

## Identification and antibiotic resistance of *Bacillus* spp. isolates from natural samples

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**Abstract:** Identification of 33 *Bacillus* spp. isolates from different environmental samples collected from the territory of Serbia was performed by sequencing of the 5'-hypervariable section of 16S rRNA gene. Eight species were identified within four phylogenetic groups: *B. pumilus*, *B. megaterium*, *B. subtilis* and *B. cereus*. Determination of their antibiotic resistance was performed using the minimum inhibitory concentration (MIC) assay. We found that just one isolate was resistant to gentamicin, 9 were resistant to clindamycin and all were resistant to vancomycin. Based on the profile of resistance, the isolates were categorized into 4 categories. *In silico* analysis of the erythromycin-resistance (*erm*) gene for clindamycin resistance showed their distribution between related and nonrelated soil and human isolates including different species of *Bacillus* genera. This finding indicates that *Bacillus* spp. from the environment could be a source of resistance to clindamycin. The potential for the presence and spread of resistance determinants in the soil and similar ecosystems exists so that monitoring of antibiotic resistance genes in nonpathogenic *Bacillus* strains from the environment is advised.

**Key words:** *Bacillus*; 5' hypervariable 16S rRNA; antibiotic resistance; erythromycin-resistance (*erm*)

### INTRODUCTION

Nature in its widest sense is an abundant source of many different species of the genus *Bacillus*. In various habitats associated with soil, in water, on and in plants, in special types of food, even in clinical specimens, *Bacillus* spp. can be found. Hallmarks of the genus are its rod-shape, endospore formation and predominantly Gram-positive aerobic bacteria. The number of described species in the latest edition of Bergey's Manual of Systematic Bacteriology reached 142 species [1]. For decades, the golden standard in molecular identification of prokaryotes was analysis of the sequence of the gene for 16S rRNA [2,3]. Although satisfactory resolution at species level for some *Bacillus* spp. can be achieved using universal primers for 16S rDNA, the majority of sequences could be gathered into phylogenetic groups at best [1]. Many species fall into several distinct phylogenetic groups, such as *B. subtilis*, *B. cereus*, *B. pumilus* and *B. sphaericus* that can be even phenotypically distinguishable, but issues

regarding identification within the group remain [4]. One solution to overcoming this problem was to focus on different variable regions within the 16S rRNA gene [5]. Goto et al. [6] identified a 275-bp-long 5'-end region of the 16S rRNA gene that is hypervariable and highly specific and can be used for more precise identification of *Bacillus* spp.

Most species of *Bacillus* have no, or a very weak, pathogenic potential and they are seldom associated with disease occurrence in humans and animals, with the exception of *B. anthracis* (the causative agent of anthrax) [7], and *B. thuringiensis* and *B. sphaericus* (that are pathogenic to invertebrates) [8]. Several other species are associated with food poisoning and opportunistic infections, as for example *B. cereus* [9]. Although antibiotic resistance is usually analyzed in the clinical context, there is growing concern about the spread of resistance genes in the environment and also evidence that clinically relevant resistance could be tracked down to the environment, to nonpathogen-

ic strains. Examples can be found in the deployment of fertilizers in agriculture that contain antibiotics as well as to the broad usage of antibiotics as growth factors in farming [10,11]. Traditionally, the spread of antibiotic resistance includes the results of analysis of human or relevant animal pathogens. However, the necessity of also considering nonpathogenic microorganisms with which pathogens interact in the environment has become obvious. In this way, it is possible to track down and eventually prevent the development of antibiotic resistance before it becomes relevant in the clinical context [12,13]. The idea that soil ecosystems could be reservoirs of antibiotic resistance for clinical pathogens is old. Benveniste and Davies [14] found similarities in resistance mechanisms between nonpathogenic soil bacteria and clinically relevant pathogenic bacteria. Evidence is now available for the presence of a vast environmental pool of genes with the potential to be captured and expressed as resistance determinants for any overused antibiotic. However, more studies are necessary to establish a strong environment-clinic connection [15].

Clindamycin is a class of antibiotics that exhibit activity by binding to the large subunit of ribosomes [16]. Resistance to clindamycin appears in three forms, and possible mechanisms include efflux pump activity encoded by *mrsA* and *mefA* genes [17,18] and inactivation of clindamycin through the activity of O-nucleotidyltransferase encoded by the *linA* gene [19]. One more mechanism involves the activity of the *erm* genes (also known as *mls* genes), which enable modification of 23S rRNA of the ribosomes [17].

This study was aimed at identifying a collection of 33 *Bacillus* spp. strains isolated from natural samples of soil, hay and manure by sequencing of the 5' hypervariable segment of the 16S rRNA gene, testing identified strains for antibiotic resistance and *in silico* analysis of the genetic determinants of clindamycin resistance in order to examine whether they represent a reservoir of resistance in the natural environment.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

The 33 *Bacillus* spp. isolates used in this work are part of a larger collection that belongs to the Laboratory

of Microbiology, Faculty of Biology in Belgrade, and were isolated from different localities in Serbia. Isolates were collected from the soil, manure and hay derived from different localities in Serbia during 1999 and 2000. The bacterial strains used in this study as reference strains were *B. subtilis* Marburg, *B. atrophaeus* ATCC 9372, *B. subtilis* ATCC 6633, *B. thuringiensis* HD1  $\Delta$ CryB from the Laboratory of Microbiology, Faculty of Biology, and *B. megaterium* OP3-4S from the Laboratory for Molecular Microbiology, Institute of Molecular Genetics and Genetic Engineering. The *Bacillus* spp. isolates and reference strains were routinely cultured in Luria-Bertani (LB) medium and in Müller-Hinton (MH) (HiMedia, Mumbai, India) for the MIC assay. Isolates and reference strains were grown under aerobic conditions at 30°C.

### DNA manipulation

Genomic DNA from the *Bacillus* spp. isolates was isolated as described earlier [20]. The 5' hypervariable region of the 16S rRNA gene, about 300 bp in length, was amplified with PCR mix (KAPA Biosystems, Boston, USA) as follows: 1x KAPA Taq ready mix, 10  $\mu$ M 16S-HV *Bacillus* spp. specific primers 16S-HV-F (5'-TGTAACGACGGCCAGTGCCTAATACATGCAAGTCGAGCG-3') and 16S-HV-R (5'-CAGGAAACAGCTATGACCACTGCTGCCTCCCGTAGGAGT-3') [6] and 1.5  $\mu$ L of DNA sample. The PCR conditions were as follows: initial denaturation step at 94°C for 5 min, followed by 30 cycles, each with 30 s of denaturation at 94°C, annealing at 50°C for 30 s and 90 s of extension at 72°C; a final extension step was at 72°C for 7 min. Prior to sequencing, the PCR products were purified with the GeneJET PCR cleanup purification Kit (Thermo Scientific) and sent to the MacroGen commercial sequencing service (Amsterdam, Netherlands).

### Phylogenetic analysis of *Bacillus* spp.

Reference sequences of *Bacillus* spp. 16S rRNA were downloaded from the GenBank database through the National Center for Biotechnology Information's BLAST search program 2.5.0 for nucleotides (<http://www.ncbi.nlm.nih.gov/>). Reference strain sequences were aligned using the CLUSTAL W multiple alignment algorithm [21]. The consensus sequence based

on the aligned reference sequences was created in BioEdit v7.1.3 [22] and aligned once more using the Clustal W algorithm for sequence editing. The isolate sequences were manually edited. The mega BLAST algorithm [23,24] was used for identification of the bacterial strains. The edited sequences and reference strain sequences from GenBank were aligned with the ClustalW algorithm in MEGA 6.0 [25]. Phylogenetic trees were constructed in MEGA 6.0 software using the neighbor-joining method based on a pairwise distance matrix with the Kimura two-parameter nucleotide substitution model. The topology of the trees was evaluated by the bootstrap resampling method with 1000 replicates and *Clostridium botulinum* NR\_036786 was included as an outgroup.

### Determination and analysis of antibiotic resistance

Antibiotic susceptibility of the selected isolates and reference strains was tested by the broth microdilution method. Overnight cultures were adjusted to optical density 0.1 that corresponds to 0.5 McFarland standard turbidity. According to the CLSI M45-P standard [22], the sensitivity of the selected isolates and reference strains was tested for clindamycin (Sigma Aldrich, tested range 0,25 - 32 µg/mL), vancomycin (Sigma Aldrich, tested range 0.5-16 µg/mL) and gentamicin (Sigma Aldrich, tested range 1-32 µg/mL). All dilutions were done in triplicate. Microtiter plates were incubated for 18 h at 30°C. The optical density was read at 600 nm using the Multiskan FC reader (Thermo Scientific). Finally, 22 µL of resazurin (concentration 0.675 mg/mL) was added to each well and incubated for 3 h at 30°C. The lowest concentration showing no change in color was defined as the MIC (minimum inhibitory concentration). The results are expressed in mg/mL. MIC values were compared to the CLSI M45-P standard [26].

### In silico analysis of erm genes

The genetic determinants responsible for clindamycin resistance were identified by analyzing data from the GenBank database and available literature. The obtained sequences were searched for homology with gene sequences deposited in GenBank through the BLAST algorithm. Sequences were aligned using the

CLUSTAL W algorithm and were presented in the MEGA 6 program using the Tamura 3-parameter model with a 50% cutoff value, and were evaluated by the bootstrap resampling method with 1000 replicates.

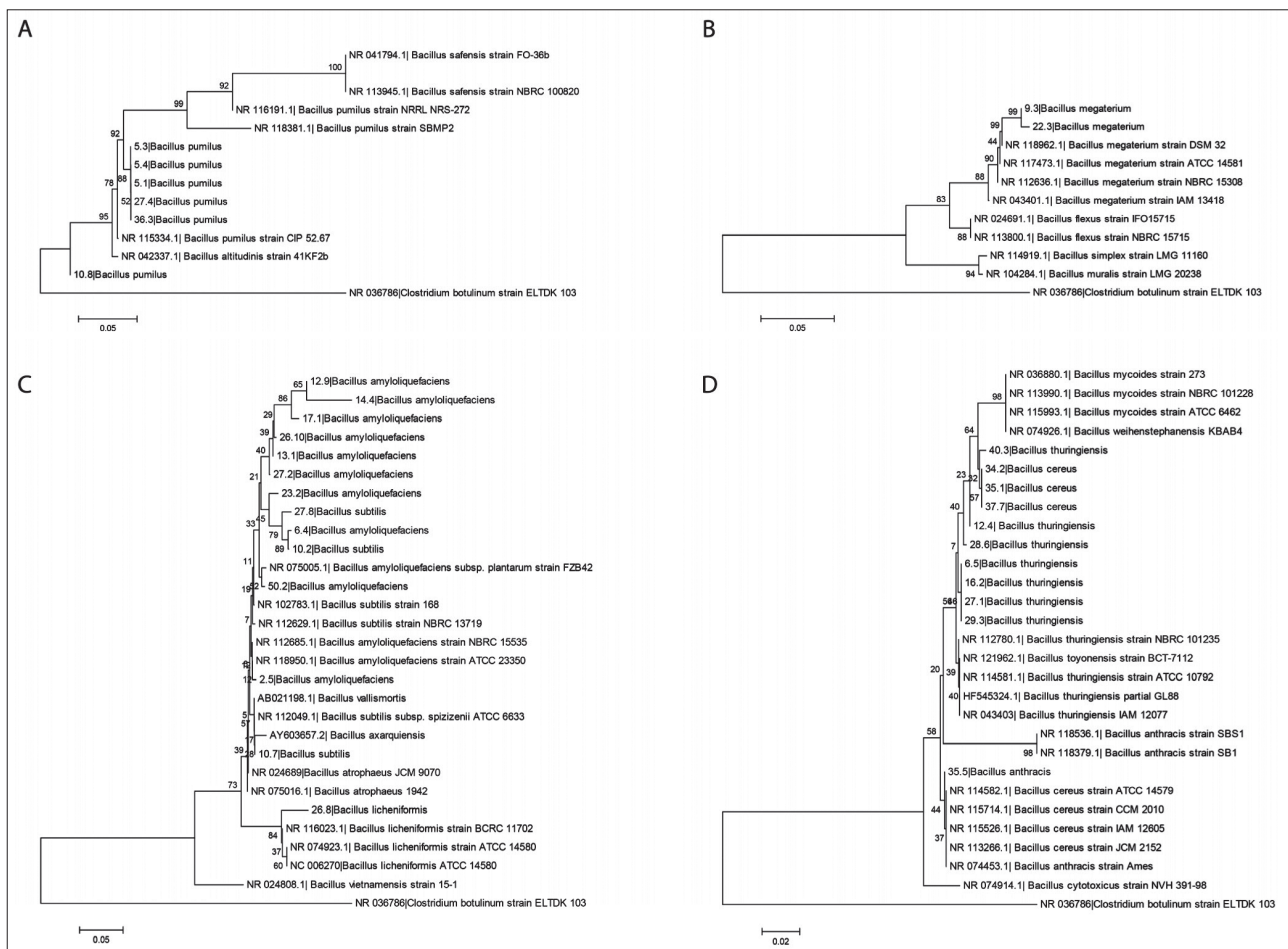
## RESULTS

### Phylogenetic analysis of *Bacillus* spp.

The total DNA from overnight cultures of 33 *Bacillus* spp. isolates was extracted and the 5' hypervariable region of 16S rRNA gene was amplified using 16S-HV primers. All isolates gave one distinct DNA band of appropriate length of about 275 bp. Purified amplified DNA was sequenced and after BLAST analysis dendrograms were constructed according to the analysis of the acquired sequences (Fig. 1). As an outliner, the sequence of the 16S rRNA gene of *Clostridium botulinum* ELTDK 103 was used. The BLAST database search revealed eight species with the following distribution: six isolates clustered with *Bacillus pumilus*, two with *Bacillus megaterium*, three with *B. subtilis*, ten with *B. amyloliquefaciens*, one with *B. licheniformis*, and three that were most similar to *B. cereus*, seven to *B. thuringiensis* and one to *B. anthracis*. Based on the analyzed sequences, four phylogenetic clusters were distinguished: *B. pumilus* (Fig. 1A), *B. megaterium* (Fig. 1B), *B. subtilis* (Fig. 1C) and *B. cereus* (Fig. 1D). Most of the isolates were derived from the soil (60%) and they belong to all four identified phylogenetic groups and 7 identified species, except *B. licheniformis*. Nine isolates from manure were scattered over the three phylogenetic groups, while only 4 isolates from hay were identified as *B. pumilus*, except one that was identified as *B. amyloliquefaciens* (Supplementary Table S1).

### Determination and analysis of the antibiotic resistance

The results of testing the antibiotic susceptibility of *Bacillus* isolates and reference strains to gentamycin (G), clindamycin (C) and vancomycin (V) are shown in the form of a heat map in Fig. 2. The determined MIC values were compared with the CLSI M45-P standard [26] and categorized as resistant (R), intermediary resistant (IR) or sensitive (S). Only one



**Fig. 1.** Phylogenetic relationships of *Bacillus* spp. isolates of **A** – *B. pumilus*; **B** – *B. megaterium*; **C** – *B. subtilis*; **D** – *B. cereus* phylogenetic groups based on the sequence of the 5' HPV 16S rRNA region. The branching pattern, which is rooted by using *Clostridium botulinum* as the outgroup, was generated by the neighbor-joining method, and the distances were calculated with the Kimura two-parameter model. Bootstrap values are given for each node as having 50% or greater support, with 1000 replicates. Bar=0.05 (except for *B. cereus* phylogenetic group where the bar=0.02) nucleotide substitutions per site. The isolate names and most similar Mega BLAST search results are shown, with the GenBank identifier next to the reference strain label.

isolate and one reference strain were intermediary resistant to gentamycin. All strains tested were sensitive to vancomycin. Twenty isolates and 4 reference strains were intermediary resistant, while 9 were resistant to clindamycin. Based on the profile of resistance, the isolates were categorized into 4 groups (categories); these are Category 1:  $G^S C^S V^S$ ; Category 2:  $G^S C^{IR} V^S$ ; Category 3:  $G^S C^R V^S$ ; Category 4:  $G^{IR} C^{IR} V^S / G^{IR} C^R V^S$ . The distribution of categories is shown in Fig. 3.

### *In silico* analysis of *erm* gene distribution

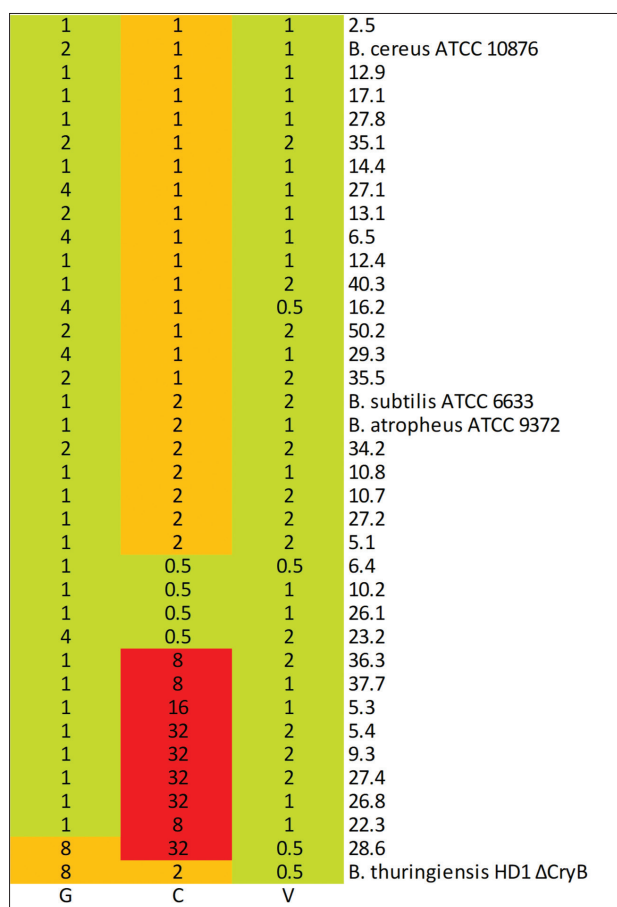
The analysis of sequence data from the existing BLAST database showed that the *erm* gene was dis-

tributed among *Staphylococcus*, *Streptococcus* and *Bacteroides* genera. Fig. 4 presents the dendrogram of resistance carriers of *erm*. As can be seen, a large group of *ermG*, *ermC* and *ermT* genes was spread across the species of *Lactobacillus*, *Bacillus*, *Staphylococcus*, *Streptococcus* and *Bacteroides*. In addition, *ermD*, *ermK* and *ermJ* genes represented in *Bacillus* spp. branched out.

### DISCUSSION

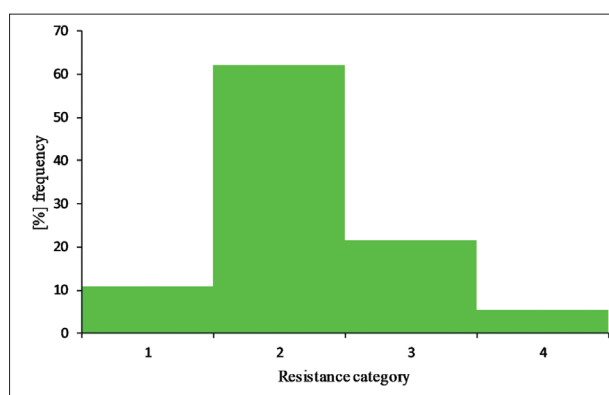
The collection of 33 isolates of *Bacillus* spp. was identified based on the sequence of the 5' hypervariable sections of the 16S rRNA gene. Using this approach,



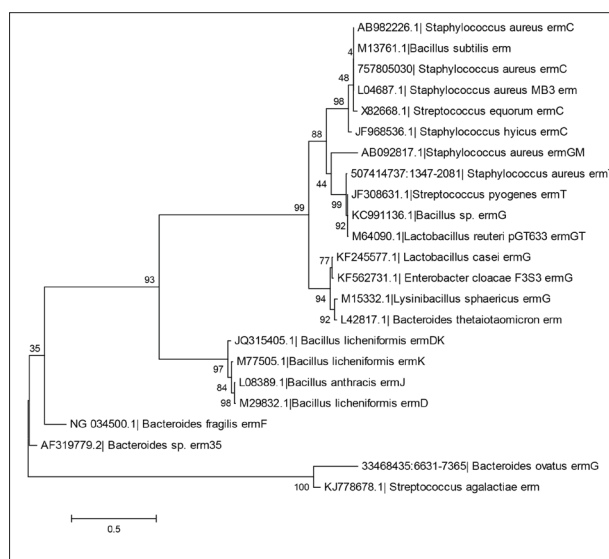


**Fig. 2.** Heat map with antibiotic susceptibility results of *Bacillus* isolates and reference strains to gentamycin (G), clindamycin (C) and vancomycin (V). MIC values are presented. According to the CLSI M45-P standard, the tested isolates and strains were categorized as: ■ – resistant, ■ – intermediary resistant and ■ – sensitive.

a clear resolution was obtained for the *B. pumilus* and *B. megaterium* phylogenetic groups. Within the *B. pumilus* group, clustering of identified isolates with related reference strains was apparent for all except isolate 10.8, although it was determined as *B. pumilus*. Additional characterization is needed to confirm this finding. In the *B. megaterium* group, isolates and reference strains clustered together, with a high percentage of sequence identity, confirming the identification of isolates 9.3 and 22.3 as *B. megaterium*. All *B. amyloliquefaciens* isolates belonged in the *B. subtilis* phylogenetic group; thus, to obtain a clear distinction between *B. subtilis* and *B. amyloliquefaciens*, additional analysis is required. Also, the sole isolate identified as *B. licheniformis* was clustered with this large group. In the work of Draganić et al. [27], the



**Fig. 3.** Distribution of isolates categorized according to antibiotic resistance. Category 1: G<sup>S</sup>C<sup>S</sup>V<sup>S</sup>; Category 2: G<sup>S</sup>C<sup>I</sup>V<sup>S</sup>; Category 3: G<sup>S</sup>C<sup>R</sup>V<sup>S</sup>; Category 4: G<sup>I</sup>C<sup>I</sup>V<sup>S</sup>/G<sup>I</sup>C<sup>R</sup>V<sup>S</sup>.



**Fig. 4.** Phylogenetic relationships of bacterial species according to the sequences of their *erm* determinants. The branching patterns, rooted by the sequences of *erm* genes of *Streptococcus agalactiae*, were constructed with the Tamura 3-parameter model. Bootstrap values are given for each node as having 50% or greater support, with 1000 replicates. Bar=0.5 nucleotide substitutions per site.

*Bacillus* isolates that belong to the same collection as the ones used in this study were identified according to the sequence of the gene for elongation factor Tu (*tuf* gene), and very satisfactory results in dividing *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* isolates were achieved. For the *B. cereus* group, isolates identified as *B. cereus* clustered amongst themselves but not together with the reference strains. On the other hand, most of the isolates determined as

*B. thuringiensis* clustered closely with the reference strains and were separated from *B. cereus* strains. Only isolate 40.3, identified also as *B. thuringiensis*, clustered with the *B. cereus* group. According to its sequence, one isolate was singled out as *B. anthracis*, but, as can be seen, sequences of numerous *B. cereus* strains from the BLAST database cluster together with it (Fig. 1D). Additional characterization is required to clarify this finding. Goto et al. [6] in their study of 5' hypervariable 16S rRNA of 69 type strains of *Bacillus* spp. showed that this sequence has very good resolution power for most species; however, the *B. cereus* group remained unresolved. Similarly, although the amplified ribosomal DNA restriction analysis (ARDRA) assay developed by Wu et al. [28] was capable of differentiating *B. subtilis* and *B. licheniformis* strains from other species of the *B. subtilis* phylogenetic cluster, it could not differentiate two species within the *B. cereus* cluster. The problem of reliable identification of *B. cereus*, *B. thuringiensis* and *B. anthracis* is ongoing and still requires a proper solution. Since this phylogenetic group contains potentially pathogenic bacteria, it was of great interest for research into *Bacillus* spp. and many approaches were applied over the years, but a reliable method is still lacking [29-31]. A similar diversity of *Bacillus* spp. isolated from marine sediment as in our study was determined using a partial sequence of 16S rRNA combined with tDNA-PCR fingerprinting in species of *B. subtilis*, *B. licheniformis*, *B. cereus* and *B. pumilus* [32] and in the research of Amin et al. [33] a similar diversity was determined for isolates from soil, identified only by their phenotypic and biochemical characteristics. Correlation between the phylogenetic affiliation and the source of isolation of *Bacillus* spp. in our research was not determined.

According to the Clinical and Laboratory Standards Institute (CLSI), which prescribes primary sensitivity tests for relevant antibiotics, testing sensitivity to vancomycin, clindamycin and gentamycin for the first tier of screening for *Bacillus* species is advised [26]. Isolates belonging to category 1 (G<sup>S</sup>C<sup>S</sup>V<sup>S</sup>) were a phylogenetically tight group of only *B. subtilis* and *B. amyloliquefaciens* isolates. The isolate 28.6 (*B. thuringiensis*) was the only one resistant to clindamycin and gentamycin. The majority of isolates from our collection showed resistance or intermediary resistance only to clindamycin. Categories 2 (G<sup>S</sup>C<sup>I</sup>V<sup>S</sup>) and 3 (G<sup>S</sup>C<sup>R</sup>V<sup>S</sup>) were most abundant amongst the strains

tested for antibiotic susceptibility. An increased incidence of resistant and intermediary resistant strains of *Bacillus* to clindamycin was reported earlier [34,35]. A similar strategy towards sensitivity analysis of *Bacillus* soil isolates to antibiotics was applied by Aslim et al. [36]. This type of approach enables a broader insight into the entrance of clinically relevant bacterial isolates into the food chain. Comparison of results of clindamycin resistance and phylogenetic affiliation of isolates showed an even distribution for category 2. All identified species, except *B. megaterium* and *B. licheniformis*, were represented with an isolate with intermediary resistance to clindamycin. On the other hand, category 3 (resistant to clindamycin) is comprised of three out of the four identified *B. pumilus* isolates, two *B. megaterium* isolates, one *B. cereus*, and a sole isolate of *B. licheniformis*. Most of the resistant and intermediary resistant isolates are not significant clinical pathogens. However, isolates 34.2 and 35.1, identified as *B. cereus*, and isolate 35.5, identified as *B. anthracis*, showed intermediary resistance, while isolate 37.7 (*B. cereus*) was resistant to clindamycin. Since these species are potential human and animal pathogens, the significance of clindamycin resistance is obvious. However, in the wider context, the resistance to clindamycin in any *Bacillus* spp. points out two important facts. First, in the ecological context, this resistance could play a role in complex inter- and intraspecies interactions in soil ecosystems [12]. In favor of this is the fact that over 80% of known antibiotics were isolated from soil microorganisms. Experimental data show that antibiotics as active substances could play a role in the modulation of gene expression and interactions in and among bacterial populations [12,37-40]. Second, within the clinical context, detected antibiotic resistance could be seen as a reservoir of resistance for clinically relevant pathogens [11-12,41]. Dworkin et al. [4] showed that conjugation between bacteria of the genus *Bacillus* and clinically relevant pathogens is possible. In addition, experimental data showed that transposons could be exchanged by conjugation between *Enterococcus faecalis* and *B. subtilis* [42], as well as between *Clostridium difficile* and *B. subtilis* [43].

The genetic determinants responsible for the resistance to erythromycin and clindamycin were detected in *C. difficile* and *Staphylococcus aureus*, and it was shown that they are actively exchanged between

these two species [44]. The *erm* genes (*ermB*, *ermF*, *ermG*, *ermC* and *ermD*), carried on transposons and plasmids, are responsible for resistance to clindamycin [45,46]. Cooper et al. [47] showed that *Lysinibacillus sphaericus* and *Bacteroides* sp. can exchange transposons with *erm* genes, proving that antibiotic resistance determinants could be transferred from soil bacteria to gut bacteria [45,47]. A considerable contribution to the dissemination of resistance genes stems from manure application to agricultural fields [11].

The data acquired *in silico* by analyzing the existing database points to a wide distribution of *erm* genes in related and unrelated soil and human bacteria. Throughout BLAST hits, *erm* genes and complete genomes of bacteria from genera *Staphylococcus*, *Streptococcus* and *Bacteroides* could be distinguished. All the elements responsible for antibiotic resistance were detected on plasmids or transposons that can be located on plasmids or chromosomes. As can be seen in Fig. 4, a large group of genes (*ermD*, *ermK* and *ermJ*) was represented in *Bacillus* spp., indicating a possible horizontal transfer between these clindamycin resistance elements. This is in agreement with literature data showing that the horizontal gene transfer of resistance genes is very common in soil ecosystems and between soil and other ecosystems [46]. In conclusion, although our study of antibiotic resistance of *Bacillus* spp. from different environments in Serbia is of limited character, it indicates that the potential for the presence and spread of resistance determinants in the soil and similar ecosystems exists and should be monitored closely.

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**Conflict of interest disclosure:** We declare that there is no actual and potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence our work.

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#### Supplementary Table S1

Available at: [http://serbiosoc.org.rs/sup/S1\\_2720.pdf](http://serbiosoc.org.rs/sup/S1_2720.pdf)