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Paenibacillus sp., as a promising candidate for development of a novel technology of inoculant production

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A bacterial strain IMBG156 producing exopolysaccharide (EPS) was isolated from siliceous rock and identified as a Paenibacillus species by partial sequencing its 16S rDNA. Paenibacillus sp. IMBG156 was used in a novel technology for inoculant production based on co-cultivating this bacterium with any bacterium of choice. Paenibacillus sp. provides in situ the bacterial cells of the inoculant with EPS, a carrier, and most likely with a source of carbon and energy. The partner bacterium designates a type of inoculant (biopesticide or biofertiliser). The strain IMBG156 does not destroy the signaling system of Gram-negative partners, based on acylated homoserine lactones, stimulates plant growth, and is rather competitive in the plant rhizosphere and soil. A prototype of the inoculant based on dual-culture Paenibacillus sp. IMBG156 — Pseudomonas sp. IMBG163 exhibits a noticeably longer shelf life than monoculture of Pseudomonas sp. IMBG163.

Key words: exopolysaccharide, inoculant, partner bacteria.

Introduction. The increasing concern on agrochemicals hazard to health and economical problems have promoted fundamental research in the area of alternative agriculture and in search for new agrobiotechnologies. Recently, tremendous efforts have been invested in studies of molecular mechanisms of plant defense [1—3].

Defense-signaling components have been discovered, and new, effective and sustainable alternatives to pesticides proposed. However, at the present time a sustainable strategy of crop defense still relies on usage of microbial biological agents able to induce system resistance in crop plants. The microbial inoculants based on the competitive, beneficial for the plant bacteria are considered as a reasonable alternative to agrochemicals [4, 5]. It is critical for the inoculant development that the inoculant product was in a formulation not only to deliver an adequate bacterial population but also to have enough product shelf life. Nowadays, the practical formulations are in use to prolong survival of Gram-negative bacteria [6—11], however, the usage of both mineral and organic carriers for bacteria makes inoculants more expensive. In our previous research we used the exopolysaccharide (EPS) mucilan produced by *B. mucilaginosus* B-4901 as an inexpensive carrier in the series of inoculants KLEPS (KLityna (a cell, Ukr.) and EPS) to prolong Gram-negative bacteria survival [12]. The inoculants enhanced crop production on poor soils and exhibited enough shelf life. In spite of advantages, the technology of KLEPS development needed a separate stage of EPS manufacture, which complicated the procedure and raised the price of the inoculant. The objectives of this study were to isolate slime-producing bacteria, following the idea to use

EPS as a carrier for inoculant development, and to simplify the two-stage technology of inoculant manufacture.

Materials and Methods. *Bacterial strains.* The microorganisms used in this work were either from our Institute collection (*Paenibacillus* sp. IMBG156, *Klebsiella oxytoca* IMBG26, Rif^r, *Pseudomonas* sp. IMBG163, *Pseudomonas* sp. IMBG168, *P. aureofaciens* IMBG288, *Agrobacterium* sp. IMBG260, *P. syringae* pv. *syringae* IMBГ295, *Xanthomonas axonopodis* pv. *phaseoli* IMBГ293) or kindly provided from other collections: *P. fluorescens* 13525 from ATCC; *Pseudomonas* sp. AP33 from A. Perebityuk (Institute of Cytology and Genetics of Belarus Academy of Sciences, Minsk); *Pantoea agglomerans* IMV56 and *Erwinia carotovora* subsp. *atroceptica* IMB9027 from R. Gvozdyak (Institute of Microbiology and Virology of NASU, Kyiv); *Agrobacterium tumefaciens* A136, *A. tumefaciens* NT1 (pTiC58ΔaccR) and *A. tumefaciens* NT1 from L. Halda-Alija (Mississippi University).

Culture conditions. The media for bacterial growth were: KB [13] and LB [14] used for all strains of bacteria, except for *Paenibacillus* sp. being cultured in the medium MZ [12]. Zeolite (10 g/l) was added to LB when needed.

Determination of EPS content. Cells of a two-day culture were centrifuged at 10,000 *g* for 30 min, EPS was extracted from supernatant by 2 v of ethanol and dried at 37 °C until stable weight was obtained.

Bacteria isolation procedures were performed using the samples of zeolite collected from Sokyrnytzya (Transkarpatian region) and fragments of silica rocks originated from Khmelnitsky region. In separate experiments 1 g of zeolite or silica rocks (fraction of 5 mm) was incubated in MZ for 48 h at 30 °C. The accumulating cultures were diluted serially and spread on selective MZ medium. Slime colonies were collected, bacteria were purified, and identified according to N. Krasilnikov [15].

Total DNA isolation was performed as recommended in [16].

Amplification, purification, and analysis of 16S rDNA. PCR primers pair of pA (8—27) and pH (1542—1523) described in [17] was used for identification of the *rrn* (16S rRNA) gene, and specific sequences of two selected isolates were detected at the annealing temperature of 52 °C and the standard concentration of $MgCl_2$ (1.5 mM). The reaction was performed with 25 pmol of each primer («Sigma», USA), 50 μM dNTP-mix, 2 U of Taq-DNA-polymerase (both reagents from «Fermentas», Lithuania). Amplification was carried out in ALF thermal cycler («Pharmacia», Sweden). Initial denaturation was performed at 98 °C for 3 min, then Taq-DNA-polymerase was added. The thermal profile involved 28 cycles of penetration temperature of 93 °C for 30 s, primer annealing at 52 °C for 30 s and 72 °C for 1 min. Amplicons were checked on a 2.0 % agarose gel. Purification of amplicons was performed with UltraClean™ PCR Clean-up™ Kit (MoBio Laboratories Inc., USA). Analysis of PCR products performed by Blast N search program (NCBI, http://www.ncbi.nlm.nih.gov). A phylogenetic tree was constructed by the program ClustalW 1.83 (http://www.genebee.msu.su/genebee.html).

Co-cultivating of partners was performed in MZ. After 30 h of co-cultivating, the serial dilutions were made for evaluation of population size. The partner cultures were detected on LB or KB agar, *Paenibacillus* sp. — on MZ plates.

Assay for production of signaling molecules. Bacterial strains (separately and in pairs with *Paenibacillus* sp.) were tested in cross-feeding assays for acylated homoserine lactones (AHLs), using the indicator strain *A. tumefaciens* A136 as recommended in [18].

Antibacterial activity was tested *in vitro* on Petri dishes by the standard agar-diffusion assay, using two layer agar with the upper layer of an indicator culture soft agar (0.4 %).

Detection of the acetylene reductase (nitrogenase) activity (ARA) was performed according to [19]. The ARA of *K. oxytoca* IMBG26 was detected with the gas chromatograph Tzvet (Cheh Republic) in 14 ml flasks where the bacterial culture was grown in an N-free medium supplemented with sucrose or EPS (final content 1.5 and 1.0 %, respectively) in the presence of 10 % acetylene within 16 h at 28 °C.

Plant growth conditions, bacteria inoculation and re-isolation. Ten wheat germinated seeds were inoculated with a washed overnight monoculture, a dual culture or a suspension of bacterial strains mixed in equal proportions (10^6 colony forming units, CFU/ml) and placed in zeolite (Sokyrnytzya deposit). Control plants were left non-inoculated. The plants were maintained under natural light at 20 °C in a growth chamber. The plants were watered once per two days. At the end of the experiment (14 days after inoculation) all plants were harvested and external root colonization was examined. Root sections of 100 mg

Table 1
Biometrical data of effects of wheat seed inoculation by Paenibacillus sp. IMBG156 on growth parameters of 30-day seedlings

Variant	Dry weight of a single plant, g	Shoot height, cm	Root length, cm
Paenibacillus sp. IMBG156	0,036±0,008a	44,856±1,212b	10,960±0,460a
Pseudomonas sp. IMBG163	0,044±0,002b	44,316±1,036b	11,080±0,457b
Pseudomonas sp. IMBG163 and *Paenibacillus* sp. IMBG156	0,045±0,003b	45,173±1,334b	11,665±0,689b
Control (water)	0,035±0,002a	40,466±1,003a	9,215±0,434a

N o t e. Error represents standard deviation. Treatment is different from the control at $p = 0.05$ as determined by Student's *t*-test. Values followed by the same letter in a column are not significantly different.

were vortexed in 0.9 % NaCl, and serial dilutions were plated on selective media LB, KB, MZ supplemented with rifampicin (50 μg/ml) when needed to discriminate between bacteria.

Statistical analysis of results. The data on biometrical parameters of wheat and bacteria survival are means from three replications. Statistical analysis was performed using SigmaPlot 8.0 software. Standard deviations were calculated for each data point.

Nucleotide sequence accession number. The sequence generated in this study has been deposited in the GenBank database under accession number AY645946.

Results and Discussion. *Phenotypic characteristics.* Two isolates (from zeolite and silica rock, designed IMBG156 and IMBG157) were characterized as aerobic Gram-positive rods of (0.2—0.5)·2.0 μm which formed spores of 0.5—0.7 μm. The spore position was preferentially central but terminal location was rarely observed. The optimal temperature of growth was 28 °C, but they grew well in a range of 10—45 °C. On the agar MZ medium they created transparent slime colonies of 10.0—13.0 mm diameter and produced 10.0—13.0 g EPS per 1 l of liquid medium. The isolates consumed carbohydrates, generated acids, and did not utilize amino acids as C and N sources. Bacteria hydrolyzed starch and could not grow anaerobically with nitrate as a respiratory substrate. We did not manage to determine the taxonomic position of the isolates with Bergy's determinative manual. To clarify their systematic position, it was practical to analyze the phylogenetic marker gene, *rrn*, encoding RNA of small subunit of ribosome (16S).

Analysis of the 16S rDNA amplicon. The 16S rDNA amplicons were obtained from IMBG156 of 1500 bp in PCR, and a sequence of 621 bp was deciphered and aligned with the most closely related bacterial sequences. The sequence shared 97 % similarity to the deposited sequence for *Paenibacillus velasolus*. In a phylogenetic tree based on sequencing data this isolate clustered with *P. velasolus*, however, more efforts were needed to prove or disprove relatedness of the isolate to *P. velasolus*, and in this study we did not use a species name.

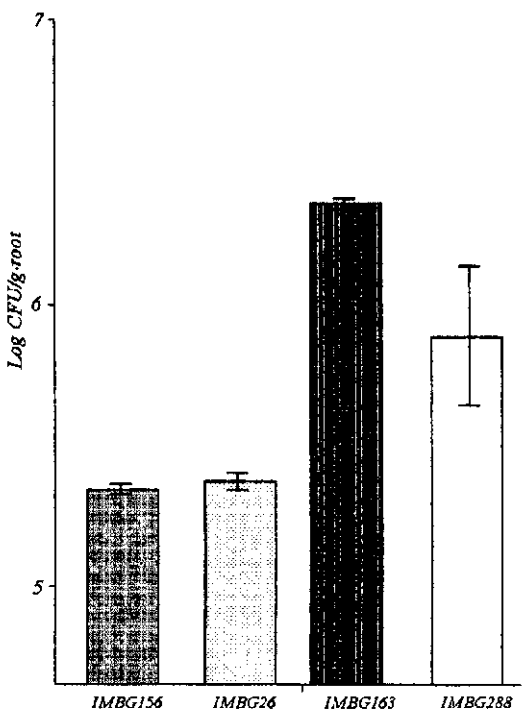

Fig 1. Average size of populations of bacterial strains associated with wheat roots: two weeks after inoculated seed planting

Effects of the bacterium on growth of wheat and survival in the plant rhizosphere. Relation to the plant of the assistant bacterium planned for the inoculant development should be at minimum neutral, but not antagonistic. The tests showed that strain IMBG156

Table 2
Co-cultivating Gram-negative bacteria with Paenibacillus sp. IMBG156

Variant	Concentration of cells in the mixed culture after the 30 h-incubation period, CFU/ml	Concentration of cells in the monoculture after the 30 h-incubation period, CFU/ml
K. oxytoca IMBG26	1.4±0.05E+9	6.8±0.04E+8
Pseudomonas sp. AP33	6.5±0.09E+9	9.5±0.41E+8
P. fluorescens ATCC 13525	1.6±0.07E+10	9.3±0.02E+8
P. aureofaciens IMBG288	3.7±0.07E+9	5.9±0.08E+8
Pseudomonas sp. IMBG163	2.5±0.09E+9	6.9±0.08E+8
P. agglomerans IMV56	5.0±0.08E+9	6.3±0.29E+8
Agrobacterium sp. IMBG260	3.7±0.07E+9	1.5±0.50E+8
Paenibacillus sp. IMBG156	—	2.4±0.03E+8

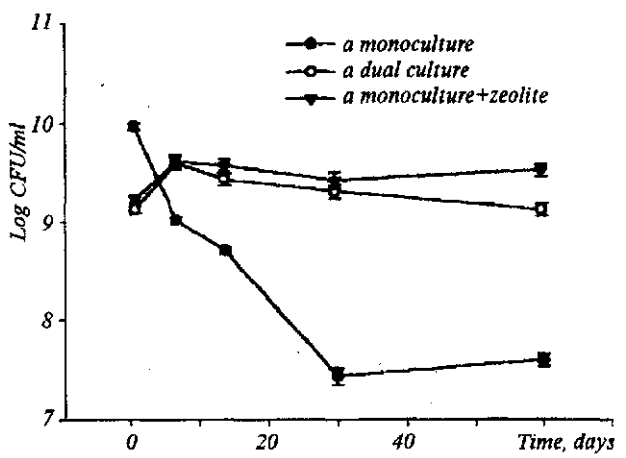

Fig. 2. Dynamics of survival of *Pseudomonas* sp. IMBG163 in dependence on culture conditions

did not cause a phytotoxic effect and increased wheat shoot height as shown in Table 1. Being applied together with *Pseudomonas* sp. IMBG163 for seed inoculation, it promoted plant growth more efficiently. In the rhizosphere of wheat inoculated by a rationally assembled consortium of plant growth promoting rhizobacteria (*Pseudomonas* sp. IMBG163, *P. aureofaciens* IMBG288, *K. oxytoca* IMBG26), the strain IMBG156 was quite competitive on background of beneficial bacteria (Fig. 1).

Co-cultivation of Paenibacillus sp. IMBG156 with bacteria of interest. With the idea to keep bacteria alive in the gel produced by bacteria within co-cultivation, *Paenibacillus* sp. and chosen Gram-negative bacteria were cultured by pairs. The results of experiments on co-cultivating showed practically the absence of restrictions in picking up partners for *Paenibacillus* sp. (Table 2). The partners gained a population size of log 9—10 CFU/ml. The critical factors in the one-step procedure of prototype inoculant manufacture were the size of population of a strain that determined a sort of inoculant, and also the concentration of EPS produced by *Paenibacillus* sp. In the case of co-cultivating IMBG156 together with *K. oxytoca* IMBG26 or *Pseudomonas* sp. IMBG163, the culture gel contained 1.4±0.05E+9 or 2.5±0.09E+9 CFU/ml of a bacterium-partner, respectively, and not less than 20.0 g/l of EPS.

A term of the inoculant shelf life is important when the inoculant is based on Gram-negative bacteria. We showed that survival of *Pseudomonas* sp. IMBG163 was prolonged in the dual culture with *Paenibacillus* sp. in comparison with the monoculture (Fig. 2). A better survival of IMBG163 was also observed in a minimal medium supplemented with zeolite. The results showed that the strain IMBG163 preserved the population size at the level of log 9 CFU/ml in both dual culture and monoculture grown in the presence of zeolite within 2 months. On the contrary, the population size of IMBG163 declined from log 10 to log 7 CFU/ml without co-inoculation by *Paenibacillus* sp. IMBG156 or addition of the mineral to the medium. This clearly demonstrated that both *Paenibacillus* and zeolite supported survival of *Pseudomonas* sp. IMBG163. *K. oxytoca* IMBG26 was able to grow and reduce acetylene (518 nM C_2H_4/h flask) in the nitrogen-free medium where 1.0 % EPS (derived from *Paenibacillus* sp. IMBG156) was used as a carbon source, and this suggested that EPS produced by *Paenibacillus* had the potential to support growth and activity of the partner.

Table 3
Antibacterial activity of dual and monocultures of Pseudomonas spp. in vitro

Inoculant	I*	II	III
Pseudomonas sp. IMBG163	4**	3	4
Pseudomonas sp. IMBG163 + *Paenibacillus* sp. IMBG156	4	3	4
Pseudomonas sp. IMBG168	4	3	4
Pseudomonas sp. IMBG168 + *Paenibacillus* sp. IMBG156	4	3	4
P. aureofaciens IMBG288	2	2	3
P. aureofaciens IMBG288 + *Paenibacillus* sp. IMBG156	2	2	3

*Indicator phytopathogenic bacteria: I — *P. syringae* pv. *syringae* IMBG295; II — *Erwinia carotovora* subsp. *atroceptica* IMB9027; III — *Xanthomonas axonopodis* pv. *phaseoli* IMBG293; **diameter of zone of suppression of indicator bacteria (mm): 2 — 10.0—20.0; 3 — 21.0—30.0; 4 — 31.0.

Detection of antibacterial activity in dual cultures. Signaling systems play a role in bacteria-bacteria and plant-bacteria communications [20]. Antibacterial activity of some pseudomonads is controlled with AHLs [21, 22]. It is well known that Gram-positive bacteria are able to destroy AHLs of Gram-negative neighbors [23]. To compose dual bacterial pairs, it is important to know that quorum signaling is not impaired by co-cultivation. Bacteria used in experiments on co-cultivation were tested in cross-feeding assays for AHL_{4-14} detection earlier [24]. Few of them produced AHLs (*Pseudomonas* sp. IMBG163, *Pseudomonas* sp. IMBG168 and *P. auerofaciens* IMBG288), and no difference was observed between mono- and dual *Pseudomonas-Paenibacillus* cultures with respect to AHL production. The results represented in Table 3 showed inhibition of pathogenic bacteria by both monocultures of pseudomonads and appropriate dual *Pseudomonas-Paenibacillus* cultures. This may mean that strain *Paenibacillus* sp. IMBG156 did not impair AHLs produced by a partner and indirectly demonstrated integrity of AHLs in dual cultures.

Paenibacillus sp. IMBG156 has been selected as a bacterium-nurse for the dual-culture technology of inoculant development, first of all, due to production of large amounts of EPS. The strain IMBG156 provided the living cells of a bacterium-partner, the second species of two-component consortium, with a carbon source and apparently caused better survival of the latter. In this study IMBG156 displayed commensal interactions in the pairs with other bacterial strains and synergistic positive impact on the plant. These results are consistent with those obtained for other bacteria acting synergistically on the plant development [25, 26]. IMBG156 was quite competitive in the plant rhizosphere bacterial community and in the soil, in contrast to the known data on the gradual replacement of *Paenibacillus* by *Pseudomonas* [27]. These additional beneficial features make the strain rather promising for application for seed inoculation in programs of plant health care and soil remediation in company with biocontrol bacteria.

The inoculants, containing both *Paenibacillus* sp. IMBG156 and a partner bacterium, can be stored for a relatively long period of time in the presence of large amounts of EPS produced *in situ* without preservatives and conventional carriers. This finding is based on a concept of keeping bacteria alive under storage of the dual culture in a gel and may be explained, first of all, by the fact that growing any Gram-negative bacterium with *Paenibacillus* sp. IMBG156 results in stimulation of EPS production which can serve both as a carbon and energy source for the bacterium-partner. In case when EPS serves as a carrier, the organisms appear to establish structured populations where cells are not aggregated. Under this condition the bacteria are positioned in a heterogeneous environment with gradients of nutrients and waste products as a consequence of diffusion and mass transport processes, and it is therefore to be expected that this heterogeneity is reflected in the physiology of the individual cells and better survival. EPS keeps up water and nutritional regime, and therefore bacterial cells are physiologically active under storage, in contrast to a dry KLEPS formulation where bacteria were dormant. The dual-culture technology based on co-cultivating the bacterium *Paenibacillus* sp. IMBG156 and any bacterium of choice is simpler and less expensive compared to the previous technology [12].

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Н. О. Козировська, В. В. Негруцька, М. В. Ковальчук, Т. М. Вознюк

Paenibacillus sp. — перспективна бактерія для створення технології виробництва бакпрепаратів для рослин

Резюме

Штам бактерій IMBG156, який продукує екзополісахарид (ЕПС), виділено з силікатної породи та ідентифіковано як Paenibacillus sp. на основі визначення первинної нуклеотидної послідовності 16S рДНК. Paenibacillus sp. IMBG156 використано у технології виробництва мікробних препаратів, яка базуться на спільному культивуванні цієї бактерії та бактерії за вибором. Paenibacillus sp. постачає in situ бактерійні клітини носієм (ЕПС) та, найвірогідніше, є джерелом вуглецю і енергії. Бактерія-партнер визначає тип препарату (біодобриво, біопестицид). Штам IMBG156 не пошкоджує сигнальної системи грамнегативних бактерій-партнерів, яка грунтується на ацильованих гомосеринлактонах, стимулює ріст рослини і є доволі конкурентною у ризосфері. У демонстраційному препараті, створеному на основі бактерій-партнерів Paenibacillus sp. IMBG156—Pseudomonas sp. IMBG163, довше зберігається необхідний титр бактерій, ніж у монокультурі Pseudomonas sp. IMBG163.

Ключові слова: екзополісахариди, бакпрепарат, бактерії-партнери.

Н. А. Козыровская, В. В. Негруцкая, М. В. Ковальчук, Т. Н. Вознюк

Paenibacillus sp. — перспективная бактерия для создания технологии производства бакпрепаратов для растений

Резюме

Штамм бактерии IMBG156, продуцирующий экзополисахарид (ЭПС), выделен из силикатной породы. Он идентифицирован как Paenibacillus sp. на основе определения первичной нуклеотидной последовательности 16S рДНК. Paenibacillus sp. IMBG156 использован в технологии производства микробиологических препаратов, основанной на совместном культивировании этой и выбранной бактерий. Paenibacillus sp. снабжает in situ клетки бактерий носителем (ЭПС) и является, наиболее вероятно, источником углерода и энергии. Бактерия-партнер определяет тип препарата (биоудобрение, биопестицид). Штам IMBG156 не повреждает сигнальной системы грамотрицательных бактерий-партнеров, которая основывается на ацилированных гомосеринлактонах, стимулирует рост растений и является достаточно конкурентоспособной в ризосфере. В демонстрационном препарате, созданном на базе бактерий-партнеров Paenibacillus sp. IMBG156—Pseudomonas sp. IMBG163, дольше сохраняется необходимый титр бактерий, чем в монокультуре Pseudomonas sp. IMBG163.

Ключевые слова: экзополисахариды, бакпрепарат, бактерии-партнеры.

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