pyocin S5) were observed most often among the producers of maximally and highly active bacteriocins.

Discussion. In previous studies, we have classified four *Pseudomonas aeruginosa* strains as producers of maximally active pyocins — UCM B-333, UCM B-335, UCM B-7, and UCM B-349, which inhibited 75—90% of closely related strains (Balko & Avdeeva, 2012). However, the aim of that previous work was only to establish the fact of the presence or absence of bacteriocins in the supernatants of all used *P. aeruginosa* strains. When the lysis zone of any transparency was detected in the area where the supernatant was applied, we confirmed the presence of antimicrobial activity, and the index of activity was equal to 1. The absence of the lysis zone was considered as the absence of activity, and the activity index was equal to 0. This approach is convenient during broad screening for bacteriocins capable of inhibiting *P. aeruginosa*. In this study, we were interested not only in the presence of antimicrobial activity but also in its intensity. It is known that the concentration of pyocins in the substrate is determined by the level of expression of their genes and correlates with the transparency of the lysis zones on a sensitive culture (Ghequire & De Mot, 2014; Tovkach, 1998; Riley & Chavan, 2007). Therefore, the activity index was evaluated as 1 only when the lysis zone in the area of bacteriocin application was completely transparent. In the case of partial or slight transparency, the activity index was estimated as part of 1. The activity spectra of bacteriocinsobtained using the proposed method of activity assessment by the lysis intensity partially differed from the data obtained by the previous method. As a result, only one strain remained in the group of maximally active pyocin producers — *P. aeruginosa* UCM B-333 — out of the four strains that we discovered earlier in this group. The other cultures were classified as highly active producers, and for some of them, the number in order of decreasing activity (NA) was changed. So, earlier, the most perspective after *P. aeruginosa* UCM B-333 (NA=1) producers included strain UCM B-335 (NA=2), then UCM B-7 (NA=3), and after it — UCM B-349 (NA=4) (Balko & Avdeeva, 2012). In this study, taking into account the lysis intensity, the NA for *P. aeruginosa* UCM B-335 remained 2, but for strain UCM B-7, it changed from 3 to 4, and for UCM B-349 — from 4 to 8. Instead, the number in order of decreasing activity for UCM B-13 was increased to 3, and for UCM B-9 — to 5. It should be noted that the used in this study method of assessment by the lysis intensity is obviously more correct when establishing the potential of practical use of bacteriocins, since it takes into account not only the ability of strains to synthesize pyocins but also phenotypically evaluates the level of their expression.

When determining the resistance of *P. aeruginosa* cultures to the action of bacteriocins, several microorganisms were assigned to the group of maximally resistant indicator strains. It should be noted that the majority of representatives of this group also belonged to maximally and highly active producers. For example, *P. aeruginosa* UCM B-333 was characterized by a low index of S — 10.4% and belonged both to the group of maximally resistant strains and to the group of producers of maximally active pyocins with the highest index of A — 75.7%. The only exception to this regularity was the *P. aeruginosa* UCM B-900. The sensitivity coefficient of this strain was 13.9, but this culture belonged to the group of mod-

erately active producers. The relative coefficient of the pyocin activity spectrum of this strain was 41.7%, which is slightly less than 50% — the criterion according to which strains were referred to the group of maximally and highly active producers. Representatives of the second group of resistance — highly resistant indicator strains also in most cases belonged to the second group of activity — moderately active producers. In this case, the exceptions were *P. aeruginosa* strains UCM B-353 and UCM B-907 — representatives of highly active producers. However, UCM B-353 was more active producer than UCM B-907 and more resistant to the action of other pyocins than that strain. It should also be noted that coefficient A of *P. aeruginosa* UCM B-907 was 52.1%, which only slightly exceeds 50% — the criterion for belonging to the group of maximally and highly active producers. Similar dependence was also observed in the third resistance group — among moderately resistant indicator strains. So, *P. aeruginosa* strains with higher resistance to the pyocin action produce bacteriocins with a wider spectrum of activity. A similar regularity was also described for carotovoricins *Pectobacterium carotovorum* (*Erwinia carotovora*) (Tovkach, 1998). This is obviously related to combined production of pyocins together with immunity proteins, which are fixed on the cell surface and protect the producer from the action of its own bacteriocins. The more pyocins are synthesized, the more immunity proteins are on the cell surface and the higher its resistance to other bacteriocins. It should be noted that the maximum correlation between the producer's resistance and the activity spectrum of its pyocins was observed for highly resistant strains and decreased with decreasing resistance of microorganisms. The partial discrepancy with some cultures may be due to the ability of microorganisms to produce other, insufficiently studied pyocins, such as, for example, our recently described microcin-II-like bacteriocins or pyocin S9 in *P. aeruginosa* UCM B-333 (Balko, 2019; Balko, 2021; Balko et al., 2022).

For *P. aeruginosa*, primers of six pyocin subtypes (S1—S5 and AP41) have been described (Dingemans et al., 2014), and primers for the amplification of pyocin M gene were designed using GenBank, KEGG, MEGA 6, BLAST, and Primer 3 program (http://primer3.ut.ee/). Based on the mentioned primers, we conducted a search for bacteriocin genes in the genomes of 40 investigated strains. It is known that pyocins are characterized by different mechanisms of influence on sensitive cultures. Thus, pyocin S1, S2, S3, and AP41 possess DNase activity, pyocin S4 has tRNase properties, and pyocin S5 inhibits microorganisms due to the ability to form pores (Soltani et al., 2021; Michel-Briand & Baysse, 2002; Riley & Chavan, 2007; Balko, 2012). According to our results, bacteriocin genes with DNase (pyocin S1, S2, and S3) and tRNase activity (pyocin S4) were detected with the same frequency in all groups of producers, while bacteriocin genes with pore-forming activity (pyocin S5) were observed most often among producers of maximally and highly active bacteriocins. Elfarash et al. (Elfarash et al., 2012) showed that DNase activity is more effective compared to tRNase activity in terms of impact on sensitive cells. Therefore, despite the significant prevalence of pyocin S4 genes among the studied strains, the presence of this bacteriocin only slightly influences the total antimicrobial activity of the substances synthesized by the producer. On the other hand, the activity spectrum of pyocin S5 is the broadest among all pyocins, which is associated with the wide spread of the FptA receptor for Fe (III) pyochelin among *P. aeruginosa*, to which this bacteriocin binds (Ghequire & De Mot, 2014). This fact testifies that one of the criteria for selecting highly active producers can be the presence of pyocin S5 genetic determinants in their genomes.

The following stage was to find out the influence of the ratio of different pyocin subtypes on producer selection. It should be noted that among 40 *P. aeruginosa* cultures, the largest number of strains — 23 (57.5%) contained pyocin S4 genes.

Pyocin S2 and S5 genes were observed somewhat less often — in 19 (47.5%) and 18 (45%) strains, respectively. The genes of pyocin S1 were localized in the genomes of 9 cultures (22.5%), while pyocin S3 — only in 7 strains (17.5%). Ghequire and De Mot (Ghequire & De Mot, 2014) described similar results and reported the presence of pyocin S4 genes in 25 strains and pyocin S5 in 20 cultures. These researchers revealed pyocin S3 and S1 genes more often — in 24 and 23 strains, respectively but rarely (in 11 cultures) — pyocin S2 genes. Also, the mentioned authors indicate the presence of pyocin AP41 genes in 17 strains (Ghequire & De Mot, 2014), while the amplicons of this pyocin were not found among the cultures we studied. This peculiarity may be related to the impossibility of simultaneous localization of pyocin AP41 and S4 genes in the genome (Ghequire & De Mot, 2014), and in the studied *P. aeruginosa* cultures, pyocin S4 genes were identified in more than half of the strains. We also failed to find amplicons of pyocin M. Ghequire and De Mot (Ghequire & De Mot, 2014) indicate that this pyocin was observed quite rarely — only in 9 strains that caused infectious pathology in humans. The presence of this pyocin gene also correlates with the detection of the exoU virulence factor (Kulasekara et al., 2006). Instead, our cultures were isolated not from humans but from the external environment — activated sludge, and this fact may explain the absence of pyocin M genes in their genomes. However, the lack of pyocin AP41 and M genes requires confirmation, which will be done in our more detailed studies in further.

It should be noted that bacteriocin genes with DNase activity were found with a similar frequency in all groups of producers. However, three pyocins possess this activity — S1, S2 and S3 (Michel-Briand & Baysse, 2002; Ohkawa et al., 1973). Therefore, we analyzed the distribution of each of these bacteriocins and showed that with widening the bacteriocin activity spectrum, the detection frequency of pyocin S1 genes increases, while the frequency of isolation of pyocin S2 and S3 genes decreases. It should be noted that among the representatives of the group of inactive producers (NA 36—40, Table 6), in most cases, only pyocin S2 genes were observed. It is known that this bacteriocin is unstable at room temperature and all manipulations for isolation and testing of activity should be carried out at 0—4 °C (Ohkawa et al., 1973). Consequently, this bacteriocin could lose its antimicrobial properties after isolation, which was observed as the lack of influence on the other *P. aeruginosa* cultures. On the other hand, pyocin S1 is much more stable in the external environment and, unlike many other pyocins, does not require the presence of ferripyoverdine receptor on the outer membrane of sensitive cells for its attachment, and therefore does not depend on the concentration of iron ions in the medium (Dingemans et al., 2013). So, the presence of pyocin S1 gene in the genome as pyocin S5 gene can be a criterion to refer the culture to highly active producer.

Moreover, 35.7% of the representatives of maximally and highly active producers with the widest spectrum of activity contained a combination of pyocin S1 and S5 genes. In the group of moderately active producers, only 13.3% of strains contained a set of these genes, and no culture with such combination of genes was found among low and inactive producers. It can therefore be concluded that the given combination of bacteriocin genes with different mechanisms of action — pyocin S1 with DNase activity and pyocin S5 with the ability to pore formation — is the most optimal for strain selection as a highly perspective bacteriocin producer. It should be noted that another similar combination of bacteriocins — pyocin S2 with DNase activity and pyocin S5 — was found among 28.6% of representatives of maximally and highly active producers. It can be assumed that pyocin S5 protects the unstable pyocin S2 from destruction, as we have previously shown for pyocin S5 and microcin-II-like bacteriocins (Balko, 2021).

Conclusions. The method of activity assessment by the lysis intensity allows one not only to determine the presence of pyocins but also to

phenotypically evaluate the level of their expression, which is an important criterion for the selection of perspective producers. Bacteriocins with a wider activity spectrum are synthesized by *Pseudomonas aeruginosa* strains with higher resistance to the action of pyocins from other cultures. The detection of gene combination of bacteriocins with different mechanisms of action, especially with DNase activity (pyocin S1) and the ability to pore formation (pyocin S5), can be considered the most optimal genotypic criterion for the selection of highly perspective pyocin producers.

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ФЕНОТИПОВІ І ГЕНОТИПОВІ КРИТЕРІЇ ДЛЯ СКРИНІНГУ ВИСОКОАКТИВНИХ ПРОДУЦЕНТІВ ПІОЦИНІВ S-ТИПУ *PSEUDOMONAS AERUGINOSA*

Бактеріоцини *Pseudomonas aeruginosa*, особливо піоцини S-типу, демонструють високу ефективність як аналоги антимікробних препаратів. Для виявлення продуцентів високоактивних піоцинів можуть бути застосовані різні методи скринінгу, однак критерії відбору перспективних штамів залишаються невідомими. **Метою** даної роботи було встановити критерії, які можуть бути використані при проведенні фенотипового і генотипового скринінгу для відбору перспективних продуцентів високоактивних піоцинів S-типу *P. aeruginosa.* **Методи.** Об'єктом дослідження були 40 штамів *P. aeruginosa.* З кожної культури отримували піоцини, визначали відносні показники спектру їх активності і чутливості продуцентів до використаних піоцинів інших штамів. Отримані результати фенотипового скринінгу порівнювали з даними генотипового скринінгу. **Результати.** Використання запропонованого методу визначення активності за інтенсивністю лізису дозволило фенотипово оцінити експресію генів піоцинів. Встановлено, що за новими критеріями до групи продуцентів максимально активних піоцинів, які пригнічують ріст понад 75% індикаторних культур, можна віднести лише один штам — *P. aeruginosa* УКМ В-333. Більшість представників максимально- і високоактивних продуцентів характеризувалися високою стійкістю до дії інших піоцинів, що можна розглядати як додатковий критерій для відбору перспективних штамів. За допомогою генетичного скринінгу встановлено, що кількість генів піоцинів в геномі не можна трактувати як чіткий критерій перспективності продуцента. Однак 50% представників продуцентів максимально- і високоактивних бактеріоцинів характеризувалися наявністю генів двох піоцинів, тоді як у 47.7% помірноактивних і 54.5% низькоактивних продуцентів частіше виявляли по одному гену піоцину. Встановлено, що з розширенням спектру активності бактеріоцинів частота виявлення генів піоцинів S1 і S5 зростає, а генів піоцинів S2 і S3 — знижується. Так, серед продуцентів максимально- і високоактивних бактеріоцинів гени піоцину S1 і S5 ідентифікували з найвищою частотою — 42.8% і 78.6%, а піоцинів S2 і S3 — з найнижчою — 28.6% та 7.1%, відповідно. Гени піоцина S4 з тРНКазною активністю виявляли з однаково високою частотою у всіх групах продуцентів. **Висновки.** Метод врахування інтенсивності лізису дозволяє не лише визначати наявність піоцинів, але й фенотипово оцінювати рівень їх експресії, що є важливим критерієм у відборі перспективних продуцентів. Бактеріоцини із ширшим спектром активності виділяють культури *P. aeruginosa*, які характеризуються вищою стійкістю до дії піоцинів інших штамів. Найбільш оптимальним генотиповим критерієм відбору високоперспективного продуцента можна вважати виявлення комбінації генів бактеріоцинів з різними механізмами дії — з ДНКазною активністю (піоцин S1) та зі здатністю до пороутворення (піоцин S5).

Ключові слова: *піоцини S-типу, Pseudomonas aeruginosa, продуценти високоактивних бактеріоцинів, фенотиповий і генотиповий скринінг.*