Isolation and Characterization of a Spore Germination Inhibitor from *Streptomyces* sp. CB-1-1, a Phytopathogen Causing Root Tumor of Melon

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The germination rate and activation conditions of spores were examined for four strains of *Streptomyces* sp., a phytopathogen causing root tumor of melon. An inhibitor was isolated from the agar-cultured material of strain CB-1-1 and then characterized. The inhibitor selectively acted on spore germination and did not affect hyphal growth, and inhibition was abolished by washing the spores in water. The inhibitor was produced by an agar culture, and most of the inhibitor existed in the spores. The IC50 value for the inhibitor was approximately 0.25 μg/ml.

Key words: *Streptomyces*; spore germination; isolation; germination inhibitor; root tumor of melon

Root tumor of melon was first found in 1982 in Kumamoto prefecture, Japan.1) This disease is characterized by the roots developing many galls, and growth-retardation and wilting in the above-ground parts of plants. After discovering the disease in melons, root tumors were also found in other cucurbitaceous plants, including cucumber, pumpkin and bottle gourd, and the disease spread to other prefectures on the islands of Kyushu, Shikoku and Honshu, Japan.2) The pathogen of this root tumor is a *Streptomyces* species, which, on the basis of morphological and physiological characteristics, is thought to be unrelated to previously described plant-pathogenic *Streptomyces* species.3)

Yoshida *et al.* have reported that about 10–20% of spores of the pathogen causing root tumor of melon, *Streptomyces* sp. B-9-1, were ungerminated spores, that a heat-shock treatment at 40 °C for 20 min increased the colony-forming rate to 110–115% compared with an untreated control, and that heat shock was more effective when the spores were placed in a 0.025% sodium dodecyl sulfate (SDS) solution.4) The stimulative effects of these treatments suggested that the spores contained a factor that inhibited their own germination, and that this inhibitor was released or decomposed by the treatments just described. We therefore attempted to identify the spore germination self-inhibitor.

*Streptomyces* spores are very small (about 1 μm in diameter), making it difficult to collect a large amount of spores. Therefore, in a previous study, we used liquid-cultured *Streptomyces* sp. B-9-1 material in our search for the spore germination inhibitor, and succeeded in isolating an inhibitor which was subsequently determined to be anthranilic acid.5) Anthranilic acid was contained in the spores and selectively inhibited spore germination. However, the inhibitory activity of anthranilic acid was weak, and the anthranilic acid content in the spores was very low. We thus concluded that, while anthranilic acid would act as a self-inhibitor of spore germination, its contribution would be small.

We therefore reexamined the germination rate and the activation conditions for spore germination with four strains of *Streptomyces* sp. that cause root tumor of melon. We report here the isolation and characterization of a germination inhibitor from *Streptomyces* sp. CB-1-1.

Materials and Methods

Microorganisms and media. Pathogenic *Streptomyces* sp. A-1, B-9-1, CB-1-1 and KM-2-1 had been previously isolated from diseased melons by Yoshida *et al.*3) An inorganic salt-starch (ISS) agar medium (Daigo Actino

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Medium No. 4, Wako Pure Chemical Industries) or yeast-starch (YS) broth medium were used for culturing the pathogen.

Collection of spores. The four strains of Streptomyces were cultured at 28 °C for 14 d on an ISS agar medium (10 ml) in Petri dishes (9 cm i.d.). Sterilized distilled water (10 ml per Petri dish) was poured into each dish, and the spores and aerial mycelia were scraped off the agar surface with an inoculating needle. The suspension was passed twice through a No. 2 filter paper to remove the mycelial fragments.

Measurement of the spore germination rate. A spore suspension (6.5 × 10⁶ spores/ml, 600 μl) was mixed with the YS broth medium (600 μl), and 200 μl of the mixture was dispensed into each of 6 wells of a 96-well microplate, after which the plate was incubated at 28 °C.

The spore germination rate was determined for each aliquot of the incubated suspension by counting the numbers of germinated and ungerminated spores with a phase contrast microscope (×600) in the same way as previously reported. To measure the germination inhibitory activity of a given fraction, a MeOH solution of the fraction (60 μl) was mixed with a spore suspension (540 μl) and the YS broth medium (600 μl), and the mixture was incubated in the wells of a microplate at 28 °C for 48 h. The absorbance at 595 nm was measured with a microplate reader (Thermo Multiscan JX), and the inhibition rate of germination was calculated as previously reported.

Heat shock treatment of the spores. A spore suspension (6.5 × 10⁷ spores/ml, 60 μl) was mixed with a 5 mM phosphate buffer (pH 7.0, 540 μl) that was maintained at 40 °C for 20 min with occasional stirring. The treated spore suspension was mixed with the YS broth medium (600 μl), and the mixture was incubated in the wells of a microplate under the same conditions as those already described. The germination rate of the spores was determined for each aliquot of the incubated suspension with a phase contrast microscope.

SDS treatment of the spores. SDS was dissolved in the 5 mM phosphate buffer, and the pH value of the solution was adjusted to 7.0 with 1 N HCl before it was autoclaved. The SDS solution (540 μl), spore suspension (6.5 × 10⁷ spores/ml, 60 μl) and YS broth medium (600 μl) were mixed, and the mixture was treated in the same way as that used for the heat shock treatment experiments. In the results section, the concentrations of the SDS solution given represent the final values in the wells.

Washing the spores with the SDS solution or water. To examine the effect of washing with the SDS solution on spore germination, a spore suspension (6.5 × 10⁷ spores/ml, 60 μl) was mixed with the SDS solution (540 μl), and incubated at 28 °C for 20 min. The suspension was centrifuged at 1,000 × g for 5 min, and the resulting supernatant was removed. The precipitated spores were resuspended in sterilized distilled water (1,200 μl). This process was repeated twice more. The precipitated spores were then resuspended in a 1/2 concentration of the YS broth medium (1,200 μl), and the mixture was treated in the same way as that used for the heat shock treatment experiment. Washing in water was conducted in the same way as washing with the SDS solution, sterilized distilled water being used instead of the SDS solution.

Effect of the inhibitor on hyphal growth. Spore suspensions were incubated in a similar manner to that used for measuring the germination inhibitory activity. An EtOAc-soluble acidic extract equivalent to 10 cultured Petri dishes was added to the bioassay suspension in wells at the beginning or 24 h after the start of incubation. The absorbance at 595 nm was measured every 12 h.

Reversibility of the effect of the inhibitor on germination. A spore suspension (6.5 × 10⁶ spores/ml, 600 μl) and the YS broth medium (600 μl) were mixed with an EtOAc-soluble acidic extract equivalent to 60 cultured Petri dishes, and the mixture incubated at 28 °C for 3 h. The suspension was centrifuged at 1,000 × g for 5 min, and the supernatant was discarded. The precipitated spores were resuspended in a 1/2 concentration of the YS broth medium (600 μl), and the suspension was incubated in the wells of a microplate at 28 °C for 45 h, before the absorbance at 595 nm was measured with a microplate reader.

Spore suspensions for the control experiments were treated in a similar manner, but without the EtOAc-soluble acidic extract or without replacement of the medium.

Detection of the inhibitor in spores and cultured materials. Spores were collected by the glass-bead-based method (originally described by DeJong and McCoy) from Streptomyces sp. CB-1-1 cultured on an ISS agar medium in the same way as that used for preparing spores for the bioassay. The EtOAc-soluble acidic extract of agar-cultured material was obtained by inoculating Streptomyces sp. CB-1-1 on to 10 Petri dishes (9 cm i.d.) each containing 10 ml of an ISS agar medium, and incubating the dishes at 28 °C for 14 d. The cultured material was macerated with acetone, and the acetone extract evaporated to an aqueous residue which was then extracted with EtOAc at pH 3.

The liquid cultured material of Streptomyces sp. CB-1-1 was obtained from a seed culture grown by transferring a loopful of Streptomyces sp. CB-1-1 from a slant culture to a 500-ml shaking flask containing 100 ml of the YS broth medium. The inoculated flask

Germination Inhibitor of Phytopathogenic Streptomyces sp.
was incubated on a reciprocal shaker (150 strokes/min) at 28 °C for 3 d, after which 0.5-ml portions of the broth were transferred to shaking flasks containing the same medium. The flasks were cultured for 6 d under the same conditions. The culture broth was filtered, and the filtrate was treated with EtOAc at pH 3 to obtain the EtOAc-soluble acidic extract.

Extracts of the spores or agar-cultured material equivalent to one agar-cultured Petri dish (10 ml of medium), or extract equivalent to 10 ml of the liquid-cultured material was dissolved in MeOH and analyzed by HPLC under the following conditions: column, Develosil ODS UG-5 (4.6 × 250 mm); solvent, 10% aq. CH₃OH; flow rate, 1 ml/min; detection, UV 300 nm.

Spectroscopic methods. Mass spectrometry was performed with a JEOL JMS-T100LC AccuTOF LC-MS instrument equipped with an electrospray ion source (ESI) under the following HPLC conditions: column, Develosil ODS UG-5 (2.0 × 250 mm); solvent, 10% aq. CH₃OH; flow rate, 0.1 ml/min. The UV spectrum was measured with a Shimadzu LC-10 HPLC system fitted with a photodiode array detector under the same HPLC conditions as those used for LC-MS.

Results and Discussion

Reexamination of the spore germination process

The spore germination rate was determined on the basis of phase contrast microscopic observations made every 12 h. Symbols: ○, Streptomyces sp. strain A-1; ●, B-9-1; △, CB-1-1; ■, KM-2-1. Error bars are ±SD.

Fig. 1. Spore Germination Rates Over Time for the Four Strains of Streptomyces sp. That Cause Root Tumor of Melon.

The spore germination rate was determined on the basis of phase contrast microscopic observations made every 12 h. Symbols: ○, Streptomyces sp. strain A-1; ●, B-9-1; △, CB-1-1; ■, KM-2-1. Error bars are ±SD.

The onset of germination and the final germination rate of the B-9-1 spores were similar to those reported by Yoshida et al., but the germination process progressed more slowly than they observed. This can be attributed to a difference in the germination conditions: Yoshida et al. observed the germination of spores on an agar medium, whereas the spores were incubated in a liquid medium in the present experiment.

Given the results of this experiment, we decided to observe the spore germination rate 48 h after incubation had begun.

Improvement of spore germination

Yoshida et al. have determined the conditions that were most effective for improving spore germination by counting the number of colonies formed. We reinvestigated the conditions that were effective for activating spore germination by directly observing spore germination with a phase contrast microscope, and compared the results for the four strains of Streptomyces.

The heat shock treatment at 40 °C for 20 min increased the germination rate slightly for all four strains, although the effect was not significant (Fig. 2, bar B). Yoshida et al. have observed that the same heat shock treatment increased the colony-forming ratio to 110–115% of that of the untreated control. These findings suggest that the heat shock treatment may have affected the growth of the germinated spores but not the germination of the spores.

Unexpectedly, incubating the spores in a 0.025% SDS solution for 48 h caused a marked decrease in the germination rates of the strain A-1, B-9-1 and KM-2-1 spores, and had a weak inhibitory effect on the strain CB-1-1 spores (Fig. 2, bar C). In a previous study, Yoshida et al. treated spores with an SDS solution for 20 min, spread the suspension on an agar plate using the dilution plate technique, and incubated the plate for 5 or 7 d until determining the germination rate by counting the colonies formed. Diluting and plating the spore suspension markedly lowered the SDS concentration, which produced different results from those obtained in the present study.

Treatment with the SDS solution for 20 min and subsequent washing with water abolished the suppression of germination, and all spores of all four strains germinated (Fig. 2, bar D). Washing in water alone also yielded complete germination (Fig. 2, bar E). Vitalization of the spores by washing in water alone was unexpected, because the surfaces of the spores and aerial mycelia are believed to be covered with hydrophobic materials.

These results suggest that the spores of strain CB-1-1 contained the largest amount of germination inhibitor among the four strains, and that the inhibitor leached into the water during washing. We thus used Strepto-
myces sp. CB-1-1 to isolate the spore germination inhibitor of the pathogen causing root tumor of melon.

Search for the inhibitor in water used to wash the strain CB-1-1 spores
The water used to wash the spores obtained from 100 cultured Petri dishes of strain CB-1-1 was treated as shown in Fig. 3. The inhibitory activity was concentrated in the EtOAc-soluble acidic fraction. Most activity was recovered from the R_f 0–0.14 fraction by preparative silica gel TLC and coincided with a peak at t_R 18.0 min from ODS-HPLC (Fig. 3). The yield was approximately 0.01 mg.

The sole inhibitor was obtained from the EtOAc-soluble acidic fraction which contained the bulk of the activity with a high collection rate with respect to activity, although the yield was very low. We subsequently examined whether the inhibitor contained in the EtOAc-soluble acidic extract acted as a self-inhibitor of spore germination before a large-scale culture and isolation of the inhibitor.

Dose response of the germination inhibitor
The inhibition of spore germination was measured by using various concentrations (5-fold serial dilutions) of the EtOAc-soluble acidic extract. The inhibition rate became lower as the concentration of the extract was decreased, the detection limit being a dose equivalent to the extract obtained from 1/62.5 of a cultured Petri dish (Fig. 4). The IC_{50} value was estimated to be a dose equivalent to the extract obtained from approximately 1/2 of a cultured Petri dish, corresponding to 0.25 mg/ml. This value indicates the new inhibitor to be 280 times stronger than anthranilic acid (70 μg/ml). This strong inhibitory activity demonstrates a true self-germination inhibitor.

The inhibitory activity of the isolated germination inhibitor was also examined on the basis of the number of agar-cultured Petri dishes (Fig. 4). The line plot overlapped with that of the EtOAc-soluble acidic extract, which means that the isolated inhibitor was the principal agent in the EtOAc-soluble acidic fraction.

Effect of the inhibitor on hyphal growth
One of the requirements for a self-inhibitor of spore germination is for it to have specific activity with respect to spores, but be completely innocuous with respect to mycelia. The effect of the inhibitor on hyphal growth
was examined by administering the inhibitor-containing EtOAc-soluble acidic fraction to a spore suspension incubated for 24 h, in which most spores had germinated, and the germination process was monitored by measuring the absorbance at 595 nm every 12 h.

The addition of the EtOAc-soluble acidic extract at the beginning of incubation did not cause any increase in absorbance (Fig. 5). When the EtOAc-soluble acidic extract was added 24 h after incubation had begun, the increase in absorbance was similar to that of the control (Fig. 5). These results show that the inhibitor in the EtOAc-soluble acidic extract acted specifically on spore germination.

Reversibility of the germination inhibitory activity

Another requirement for a self-inhibitor of spore germination is reversibility of the inhibitory action. We examined whether the inhibitor in the EtOAc-soluble acidic fraction met this requirement by incubating spores in the presence of the inhibitor for 3 h, washing the spores and then subjecting them to further incubation.

The presence of the EtOAc-soluble acidic extract throughout the incubation period entirely inhibited spore germination, because the absorbance at 595 nm did not change from the value at the start of incubation (Fig. 6, black bar). However, with subsequent washing after 3 h of exposure to the inhibitor, there was an increase in absorbance of the spore suspension (Fig. 6, gray bar). Thus, the inhibitor in the EtOAc-soluble acidic extract reversibly inhibited spore germination, and did not inactivate the spores.

Detection of the inhibitor in spores, and in the agar- and liquid-cultured material

Determining whether the inhibitor was present in only the spores, or also in the agar-cultured or liquid-cultured material is an important issue for mass production of the inhibitor and for determining the authenticity of the inhibitor. The spore suspensions used for these experiments may possibly have included the contents of mycelia or metabolites effused into the medium. We thus collected spores by rolling glass beads around on the surface of agar cultures, thereby attaching the spores to the glass beads. The inhibitor content was compared among the spores, and agar-cultured and liquid-cultured materials.

HPLC analyses of the extracts showed that the extract from the agar-cultured material had the highest inhibitor content, but that most of the inhibitor existed in the agar-cultured medium.
spores (Fig. 7). No inhibitor was detected in the liquid-cultured material.

These results show the inhibitor to be a true self-germination inhibitor. No substance interfering with isolation of the inhibitor was present in the EtOAc-soluble acidic extract of the agar-cultured material, leading us to decide to prepare the inhibitor from the agar-cultured material.

Isolation of the inhibitor from plate-cultured material
The inhibitor was isolated from agar-cultured material from 3,500 Petri dishes as shown in Fig. 8 by using an ODS-HPLC analysis of the inhibitor peak. Preparative HPLC was performed with the improved solvent system of 10% aq. CH$_3$CN plus 0.1% trifluoroacetic acid, and we succeeded in isolating 0.25 mg of the active agent.

Physico-chemical properties of the inhibitor
ESI-MS analyses of the inhibitor revealed a peak at $m/z$ 182 ($M + H$)$^+$ or $m/z$ 180 ($M − H$)$^-$, and HR-MS in the negative ion mode afforded the accurate mass for the ($M − H$)$^-$ ion as 180.0407 (calcd. 180.0409), which corresponds to the molecular formula C$_7$H$_7$N$_3$O$_3$. The inhibitor had a characteristic UV spectrum with absorption maxima at 263, 318 and 328 nm (Fig. 9). $^{13}$C-NMR and $^1$H-NMR spectra for the inhibitor would have been helpful for a structural analysis; however, these spectra could not be obtained because of the limited amount of the sample available. The inhibitor probably had a heterocyclic structure with poor connectivity between protons. A search of the SciFinder database for the molecular formula and narrowing down the candidates from the features of the UV spectrum did not afford a possible structure. Germidicin, a germination inhibitor of *Streptomyces viridochromogenes*, or other known...
fungal inhibitors did not suit the spectral characteristics of this inhibitor.

We are now performing a microscale structural analysis combining computational chemistry and synthetic/degradative reactions to further characterize this inhibitor.

References


