

DOI: <https://doi.org/10.23649/JAE.2023.39.5>

EVALUATION OF THE CAPABILITIES OF THE RAPD-PCR METHOD FOR THE GENETIC IDENTIFICATION
OF *B. SUBTILIS* STRAINS

Research article

Terletskiy V.P.^{1,*}

¹ ORCID : 0000-0003-4043-3823;

¹ Pushkin Leningrad State University, Saint-Petersburg, Russian Federation

* Corresponding author (valeriter[at]mail.ru)

Abstract

Currently, special attention is paid to biological methods of plant protection. Widely used bacteria that are antagonists of phytopathogens include *B. subtilis*. The bacterium is an active component of many biopreparations. The selection of PCR primers for genotyping and identification of commercially used strains of this bacterium remains an urgent task. This study identified the most effective primers for performing RAPD-PCR on a group of bacterial isolates in order to identify their genetic diversity. It was found that the OPA-3 primer is capable of identifying 4 different genotypes, and OPL-12 – three. Other three primers including OPM-15, M13 and ERIC1/Eric2 did not produce satisfactory results and cannot be recommended for routine use with isolates of *B. subtilis*. At the same time, reproducibility of the results and good agreement between the data obtained with two selected primers in different experiments were observed.

Keywords: RAPD-PCR, *Bacillus subtilis*, genotyping.

ОЦЕНКА ВОЗМОЖНОСТЕЙ МЕТОДА RAPD-PCR ДЛЯ ГЕНЕТИЧЕСКОЙ ИДЕНТИФИКАЦИИ
ШТАММОВ *B. SUBTILIS*

Научная статья

Терлецкий В.П.^{1,*}

¹ ORCID : 0000-0003-4043-3823;

¹ Ленинградский государственный университет имени А. С. Пушкина, Санкт-Петербург, Российская Федерация

* Корреспондирующий автор (valeriter[at]mail.ru)

Аннотация

В настоящее время особое внимание уделяется биологическим методам защиты растений. К широко используемым бактериям, являющимся антагонистами фитопатогенов, относится *B. subtilis*. Эта бактерия является активным компонентом многих биопрепаратов. Актуальной задачей остается подбор ПЦР-праймеров для генотипирования и идентификации коммерчески используемых штаммов этой бактерии. В данной работе были определены наиболее эффективные праймеры для проведения RAPD-ПЦР на группе бактериальных изолятов с целью выявления их генетического разнообразия. Установлено, что праймер OPA-3 способен идентифицировать 4 различных генотипа, а OPL-12 – три. Остальные три праймера, включая OPM-15, M13 и ERIC1/Eric2, не дали удовлетворительных результатов и не могут быть рекомендованы для проведения рутинной работы с изолятами *B. subtilis*. В то же время наблюдалась воспроизводимость результатов и хорошее совпадение данных, полученных с двумя выбранными праймерами в разных экспериментах.

Ключевые слова: RAPD-PCR, *Bacillus subtilis*, генотипирование.

Introduction

The natural features of *Bacillus subtilis* strains (wide biodiversity within the species, the ability for sustainable growth in various media, symbiotic properties, high antagonistic activity, production of a number of hydrolytic enzymes and antibiotics of different chemical classes, resistance to adverse environmental factors and ecological plasticity) determined their prospects to obtain new biological products to protect plants from diseases of fungi and bacterial origin [1], [2], [3]. *B. subtilis* is capable of producing various hydrolytic enzymes, due to which lysis of the cell wall of the phytopathogenic fungus and bacteria occurs [4]. Due to the peculiarities of the organization of the genome, which was a consequence of the need to adapt to changing environmental conditions, *B. subtilis* has high genetic plasticity within its strains, which has made it a convenient object for numerous studies [5], [6], [7]. Currently, the bacterium is widely used in agriculture as a biofertilizer and an antagonist to soil-borne plant infections, being part of many commercially used biopreparations [8].

Revealing genetic variations in bacteria populations can be achieved by application of genotyping techniques. In addition, bacterial genotyping is used to identify strains, identify antibiotic resistance genes and virulence genes. One of the most effective and fast method for genotyping is PCR, which allows the amplification of polymorphic regions in the genomes being studied. This approach makes it possible to quickly identify bacterial strains, that makes it possible to identify the spread of infections, determine the sources of the pathogen in environmental objects, and certify commercially used strains of antagonistic bacteria that are used in the development of environmentally friendly biopreparations. PCR-based genotyping methods have significant advantages over many other methods for identifying genetic profiles of strains. In particular, the pulsed-field gel electrophoresis (PFGE) method requires expensive specialized equipment and analysis takes a long time. Methods for identifying bacterial strains using sequencing of specific DNA regions, such as multilocus sequence typing

(MLST), often do not provide a sufficiently high resolution, since they are based on sequencing only several conserved housekeeping genes. Work is constantly being carried out to develop genotyping methods based on the use of polymerase chain reaction. Among the fastest and simplest genotyping methods is RAPD-PCR, which is based on the use of short primers for carrying out a polymerase chain reaction [9], [10].

Research methods and principles

The research object was 14 *B. subtilis* isolates isolated from environmental objects and grown in the laboratory of Microbiological Plant Protection of the All-Russian Institute of Plant Protection. A literature search was conducted to get optimal primers for PCR. We analyzed genotyping data, discriminatory ability, quantity and quality of amplified DNA fragments obtained previously on a number of strains of this bacterial species. As a result of the search, three short RAPD-PCR primers were identified (OPA-3, OPL-12 and OPM-15), along with forward and reverse primers ERIC, which are used to amplify a conserved region of the genome of enterobacteria of different species (ERIC1 and ERIC2), as well as primer M13, which detects minisatellite DNA in bacteria and animals (see table 1).

Table 1 - Nucleotide sequences in the primers used for genotyping *B. subtilis* isolates

DOI: <https://doi.org/10.23649/JAE.2023.39.5.1>

Primer	Nucleotide sequence	T denaturation (15 sec), °C	T annealing (15 sec), °C	T elongation (60 sec), °C
OPA-3	AGTCAGCCAC	95	37	72
OPL-12	GGGCGGTACT	95	37	72
OPM-15	GACCTACCAC	95	37	72
ERIC1	ATGTAAGCTCC T GGG GATTCAC	95	37	72
ERIC2	AAGTAAGTGA CT GGG GTGAGCG	95	37	72
M13	GAGGGTGGCG GT TCT	95	37	72

The primers were first checked simultaneously against several publications in the scientific literature to eliminate errors in the nucleotide sequence, and then checked against the NCBI database to confirm their specificity with respect to the strains of bacteria being studied and additional control of the nucleotide sequence.

The mechanism for detecting genomic DNA variability using PCR with ERIC primers is the amplification of a DNA section that has different lengths in individual bacterial strains. RAPD primers detect differences in DNA at the species level and often discriminate even strains at subspecies level in bacteria. The mechanism for detecting differences lies in different primer binding sites in different species and strains, which leads to the amplification of DNA fragments of various lengths.

The reaction mixture for PCR for 10 samples contained the following components:

- 103 µl of distilled water
- 14 µl of producer suggested PCR buffer
- 14 µl MgCL₂ (25 mM, 2.5 mM of final concentration)
- 3 µl dNTP (5 mM, 100 µM of final concentration)
- 3 µl primer (20 µM, 0.4 µM of final concentration)
- 3 µl Hot Start Taq polymerase (5u/µl).

After mixing the components, the mixture was added to 10 PCR tubes in a volume of 14 µl in each. Then 1 µl of genomic DNA isolated from bacterial strains was added to these tubes. At the primer annealing temperature recommended in the literature for ERIC primers of 52°C, surprisingly, no amplification occurred, and this circumstance required lowering the temperature to 37°C. Before the first PCR cycle, a long primary denaturation step was carried out (once) at 95°C for 5 minutes, and after amplification, a long elongation step was carried out at 72°C for 3 minutes. The results of genotyping using the RAPD method were carried out twice, and in both cases the results were the same, which indicates the reproducibility of the selected conditions for conducting the DNA fragment amplification reaction. Preliminary experiments have shown that the optimal concentration of magnesium chloride in the mixture is 2.5 mM. At this concentration, active synthesis of PCR products occurred without the appearance of an excessive trail of nonspecific fragments (Fig.1). Optimal annealing temperature for all primers was 37°C.

After completion of PCR, the amplified DNA was transferred into wells for agarose electrophoresis in the presence of ethidium bromide (1.5% agarose, Tris-acetate buffer). Electrophoresis is carried out at 100V for 3 hours in a chamber with a distance between electrodes of 20 cm (voltage gradient 5 V/cm). GeneRuler (ThermoFisher™) was used as a marker to determine the lengths of DNA fragments that were amplified as a result of PCR. Visualization of the results of separation of amplified fragments was carried out in a gel documentation system under ultraviolet light. Analysis of the number and distribution of DNA fragments in the gel was carried out visually in relation to DNA fragments of marker DNA.

Main results

The OPM-15 primer did not result in the amplification of discernible DNA fragments in the *Bacillus subtilis* genome, and the ERIC primer pair produced a monomorphic pattern consisting of two DNA fragments in all isolates. Primer M13 also did not lead to the formation of clearly distinguishable amplifications. The primers OPA-3 and OPL-12 turned out to be the most informative for *B.subtilis* (fig. 1).

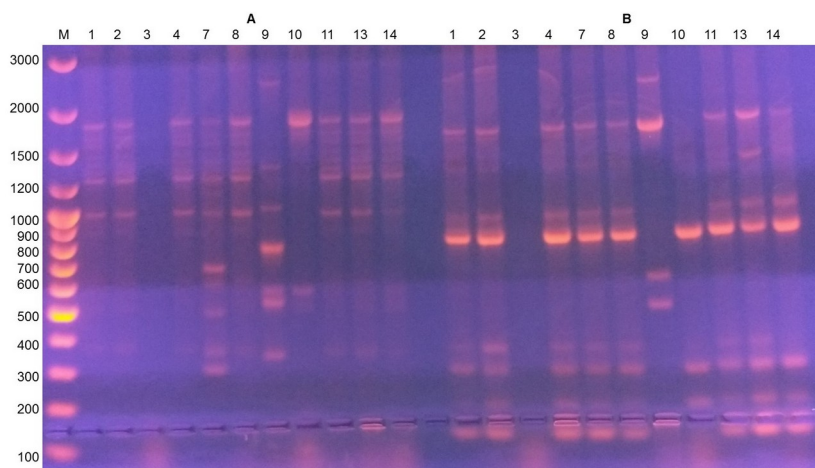


Figure 1 - Genotyping *B.subtilis* strains using primer OPA-3 (left, A) and primer OPL-12 (right, B)
DOI: <https://doi.org/10.23649/JAE.2023.39.5.2>

Note: M - marker of DNA fragment length (GeneRuler 100 bp, Thermo Fisher Scientific)

No amplification occurred in isolate number 3. The OPA-3 primer revealed 4 different genotypes among all isolates, with the dominant cluster comprising isolates 1, 2, 4, and 8, while isolates 7, 9, and 10 were unique, i.e. they differed from the dominant cluster and from each other. A similar picture was observed when using the OPL-12 primer. The difference was that in addition to isolates 1, 2, 4, and 8, the dominant cluster also included isolate 7. Accordingly, the remaining isolates 9 and 10 were unique. In total, the latter primer revealed three genotypes.

Thus, these two primers can be successfully used for genotyping and identification (certification) of *Bacillus subtilis* strains, since they are capable to amplify several polymorphic DNA fragments for subsequent visualization.

Conclusion

1. The optimal temperature for genotyping *B. subtilis* using primers OPA-3 and OPL-12 is 37°C, the concentration of magnesium ions is 2.5 mM;
2. When genotyping *B. subtilis* strains, the short RAPD primers OPA-3 and OPL-12 were the most informative as compared with three other primers;
3. PCR genotyping of *B. subtilis* strains used as an active component in biopreparations allows certification of these strains to confirm their purity and genetic uniformity.

Финансирование

Работа выполнена по теме Государственного задания 0665-2019-0019 «Разработка эколого-генетических основ отбора штаммов микробов-антагонистов, энтомопатогенных грибов и нематод; разработка технологий получения и применения новых полифункциональных препаратов для контроля численности вредных организмов (вредители, возбудители болезней) и повышения супрессивности почвы».

Конфликт интересов

Не указан.

Рецензия

Все статьи проходят рецензирование. Но рецензент или автор статьи предпочли не публиковать рецензию к этой статье в открытом доступе. Рецензия может быть предоставлена компетентным органам по запросу.

Funding

The studies were carried out as part of the implementation of project No.0665-2019-0019 «Development of Ecological and Genetic Basis for Selection of Strains of Microbial Antagonists, Entomopathogenic Fungi and Nematodes; Development of Technology of Generation and Application of New Polyfunctional Biopreparations for Control over Harmful Organisms (Pests, Pathogens) and for Increasing Soil Suppressiveness».

Conflict of Interest

None declared.

Review

All articles are peer-reviewed. But the reviewer or the author of the article chose not to publish a review of this article in the public domain. The review can be provided to the competent authorities upon request.

Список литературы на английском языке / References in English

1. Berić T. RAPD Analysis of Genetic Diversity and Qualitative Assessment of Hydrolytic Activities in a Collection of *Bacillus* sp. isolate / T. Berić, M. Urdaci, S. Stanković [et al.] // Archives of Biological Sciences. — 2009. — Vol. 61(4). P. 645-652. DOI:10.2298/ABS0904645B.
2. Kaspar F. Bioactive Secondary Metabolites from *Bacillus subtilis*: A Comprehensive Review / F. Kaspar, P. Neubauer, M. Gimpel // J. Nat. Prod. — 2019. — Vol. 82(7). — P. 2038-2053. DOI: 10.1021/acs.jnatprod.9b00110.
3. Abdelmoteleb A. New *Bacillus subtilis* Strains Isolated from *Prosopis glandulosa* Rhizosphere for Suppressing *Fusarium* Spp. and Enhancing Growth of *Gossypium hirsutum* L. / A. Abdelmoteleb, L. Moreno-Ramírez, B. Valdez-Salas [et al.] // Biology (Basel). — 2022. — Vol. 12(1). P. 73. DOI: 10.3390/biology12010073.
4. Maleki F. Consolidated Bioprocessing for Bioethanol Production by Metabolically Engineered *Bacillus subtilis* Strains / F. Maleki, M. Changizian, N. Zolfaghari [et al.] // Sci. Rep. — 2021. — Vol. 11(1). — P. 13731. DOI: 10.1038/s41598-021-92627-9.
5. Pinchuk I.V. Amicoumacin Antibiotic Production and Genetic Diversity of *Bacillus subtilis* Strains Isolated from Different Habitats / I.V. Pinchuk, P. Bressollier, I.B. Sorokulova [et al.] // Res. Microbiol. — 2002. Vol. 153(5). — P. 269-276. DOI: 10.1016/s0923-2508(02)01320-7.
6. Matarante A. Genotyping and Toxigenic Potential of *Bacillus subtilis* and *Bacillus pumilus* Strains Occurring in Industrial and Artisanal Cured Sausages / A. Matarante, F. Baruzzi, P.S. Cocconcelli [et al.] // Appl. Environ. Microbiol. — 2004. — Vol. 70(9). — P. 5168-5176. DOI: 10.1128/AEM.70.9.5168-5176.
7. Lefevre M. Safety Assessment of *Bacillus subtilis* CU1 for Use as a Probiotic in Humans / M. Lefevre, S.M. Racedo, M. Denayrolles [et al.] // Regul. Toxicol. Pharmacol. — 2017. — Vol. 83. — P. 54-65. DOI: 10.1016/j.yrtph.2016.11.010.
8. Lee B.C. High Antimicrobial Activity of Lactoferricin-expressing *Bacillus subtilis* Strains / B.C. Lee, J.C. Tsai, C.W. Hung [et al.] // Microb. Biotechnol. — 2022. — Vol. 15(6). — P. 1895-1909. DOI: 10.1111/1751-7915.14026.
9. Kwon G.H. Development of a RAPD-PCR Method for Identification of *Bacillus* Species Isolated from Cheonggukjang / G.H. Kwon, H.A. Lee, J.Y. Park [et al.] // Int. J. Food Microbiol. — 2009. — Vol. 129(3). — P. 282-287. DOI: 10.1016/j.ijfoodmicro.2008.12.013.
10. Khowal S. A Report on Extensive Lateral Genetic Reciprocation between Arsenic Resistant *Bacillus subtilis* and *Bacillus pumilus* Strains Analyzed Using RAPD-PCR / S. Khowal, M.Z. Siddiqui, S. Ali [et al.] // Mol. Phylogenet. Evol. — 2017. — Vol. 107. — P. 443-454. DOI: 10.1016/j.ympev.2016.12.010.