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FUNDAMENTAL BASIS OF CREATION OF PROBIOTIC WITH PROVITAMIN ACTIVITY BASED ON STRAINS *BACILLUS AMYLOLIQUEFACIENS* IMV B-7513 AND IMV B-7525

*Dysbiotic conditions prevail in both humans and animals. Intestinal dysbiosis is accompanied, in addition to the quantitative and qualitative changes of the microflora, by immunosuppression and metabolic disorders, such as avitaminosis A. Therefore, the search and study of microorganism strains, which may be used for correction of dysbiosis and allow to normalize the homeostasis of a macroorganism, are highly relevant today. In relation to that the use of carotene synthesizing strains of bacteria of *Bacillus* genus is very perspective. This would allow connecting in one preparation the properties of probiotics and ability to compensate the deficiency of vitamin A in a macroorganism.*

*The article presents the analysis of literature data along with the results of own research concerning carotene synthesizing activity of bacteria of *Bacillus* genus, the nature of their carotenoid pigments and their function in a macroorganism. It was determined that carotenoid pigments of *B. amyloliquefaciens* IMB B-7513 and IMB B-7525 strains were presented by a complex with lipids and carbohydrates, and belonged to C_{30} -apocarotenoids, including apo-8'-phytofluene, apo-8'- ζ -carotene, apo-8'-neurosporene and apo-8'- β -carotene-3-ol. A possible way of carotene synthesis was proposed. The end product of its pathway synthesis probably is apo-8'- β -carotene-3-ol, which is able to be metabolized into vitamin A. Earlier it was shown that the investigated strains of bacteria are safe for warm-blooded animals and exhibit probiotic and provitamin activity in vivo.*

*The obtained results provide a fundamental basis for creation of a probiotic preparation with provitamin activity based on carotene producing strains *B. amyloliquefaciens* IMV B-7513 and B-7525.*

*K e y w o r d s: bacteria of the genus *Bacillus*, carotenoid pigments, probiotic features, provitamin activity.*

Dysbiotic conditions present a serious problem being widespread among people, agricultural animals and birds. The solution of this problem involves an application of probiotics based on living cultures of representatives of normal microflora of gastrointestinal tract. In recent years probiotics consisting of bacteria of *Bacillus* genus have become widely used.

Usually intestinal dysbiosis is accompanied by quantitative and qualitative changes of microbiota by immunosuppression and metabolic violations, in particular by avitaminosis A. Therefore, a research is necessary to identify and study microorganisms, the use of which for treatment of dysbiosis will allow to normalize the homoeostasis of macroorganism. In relation to that it is very relevant to use carotene synthesizing activity of bacteria of the *Bacillus* genus, that would allow to unite in one preparation the properties of probiotics and the ability to compensate for the deficiency of vitamin A in a macroorganism.

Carotenoids are a group of natural pigments of yellow, orange or red colors. They are synthesized by plants, protozoa (*Dunaliella salina*), fungi (*Blakeslea trispora*) and bacteria (*Streptomyces*, *Staphylococcus* spp., etc.). Some species of bacteria of the *Bacillus* genus, particularly *B. indicus*, *B. vedderi*, *B. jeotgali*, *B. okuhidensis*, *B. clarkii*, *B. pseudofirmus*, *B. firmus*, have yellow and orange pigmentation of vegetative cells and spores, due to the synthesis of various carotenoids. These pigments are referred to isoprenoid substances. They contain in its structure molecules of isopentenyl diphosphate, and are usually represented by apo- or diapocarotenoids with lipophilic properties [1, 2]. Unlike eukaryotes, bacterial carotenoids are associated with cytoplasmic membrane. They are mainly bound to proteins, lipids and carbohydrates.

Thus, using mass spectrometry methyl-1-(6-C_{10:0})-glycosyl-3.4-dehydro-apo-8'-lycopenoate, methyl-1-glycosyl-3.4-dehydro-apo-8'-lycopenoate, methyl-1-(6-C_{10:0})-glycosyl-3.4-dehydro-apo-8'-lycopene and 1-glycosyl-3.4-dehydro-apo-8'-lycopene were identified in vegetative cells and spores of strain *B. indicus* HU36 [3]. Carotenoids of the vegetative cells had yellow coloring and spores pigmentation was orange.

It is known that the vegetative cells and spores of bacteria of the genus *Bacillus* contain not only the C₃₀-carotenoids, but C₄₀-1-HO-demethylspheroidene and keto/hydroxy- γ -carotene derivatives. Also in the cells and spores of these bacteria neurosporene was found [1].

Despite the presence of a large number of published scientific papers on bacilli pigments, few studies are conducted about carotene synthesizing activity of bacteria of the genus *Bacillus*, properties of bacterial carotenes. There are no data on the patterns of carotenoid pigments biosynthesis, their metabolic transformation in a macroorganism. The methods of extraction, qualitative and quantitative analysis of these pigments are imperfect.

Several schemes of synthesis of carotenes in various representatives of *Bacillus* genus were suggested [1, 3] and different ways of formation of pigments in vegetative cells and spores of these bacteria were observed.

The strains *B. amyloliquefaciens* IMV B-7513 and IMB B-7525 from the collection of antibiotics department of the D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Science of Ukraine were the objects of our investigation. These strains synthesize orange pigment after cultivation on meat-peptone and tryptone soy agar.

The class determination and carotenes identification were performed by means of thin layer chromatography (TLC) and high performance liquid chromatography with mass spectrometry (LC-MS) on liquid chromatography Agilent Technologies 1200 with mass-detector Agilent Technologies G1956B. Chromatographic column Zorbax SB-C18 (2.1×150 mm, 3.5 μ m) was used for pigment separation. The mobile phase included acetone and formic acid. Detection was done by diode-array detector with specter detection range 191–700 nm. Ionization was conducted in ESI and APSI modes with the fixation of positive ions in SCAN mode within the range of 100–1200 m/z [4].

The presence of the gene encoding phytoene synthase which was required for the first steps of carotenoid synthesis was determined by PCR. [5]. The nucleotide sequences of primers for fragment gene amplification were picked using programs MEGA 5.0 and Primer 3.

The evaluation of *yis P* gene expression was performed by quantitative real

time PCR using q Tower 2.2 («Analytik Jena AG», Germany). The relative level of gene expression was calculated with $2^{-\Delta\Delta Ct}$ method [6]. The expression of 16S rRNA was used as an endogenous control.

Statistical analysis of the results was carried out by determination of mean arithmetic values, their standard deviation and mean square error with significance levels 0.05 and 0.01.

An optimal system for pigments extraction from strains *B. amyloliquefaciens* IMV B-7513 and IMV B-7525 was chosen for the investigation of their nature. It was established that full extraction of pigments from bacterial biomass is achieved by using chloroform and methanol solution (2:1). The standard systems for extraction of carotenoids from plants, streptomycetes and fungi were ineffective because of the differences and specificity of the structure of bacilli carotenoids. The obtained pigment extracts gave positive qualitative reaction with FeSO_4 , H_2SO_4 , SbCl_3 and specific for carotenes absorption maxima. However, absorption maxima comparatively to β -, γ - and ϵ -carotenes shifted to the short-wave region.

The extracts were separated by TLC using an adapted system – chloroform:methanol:ethyl acetate (1:1:2). The treatment of chromatograms with group reagents showed that pigments were located in complex with lipids and proteins. Pigments of the investigated strains were divided into 3 different components with R_f 0.3; 0.7 and 0.9.

Since the obtained data could be used to assign the pigments of the investigated strains to the carotenes but not to a specific class, we have determined the presence of the gene, responsible for production of the initial product of the carotene synthesis pathway, in the genomes of the investigated strains of the *Bacillus* genus. Carotenoid synthesis in all carotene synthesizing organisms begins with the formation of phytoene by phytoene synthase [7]. In species *B. subtilis*, *B. amyloliquefaciens* and *B. methylotrophicus* this enzyme is encoded by the gene *yisP*. The presence of *yisP* gene was determined in the genomes of both investigated strains after molecular genetic analysis (Fig. 1), moreover the level of *yisP* expression was higher in the strain *B. amyloliquefaciens* IMV B-7513 than in the *B. amyloliquefaciens* IMV B-7525 (Fig. 2).

Fig. 1. Electrophoregram of phytoene synthase gene amplification (*yisP*) of the *Bacillus* genus bacteria. M – marker, 1 – *B. amyloliquefaciens* IMV B-7513, 2 – *B. amyloliquefaciens* IMV B-7525

Fig. 2. The relative level of transcription activity of phytoene synthase gene of the strains *B. amyloliquefaciens* IMV B-7513 (A), IMV B-7525 (B), control (B)

Thus, the presence of *yisP* indicates that the synthesis of carotenoids by the investigated strains could include two pathways with the production of C_{30} or C_{40} carotenes.

For further identification of pigments HPLC-MS analyzes of carotene extracts of strains *B. amyloliquefaciens* IMV B-7513 and IMV B-7525 was performed. As a result three compounds were determined. Their molecular masses and maxima of absorption spectra corresponded with apo-8- ζ -carotene, apo-8-neurosporene and apo-8'- β -carotene-3-ol which was bound with glucose and a $C_{8:0}$ fatty acid (Table 1). Moreover, during the strain cultivation with diphenylamine, a well-known inhibitor of phytoenedesaturases, the apophytofluene was identified in the extract.

Table 1

Physical and chemical characterization of carotenoid strains
***B. amyloliquefaciens* IMV B-7513 and IMV B-7525**

No	Elution time, min	λ max, nm	Molecular weight	Substance
1	17.4	380, 398, 422	405	apo-8- ζ -carotene
2	18.9	415, 440, 465	498	apo-8-neurosporene
3	22.6	395, 418, 440	723	($C_{8:0}$)-glycosil- apo-8'- β -carotene-3-ol
4*	18.4	330, 345, 364	565	apo-8-phytofluene

* - Data were obtained as a result of diphenylamine inhibition analysis.

It should be noted that in the pigment extracts of strains *B. amyloliquefaciens* IMV B-7513 and IMV B-7525 apolycopene were not found. However, according to the literature data, the microorganisms of the *Bacillus* genus often form apolycopene derivatives linked with sugar and fatty acid residues during the synthesis of C_{30} -apocarotenoids [8, 9]. Therefore, we have assumed that in the investigated strains *B. amyloliquefaciens* after the neurosporene formation

dehydrogenation and cyclization or only cyclization of the isoprenoid chain occurred with the formation of apo-8'- β -carotene-3-ol. This phenomenon requires further investigations using molecular genetic techniques.

In addition to the antioxidant function, carotenoid pigments perform photo and radioprotective activity, are involved in photosynthetic process. In the body of warm-blooded animals some carotenes serve as the provitamin A. Nonoxidased carotenoids also have antioxidant, antitumor and immunostimulatory activity [10]. Some apo-carotenoids reveal similar properties to C_{40} carotenoids, for example provitamin activity [11, 8].

Considering the abovementioned and the fact that the carotenes in a macro-organism may exhibit prebiotic properties, a few of veterinary medications were created based on carotene synthesizing streptomycetes and yeasts. Their application can significantly increase the growth rate of animals, improve their physiological state, prevent diseases, including those associated with metabolic disorders [12]. However, none of the known drugs combines provitamin and probiotic properties simultaneously.

So, strains *B. amyloliquefaciens* IMV B-7513 and IMV B-7525 synthesize C_{30} -apocarotenoids. The end product of their synthesis pathway probably is apo-8'- β -carotene-3-ol, which according to the literature [6], is able to be metabolized into vitamin A. Based on our data, we propose the following way of carotene synthesis by the investigated bacillus strains (Fig. 3).

Fig. 3. Possible way of carotene synthesis by *B. amyloliquefaciens* IMV B-7513 and IMV B-7525

Thus, we isolated carotenoid pigments of strains *B. amyloliquefaciens* IMV B-7513 and IMV B-7525 for the first time. It was established that they represent a complex with lipids and carbohydrates and are related to C_{30} -apocarotenoids, particularly apo-8-phytofluene, apo-8- ζ -carotene, apo-8-neurosporene and apo-8'- β -carotene-3-ol.

Our data provide a fundamental base for using carotene producing bacillus strains as potential components of biological products with provitamin activity. The necessity of such drugs exists not only in medicine but in veterinary due to the diseases of humans, mammals and birds caused by the deficiency of vitamin A, induced by intestine dysbiosis so widespread nowadays. The probiotics based on *Bacillus* strains have been widely used for correction of dysbiotic states in recent years. Synthetic vitamin A or β -carotene that is biotechnologically obtained and is not totally metabolized in the body is used for the prevention and treatment of vitamin A deficiency. Attempts to combine probiotic properties of bacteria and carotene synthesizing abilities in one product have recently emerged. There is a probiotic drug based on strain *Bacillus indicus* HU 36, which produces C_{30} -diapocarotenes [12], characterized only by antioxidant, anticancer and immunomodulating activities.

Information about the simultaneous display of probiotic and provitamin activity by bacilli in world literature is not available. We determined that strains *B. amyloliquefaciens* IMB B-7513 and B-7525 IMB in addition to the ability to synthesize carotenes are also characterized by probiotic properties. In particular, these probiotic properties include the antagonistic activity against opportunistic microorganisms, resistance to low pH levels and concentrations of bile acids, low adhesive and high enzymatic (proteo-, lipolytic, xylano-, cellulolytic, pectinesterase) activity [13]. Strains are harmless for warm-blooded animals [14]. After *per os* ingestion of strains (separately or in a mix) qualitative and quantitative composition of intestinal microbiocenosis in mice and birds is normalised, the microstructure of organs is restored, vitamin A is accumulated in the liver, the cellular immunity is activated [15, 16].

Thus, the study of carotene synthesizing activity of the representatives of *Bacillus* genus highly relevant. These bacteria are characterized by a marked antagonism against opportunistic and pathogenic microorganisms, immunostimulating effect and high biosynthetic activity, manufacturability in the production. We have not only determined the identity of carotenoid pigments of strains *B. amyloliquefaciens* IMV B-7513 and IMV B-7525 to C_{30} -apocarotenes, but also we have developed the fundamentals for creation of a new Ukrainian probiotic preparation with provitamin activity.

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**ФУНДАМЕНТАЛЬНІ ОСНОВИ СТВОРЕННЯ ПРОБІОТИЧНОГО
ПРЕПАРАТУ З ПРОВІТАМІННОЮ АКТИВНІСТЮ НА ОСНОВІ ШТАМІВ
BACILLUS AMYLOLIQUEFACIENS IMB B-7513 ТА IMB B-7525**

Резюме

Дисбіотичні стани широко поширені як у людей, так і у тварин. Дисбіоз кишківника супроводжується, окрім кількісних і якісних змін мікрофлори, імуносупресією і метаболічними порушеннями, зокрема авітамінозом А. Тому актуальним є пошук і дослідження штамів мікроорганізмів, використання яких для корекції дисбіозів дозволить нормалізувати гомеостаз макроорганізму. У цьому зв'язку вельми перспек-

тивним є використання каротинсинтезувальних штамів бактерій роду *Bacillus*, що дозволить поєднати в одному препараті властивості пробіотиків і здатність компенсувати дефіцит вітаміну А в макроорганізмі.

У статті проаналізовано дані літератури і представлено результати власних досліджень щодо каротинсинтезувальної здатності бактерій роду *Bacillus*, природи їх каротиноїдних пігментів та їх функції в макроорганізмі. Встановлено, що каротиноїдні пігменти штамів *B. amyloliquefaciens* IMB B-7513 та IMB B-7525, представлені комплексом з ліпідами та вуглеводами, відносяться до C₃₀-апокаротиноїдів, зокрема, апо-8'-фітофлуену, апо- ζ -8'-каротину, апо-8'-нейроспорину та апо-8'- β -каротен-3-олу. Запропоновано можливий шлях їх синтезу, кінцевим продуктом якого є апо-8'- β -каротен-3-ол, що здатний метаболізуватись у вітамін А. Раніше в дослідях *in vivo* показано, що штами безпечні для теплокровних, проявляють пробіотичну і провітамінну активність.

Отримані результати є фундаментальною основою створення на основі каротинсинтезувальних штамів *B. amyloliquefaciens* IMB B-7513 та IMB B-7525 пробіотичного препарату з провітамінною активністю.

Ключові слова: бактерії роду *Bacillus*, каротиноїдні пігменти, пробіотичні властивості, провітамінна активність.

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ФУНДАМЕНТАЛЬНЫЕ ОСНОВЫ СОЗДАНИЯ ПРОБИОТИЧЕСКОГО ПРЕПАРАТА С ПРОВИТАМИННОЙ АКТИВНОСТЬЮ НА ОСНОВЕ ШТАММОВ *BACILLUS AMYLOLIQUEFACIENS* IMB B-7513 ТА IMB B-7525

Резюме

Дисбиотические состояния широко распространены как у людей, так и у животных. Дисбиоз кишечника сопровождается, кроме количественных и качественных изменений микробиоты, иммуносупрессией и метаболическими нарушениями, в частности авитаминозом А. Поэтому актуальным является поиск и исследование штаммов микроорганизмов, использование которых для коррекции дисбиозов позволит нормализовать гомеостаз макроорганизма. В связи с этим весьма перспективным является использование каротинсинтезирующих штаммов бактерий рода *Bacillus*, что позволило бы соединить в одном препарате свойства пробиотиков и способность компенсировать дефицит витамина А в макроорганизме.

В статье проанализированы данные литературы и представлены результаты собственных исследований о каротинсинтезирующей активности бактерий рода *Bacillus*, природе их каротиноидных пигментов и их функции в макроорганизме. Установлено, что каротиноидные пигменты штаммов *B. amyloliquefaciens* IMB B-7513 и IMB B-7525, представленные комплексом с липидами и углеводами, относятся к C₃₀-апокаротиноидам, в частности, апо-8'-фітофлуену, апо- ζ -8'-каротину, апо-8'-нейроспорину и апо-8'- β -каротен-3-олу. Предложен возможный путь их синтеза, конечным продуктом которого является апо-8'- β -каротен-3-ол, способный метаболізируватись в вітамін А. Ранее в опытах *in vivo* показано, что штаммы безопасны для теплокровных, проявляют пробиотическую и провитаминную активность.

Полученные результаты являются фундаментальной основой создания на основе

каротинсинтезирующих штаммов *B. amyloliquefaciens* ИМВ В-7513 и ИМВ В-7525 пробиотического препарата с провитаминной активностью.

Ключевые слова: бактерии рода *Bacillus*, каротиноидные пигменты, пробиотические свойства, провитаминная активность.

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