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A new *Peribacillus simplex* d27.3 strain mediates antimicrobial activity through a combination of secondary metabolites, including fengycins

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Abstract

Soil is a classical source of beneficial microorganisms. Soil microbiomes provided the overwhelming majority of antibiotic-producing strains, biocontrol agents, probiotics, and plant-protecting bacteria. The functionality of strains isolated from various soil samples is predetermined by the biosynthetic potential encoded in their genomes. Here, we describe a novel *Peribacillus simplex* d27.3 strain isolated from the soil sample of a pine forest in the Republic of Dagestan, Russia. *P. simplex* d27.3 displayed antibiotic activity against gram-positive bacteria and fungi while being inactive against the model hypersensitive gram-negative strain *E. coli* Δ*lptD.* Metabolomic analysis revealed that antimicrobial activity was partially mediated by the fengycin lipopetides (C-16 fengycin A, C-17 fengycin A, and C-16 fengycin B). In addition, the *P. simplex* d27.3 strain was found to produce other hydrophilic and more hydrophobic antimicrobials yet to be described. Thus, the *P. simplex d27.3* strain is a producer of useful antimicrobial compounds with a high potential for application in biotechnology and agriculture.

Keywords: *Peribacillus simplex*, fengycins, antibiotics, soil microbiome.

Introduction

Bacillus spp. and related bacteria are ubiquitous in nature and can be found in soil samples (Saxena et al., 2020), in the air (Yoo et al., 2019), in the animal-associated microbiota (Baranova et al., 2022), and in plant endophyte communities (Lopes, Tsui, Goncalves, and de Queiroz, 2018). These bacteria, especially in spore form, are resistant to harsh environmental conditions, including heat, low moisture, and high acidity (Nicholson et al., 2000).

Bacillus are not only widespread, but they also occupy a variety of ecological niches, become predominant cultures in their habitats, and modulate microbial communities (Kiesewalter, Lozano-Andrade, Strube, and Kovacs, 2020). An important feature of *Bacillus* species is their ability to produce structurally different antagonistic secondary metabolites (Kiesewalter, Lozano-Andrade, Strube, and Kovacs, 2020). Bacteria related to the *Bacillus subtilis* group are capable of secreting ribosomal peptides (RPs), volatile compounds, polyketides (PKs), non-ribosomal peptides (NRPs), hybrids between PKs and NRPs, including siderophores and lipopeptides (Caulier et al., 2019). Lipopeptides from *Bacillus* and *Paenibacillus spp.* are linear or cyclic

peptides synthesized by nonribosomal peptide-synthetase (NRPS) enzymes and containing a lipophilic hydrocarbon tail on the N-terminus (Cochrane and Vederas, 2016). Lipopeptides are a diverse group, including linear or cyclic, cationic or uncharged molecules: polymyxins, octapeptins, polypeptins, iturins, surfactins, fengycins, fusaricidins, tridecaptins, and kurstakins. Each class has numerous isoforms and homologues with different activities (Cochrane and Vederas, 2016).

Bacillus species are widely used as probiotic strains due to their stability and diverse secondary metabolism (Saxena et al., 2020). In animals, apart from pathogen protection, these bacteria provide the host with digestive enzymes and vitamins and have immunomodulatory effects (Luise et al., 2022). *Bacillus* species that colonize plant roots mediate the interaction of the plant with the soil microbiome and also participate in the regulation of phytohormones and other metabolites (Hashem, Tabassum, and Fathi Abd Allah, 2019).

Members of the *Bacillaceae* family not belonging to the *Bacillus* genus, including *Peribacillus simplex*, share a lot of common features with the well-known species such as *B. subtilis*, *B. thuringiensis, B. amyloliquefaciens* and *B. venlezensis* (Manetsberger et al., 2023). They have also been widely used in agriculture. However, they remain less studied than the relative *Bacillus* species (Manetsberger et al., 2023).

Materials and methods

Soil sampling and isolation of *Bacillus* species

A composite soil sample was collected from the upper soil layer at a depth of 3–5 cm in a pine forest of the Republic of Dagestan, Russia (42°24′24.1″N 46°54′20.2″E) using the "envelope" method. For the isolation of spore-forming bacteria, we dissolved 0.2 g of the soil sample in 2 ml of sterile water and homogenized it by vigorous stirring for 10 min. The homogenate was then heated at 80 °C for 30 min to eliminate non-spore-forming microorganisms and vegetative *Bacillus* cells (Tenssay, Ashenafi, Eiler, and Bertilson, 2009). After serial ten-fold dilution, 200 µl of each dilution (10x, 100x, and 1000x, respectively) was plated on the solid T3 nutrient medium (Travers, Martin, and Reichelderfer, 1987) with 20 g/L of agar for stimulating sporulation. Plates were incubated at 28 °C for 72 h. The colony of interest was selected by morphological analysis with further series of subculturing on fresh solid T3 nutrient medium (Travers, Martin, and Reichelderfer, 1987) until the culture was completely purified.

Staining parasporal bodies and vegetative cells

Toxin-containing parasporal bodies and vegetative cells were observed by light microscopy of an isolate culture stained according to the method described by Rampersad, Khan, and Ammons (2002). A bacterial culture mixed with a drop of water was applied to a microscope slide and left until completely dry. Then the slide was stained with 0.133 % Coomassie blue dye in 50 % acetic acid for 5–10 min, washed with distilled water, and dried. Parasporal bodies and vegetative cells of the strain were observed under oil immersion objective with a 100x magnification.

Identification of the *Bacillus* strain

The isolate of interest, called d27.3, was identified through the sequencing of the *gyrB* gene. The bacterial culture was grown for 1 day on a commercial LB (Luria-Bertani) broth (VWR International, UK) with the addition of 20 g/L of agar, then suspended in 150 µl of TE buffer (Tris-EDTA+H_2O, pH 7.5), incubated at 99 °C for 10 min, and centrifuged at +4 °C for 15 minutes. The resulting supernatant was used to amplify the *gyrB* gene using primers gyrB_F (5'-CTTGAAGGACTAGARGCAGT-3') and gyrB_Rf (5'-CCTTCACGAACATCYTCACC-3') by PCR (Punina et al., 2013). The volume of the reaction mixture was 20 µl, including 0.8 µl of bacterial DNA, 10 µl of Fermentas DreamTaq PCR master mix (Thermo Fisher Scientific, USA), and 0.3 µl of each primer. PCR was carried out according to the following program: 3 min at 94 °C (initial denaturation); 30 sec at 94 °C (30 denaturation cycles); 1.5 min at 72 °C (elongation); 7 min at 72 °C (final elongation); and storage stage at 12 °C. PCR products were verified by electrophoresis in a 1 % agarose gel stained with 0.002 % ethidium bromide by comparison with the λ DNA/HindIII marker (Thermo Fisher Scientific, USA). The purified PCR products were sequenced using the Sanger method in the Collective Use Center "Genome Technologies, Proteomics, and Cell Biology" (FSBSI ARRIAM). A search for homologous sequences was done using the BLAST tool of the NCBI Genbank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990).

Agar overlay assay

Antagonistic *Bacillus* strains were grown overnight in liquid cultures at 30 °C. Taken from those cultures, 2 µL aliquots were plated on 2YT (BD, Franklin Lakes, NJ, USA) agar plates to form regular colonies. The colonies were cultivated at 30 °C, followed by chloroform vapor treatment for 5 min. The agar overlay assay was performed using soft agar (8 g/L tryptone, 5 g/L yeast extract, 2.5 g/L NaCl, 0.5 % agar). Soft agar was melted, cooled to 40 °C, and inoculated with an overnight culture of the target microorganism using a 1 : 1000 dilution. Next, 7 mL of the resulting liquid soft agar was used to overlay *Bacillus* colonies. Agar plates were incubated overnight at 30 °C after soft-agar solidification. Clearance zones were measured in three biological replicates.

Isolation of the antibiotic compound

B. simplex d27.3 was cultivated in BHI medium (BD, USA) at 30 °C using 750 mL flasks in 100 mL with 250 rpm shaking. The cells were centrifuged at 10,000× g for 10 min, and the supernatant was filtered using a Millistak + HC Pod Depth Filter (Millipore, Billerica, MA, USA). To identify the active compounds, two steps of purification were performed. In the first step, the supernatant was purified by SPE with LPS-500 sorbent (Technosorbent, Russia). The fractions exhibiting antistaphylococcal activity were freeze-dried, dissolved in 10 % DMSO, and fractionated on a RP-HPLC Symmetry C18 5 µm 4.6 × 150 mm (Waters) RP-HPLC column using buffers A (10 mM NH_4OAc, 5 % ACN, pH 5.0) and B (10 mM NH4OAc, 80 % ACN, pH 5.0), flow rate of 5 mL/min, gradient of 0–5 min (0 % B), 5–10 min (0–32 % B), 10–40 min (32–74 % B), 40–50 min (74–100 % B). Absorbance was monitored at 214 and 260 nm.

Fraction activity measurement

Inhibition of bacterial cell growth was measured by a doubling dilution of medium and chromatography fractions in 2YT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) inoculated with *S. aureus* OD_{600} = 0.002. After overnight incubation at 30 °C, *S. aureus* growth was analyzed by GFP fluorescence (λex/λem = 488/513 nm) and OD_{600} using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific).

Mass spectrometry analysis

LC-MS analysis was carried out on an Ultimate 3000 RSLC nano HPLC system connected to an Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific). Samples were separated on a Luna C18(2)-HST 2.5 um LC column 100×2.1 mm (Phenomenex) at a 200 ul/min flow rate. Separation was done by a linear gradient of 90 % acetonitrile in water, 10 mM ammonium formate, 0.1 % FA (Buffer B) in 99.9 % H2O, 10 mM ammonium formate, 0.1 % FA (Buffer A). UV data was collected at 215 and 260 nm. MS1 and MS2 spectra were recorded with HCD fragmentation at 30K and 15K resolution, respectively.

Results

Identification of *Peribacillus simplex* isolate

A clone of bacteria designated as d27.3 forming thermoresistant spores was isolated from the soil sample collected in a forest in the Republic of Dagestan, Russia. After 24 hours of cultivation at +28 °C on the LB (Luria-Bertani) Broth, Miller (VWR International 63 Ltd., Poole, UK) containing agar, the strain formed moderate yellow-white colonies with mucoid texture, round form, entire margins, and convex elevation (Fig. 1B). On the third day of cultivation at +28 °C on solid nutrient medium T3 (Travers, Martin, and Reichelderfer, 1987), rod-shaped vegetative cells and oval-shaped spores were observed, thus suggesting that the isolate belongs to the *Bacillus* group (Fig. 1A). Further identification of the taxonomy of the isolate has been performed by *gyrB* locus amplification and sequencing. The results of BLAST tool analysis have shown that the closest genome to the *gyrB* sequence is the genome of *Peribacillus simplex* strain SH-B26 deposited in the NCBI database, ID GCF_001578185.1. Thus, we might conclude that the d27.3 isolate belongs to the *P. simplex* species. The isolate has been deposited in the joint Russian Collection of

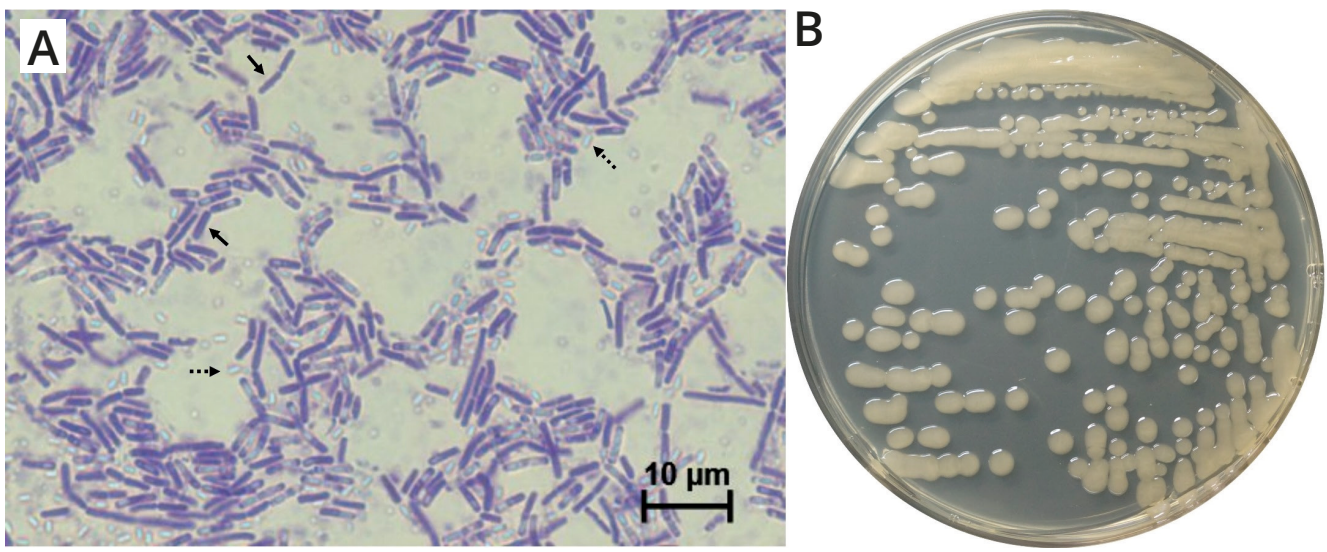

Fig. 1. The transition of the d27.3 strain from vegetative to sporulating culture after three days of cultivation on T3 agar nutrient medium stained with Coomassie brilliant blue (100× objective) — A, and the morphology of the d27.3 strain's colonies after one day of cultivation on LB agar nutrient medium — B. Black solid arrows show vegetative cells, black dotted arrows show spores.

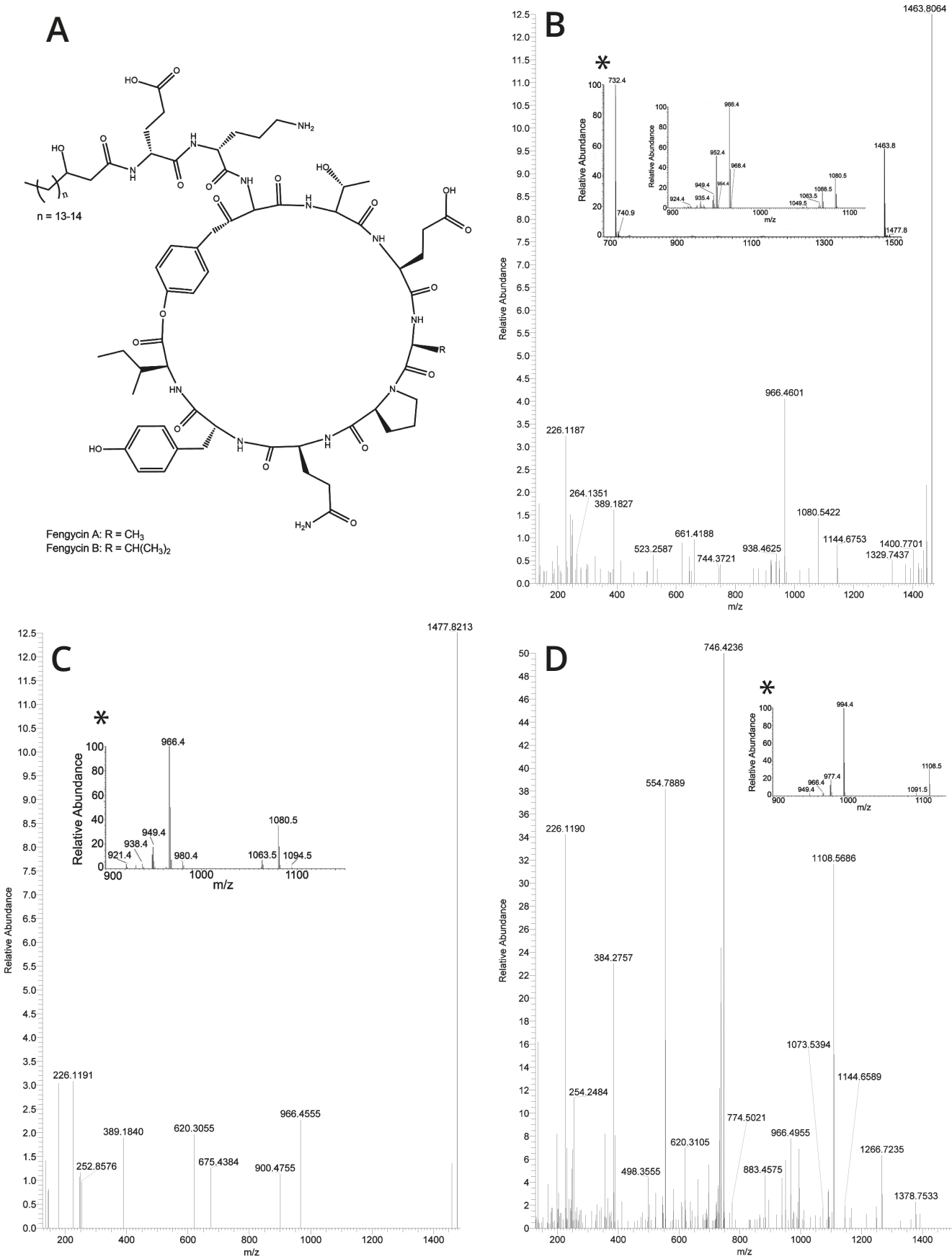

Fig. 2. Identification of metabolites of strain d27.3 by MS/MS tandem mass spectrometry. A — Fengycin structure; B–D — The positive mode HCD mass spectra of fengycins. The parent ion of the C16-Fengycin A 1463.8064 [M+H] — B. The parent ion of the C17-Fengycin A 1477.8213 [M+H] — C. The parent ion of the C16-Fengycin B 1491.8379 [M+H] — D. The reference mass spectra of the corresponding compounds are marked with an asterisk (*) (Medeot, Fernandez, Morales, and Jofre, 2020).

Agricultural Microorganisms (RCAM) at the All-Russia Research Institute for Agricultural Microbiology in Saint Petersburg (http://62.152.67.70/cryobank/login.jsp).

Antibiotic activity of *Peribacillus simplex* d27.3

Agar overlay assay was performed to reveal antibiotic or antifungal activity of the strain. We have chosen *E. coli* Δ*lptd* as a hypersensitive model of a gram-negative bacterium. In gram-negative bacteria, LptD is involved in the transport of outer membrane lipopolysaccharides through the periplasmic space and subsequently outward through the outer membrane (Sperandeo et al., 2008). Mutations in the *lptd* gene lead to increased permeability of the cell wall. *S. aureus* was chosen as a gram-positive model pathogen and *C. albicans* as a fungal pathogen. Clearance zones were observed against *S. aureus* (10.6 ± 2.2 mm) and *C. albicans* (7.3 ± 1.9 mm). On the other hand, the strain exhibited no effect against the sensitive gram-negative model strain.

Identification of fengycins as bioactive compounds of *Peribacillus simplex* d27.3

To identify the bioactive compounds secreted by *Peribacillus simplex* d27.3, the strain was cultivated in BHI medium. The compounds were purified in two steps, including SPE with LPS-500 sorbent and C18 RP-HPLC. After extraction with LPS-500, the fraction eluted with 80 % ACN was freeze-dried for further HPLC analysis. However, the content of active substances in this fraction accounted for nearly 20 % of the total activity. Another 20 % of the active metabolites did not bind to the sorbent while nearly 60 % of activity was discovered in the fraction eluted under acidic conditions (100 % ACN with 0.1 % TFA).

The resulting LPS fraction was subjected to HPLC. The most active were the fractions corresponding to the ACN content of 51–52 % and 52–53 % (33 min and 34 min fractions). The HPLC-purified fractions eluted at 33 and 34 minutes exhibiting anti-staphylococcal activity were subjected to liquid chromatography-mass spectrometry analysis to determine the active compounds produced by the strain. Metabolomic analysis revealed fengycins were the major compounds in the fractions: C-16 fengycin A corresponding to fraction 33 min, and C-16 fengycin A, C-17 fengycin A, and C-16 fengycin B detected in fraction 34 min (Fig. 2A and Figs S1, S2 (Supplements)).

We used MS/MS spectral analysis to confirm the identity of fengycins (Figs 2B–D). The mass spectra of the first LC-fraction showed peaks at m/z 1463.80 and 1477.82. The mass spectra of the second LC-fraction showed a major peak at m/z 1491.83 trace amounts of m/z 1505.84. According to the m/z values reported by Medeot, Fernandez, Morales, and Jofre (2020), the observed main product ions at m/z 966.4 and 1080.5 correspond to fengycin A, while product ions at m/z 966.4 and 1108.5 correspond to fengycin B. The observed mass difference of 14 Da indicates the length of fatty acid fragments.

Thus, *P. simplex* d27.3 strain produces a set of fengycin lipopeptides known to possess antifungal activity (Vanittanakom, Loeffler, Koch, and Jung, 1986), which agrees with the results obtained in our work. Though fengycines exhibit low cytotoxicity (Deleu, Paquot, and Nylander, 2008) probably due to cellular membrane disruption effects (Deleu, Paquot, and Nylander, 2005; 2008), they are able to decrease cell proliferation in human cancer models (Yin et al., 2013). Moreover, fengycins suppress intestinal colonization by *Staphylococcus aureus* acting as quorum sensing modulators (Piewngam et al., 2018), thus possessing a wide repertoire of beneficial activities. However, fengycins are not the only active components secreted by the *P. simplex* d27.3 strain. *Bacillus* and *Bacillus*-related species are well known for their broad functionality, including biocontrol, probiotic, and antimicrobial activity. The landscape of functionality is predetermined by the biosynthetic potential of a particular strain that can be mediated by various biologically active secondary metabolites (Sumi, Yang, Yeo, and Hahm, 2015; Manetsberger et al., 2023; etc).

Conclusion

Here we report a new *Peribacillus simplex* d27.3 strain isolated from a forest soil sample in Russia, Republic of Dagestan. *P. simplex* d27.3 has a broad activity spectrum against gram-positive bacteria and fungi. The antagonistic activity of *P. simplex* d27.3 was mediated by a combination of metabolites, including well-known fengycins and new antimicrobials yet to be identified. A more detailed investigation of the *P. simplex* d27.3 genome and metabolome will provide clues for its functionality and practical application. The data obtained suggest that *P. simplex* d27.3 has a high potential as a biocontrol agent against common phytopathogens and may provide practical advantages in agriculture.

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