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ON DDSL-BASED GENOTYPING OF POTATO BACTERIOSIS AGENTS, THEIR ANTAGONISTS AND MICROBIAL BIODESTRUCTORS FOR PLANT PROTECTION AND ECOTECHNOLOGIES

V.P. TERLETSKIY[1, 2] ✉, A.M. LAZAREV[1], I.I. NOVIKOVA[1], I.V. BOJKOVA[1], V.N. ZEYRUK[3]

[1]*All-Russian Research Institute of Plant Protection*, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail valeriter@mail.ru (✉ corresponding author), allazar54@mail.ru, irina_novikova@inbox.ru, irina_boikova@mail.ru;
[2]*Pushkin Leningrad State University*, 10, Petersburg Sh., St. Petersburg—Pushkin, 196605, e-mail valeriter@mail.ru;
[3]*Lorkh All-Russian Research Institute of Potato Farming*, 23, ul. Lorkha, pos. Korenevo, Lyubertsy Region, Moscow Province, 140051 Russia, e-mail vzeyruk@mail.ru;
ORCID:
Terletskiy V.P. orcid.org/0000-0003-4043-3823
Lazarev A.M. orcid.org/0000-0002-4282-0141
Novikova I.I. orcid.org/0000-0003-2816-2151
Bojkova I.V. orcid.org/0000-0001-6268-7301
Zeyruk V.N. orcid.org/0000-0002-2818-2141

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Abstract

Intensification of agricultural and industrial production necessitates environmentally friendly technologies to prevent human habitat from chemical pollutions. Microbial producers of biologicals for biocontrol of plant pathogens and hydrocarbon destructors for bioremediation are characterized by high spontaneous genetic variability which can lead to a change in their activity. Therefore, in stabilizing selection, it is necessary to confirm strain affiliation. Here, we presents data on the application of the double digest and selective label (DDSL) technique developed by us to study the genetic profiles of plant pathogenic agents of the genera *Pseudomonas*, *Pectobacterium*, their antagonists *Bacillus subtilis* (*Bs*), and the hydrocarbon destructors of the genus *Pseudomonas*. The study confirmed high biological efficiency of two selected *Bs* strains, the M-22 and I5-12/23 against bacterial diseases of stored potato tubers. In addition, destructors from the genus *Pseudomonas* were genetically identified. The aim of the study was to evaluate genetic diversity among *Pseudomonas*, *Pectobacterium*, and *Bs* strains to select effective microbial antagonists and hydrocarbon destructors. The DDSL technique uses two restriction endonucleases for bacterial genomic DNA digestion. Taq DNA polymerase supplemented into reaction mix provides simultaneous labeling DNA fragments by biotinylated deoxycytidine triphosphate (Bio-dCTP). Only fragments digested with one of the restriction enzymes producing fragments with 3′-recessed ends are subjected to labeling. The second restriction enzyme produces only blunt ends which can not bind Bio-dCTP tag. As a result of DDSL reaction 20 to 50 clear DNA fragments are visualized on the filter, and their quantity and distribution are characteristic for each bacterial strain. Genotyping allows generating genetic profile for each bacterial strain, i.e., assigning a "bar-code" to the bacteria that identifies a given microbe with confidence. Genotyping *P. atrosepticum* D822 и G784 allows for identification of about 50 DNA fragments more than 20 % of which were specific for only one of the compared strains. We used two pairs of restriction enzymes — XbaI/DraI and XbaI/Eco24I. Our results indicate on equal discriminatory ability of these two enzyme combinations when compared *P. atrosepticum* strains D822 и G784. We noted some advantage of XbaI/DraI enzymes because of its ability to identify differences in genetic profiles in a range of longer DNA fragments. The optimal enzymes for *Pseudomonas* genus genotyping were restriction endonucleases BcuI/Eco32I, for *Bs* the first restriction enzyme was SgsI (39 cleavage sites), the second was Eco32I reducing the size of the obtained DNA fragments. High antagonistic activity of *B. subtilis* strain I5-12/23 which belongs to I genotypic group was shown in laboratory experiments with artificial contamination of potato tubers with *P. atrosepticum* 1944 и *P. carotovorum* subsp. *carotovorum* 481. Index of development of bacterial soft rot disease after treatment of infected tubers by *B. subtilis* strain I5-12/23 was 0-0.02, in control

the index was 4.04. Biological activity of this strain after treatment of infected tubers was up to 100 % whereas chemical fungicide Maxim KS gave rise to only 77.7 % value. Experiments conducted in potato storage houses confirmed high biological activity of two selected bacillus strains against bacterial diseases during potato tuber storage. *B. subtilis* strain I5-12/23 demonstrated highly expressed antagonistic activity against causal agents of bacterial soft rot, ring rot as well as fusarium dry rot potato diseases. Significant suppression of potato tuber diseases in comparison with control after treatment by selected antagonist strains was demonstrated, and this effect was comparable with that of chemical standard Maxim KS. Percentage of healthy tubers treated by bacillus strains was in the range of 30.4-35.5 % whereas in control this value did not exceed 13.3 %. Thus, yield of healthy products compared to control was 2.7 times higher. The most efficient *B. subtilis* strain I5-12/23 effectively suppressed causal agents for ring rot and fusarium dry rot potato diseases. Prevalence of ring rot disease was 2.6-2.9 %, fusarium dry rot — 1.5-3.0 %, the values which are significantly lower than those in samples treated by *B. subtilis* M-22 and Maxim KS (4.8 and 3.5 %; 9.0 and 4.1 %, respectively). After genotyping, destructor strains of the genus *Pseudomonas* utilizing difficult-to-oxidize compounds, including heavy oil fractions and polyaromatic hydrocarbons (benzopyrene, chrysene, phenanthrene, anthracene, chrysene, naphthalene) have been deposited in the VIZR collection. The range of their activity is enough to compose associations for utilizing specific pollutants. Thus, DREAM genotyping identifies bacterial strains to confirm their origin in the course of development and use of biological products for various purposes.

Keywords: restriction endonucleases, genotyping, plant pathogens, microbial antagonists, biodestructors, *Pectobacterium*, *Pseudomonas*, *Bacillus subtilis*, *Solanum tuberosum* L., potato

Preservation of the environment in the intensification of agricultural and industrial production is one of the global challenges today. This problem could be addressed by using the environmentally friendly technologies, including biocontrol of plant pathogens and bioremediation.

Substantial crop losses from bacteriosis constitute a serious problem for potato growing in the Russian Federation. The most common disease is considered to be black stem, or soft rot. Its main pathogen is microorganisms from the genus *Pectobacterium* (syn. *Erwinia*) [1-3]. Semi-parasitic bacteria of the genus *Pseudomonas* (*Ps. fluorescens* and *Ps. marginalis*), producing pectolytic enzymes, also actively participate in damaging and subsequent decomposition of tubers, especially during the winter storage of crops.

The methods of phytosanitary monitoring at the population level make it possible to identify both the strains of pathogens that determine the intensity of the infectious process in the ecosystem, and suppressor microorganisms introduced to control it [4, 5]. Genotyping of microorganisms is used to study the genetic profiles of bacterial strains for their identification and individualization [3, 6, 7-9]. Based on genotyping results, each strain can be assigned a “bar-code”, which is extremely important for identifying the types of bacteria and their antagonists [6]. Following the introduction of an antagonist microorganism into the rhizo or phyllosphere of a plant, its presence in the microbial community is confirmed by molecular-genetic methods, with determination of the degree and duration of dominance, which will ensure effective suppression of the population density of phytopathogenic species and the dynamic stability of soil microbiocenoses. For in-depth identification of pathogens, it is important to carry out genotyping during the phytosanitary control of potato diseases caused by the bacteria *Pectobacterium* and *Dickeya* [10-12].

Studies have shown that genotyping allows quick and highly accurate identification of collection strains of the genus *Bacillus*, which are similar in morphological and cultural features, but differ in the composition of complexes of metabolic products. It can be used to recognize individual strains and groups of closely related *Bs* strains and identify introduced suppressor microorganisms, which provides grounds to recommend genotyping both for controlling the origin of strains and for phytosanitary monitoring [13-15].

Currently, antagonists of pathogens and biological products based on them

are successfully used to regulate the density of populations of phytopathogenic species in agrobiocenoses [16-18]. The high biological activity of biological products has been shown in many agricultural crops [19-22]. The selection of biological products based on phytosanitary monitoring data, taking into account biological characteristics and the spectrum of action of producer strains, largely determines the effectiveness of microbiological and integrated protection of potatoes from diseases [23, 24]. Methods of blocking chemical signals of communication (QS, quorum sensing) in pathogen populations due to the release of certain inhibitors by antagonist bacteria are of interest [25, 26].

The most effective and widely used (about 90-95% of the biopesticide market) means of combating diseases are preparations based on gram-positive spore-forming bacteria of the *Bacilliaceae* family, capable of synthesizing biologically active compounds of various chemical nature [27-29].

The density of populations of microorganisms — suppressors and antagonists of plant pathogens introduced into the soil microbiocenosis is of great importance [30-33]. In this regard, to predict the intensity of disease development, to assess the effectiveness of biological control of populations of phytopathogenic species, and to adjust the regulations for the use of biological products, it is necessary to determine and identify introduced strains of suppressor microorganisms.

Another important area of application of the genetic certification of microorganisms is associated with environmental pollution with hydrocarbons (leakage during oil production, oil spills after accidents at pipelines and oil refineries). The designated problem has been exacerbated in recent years, while bacterial hydrocarbon destructors consuming the carbon of such products in their metabolism are increasingly used. Selection and genetic certification of the most active destructor strains makes it possible to more effectively address such environmental issues.

Based on our previously proposed double digest and selective label (DDSL) technique, a method for genetic identification and certification of strains of *Bacillus subtilis* (*Bs*) and some representatives of the genus *Streptomyces*, being promising antagonists of phytopathogens, was developed [6]. This method was previously tested on strains that differ in physiological and biochemical characteristics and the composition of secondary metabolites, which determine the antibiotic activity. Its high resolution has also been proven.

Here, we present data on application of DDSL technique to study the genetic profiles of plant pathogenic agents of the genera *Pseudomonas*, *Pectobacterium*, and their antagonists (*Bacillus subtilis*). The outcomes can be used in the development of biological products against phytopathogens based on their antagonists and to control the origin of strains. The study confirmed high biological efficiency of two selected *Bs* strains (M-22 and I5-12/23) against bacterial diseases of stored potato tubers in production. In addition, we were the first to identify (certify) the strains of bacteria-destructors (genus *Pseudomonas*), suitable for addressing the environmental problems.

The aim of the study was to evaluate genetic diversity among *Bacillus*, *Pectobacterium* and *Pseudomonas* strains to select the most effective microbial antagonist destructor strains.

Materials and methods. The strains of plant pathogenic bacteria used in the work were certified and are included in the State collection of phytopathogenic microorganisms and their pests of the Vavilov All-Russian Research Institute of the Plant Industry (registered in WFCC WDCM 760, Japan, 01.28.1998). Strains *P. atrosepticum* D822 and G784 were isolated from the infected potato tubers in the Leningrad Province. Certified strains *P. atrosepticum* 1944 and *P. carotovorum* subsp. *catovorum* 481 were obtained from the State collection of phytopathogenic

microorganisms and plant varieties-identifiers (differentiators) of pathogenic strains of microorganisms of the All-Russian Research Institute of Phytopathology. The certified *Ps. fluorescens* 894 was obtained from the All-Russian collection of microorganisms, Skryabin Institute of Biochemistry and Physiology of Microorganisms (IBPM RAS). Strain *Ps. marginalis* (previously a member of the group *Ps. xanthochlora*) is a part of the collection of plant pathogenic microorganisms of Lorkh All-Russian Research Institute of Potato Farming.

Bacteria were isolated by methods generally accepted in plant bacteriology [34] from potato plants with symptoms of bacterial rot. Bacteria were identified using the conventional physiological, biochemical, and molecular techniques (polymerase chain reaction, PCR) [34]. For the genus *Pseudomonas*, the primary analysis was carried out using the LOPAT system (levan, oxidase, potato soft rot, arginine dihydrolase, hypersensitivity reaction), as well as for the fluorescent pigment on King B medium, for the genus *Pectobacterium* (pathotypes *carotovora* and *atroseptica*) by liquefaction of pectate potato tissue.

Double digest and selective label (DDSL) technique was used for genotyping of bacteria genus *Pectobacterium* and *Pseudomonas* [7, 8]. During preliminary application of this method on two strains of *Pectobacterium* and two strains of *Pseudomonas*, an in silico search was carried out (http://in-silico.ehu.eus/) showing that the best first restriction enzyme for microorganisms of the genus *Pseudomonas* is BcuI (A↓CTAGT, 78 cleavage sites in the reference genome of *P. fluorescens* Pf-5, Thermo Fisher Scientific, Inc., USA), and for representatives of the genus *Pectobacterium* XbaI (T↓CTAGA,, 87 cleavage sites in the reference genome of *P. carotovorum* subsp. *catovorum* , Thermo Fisher Scientific, Inc., USA). The first enzyme is characterized by a smaller number of DNA cleavage sites with 3'-truncated ends, capable of binding labeled Bio-dCTP. The second enzyme, which cleaves DNA at a greater number of restriction sites, produces only blunt ends which cannot bind the tag.

As a result, the reaction mixture after enzymatic digestion contains a limited number of labeled DNA fragments that can be separated and visually recognized. In relation to bacteria of the genus *Pseudomonas*, Eco32I should be considered the best second enzyme (GAT↓ATC, 1556 cleavage sites in the reference genome, Thermo Fisher Scientific, Inc., USA), for the microorganisms of genus *Pectobacterium* DraI (TTT↓AAA, 1332 cleavage sites, Thermo Fisher Scientific, Inc., USA). Enzyme pairs are compatible in the same reaction buffer Msp20I (TGG↓CCA, 1028 cleavage sites, Thermo Fischer Scientific, Inc., USA) and Eco24I (GRGCY↓C, 1314 cleavage sites, Thermo Fischer Scientific, Inc., USA) [6].

Bs strains were analyzed using a pair of enzymes SgsI and Eco32I. The *B. subtilis* genome has 39 cleavage sites (GG↓CGCGCC) for the first enzyme, thus forming sticky ends that are able to bind Bio-dCTP using Taq polymerase. This pair of enzymes is compatible in R buffer (Thermo Fischer Scientific, Inc., USA).

For the DDSL reaction, 15 μl of distilled water, 2 μl of bacterial DNA, 2 μl of a buffer suitable for both restriction endonucleases, and 1 μl of a preprepared mixture, including restriction endonucleases, a Bio-dCTP tag and DNA polymerase, for instance Taq-polymerase, were mixed in an Eppendorf tube [6, 12].

The reaction was carried out in a Termit solid-state thermostat (DNA-Technologies LLC, Russia) at 37 °C for 1-2 h. Tris-acetate buffer and 0.8% agarose gel were used for electrophoresis, the voltage was 1.5 V/cm. Vacuum transfer of DNA fragments onto a nylon filter was carried out immediately after electrophoresis in a VacuGene XL Vacuum Blotting System™ (GE Healthcare, USA). In this case, the transfer of DNA from a double-stranded state to a single-stranded state was not required, since there was no molecular

hybridization. The detection of DNA fragments on the filter was based on the detection of the enzymatic activity of alkaline phosphatase in the presence of substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride (NBT, Thermo Fischer Scientific, Inc., USA). Unbound tag was washed in buffer with maleic acid and salt.

Bs strains M-22 and 5-I-12/23, used in this work, were previously selected based on high antagonistic activity and deposited in the State collection of plant pathogenic microorganisms and their pests of the Vavilov All-Russian Research Institute of the Plant Industry. The bacteria were grown at 28 °C for 72 h (a nutrient medium containing 30 g/l maize extract, 15 g/l molasses; pH 7.2; a Bio-san OS-20 laboratory shaker, Diaem, Russia, 220 rpm; 750 ml flasks with 100 ml of medium). Sampling and assessment of culture growth were carried out using an Axio Imager A-2 light microscope (Karl Zeiss, Germany) once a day. The titer of viable cells was measured by serial dilutions followed by inoculation on the SPA medium and counting the colonies.

To assess the biological activity of the *Bs* 5-I-12/23 strain with artificial infection, 10 intact visually healthy potato (*Solanum tuberosum* L.) tubers (cv. Sante) were selected in 5 replicates for each variant. Mechanical damages 10 mm deep were made to their surface with a scalpel, into which 0.3 ml of a mixture of 1-day-old cultures of *P. atrosepticum* 1944 and *P. carotovorum* subsp. *catovorum* 481 (10^6 CFU/ml) in sterile distilled water was inoculated. Then the tubers were treated with the culture liquid of the *Bs* 5-I-12/23 strain with a titer of 10^8 and 10^9 CFU/ml. In the control, the tubers were treated with water, in the standard with the contact fungicidal dressing agent Maxim, KS (0.2 l/t) (Syngenta AG, Switzerland). Development of infections was assessed on day 8-10. The lesion index was calculated using the formula:

$$x = \frac{dh}{100},$$

where d is the diameter of the decay zone (lesion), mm, h is the depth of the decay zone, mm.

The biological activity of *Bs* strains 5-I-12/23 and M-22 during storage in a pile of potato tubers was studied in the winter period of 2018-2019 in a potato storage facility (Lorkh All-Russian Research Institute of Potato Farming, Moscow Province, Kraskovo village) based on the dynamics of disease development. To assess the antagonistic activity of the strain, a preliminary psychopathological analysis of potato tubers was carried out (GOST 33966-2016 “Seed potatoes. Technical conditions and methods for determining the quality”. Moscow, 2020) [35]. To assess the biological activity of the strain under conditions that provoke rot development, tubers of cv. Udacha were used. Before placing for storage, the tubers were treated with the *Bs* 5-I-12/23 strain culture liquid (a titer of 10^8 and 10^9 CFU/ml). The control tubers were treated with water. The standards were treated with the *Bs* M-22 culture liquid (a titer of 10^9 CFU/ml) and the contact fungicidal dressing agent Maxim, KS (0.2 l/t) (Syngenta AG, Switzerland), recommended for the treatment of seed potatoes before storage to protect them from rot of various etiologies, including wet rot. Tuber sample weighted 5 kg, the working solution rate was 3 l/t, the experiment was repeated 10 times. After drying, the treated material in nets was placed for storage in a potato mound at a depth of 20-30 cm from the surface under high humidity and temperature to provoke the infection present in the tubers and to reveal the effectiveness of the studied strain. Tuberous infections were counted after 6 months of storage. The prevalence of common scab, ring rot, late blight and fusarium blight was assessed by the proportion (%) of diseased tubers. The biological efficiency was calculated by the formula:

$$BE = \frac{a - b}{a} \cdot 100\ \%,$$

where BE is the decrease in the prevalence or development of the disease to control, %; a is the prevalence or development of the disease in the control, %; b is the prevalence or development of the disease in the experimental variant, %.

Statistical data processing was performed by methods of analysis of variance using the Statistica 6.0 software package (StatSoft, Inc., USA). Methods of parametric statistics were used for statistical processing. Mean values (*M*), standard errors of means (±SEM) and their 95% confidence intervals were calculated by Student's *t*-test.

Results. Genotyping by DDSL-technique can be briefly described as the sequential execution of the following steps: 1) cleavage of genomic DNA by two restriction endonucleases simultaneously and labeling of individual DNA fragments (containing 3′-truncated ends) with a biotin residue; 2) electrophoretic separation of DNA fragments by length; 3) vacuum transfer of DNA in distilled water to a filter; 4) identification of fragments labeled with biotin in a color chemical reaction [6].

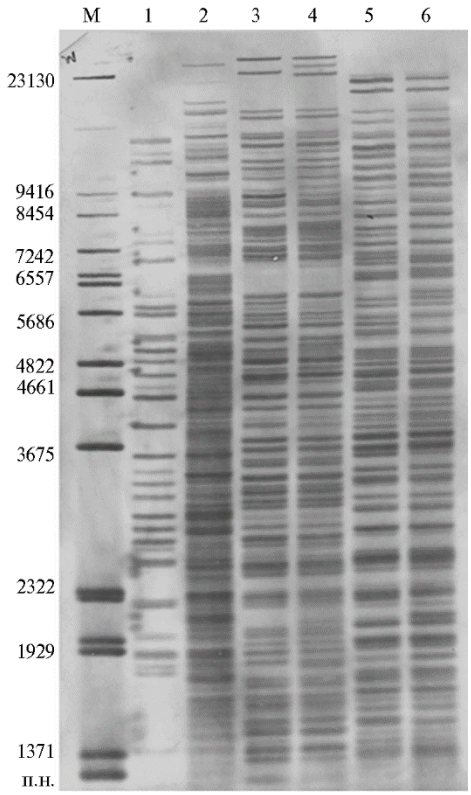

Fig. 1. Genotyping of strains of the genera *Pseudomonas* and *Pectobacterium* by double digest and selective label (DDSL) technique: 1 — *Ps. fluorescens* 894, 2 — *Ps. marginalis*, 3 and 5 — *P. atrosepticum* Д822, 4 and 6 — *P. atrosepticum* Г784. Restriction endonucleases are BcuI-Eco32I (tracks 1 and 2), XbaI-DraI (tracks 3 and 4) and XbaI-Eco24I (tracks 5 and 6). M is a DNA fragment length marker [12].

The DDSL-technique was successfully tested on *Ps. aeruginosa*, *Staphylococcus aureus*, *Salmonella* spp. and other causative agents of infectious diseases of interest in medical and veterinary practice [7, 8]. The use of the DDSL test also made it possible to identify genetic profiles in microorganisms of the genera *Pectobacterium* and *Pseudomonas*, which characterize the individuality of each of them (Fig. 1) [12]. For example, we noted significant differences between *Ps. fluorescens* and *Ps. marginalis*. *P. atrosepticum* strains D822 and G784 were compared based on two combinations of XbaI-DraI and XbaI-Eco24I enzymes in Tango™ reaction buffer (Thermo Fischer Scientific, Inc., USA). These pairs of enzymes helped us to detect about 50 DNA fragments. Despite certain interspecific differences in these species, genotyping revealed a group of identical DNA fragments, which indicates a generic phylogenetic closeness. Both combinations of restriction enzymes revealed a certain differentiation between *P. atrosepticum* strains D822 and G784 in terms of genetic profiles.

Therefore, genomic analysis by the DDSL-technique quantitatively shows both interspecies differences and genetic variations within a species at the level of individual strains [12]. For example, a comparison of microorganisms of the genus

Pectobacterium revealed more than 10 DNA fragments characteristic of each of them. Analysis of their genetic profiles allows us to conclude about the advantage of using the XbaI-DraI pair which detects, along with short fragments, longer ones, reaching 23000 bp. and grouped at the top of the filter (see Fig. 1).

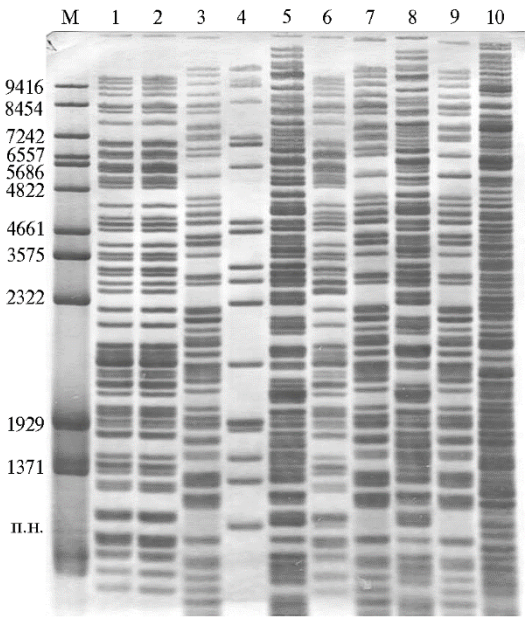

Fig. 2. Genotyping of strains destructors of hydrocarbons from the genus *Pseudomonas* by double digest and selective label (DDSL) technique using BcuI-Eco32I restriction enzymes: 1 — 1-1, 2 — 10-1, 3 — 14-2, 4 — ку-1, 5 — ко-1, 6 — Pp-2, 7 — lb 3-2, 8 — Pp-5, 9 — Pp-7, 10 — P.Pol Г+. M is a DNA fragment length marker [12]

The work was continued on a group of bacterial strains destructors of hydrocarbons from the genus *Pseudomonas* for their genetic certification. Earlier genotyping of clinical strains of *Pseudomonas aeruginosa* showed the effectiveness of the DDSL technique in identifying the pathways of infection and identification of bacterial strains circulating in products [7]. A pair of BcuI-Eco32I enzymes, compatible in the same reaction buffer, allows for clear visualization of more than 40 DNA fragments (Fig. 2).

Our study of bacteria destructors of the genus *Pseudomonas*, characterized by the ability to assimilate carbon from technogenically contaminated soil and water ecosystems in different climatic zones, revealed a significant genetic diversity of strains. On their basis, stable associations were created that effectively and quickly oxidize various toxicants in a short time in different environmental conditions. The formed collection of biodegradants (VIZR) includes strains that utilize difficult-to-oxidize compounds, including heavy oil fractions and polyaromatic hydrocarbons (in particular, benzopyrene, chrysene, phenanthrene, anthracene, chrysene, naphthalene), which makes it possible to compose bacterial associations for the utilization of various pollution.

The genotyping methodolofy we have developed based on DDSL technique was also successfully tested for the identification of 13 *Bs* strains (Table 1) [6], which have high antagonistic activity against a wide range of phytopathogenic fungi and bacteria and are part of the State collection of phytopathogenic microorganisms and their pests (Vavilov All-Russian Research Institute of the Plant Industry) [6, 8]. Currently, the collection includes more than 8000 strains of microorganisms, including more than 200 selected strains with high polyfunctional activity which are promising producers of biological products for various purposes [31].

Using the DDSL technique, we identified 7 groups of genotypically identical clusters and unique *Bs* strains: group 1 — 147/48/314, 110/723, 85/3/8, group 2 — 1-I, 2-I, 3 -I, 4-I, 5-I-12/23, group 3 — FR-318, group 4 — FR-327, group 5 — 1-K, group 6 — V-10, and group 7 — M-22. The noted distribution by genotypes is somewhat different from the previously obtained data, when we identified only six groups of genotypes [6]. In the genotyping performed

in this study, the M-22 strain, which is used in commercial biological products for plant protection, had a unique genotype that differs from all others. In previous work [6], this microorganism fell into a large cluster of genetically identical strains. Further re-culturing confirmed that there was a contamination of the M-22 strain with bacteria of group I. Otherwise, the genotypes of the analyzed bacteria coincided with those described by us in our previous work.

1. Characterization of *Bacillus subtilis* strains selected for genotyping by double digest and selective label (DDSL) technique [6]

Strain	Extraction site, source	Biological activity
B. subtilis B-10	Leningrad province (Russia), zoo-manure	Fungicidal, phytoregulatory
B. subtilis M-22	Ukraine, air medium	Fungicidal, bactericidal, phytoregulatory
B. subtilis 1-И	India, surface of cucumber seeds	Fungicidal
B. subtilis 2-И	India, surface of wheat seeds	Fungicidal
B. subtilis 3-И	India, surface of wheat seeds	Fungicidal
B. subtilis 4-И	India, surface of bean seeds	Fungicidal
B. subtilis 5-И-12/23	India, surface of tomato seeds	Fungicidal, bactericidal, phytoregulatory
B. subtilis 1-К	China, cucumber rhizosphere	Fungicidal
B. subtilis ФР-318	Collection of the All-Russian Research Institute for Agricultural Microbiology	Phosphate mobilizing
B. subtilis ФР-327	Collection of the All-Russian Research Institute for Agricultural Microbiology	Phosphate mobilizing
B. subtilis var. *niger* 147/48/314	Collection of the All-Russian Research Institute for Agricultural Microbiology	Fungicidal
B. subtilis var. *niger* 110/723	Collection of the All-Russian Research Institute for Agricultural Microbiology	Fungicidal
B. subtilis var. *niger* 85/3/8	Collection of the All-Russian Research Institute for Agricultural Microbiology	Fungicidal

Noteworthy is the group of strains 1-I, 2-I, 3-I, 4-I, 5-I-12/23 which did not differ genetically from each other, although they were isolated from different sources and had some differences in antagonistic activity. This could be due to the genetic and evolutionary proximity of the strains with the simultaneous presence of different genes that determine the biological properties. In other words, genomes can be similar, but differ in a small number of structural genes. With the appearance of an increasing number of changes in the genome in the process of evolution or selection for certain traits, visible differences appear in the genomic DNA. We compared the DNA fragments on the filter visually. Existing programs for comparing genetic profiles, for example BioNumerics™ (https://www.applied-maths.com/down-load/software) can be used when a large number of compared strains. According to the instructions for the specified program, the final stage of the computer comparison is the visual control of the coincidence of the fragments. In our case, given the small number of strains, preference was given to visual assessment. Differences in the number and distribution of DNA fragments suggest that the samples are genetically distinct strains. If the DNAs in the samples do not differ, there is a possibility that they may have a different genetic profile when using a different genotyping method.

Our studies have shown that the DDSL technique makes it possible to identify both strains of bacteriosis pathogens during the development of epiphytotics and antagonistic microorganisms introduced into the agrocenosis, which will allow us to further study the features of the parasite-host relationship in the plant-plant pathogen-antagonist system at the population level.

The study of *Bs* activity against pathogens of bacterial and fungal potato diseases in vitro on a wide range of test cultures showed that the *Bs* I-5-12/23 strain is the most promising for protection against bacterioses [32]. In our tests with artificial inoculation of tubers with *P. atrosepticum* 1944 and *P. carotovorum* subsp. *catovorum* 481, there was a significant decrease in the infection of potatoes

during storage after treatment of infected plant material with *Bs* I-5-12/23 (Table 2). The index of development of soft bacterial rot was 0-0.2 vs. 4.04 in the control. The biological efficiency of the chemical fungicide Maxim, KS was 77.7% while the treatment of tubers with a suspension of the *Bs* strain I-5-12/23 led to 100%.

2. Biological effectiveness of *Bacillus subtilis* I-5-12/23 strain against *Pectobacterium atrosepticum* 1944 and *P. carotovorum* subsp. *catovorum* 481 on potato (*Solanum tuberosum* L.) cv. Sante tubers after artificial inoculation ($N = 5$, $n = 10$, $M \pm$SEM)

Variant	Disease prevalence, %	Disease development index	Biological effectiveness, %
Control (nwithout treatment)	80.7±4.3	4.04±1.8	
B. subtilis, 10^9 CFU/ml	0	0	100
B. subtilis, 10^8 CFU/l	20.4±2.8	0.02±0.01	99.5±0.3
Maxim, KS, 0.2 l/g (standard)	80.1±6.6	0.9±0.1	77.7±0.2
LSD_{05}	9.8	0.1	0.5

Comparison of the effectiveness of *Bs* strains I-5-12/23 and M-22 in storage conditions showed that the former had a more pronounced antagonistic activity against ring rot, foot rot, and fusarium (Table 3). We did not observe any significant differences in the effectiveness of the preparations with respect to ordinary scab.

3. Biological effectiveness of *Bacillus subtilis* I-5-12/23 and M-22 strains against diseases of potato (*Solanum tuberosum* L.) cv. Udacha tubers during storage ($M \pm$SEM, Moscow Province, Kraskovo, winter of 2018-2019)

Variant	Portion of tuber, %					
	healthy	infected				
		total	ordinary scab	ring rot	foot rot	fusariosis
Control (water)	13.3±1.1	86.7±2.5	52.3±2.2	7.4±0.3	11.8±1.2	15.2±1.5
B. subtilis M-22, 10^9 CFU/ml	30.4±1.9	69.6±3.3	47.3±1.7	4.8±0.2	8.5±0.7	9.0±0.8
B. subtilis И-5-12/23, 10^9 CFU/ml	32.4±1.7	67.6±1.2	53.8±4.5	2.6±0.1	8.2±0.3	3.0±0.1
B. subtilis И-5-12/23, 10^8 CFU/ml	35.5±2.0	64.5±2.6	55.7±3.7	2.9±0.2	4.4±0.2	1.5±0.1
Maxim, KS, 0.2 l/g	30.0±1.5	70.0±4.3	54.1±3.3	3.5±0.1	8.3±0.4	4.1±0.2
LSD_{05}	2.3	5.4	6.6	0.4	1.4	0.2

Analysis of the obtained data indicates that the biological effectiveness of *Bs* strains against potato tuber diseases during storage is comparable to the effectiveness of a chemical fungicide. The rate of healthy tubers in the control did not exceed 13.3%, after treatment with *Bs* strains reached 30.4-35.5%, which increased the yield of healthy products by more than 2 times.

It should be noted that in the genotyping of plant pathogenic bacteria, antagonistic microorganisms and biodestructors by our proposed DDSL technique, the pairs of restriction endonucleases are used which require 1-2 hours to develop the reaction. In recent years, commercial preparations of similar enzymes with a shorter incubation time (up to 5 min, the so called fast digest), for example, restriction endonucleases from Thermo Fischer Scientific, Inc. (USA), have appeared. They give a faster result, but this is associated with a certain risk of incomplete cleavage of genomic DNA, which distorts the appearance of the genetic profile.

Various methods of genotyping pseudomonads are discussed in the literature. In particular, when genotyping 232 isolates using the pulsed-field gel electrophoresis (PFGE) method and multiple locus variable-number tandem repeat analysis (MLVA), the results were only 91% consistent [36]. These data once again emphasize the need for the use of high-resolution methods that allow the detection of a large number of DNA fragments in the analyzed genomes. Genotyping of species of the genus *Pectobacterium* is often carried out using multilocus sequencing of various numbers of housekeeping genes, from four [37] to thirteen [38].

So, the method of genetic certification developed by us (double digest and selective label technique, DDSL) helps to reveal and unambiguously identify strains of plant pathogenic bacteria of the genera *Pectobacterium* and *Pseudomonas* and their antagonists in the phytosanitary monitoring during epiphytoties of bacteriosis, which, in particular, is necessary for development of phytosanitary technologies for growing and storing potatoes. In addition, the DDSL technique genetically identifies strains of biodestructors from the genus *Pseudomonas*, which allows for identity control of strains during their commercial use (certification). Genotyping by the DDSL technique visualizes a large number of DNA fragments (in this work more than 40), the distribution of which characterizes and individualizes the bacterial strain. For each type of microorganism, a preliminary selection of restriction enzymes is required, which produce the optimal number and size of DNA fragments on the filter. The combination of the DDSL technique and the assessment of biological activity showed that of the studied *Bacillus subtilis* (*Bs*) strains, the *Bs* I-5-12/23 has the most pronounced antagonistic properties against the causative agents of ring rot, foot rot and fusarium disease. Postharvest treatment of potatoes with itaqueous suspension of *Bs* I-5-12/23 also revealed high biological effectiveness with a significant decrease in the damage to tubers during storage compared to the chemical preparation Maxim, KS. The DDSL technique can be also used to study populations of microorganisms in natural and artificial biocenoses.

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