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DETECTION OF PLANTARICIN GENES IN STRAINS OF *LACTOBACILLUS PLANTARUM* – ANTAGONISTS OF PHYTOPATHOGENIC BACTERIA

*The **aim** of investigation was to detect the presence of genes responsible for bacteriocin synthesis in strains of Lactobacillus plantarum with the clear antagonistic effect against the gram-negative phytopathogens. **Methods**. To reveal eleven genes involved in plantaricin synthesis the polymerase chain reaction was used. To test the ability to synthesize bacteriocins as antagonistic compounds the experiments with the lawns of test-strains Listeria ivanovii INRA, Rhizobium radiobacter C58, Ralstonia solanacearum B-1109-UCM, Erwinia carotovora ZM1, Rhizobium vitis ОНУ 389, R. vitis ОНУ 388, R. vitis 379 and R. rhizogenes 15834 were used. **Results**. In genomes of the tested L. plantarum strains the genes plnD, plnEF, plnG, plnI, plnN were present, but the genes plnA, plnB, plnC, plnW were not revealed. Applying the cultural liquids of lactobacilli on the lawns of the test-strains has shown that the cell-free cultural liquid with the initially low pH (4.1–4.3) caused the zones of growth inhibition on the lawns of all test-strains. The neutralized cell-free cultural liquid did not affect the growth of the test-strains. **Conclusion**. Although the tested strains L. plantarum ONU 87, L. plantarum ONU 206 and L. plantarum ONU 991 possessed some genes of plantaricin regulon, in the investigations in vitro they caused the inhibition of the phytopathogens and listerias due to the low pH of the cell-free cultural liquids but not due to the synthesis of bacteriocins. The combination of the genes of plantaricin regulon of L. plantarum ONU 206 and L. plantarum ONU 991 resembled that in L. plantarum J23 described in literature.*

Key words: Lactobacillus plantarum, antagonists, bacteriocins, phytopathogens.

Lactobacillus plantarum bacteria isolated from plant surfaces and dairy products are characterized by the clear antagonistic activity against some phytopathogens [4]. The ability of *L. plantarum* to inhibit bacteria and fungi is strain-specific [15].

Antagonistic activity of lactobacilli is the result of the effects of organic acids, hydrogen peroxide, microbial competition and bacteriocin synthesis [14, 16]. Although the bacteriocin action is described in details only as an effect on gram-positive bacteria, the cases of inhibition of gram-negative bacteria are also known in literature [12, 13]. This makes lactobacilli especially perspective for plant protection as the majority of phytopathogens are the gram-negative bacteria.

In our previous investigation, the use of *Lactobacillus plantarum* ONU 87, *Lactobacillus plantarum* ONU 206 and *Lactobacillus plantarum* ONU 991 strains in plant protection on a model of *Kalanchoe daigremontiana* Mill. was described. Not only the supernatants with low pH but also the supernatant of *L. plantarum* ONU 206 culture

with pH 6.5 caused the inhibitory effect on tumor formation decreasing the amount of infected samples in 70.6% [8]. Basing on the results of investigation, it was supposed that the inhibitory effect of metabolism products from the cultural supernatant of lactobacilli was caused not only by lactic acid, but also by bacteriocins.

Several types of plantaricin regulons are described in literature [6; 11]. The most studied is the regulon of *L. plantarum* C11. The regulatory operon *plnABCD* encodes an inducible peptide-pheromone PlnA, a hystidin-kinase PlnB and the response regulators PlnC and PlnD. The operons *plnEFI* and *plnJKLR* encode two-peptide bacteriocins PlnEF and PlnJK and the corresponding immunity proteins. Operon *plnGHSTUVW* encodes the proteins of ABC transport system necessary for the processing and secretion of a bacteriocin [1; 6; 13].

According to this, the aim of investigation was to detect the presence of genes responsible for bacteriocin synthesis in strains of *Lactobacillus plantarum* with the clear antagonistic effect against the gram-negative phytopathogens *Rhizobium radiobacter*, *R. vitis, R. rhizogenes, Ralstonia solanacearum, Erwinia carotovora*.

Materials and Methods

Strains from the Collection of Microbiology, Virology and Biotechnology Chair of Odesa National I.I. Mechnykov University – *L. plantarum* ОНУ 87, *L. plantarum* ONU 206, *L. plantarum* ONU 991 isolated from dairy products, were brought to the assays.

To test lactobacilli on the ability for the synthesis of bacteriocins, the test-strain *Listeria ivanovii* INRA was used because listerias are the classical test-objects for the detection of bacteriocinogenic activity in lactic acid bacteria. The *Listeria ivanovii* strain was kindly provided by Dr. Thomas Haertle (INRA, Nantes, France). Phytopathogens *Rhizobium radiobacter* C58, *Ralstonia solanacearum* B-1109-UCM і *Erwinia carotovora* ZM1 were kindly provided by the Collection of D.K. Zabolotny Institute of Microbiology and Virology (Kyiv, Ukraine), *Rhizobium vitis* ONU 389 and *R. vitis* ОНУ 388 – by the Collection of Microbiology, Virology and Biotechnology Chair of Odesa National I.I. Mechnykov University, *R. vitis* 379 and *R. rhizogenes* 15834 – by the Collection of Microorganisms of Institute of Agricultural Microbiology, Saint-Petersburg, Russia.

Lactobacilli were cultivated overnight at 37 °C in MRS broth [5]. DNA was isolated by the kit "DNA sorb" (ZNII Epidemiology, Russia). The presence of *pln* locus genes was detected by the method of polymerase chain reaction (PCR): *plnA* [6; 10], *plnB*, *plnC*, *plnD* [6], *plnEF* [1; 6], *plnI*, *plnK* [6], *plnJ* [1; 6], *plnG*, *plnN* [6] and the structural plantaricin W gene [1]. Amplification was carried out by the parameters proposed by Ben Omar et al. (2008) [2]. PCR products were detected by electrophoresis in 1.5% agarose. The markers of molecular weight pUC19/MspI (501, 404, 331, 242, 190, 147, 111 b.p.) and pBR322 DNA/AluI (908, 659, 521, 403, 281, 257 b.p.) were used (Fermentas, Lithuania).

To reveal the antagonistic effect, the overnight cultures of lactobacilli were centrifuged and filtered through 0.22 μm Millipore filters. Phytopathogens and listerias were cultivated overnight at 37 °C in LB broth [3], and after used for the preparing of lawns and testing by the well-diffusion method. The cell-free supernatants both with the initial low pH and neutralized with 1 M NaOH were brought to the wells. The zones of growth inhibition were detected after the overnight cultivation at 37 ºC.

Results and Discussion

The next results of the search for the genes responsible for bacteriocin synthesis in studied *L. plantarum* strains isolated from the home-made dairy products, were obtained (Tab. 1).

Table 1

Presence of the genes involved in plantaricin synthesis in *L. plantarum* strains from dairy products

Strain	plnA	plnB	plnC	plnD	plnEF	plnG	plnI	plnJ	plnK	plnN	plnW
L. plantarum ОНУ 87	-	-	-	+	+	+	+	+	+	+	-
L. plantarum ОНУ 206	-	-	-	+	+	+	+	+	-	+	-
L. plantarum ОНУ 991	-	-	-	+	+	+	+	-	-	+	-

As the obtained data show, none of the studied strains possess all the tested genes of plantaricin regulon. Genes of the recognition system responsible for the synthesis of an inducible peptide-pheromone PlnA, a histidine protein kinase PlnB and a response regulator PlnC were absent. Also none of the strains contained the *plnW* gene. This gene was described in literature only for the one strain, and it was found out that this sequence is rare in *L. plantarum* [7]. Thus, our obtained data coincide with the data of literature.

All the strains possessed the genes of plantaricin synthesis *plnEF* and the gene encoding own immunity against it (*plnI*) [13], and that allowed us to suppose that the studied strains isolated from dairy products could produce the bacteriocin (Fig. 1).

In the strains isolated from dairy products, gene *plnK* was revealed only in one strain – *L. plantarum* ONU 87, but genes *pln*D, *plnEF*, *plnN*, *plnI* were found in all of the three strains mentioned in the table (Fig. 2).

Lack of genes *plnA*, *plnB*, *plnC* and *plnK*, but the presence of *plnD* allows to suppose that strains *L. plantarum* ONU 206 and *L. plantarum* ONU 991 are similar to the strain *L. plantarum* J23described in the literature possessing operon *plNC8IF-NC8HK-D* encoding an inducible peptide PLNC8IF, a hystidine kinase PLNC8HK and response regulator PlnD instead of the regulatory operon *plnABCD*. Besides, this strain lacks the gene *plnK* [11].

The presence of genetic sequences indicates the potential ability of *L. plantarum* strain to produce plantaricin, but in some cases a strain possessing all necessary genes does not secrete active bacteriocin in a medium, or its synthesis is non-regulatory [9]. To check the ability to synthesize bacteriocins as antagonistic compounds, tests on the lawns of indicator-strains were carried out. Applying cultural supernatants of the studied strains of *L. ivanovii* INRA, *Rhizobium radiobacter* C58, *Ralstonia solanacearum* B-1109-UCM, *Erwinia carotovora* ZM1, *Rhizobium vitis* ONU 389, *R. vitis* ONU 388, *R. vitis* 379 and *R. rhizogenes* 15834 we found out that only the supernatants with initial low pH (4.1–4.3) caused zones of growth inhibition on the lawns of all indicator-strains.

Neutralized cultural supernatant did not affect the growth of test-strains. This allowed us to make a conclusion that inhibition of phytopathogens and listerias was the result of low pH of cultural supernatants.

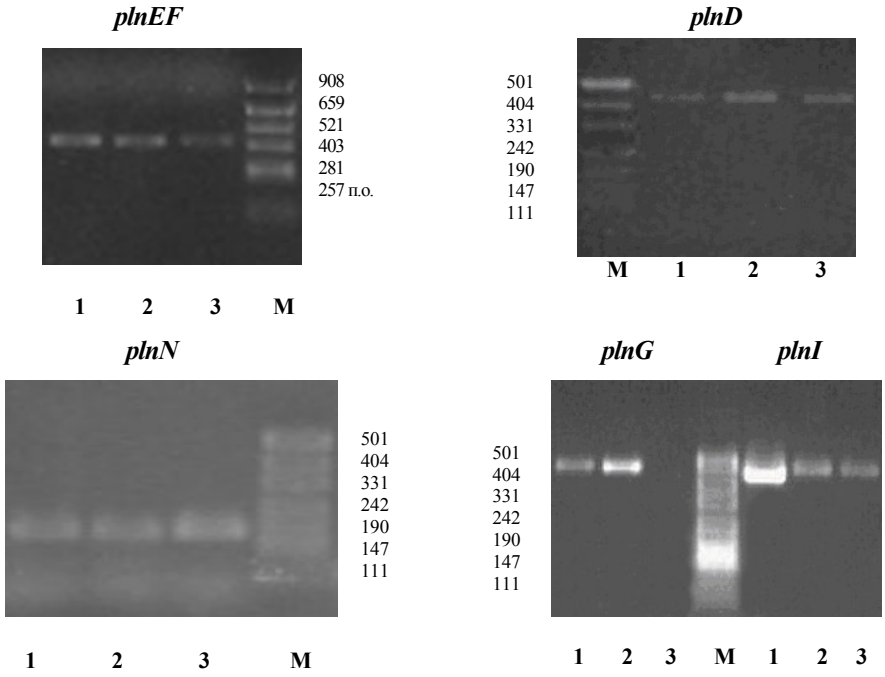

Fig. 1. Electrophoregram of amplification products of PCR with primers to genes *plnEF* (amplicon size 428 b.p., M – marker of molecular weight pBR322 DNA/AluI, Fermentas, Lithuania), *plnD* (amplicon size 414 b.p., M – marker of molecular weight pUC19/MspI, Fermentas, Lithuania), *plnN* (amplicon size 146 b.p., M – marker of molecular weight pUC19/MspI, Fermentas, Lithuania), *plnG* (amplicon size 453 b.p., M – marker of molecular weight pUC19/MspI, Fermentas, Lithuania) and *plnI* (amplicon size 450 b.p., M – marker of molecular weight pUC19/MspI, Fermentas, Lithuania), with DNA of the *Lactobacillus plantarum* strains: 1 – strain ONU 991; 2 – strain ONU 206; 3 – strain ONU 87.

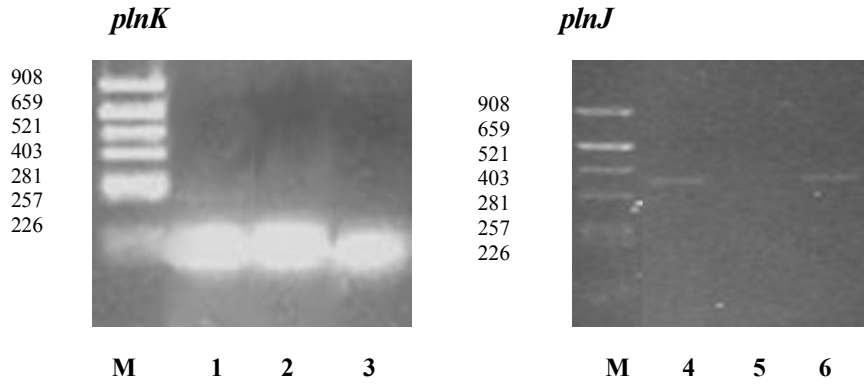

Fig. 2. Electrophoregram of amplification products of PCR with primers to gene *plnK* (amplicon size 246 b.p., M – marker of molecular weight pBR322 DNA/AluI, Fermentas, Lithuania) with DNA of the strains *Lactobacillus plantarum* (1 – strain ONU 87; 2 – strain ONU 206; 3 – strain ONU 991); gene *plnJ* (amplicon size 475 b.p., M – marker of molecular weight pBR322 DNA/AluI, Fermentas, Lithuania): 4 – strain ONU 87; 5 – strain ONU 991; 6 – strain ONU 206)

Thus, the presence of genetic sequences indicates the potential ability of the strain to synthesize bacteriocins but did not allow to make a preliminary conclusion about the active production of antagonistic substances into a medium.

Fig. 3. Zones of growth inhibition on *R. vitis* ONU 388 lawn caused by the supernatants of *L. plantarum* ONU 87, ONU 206 and ONU 991 (from left to right)

Although the tested strains *L. plantarum* ONU 87, *L. plantarum* ONU 206 and *L. plantarum* ONU 991 possessed some genes of plantaricin regulon, in investigations *in vitro* they caused the inhibition of the phytopathogens and listerias due to the low pH of the cell-free cultural liquids but not due to the synthesis of bacteriocins.

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ВИЯВЛЕННЯ ГЕНІВ ПЛАНТАРИЦИНІВ У ШТАМІВ *LACTOBACILLUS PLANTARUM* – АНТАГОНІСТІВ ФІТОПАТОГЕННИХ БАКТЕРІЙ

Реферат

Метою *дослідження було виявити наявність генів синтезу бактеріоцинів у штамів Lactobacillus plantarum, які мають виражений антагоністичний вплив проти грамнегативних фітопатогенів.* ***Методи****. Для виявлення одинадцяти генів, задіяних у синтез плантарицинів, використовували полімеразну ланцюгову реакцію. Для перевірки здатності до синтезу бактеріоцинів як антагоністичних речовин здійснювали тестування на газонах тест-штамів Listeria ivanovii INRA, Rhizobium radiobacter C58, Ralstonia solanacearum B-1109-UCM, Erwinia carotovora ZM1, Rhizobium vitis ОНУ 389, R. vitis ОНУ 388, R. vitis 379 і R. rhizogenes 15834.* ***Результати****. В геномах досліджених штамів L. plantarum були відсутніми гени plnA, plnB, plnC, plnW, але присутні гени plnD, plnEF, plnG, plnI, plnN. Нанесення культуральних рідин лактобацил на газони тест-штамів показало, що лише культуральна рідина з первинним низьким pH (4,1–4,3) спричиняла зони затримки росту на газонах усіх тест-штамів. Нейтралізована культуральна рідина не впливала на ріст тест-штамів.* ***Висновок****. Досліджені штами L. plantarum ОНУ 87, L. plantarum ОНУ 206 і L. plantarum ОНУ 991, хоча й містили низку генів плантарицинового регулону, у дослідах in vitro спричиняли пригнічення фітопатогенів та лістерій за рахунок низьких значень pH культуральної рідини, а не за рахунок синтезу*

бактеріоцинів. За складом генів плантарициновий регулон штамів L. plantarum ОНУ 206 та L. plantarum ОНУ 991 був найближче до описаного у літературі штаму L. plantarum J23.

Ключові слова: Lactobacillus plantarum, антагоністи, бактеріоцини, фітопатогени.

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ВЫЯВЛЕНИЕ ГЕНОВ ПЛАНТАРИЦИНОВ У ШТАММОВ *LACTOBACILLUS PLANTARUM* – АНТАГОНИСТОВ ФИТОПАТОГЕННЫХ БАКТЕРИЙ

Реферат

Целью *исследования было выявление наличия генов синтеза бактериоцинов у штаммов Lactobacillus plantarum, которые имеют выраженный антагонистический эффект против грамнегативных фитопатогенов.* ***Методы****. Для выявления одиннадцати генов, задействованных в синтезе плантарицинов, использовали полимеразную цепную реакцию. Для проверки способности к синтезу бактериоцинов как антагонистических веществ осуществляли тестирование на газонах тест-штаммов Listeria ivanovii INRA, Rhizobium radiobacter C58, Ralstonia solanacearum B-1109-UCM, Erwinia carotovora ZM1, Rhizobium vitis ОНУ 389, R. vitis ОНУ 388, R. vitis 379 и R. rhizogenes 15834.* ***Результаты****. В геномах исследованных штаммов L. plantarum отсутствовали гены plnA, plnB, plnC, plnW, но были выявлены гены plnD, plnEF, plnG, plnI, plnN. Нанесение культуральных жидкостей лактобацилл на газоны тест-штаммов показало, что только культуральная жидкость с первичным низким pH (4,1–4,3) приводила к появлению зон задержки роста на газонах всех тест-штаммов. Нейтрализованная культуральная жидкость не влияла на рост тест-штаммов.* ***Вывод****. Исследованные штаммы L. plantarum ОНУ 87, L. plantarum ОНУ 206 и L. plantarum ОНУ 991, хотя и содержали ряд генов плантарицинового регулона, в опытах in vitro вызывали угнетение роста фитопатогенов и листерий за счет низких значений pH культуральной жидкости, а не в результате синтеза бактериоцинов. По составу генов плантарициновий регулон штаммов L. plantarum ОНУ 206 и L. plantarum ОНУ 991 был ближе к описанному в литературе штамму L. plantarum J23.*

Ключевые слова: Lactobacillus plantarum, антагонисты, бактериоцины, фитопатогены.

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