ISSN 1330-9862
(FTB-2722)

original scientific paper

Antimicrobial Activity of *Bacillus* sp. Natural Isolates and Their Potential Use in the Biocontrol of Phytopathogenic Bacteria

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Received: January 25, 2011
Accepted: August 31, 2011

Summary

Screening of 203 *Bacillus* sp. natural isolates for antimicrobial activity against phytopathogenic bacteria showed that 127 tested strains inhibit at least one sensitive strain, which illustrates their potential use as biocontrol agents. Among them, 104 isolates showed significant antagonism against *Xanthomonas oryzae* pv. *oryzae*, and only one of these (VPS50.2) synthesizes bacteriocin. An additional screening tested whether 51 isolates contained genes involved in the biosynthesis of lipopeptides of the iturin and surfactin classes. Results show that 33 isolates harbour the operon for iturin biosynthesis, and six of them carry the *sfp* gene, responsible for the biosynthesis of surfactin. Lipopeptide purification from the supernatant of isolate SS12.9 (identified as *B. subtilis* or *B. amyloliquefaciens*) was performed using ethyl acetate extraction, ultrafiltration and reversed phase HPLC. Mass spectrometry analysis confirmed that isolate SS12.9 produces a substance of the iturin class with potential for biocontrol of *X. oryzae* pv. *oryzae*.

Key words: *Bacillus* sp., *Xanthomonas oryzae* pv. *oryzae*, lipopeptides, iturin, bacteriocin

Introduction

The interest for biological control of plant pathogens has increased over the past decade, especially because of the importance of using environmentally friendly alternatives to the extensive use of chemical pesticides for combating crop diseases (*1*). Biocontrol of plant diseases is not only an alternative to chemical pesticides, but it may also provide control of diseases that cannot be managed (or only partially) by other control strategies (*2*). The use of beneficial microorganisms (biopesticides) is considered as one of the most promising methods for more rational and safe crop management practices. Most of the bacterial strains exploited as biopesticides belong to the genera *Agrobacterium*, *Pseudomonas* and *Bacillus* (*3*).

Most of the species from the genus *Bacillus* are considered as safe microorganisms and they possess remarkable abilities to synthesize many substances that have been successfully used in agriculture and for industrial purposes (*4*). The secondary metabolites produced by several species and strains of the genus *Bacillus* have been found to show antibacterial or antifungal activity against different phytopathogens (*1*,*5*,*6*). Members of *Bacillus* sp. are generally soil-inhabiting bacteria or exist

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as epiphytes and endophytes in the spermosphere and rhizosphere. In addition, they grow fast in liquid culture and form extremely resistant spores with high thermal tolerance, which makes these bacteria ideal candidates for use as biocontrol agents. This resulted in increased development and implementation of biological products with antimicrobial activity based on *Bacillus* strain or their metabolites as alternative or supplementary methods to chemicals for plant disease control (*3*).

Among many structurally diverse antimicrobial compounds that are attributed to *Bacillus subtilis* (*4*), cyclic lipopeptides (LPs) of the surfactin, iturin and fengycin families have well-recognized potential for use in biotechnology and biopharmaceutical applications because of their surfactant properties (*1*). Iturins consist of a peptide ring of seven α-amino acid residues, with the constant chiral sequence LDDLLDL and common presence of D-Tyr2, closed by a β-amino fatty acid with 14 to 17 carbon atoms (*7*). Although it has been shown that they exhibit a strong activity against pathogenic fungi and yeasts, their antibacterial activity has not been intensively investigated so far.

In this study we performed the screening of 203 natural isolates of *Bacillus* for antagonism against phytopathogenic bacteria. *Bacillus* strains were isolated from soil, manure and hay samples from different locations in Serbia (*8*). The purpose of this study is to identify the most promising strains for potential use in biocontrol, including the preliminary analysis of the structure of antimicrobial compounds and the presence of the genes responsible for their biosynthesis in the tested strains.

Materials and Methods

Test organisms and culture conditions

To determine the antimicrobial activity of the isolated *Bacillus* strains, 9 bacterial species were used as indicator strains. The phytopathogenic bacteria used for the test were *Burkholderia glumae, B. cepacia, B. plantarii, Erwinia carotovora, Pseudomonas fuscovaginae, P. aeruginosa, Agrobacterium tumefaciens, Xanthomonas oryzae* pv. *oryzae*, *Ralstonia solanacearum*, as well as *Bacillus subtilis* 168 and *Escherichia coli*. All bacteria, except *X. oryzae* pv. *oryzae*, were grown aerobically in Luria Bertani (LB) broth. Agar plates were made by adding 1.5 % agar (Torlak, Belgrade, Serbia) to the liquid medium. Bacteria were incubated at 30 °C (37 °C for *P. aeruginosa* and *E. coli*) for 24 h. *X. oryzae* pv. *oryzae* was maintained on PSA plates (in g/L: peptone 10, sucrose 10, and agar 15) at 30 °C for 24 h.

Isolation and preliminary characterization of Bacillus sp. strains

Natural isolates of *Bacillus* were isolated as described previously (*8*). The method for isolating various *Bacillus* strains was based on the resistance of their endospores to elevated temperatures. Test tubes containing a mixture of approx. 1 g of sample (hay, soil or manure) and 1 mL of nutrient broth were placed in water bath at 80 °C for 10 min to separate the endospores from vegetative cells (*9*). Concentrated samples, as well as two following decimal dilutions were spread on the surface of LB plates and incubated at 30 °C for 48 h. The distinct single colonies were subcultured onto fresh LB plates. The subcultured bacterial isolates were preliminary characterized by microscopic appearance, Gram staining and catalase test.

Screening of isolates for antimicrobial effect against plant pathogens

The bacterial isolates characterized as members of the genus *Bacillus* were screened for antagonism against five phytopathogenic microorganisms (*B. glumae*, *B. cepacia*, *E. carotovora*, *P. fuscovaginae* and *X. oryzae* pv. *oryzae*). In the primary screening, all 203 strains were grown in LB broth at 30 °C in a rotary shaker (200 rpm) overnight. The culture broth was centrifuged at 12 000 rpm for 10 min and the cell-free supernatant was tested. Well diffusion inhibition assay was conducted as described previously (*10*). The indicator strains were grown overnight according to their specific growth requirements. Top agar was made by adding 0.7 % agar to either LB or PSA plates. To create an overlay, 600 μL of indicator strain (approx. 10^6 CFU/mL) were added to 6 mL of LB top agar (PSA top agar for *X. oryzae* pv. *oryzae*) and cooled to 45 °C. The mixture was overlaid onto LB (or PSA) plates with positioned sterile corks of 7 mm in diameter and allowed to completely solidify. The wells were made by taking out the corks. Then, 50 μL of the culture supernatant were added to the wells and all plates were then incubated overnight at the optimal growth conditions for the indicator strain. Alternatively, the supernatants were concentrated up to five times by evaporation in order to obtain larger and more visible zones of inhibition of sensitive strains.

Secondary screening was done with the 25 most promising selected strains. Agar well diffusion assay was performed as described above, except that a greater number of indicator strains was used (Table 1) and the supernatants collected for the test were filtered through 0.45-μm filter in order to obtain cell-free extracts. In addition, proteinaceous nature of an antimicrobial compound in the supernatant was tested by using Pronase E (AppliChem GmbH, Darmstadt, Germany) as follows. During well diffusion assay, when aliquots of the tested samples were added to the well, a small amount of crystalline Pronase E was placed on the edge of the well with sterile toothpick. Radii of inhibition zones were measured from the edge of well and expressed in mm. Moreover, the absence of inhibition around the Pronase E crystal was monitored.

Antimicrobial activity and growth

Antimicrobial activity was monitored during the growth cycle of *Bacillus* sp. strain SS12.9 in LB medium. Viable counts of heat-treated bacteria at 80 °C for 10 min and the number of spores were recorded, and the supernatant was tested for activity on indicator bacterial strains *E. carotovora* and *X. oryzae* pv. *oryzae* in well diffusion assay as described earlier.

DNA isolation

Genomic DNA from *Bacillus* strains was prepared as described earlier (*11*). Briefly, after centrifugation and two washes in TE buffer (10 mmol/L Tris–HCl, pH=8,

Table 1. Antimicrobial spectrum of *Bacillus* sp. natural isolates

Indicator *Bacillus* sp.	*Burkholderia glumae*	*Burkholderia cepacia*	*Erwinia carotovora*	*Pseudomonas fuscovaginae*	*Xanthomonas oryzae* pv. *oryzae*	*Agrobacterium tumefaciens*	*Bacillus subtilis* 168	*itu* operon	*sfp* gene
SS6.4	5	8	2	–	6	–	6	*	
SS6.5	5	–	5	–	6	2	12		
SS6.6	5	10	2	–	12	7	8	*	
SS8.2	5	5	2	–	4	5	12	*	
SS9.2	5	10	–	–	10	5	–	*	
SS9.4	5	–	–	5	–	–	–		
SS11.3	3	5	–	–	11	5	–	*	
SS12.9	9	5	5	–	12	3	3	*	
SS17.2	5	7	3	–	10	2	3	*	
SS17.4	3	5	3	–	10	5	3	*	
SS18.3	2	2	3	–	10	5	3	*	
SS19.2	5	2	3	–	9	4	–		
SS21.3	5	2	–	–	9	5	8	*	
SS23.1	9	5	–	5	9	5	10	*	
SS26.9	9	3	2	5	8	5	12	*	
SS27.3	9	–	2	5	9	5	15		
SS28.2	–	3	2	2	8	5	12	*	
SS29.4	9	3	5	–	9	5	12	*	
SS30.3	–	5	5	2	8	5	12		
SS32.7	–	10	5	–	10	5	12		
SS38.1	–	10	2	–	8	–	12	*	*
SS38.3	–	10	2	–	8	–	12	*	
SS40.3	–	–	2	–	8	–	–		
VPS50.2	–	4 (P)	–	–	–	–	4 (P)	**B**	**B**
VPS50.4	–	2	2	–	8	–	–		

Radii of inhibition zones are shown in mm; P: part of zone of inhibition missing in a place where Pronase E was added; **B**: bacteriocin production; *operon/gene detected, amplified and sequenced

and 1 mmol/L EDTA), the cells were resuspended in 1 mL of a lysis buffer (50 mmol/L Tris, pH=8, 1 mmol/L EDTA and 25 % sucrose) containing 20 μg/mL of lysozyme (Serva GmbH, Heidelberg, Germany) and incubated at 37 °C for 45 min. The reaction was stopped using 1 mL of EDTA (250 mmol/L, pH=8) for 5 min. The samples were then treated with 400 μL of 20 % SDS and 20 μL of 20 mg/mL of Proteinase K (Sigma-Aldrich, St. Louis, MO, USA) solution. The mixture was incubated at 65 °C until it became clear and less viscous, and then the phenolchloroform extraction was performed. DNA was precipitated in ethanol and resuspended in 100 μL of TE buffer with the volume of RNase of 10 μL (10 mg/mL).

Identification of selected antagonistic bacteria

The isolates characterized as *Bacillus* species that showed clear antagonism against some of the tested phytopathogenic bacteria were subjected to further identification on the basis of phenotypic and physiological characteristics using API test system (bioMérieux, Marcy l'Etoile, France) and analysis of the 16S rDNA sequence.

To obtain 16S rDNA sequences, polymerase chain reaction (PCR) was carried out using primers $P1_{16S}$ (5'--GAATCTTCCACAATGGACG-3') and $P2_{16S}$ (5'-TGACGGGCGGTGTGTACAAG-3') and PCR protocol as follows: 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 68 °C for 30 s and 72 °C for 10 min. Purified PCR fragments were sequenced with both primers and compared with 16S rRNA gene sequences in the public database using BLAST.

API 50 CHB analysis was conducted according to the manufacturer's instructions.

Detection of iturin operon and sfp gene

DNA from selected strains was isolated as described earlier. PCR mix was as follows: 1 μL of DNA, 2.5 U of KAPA Long Range DNA Polymerase (KAPA Biosystems, Woburn, MA, USA), 5 μL of 5× KAPA LR buffer (without Mg^{2+}), 1.75 μL of $MgCl_2$ (25 mM), 0.75 μL of deoxynucleoside triphosphate solution (10 mM each) and 1 μL of each primer, forward ITUP1-F (5'-AGCTTAGGGAACAATTGTCATCGGGGCTTC-3' positioned from nucleotide 15353 to 15383 of the iturin A operon sequence, DDBJ/EMBL/GenBank accession no. AB050629) and reverse ITUP2-R (5'-TCAGATAGGCCGCCATATCGGAATGATTCG-3', complementary sequence positioned from nucleotide 17326 to 17355 of AB050629), which are able to detect a 2-kb region that includes the intergenic sequence between *ituA* and *ituB* (*12*). PCR was conducted

according to the KAPA Long Range manufacturer's instructions. These conditions include an autoextension step, lower extension temperature, and were as follows: 94 °C for 2 min, 10 cycles of 94 °C for 15 s, 45 °C for 15 s and 68 °C for 3 min, and then 25 cycles of 94 °C for 15 s, 45 °C for 15 s, 72 °C for 3 min and 72 °C for 10 min. Amplification was accomplished in a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany).

A 675-bp fragment from the *sfp* gene (corresponding positions 167–841, GenBank accession no. X63158) from *B. subtilis* encoding 4'-phosphopantetheinyl transferase, the enzyme involved in the biosynthesis of surfactin (*13*), was targeted for amplification using two oligonucleotides: P17 (5'-ATG AAG ATT TAC GGA ATT TA-3') and P18 (5'-TTA TAA AAG CTC TTC GTA CG-3'). PCR conditions used were as described for the detection of iturin operon. PCR products were analyzed by agarose gel electrophoresis on 1 % agarose gels containing 0.5 μg/mL ethidium bromide in 1×TAE buffer (2 mol/L Tris base, 1 mol/L glacial acetic acid, 50 mmol/L EDTA, pH=8), at constant voltage of 80 V for 1 h and visualized by CCD camera BioDocAnalyze Darkhood (Biometra, Goettingen, Germany). All obtained PCR fragments were sequenced by using Macrogen sequencing service (Macrogen Inc., Seoul, Korea).

Ethyl acetate extraction of lipopeptides

Lipopeptide substances with antimicrobial activity were isolated from the overnight culture of *Bacillus* SS12.9. The cells were removed by centrifugation (8000 rpm, 10 min), and 500 mL of cell-free supernatant were used for extraction with ethyl acetate in a ratio of 1:1. The ethyl acetate fraction was collected and dried in a rotary evaporator, and the precipitate was resuspended in 50 mM sodium phosphate buffer, pH=6.8. The obtained ethyl acetate fraction was subjected to ultrafiltration through a 3-kDa molecular cut-off filter (Millipore, Billerica, MA, USA) and fractions containing compounds with molecular mass lower than 3 kDa were collected and tested in a well diffusion assay against *X. oryzae* pv. *oryzae*.

Purification of lipopeptides

Antimicrobial lipopeptide produced by the isolate SS12.9 was purified using the method described previously (*14*). The ethyl acetate fraction obtained after ultrafiltration through a 3-kDa membrane (500 μL) was applied on a reversed phase HPLC column (Supelcosil LC-18, 25 cm×10 mm, 5 μm, Supelco, St. Louis, MO, USA). An isocratic mobile phase consisting of acetonitrile and 10 mM ammonium acetate (40:60) with a flow rate of 2.5 mL/min was used, and the absorption was monitored at 260 nm. All fractions obtained after chromatography were evaporated, dissolved in distilled water and tested for the activity against *X. oryzae* pv. *oryzae*. Mass spectrometry analysis was performed using a MALDI-TOF/TOF mass spectrometer (Model 4800, Applied Biosystems, Carlsbad, CA, USA).

Results

Isolation and characterization of antagonistic Bacillus strains

It is of interest to determine the *in vitro* antagonistic potential of several natural isolates of *Bacillus* sp. against several phytopathogenic bacteria. For this reason, 203 *Bacillus* strains were isolated from 32 localities and from three ecological niches in Serbia. Among them, 23 strains were isolated from hay and straw, 118 from soil and 62 from manure samples. Distinct colonies were subcultured on LB agar plates and selected on the basis of their morphology. All isolates proved to be Gram-positive, endospore forming, catalase-positive rods.

The 203 *Bacillus* isolates were initially screened for antagonism against several phytopathogenic bacteria; 127 were found to display a significant level of antagonism against at least one bacterial species, and 104 showed antagonism against *X. oryzae* pv. *oryzae*. Twenty five isolates were chosen for a second round of screening of antagonism against seven target bacterial strains and the results are shown in Table 1. None of the supernatants of the *Bacillus* strains showed any activity against *B. plantarii, P. aeruginosa, E. coli* or *R. solanacearum*; however, most exhibited very strong antimicrobial activity against *X. oryzae* pv. *oryzae*. In addition, some strains displayed inhibition against *B. subtilis* 168, *B. cepacia, B. glumae, E. carotovora, A. tumefaciens* and *P. fuscovaginae*. It was established that only the supernatant from isolate VPS50.2 was sensitive to Pronase E, indicating that this strain most probably produced a bacteriocin. The antagonistic effects of supernatants from isolate VPS50.2 against *B. subtilis* 168 and from isolate SS12.9 against *X. oryzae* pv. *oryzae* are presented in Fig. 1.

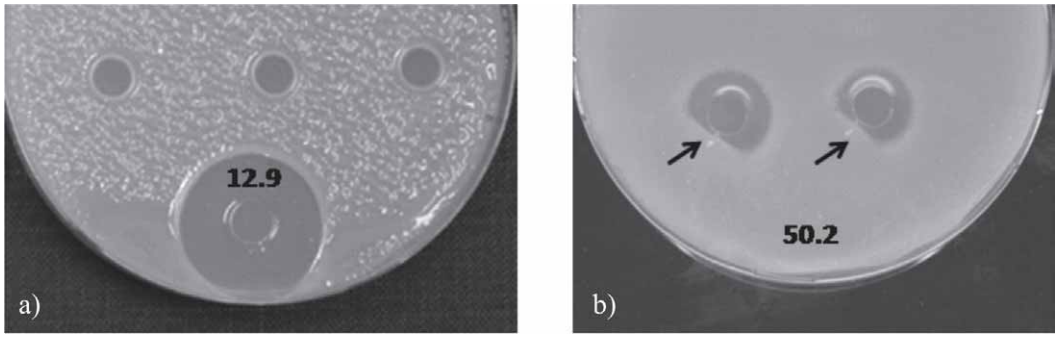

Fig. 1. Inhibition of growth of: a) *X. oryzae* pv. *oryzae* by *Bacillus* sp. isolate SS12.9 and b) of *Bacillus subtilis* 168 by *Bacillus* sp. isolate VPS50.2. Concentrated cell-free supernatant of the culture was used in the test. Arrow – position of the crystal of Pronase E

Detection of iturin operon and sfp gene

To investigate whether *Bacillus* isolates, which displayed antagonistic activity, possessed either the lipopeptide iturin biosynthesis locus and/or the *sfp* gene, responsible for biosynthesis of surfactin, DNA from *Bacillus* isolates was subjected to screening for the presence of iturin operon and *sfp* gene. As can be seen in Fig. 2a, most of the screened isolates (33) gave a single clear PCR product of approx. 2 kb when ITUP1 and ITUP2 primers were used, indicating the most likely presence of the iturin operon in their chromosome. For isolates SS16.5 and SS34.7, the size of the PCR products was larger than expected. 16 PCR products from various isolates were then sequenced, as listed in Table 1: all sequences derived from samples that had a discreet band of 2 kb exhibited 95–100 % DNA homology with *Bacillus* sp., which is known to produce iturin (*12*). The sequence of the larger fragment from isolate 16.5 showed 89 % similarity with a sequence present in the genome of *B. pumilus* SAFR-032; this sequence had no homology with the genes involved in the production of antimicrobial lipopeptides.

Most of the isolates did not result in any PCR products in the reaction with specific primers for *sfp* gene. Only the genomic DNA of seven isolates resulted in the amplification of DNA fragments using *sfp* primers (Fig. 2b). The products of the expected size (675 bp) from isolates SS13.3, SS17.1 and SS17.2 were sequenced, and the sequence comparisons showed 99, 97 and 97 % homology, respectively, between these fragments and phosphopantetheinyl transferase gene in the database from the known surfactin producers (*15*). It was concluded that these three strains most probably had the potential of synthesizing surfactin.

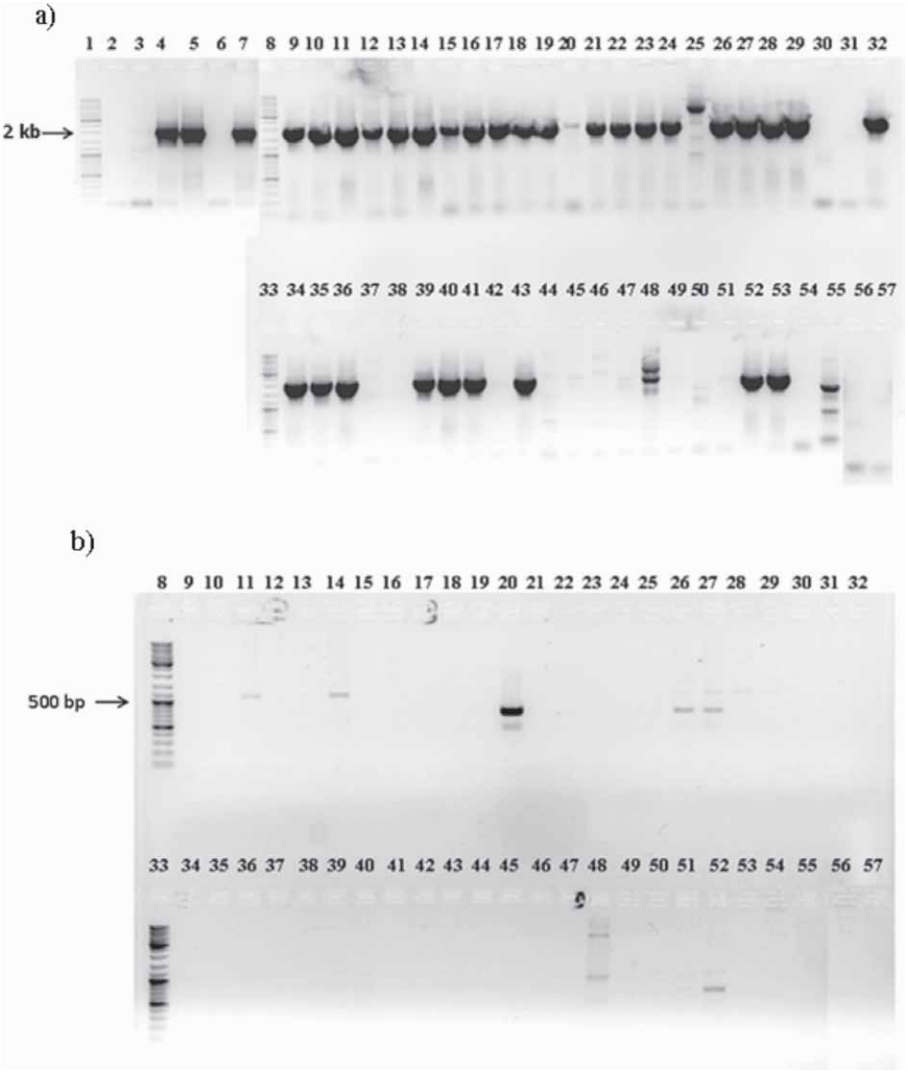

Fig. 2. Amplification products obtained with PCR primers from: a) iturin operon and b) *sfp* gene from *Bacillus* isolates. The same labelling was used for both pictures. In Fig. 2b first 7 lanes were omitted (all negative). Lanes: **1**. DNA ladder, **2**. SS2.14, **3**. SS2.15, **4**. SS6.1, **5**. SS6.4, **6**. SS6.5, **7**. SS6.6, **8**. DNA ladder, **9**. SS8.2, **10**. SS9.2, **11**. SS10.3, **12**. SS10.4, **13**. SS11.3, **14**. SS11.4, **15**. SS12.6, **16**. SS12.7, **17**. SS12.8, **18**. SS12.9, **19**. SS13.2, **20**. SS13.3, **21**. SS14.3, **22**. SS14.4, **23**. SS15.4, **24**. SS16.1, **25**. SS16.5, **26**. SS17.1, **27**. SS17.2, **28**. SS17.4, **29**. SS18.3, **30**. SS19.1, **31**. SS19.2, **32**. SS19.4, **33**. DNA ladder, **34**. SS20.2, **35**. SS20.3, **36**. SS21.3, **37**. SS21.5, **38**. SS22.3, **39**. SS23.1, **40**. SS26.9, **41**. SS27.3, **42**. SS28.2, **43**. SS29.4, **44**. SS30.3, **45**. SS32.7, **46**. SS34.4, **47**. SS34.5, **48**. SS34.7, **49**. SS35.1, **50**. SS35.4, **51**. SS36.4, **52**. SS38.1, **53**. SS38.3, **54**. SS40.3, **55**. VPS50.2, **56**. *B. subtilis* 168, **57**. control sample without DNA

Antimicrobial activity during growth

The antimicrobial activity detected in several *Bacillus* isolates was dependent on the growth phase. To establish whether the same is true for strain SS12.9, antimicrobial activity was determined during various growth phases by taking samples of supernatants every two hours during the growth in LB medium, and tested for their activity using *X. oryzae* pv. *oryzae* as indicator strain. Changes in cell numbers (viable counts) and spores were recorded as well as the size of the zones of inhibition on indicator strains, as shown in Fig. 3. Antagonistic activity was observed after 12 h of growth, reaching maximum in 18 h. Almost the same level of activity was recorded after 48 h, and no significant decrease was observed after 120 h. Similar results were obtained when *E. carotovora* was used as indicator strain (data not shown).

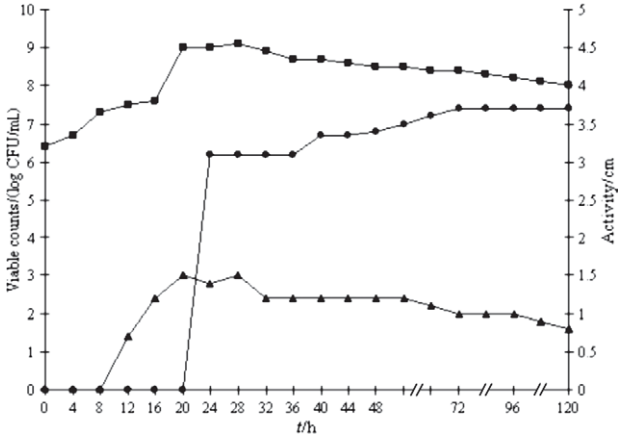

Fig. 3. Production of antimicrobial substance during growth and sporulation of *Bacillus* sp. isolate SS12.9. (■) Viable counts (log CFU/mL), (●) spores (log CFU/mL) and (▲) antimicrobial activity (radii of zones of inhibition in mm). *X. oryzae* pv. *oryzae* was used as indicator strain

Taxonomical analysis of Bacillus SS12.9 and VPS50.2

Following the studies of bacterial antagonisms described above, it was decided to further characterize the SS12.9 and VPS50.2 *Bacillus* strains. Identification of *Bacillus* isolate SS12.9 using API 50 CHB kit and API WEB software showed 93.8 % similarity with *B. subtilis* species. Isolate VPS50.2 exhibited 99.9 % identity with *B. licheniformis*. Homology analysis of nucleotide sequences derived from PCR-amplified 16S rDNA fragments revealed that isolate SS12.9, with 99 % identity, could be designated as *B. subtilis* and *B. amyloliquefaciens*. For VPS50.2 isolate, homology analysis revealed 100 % identity with *B. licheniformis*. It was concluded that strain SS12.9, according to the two methods used for the determination (API 50 CHB kit and homology analysis of nucleotide sequences of 16S rDNA), can be designated as *B. subtilis*, whereas strain VPS50.2 was *B. licheniformis*.

Purification and characterization of lipopeptide from Bacillus sp. SS12.9

Antimicrobial lipopeptide was isolated from cell-free culture supernatant of strain SS12.9 by using ethyl acetate extraction. After evaporation, the ethyl acetate fraction was subjected to ultrafiltration through 3-kDa molecular cut-off filter and filtrate containing lipopeptide with high antimicrobial activity against *X. oryzae* pv. *oryzae* was used for further purification on reversed phase HPLC. All fractions from the reversed phase HPLC were tested in agar well assay using *X. oryzae* pv. *oryzae* as the sensitive strain, and the antimicrobial activity was detected in a single peak within the second half of the chromatogram (Fig. 4). Mass spectrometry analysis of the HPLC fraction with the antimicrobial activity showed that antimicrobial compound within the peak has a mass number of m/z=1029.47. Additional analysis that included the fragmentation of the obtained compound showed that it consists of seven distinct amino acids (Pro, Val, Asn, Arg, Phe, Tyr and Arg), strongly suggesting that this compound belongs to the iturin family of antimicrobial lipopeptides.

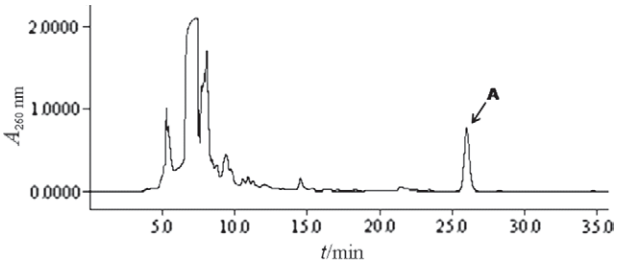

Fig. 4. HPLC chromatogram of ethyl acetate fraction from *Bacillus* sp. SS12.9 culture supernatant. Column: Supelcosil LC-18, 25 cm×10 mm, 5 μm, Supelco; eluent: acetonitrile and 10 mM ammonium acetate (40:60); flow rate: 2.5 mL/min; detection: 260 nm. **A –** the peak with antimicrobial activity against *X. oryzae* pv. *oryzae*

Discussion

Many strains of *Bacillus* genus and/or its metabolites are believed to be promising for an alternative or supplementary method to chemical plant protection (*16, 17*). In this work, a collection of 203 *Bacillus* isolates from soil, manure and hay samples were used as the source for identification of strains with antimicrobial activity. The preliminary screening, performed with five phytopathogenic bacteria used as indicator strains (*B. glumae*, *B. cepacia*, *E. carotovora*, *P. fuscovaginae* and *X. oryzae* pv. *oryzae*) showed that many strains (127) from the collection exhibit antagonistic activity against at least one indicator strain. The species that was most sensitive to antimicrobial compounds from natural isolates of *Bacillus* was *X. oryzae*, with 104 isolates that showed significant level of inhibition during screening. The high percentage of the tested strains that inhibit the important rice pathogen *X. oryzae* pv. *oryzae* (*18*) illustrates the potential use of *Bacillus* natural isolates as biocontrol agents. From all 203 isolates that were tested, only the activity of the supernatant from strain VPS50.2 was sensitive to Pronase E treatment, indicating that antimicrobial substance produced by this strain has a proteinaceous nature and that it most likely belongs to bacteriocins. Isolate VPS50.2 was identified as *B. licheniformis*, and it is believed that this is a strain that merits further investigations, since within

the *Bacillus* genus a relatively small number of bacteriocin-producing strains have been identified thus far.

Since the production of antimicrobial lipopeptides synthesized in a nonribosomal manner is one of the possible ways for *Bacillus* isolates to exert their antimicrobial action, we performed the screening of part of the collection for the presence of operon for iturin biosynthesis and *sfp* gene, responsible for the production of surfactin. Most of the isolates (33 out of 51) were identified as iturin producers (Fig. 2a), and all of them showed very intense inhibitory effect against *X. oryzae* pv. *oryzae*. Further characterization of strains that possess both iturin operon and *spf* gene (isolate SS17.2, presented in Table 1) will be of particular interest, since the strains that produce iturins and surfactins could have wider possibilities in the control of plant diseases.

It was determined that the isolate *Bacillus* sp. SS12.9 belongs to the species of *B. subtilis*. The antibacterial activity of this strain could be detected early in the logarithmic growth phase reaching the maximum at the beginning of the stationary phase, with no significant decrease during prolonged incubation of 120 h (Fig. 3). It therefore seems that sporulation had no effect on the production of antimicrobial substance, which was very stable even in the culture after several days of incubation.

Finally, the antimicrobial lipopeptide produced by the strain SS12.9 was isolated from the culture supernatant and purified. Mass spectrometry analysis confirmed that the lipopeptide most likely belongs to iturin group, since it consists of seven amino acid residues including invariable tyrosine at the position 2 in the ring (7). This study demonstrates that the *Bacillus* strains characterized here merit further investigation for possible use as biocontrol agents of bacterial plant diseases.

Conclusions

The results of this study show that many strains of the collection of *Bacillus* natural isolates have strong antimicrobial activity against important phytopathogenic bacteria. According to the obtained results, in most strains this activity is the result of the production of nonribosomally synthesized cyclic lipopeptides from iturin and in some cases surfactin family. On the other hand, the production of bacteriocins is not that common among the strains from our collection, since we identified only one isolate that synthesizes a bacteriocin-like substance. Also, an interesting result of this work is a very strong antagonistic effect of *Bacillus* isolates against *Xanthomonas oryzae* pv. *oryzae*, which was detected in about 50 % of the tested strains. The available data concerning the biological control of this important rice pathogen are very limited, so further experimental work on this topic will be of great interest.

Acknowledgments

This work was supported by the Ministry of Education and Science, the Republic of Serbia (Grant No. 173026) and by International Centre for Genetic Engineering and Biotechnology, Italy (Grant CRP-YUG06/01).

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