Short Communication

CHARACTERIZATION OF *BACILLUS SUBTILIS* YB8, COPRODUCER OF LIPOPEPTIDES SURFACTIN AND PLIPASTATIN B1

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Biological control of soilborne plant pathogens by antagonistic microorganisms offers a practical supplement or alternative to many disease management strategies that depend heavily on chemical pesticides. We have isolated *Bacillus subtilis* YB8 from a bark compost as a suppressive bacterium against phytopathogenic fungi (11). The number of this bacterium in the compost was 1.3×10^6 (cfu/g dry solid) and this was one to two orders higher than those of other bacteria from different suppressive composts (11). The higher stability observed in the natural environment will be a suitable characteristic when this bacterium is used as a biological pesticide.

When YB8 formed a colony on an L agar plate on which 20 μ l of tributyrin was spread, a clear halo appeared around the colony. This surface-active substance was purified and determined to be surfactin (1, 7) as follows: The acid precipitate of the culture supernatant was collected and extracted with methanol. The extract was separated in the HPLC system (6) described previously, and a clear peak of surfactin was detected in comparison with the authentic sample purchased from Wako Pure Chemical Ind., Ltd., Osaka, Japan. The IR spectrum, amino acid analysis and molecular weight determination by FAB-MS of the peak sample also revealed that the substance was surfactin (data not shown). The structure of surfactin as a potent surface-active reagent (3) and antibiotic (2) is shown as follows:

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Although purified sample of surfactin could not make any inhibitory zone on a plate which contained a phytopathogenic fungus, *Fusarium oxysporum* f. sp. *lycopersici* race J1 SUF119, which causes the crown and root rot of tomato (11), the colony of YB8 made a clear zone inhibiting the growth of the fungus. This suggests that YB8 produces antifungal substance(s) extracellularly.

The antifungal substance was isolated from the culture supernatant of *B. subtilis* YB8 cultivated in ACS medium (15) for 5 days at 30°C. This medium contained sucrose 100 g, citric acid 11.7 g, Na₂SO₄ 4 g, yeast extract 5 g, (NH₄)₂-HPO₄ 4.2 g, KCl 0.76 g, MgCl₂·6H₂O 0.420 g, ZnCl₂ 0.0104 g, FeCl₃·6H₂O 0.0245 g, MnCl₂·4H₂O 0.0181 g in 1 l, and was adjusted to pH 6.9 with NH₄OH. Purification of the antifungal substance was carried out as follows by modifying the methods developed for purification of fengycin (15) or plipastatins (14). The supernatant (4 l) was precipitated at pH 2. The precipitate was collected by centrifugation and was extracted with 95% ethanol. The extract was mixed with 10% charcoal (w/v), stirred for 60 min and filtered through a suction funnel. The charcoal was stirred with 1 l of chloroform-methanol-H₂O (65:25:4) for 60 min followed by the suction treatment, and the eluate was concentrated and dissolved in 4 volumes of propanol (v/v). This solution was charged on a propanol-filled column of silica gel (Silica gel 60; Merck, Darmstadt, Germany), and was followed by successive elution with one equal column-volume of propanol, 2 equal column-volumes of 90% propanol and 2.5 equal column-volumes of 80% propanol. These fractions were bioassayed on a agar plate containing the fungus. The active eluate from the 80% propanol elution was further purified by a reversed-phase preparative HPLC using a PREP-ODS (GL Sciences, Tokyo, Japan; 2 cmφ × 25 cm, flow rate 10 ml/min, detection at 205 nm) with a 3:4 mixture of acetonitrile and 5 mM ammonium acetate. The main peak with the highest antifungal activity was passed through a Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column with 80% methanol and 3 mg of the purified antifungal substance was obtained.

The IR spectrum of the purified antifungal substance molded in KBr showed the characteristic absorption of peptide bonds (1,654 and 1,543 cm⁻¹) and lactone carbonyl absorption (1,753 cm⁻¹). The hydrolysate of the substance was analyzed using an amino acid analyzer. The amino acid composition was determined to be comprised of Val (1), Thr (1), Glu (3), Pro (1), Ile (1), Tyr (2) and Orn (1) residues. The protonated molecular ion peak of this compound was displayed at *m/z* 1491.9 by FAB-MS analysis. From these results, the antifungal substance was considered to coincide with plipastatin B1, which had been reported as a new inhibitor of phospholipase A₂ (14), and its structure is as follows:

B. subtilis YB8 was the first bacterium which was the coproducer of two

lipopeptide antibiotics, surfactin and plipastatin B1. The antifungal effect of plipastatin B1 was already reported as follows: This substance inhibited the growth of *Alternaria mali* (apple leaf spot pathogen), *Botrytis cinerea* (gray mold pathogen) and *Pyricularia oryzae* (rice blast pathogen), and considerable high protective value against *Helminthosporium* leaf spot was also observed in the pot test (16).

The time course of the production of surfactin and plipastatin B1 is shown in Fig. 1. Fresh overnight culture in ACS medium was inoculated into eight of the 40 ml each of ACS medium per Erlenmeyer flask (200 ml). The cultivation was operated at 30°C, and each flask was taken out periodically and the analyses of surfactin and plipastatin B1 were conducted as follows: The precipitate of the acidified supernatant was collected by centrifugation and was extracted with methanol. The filtered extracted solution was analyzed by reversed-phase HPLC. The HPLC condition for surfactin quantification was followed as described previously (6). Plipastatin B1 was detected and quantified by reversed-phase HPLC system newly developed in this work: The filtrate described above was injected into an HPLC column (ODS-2, 4.6 mm ϕ $\times$ 250 mm, GL Sciences). The system was operated at a flow rate of 1.0 ml/min with acetonitrile-ammonium acetate (10 mM) 1:1 (v/v). The elution pattern was monitored at 205 nm. The concentration of plipastatin B1 was determined by a calibration curve made by the purified sample.

Surfactin was already accumulated in the culture medium at the beginning of the stationary growth phase (16 h) while little plipastatin B1 was produced at this time. As these two substances were extracted simultaneously from the same precipitate and the same methanol extract was used for the HPLC analysis, the experimental deviation or error for this quantitative comparison was very small. Both substances reached a maximum at 2 to 3 days of incubation and the same level

Fig. 1. Time course of production of surfactin and iturin A by *Bacillus subtilis* YB8 in ACS medium.

Growth was monitored at 660 nm and is indicated by closed triangles. The amount of surfactin and plipastatin B1 production is shown by closed rectangles and circles, respectively.

was kept at least 5 days. In the time course of the plipastatin production by *Bacillus cereus* BMG302-fF67, the maximum peak of plipastatin production was observed at one day and the gradual decrement with a pH change to alkaline was reported (14).

We have reported several *B. subtilis* as a potential biological control agent (12, 13). Among them, *B. subtilis* RB14 produces iturin A (10) and surfactin and a synergistic suppressive effect of the two lipopeptides against the growth of plant pathogens was described (5). Here, *B. subtilis* YB8, will be another candidate of microbial pesticides by producing surfactin and plipastatin which have a different suppressive spectrum to plant pathogens, and there might be a synergistic suppressive effect of the two lipopeptides against the growth of a plant pathogen.

As no biochemical or genetic analysis of an plipastatin producer is reported, molecular cloning for the production of this compound from *B. subtilis* YB8 is important for the understanding of the lipopeptide antibiotics. Usually antibiotic biosynthesis is carried out through physiologically, biochemically and genetically complex mechanisms, thus the establishment of a host-vector system in an original antibiotic producer is important for cloning antibiotic production gene(s).

Although transformation of *B. subtilis* YB8 with plasmid DNA by a competent cell method was unsuccessful, an electroporation method (9) developed for an iturin A producer was employed for this coproducer. About 10^5 transformants were obtained from 1 μ g of plasmid pC194 (4) or pUB110 (8). This transformation efficiency is high enough for shotgun cloning of the gene(s), and isolation of a negative producer by mutagenesis is now in progress.

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