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SCREENING FOR THE PRESENCE OF BIOSYNTHETIC GENES FOR ANTIMICROBIAL LIPOPEPTIDES IN NATURAL ISOLATES OF *BACILLUS* SP.

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Abstract - A collection of 205 natural isolates of *Bacillus* was tested for the presence of genes for biosynthesis of antimicrobial lipopeptides, iturin, surfactin, fengycin and bacillomycin D. For the detection of iturin producers by PCR screening, we used forward ITUP1-F and reverse ITUP2-R primers which are capable of detecting a 2-kb region that includes the intergenic sequence between the *ituA* and *ituB* genes. A 675-bp fragment from the gene *sfp* from *B. subtilis* encoding 4'-phosphopantetheinyl transferase involved in the biosynthesis of surfactin was targeted for amplification by using primers P17 and P18. Other two pairs of primers were BACC1F and BACC1R for bacillomycin D and FEND1F and FEND1R for potential fengycin producers, respectively. The results of the screening showed that the majority of tested strains had more than one biosynthetic operon, since 81% possessed the genes for bacillomycin D production, 54% for surfactin, 38% for iturin and 25% for fengycin production.

Key words: *Bacillus,* iturin, surfactin, fengycin, bacillomycin

INTRODUCTION

Many species of the genus *Bacillus* produce lipopeptides with antagonistic activity against bacteria, fungi or animal cells. *Bacillus* lipopeptides, which can be cyclic or linear, mainly consist of 7 to 11 amino-acid residues linked to β-amino or β-hydroxy fatty acids. Because of their amphiphilic nature, most act as biosurfactants. The length of the hydrocarbon chain of fatty acids can be different, and the cyclic structure of lipopeptides prevents the cleavage of their peptide bonds by proteolytic enzymes. The mechanism of the antagonistic action of lipopeptides is based on their interaction with the cell membrane and formation of pores or, at higher concentrations, the solubilization of the membrane, (Deleu et al., 2005). Antimicrobial lipopeptides are synthesized in a nonribosomal manner. The strains that produce them have established their place in the biological control of plant pathogenic bacteria and fungi (Pengnoo et al., 2000; Abanda-Nkpwatt et al., 2006).

Lipopeptides of the iturin family, such as iturin, mycosubtilin and bacillomycin, consist of seven amino-acid residues circularized with β-amino fatty acid (Peypoux et al., 1978). Iturins are characterized by the chiral sequence LDDLLDL within the amino-acid ring and D-Tyr2 (Magnet-Dana and Peypoux, 1994). Beside its antimicrobial effect, Iturin A shows a high degree of thermostability, retaining 100% of biological activity after heating at 100°C for 30 min (Yu et al., 2002). The antagonistic effects of the iturin cyclic peptides are the result of their interaction with the cell membrane and formation of pores (Magnet-Dana and Peypoux, 1994). A similar effect was observed in the action of mycosubtilin which in-

fluences the permeability of the membranes of yeast cells (Besson and Michel, 1989). The Tyr residue at position 2 in the peptide ring of peptides from the iturin family has a significant role in the mechanism of pore formation in target cells (Harnois et al., 1989, Volpon et al., 1999).

The *Bacillus* strains that produce iturins showed antifungal activity against some important plant pathogens, proving their potential in the application of biological control (Han et al., 2005; Arrebola et al., 2010).

Strains of other *Bacillus* species also produce lipopeptides that belong to the surfactin family. This group consists of surfactin and its analogs lichenysins and pumilacidins. Similar to iturins, they have a cyclic structure of seven amino acids, however, the ring is closed with β-hydroxy fatty acid with a different length of hydrocarbon chain, and they act as very powerful biosurfactants (Arima et al., 1968; Nagai et al., 1996). Lichenysin A is produced by *Bacillus licheniformis,* and beside the determination of its structure, the biosynthetic operon of this lipopeptide has also been identified (Yakimov et al., 1995; 1998).

The fengycin family of lipopeptides also includes plipastatins. Their structure is somewhat different to that of other lipopeptides. They consist of 10 amino-acid residues, and their structure contains a ring of eight amino-acid residues linked to a dipeptide associated with the β-hydroxy fatty acid. Within this family of lipopeptides, the composition of the stereoisomers of amino-acid residues can be different (Volpon et al., 2000). Some of the bacterial strains producing these lipopeptides display strong antagonistic effects against different organisms. *Bacillus amyloliquefaciens* strain GA1, which exhibits strong antifungal activity, contains the biosynthetic genes for eight antimicrobial agents, including lipopeptides from the surfactin, fengycin and iturin families, as well as macrolactin, difficidin, bacillaene, bacilysin and bacillibactin (Arguelles-Arias et al., 2009). The strain *Bacillus thuringiensis* CMB26 produces the analog of fengycin with bactericidal, fungicidal and insecticidal effect (Kim et al., 2004). These and many other strains have a significant potential in the biological control of plant diseases.

In this study, we performed a screening for the presence of biosynthetic genes for the antimicrobial lipopeptides iturin, surfactin, fengycin and bacillomycin D in natural isolates of *Bacillus* from soil samples that were taken from different locations in Serbia, in order to identify the strains with the capacity for application in biological control.

MATERIALS AND METHODS

Media for bacterial growth and culture conditions

Natural isolates of *Bacillus* sp. were grown aerobically in Luria Bertani (LB) broth, containing 1.0% Bacto-tryptone, 0.5% yeast extract, 1.0% NaCl, and maintained on LB plates. Agar plates were made by adding 1.5 % (wt/vol) agar (Torlak, Belgrade, Serbia) to the liquid medium. Bacteria were incubated at 30°C for 24 h. All strains used in this study are given in Table 2.

Isolation of DNA

Genomic DNA from the *Bacillus* strains was isolated as described earlier (Le Marrec et al., 2000). After centrifugation of the culture and two washes in TE buffer (10 mmol Tris-HCl (pH 8·0), 1 mmol EDTA), cells were resuspended in 1 ml lysis buffer (50 mmol Tris (pH 8·0), 1 mmol EDTA; 25% sucrose) containing 20 μg ml of lysozyme (Serva) and incubated for 45 min at 37°C. The reaction was stopped with 1ml EDTA (250 mmol pH 8·0) for 5 min. The samples were then treated with 400 μl of 20% (w/vol) SDS and 20 μl of a 20 mg/ml of Proteinase K (Sigma) solution. The mixture was incubated at 65°C until it became clear and less viscous, followed by phenol-chloroform extraction. DNA was precipitated in ethanol and resuspended in 100 μl of TE buffer with 10 μl of RNase (10 mg/ml).

PCR identification of the lipopeptide genes

The oligonucleotides used in the PCR amplifications

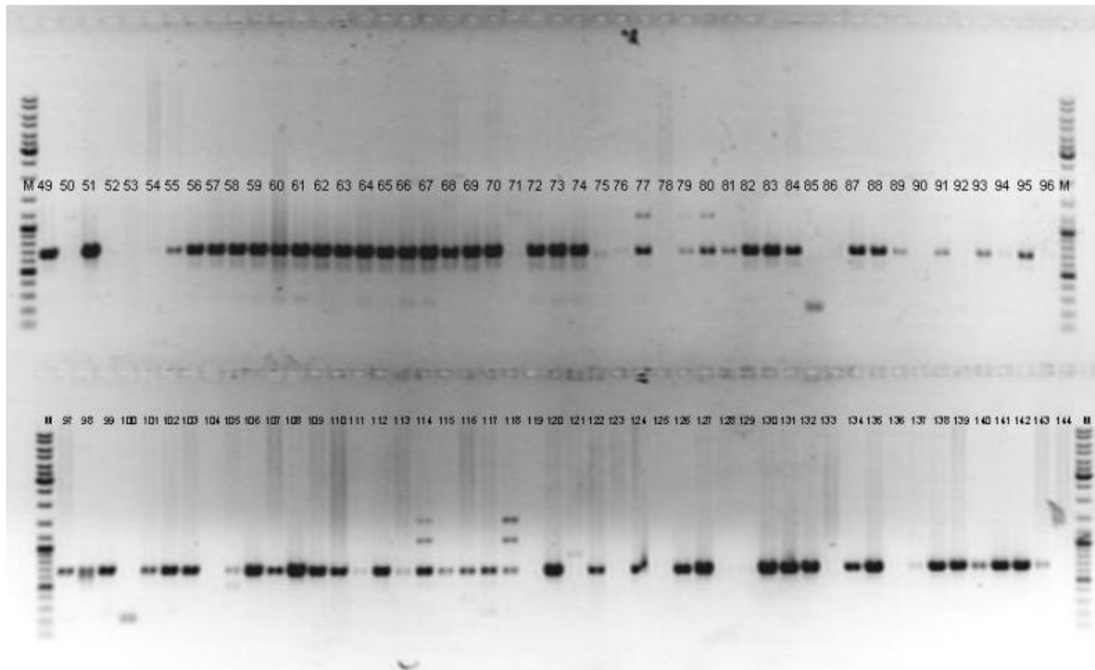

Figure 1. Screening of the presence of *sfp*, biosynthetic gene for surfactin. The number of the sample corresponds to the strain number in Table 2 (M-DNA ladder).

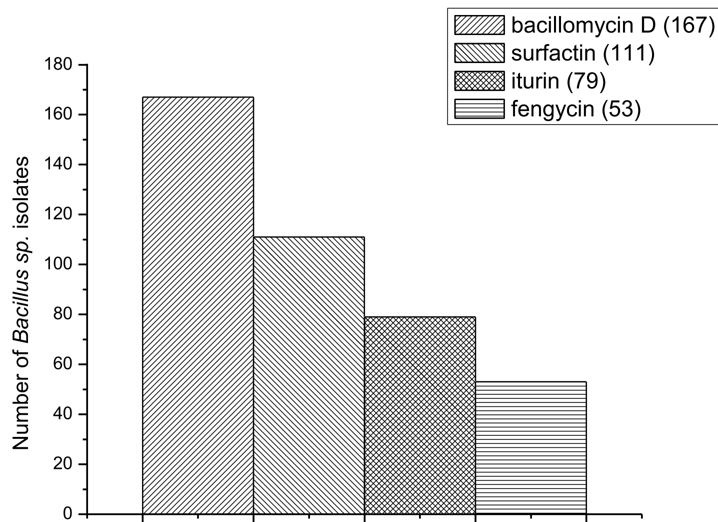

Figure 2. Number of isolates with detected biosynthetic operons for antimicrobial lipopeptides among the total number of 205 *Bacillus* strains.

are listed in Table 1. Briefly, the 30 µl reaction contained 50-100 ng/µl of DNA, 1 U of KAPA Taq DNA Polymerase (Kapa Biosystems, Woburn, U.S.A.), 200 µM of each deoxynucleoside triphosphate, 0.4 µM of an appropriate primer pair and 1 x Kappa buffer with Mg^{2+}. The initial denaturation for the *fenD* and bacillomycin D gene was at 94°C for 3 min; 45 cycles for the *fenD* gene at 94°C for 1 min, at 59°C for 1 min,

Table 1. Primers for PCR detection of biosynthetic genes for antimicrobial lipopeptides from natural isolates of *Bacillus* sp.

Lipopeptide	Primer name	Sequence	Position/Accession number in GenBank	Reference
Fengycin	FEND1F	5'- TTTGGCAGCAGGAGAAGTT -3'	3687-3706, AJ011849	Ramarathnam et al., 2007
	FEND1R	5'- GCTGTCCGTTCTGCTTTTTC-3'	4650-4631, AJ011849	Ramarathnam et al., 2007
Bacillomycin D	BACC1F	5'- GAAGGACACGGCAGAGAGTC-3'	34274-34293, AY137375.1	Ramarathnam et al., 2007
	BACC1R	5'- CGCTGATGACTGTTCATGCT-3'	35148-35129, AY137375.1	Ramarathnam et al., 2007
Iturin	ITUP1-F	5'-AGCTTAGGGAACAATTGTCATCGGGGCTTC-3'	15353-15383, AB050629	Tsuge et al., 2005
	ITUP2-R	5'-TCAGATAGGCCGCCATATCGGAATGATTCG-3'	17326-17355, AB050629	Tsuge et al., 2005
Surfactin	P17	5'-ATGAAGATTTACGGAATTTA-3'	167-186, X63158	Hsieh et al., 2004
	P18	5'-TTATAAAAGCTCTTCGTACG-3'	841-822, X63158	Hsieh et al., 2004

followed by a 72°C extension for 1 min for 45 s; 35 cycles for bacillomycin D were at 94°C for 1 min, at 60°C for 30 s, and extension at 72°C for 1 min. The final extension for both genes was at 72°C for 6 min. The initial denaturation for the iturin A operon sequence and the *sfp* gene was at 94°C for 5 min; the final extension was at 72°C for 10 min. Thirty cycles for the iturin A operon sequence were at 94°C for 30 s, 60°C for 30 s, and a 72°C extension for 2 min, 30 s. Thirty cycles for the *sfp* gene were at 94°C for 30 s, 43°C for 30 s, and a 72°C extension for 1 min. The PCR products were analyzed by electrophoresis in a 1% agarose gels containing 0.5 µg/mL ethidium bromide in 1 x TAE buffer (2 mol Tris base, 1 mol glacial acetic acid, 50 mmol EDTA, pH 8.0). The products were resolved at 7 V/cm for 1 h in the presence of a 0.1 volume of loading dye (50% glycerol, 1x TAE, 0.3 % orange G). The gels were photographed under UV illumination (Biometra, Goettingen, Germany). The expected PCR products were gel purified (Qiagen, Germany) and sequenced (Macrogene sequencing service, Amsterdam, Netherlands).

RESULTS AND DISCUSSION

The natural isolates of *Bacillus* sp. were isolated from the soil samples from different locations in Serbia and the results for the isolation of the strains and determination to the level of genus were published earlier (Stanković et al., 2007). In addition, part of the collection was subjected to screening for presence of biosynthetic genes for iturin and surfactin (Berić et al., 2012). According to these results, limited to 51 isolate, 33 of them harbored the operon for iturin biosynthesis, and six of them contained the *sfp* gene, responsible for the biosynthesis of surfactin. In addition, the antimicrobial spectrum for these strains was determined, including the most common bacterial plant pathogens (Berić et al., 2012). In order to gain insight into the presence of biosynthetic genes for the whole collection of *Bacillus* natural isolates, in this work we performed PCR screening for those genes, including the most important antagonistic lipopeptides. Part of the screening is presented in Fig. 1, and the results of the whole screening of 205 strains are summarized in Table 2. As presented in Table 1 we used forward ITUP1-F and reverse ITUP2-R primers for the detection of iturin producers, which can amplify a 2 kbp region that includes parts of the *ituA* and *ituB* genes and the intergenic sequence between them. A 675 bp fragment from the gene *sfp* from *B. subtilis* encoding 4'-phosphopantetheinyl transferase involved in the biosynthesis of surfactin was targeted for amplification by using primers P17 and P18. Another two pairs of primers were BACC1F and BACC1R for the detection of bacillomycin D and FEND1F and FEND1R for potential fengycin producers, respectively. All obtained PCR fragments were of expected size (part of the screening is presented in Fig. 1. The nucleotide sequence homologies among fragments from the genes with published sequences and our fragments were between 98% and 100% (data not shown). These results suggest that the

Table 2. List of strains of *Bacillus* sp. used in this study with the results of PCR screening of biosynthetic genes for antimicrobial lipopeptides.

Number	Strain	FEND	BACC	ITUP	Sfp	Number	Strain	FEND	BACC	ITUP	Sfp
1.	1.1	-	-	-	+	53.	12.3	-	+	-	-
2.	2.1	-	+	-	+	54.	12.4	-	-	-	-
3.	2.2	-	+	-	+	55.	12.5	-	+	-	+
4.	2.3	-	-	-	+	56.	12.6	-	+	+	+
5.	2.4	-	+	+	+	57.	12.7	-	+	+	+
6.	2.5	-	+	+	+	58.	12.8	-	+	+	+
7.	2.6	-	+	-	+	59.	12.9	-	+	+	+
8.	2.7	-	-	-	-	60.	13.1	-	+	+	+
9.	2.8	-	+	-	+	61.	13.2	-	+	+	+
10.	2.9	-	+	+	+	62.	13.3	-	+	+	+
11.	2.10	-	+	-	+	63.	15.2	-	+	+	+
12.	2.11	-	-	-	+	64.	15.4	-	+	+	+
13.	2.12	-	+	-	+	65.	13.4	-	+	+	+
14.	2.13	-	-	-	+	66.	14.3	+	+	+	+
15.	2.14	+	-	-	+	67.	14.4	-	+	+	+
16.	2.15	-	-	-	-	68.	14.6	-	+	-	+
17.	3.1	-	+	-	+	69.	16.1	-	+	+	+
18.	3.2	-	+	-	+	70.	16.2	+	+	-	+
19.	4.1	-	+	-	+	71.	16.4	-	-	-	-
20.	5.1	-	+	-	+	72.	17.1	-	+	+	+
21.	5.2	-	-	-	+	73.	17.2	-	+	+	+
22.	5.3	-	+	-	+	74.	16.5	-	+	+	+
23.	5.4	-	-	-	+	75.	17.3	-	+	-	-
24.	6.1	-	+	+	+	76.	17.4	-	+	-	-
25.	6.2	+	+	+	+	77.	18.1	-	+	+	+
26.	6.3	-	+	+	+	78.	18.2	+	+	-	-
27.	6.4	-	+	+	+	79.	18.3	-	+	+	+
28.	6.5	-	-	-	+	80.	18.4	-	+	+	+
29.	6.6	-	+	+	+	81.	19.1	-	+	-	+
30.	7.1	-	+	+	+	82.	19.2	-	+	+	+
31.	8.1	-	+	+	+	83.	19.3	+	-	-	+
32.	8.2	-	+	+	+	84.	19.4	+	+	-	+
33.	8.3	-	+	+	-	85.	20.1	-	+	+	-
34.	8.4	-	+	+	-	86.	20.2	-	+	-	-
35.	9.1	-	+	-	-	87.	20.3	-	+	+	+
36.	9.2	-	+	+	+	88.	20.4	-	+	+	+
37.	9.3	-	+	+	+	89.	21.1	-	+	-	+
38.	9.4	-	-	-	+	90.	21.2	-	+	-	-
39.	10.1	-	+	+	+	91.	21.3	-	+	+	+
40.	10.2	-	+	+	+	92.	21.4	-	+	-	-
41.	10.3	-	+	+	+	93.	21.5	-	+	-	+
42.	10.4	-	+	+	-	94.	21.6	-	+	-	-
43.	10.5	-	+	-	-	95.	21.7	+	+	+	+
44.	10.6	-	+	-	-	96.	22.1	-	+	-	-
45.	10.7	+	+	+	+	97.	22.2	-	+	-	+
46.	10.8	-	+	+	-	98.	22.3	-	+	+	+
47.	11.1	-	+	-	+	99.	22.4	-	+	+	+
48.	11.2	-	+	-	+	100.	22.5	-	-	+	-
49.	11.3	-	+	+	+	101.	23.1	-	+	+	+
50.	11.4	-	+	+	-	102.	23.2	-	+	+	+
51.	12.1	+	+	-	+	103.	23.3	-	+	-	+
52.	12.2	-	+	-	-	104.	23.4	-	-	-	-

Table 2. *Continued*

Number	Strain	FEND	BACC	ITUP	Sfp	Number	Strain	FEND	BACC	ITUP	Sfp
105.	24.1	-	+	-	+	156.	33.2	-	+	-	-
106.	26.1	+	-	-	+	157.	33.3	-	+	-	-
107.	26.2	+	-	-	+	158.	33.4	-	-	-	-
108.		+	+	-	+	159.	33.5	+	+	-	-
109.	26.3	+	+	-	+	160.	33.6	+	+	-	-
110.	26.4	+	+	-	+	161.	33.7	+	+	-	-
111.	26.5	-	-	+	+	162.	33.8	+	+	-	+
112.	26.6	-	-	-	+	163.	34.1	+	+	-	+
113.	26.7	+	+	-	+	164.	34.2	+	+	-	+
114.	26.8	-	+	+	+	165.	34.3	+	+	-	+
115.		-	+	-	+	166.	34.4	+	+	-	-
116.	26.9	+	+	-	+	167.	34.5	-	+	-	-
117.	26.10	+	+	+	+	168.	34.6	-	-	-	-
118.	27.1	-	+	+	+	169.	34.7	-	+	-	-
119.	27.2	-	-	-	-	170.	35.1	-	+	+	-
120.	27.3	-	-	+	+	171.	35.2	-	+	+	-
121.		-	+	+	-	172.	35.3	-	+	+	-
122.	27.4	-	-	-	+	173.	35.4	+	+	+	+
123.	27.5	+	+	-	-	174.	35.5	-	-	-	-
124.	27.6.1	-	-	-	+	175.	35.6	-	+	-	-
125.	27.8	+	-	-	-	176.	35.7	-	+	-	-
126.	27.9	-	-	-	+	177.	35.8	-	+	-	-
127.	28.1	+	-	-	+	178.	36.1	-	+	-	-
128.	28.2	-	+	+	-	179.	36.2	-	+	-	-
129.	28.3	-	+	-	-	180.	36.3	-	+	+	+
130.	28.4	-	+	-	+	181.	36.4	-	-	-	-
131.	28.5	+	+	+	+	182.	36.5	-	+	+	+
132.	27.7.1	+	+	-	+	183.	36.6	-	+	-	-
133.	28.6	+	+	-	-	184.	37.1	-	+	-	-
134.	29.1	-	+	+	+	185.	37.2	-	+	-	-
135.	29.2	-	-	-	+	186.	37.3.	-	+	-	-
136.	29.3	-	-	-	-	187	37.4	-	+	+	-
137.	29.4	-	+	+	-	188.	37.5	-	+	-	-
138.	29.5	+	+	-	+	189.	37.6	-	+	-	-
139.	29.6	+	+	-	+	190.	37.7	-	+	-	-
140.	29.7	-	+	-	+	191.	38.1	+	+	-	-
141.	30.1	-	-	-	+	192.	38.2	+	+	+	+
142.	30.2	+	+	-	+	193.	38.3	+	+	+	+
143.	30.3	-	-	-	+	194.	38.4	+	+	+	+
144.	30.4	+	-	-	-	195.	39.1	+	+	+	+
145.	30.5	-	+	-	-	196.	39.2	-	+	-	+
146.	30.6	-	+	-	-	197.	39.3	+	+	+	+
147.	30.7	+	+	-	-	198.	40.1	-	-	-	+
148.	31.1	+	+	-	+	199.	40.2	+	+	+	+
149.	31.2	+	+	-	-	200.	40.3	+	+	-	+
150.	31.3	-	+	-	-	201.	40.5	+	+	-	+
151.	32.1	+	-	-	+	202.	40.6	+	+	+	+
152.	32.3	+	+	-	+	203.	50.1	-	-	-	+
153.	32.4	-	+	+	+	204.	50.2	-	+	+	+
154.	32.5	+	-	-	+	205.	50.4	-	-	-	+
155.	32.6	-	+	-	-						
	32.7										
	32.8										
	33.1										

biosynthetic genes for antimicrobial lipopeptides have a highly conserved structure among *Bacillus* strains of different origin. The distribution of these genes showed that the majority of our strains can produce more than one antimicrobial lipopeptide (Table 1), which is in accordance with literature data (Arguelles-Arias et al., 2009). In addition, the screening of our collection showed that 81% of the isolates possessed the genes for bacillomycin D production, 54% for surfactin, 38% for iturin and 25% for fengycin (Fig. 2). We did not observe a correlation between the distribution of the genes and the origin of the strains, considering the regions from which the samples were taken. Our preliminary data show that many of these strains have a strong antagonistic effect against some important bacterial plant pathogens, especially the bacteria from the genus *Xanthomonas* (Berić et al., 2012), as well against some post-harvest fungal pathogens (unpublished results). However, further experiments will show which lipopeptides are actually produced by the strains of interest, since the presence of biosynthetic operons does not mean that all of them are active.

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