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Antifungal activity and gene expression of lipopeptide antibiotics in strains of *Bacillus* genus

A. Yu. Grabova[1], I. V. Dragovoz[1], L.B. Zelena[1], D. M. Tkachuk[2], L. V. Avdeeva[1]

[1] D. K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine
154, Academika Zabolotnoho Str., Kyiv, Ukraine, 03680

[2] ESC "Institute of Biology",
Taras Shevchenko National University of Kyiv
64/13, Volodymyrska Str., Kyiv, Ukraine, 01601
gau.imv@ukr.net

Aim. To research the antifungal activity and gene expression of lipopeptide antibiotics in strains of genus *Bacillus*. **Methods.** Deferred antagonism method, PCR, qRT-PCR, MALDI-TOF mass spectrometry. **Results.** It was revealed that *Bacillus* sp. strains C6 and Lg37s had the highest antifungal activity among the five tested strains. Based on the molecular genetic methods, it was shown that the expression of genes of lipopeptide antibiotics, related to the fengycin family, occurred in all these strains. At the same time, gene expression of cyclolipopeptide iturin was found in the *Bacillus* sp. strains C6 and Lg37s. It was determined that *Bacillus* sp. C6 strain had the highest level of expression of the fengycin operon`s genes, whereas the lowest level was observed in *Bacillus* sp. C10 strain. By means of MALDI-TOF mass spectrometry, the presence of fengycins in the cell-free cultural fluid of *Bacillus* sp. C6 strain was detected. **Conclusion.** The direct correlation between the level of antifungal activity and the fengycin synthetases expression has not been disclosed. A higher level of antagonism detected for two *Bacillus* strains is more likely associated with the expression and subsequent synthesis of fengycin and iturin.

K e y w o r d s: bacteria of genus *Bacillus*, antifungal activity, MALDI-TOF, lipopeptide antibiotics.

Introducion

Most bacterial species of genus *Bacillus* are non-pathogenic microorganisms that are able to synthetize the wide range of biologically active substances for agricultural and industrial purposes [1]. A lot of *Bacillus* strains can stimulate the growth and development of plants; therefore, they are referred to PGPR (plant-growth promoting rhizobacteria), and they are able to promote plants protection against pathogens via various exometabolites and mechanisms of action [2]. It is known that 4–5 % of the genome of *B. subtillis*, one of the most well studied microorganisms at this moment, contain information about the synthesis of more than 20 antibiotic compounds, amongst which the cyclic lipopeptide antibiotics are the most important [3]. The families of iturins, fengycins and surfactins are related to them. These families represent isoforms that differ in terms of length and degree of fatty acids side chains branching along with amino acids presented in a cyclic peptide [4]. Fengycins consist of one β-fatty hydroxyacid in the side chain and ten amino acids, wherein there is a characteristic Ala-Val dimorphism in the sixth position of peptide ring. Due to their amphiphilic nature, the main target of their activity is a cell membrane, the permeability of which they disrupt [5]. It is also known that fengy-

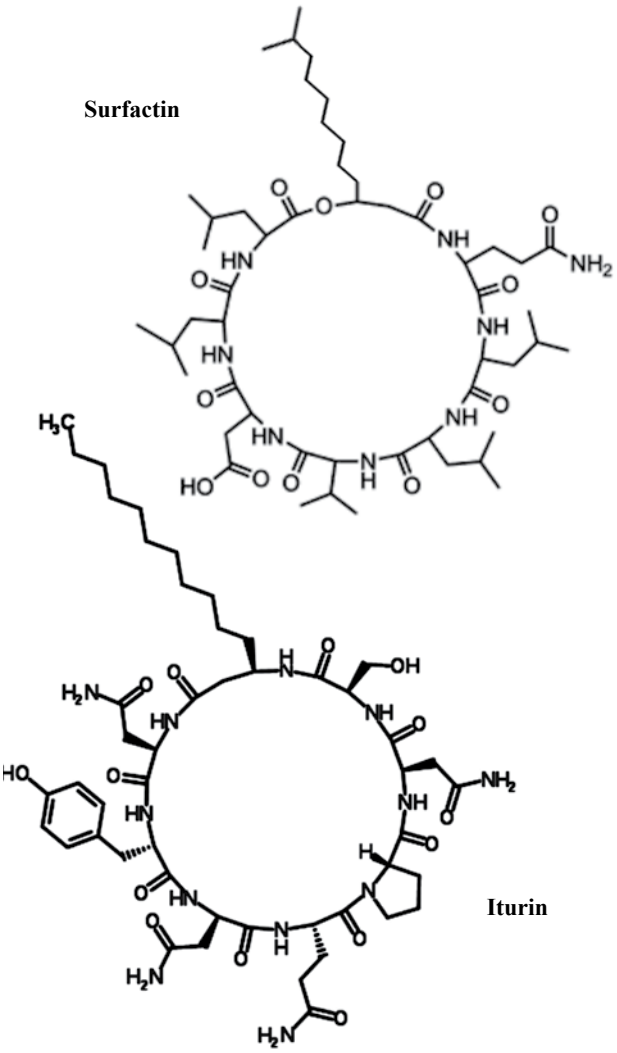
cins possess a high fungitoxic activity. Iturins are cyclic lipopeptide antibiotics that contain one β-amino fatty acid and seven α-amino acids. In the protein fragment of iturins, D-tyrosine locates in the second position, and two extra D-amino acids are in the third and sixth positions. Antibiotics of the iturin family have high hemolytic, antifungal and antibacterial (in a lesser degree) activities, but they lack for the antiviral activity [3, 6]. The surfactin family includes different structural variants, but all surfactins are pentapeptides linked to β-fatty hydroxyacid and have lactonic rings [7]. They are strong biosurfactants with high emulsifying and foaming activities. Owing to their amphiphilic properties, they can upset the permeability of biological membranes by embedding into a lipid layer. It is known that the lipopeptide antibiotics of these families can enhance the activities of each other and thus affect a phytopathogen in a synergistic way [8].

The synthesis of cyclopeptides related to the families of iturins and fengycines is indicative for *B. amyloliquefaciens* [6].

The five *Bacillus* strains with average and high level of antagonism against phytopathogenic micromycetes have been previously collected perviously collected [9]. The purpose of this study was to analyze both the antifungal activity and the expression of lipopeptide antibiotic synthesis genes in selected strains.

Materials and Methods

The objects of the study were five bacterial strains: *Bacillus* sp – C1, C6, C10, Lg24s and Lg37s from the collection of the Department of antibiotics of DK



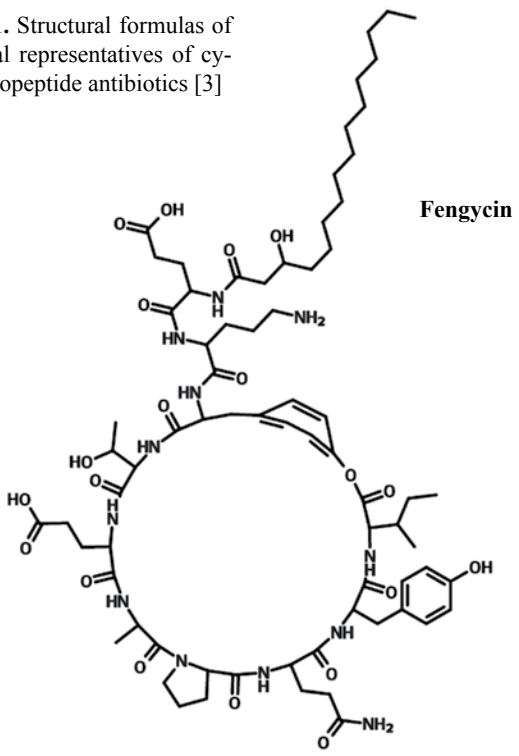


Fig. 1. Structural formulas of typical representatives of cyclolipopeptide antibiotics [3]

Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine. Phytopathogenic micromycetes *Fusarium culmorum* 50658, *F. lactis* 50719, *F. solani* 50718, and *Alternaria alternata* 16873, which were provided by the Department of physiology and taxonomy of micromycetes of DK Zabolotny Institute of Microbiology and Virology of NAS of Ukraine, were used in this study.

The level of antagonism of bacilli against phytopathogenic fungus was studied by the deferred antagonism method in accordance with the Burova's modification [10]. The results were interpreted on the basis of inhibition zones of the tested culture at 12th day of incubation. Based on the inhibition zone values, a conclusion in respect of antagonism has been made: 0 mm – the absence of antagonism between the tested microorganisms, less than 10 mm – poor antagonism, 10-20 mm – medium antagonism, more than 20 mm – high antagonism. The experiments were conducted in triplicate. Statistical data processing was carried out by Microsoft Excel.

For the extraction of lipopeptides, bacteria were cultivated by the batch method under the conditions described in the study [11]. The liquid culture was centrifuged for 30 min at 10000 RPM and +4 °C. Cell-free culture liquid (CL) was acidified with HCl to pH = 2.5, and then was centrifuged for 30 min at 10000 RPM and +4 °C. The obtained pellet was extracted with 80 % ethanol three times, and then the combined extract was evaporated in the rotary evaporator at +55 °C. The dried residue was successively washed with ethyl acetate and acetone, and dissolved in 5 mL of methanol, and then was used as the source of antibiotic compounds [12]. Methanol extract was purified using thin layer chromatography on silica gel 60 F254 plates (with stacking layer; Merck, Darmstadt, Germany). The plates were visualized in the solvent system of chloroform/methanol/water (65:25:4) during 90 min at room temperature. After drying out, one plate was placed in a desiccator with crystalline iodine for 5 min, and then the plate was immersed into 1 % aqueous solution of starch in order to visualize lipopeptides' spots. The silica gel from other plates were removed in the corresponding zones based on the Rf values and extracted with methanol [13]. The evaporated extracts were used for MALDI-TOF-MS analysis.

Mass spectrometric analysis was performed at the shared use center "Matrix-assisted laser desorption/ionization" at the Chuiko Institute of Surface Chemistry of the NAS of Ukraine. Analysis of the lipopeptide fraction of exometabolites of *Bacillus* sp. C 6 strain was performed using Autoflex-II mass spectrometer (Bruker Daltonics, Germany). The measurement was performed in the range from 1000 to 1500 m/z. The H+-matrix ionization with CHCA (α-cyano-4-hydroxycinnamic acid) ("Sigma-Aldrich", USA) was applied. The matrix reagent was prepared by dissolving CHCA (10 mg/mL) in the mixture of the equal volumes of acetonitrile and 0.2 % water solution of trifluoroacetic acid ("Sigma-Aldrich", USA). The reflectory mode of work (in a positive mode) of time-of-flight detector along with applied voltage of 20 kV was utilized. The spectra were processed by using FlexAnalysis software ("Bruker Daltonics", Germany). The monoisotopic values of protonated molecules were measured. The obtained mass spectra represented the averaged values of 150 individual spectra of analyzed compound [14].

The total DNA was extracted from the overnight culture of *Bacillus* cells by using DNA-sorb-B kit ("Amplisens", Russia) according to manufacturer's instructions. The content of the reaction mixture and conditions of the amplification reaction with primers targeted to the genes of fengycin synthesis are provided in the study [14]. The primer sequences targeted to the three genes of fengycin synthesis (*fenA, fenD, fenE*) were designed by using Primer3 software based on the nucleotide sequences of these genes that are provided in the GeneBank along with KEGG (Kyoto Encyclopedia of Genes and Genomes) databases for the species of *Bacillus* genus. (*fenA1*: 5'-GACA GGGGCTGTCTCTGAAG-3', *fenA2*: 5'-TGCATC CCTGATAAAAA-GGC-3'; *fenD1*: 5'-TTGTAACA GAGCGGCATCAG-3', *fenD2*: 5'-TGATAG-CAGA CGTCAAACGC-3'; *fenE1*: 5'-GCGCTCTTTAAC CAGTTTGC-3', *fenE2*: 5'-AAACGTCGTATTTTCC

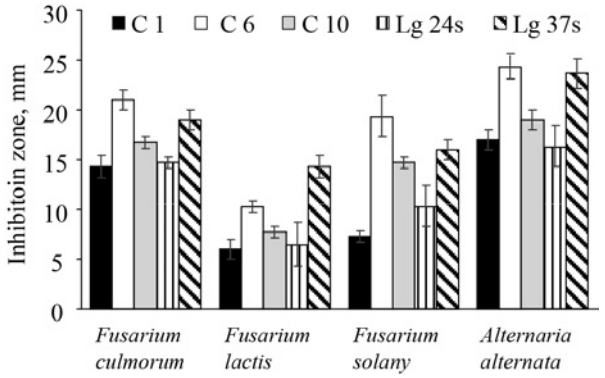


Fig. 2. Antagonism of *Bacillus* strains against phytopathogenic micromycetes

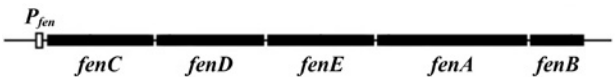


Fig. 3. Structure of fengycin operon [22]

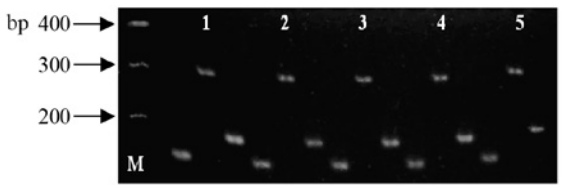


Fig. 4. Electrophoregram of the amplification products with primers targeted to fengycin synthetase genes and DNA isolated from different strains of *Bacillus* sp.:
M - molecular weight marker,
1 – C 1, 2 – C 6, 3 – C 10, 4 – Lg 24s, 5 – Lg 37s;
286 b. p. – *fenA* gene fragment, 132 b. p. – *fenD* gene fragment, 168 b. p. *fenE* gene fragment

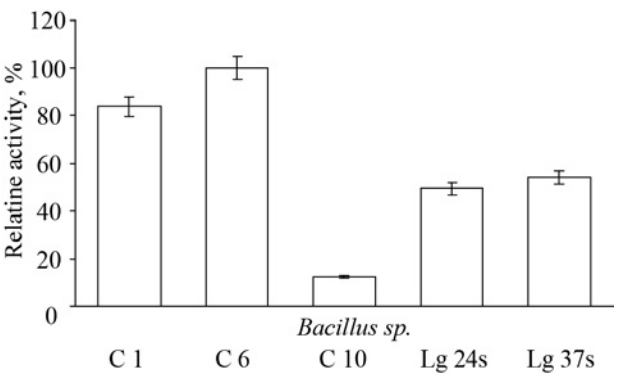


Fig. 5. The relative total expression of fengycin synthetase genes (*fenA, fenD, fenE*) in *Bacillus* strains

Note: 100 % corresponds to the highest level of gene expression. The observed difference between the expression of genes is statistically significant ($p < 0.05$)

GTCG-3'). The sequences of the primers targeted to the *ItuD* gene were listed in the study [15].

For the extraction of total RNA from the overnight culture of bacterial cells, Trizol RNA Prep kit ("Neogene", Ukraine) was used followed by the treatment with DNAse I ("Fermentas", Lithuania). cDNA was synthetized from 1 μg of RNA by using GenPak® MasterMixCore kit ("Neogene", Ukraine). Real-time PCR was performed on the qTower 2.2 thermal cycler (Analytik Jena AG, Germany) under conditions described in the study [16]. The estimation of the relative expression level of gene was conducted according to the $2^{-\Delta\Delta Ct}$ method [17]. The expression of the 16S rRNA gene was assessed as an endogenic control [16].

Results and Discussion

Previously, the *Bacillus sp.* strains C1, C6, C10, Lg24s and Lg37s with relatively high fungicidal effect against phytopathogenic fungi were chosen. In order to confirm the antifungal activity of the selected strains, their antagonism against other relevant micromycetes, which infect and cause diseases of agricultural crops, was studied as well. The results are shown in Figure 2.

It was shown that the most susceptible to the effect of bacilli is *A. alternata* (inhibition zones are in the range of 16.3-24.3 mm), and the least susceptible is *F. lactis* (inhibition zones are in the range of 6-14.3 mm). The highest antagonism with respect to *F. culmorum, F. solani* and *A. alternata* was demonstrated by *Bacillus* sp. C6 strain, and with respect to *F. lactis* – by *Bacillus* sp. Lg37s strain. The obtained results indicated that both *Bacillus* sp. C6 and Lg37s strains have the highest level of antagonistic activity against the relevant phytopathogenic fungi.

Based on the obtained results, we made an assumption that the differences in the antifungal activity of the strains may derive from different level of the antibiotic compounds synthesized by them.

One of such compounds is the cyclolipopeptide fengycin. It is known that, among other biosurfactants, the antibiotics of fengycin family are characterized by a pronounced fungicidal activity [3, 6, 18, 19].

Fengycin is synthesized using non-ribosomal enzyme complex, which is composed of five fengycin synthetases (FenC, FenD, FenE, FenA and FenB). Previously, it was shown that these enzymes are encoded by the genes which are the part of *fen* operon, transcription of which occurs from one promoter *fenp* (Fig. 3) [20].

It is suggested in the prior art to use DNA-markers in order to detect lipopeptide producers (in particular, markers to *fenD* gene in order to detect fengycin synthetics) [21].

Using specific primers the genes of three fengycin synthetases: *fenA, fenD, fenE* were identified in all strains in question (Fig. 4).

For quantitative evaluation of the transcriptional activity level of these genes, RT-PCR was performed, the results of which were indicative for the expression of three genes in all strains. The comparative analysis of the total activity for three genes of the fengycin operon showed that the highest level of expression among the five strains was observed in the strain *Bacillus* sp. C6, and the least – in *Bacillus* sp. C10 (Fig. 5).

It should be noted that there was no direct correlation between the levels of transcriptional activity of the fengycin synthetase genes and antifungal activity of *Bacillus* strains. Thus, the expression in *Bacillus* sp. Lg37s strain was twice as small as maximum, whereas the antagonistic activity of this strain was at the same level as for *Bacillus* sp. C6, but for *F. lactis*, it exceeded the antagonistic activity of *Bacillus* sp. C6. In the meantime, the level of fengycin genes transcription for *Bacillus* sp. C1 was less than maximum by just 16 %, but the antagonistic activity with regard to micromycetes was lower than that of *Bacillus* sp. C6.

Therefore, the ascertained fact of the lack of direct correlation between the level of antifungal activity and the expression of fengycin synthetases suggests another explanation the difference between the strains in the manifestation of antagonism. Since there are literature data declaring that the simultaneous action of cyclolipopeptides from different families in bacilli may enhance their biologic efficiency,

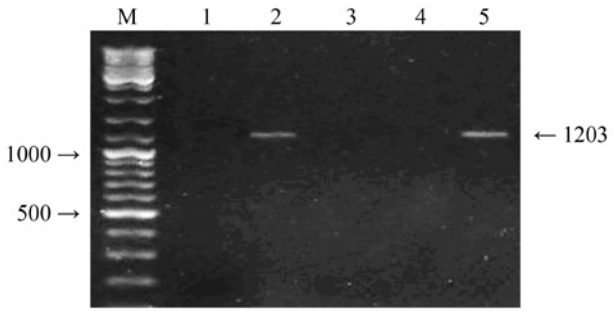


Fig. 6. Electrophoregram of the results of RT-PCR with primers targeted to *ItuD* gene: *M* – molecular weight marker, 1 – *Bacillus* sp. C1, 2 – *Bacillus* sp. C6, 3 – *Bacillus* sp. C10, 4 – *Bacillus* sp. Lg24s, 5 – *Bacillus* sp. Lg37s

we assumed that the high level of antagonism revealed for *Bacillus* sp. C6 and Lg37s strains may be associated with the synthesis of lipopeptide antibiotics of other families. Based on the PCR analysis, it was discovered that the gene of iturin synthesis was present in all tested strains. However, the fact of the gene presence *per se* does not allow a statement about its expression. In order to confirm this hypothesis, the reverse transcription PCR (RT-PCR) targeted to *ituD* gene was conducted (Fig. 6).

It was revealed that the expression of the gene of iturin synthesis was present in two strains, *Bacillus* sp. C 6 and *Bacillus* sp. Lg 37s. Therefore, the difference in antifungal activity of the tested *Bacillus* strains is probably associated with the expression and subsequent synthesis of a wider spectrum of antibiotic compounds, and their simultaneous fungitoxic action (additive effect), which is in line with literature data [8].

Additionally to the discovery of the genes of lipopeptide antibiotics biosynthesis via PCR, MALDI-TOF mass spectrometry is a recognized and widely used method of screening the producers of these compounds [23]. For the *Bacillus* sp. C6 strain, which expresses the genes of fengycin synthetases in the most active mode, the mass spectrometric analysis for the lipopeptide fraction of exometabolites was conducted (Fig. 7).

It was shown that the fengycin peaks for different homologs that correspond to these compounds in MALDI-TOF mass spectrum lie within the range

Fig. 7. MALDI-TOF mass spectrum of lipopeptide fraction of exometabolites for *Bacillus* sp. C6.

from 1471.9 to 1545.8 m/z. The comparison of m/z values experimentally obtained in the analysis of mass spectra of fengycins with analogical literature data is provided in the Table 1 [4, 24–27]

The major peaks in the m/z range from 1471.9 to 1517.9 might correspond to fengycins` isoforms that contain fatty β-hydroxyacid with the hydrocarbon chain of 15–17 carbons in length. In case when m/z = = 1490.0, a double bond is present in the fatty acid chain, which consists of 16 carbon atoms [4]. Ions detected at m/z 1490 and 1518 are likely to be a hydrolyzed form of Na+ adducts of C15 and C17 homologs of fengycin A [26]. The obtained data confirmed and corroborated the results of the molecular genetic analysis of the expression of three fengycin synthesis genes in *Bacillus* sp. C6 strain.

Conclusions

Therefore, it has been revealed that the most active antagonists of phytopathogenic micromycetes amongst the five studied strains are *Bacillus* sp. C6 and Lg37s strains. Using molecular genetic analysis, it has been shown that all studied *Bacillus* strains express the genes for synthesis of the fengycin family

Table 1. **Correspondence of MALDI-TOF mass spectra to known fengycins**

MALDI-TOF peaks obtained in experimental way, m/z	MALDI-TOF peaks from literature, m/z	Fengycin	Amino acid in position 6
1471.9	1471.8	C15fen [M+Na]	ALA
1485.9	1485.8	C16fen [M+Na]	ALA
1490.0	1490.0	C15fenA [M+H2O+Na]	
	1489.9	C16fenB [M+H]	VAL
1499.9	1499.8	C15fen [M+Na]	VAL
	1499.8	C17fen [M+Na]	ALA
1513.9	1513.8	C16fen [M+Na]	VAL
1517.9	1518.0	C17fenA [M+H2O+Na]	

cyclolipopeptides. At that, the level of expression of these genes is the greatest in *Bacillus* sp. C6 strain and the lowest in *Bacillus* sp. C1 strain. In spite of the significant difference between the strains of *Bacillus* sp. C6 and Lg37s in terms of fengycin synthetases` expression level, they did not vary significantly with regard to antifungal activity (except antagonism to *F. lactis*). Apart from fengycins, these two strains have transcription of the gene for synthesis of antibiotics related to the iturin family. By applying MALDI-TOF mass spectrometry, it has been confirmed that lipopeptide antibiotics of the antagonistic strain *Bacillus* sp. C6 relates to the fengycin family. Presumably, the antifungal activity of bacilli depends on the spectrum of synthesized cyclolipopeptides. The role of these compounds in performing the biocontrol using the "phytopathogenic micromycete - plant" model systems is the subject of our further research.

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Протигрибкова активність та експресія генів ліпопептидних антибіотиків у штамів бактерій роду *Bacillus*

Г. Ю. Грабова, І. В. Драговоз, Л. Б. Зелена, Д. М. Ткачук, Л. В. Авдєєва

Мета. Дослідити протигрибкову активність та експресію генів ліпопептідних антибіотиків у штамів бактерій роду *Bacillus*. **Методи.** Метод відстроченого антагонізму, PCR, qRT-PCR, MALDI-TOF мас-спектрометрія. **Результати.** З п'яти досліджених штамів найбільшу протигрибкову активністю показали штами *Bacillus* sp. C6 і Lg37s. Визначено, що у всіх штамів відбувається експресія генів ліпопептідних антибіотиків сімейства фенгіцінов. Для штамів *Bacillus* sp. C6 і Lg37s показана експресія гену синтезу циклоліпопептіда ітуріна, найбільш активно експресуються гени фенгіцінового оперона у штаму *Bacillus* sp. C 6, найменш – у *Bacillus* sp. C 10. За допомогою MALDI-TOF мас-спектрометрії виявлено наявність фенгіцінов в безклітинній культуральній рідини штаму *Bacillus* sp. C6. **Висновки.** Прямої залежності між рівнем протигрибкової активності і експресією фенгіцінсінтетаз не виявлено. Більш високий рівень антагонізму, виявлений у двох штамів бацил, ймовірно, пов'язаний з експресією генів та подальшим синтезом фенгіціну та ітуріну.

Ключові слова: бактерії роду *Bacillus*, протигрибкова активність, MALDI-TOF, ліпопептідні антибіотики.

Антигрибковая активность и экспрессия генов липопептидных антибиотиков у штаммов бактерий рода *Bacillus*

А. Ю. Грабова, И. В. Драговоз, Л. Б. Зеленая, Д. М. Ткачук, Л. В. Авдеева

Цель. Исследовать антигрибковую активность и экспрессию генов липопептидных антибиотиков у штаммов бактерий рода *Bacillus*. **Методы.** Метод отсроченного антагонизма, PCR, qRT-PCR, MALDI-TOF масс-спектрометрия. **Результаты.** Из пяти исследованных штаммов наибольшей антигрибковой активностью обладают штаммы *Bacillus* sp. C6 и Lg37s. Показано, что у всех штаммов происходит экспрессия генов липопептидных антибиотиков семейства фенгицинов. Для штаммов *Bacillus* sp. C6 и Lg37s показана экспрессия гена циклолипопептида итурина, наиболее активно экспрессируются гены фенгицинового оперона у штамма *Bacillus* sp. C6, наименее – у *Bacillus* sp. C10. С помощью MALDI-TOF масс-спектрометрии обнаружено наличие фенгицинов в бесклеточной культуральной жидкости штамма *Bacillus* sp. C 6. **Выводы.** Прямой зависимости между уровнем антигрибковой активности и экспрессией фенгицинсинтетаз не установлено. Более высокий уровень антагонизма, обнаруженный у двух штаммов бацилл, вероятно, связан с экспрессией и дальнейшим синтезом фенгицина и итурина.

Ключевые слова: бактерии рода *Bacillus*, антивогрибковая активность, MALDI-TOF, липопептидные антибиотики.

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