

<https://doi.org/10.15407/microbiolj87.02.071>

O.B. BALKO^{1*}, O.I. BALKO¹, N.G. SHELKOVA², V.G. VOITSEKHOVSKY², L.V. AVDEEVA¹

¹ D.K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine,
154 Akademika Zabolotnoho Str., Kyiv, 03143, Ukraine

² Bogomolets National Medical University,
13 T. Shevchenko boul., Kyiv, 01601, Ukraine

*Author for correspondence; e-mail: oleksandralko@gmail.com

CREATION OF PYOCIN COMPOSITION AGAINST LABORATORY AND CLINICAL PSEUDOMONAS AERUGINOSA STRAINS

Pseudomonas aeruginosa belongs to the group of ESKAPE pathogens, which most often cause nosocomial infections and are characterized by a high level of antibiotic resistance. Bacteriocins are considered to be one of the promising substances that can be used as an additional means of influencing *P. aeruginosa* strains with multiple antibiotic resistance. The **aim** of this work was to evaluate the possibility of creating an effective composition from the minimum quantity of pyocins capable of inhibiting the growth of laboratory and clinical *P. aeruginosa* strains. **Methods.** The object of the study was *P. aeruginosa* bacteriocins (pyocins) isolated from 10 highly active producers. Analysis of pyocin's activity and subsequent selection of the composition were carried out after assessing its influence on 51 cultures, which included laboratory strains, isolated from plant cultures, and clinical *P. aeruginosa* strains. **Results.** It was established that according to activity spectrum of the studied bacteriocins, pyocins 335, 333, 330, 13, and 332 can be referred to the most promising. To inhibit the growth of laboratory *P. aeruginosa* strains, it is sufficient to use pyocins 335 and 333 added with a third pyocin into the composition — 13 or 332. As for cultures isolated from plants, only pyocins 330 and 332 were characterized by high activity. In contrast, most pyocins influenced clinical isolates, which were multidrug-resistant to widely used antibiotics. The average activity of the selected pyocin composition 332+333+335 against laboratory cultures was 204.8×10^3 AU/mL and against clinical strains — 153.6×10^3 AU/mL. **Conclusions.** Thus, for the first time, the possibility of creating an effective composition of three pyocins that is capable of affecting both laboratory and clinical *P. aeruginosa* strains has been shown. The most optimal combination includes pyocins 332, 333, and 335, which contain S1, S2, S4, S5, and S9 bacteriocin subtypes. These substances do not exhibit mutual antagonism, bind to different receptors, and, due to DNase, tRNase, and pore-forming activities, inhibit the growth of all 51 cultures studied, represented by laboratory and isolated from plant strains, as well as clinical multidrug-resistant *P. aeruginosa* isolates.

Keywords: pyocins, composition creation, *Pseudomonas aeruginosa*, antibiotic resistance, clinical multidrug-resistant isolates, laboratory strains.

Citation: Balko O.B., Balko O.I., Shelkova N.G., Voitsekhovskiy V.G., Avdeeva L.V. Creation of Pyocin Composition Against Laboratory and Clinical *Pseudomonas aeruginosa* Strains. *Microbiological journal*. 2025 (2). P. 71–84. <https://doi.org/10.15407/microbiolj87.02.071>

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It is known that *Pseudomonas aeruginosa* belongs to ESKAPE pathogens (Arbune et al., 2021). Microorganisms of this group most often cause nosocomial infections and are characterized by a high level of antibiotic resistance (Arbune et al., 2021). *P. aeruginosa* strains induce infectious diseases that are difficult to treat (Amann et al., 2019). To overcome the resistance of microorganisms, a search for new drugs is conducted, existing medicines are modified, and antibiotics that have not been used for a long time are returned to clinical practice (Guliy et al., 2014). However, the presence of various resistance mechanisms in *P. aeruginosa* strains to currently widely used antibiotics complicates the prospect of their use in the future (Elfadadny et al., 2024).

One of the promising substances that can be used as additional remedies in the struggle against resistant *P. aeruginosa* strains are bacteriocins (Ahmed et al., 2023; Balko, 2012). They are antimicrobial protein or peptide agents, analogs of narrow-spectrum antibiotics, and affect only closely related strains (Lopetuso et al., 2019). These substances participate in bacterial antagonism and are widely distributed in the microbial world (Zhao et al., 2021; Versluis et al., 2018; Kotsofliak et al., 2004). Bacteriocins of each bacterial species have their specific name, and appropriate substances of *P. aeruginosa* are indicated as pyocins (Balko et al., 2020).

P. aeruginosa pyocins can be divided into high molecular weight analogs of phage-tails with a molecular weight (m.w.) near 1–10 MDa (R- and F-type pyocins), colicin-like S-type pyocins with m.w. from 20 to 100 kDa, and low molecular mass microcin-II-like bacteriocins with m.w. up to 10 kDa (Charkhian et al., 2024; Cubillo-Komarova et al., 2024). Under investigation with experimental animals, it has been shown that S-type bacteriocins are safe and effective for treating diseases caused by *P. aeruginosa* (Soltani et al., 2021). Previously, we have selected producers of highly active S-type pyocins that inhibited the growth of more than 75% of the tested cultures

(Balko & Avdeeva, 2012). Cultivation conditions have been optimized to maximize the activity of these substances (Balko et al., 2013b). It should be noted that pyocins consist of two subunits — the bacteriocin itself, which is responsible for antimicrobial activity, and the immunity protein, which is incorporated into the cell wall of bacterium-producer and protects it from lysis by its own substances (Atanaskovic et al., 2020). As a result, some strains remain resistant to the action of certain types of bacteriocins. The solution to this problem may be the use of not one, even highly active pyocin, but a certain composition of several bacteriocins to provide an effect on a wide range of *P. aeruginosa* strains.

Therefore, the **aim** of this work was to evaluate the possibility of creating an effective composition from a minimum quantity of pyocins capable of inhibiting the growth of laboratory and clinical *Pseudomonas aeruginosa* strains.

Materials and Methods. The objects of this study were pyocins isolated from ten highly active *P. aeruginosa* producer strains — UCM B-6, UCM B-7, UCM B-9, UCM B-13, UCM B-330, UCM B-332, UCM B-333, UCM B-335, UCM B-349, and UCM B-353 from the Ukrainian collection of microorganisms (UCM, Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine) (Pidgorskyi et al., 2007).

Isolation of bacteriocins (pyocins). To obtain *P. aeruginosa* pyocins, nalidixic acid was added to a suspension of strain-producers in the logarithmic growth phase (final concentration 100 µg/mL). After further incubation for 3 h, the induction was stopped by the addition of chloroform. The pyocins were purified from bacterial detritus by low-speed centrifugation at 4000 g for 30 min and subsequently by centrifugation in Amicon Ultra-15 Centrifugal Filter Units (100 KDa). The obtained supernatants were aseptically removed and stored in closed containers at 4–6 °C. Chloroform was used as a preserving agent (Balko et al., 2019). The pyocins were marked according

to the numbers of their producer strains. For example, bacteriocins from *P. aeruginosa* UCM B-333 were designated as pyocin 333, from *P. aeruginosa* UCM B-335 — as pyocin 335, etc.

Compositions 332+333, 332+335, 333+335, and 332+333+335 were obtained by mixing equal volumes of pyocins 332, 333, and 335.

Antimicrobial activity of pyocins was tested by the «two-layer agar» method (Balko et al., 2013b). As indicator cultures, there were used forty laboratory *P. 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, two strains isolated from plants: *P. aeruginosa* UCM B-1107 and UCM B-1108, and nine clinical isolates *P. aeruginosa*. Laboratory strains were obtained from UCM. Most of these strains were isolated from soil and activated the sludge, strains UCM B-1107 and UCM B-1108 — from a rot-affected onion bulb (*Allium cepa*) (Pidgorskyi et al., 2007). Clinical isolates were isolated from different biotopes of patients (wounds, blood, ear, bronchi, abscess) and obtained from the Department of Microbiology and Parasitology with the Basics of Immunology of the Bogomolets National Medical University. The belonging of these strains to certain species was established based on phenotypic properties using a computer program for identification of *Pseudomonas* genus bacteria (Kotsofliak et al., 2003).

The activity of *P. aeruginosa* bacteriocins against the used indicator cultures was assessed by the transparency of the formed lysis zones in the area of bacteriocin application (Balko et al., 2024). If the zone of lysis was completely transparent, pyocin activity was interpreted as 1, in the case of non-absolute transparency — 0.75. Both of these indices were evaluated as high pyocin activities. If partial transparency was observed in the lysis zone, the activity was designated as 0.5 and interpreted as medium. In the case of slight transparency, the activity was estimated as 0.25 and was considered low. The absence of a growth

inhibition zone was interpreted as the absence of antimicrobial activity, designed as 0.

Quantitative indices of pyocins and their composition' activities were determined by the serial two-fold dilutions method. The activity of the substances was defined by the maximum dilution capable of causing lysis zone formation. The obtained results were counted for 1 ml of studied lysate and expressed in activity units — AU/mL or for convenience in $\times 10^3$ AU/mL (Balko & Avdeeva, 2012).

The average activity of single pyocins and their compositions against indicator strains was determined as the sum of the activity indices of bacteriocins used divided by the number of studied cultures.

The sensitivity of clinical *P. aeruginosa* strains to antibiotics was determined using the disk diffusion method, which was performed in accordance with the EUCAST requirements (EUCAST Disk Diffusion Method, 2024). For this purpose, 24 h culture of microorganisms was added to a 0.85% NaCl solution, and suspension was prepared by the turbidity standard of 0.5 McFarland Units, which corresponds to 1×10^8 CFU/mL. The obtained suspension was laid on the surface of Mueller-Hinton agar, after which antibiotic disks were imposed. The plates were cultured at 37 °C for 16—20 h. Antimicrobial activity was assessed by the appearance of growth inhibition zones around the applied disks. The strain's belonging to the group of susceptible, moderately susceptible, or resistant to certain antibiotic was assessed according to breakpoints Eucast ver. 14.0 (The European Committee on Antimicrobial Susceptibility Testing, 2024), CLSI in the M100 33rd ed., or FDA STIC website (Clinical and Laboratory Standards Institute, 2024; FDA Susceptibility Test Interpretive Criteria, 2024).

All experiments were carried out at least in two-three technical repeats; the results obtained were processed using standard software Microsoft Office Excel 2007.

Results. Ten highly active pyocins were obtained from *Pseudomonas aeruginosa* strains. These bacteriocins were characterized by high, medium, and low activity against 40 laboratory *P. aeruginosa* strains (Table 1). It should be noted that pyocin 333 showed high activity against the largest number of cultures tested — 30 strains. However, pyocins 330 and 332 were characterized by the highest average activity (affected 10 strains), and pyocins 335 and 330 — by the highest low activity (influenced 3 cultures). It should be noted that for the selection of a composition capable of affecting all strains of *P. aeruginosa*, not only the activity in the action on sensitive cultures is important but also the ability to inhibit the growth of a larger number of strains. Therefore, the prospects of pyocins for inclusion in a composition were assessed by their total activity — the number of strains affected by the studied pyocin with any activity. According to this criterion, pyocins 335, 333, 330, 13, and 332 were considered the most promising.

Table 1. Division of pyocins by activity against 40 laboratory *Pseudomonas aeruginosa* strains

Pyocin	Sensitivity of <i>Pseudomonas aeruginosa</i> strains to pyocins			
	High	Medium	Low	Total
335	28	6	3	37
333	30	4	1	35
330	22	10	3	35
13	28	6	0	34
332	23	10	1	34
9	25	7	0	32
7	29	2	0	31
353	24	6	0	30
6	28	1	0	29
349	24	5	0	29

Here and in Tables 2, 3: High activity — the number of strains, applying on which pyocin formed a lysis zone with transparency 1 or 0.75; medium activity — 0.5, and low activity — 0.25. Total activity — the number of strains affected by studied pyocin with any activity.

It can be noted that laboratory strains were characterized by different sensitivity to the pyocins used (Table 2). Thus, all ten pyocins showed exceptionally high activity against eleven strains of *P. aeruginosa*, for example, UCM B-2, UCM B-3, UCM B-5, etc. In contrast, *P. aeruginosa* UCM B-336 was affected only by pyocins 335, 353, and 330 with medium activity. Only pyocins 353, 349, and 332 with medium activity, and pyocin 333 with low activity influenced *P. aeruginosa*

UCM B-900. When analyzing the possibility of using selected promising pyocins, the following regularities were discovered. Pyocin 335 inhibited the growth of most of the tested cultures, except *P. aeruginosa* UCM B-335, B-337, and B-900, whereas pyocin 333 did not affect *P. aeruginosa* UCM B-333, B-335, B-336, B-338, and B-343 at all. This indicates that the application of only these two pyocins will not be effective enough since both of them do not inhibit the growth of *P. aeruginosa* UCM B-335. Pyocin 330 did affect the mentioned culture either. Therefore, widening the total spectrum of activity is possible by including a third pyocin into the composition — 13 or 332. Both of them were active against *P. aeruginosa* UCM B-335, and at the same time, pyocin 332 was characterized by high activity and pyocin 13 — by medium activity.

Subsequently, the action of ten highly active pyocins was tested against strains *P. aeruginosa* UCM B-1107 and B-1108, which were isolated from plants (Table 3A). It can be noted that pyocins 335 and 333 selected at the previous stage did not affect these strains. In contrast, pyocins 330 and 332 were characterized by high activity. Another bacteriocin, namely pyocin 13, which was also considered a potential component for the composition, inhibited the growth of only *P. aeruginosa* UCM B-1108 and showed low activity. The above indicates that pyocins 335, 333, and 332 should be included in the composition of bacteriocins with the minimum set of active substances and the widest possible spectrum of activity against laboratory strains of various origins.

The potential application of the selected composition was also tested against clinical isolates of *P. aeruginosa* (Table 3B). It was found that all bacteriocins were characterized by high activity against 5 *P. aeruginosa* cultures, for example, 2220, 2222, etc. Concerning strains 216, 3392, and 3458, most pyocins were also highly active. Isolate 3523 was

characterized by the lowest sensitivity; only bacteriocins 349, 330, 335, and 332 affected it. It can be noted that the possibility of using the selected composition of pyocins — 335, 333, and 332 — against clinical strains is also quite effective, since these substances inhibited the growth of all the mentioned cultures, including *P. aeruginosa* 3523.

Table 2. Sensitivity of laboratory *Pseudomonas aeruginosa* strains to pyocins

Str.	Pyocin activity			Str.	Pyocin activity		
	High	Medium	Low		High	Medium	Low
B-1	333, 332, 6, 349	335, 330, 9,7, 353	—	B-335	332	13, 9, 349	—
B-2	All	—	—	B-336	—	335, 330, 353	—
B-3	All	—	—	B-337	330, 13	333, 9, 353, 6	—
B-5	All	—	—	B-338	13	335, 332, 9, 349	—
B-6	335,333, 7	—	—	B-339	333, 13, 7, 6, 349	335, 330, 9, 353	—
B-7	333, 332, 349	—	335, 330	B-340	335, 333, 330, 13, 9, 7, 353, 6, 349	—	—
B-8	All	—	—	B-341	All	—	—
B-9	335, 7, 349	333, 13	330, 332	B-342	All	—	—
B-10	335,333, 13, 332, 9,7, 353, 6,349	330	—	B-343	349	335, 330, 13, 332, 9, 7	—
B-12	All	—	—	B-344	All	—	—
B-13	335, 333	332, 349	330	B-345	333, 330, 13, 9, 7, 353, 6	335, 332	—
B-14	335,333, 330, 13, 332, 9,7, 353, 6	—	—	B-346	All	—	—
B-15	335, 333, 13, 332, 9,7, 353, 6	330	—	B-347	335, 13, 332, 7, 6, 349	333, 330, 9, 353	—
B-16	335,333, 330, 13, 9,7, 353, 6,349	332	—	B-348	All	—	—
B-329	335, 330, 9, 7, 353, 6, 349	333, 13, 332	—	B-349	335, 333, 330, 13, 332, 9, 7, 353, 6	—	—
B-330	333	13, 332, 349	335	B-351	335, 333, 330, 13, 332, 9, 7, 353, 6	—	—
B-331	All	—	—	B-352	335, 333, 330, 13, 332, 9, 7, 353, 6	—	—
B-332	335, 333, 330, 13, 9, 7, 353, 6, 349	—	—	B-353	335, 333, 13, 9, 7, 6	330, 332	—
B-333	349	330, 13, 332	335	B-900	—	332, 353, 349	333
B-334	335, 333, 13, 332, 9, 7, 353, 6, 349	330	—	B-907	335, 333, 330, 13, 332, 9, 7, 353, 6	—	—

Here and in Table 3: Pyocins are ranged in order of activity decrease established by the transparency of formed lysis zone; All — sensitivity to all ten studied pyocins — 335, 333, 330, 13, 332, 9, 7, 353, 349; «-» — the absence of active pyocins with corresponding activity; Str. — strains.

It is known that in most cases, clinical strains are characterized by resistance to antibiotics (Akeruke et al., 2023). Testing the sensitivity of nine clinical *P. aeruginosa* strains, we also revealed their significant antibiotic resistance (Table 4). It can be noted that only ofloxacin and lomefloxacin retained activity against the indicated strains, while the sensitivity to other drugs in different isolates varied significantly. All cultures were resistant to piperacillin, cefepime, and amikacin, and about half of them — to ciprofloxacin and tobramycin. These antibiotics belong to different groups — penicillins, cephalosporins, aminoglycosides, and quinolones. Taking into account resistance to drugs of three or more groups, the above isolates should be considered as multidrug-resistant strains (Magiorakos et al., 2012). Thus, the studied pyocins and their compositions are capable of inhibiting the growth of not only laboratory but also clinical, multidrug-resistant *P. aeruginosa* strains.

To more thoroughly assess the effect of the selected substances on *P. aeruginosa* cultures, their activity was determined in absolute indices. It was found that the effect of pyocins on laboratory strains *P. aeruginosa* UCM B-3 and B-10 ranged from 204.8 to 819.2×10³ AU/mL (Table 5A). The activity of these substances against clinical iso-

lates was somewhat lower. Thus, the average activities of pyocins 332, 333, and 335 against *P. aeruginosa* UCM B-3 and B-10 were 614.4, 409.6, and 307.2×10³ AU/mL, while against clinical strains — 230.4, 225.6, and 30.4×10³ AU/mL, respectively. The sensitivity of *P. aeruginosa* 3523 was atypical and was not taken into account. The effect of various combinations of selected bacteriocins on microorganisms was subsequently tested. Two- and three-component mixtures of pyocins affected laboratory strains with activity from 204.8 to 409.6×10³ AU/mL, while these mixtures influenced clinical isolates with activity from 51.2 to 409.6×10³ AU/mL (Table 5B). The average activities of pyocin compositions 332+333, 332+335, 333+335, and 332+333+335 against laboratory cultures were 307.2, 204.8, 204.8, and 204.8×10³ AU/mL, and against clinical cultures — 256, 76.8, 76.8, and 153.6×10³ AU/mL, respectively. The above indicates the possibility of using a selected pyocins composition to inhibit the growth of *P. aeruginosa* strains of various origins, although the effectiveness of their action on multidrug-antibiotic-resistant strains is somewhat lower.

Discussion. It is known that *P. aeruginosa* is characterized by the multiplicity of bacteriocin

Table 3. Pyocin sensitivity of isolated from plants and clinical *Pseudomonas aeruginosa* strains

Strains		Activity of pyocins		
		High	Medium	Low
A. Isolated from plants	B-1107	330, 332	9	353
	B-1108	330, 332	9	13, 353, 349
B. Clinical	2220	All	—	—
	2222	All	—	—
	3523	—	330, 349	335, 332
	216	335, 333, 330, 332, 7, 353, 6	13, 9	—
	1683	All	—	—
	1806	All	—	—
	2914	All	—	—
	3392	335, 333, 330, 13, 332, 9, 7, 353, 6	349	—
3458	335, 333, 330, 13, 332, 9, 7, 353, 6	—	—	

Table 4. Sensitivity of clinical *Pseudomonas aeruginosa* strains to antibiotics

Antibiotics	Clinical <i>Pseudomonas aeruginosa</i> strains								
	2220	2222	3523	216	1683	1806	2914	3392	3458
PIP*	R	R	R	R	R	R	R	R	R
TZP*	I	I	I	I	I	I	I	I	I
TCC*	I	I	I	I	I	I	I	I	I
FEP*	R	R	R	R	R	R	R	R	R
CZA*	R	S	S	S	S	S	R	S	S
IPM*	R	I	I	I	I	I	I	R	I
MEM*	R	S	I	R	I	I	I	R	S
ATM*	I	I	I	R	I	I	I	I	R
CIP*	I	I	I	I	R	R	R	I	R
LVX*	R	I	I	I	I	I	R	R	I
OFX**	S	S	S	S	S	S	S	S	S
LOM**	S	S	S	S	S	S	S	S	S
AMK*	R	R	R	R	R	R	R	R	R
TOB*	R	S	S	R	R	I	S	R	S
GEN***	S	S	S	S	R	R	I	S	S

S — sensitive, I — intermediate, R — resistant. Penicillins: PIP — piperacillin 30 µg, TZP — piperacillin-tazobactam 30—6 µg, TCC — ticarcillin-clavulanate 75—10 µg. Cephalosporins: FEP — cefepime 30 µg, CZA — ceftazidime-avibactam 10—4 µg. Carbapenems: IPM — imipenem 10 µg, MEM — meropenem 10 µg, ATM — aztreonam 30 µg. Quinolones: CIP — ciprofloxacin 5 µg, LVX — levofloxacin 5 µg, OFX — ofloxacin 5 µg, LOM — lomefloxacin 10 µg. Aminoglycosides: AMK — amikacin 30 µg, TOB — tobramycin 10 µg, GEN — gentamicin 10 µg. *Eucast ver. 14.0; **CLSI in the M100 — 33rd ed., ***FDA STIC website.

Table 5. Activity indices of single pyocins (A) and their composition (B) against clinical and laboratory *Pseudomonas aeruginosa* strains

A		Activity of piocins, ×10 ³ AU/mL			B		Activity of pyocin compositions, ×10 ³ AU/mL			
		332	333	335			332+333	332+335	333+335	332+333+335
Laboratory strains	B-3	819.2	409.6	204.8	Laboratory strains	B-3	409.6	204.8	204.8	204.8
	B-10	409.6	409.6	409.6		B-10	204.8	204.8	204.8	204.8
Clinical strains	2220	102.4	102.4	25.6	Clinical strains	2914	409.6	102.4	51.2	204.8
	2222	102.4	51.2	25.6		3392	102.4	51.2	102.4	102.4
	3523	3,2	—	6,4						
	216	204.8	409.6	25.6						
	1683	102.4	409.6	25.6						
	1806	102.4	204.8	12.8						
	2914	819.2	409.6	51.2						
	3392	204.8	204.8	51.2						
	3458	204.8	12.8	25.6						

production (Balko et al., 2020). This means that each strain can simultaneously synthesize more than one subtype of bacteriocins. We previously have shown that *P. aeruginosa* UCM B-332 synthesizes pyocins of the S2 and S5 subtypes, UCM B-335 — S4 and S5 subtypes, and UCM B-333 — S1, S5, and S9 subtypes (Balko et al., 2024), and also produce microcin-II-like bacteriocins (Balko, 2019). It can be noted that all pyocin stocks with a broad spectrum of action selected for the composition contained at least two bacteriocins, one of which belonged to the S5 subtype. It was established that the ability of a producing strain to secrete S5 subtype pyocin indicates high activity of its bacteriocins (Balko et al., 2024). According to the results of this study, the presence of S5 subtype pyocin may additionally indicate a wide spectrum of action of synthesized bacteriocins. This conclusion is consistent with the results obtained by other authors (Elfarash et al., 2014).

It should be noted that pyocins 332, 333, and 335 differed from each other in the second subtype of bacteriocins in their compositions. Thus, pyocin 332 contained S2 subtype bacteriocin, pyocin 333 — S1 and S9 subtypes, and pyocins 335 — S4 subtype (Balko et al., 2024). It is known that among the colicin-like bacteriocins of *P. aeruginosa*, the S2 subtype pyocin is characterized by the highest activity (Elfarash et al., 2012; Ohkawa et al., 1973). By our results, the average activity was also higher in pyocin 332, which contained the S2 subtype bacteriocin. The lowest activity was peculiar to S4 subtype pyocins, characterized by tRNase properties (Parret & De Mot, 2000). This feature may be connected with the need of these substances to penetrate the cytoplasm and subsequently destroy tRNA, the absence of which is not so critical for bacterial cell survival as DNA destruction or the formation of pores in the cell wall caused by the action of other bacteriocins subtypes (Elfarash et al., 2012). In these studies, pyocin 335, which contained S4 subtype bacteriocin, was also characterized by lower average activity compared to pyocins 332 and 333.

According to the known physicochemical properties of S2 subtype bacteriocin, they are unstable in the external environment and after the isolation of these particles, and all manipulations are recommended to be carried out with the maintenance of the temperature regime within 0—4 °C (Ohkawa et al., 1973). In this study, we isolated all pyocins, including those containing S2 subtype bacteriocins, under the same temperature conditions. At the same time, the high activity of these substances remained. This peculiarity can be explained by the stabilizing effect of microcin-II-like bacteriocins. The production of these substances is a characteristic feature of *P. aeruginosa* strains (Ghequire & De Mot, 2014). We previously showed that the separation of a mixture of microcin-II-like bacteriocins and subtype S1 pyocin into individual substances leads to a rapid loss of activity of the latter. Instead, the use of unfractionated initial lysate ensured the stability of bacteriocins and minimal loss of their activity (Balko, 2021). The above indicates that to maintain a high antimicrobial activity of pyocins, it is advisable to include them in the composition in an unpurified form.

In this study, it was found that the use of one bacteriocin was characterized by higher activity indicators than two ones. For example, the activity of pyocin 333 against *P. aeruginosa* UCM B-3 was 409.6×10^3 AU/mL, and pyocin 335 — 204.8×10^3 AU/mL. The mixture 333+335 contained half the volume of either mentioned pyocin, therefore its activity should amount 307.2×10^3 AU/mL but it was 204.8×10^3 AU/mL. It should be noted that the components of pyocin 333 are bacteriocins of the S1 and S5 subtypes, and pyocin 335 is composed of the S4 and S5 subtypes. In previous studies, pyocins of the above subtypes were detected in *P. aeruginosa* UCM B-342 (S1+ S3+ S4+ S5), which is classified as a producer of highly active bacteriocins, and in *P. aeruginosa* UCM B-344 (S1+ S3+ S4+ S5) and B-345 (S1+ S4+ S5) — producers of moderately active bacteriocins (Balko et al., 2024). Therefore, the reason for

the observed difference is not related to antagonism between different subtypes of pyocins in the mixture. It is also known that S5 subtype pyocins inhibit bacterial growth through pore-forming activity after binding to the FptA receptor (Ghequire & De Mot, 2014), pyocins of the S2 and S4 subtypes — due to their DNase and tRNase properties, respectively, after binding to the FpvAI receptor (Denayer et al., 2007). S1 subtype pyocins are characterized by DNase activity, however, the receptor to which they bind remains unknown but it is not related to the FpvA-type receptor (Ghequire & De Mot, 2014). Thus, S1, S4, and S5 subtypes bacteriocins are characterized by different mechanisms of action and affect sensitive cells after the attachment to different receptors. The above, as well as the multiplicity of bacteriocin production in *P. aeruginosa*, refute the possibility of mutual antagonism when pyocins of different subtypes are used simultaneously. It can be assumed that in the case of mixing several pyocins in a composition, bacteriocins of the same subtype, which bind to similar receptors, will be simultaneously detected. As a result, the number of active particles per cell will decrease. For lysis of a single bacterial cell, about 200 particles of S-type pyocins are required, while with fewer particles, the cell can remain viable (Balko, 2012). Since the bacteriocins mixture was obtained by combining equal volumes of three pyocins, the mixture contains other quantities of certain bacteriocin subtype particles than the same volume of the initial lysate. This may be the reason for the differences found between the activities of initial pyocins and their mixtures.

Nevertheless, it is known that after the synthesis of the bacteriocin, the immunity protein builds into the cell wall of the producer strain and protects it from the damaging effects of its own bacteriocin (Atanaskovic et al., 2020). As a result, if bacteria have genes for a certain subtype of pyocins, these cultures will be resistant to the action of this subtype of bacteriocins synthesized by other strains. Therefore, despite the higher activity of individual

pyocins compared to their mixture, the use of the composition can be considered more promising. Thus, our composition of pyocins 332+333+335 contains particles of five different subtypes — S1, S2, S4, S5, and S9, which significantly reduces the probability that sensitive cultures would have a similar set of immune proteins that would provide resistance to this mixture. Also, the presence of different action mechanisms — DNase, tRNase, and pore-forming activities — will allow pyocin compositions to provide a complex antimicrobial effect on bacteria.

Comparing the effect of bacteriocins on *P. aeruginosa* strains of different origins, it was found that the average activities of pyocins 332, 333, and 335 against multidrug-resistant isolates were 2.7, 1.8, and 10.1 times lower, respectively, compared to the effect on laboratory cultures. Three groups of antibiotic resistance mechanisms have been described for *P. aeruginosa*: intrinsic, acquired, and adaptive (Fernández-Billón et al., 2023). It can be allowed that such intrinsic and acquired mechanisms as a low outer membrane's permeability (Langendonk et al., 2021), presence of chromosomally encoded β -lactamases (Kovalchuk et al., 2019), horizontal gene transfer of aminoglycoside-modifying enzymes and plasmid-encoded β -lactamases (Tooke et al., 2019; Ramirez & Tolmasky, 2010), and modification of genes and porin proteins (Hooper & Jacoby, 2016) — in this case, do not cause increased resistance of cultures to bacteriocins. An important role in *P. aeruginosa* defense is played by an adaptive mechanism, which is realized through the ability to form biofilms (Alfarras & Al-Daraghi, 2024; Balko et al., 2018). We have established that biofilm formation ensures resistance to the action of antimicrobial substances as early as 15 min after the contact of microorganisms with a dense substrate (Balko et al., 2017). It has previously been shown that *P. aeruginosa* strains differ in the intensity of biofilm formation (Balko et al., 2013a). Strains exposed to antimicrobial substances form a more stable biofilm compared to laboratory cultures (Alfarras

& Al-Daraghi, 2024; Ahmed & Abdul Muhsin, 2024). Therefore, it can be assumed that the variability of pyocin effects on clinical strains, especially on *P. aeruginosa* 3523, is associated with the peculiarities of their biofilm formation. Hopefully, these regularities will be investigated further. As already noted, the highest difference between the average activities against laboratory and clinical strains was observed in pyocin 335, which contains bacteriocins with tRNase (S4) and pore-forming (S5) properties, significantly lower — in pyocin 332, which includes bacteriocins with DNase (S2) and pore-forming (S5) activity, and the lowest — in pyocin 333, which is a complex of two bacteriocins with DNase (S1 and S9) and pore-forming (S5) properties. The above indicates that the presence and quantity of particles with DNase activity among the active substances may be decisive. It is known that one of the important components of the biofilm is eDNA, which determines the stability of microorganisms in the external environment (Okshevsky et al., 2015). Therefore, the particles with DNase properties present in the pyocin mixture c destroy eDNA and improve access of bacteriocins of other subtypes to bacterial cells. This statement may be confirmed by the reduction in the difference in the effects on laboratory and clinical strains of a single pyocin, a mixture of two pyocins, and a selected composition. Thus, the average activity of pyocin 335 (S4+S5) against clinical isolates was 10.1 times lower than against laboratory cultures. The similar activity of the pyocin 333+335 mixture (S1+S5+S9 and S4+S5) was only 2.7 times lower, and the smallest difference in effect was noted when using the pyocin 332+333+335 (S2+S5 and S1+S5+S9, and S4+S5) composition and was 1.3 times. The above indicates that the use of a mixture of bacteriocins with different action mechanisms is more effective compared to the application of a single pyocin.

Roulová et al. revealed that multidrug-resistant strains of *P. aeruginosa* are most often characterized by resistance to ciprofloxacin (30.5%), gentamicin (28.8%), and meropenem (27.2%),

somewhat less often to ceftazidime (11.5%), amikacin (11.5%), and piperacillin-tazobactam (11.5%), and in 8.5% — to aztreonam (Roulová et al., 2022). Other researchers indicate that a currently high rate of *P. aeruginosa* resistance to ciprofloxacin, imipenem, and piperacillin-tazobactam remains, although resistance to amikacin, imipenem/relebactam, ceftolozane/tazobactam, and ceftazidime/avibactam is partially decreased (Sastre-Femenia et al., 2023). It should be noted that resistance indices vary significantly in different regions according to the frequency and priority of antibiotics application (Garmasheva et al., 2018). Analyzing the obtained data, we can note that resistance to piperacillin, cefepime, and amikacin was detected in all tested strains, and to ciprofloxacin and tobramycin — in 44% of strains, to meropenem and levofloxacin — in 33%, and to ceftazidime-avibactam, imipenem, aztreonam, and gentamicin — in 22% of cultures. Similar results have been reported by De Oliveira et al., who indicate that among *P. aeruginosa* strains, the most common is resistance to first- and second-generation cephalosporins, piperacillin-tazobactam, aminoglycosides, quinolones, carbapenems and polymyxins (De Oliveira et al., 2020). It can be noted that the clinical isolates we used were also characterized by moderate sensitivity to most antibiotics (marked in Table 4 as I), which allows the use of these drugs only in the maximum permissible concentration. The sensitivity of all isolates was found exclusively to ofloxacin and lomefloxacin (marked in Table 4 as S). However, these drugs are recommended for use only in the CLSI and FDA protocols (Clinical and Laboratory Standards Institute, 2024; FDA Susceptibility Test Interpretive Criteria, 2024), while the EUCAST, whose recommendations apply to medical institutions in European countries, including Ukraine, does not allow prescription of these antibiotics (The European Committee on Antimicrobial Susceptibility Testing, 2024). This indicates the limited number of antimicrobial drugs available for the treatment of infections caused by *P. aeru-*

ginosa. Therefore, the ability of the pyocin composition to suppress not only laboratory but also clinical strains can be regarded as a prospect for its further application in clinical practice.

Thus, for the first time, the possibility of creating an effective composition of three pyocins that is capable of affecting both laboratory and clinical *Pseudomonas aeruginosa* strains has been shown. The most optimal is the combination of pyocins 332, 333, and 335, which contains S1, S2, S4, S5, and S9 bacteriocin subtypes. These substances do not exhibit mutual antagonism, bind

to different receptors, and, due to DNase, tRNase, and pore-forming activities, inhibit the growth of all 51 cultures studied, represented by laboratory and isolated from plants strains, as well as clinical, multidrug-resistant *P. aeruginosa* isolates. The data obtained can be used for the further development of therapeutic agents for local external application in the form of ointment, gel, or spray to prevent infectious processes caused by *P. aeruginosa* on wounds or burn surfaces.

Conflicts of interest. The authors declare that there are no conflicts of interest.

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Received 15.01.2025

О.Б. Балко¹, О.І. Балко¹, Н.Г. Шелкова², В.Г. Войцеховський², Л.В. Авдєєва¹

¹ Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України,
вул. Академіка Заболотного, 154, Київ, 03143, Україна

² Національний медичний університет імені О.О. Богомольця,
бульвар Тараса Шевченка, 13, Київ, 01601, Україна

ПІДБІР КОМПОЗИЦІЇ ПІОЦИНІВ ДЛЯ ПРИГНІЧЕННЯ РОСТУ ЛАБОРАТОРНИХ І КЛІНІЧНИХ ШТАМІВ *PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa відносять до групи ESKAPE патогенів, які найчастіше спричиняють внутрішньо-лікарняні захворювання і характеризуються високим рівнем стійкості до антибіотиків. Одними із перспективних речовин, які можуть бути використані як додаткові засоби впливу на штами *P. aeruginosa* з множинною стійкістю до антибіотиків вважаються бактеріоцини. **Метою** даної роботи було оцінити можливість створення ефективної композиції з мінімальної кількості піоцинів, здатної пригнічувати ріст лабораторних і клінічних штамів *Pseudomonas aeruginosa*. **Методи.** Об'єктом дослідження були бактеріоцини *P. aeruginosa* (піоцини), отримані з 10 високоактивних продуцентів. Аналіз активності піоцинів та наступний підбір композиції здійснювали після оцінки їх впливу на 51 культуру, до яких належали лабораторні штами, виділені з рослин, і клінічні ізоляти *P. aeruginosa*. **Результати.** Встановлено, що за спектром активності досліджуваних бактеріоцинів до найбільш перспективних можна віднести піоцини 335, 333, 330, 13 і 332. Для пригнічення росту лабораторних штамів *P. aeruginosa* достатньо використання піоцинів 335 і 333, а також введення в композицію третього — 13 або 332. Щодо виділених із рослин культур, високою активністю до них характеризувалися лише піоцини 330 і 332. Натомість на клінічні ізоляти, які виявились множинно-резистентними до широко застосовуваних антибіотиків, впливала більшість піоцинів. Усереднена активність підібраної композиції піоцинів 332+333+335 щодо лабораторних культур становила 204.8×10^3 ОА/мл, а щодо клінічних штамів — 153.6×10^3 ОА/мл. **Висновки.** Таким чином, уперше показано можливість створення ефективної композиції з трьох піоцинів, яка здатна впливати як на лабораторні, так і на клінічні штами *Pseudomonas aeruginosa*. Найбільш оптимальним є поєднання піоцинів 332, 333 і 335, які містять бактеріоцини підтипів S1, S2, S4, S5 і S9. Дані речовини не проявляють взаємного антагонізму, зв'язуються з різними рецепторами і завдяки ДНКазній, тРНКазній і пороутворюючій активностям пригнічують ріст усіх досліджених 51 культур, представлених лабораторними і виділеними з рослин штамми, а також клінічними, множинно-резистентними ізолятами *P. aeruginosa*.

Ключові слова: піоцини, створення композиції, *Pseudomonas aeruginosa*, стійкість до антибіотиків, клінічні множинно-резистентні ізоляти, лабораторні штами.