APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1996, p. 4081–4085 Vol. 62, No. 11
0099-2240/96/$04.00+0

Biocontrol of *Rhizoctonia solani* Damping-Off of Tomato with *Bacillus subtilis* RB14

ORIE ASAKA AND MAKOTO SHODA*

Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama, 226 Japan

Received 18 September 1995/Accepted 31 August 1996

***Bacillus subtilis* RB14, which showed antibiotic activities against several phytopathogens in vitro by producing the antibiotics iturin A and surfactin, was subjected to a pot test to investigate its ability to suppress damping-off of tomato seedlings caused by *Rhizoctonia solani*. To facilitate recovery from soil, *B. subtilis* RB14-C, a spontaneous streptomycin-resistant mutant of RB14, was used. Damping-off was suppressed when the culture broth, cell suspension, or cell-free culture broth of RB14-C was inoculated into soil. Iturin A and surfactin were recovered from the soils inoculated with the cell suspension of RB14-C, confirming that RB14-C produced them in soil. The gene *lpa-14*, which was cloned from RB14 and required for the production of both antibiotics, was mutated in RB14-C, and a mutant, RΔ1, was constructed. The level of disease suppressibility of RΔ1 was low, but RΔ1(pC115), a transformant of RΔ1 with the plasmid pC115 carrying *lpa-14*, was restored in suppressibility. These results show that the antibiotics iturin A and surfactin produced by RB14 play a major role in the suppression of damping-off caused by *R. solani*. RB14-C, RΔ1, and RΔ1(pC115) persisted in soil during the experimental period and were recovered from the soil, mostly as spores.**

The overuse of chemical pesticides has caused soil pollution and harmful effects on human beings. Accordingly, biological control of soilborne diseases has been attracting attention. Many reports or reviews in this area have already appeared (4–9, 18–20, 40). So far, gram-negative bacteria, especially *Pseudomonas* strains, have been intensively investigated as biological control agents with regard to the production of antimicrobial metabolites (12, 23, 32, 33), genetic analysis and regulation of some metabolites (17, 18, 39), and ecological fitness of soil (3, 21, 24, 32, 33). The gram-positive bacteria, like *Bacillus* spp., however, have been studied less intensively than the gram-negative bacteria (31). *Bacillus subtilis* is considered to be a safe biological agent (2, 4, 14, 25, 26, 28, 35, 37), but evaluation of *B. subtilis* has focused primarily on the degree of disease suppression. The population dynamics and mechanism of suppression in soil have not been as extensively investigated (1, 20, 30, 38).

B. subtilis RB14, which was isolated in our laboratory, produces the antibiotics iturin A and surfactin (11). Iturin A is a cyclolipopeptide containing seven residues of α amino acids (L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser-) and one residue of a β amino acid (Fig. 1A). The antibiotic activity of iturin A is very strong. The other cyclic lipopeptide, surfactin, contains a β-hydroxy fatty acid connected by an ester peptide linkage to seven cyclic α amino acid residues (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu-) (Fig. 1B). Surfactin has weak antibiotic activity but is a potent surfactant. We have already established the methods to recover RB14 from soil and then to distinguish vegetative cells from spores of RB14 and to quantify iturin A and surfactin by high-performance liquid chromatography (HPLC) after extraction from soil (13).

Therefore, for this study, our goals were (i) to assess whether *B. subtilis* RB14 would make an effective biological control agent by testing it against the damping-off of tomato seedlings caused by *Rhizoctonia solani*, (ii) to elucidate RB14's suppressive mechanism by biochemical analysis and genetic manipulation, and (iii) to assess the basic ecological behavior of RB14 in soil.

* Corresponding author. Mailing address: Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta 4259, Midori-ku, Yokohama, 226 Japan. Phone: (81) 45-924-5274. Fax: (81) 45-924-5276. Electronic mail address: mshoda@res.titech.ac.jp.

MATERIALS AND METHODS

Microorganisms and plasmid. The bacterial strains and plasmid used are listed in Table 1. *B. subtilis* RB14, which was isolated originally from compost, produces iturin A and surfactin (11). To facilitate recovery from soil, *B. subtilis* RB14-C, a spontaneous streptomycin-resistant mutant of RB14, was selected. The growth rate and antifungal activity of RB14-C were confirmed to be the same as those of the parental strain RB14.

B. subtilis RΔ1, derived from RB14 (10), is deficient in the *lpa-14* locus associated with the production of iturin A and surfactin (13) and therefore does not produce either antibiotic.

B. subtilis RΔ1(pC115), a transformant of RΔ1 with the plasmid pC115 carrying *lpa-14* (13), restored the production of iturin A and surfactin. The average concentrations of iturin A and surfactin produced in a liquid medium by the bacterial strains in several experiments are shown in Table 1. The level of iturin A production by RΔ1(pC115) was almost the same as that of RB14, but that of surfactin was only about one-eighth that of RB14.

The pathogenic fungus *R. solani* K-1 was isolated from cockscomb at the Kanagawa Horticultural Experiment Station, Kanagawa, Japan (27). The anastomosis grouping of K-1 is AG-4. *R. solani* K-1 falls into the praticola type as a severe damping-off pathogen of many plants (36).

Media. L medium contains (per liter) 10 g of Polypepton (Nihon Pharmaceutical Co., Tokyo, Japan), 5 g of yeast extract, and 5 g of NaCl, adjusted to pH 7.0. Number 3 medium used for antibiotic production contains (per liter) 10 g of Polypepton, 10 g of glucose, 1 g of KH_2PO_4, and 0.5 g of $MgSO_4 \cdot 7H_2O$, adjusted to pH 6.8. To cultivate the strains or select the transformants from soil, chloramphenicol, erythromycin, or streptomycin was added at a concentration of 5, 5, or 100 μg/ml, respectively. Each medium was solidified with 2.0% agar, when necessary. PDA medium (Eiken Chemical Co., Tokyo, Japan) containing (per liter) 200 g of potato infusion, 20 g of glucose, and 15 g of agar, adjusted to pH 5.6, was used for stock culture of *R. solani* K-1. PDP medium containing (per liter) 200 g of potato infusion, 20 g of glucose, and 1 g of Polypepton (pH 5.6) was used for cultivation of *R. solani* K-1.

Soil treatments. The soil used in this study was a low humic andosol taken from a field at the Kanagawa Horticultural Experiment Station (27). The soil was sieved through an 8-mesh (about 2-mm-pore-size) screen and air dried. The soil and vermiculite were mixed in the ratio of 4:1 (wt/wt) and nutrient amended so that the final concentrations of N, P_2O_5, and K_2O were 70 mg, 240 mg, and 70 mg per 100 g of dry soil, respectively. The prepared soil was kept in plastic bags at room temperature. The soil was put into a sterilizable polypropylene bag and autoclaved for 60 min at 121°C four times at 12-h intervals. The main characteristics of the soil thus prepared were as follows: texture, low humic andosol; moisture content, 12.7%; maximum water-holding capacity, 137 g/100 g of dry

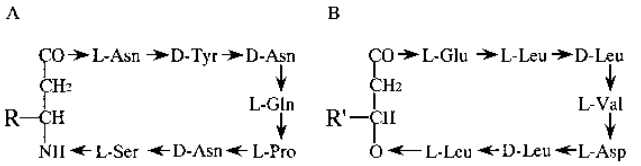

FIG. 1. Structures of iturin A (A) and surfactin (B). R denotes one of the following aliphatic chains: $CH_3(CH_2)_{10}$-, $CH_3CH_2CH(CH_3)(CH_2)_8$-, $(CH_3)_2CH(CH_2)_9$-, $CH_3(CH_2)_{12}$-, and $(CH_3)_2CH(CH_2)_{10}$-. R′ denotes $(CH_3)_2CH(CH_2)_9$-.

soil; pH 5.9; and bulk density, 0.522 g/cm^3. The measurement of these properties followed previously described methods (16). Sterilized soil (150 g) was put into a plastic pot with a diameter of about 90 mm, and the moisture was kept at 60% of the maximum water-holding capacity by the daily addition of sterilized water.

Inoculation of *R. solani* into soil. Fifty milliliters of sterile PDP medium in 200-ml Erlenmeyer flasks was inoculated with 5-mm plugs taken from a PDA petri dish culture of *R. solani* K-1. The flasks were incubated without shaking in the dark at 30°C for 1 week. The mycelial mats, on the surface of the medium, were then homogenized (4,000 rpm, 2 min) in sterile water and inoculated into the soil at the ratio of one-eighth piece of the mat to one pot 5 days before planting the germinated tomato seeds. After inoculation, the pots were incubated at 30°C and the moisture was kept at 60% of the maximum water-holding capacity.

Application of RB14-C and its derivative strains to soil. *B. subtilis* RB14-C was incubated for 16 h in L medium with streptomycin at 30°C in a shaker, and then 1 ml of the culture broth was inoculated into 100 ml of number 3 medium with streptomycin in an Erlenmeyer flask. The flask was shaken for 5 days at 30°C. Twenty milliliters of culture broth of RB14-C was mixed with 150 g of soil in a pot. For the treatment consisting only of cell suspension, culture broth was centrifuged (8,000 × *g*, 10 min, 4°C) and the sedimented cells were washed in 0.85% NaCl (pH 7.0) and then centrifuged again under the same conditions. The washed cells were suspended in sterile distilled water, and 20 ml of the cell suspension was mixed with 150 g of soil. For the treatment consisting of centrifuged culture broth, 20 ml of supernatant, after the centrifugation described above, was mixed with 150 g of soil. The three inoculations into soil were carried out simultaneously 3 days before planting the germinated tomato seeds. For each treatment, two to six pots were prepared and experiments were repeated at least three times.

For comparing the suppressive effects of cell suspensions of RB14-C, RΔ1, and RΔ1(pC115), each strain was cultivated as follows: 3 ml of each culture broth, after 16 h at 30°C in L medium with appropriate antibiotics, was inoculated into 300 ml of fresh L medium with the antibiotics and cultivated for 24 h. The cells were collected by centrifugation (8,000 × *g*, 10 min, 4°C), washed in 0.85% NaCl solution, and then centrifuged again. Then, 20 ml of each cell suspension in 0.85% NaCl solution was inoculated into the 150 g of soil. These inoculations were also carried out 3 days before planting the germinated tomato seeds.

Cell recovery from soil and counting of viable cell number. The soils were sampled both before planting and at 14 days after planting. In the latter sampling, the roots and surrounding soil were sampled together. Three grams of soil was suspended in 8 ml of 0.85% NaCl solution (pH 7.0) in a 50-ml Erlenmeyer flask and then shaken for 15 min at 140 strokes per min at room temperature. The suspension was serially diluted in 0.85% NaCl solution and plated onto L agar plates containing the proper antibiotics. The plates were incubated at 37°C, viable cells were counted after 12 h, and the cell number was expressed as the total cell number. To determine the number of spores, 1 ml of the suspension was heated for 15 min at 80°C, serially diluted, and spread onto L agar plates. All data are expressed as log CFU per gram of dry soil.

TABLE 1. Bacterial strains and plasmid

Strain or plasmid	Phenotype or plasmid marker[a]	Production (μg/ml)[b]		Reference
		Iturin A	Surfactin	
B. subtilis				
RB14		140	335	11
RB14-C	Sm^r	127	308	This work
RΔ1	Em^r	0	0	10
RΔ1(pC115)	Em^r Cm^r	152	41	13
Plasmid pC115	Cm^r, 4.2 kb, *lpa-14*$^+$			13

[a] Sm^r, streptomycin resistance; Em^r, erythromycin resistance.
[b] Each strain was cultivated in number 3 medium at 30°C for 5 days, and concentrations of iturin A and surfactin were determined by HPLC as described in Materials and Methods.

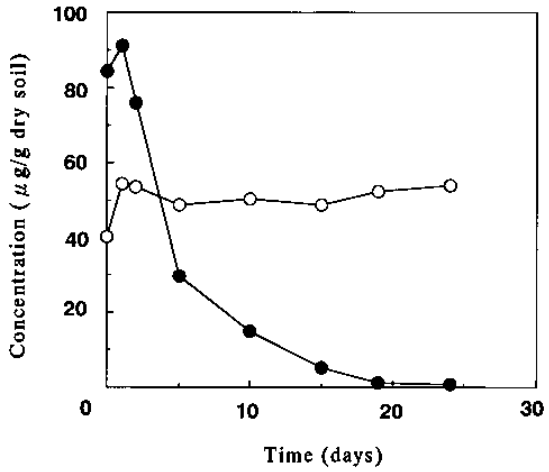

FIG. 2. Stability of iturin A (●) or surfactin (○) in soil. The centrifuged culture broth of RB14-C was mixed with soil in a pot and incubated at 30°C. Sterilized water was supplied daily to maintain soil water content at 60% of the maximum water-holding capacity. On each day, 3 g of soil was sampled and suspended in 21 ml of a mixture of acetonitrile–3.8 mM trifluoroacetic acid (4:1 [vol/vol]) to extract iturin A and surfactin. Each concentration was determined by HPLC as described in Materials and Methods.

Quantitative analysis of iturin A and surfactin recovered from soil. Three grams of soil was suspended in 21 ml of a mixture of acetonitrile–3.8 mM trifluoroacetic acid (4:1 [vol/vol]) in a 50-ml Erlenmeyer flask and then shaken for 1 h (140 strokes per min, room temperature). The soil in the suspension was then removed with filter paper (Toyo Roshi Co., Ltd., Tokyo, Japan), and the filtrate was evaporated. The precipitate was extracted with 2 ml of pure methanol for 2 h. After the extracted solution was centrifuged at 10,000 × *g* for 2 min, the supernatant was filtered through a 0.2-μm-pore-size polytetrafluoroethylene membrane (JP020; Advantec, Ltd., Tokyo, Japan) and injected into an HPLC column (octyldecyl silanolate-2, 4.6 mm [diameter] by 250 mm; GL Sciences, Tokyo, Japan). The system was operated at a flow rate of 1.0 ml/min with acetonitrile–10 mM ammonium acetate (3:4 [vol/vol]) for measurement of iturin A. The elution was monitored at 205 nm. Surfactin was analyzed with the same HPLC column used for identification of iturin A. The system was operated at a flow rate of 1.5 ml/min and monitored at 205 nm with the solvent acetonitrile–3.8 mM trifluoracetic acid (80:20 [vol/vol]) (13). The concentration of iturin A was determined with a calibration curve made with each purified component, and the total amount of five homologs of iturin A, as shown in Fig. 1, was used as the concentration of iturin A. The concentration of surfactin was determined with authentic surfactin purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Lower limits for detection of iturin A and surfactin by HPLC were about 0.30 μg/ml. Therefore, when the concentrations of both antibiotics were lower than the limit of detection, we judged them to be not detectable. It had been confirmed beforehand that no peak for iturin A or surfactin was observed in nontreated soil, and about 70 to 90% of iturin A and surfactin could be recovered from the soil by this method.

Plant growth. Seeds of tomato (Ponderosa) were surface disinfected for 1 min with 70% ethanol, rinsed five times with sterile distilled water, and then surface disinfected again for 5 min with 0.5% sodium hypochlorite. After at least 10 rinses with sterile distilled water, the seeds were germinated on a 2% agar plate at 30°C for 2 days. Each pot was sown with nine germinated seeds and placed in a growth chamber at 30°C with 80% relative humidity under 16 h of light (about 12,000 lux). At least two pots were prepared with each treatment. The moisture content was kept at 60% of the maximum soil water-holding capacity. After 2 weeks, the percentage of diseased seedlings per pot was determined. Furthermore, the shoots were clipped off at the soil surface level and the lengths and dry weights of the shoots were measured.

Stability of iturin A and surfactin in soil. To observe the stability of iturin A and surfactin in soil without plants, 20 ml of the centrifuged culture broth of RB14-C, which was prepared as described above, was mixed with 150 g of soil in a pot and incubated in a growth chamber as with the plant test described above. Iturin A and surfactin were recovered from two samples taken periodically from one pot, and the concentration of each was determined. The deviation of the concentrations for the duplicate samples was less than 5%, and the average concentrations are shown in Fig. 2.

Determination of plasmid stability. The stability of pC115 in RΔ1(pC115) in soil was determined as follows: 100 colonies which appeared on the L agar

TABLE 2. Effects of different treatments of *B. subtilis* RB14-C cultures on the suppression of damping-off of tomato plants caused by *R. solani* K-1 14 days after planting[a]

Treatment		Shoot length (mm)	Shoot weight (mg [dry wt]/pot)	Diseased plants (%)
R. solani	RB14-C			
+		13.1[b]	77[b]	85.2[b]
+	Culture broth	77.4[d]	502[c]	16.7[c]
+	Cell suspension	66.9[c]	574[c]	27.8[c]
+	Centrifuged culture broth	82.4[d]	651[c]	11.1[d]
−		83.1[d]	626[c]	0[e]

[a] For each treatment, each datum is an average of results from two to six pots (which contain nine seeds per pot) from experiments repeated three times. Means in any column with different letters are significantly different ($P = 0.05$) according to Fisher's protected least significant difference analysis.

TABLE 3. Concentrations of iturin A and surfactin recovered from treated soils 0 and 14 days after treatment

Treatment with RB14-C	Concentration of indicated antibiotic (μg/g of dry soil)[a] at:			
	0 days		14 days	
	Iturin A	Surfactin	Iturin A	Surfactin
Culture broth	19.4	18.1	5.3	18.1
Cell suspension	0.58	4.73	1.78	0.89
Centrifuged culture broth	19.5	18.0	1.24	10.7
None	ND[b]	ND	ND	ND

[a] Each value is an average of results from two pots in one experiment.
[b] ND, not detected. No peaks for iturin A or surfactin were observed by HPLC.

medium with erythromycin and were judged to be RΔ1(pC115) by their surface appearance were randomly selected and transferred by replica plating onto L agar medium with and without chloramphenicol. That a fraction of bacteria expressed chloramphenicol resistance (Cm^r) and therefore carried pC115 among the 100 colonies was considered the indicator of pC115 stability. The existence of plasmid pC115 in the Cm^r colonies was confirmed by extraction of the plasmid from 10 randomly chosen colonies.

Statistical analysis. Each plant test was repeated at least three times, and each mean of data was analyzed by Fisher's analysis of variance. The means ± standard deviations of the population densities were calculated with logarithmically transformed values.

RESULTS

Suppressive effect of culture broth of *B. subtilis* RB14-C. The suppressive effect of the cells and culture broth of *B. subtilis* RB14-C on the damping-off of tomato seedlings caused by *R. solani* is shown in Table 2. In the pots which were not infested with *R. solani*, all tomato seedlings grew normally and no disease appeared. However, in the pots infested only with *R. solani*, the percentage of diseased plants was 85.2% and the tomato shoot dry weights and shoot lengths were decreased markedly. With the RB14-C culture broth treatment, the percentage of diseased plants decreased to 16.7% and both the shoot dry weights and the shoot lengths significantly increased. The differences in shoot length, shoot weight, and percent diseased plants after five treatments were statistically significant at $P = 0.05$.

The cells of RB14-C were recovered from soil in the pots. On day 0, the total cell number was 7.76×10^7 CFU/g of dry soil and the spore number was 1.25×10^8 CFU/g of dry soil. On day 14, the final day of the test, the total cell number was 4.56×10^7 CFU/g of dry soil and the spore number was 4.78×10^7 CFU/g of dry soil. A slight decrease in cell number was observed, and most of the viable cells of RB14-C existed as spores.

The amounts of iturin A and surfactin recovered from soils on days 0 and 14 are shown in Table 3. Each value is an average of results from two pots in one experiment. Significant amounts of iturin A and surfactin were recovered on day 14. The decrease in the amount of iturin A was larger than that of surfactin in spite of the existence of similar amounts of the two substances on day 0. As presented in Fig. 2, the level of recovery of surfactin was stable over the duration of the test period (24 days) whereas that of iturin A gradually decreased.

Suppressive effects of cell suspension and centrifuged culture broth of *B. subtilis* RB14-C. Cell suspensions or the centrifuged culture broth of *B. subtilis* RB14-C was inoculated into soil, and the suppressive effects were compared (Table 2). When the centrifuged culture broth was inoculated, the suppressive effect was the same as that of the nonfractionated culture broth. The percentage of diseased plants was 11.1%, and the level of plant growth was restored to that of the control. The concentration of iturin A decreased more rapidly than that of surfactin in both broth treatments (Table 3).

When the cell suspension of RB14-C was inoculated into soil, a suppressive effect was observed (Table 2). On day 0, the total cell number was 3.91×10^7 CFU/g of dry soil and the spore number was 3.34×10^7 CFU/g of dry soil. On day 14, the total cell number was 2.73×10^7 CFU/g of dry soil and the spore number was 3.06×10^7 CFU/g of dry soil. The slight decrease in cell number and the existence of most of the viable cells of RB14-C as spores were similar to the results of the culture broth treatment.

Comparison of suppressive effects by RB14-C, RΔ1, and RΔ1(pC115). To ascertain that the suppressive effect of RB14-C was related to iturin A and surfactin, cell suspensions of RB14-C, RΔ1, and RΔ1(pC115) were inoculated into soil and the suppressive effects were compared (Table 4). Washed cell suspensions were used in this experiment to minimize the effect of carryover of iturin A and surfactin from the culture broth and to clarify the effect of the cells themselves. In soil infested with *R. solani*, the percentage of diseased plants was 85.2% and the tomato shoot dry weights and the shoot lengths were significantly less than those of the healthy control. When RB14-C was inoculated into soil, the percentage of diseased plants was reduced to 24.1% and the shoot dry weights and shoot lengths were significantly greater than those of the nontreated pots infested with *R. solani*. In soil inoculated with RΔ1, the percentage of diseased plants was 64.8%, which was significantly higher than that of pots treated with RB14-C, and the shoot dry weights and the shoot lengths also were significantly reduced.

TABLE 4. Effects of strains of RB14-C and its derivatives on the suppression of damping-off of tomato plants caused by *R. solani* K-1 14 days after planting[a]

Treatment		Shoot length (mm)	Shoot weight (mg [dry wt]/pot)	Diseased plants (%)
R. solani	Bacterium			
+		17.2[b]	129[b]	85.2[b]
+	RB14-C	70.7[c]	560[c]	24.1[c]
+	RΔ1	37.2[b]	312[b]	64.8[b]
+	RΔ1(pC115)	69.2[c]	536[c]	29.4[c]
−		95.9[c]	704[c]	0[c]

[a] For each treatment, each datum is an average of results from experiments repeated three times with two pots (which contain nine seeds per pot). Means in any column with different letters are significantly different ($P = 0.05$) according to Fisher's protected least significant difference analysis.

TABLE 5. Populations of *B. subtilis* RB14-C, RΔ1, and RΔ1(pC115) in soils

Strain	Cell number (log CFU/g of dry soil)[a] at:			
	0 days		14 days	
	Total	Spore	Total	Spore
RB14-C	8.12 ± 0.23	8.08 ± 0.21	8.10 ± 0.09	7.94 ± 0.14
RΔ1	7.81 ± 0.07	7.84 ± 0.09	7.73 ± 0.26	7.79 ± 0.18
RΔ1(pC115)	7.83 ± 0.12	7.69 ± 0.18	7.67 ± 0.20	7.65 ± 0.27

[a] Each value is the mean (± the standard deviation) from three independent experiments with two replicates (pots) per experiment.

When RΔ1(pC115) was inoculated, the disease was considerably suppressed, with 29.6% diseased plants. Both shoot dry weight and shoot length were restored to values comparable to those found after the RB14-C treatment, showing that the suppressive effect of RΔ1(pC115) was equivalent to that of RB14-C. The values for shoot length and shoot weight and the percent diseased plants after three treatments also were statistically significant at $P = 0.05$.

The populations of these strains at the beginning and the end of the plant test are shown in Table 5. The initial cell numbers showed only a slight difference, and most of the viable cells existed as spores both on day 0 and on day 14. Spores of all three strains were stable in soil, with similar persistence in the rhizosphere.

The stability of plasmid pC115 in RΔ1(pC115) added to soil also was determined. On day 0, its stability was 82.7% in total cells and 83.3% in spores, while on day 14, the plasmid was present in 76.3% of total cells and in 79.7% of spores. This indicates that pC115 was stably maintained in RΔ1 in soil.

Iturin A and surfactin were recovered from the soil as shown in Table 6. Significant amounts were recovered from soil both on day 0 and on day 14 when the cell suspension of either RB14-C or RΔ1(pC115) was introduced. However, neither iturin A nor surfactin was detected by HPLC when the cell suspension of RΔ1 was inoculated into soil.

DISCUSSION

The culture broth, cell suspension, and centrifuged culture broth of *B. subtilis* RB14-C clearly showed a suppressive effect on the incidence of damping-off of tomato caused by *R. solani* in vivo (Table 2). When plants were treated with culture broth of RB14-C and without *R. solani*, the growth of the tomato plants was the same as that of the control, indicating that RB14-C imparts no growth-enhancing activity to the plant.

TABLE 6. Concentrations of iturin A and surfactin recovered from soil when cell suspensions of RB14-C, R1Δ, or RΔ1(pC115) were introduced into soil

Strain	Concentration of indicated antibiotic (μg/g of dry soil)[a] at:			
	0 days		14 days	
	Iturin A	Surfactin	Iturin A	Surfactin
RB14-C	0.73 ± 0.31	3.75 ± 0.16	0.49 ± 0.21	4.36 ± 0.15
R1Δ	ND[b]	ND	ND	ND
RΔ1(pC115)	0.61 ± 0.4	1.73 ± 0.1	0.40 ± 0.2	2.02 ± 1.0

[a] Each value is the mean (± the standard deviation) from three independent experiments with two pots for each treatment.

[b] ND, not detected. The determined concentrations were lower than the limit of detection by HPLC.

When culture broth or centrifuged culture supernatant was added to soil, the initial concentration of iturin A and surfactin was about 20 μg/g of dry soil. Both substances were recovered from soil after 14 days. However, the concentration of iturin A was reduced to 1 to 5 μg/g of dry soil and the concentration of surfactin was maintained at more than 10 μg/g of dry soil. This suggests a difference in persistence in soil. Therefore, the levels of recovery of iturin A and surfactin from soil were determined after addition of centrifuged culture supernatant to soil in the absence of *R. solani* (Fig. 2). Surfactin was again more persistent than iturin A in soil. We do not know if the reduced amount of iturin A was caused by leaching from soil during watering, by possible biodegradation by airborne microorganisms or residual *B. subtilis* cells introduced during the experiment, or by irreversible binding to soil materials or humic acids. Since concentrations of iturin A and surfactin in centrifuged culture supernatant were stably maintained for more than 1 month, such instability was probably related to the soil environment. In spite of the lower concentration of iturin A over time, our results showed that iturin A is effective for suppression of the disease. The persistence of surfactin in soil may contribute to suppressiveness by its synergistic effect on the antifungal activity of iturin A (11, 22).

The contributions of iturin A and surfactin to the suppressiveness of RB14-C in soil were confirmed with a mutant, RΔ1, which is deficient in production of iturin A and surfactin. RΔ1 exhibited weak suppressiveness of damping-off as shown in Table 4. RΔ1(pC115), a transformant of RΔ1 containing the plasmid pC115, which carries the *lpa-14* gene, was restored for production of iturin A and partially restored for production of surfactin (Table 1). The complemented mutant exhibited a level of suppressive activity almost equal to that of the parental strain RB14-C. From these experiments, we infer that the major mechanism of suppression by RB14-C is production of the antibiotics iturin A and surfactin. Although the amount of surfactin detected in soil inoculated with RΔ1(pC115) was slightly lower than that of soil inoculated with RB14-C (Table 6), the suppressive ability of the transformant was almost the same as that of RB14-C. This also suggests that iturin A plays a role more important than that of surfactin in suppression of *R. solani* damping-off in soil.

The percentage of diseased plants in soil treated with RΔ1 (64.8%) was significantly lower than that of the control treatment of soil with *R. solani* (85.2%), and the shoot lengths and shoot weights were significantly higher than those of the control (Table 4). As neither iturin A nor surfactin was detected on days 0 and 14 (Table 6), other determinants, such as unknown antibiotics, lytic enzymes, or siderophores (15), may be involved in the activity of RΔ1; this may be clarified by more complete characterization of the mutant. Similar phenomena were reported for *Pseudomonas fluorescens* strains which produce siderophore (21) or the antibiotic phenazine-1-carboxylate (32) in that they retained suppressiveness even when mutants lacking the production of these substances were prepared.

The cells of RB14-C recovered from soil existed mostly as spores, and the population of the bacterium in the soil was on the order of 10^7 CFU/g of dry soil after 14 days. We previously showed that when vegetative cells of *B. subtilis* were inoculated into soil, sporulation occurred quickly, mainly because of the oligotrophic condition of the soil. The population was stabilized at the concentration of the spore number, and almost no change was observed for 30 days (34). In this experiment, the initial populations of RB14-C and its derivatives were also well maintained in soil and most of the cells existed as spores (Table 5).

Although *B. subtilis* is not considered a representative rhizosphere species, like *Pseudomonas* spp., the rhizosphere population density, as well as the persistence of the bacterium in soil, is an important factor in suppression of damping-off caused by *R. solani*. As the difference between the total cell number and spore number, which corresponds to the vegetative cell number, was small and the inoculation level of 10^7 to 10^8 CFU/g of soil in these experiments was rather large (Table 5), it is difficult to estimate a threshold population density of RB14-C to show significant suppression of plant pathogens, which was reported as 10^5 CFU/g of root in the case of *Pseudomonas* spp. (29). However, the facts that most of the viable cells recovered from soil were in the form of spores and that spores have no ability to produce antibiotics suggest that the spores germinate in the rhizosphere and that eventually the antibiotics are produced, although the exact number of vegetative cells was not determined.

B. subtilis organisms are stable in soil as spores, and this is advantageous for the use of this bacterium as a biocontrol agent mainly because of the spore's stability and ease of handling. This study showed that treatment with the culture broth, cell suspension, or centrifuged culture broth will be effective as a biological control.

ACKNOWLEDGMENTS

We thank S. Fujiwara and N. Kita, Kanagawa Prefectural Agricultural Research Institute, for supplying the soil and for valuable suggestions for the experiment. We appreciate Tsuyoshi Hata's cooperation in this study.

REFERENCES

1. **Acea, M. J., C. R. Moore, and M. Alexander.** 1988. Survival and growth of bacteria introduced into soil. Soil Biol. Biochem. **20:**509–515.
2. **Baker, C. J., J. R. Stavely, and N. Mock.** 1985. Biocontrol of bean rust by *Bacillus subtilis* under field conditions. Plant Dis. **69:**770–772.
3. **Carrol, H., Y. Moënne-Loccoz, D. N. Dowling, and F. O'Gara.** 1995. Mutational disruption of the biosynthesis genes coding for the antifungal metabolite 2,4-diacetylphloroglucinol does not influence the ecological fitness of *Pseudomonas fluorescens* F113 in the rhizosphere of sugarbeets. Appl. Environ. Microbiol. **61:**3002–3007.
4. **Chang, I., and T. Kommedahl.** 1968. Biological control of seedling blight of corn by coating kernels with antagonistic microorganisms. Phytopathology **58:**1395–1401.
5. **Cook, R. J.** 1990. Twenty-five years of progress towards biological control, p. 1–14. *In* D. Hornby (ed.), Biological control of soil-borne plant pathogens. C.A.B. International, Wallingford, United Kingdom.
6. **de Freitas, J. R., and J. J. Germida.** 1991. *Pseudomonas cepacia* and *Pseudomonas putida* as winter wheat inoculants for biocontrol of *Rhizoctonia solani*. Can. J. Microbiol. **37:**780–784.
7. **de la Cruz, A. R., A. R. Poplawsky, and M. V. Wiese.** 1992. Biological suppression of potato ring rot by fluorescent pseudomonads. Appl. Environ. Microbiol. **58:**1986–1991.
8. **Geels, F. P., and B. Schippers.** 1983. Reduction of yield depressions in high frequency potato cropping soil after seed tuber treatments with antagonistic fluorescent *Pseudomonas* spp. Phytopathol. Z. **108:**207–214.
9. **Gutterson, N.** 1990. Microbial fungicides: recent approaches to elucidating mechanisms. Crit. Rev. Biotechnol. **10:**69–91.
10. **Hiraoka, H., T. Ano, and M. Shoda.** 1992. Molecular cloning of a gene responsible for the biosynthesis of the lipopeptide antibiotics iturin and surfactin. J. Ferment. Bioeng. **74:**323–326.
11. **Hiraoka, H., O. Asaka, T. Ano, and M. Shoda.** 1992. Characteristics of *Bacillus subtilis* RB14, coproducer of peptide antibiotics iturin A and surfactin. J. Gen. Appl. Microbiol. **38:**635–640.
12. **Homma, Y., and T. Suzui.** 1989. Role of antibiotic production in suppression of radish damping-off by seed bacterization with *Pseudomonas cepacia*. Ann. Phytopathol. Soc. Jpn. **55:**643–652.
13. **Huang, C. C., T. Ano, and M. Shoda.** 1993. Nucleotide sequence and characteristics of the gene, *lpa-14*, responsible for biosynthesis of the lipopeptide antibiotics iturin A and surfactin from *Bacillus subtilis* RB14. J. Ferment. Bioeng. **76:**445–450.
14. **Hwang, S. F., and P. Chakravarty.** 1992. Potential for the integrated control of *Rhizoctonia* root-rot of *Pisum sativum* using *Bacillus subtilis* and a fungicide. J. Plant Dis. Prot. **99:**626–636.
15. **Ito, T., and J. B. Neilands.** 1958. Products of "low iron fermentation" with *Bacillus subtilis*: isolation, characterization and synthesis of 2,3-dihydroxybenzoylglycine. J. Am. Chem. Soc. **80:**4645–4647.
16. **Katayama, A., M. Hirai, M. Shoda, and H. Kubota.** 1986. Factors affecting the stabilization period of sewage sludge in soil with reference to the gel chromatographic pattern. Soil Sci. Plant Nutr. **32:**383–395.
17. **Kraus, J., and J. E. Loper.** 1995. Characterization of a genomic region required for production of the antibiotic pyoluteorin by the biological control agent *Pseudomonas fluorescens* Pf-5. Appl. Environ. Microbiol. **61:**849–854.
18. **Laville, J., C. Voisard, C. Keel, M. Maurhofer, G. Defago, and D. Haas.** 1992. Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proc. Natl. Acad. Sci. USA **89:**1562–1566.
19. **Lemanceau, P., P. A. H. M. Bakker, W. J. de Kogel, C. Alabouvette, and B. Schippers.** 1992. Effect of pseudobactin 358 production by *Pseudomonas putida* WCS358 on suppression of fusarium wilt of carnations by nonpathogenic *Fusarium oxysporum* Fo47. Appl. Environ. Microbiol. **58:**2978–2982.
20. **Liang, L. N., J. L. Sinclair, L. M. Mallory, and M. Alexander.** 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. Appl. Environ. Microbiol. **44:**708–714.
21. **Loper, J. E.** 1988. Role of fluorescent siderophore production in biological control of *Pythium ultimum* by a *Pseudomonas fluorescens* strain. Phytopathology **78:**166–172.
22. **Maget-Dana, R., L. Thimon, F. Peypoux, and M. Ptak.** 1992. Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. Biochimie **74:**1047–1051.
23. **Maurhofer, M., C. Keel, U. Schnider, C. Voisard, D. Haas, and G. Defago.** 1992. Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. Phytopathology **82:**190–195.
24. **Mazzola, M., R. J. Cook, L. S. Thomashow, D. M. Weller, and L. S. Pierson III.** 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl. Environ. Microbiol. **58:**2616–2624.
25. **Merriman, P. R., R. D. Price, J. F. Kollmorgen, T. Piggott, and E. H. Ridge.** 1974. Effect of seed inoculation with *Bacillus subtilis* and *Streptomyces griseus* on the growth of cereals and carrots. Aust. J. Agric. Res. **25:**219–226.
26. **Olsen, C. M., and K. F. Baker.** 1968. Selective heat treatment of soil, and its effect on the inhibition of *Rhizoctonia solani* by *Bacillus subtilis*. Phytopathology **58:**79–87.
27. **Phae, C. G., M. Shoda, N. Kita, M. Nakano, and K. Ushiyama.** 1992. Biological control of crown and root rot and bacterial wilt of tomato by *Bacillus subtilis* NB22. Ann. Phytopathol. Soc. Jpn. **58:**329–339.
28. **Pusey, P. L., and C. L. Wilson.** 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. Plant Dis. **68:**753–756.
29. **Raaijmakers, J. M., M. Leeman, M. M. P. van Oorschot, I. van der Sluis, B. Schippers, and P. A. H. M. Bakker.** 1995. Dose-response relationships in biological control of Fusarium wilt of radish by *Pseudomonas* spp. Phytopathology **85:**1075–1081.
30. **Siala, A., and T. R. Gray.** 1974. Growth of *Bacillus subtilis* and spore germination in soil observed by a fluorescent-antibody technique. J. Gen. Microbiol. **81:**191–198.
31. **Silo-suh, L. A., B. J. Lethbridge, S. J. Raffel, H. He, J. Clardy, and J. Handelsman.** 1994. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. Appl. Environ. Microbiol. **60:**2023–2030.
32. **Thomashow, L. S., and D. M. Weller.** 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. J. Bacteriol. **170:**3499–3508.
33. **Thomashow, L. S., D. M. Weller, R. F. Bonsall, and L. S. Pierson III.** 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Appl. Environ. Microbiol. **56:**908–912.
34. **Tokuda, Y., T. Ano, and M. Shoda.** 1995. Survival of *Bacillus subtilis* NB22 and its transformant in soil. Appl. Soil Ecol. **2:**85–94.
35. **Turner, J. T., and P. A. Backman.** 1991. Factors relating to peanut yield increases after seed treatment with *Bacillus subtilis*. Plant Dis. **75:**347–353.
36. **Ushiyama, K., J. Nishimura, and N. Aono.** 1987. Damping-off of feather cockscomb (*Celosia argentea* L. var. *cristata* O. Kuntze) caused by *Rhizoctonia solani* Kuhn. Bull. Kanagawa Hortic. Exp. Stn. **34:**33–37.
37. **Utkhede, R. S.** 1984. Antagonism of isolates of *Bacillus subtilis* to *Phytophthora cactorum*. Can. J. Bot. **62:**1032–1035.
38. **van Elsas, J. D., A. F. Dijkstra, J. M. Govaert, and J. A. van Veen.** 1986. Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. FEMS Microbiol. Ecol. **38:**151–160.
39. **Vincent, M. N., L. A. Harrison, J. M. Brackin, P. A. Kovacevich, P. Mukerji, D. M. Weller, and E. A. Pierson.** 1991. Genetic analysis of the antifungal activity of a soilborne *Pseudomonas aureofaciens* strain. Appl. Environ. Microbiol. **57:**2928–2934.
40. **Weller, D. M.** 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. **26:**379–407.