Short Communication

CHARACTERIZATION OF *BACILLUS SUBTILIS* RB14, COPRODUCER OF PEPTIDE ANTIBIOTICS ITURIN A AND SURFACTIN

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(Received July 31, 1992)

Concerns about potentially harmful effects of long-term treatment of chemical insecticides and pesticides and the emergence of pathogens resistance to these agents make the use of natural bacterial antagonists specific to the pathogens—i.e. biological control of plant diseases—attractive to protect the environment.

Bacillus subtilis, a Gram-positive, spore-forming bacterium, has proven safe over many years, because it is a non-pathogenic bacterium (3) and is even consumed in large quantities as the Japanese food natto (5). In recent years, there has been considerable interest in using *B. subtilis* as a biocontrol agent because of its ability to antagonize and repress plant diseases (13, 14, 16). We have also isolated several *B. subtilis* strains from suppressive composts, and shown that the bacteria were actually effective against plant pathogens (10–12). Whereas no correlation was usually observed between the size of the inhibition zone in vitro and the protective effect in glasshouse trials (16), we have observed a positive correlation between the strength of inhibition in vitro and the one of suppressiveness in vivo in our screening system (10, 11). Isolating another bacterium with much stronger suppressiveness will be stimulating and beneficial for the biological control of plant diseases. A newly isolated bacterium *B. subtilis* RB14 inhibited the growth of *Fusarium oxysporum* more significantly than any other bacteria isolated. In this paper, we describe the characterization of the bacterium RB14 which produces lipopeptides, surfactin and iturin A.

Suppressive effects in vitro on various phytopathogenic microorganisms by *B. subtilis* RB14 were tested as shown in Table 1. Ten phytopathogenic fungi were grown on potato-dextrose agar (PDA) medium (potato-dextrose agar 39 g, distilled

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Table 1. Suppressive effect of *B. subtilis* RB14 against plant pathogens in comparison with *B. subtilis* NB22 in vitro.

Phytopathogens	Name of plant disease	Inhibition zone of RB14 in		Inhibition zone of NB22 in	
		PDA medium (mm)	L medium (mm)	PDA medium (mm)	L medium (mm)
Fungi					
<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i> race J1 SUF119	Fusarium wilt of tomato	13	13	9	13
<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i> race J2		13	16	8	12
<i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i> KEF2R-1	Sheath blight of rice Brown spot of rice	11	14	8	12
<i>Rhizoctonia solani</i> AG-4		38	17	33	15
<i>Cochliobolus miyabeanus</i> NIAES 5425		32	30	31	20
<i>Alternaria mali</i> IFO 8984		27	23	29	21
<i>Cercospora kikuchii</i> NIAES 8984	Purple seed stain of soybean	27	30	24	25
<i>Pyricularia oryzae</i> IFO 5279	Blast of rice	13	19	10	15
<i>Pyricularia oryzae</i> NIAES 5001	Verticillium wilt of tomato	40	27	30	26
<i>Verticillium dahliae</i> Klebahn V-3		22	23	14	20
Bacterium					
<i>Pseudomonas syringae</i> pv. <i>lachrymans</i> P1-7415	Angular leaf spot of cucumber	— ^a	12	— ^a	9

^a Not tested.

water 1 l, pH 5.6) at 30°C for 5 days and then suspended in sterile distilled water. A portion of this suspension was mixed into L agar or PDA plate. After spotting an isolated bacterium at the center of this plate, the suppression by the bacterium was investigated by observing the sizes of inhibitory zones made on the lawn of the fungus. A phytopathogenic bacterium was grown by shaking in L broth at 30°C for 1 day. The cell suspension was diluted and mixed into L agar medium plates. All the tested microorganisms were clearly suppressed by the newly isolated *B. subtilis* RB14.

The suppressive substances were isolated from the culture supernatant of *B. subtilis* RB14 grown in No. 3 medium (10 g of Polypepton, 10 g of glucose, 1 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 l of distilled water [pH 7.0]) for 5 days at 30°C. The supernatant was precipitated at pH 2. The precipitate was collected by centrifugation and was extracted with methanol. The extract was analyzed by thin-layer chromatography (TLC) on silica-gel plates 60F₂₅₄ (Merck, Darmstadt, Germany) with chloroform-methanol-water (65:25:5, vol/vol). Components were visualized by spraying the plates with 10% H_2SO_4 and heating at 110°C for 45 min. Two main spots were detected. The *R_f* values of these spots were 0.63 and 0.38. The *R_f* value 0.38 corresponded to that of iturin A purified in this laboratory (10), and was confirmed to be iturin A by HPLC analysis, which was operated by a reverse-phase column Inertsil ODS-2 (GL Sciences Inc., Tokyo, 4.6 ϕ × 250 mm) with acetonitrile-ammonium acetate (10 mM) (2:3, vol/vol).

Surfactin (1) produced by *B. subtilis* is well known as a potent surface-active agent and as an antibiotic (2, 4). The *R_f* value of the purified surfactin (Wako Pure Chemical Ind. Ltd., Osaka) was shown to be 0.63, which was the same *R_f* value of the other spot. This substance was identified as surfactin by the following analyses.

The precipitate at pH 2.0 described above was dissolved in distilled water (adjusted to pH 7.0 with NaOH). The suspension was kept overnight at 4°C and then extracted with the same volume of dichloromethane for 30 min at room temperature. Then, the dichloromethane phase was removed. After the extraction was repeated 4 times, all the dichloromethane phase was evaporated. The white precipitate was used for the further analyses. The IR spectrum of the substance molded in KBr showed the characteristic IR absorption of surfactin previously reported (1). Strong bands characteristic of peptides at 3300 cm^{-1} , at 1650 cm^{-1} , and at 1520 cm^{-1} were clearly observed. The bands at 2960 cm^{-1} , 2930 cm^{-1} , 2850 cm^{-1} reflected aliphatic chains ($-\text{CH}_3$, $-\text{CH}_2-$) of the fraction. The band at 1730 cm^{-1} was due to lactone carbonyl absorption. These results indicated that the product had an analogous structure to surfactin.

The dichloromethane extract described above was analyzed by the same HPLC column used for iturin detection by monitoring at 205 nm with the solvent acetonitrile-acetic acid (1%) (68:32, vol/vol). Two main peaks were detected at retention times 53 min and 70 min. The authentic sample of surfactin was detected at retention time 70 min. Two main peaks with retention times 53 min and 70 min were collected, and the hydrolysate of each component was analyzed by an amino

Fig. 1. Suppressive effect in vitro of a newly isolated bacterium *B. subtilis* RB14 (A) on *Fusarium oxysporum* in comparison with the previously isolated *B. subtilis* NB22 (B) (10,11).

acid analyzer, respectively. The amino acid composition of each component was determined to be Asp, Glu, Val, Leu in the molar ratio of 1 : 1 : 1 : 4, which was the same value expected from surfactin (1,8). The molecular ion peaks of the compounds with HPLC-retention times 53 min and 70 min were detected by FAB-MS at 1044 and 1058, respectively. As the presence of sodium ions were detected by the yellow color in flame reaction, these molecular ion peaks were considered to be $(M + Na)^+$ formed by the attachment with Na^+ , as reported by Jenny et al. (7). These results lead to the molecular weight 1022 for the compound with retention time 53 min, and 1036, which is the same molecular weight as surfactin, for the compound with retention time 70 min. The mass difference of 14 units between the two was thought to be attributed to the one unit of methylene residue. All these results described above indicated that the strain *B. subtilis* RB14 produced the bio-surfactant surfactin as well as iturin A.

A phytopathogenic fungus, *F. oxysporum* f. sp. *lycopersici* race J1 SUF119 (11), which causes the crown and root rot of tomato, was inhibited more significantly by this RB14 than by *B. subtilis* NB22, which was found to produce iturin A previously at about 100 ppm (10,11) (Fig. 1). RB14 inhibited the growth of other phytopathogens also more significantly than NB22 (Table 1).

As *B. subtilis* RB14 coproduces iturin A at about 100 ppm and surfactin at about 200 ppm in No. 3 medium, the significant inhibition of plant pathogens by RB 14 was considered to be attributed to the synergistic effect of the two compounds, iturin A and surfactin. Although surfactin itself did not inhibit the growth of a phytopathogenic fungus *F. oxysporum* at least at the concentration of 100 μ g/ml, it greatly enhanced the antibiotic activity of iturin as shown in Table 2. The synergistic effects of these two lipopeptides first found in this work may explain why this bacterium showed a wide suppressive spectrum against phytopathogens as shown in Table 1.

Coproduction of surfactin and iturin A by *B. subtilis* has been reported by others (15). However, neither the suppressive effect against phytopathogenic

Table 2. Effects of iturin A and surfactin on the growth of phytopathogenic fungus *F. oxysporum*.

Lipopeptide(s) added	Growth of <i>F. oxysporum</i> ^a					
	Concentration of lipopeptide (μg/ml)					
	0	6.25	12.5	25	50	100
Iturin A	+++	n.t.	+	+	+	—
Surfactin	+++	+++	+++	+++	+++	+++
Iturin A and 100 μg/ml of surfactin ^b	+++	n.t.	+	—	—	n.t.

^a *F. oxysporum* was spotted at the center of the plate containing lipopeptide(s), and the degrees of growth inhibition on PDA were determined after 7 days incubation at 28°C according to the following levels: + + +, no inhibition; +, significant inhibition; —, no growth; n.t., not tested.

^b Only iturin A concentration was varied, and the surfactin was fixed at the concentration of 100 μg/ml.

microorganisms nor the idea of biological control of them has been demonstrated. Surfactin is already known as a potent surface-active agent (4) and as an antibiotic against some bacteria (2), but is not effective against phytopathogenic fungus *F. oxysporum* (Table 2). The reason why the antifungal antibiotic activity of iturin was enhanced in the presence of surfactin is being investigated by physicochemical method, but the structural similarities between the two compounds (1, 6, 8, 9) may enhance the destructive activity of iturin to the cell membrane of plant pathogens. This will help the suppressive effect against plant pathogens even in soil where this bacterium was treated as a biological control agent to replace chemical fungicides.

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