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PAENIBACILLUS POLYMIXA, AS A CORE DIAZOTROPHIC ENDOLICHENIC NON PHOTOSYNTHETIC BACTERIOBIOME REPRESENTATIVE OF CORTICOLOUS *PHYSCIA BIZIANA* THALLUS

M. G. GHARIBYAN [1*], R. R. SARGSYAN [1,2**], H. H. PANOSYAN [1***]

[1] *Chair of Biochemistry, Microbiology and Biotechnology, YSU, Armenia*
[2] *Lichen Research and Conservation Group, Takhtajyan Institute of Botany of NAS of the Republic of Armenia*

The unique organization of lichen thallus provides still unexplored environment for microbial communities. The study aimed to isolate and identify endolichenic non photosynthetic diazotrophic bacteria from the thallus of corticolous lichen species *Physcia biziana*. Two strains of chemoorganoheterotrophic endospore-forming bacteria were isolated and identified based on 16S rRNA gene sequence analysis as *Paenibacillus polymyxa* (> 93.54% similarity). Characterization of cultivable strains suggest the involvement of associated bacteria in nitrogen cycling. This study highlights the significance of studying the endobacteriobiome of lichens, as it is an important aspect of their multicomponent natural biofilm.

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***Keywords*:** *Physcia biziana*, endolichenic bacteriobiome, *Paenibacillus polymixa*, 16S rRNA genes.

Introduction. Lichens represent some of the oldest and most diverse symbiotic lifestyles on Earth. Lichen thallus is a multicomponent mutualistic symbiotic structure composing of mycobiont (fungus), photobiont (cyanobacterium/alga), and non-photosynthetic bacteriobiont (third symbiotic partner) [1, 2]. Lichen bacterial associations were first studied based on traditional cultivation techniques in the last century [3–5]. The dominating genera isolated and identified based on phenotypic features were mainly *Azotobacter*, *Pseudomonas*, *Beijerinckia*, *Bacillus* and *Clostridium* [5]. Although information on lichen thallosphere and thallus-associated bacteribiomes has been known for a long time, their microbial communities is not yet well characterized. A series of research have been undertaken to affiliate the third symbiotic partners of lichens using up-to-date culture-independent approaches [5, 6]. Comparative omics studies in recent years have revealed that lichens are furnished with a complex bacterial endomicrobiome, forcing the redefining of lichen as a multicomponent natural biofilm. It was shown that more than 800 types of bacteria can contribute to the bacterial microbiome of a single lichen individual [1].

The lichen bacteriobiome consists of two parts, core (more stable part) and specific microbial (variable part depends on species) communities [7]. In many so

* E-mail: manehgharibyan@gmail.com
** E-mail: razmiksargsyan@ysu.am
*** E-mail: hpanosyan@ysu.am

far studied lichens, Proteobacteria form the largest and metabolically most active bacterial class. The other dominant phyla found in lichens were Actinobacteria, Bacteroidetes, Firmicutes, Deinococcus and Verrucomicrobia [1, 6, 8–10]. The lichen species is probably the best predictor of its microbiome composition [11].

The lichen microbiome may contribute multiple functions to the lichen symbiotic system [1, 2]. The endolichenic bacteriobiome participates in diazotrophy, nutrient supply by uptake and/or assimilation of biogenic elements, decomposition of macromolecules, produces hormones and vitamins that stimulate thallus growth and photosynthesis, protects lichens from abiotic and biotic stresses by detoxification reactions and the production of antibacterial substances [1]. Therefore, it is important to study lichen microbiomes to understand their ecological and functional role in the symbiosis.

More than 600 lichen species have been described in Armenia, but their microbial coexistence have not been studied enough and evaluated [12]. The diazotrophic non photosynthetic bacterial diversity of lichens found in Armenia have been investigated since 1960s. Bacteria belonging to genera *Azotobacter, Bacillus* and *Pseudomonas* have been isolated and characterized from most distributed in Armenian lichens like *Parmella, Lecanora, Ramalina, Physcia* [4]. Considering development of methodological approaches and actuality of investigation of bacterial composition of lichens we aimed in this study to isolate diazotrophiic endolichenic bacterial composition from one of the most common lichens in the territory of Armenia, *Physcia biziana*, based on cultivable methods and to identify the isolates based on phylogenetic analysis of 16S rRNA genes.

Materials and Methods.

***Collection of Samples*.** The object of study were the samples of corticolous lichen thalli collected from the surroundings of "Jrvezh" forest park (N 40°11'20" E 44°36'30") in Yerevan in September 2019. The area mostly covered by temperate deciduous forests predominating by Oriental Beech (*Fagus orientalis*), Hornbeam (*Carpinus* spp.) and Oak (*Quercus* spp.). The samples were packed in sterile plastic bags and transferred to the laboratory. where they were cleaned of soil debris, other elements and identified according to the identification key as a species belonging to the genus *Physcia – P. biziana* [12].

***Isolation of Diazotrophic Bacteria*.** To isolate the non-photoautotrophic bacteria, lichen thalli were washed with running tap water two times to remove soil particles and other debris. For the surface sterilization process was chosen method described by Li et al. [13] with some modification. The washed samples were immersed in 70% ethanol for 5 *min* in aseptic conditions, then were washed by sterilized distilled water two times. To ensure disinfection the lichen thalli were dipped in 0.9% NaClO two times for 30 *s* each, then rinsed with sterilized distilled water 5 times for 30 *s*, subsequently treated lichen thalli were left for desiccation. After drying, samples were transferred to 10% $NaHCO_3$ for 10 *min* to complete the sterilization.

After sterilization of the outer surface the thalli were cut into 0.5 cm^2 pieces under aseptic conditions with sterile lancet. Then to isolate atmospheric nitrogen fixating bacteria, thalli pieces were placed on the surfaces of the medium containing (*g*/*L*): malic acid, 5.0; K_2HPO_4, 0.5; $MgSO_4$, $7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, $2H_2O$,

0.02; micronutrient solution ($CuSO_4$, $7H_2O$, 0.12; H_3BO_3, 1.40; Na_2MoO_4, H_2O, 1.0; $MnSO_4$, H_2O, 1.175). Complete volume to 1000 *mL* with distilled water, 2 *mL* bromothymol blue (5 *g*/*L* in 0.2 *N* KOH), 2 *mL*; FeEDTA (solution 16.4 *g*/*L*), 4 *mL*; vitamine solution (biotin, 10 *mg*; pyridoxal-HCl, 20 *mg*), 1 *mL*; KOH, 4.5 *g*; and 15 *g* agar to make a solid medium. Lichen thalli were macerated also into homogenized pieces using a mortar followed by serial dilution of up to 10^{-5} in microcentrifuge tubes. Thereafter, 20 μL of ground lichen thalli solution was inoculated into the solid medium mentioned above. All inoculated plates were incubated at 28°C for 5 days. The bacteria colony characteristics such as color, texture, and shape, were observed and single colonies were collected and restreaked on the same agar plates for purification by incubating 3 days. All isolates are maintained in the culture collection of microbes at Chair of Biochemistry, Microbiology and Biotechnology, YSU.

Identification of Isolated Bacteria. Slides were prepared from purified cultivated bacteria for microscopy. Gram staining and spore staining were also performed to determine their shape by Peshkov's differential method using Loeffler's methylene blue as a dye. Catalase and oxidase activity of the isolates were determined using widely obtained methods [14].

DNA Extraction from Isolated Bacteria. Genomic DNA was isolated from bacterial cells using by Gene-EluteTM Bacterial Genomic DNA Kit ("Sigma Aldrich"). The integrity of the isolated DNA was checked by agarose gel electrophoresis, which was performed for 40 *min* at 80 *V* in a 1% agarose gel using Tris-Acetate-EDTA (TAE) as a buffer and ethidium bromide as a fluorescent marker. The genomic DNA was eluted with 50 μL of Tris-EDTA bufer (TE) and used as a template in the PCR assays.

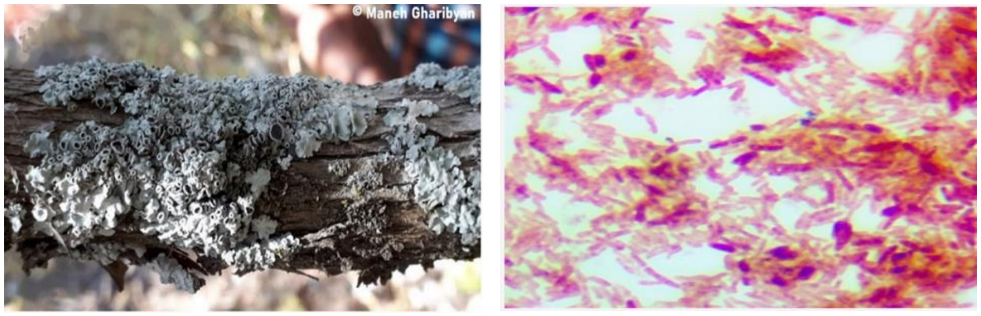
Polymeric Chain Reaction (PCR). 16S rRNA genes were amplifed using universal primer pairs 27f (5′-GAGTTTGATCCTGGC TCA-3′) and 1525r (5′-GAAAGGAGGAGATCCAGCC-3′) (*Escherichia coli* numbering). PCR mixtures used for amplifcation of sequences contained 10 *ng* DNA, 5 μL 10×PCR bufer, 5 μL 10 *mM* dNTP (dATP, dGTP, dCTP, and dTTP), 1 μL of each primer (25 *pmol*/μL), 1.5 *mM* $MgCl_2$, 0.2 μL Taq DNA polymerase and sterile water to make up a final volume of 50 μL. PCR amplification was conducted using a DNA Engine thermocycler (BIO-RAD). First, the templates were denaturized for 3 *min* at 96°C, then 30 cycles of the following steps were completed: denaturation for 30 *s* at 96°C, annealing for 30 *s* at 55°C and extension for 2.5 *min* at 72°C. The 30 cycles were followed by a final 10 *min* extension at 72°C. PCR products were viewed under UV light after standard ethidium bromide gel electrophoresis. PCR products were purified using the GenElute™ PCR Cleanup Kit ("Sigma Aldrich").

Sequencing and Phylogenetic Analysis. Bacterial DNA associated with lichen thallus was sent to the University of Bergen in Norway for phylogenetic analysis. Each isolate's 16S rRNA sequence was sequenced using the BigDye Terminator kit ("Perkin Elmer") on an ABI PRISM capillary sequencer as a phylogenetic marker. The pair of unique primers 27F (5' GAGTTTGATCCTGGCTCA 3') and 1492R (3' GAAAGGAGGAGATCCAGCC 5') was used for the amplification of 16S r-RNA genes. Sequences were processed with Chromas (version 2.6.6) software. Reverse sequences were aligned with the Bioedit Sequence Alignment

Editor tool. Consensus sequences obtained were aligned with the closest bacterial sequence in the NCBI (National Center for Biotechnology Information) gene bank using the nucleotide BLAST tool, thus identifying them to species. Alignment for phylogenetic analysis of 16S rRNA genes was made using ClustalW 4 [15]. Phylogenetic tree of the strains was constructed using the neighbor-joining method with the MEGA X software [16]. Confidence in branching points was determined by bootstrap analysis (1000 replicates).

Nucleotide Sequence Accession Numbers. The 16S rRNA gene sequence was deposited to GenBank and the accession number assigned was as follows: PP330953.

Results and Discussion. Collected corticolous lichen thalli identified based on standard methods as *P. biziana* were used to isolate endolichenic diazotrophic bacteria. Two aerobic chemoheterotrophic diazotrophic strains designated 1.1 and 1.2 have been isolated on used medium after incubation of 5 days at 28°C. The microscopic examination of their fixed preparations, after Gram and Peshkov staining, showed that all isolates were aerobic, oxidase, catalase positive, Gram postive rod-shaped bacteria and had oval endospores (Fig. 1).



A B

Fig. 1. A) *P. biziana* as an epyphite of *Fagus orientalis* in "Jrvezh" forest park; B) Peshkov stained endospores of strain 1.1 (magnification rate ×2000).

The 16S ribosomal gene of the isolates was partially sequenced. Identification by BLASTn searches for significant sequence similarity revealed that both phylotypes represent the genus *Paenibacillus* (Firmicutes). As a result, two strains isolated from the species *P. biziana* were closely related to the species *Paenibacillus polymixa* (see Table). The isolate 1.1 had 93.54% sequence similarity to *P. polymixa,* while isolate 1.2 demonstrated 94.67% similarity to *P. polymixa.*

BLAST results of 16S rRNA gene sequences of isolates and accession numbers

Strain	Sequence length, *bp*	Closest match taxonomic affiliation, phylotype accession No	% Similarity to closest match	Accession No
1.1	999	*P. polymyxa* ZF129 (CP040829.1)	93.54%	PP330953
1.2	1492	*P. polymyxa* 2F129 (CP040829.1)	94.67%	NA*

* NA – not availble.

Neighbor-joining evolutionary distance trees based on 16S rRNA gene sequences for isolated strains and selected reference sequences from Gen Bank were constructed (Fig. 2). A monophyletic clade of closely related phylotypes can be clearly identified in the tree. Both strains constitute a part of the cluster with *P. polymyxa* (EF656457.1) and *Paenibacillus jamilae* (MH017390.1). The positions of the strains in the phylogenetic tree clearly indicate that the studied strains belong to *P. polymyxa*.

P. polymyxa is a diazotrophic bacterium, some strains of which synthesize polymyxin antibiotic compounds [17]. It lives also in the rhizosphere and inside plants (i.e., endophytic lifestyle). The effectiveness of complexes of surfactant compounds produced by *P. polymyxa* against biofilms of *Bacillus subtilis, Micrococcus luteus, Pseudomonas aeruginosa, Shaphylococcus aureus, Streptococcus bovis* bacteria has been proven [18]. All this suggests that the possible role of this bacterium in lichen is the fixation of atmospheric nitrogen, the stimulation of lichen growth and possibly the production of several secondary metabolites that endow the lichen with resistance to abiotic and biotic stresses in the environment.

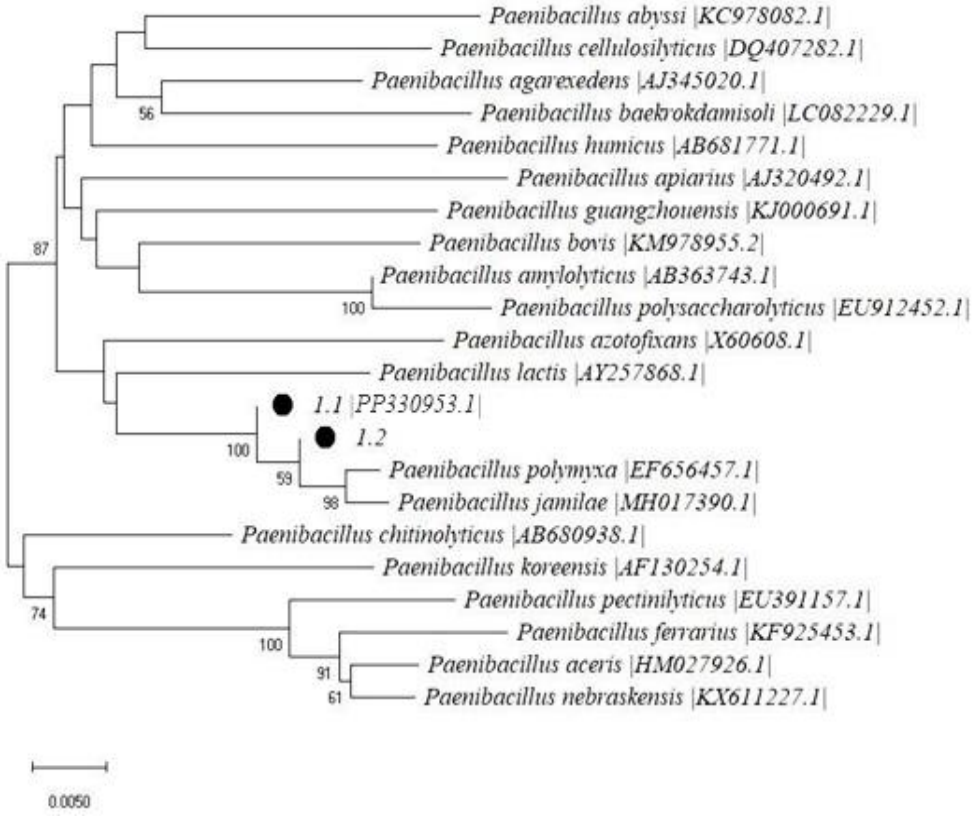


Fig. 2. Phylogenetic tree based on nearly complete 16S rRNA gene sequences, showing the relationships between isolated of *Paenibacillus* strains obtained from *Physcia biziana* thalli and closely related species. Evolutionary analyses were conducted in MEGAX using the neighbor-joining method. The percentage of replicate trees (> 50%) in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Scale bar represents 0.005 substitutions per site.

The lichen microbiome is identified as a surprisingly abundant and structurally integrated element of the classical lichen symbiosis. The diversity of lichen-associated non phototrophic diazotrophic bacterial community of *P. biziana* was, to our knowledge, for the first time investigated by a culture-dependent approach. The *P. biziana* lichen samples were chosen, because it was poorly studied and one of the most distributed corticolous lichens in Armenia. It is not surprising to find also *Paenibacillus* phylotypes within the microbial community of terrestrial lichens. Paenibacilli belong to the Firmicutes phylum and are one of the four most frequent and isolated phyla in the lichen-associated microbiome [1, 5, 10, 19]. All those findings confirmed paenibacilli as a core endolichenic bacteriobiome representatives, and were found for many corticolous and saxicolous lichens. Thus, different species of *Paenibacillus*, like *Paenibacillus pabuli, Paenibacillus agarexedens, Paenibacillus amylolyticus*, have been isolated from the lichens belonging to genus *Cladonia* (*Cladonia coccifera, Cladonia rangiferina, Cladonia digitate*) [20]. *P. pabuli* and *P. amylolyticus* as endolichenic microbes were isolated from *Hypogymnia physodes*, while *Paenibacillus mendelii* and *Paenibacillus phyllosphaerae* were found in thalli of *Pseudevernia furfuracea* [5]. Recently the microbial community associated with the crustose lichen *Rhizocarpon geographicum* L. (DC.) living on oceanic seashore have been also investigated. A large source of diversity revealed by using multiple isolation methods, among which *Paenibacillus etheri*, was the most abundant bacterial species found at the species level [10]. Another species *Paenibacillus odorifer* isolated from *R. geographicum* has been characterrized as a producer of polysaccharide that exhibited remarkable antioxidant and cytotoxic activities [8]. A clear statement cannot be made about the ecological role of lichen-inhabiting bacteria. It is, nevertheless, interesting that many strains are capable of growing readily on N-free media. In the case of *Paenibacillus*, this agrees with data about strain-specific N-fixing capacities in this genus. Eight strains are known in this genus as nitrogen fixers [21]. Our data would be in accordance with a nitrogen-fixing role of lichen-associated bacterial strains, but further experiments are needed to assess whether fixed nitrogen is available in significant amounts to the symbionts.

Conclusion. In this paper, endolichenic non photosynthetic diazotrophic bactriobiome of corticolous lichen *Physcia biziana* have been studied using culture-dependent methods. Two diazotrophic endospore-forming bacterial strains have been isolated and based on 16S rRNA gene sequence analysis identified as *Paenibacillus polymyxa*. This finding suggests the possible role of *Paenibacillus polymyxa,* as a core diazotrophic endolichenic bactriobiome representative, in fixation of atmospheric nitrogen.

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Մ. Գ. ՂԱՐԻԲՅԱՆ, Ռ. Ռ.ՍԱՐԳՍՅԱՆ, Հ. Հ. ՓԱՆՈՍՅԱՆ

PAENIBACILLUS POLYMIXA ԴԻԱԶՈՏՐՈՖ ԷՆԴՈԹԱԼՈՄԱՅԻՆ ՈՉ ՖՈՏՈՍԻՆԹԵՏԻԿ ԲԱԿՏԵՐԻԱՆ, ՈՐՊԵՍ *PHYSCIA BIZIANA* ԷՊԻԼԻԹԱՅԻՆ ՔԱՐԱՔՈՍԻ ԲԱԿՏԵՐԻԱԲԻՈՄԻ ՆԵՐԿԱՅԱՑՈՒՑԻՉ

Քարաքոսային թալոմը շնորհիվ իր մենահատուկ կառուցվածքի դեռևս չբացահայտված մանրէային համակեցությունների կենսամիջավայր է: Հետազոտության նպատակն է եղել *Physcia biziana* էպիլիթային քարաքոսի էնդոթալոմային ոչ ֆոտոսինթետիկ դիազոտրոֆ բակտերիաների մեկուսացումն ու նույնականացումը: Մեկուսացվել և 16S ռՌՆԹ գեների հաջորդականության վերլուծության հիման վրա նույնականացվել են որպես *Paenibacillus polymyxa* (>93.54% նմանություն) քեմոօրգանոհետերոտրոֆ էնդոսպոր առաջացնող բակտերիաների երկու շտամներ: Ստացված շտամների նկարագրությունը թույլ է տալիս ենթադրել տվյալ բակտերիաների դերը քարաքոսի ազոտային սննդառության գործընթացում: Այս ուսումնասիրությունը ընդգծում է քարաքոսերի էնդոբակտերիոբիոմի որպես բազմաբաղադրիչ բնական կենսաշերտի ուսումնասիրության կարևորությունը:

М. Г. ГАРИБЯН, Р. Р. САРГСЯН, О. А. ПАНОСЯН

PAENIBACILLUS POLYMIXA КАК ВАЖНЫЙ ДИАЗОТРОФНЫЙ ЭНДОФИТНЫЙ НЕФОТОСИНТЕЗИРУЮЩИЙ ПРЕДСТАВИТЕЛЬ БАКТЕРИОБИОМА ТАЛЛОМА ЭПИЛИТНОГО ЛИШАЙНИКА *PHYSCIA BIZIANA*

Уникальная организация таллома лишайника представляет собой до сих пор неизученную среду для микробных сообществ. Целью исследования было выделение и идентификация доминирующих эндолихенных нефотосинтезирующих бактерий из таллома эпилитного лишайника *Physcia biziana*. Два штамма хемоорганогетеротрофных эндоспорообразующих бактерий были выделены и идентифицированы на основе анализа последовательности генов 16S рРНК как *Paenibacillus polymyxa* (сходство > 93.54%). Характеристика культивируемых штаммов позволяет предположить участие ассоциированных бактерий в круговороте азота. Данное исследование подчеркивает важность изучения эндобактериобиома лишайников, поскольку он является важным аспектом их многокомпонентной природной составляющей.