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Pyoverdin$_{2112}$ of *Pseudomonas fluorescens* 2112 Inhibits *Phytophthora capsici*, a Red-Pepper Blight-Causing Fungus

LEE, EUN-TAG[1], SI-KYU LIM[1], DOO-HYUN NAM[1,2], YONG-HO KHANG[1,3], AND SANG-DAL KIM[1,3]*

[1]*Institute of Biotechnology,* [2]*Department of Pharmacy, and* [3]*Department of Applied Microbiology, Yeungnam University, Gyongsan 712-749, Korea*

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Abstract A bacterium, *Pseudomonas fluorescens* 2112, that is antagonistic against a red-pepper blight-causing fungus, *Phytophthora capsici*, was isolated from the local soil of Gyongju, Korea. This strain formed an orange-colored clear halo zone on chrome azurol S (CAS) blue agar, suggesting the production of a siderophore in addition to an antifungal antibiotic. The optimal culture conditions for siderophore production by *P. fluorescens* 2112 were 30-h cultivation at 25°C and pH 6.5 in King's B medium. The presence of 20 µg/ml of Fe^{3+} ion or EDDHA promoted the production of siderophore in King's B medium. The siderophore was purified from culture broth by CM-Sephadex C-25 and Sephadex G-25 column chromatographies. The UV spectra of the purified siderophore was the same as that of pyoverdins or pseudobactins. The molecular mass was 1,958 Da determined by FAB-mass spectrometer, and the amino acid composition analysis showed that the purified siderophore consisted of glycine/threonine/serine/glutamic acid/alanine/lysine with the molar ratio of 3:2:1:1:1:1. DL-Threo-β-hydroxyaspartic acid and N^{δ}-hydroxyornithine, two of the essential constituents of pyoverdin, were also found. The purified siderophore pyoverdin showed strong *in vitro* and *in vivo* antagonistic activities against phytophthora blight-causing *P. capsici*. Especially in an *in vivo* pot test, the siderophore protected red-pepper *Capsicum annum* L. very well from the attack of *P. capsici*. These results indicated that the purified siderophore of *P. fluorescens* 2112 played a critical role in the biocontrol of the red-pepper blight disease, equivalent to treatment by *P. fluorescens* 2112 cells.

Key words: Antagonistic bacterium, *Pseudomonas fluorescens* 2112, antifungal, siderophore, biocontrol, pyoverdin

There are three main biological means to control phytopathogenic fungi. They include the cell wall lysis of phytopathogenic fungi by the bacterial chitinases [10, 13, 17, 24], the direct inhibition of the life cycle of phytopathogenic fungi by antifungal antibiotics produced by bacteria [2, 11, 14-16], and the competitive antagonism of plant growth-promoting rhizobacteria (PGPR) by the produced siderophores (Fe^{3+} specific-binding material) [20].

Siderophore mediating the iron absorption in a rhizosphere is a ferric iron-chelating organic compound, which is water-soluble and has a low molecular mass, having yellow-green fluorescent chromophores connected with the peptide chain [4, 26, 28, 30]. In agreement with the chemical structure, siderophore compounds are divided into two groups; catechol amides and hydroxamate derivatives.

Iron is one of the essential factors for the spore germination and germ tube elongation of the phytopathogenic fungi. Nonlimitation of iron concentration in soil may cause permeation or infection of the phytopathogenic fungi, producing plant-rot. In this aspect, PGPR colonies producing extracellular siderophores can inhibit the growth of phytopathogenic fungi through competitive predominance over the ferric ions in the rhizosphere, thereby enhancing the growth of plants and the yield of agricultural crops.

These days, PGPR strains producing siderophores have been extensively explored for application to plant rhizospheres in order to control plant diseases and/or enhance plant growth [18, 19, 20, 25]. Actually, multifunctional PGPR strains having another biocontrol mechanism in combination with siderophore production would be a more effective biocontrol measure in agriculture.

Therefore, we earlier isolated and identified a new PGPR strain, *Pseudomonas fluorescens* 2112 [14], having multifunctional biocontrol ability, and were able to produce a siderophore as well as an antibiotic 2,4-diacetylphloroglucinol [15]. Herein, we report on the purification and characterization of the siderophore from this strain, and also *in vitro* and *in vivo* antagonistic activity of the purified siderophore against red-pepper blighting fungus, *Phytophthora capsici*.

*Corresponding author
Phone: 82-53-810-2395; Fax: 82-53-811-4319;
E-mail: sdkim@yumail.ac.kr

MATERIAL AND METHODS

Strains

Pseudomonas fluorescens 2112, a multifunctional antagonistic strain against red-pepper rotting fungi, *Phytophthora capsici*, that produces an antifungal antibiotic and a siderophore, was isolated from a local soil of Gyongju, Korea [14, 15]. This antagonistic strain was grown typically at 30°C for 30 h on King's B (KB) medium (pH 6.5). All the media used were deferrated by extraction with 3% (w/w) 8-hydroxyquinoline in a chloroform solution for 2 days, according to the procedure described earlier [29].

Phytopathogenic fungi, *Fusarium oxysporum* and *F. solani*, were grown at 28°C on potato-dextrose agar (PDA) plate. A spore suspension of the *Fusarium* species was obtained by sieving culture broth aseptically through 10 layers of sterile cheesecloth after being grown in potato dextrose broth (PDB) for 5 days, collected by centrifugation, and washed 5 times with sterile 0.1 M $MgSO_4$ solution. The zoospore of *P. capsici* was made by scraping hyphae with bent glass after growing on V8 juice agar for 5 days, followed by exposing to a fluorescent lamp at 28°C for 2 days, and finally collected in 5 ml of sterile water per plate.

Siderophore Activity

The product of siderophore in the culture broth was determined by a chrome azurol S (CAS) colorimetric assay [27]. Deferoxamine mesylate was employed as a standard in this assay. On the other hand, a CAS-shuttle assay [27] was also applied for fast detection of siderophore activity. The addition of 4 mM 5-sulfosalicylic acid to the CAS assay solution made the iron-dye complex more unstable at 37°C, thereby bringing rapid decolorization within 20 min. One unit of siderophore activity was defined as the amount to decrease 0.001 A_{630}/sec at the initial decolorization stage.

Siderophore Typing

In order to ascertain the type of siderophore, the Arnow phenolic acid assay [1] and Csaky hydroxylamine/hydroxamic acid assay [7] were performed. For the identification of catechol-phenolic type siderophore, 2,3-dihydroxybenzoic acid (2,3-DHBA) was used as a standard. For the confirmation and quantitative assay of hydroxamate type, hydroxylamine HCl was employed as a standard.

Purification of Ferric Siderophore

Ferric siderophore was purified as described previously [18, 22]. Bacterial cells were grown in King's B broth at 30°C at 180 rpm for 40 h, and 500 mg/l of $FeCl_3$ was added as a supplement. After removing cells by centrifugation, the ferrated reddish-brown culture broth was concentrated 50-fold by evaporation. The proteinous materials in the broth were precipitated by saturated sodium chloride, and then the supernatant was extracted twice with an equal volume of chloroform-phenol (1:1, v/v). After adding 3 volumes of diethyl ether to the organic extract, the ferric siderophore was re-extracted several times with deionized water until no more color was observed in the organic phase. The aqueous extract was washed 3 times with an equal volume of diethyl ether, and dried under low pressure.

The crude ferric siderophore dissolved in 0.05 M sodium acetate buffer (pH 6.5) was purified with a CM-Sephadex C-25 column (2.5×90 cm). The major reddish-brown fractions were concentrated, and again subjected to the same column chromatography to increase its purity. The collected fractions containing ferric siderophore were concentrated by lyophilization and stored at -20°C in the dark. The purified ferric siderophore was identified by thin-layer chromatography (TLC) on Silica gel 60 F_{254} plates (Merck GmBH, Germany), which were developed with ethanol/water (70:30, v/v) and visualized with iodine vapor or 0.2% ninhydrin solution.

Preparation of Iron-Free Siderophore

To obtain an iron-free siderophore, the purified ferric siderophore was mixed with 1.0 M ethylenediamine tetra acetic acid (EDTA) for 1 h [22], and the mixture was loaded onto a Sephadex G-25 gel filtration column (2.5×90 cm). The iron-free siderophore was eluted with deionized water, and the major yellow-green fractions were collected, monitored by absorbance at 367 nm, and then concentrated by lyophilization. The purified iron-free siderophore was confirmed by TLC assay using Silica gel 60 F_{254} plates, which were developed with chloroform/acetic acid/ethanol (90:5:5, v/v/v) and detected by staining with iodine vapor or 0.2% ninhydrin solution after exposing to 0.1 M ferric chloride solution or UV irradiation.

Characterization of the Purified Siderophore

The absorption spectra of the purified ferric or the iron-free siderophore were measured by a Hitachi U-2000 spectrophotometer. The molecular weight of siderophore was determined by a fast atom bombardment (FAB) mass spectrometer (JM S-AX505H, Jeol). The amino acid composition of the siderophore was determined by a Biochrom 20 amino acid analyser (Pharmacia LKB, Sweden). For the identification of β-threo-hydroxyaspartic acid or N^{δ}-hydroxyornithine in the siderophore, DL-threo-β-hydroxyaspartic acid treated with 6 N HCl or rodotoruric acid ([cyclo N^{δ}-acetyl-N^{δ}-hydroxyl-L-ornitine]$_2$) treated with 47% HI was employed.

In Vitro and *In Vivo* Antifungal Test

An *in vitro* antifungal test of the purified siderophore was carried out by the paper disk method. The 6 mm paper disks soaked with 120 μl of sterilized siderophore (50 mg/

ml) were placed on the center of a PDA plate, where zoospores or spores of phytopathogenic fungi, *P. capsici*, *F. solani*, and *F. oxysporum*, were scraped with a bent glass rod. Antifungal activity was determined by the size of the clear zone around the paper disks after 3 to 5 days of incubation at 28°C.

For *in vivo* antifungal assay of the purified siderophore against *P. capsici*, red-pepper plant *Capsicum annum* was grown in a pot having a soil/compost/sand mixture (2:1:1, w/w/w). Plants were grown in the constant temperature-humidity chamber at 28°C with 60% humidity. Zoospores of *P. capsici* were inoculated into a V8 juice plate (5.3×10^2 spores/ml), and the purified siderophore solution was spread onto the same pot. Outbreaks of red-pepper rotting on the plant occurred everyday.

RESULTS AND DISCUSSION

Siderophore Production by *P. fluorescens* 2112

In order to select an antagonistic strain having a strong siderophore activity, 4 isolates of *Pseudomonas* sp. strains forming orange-colored halo zones were tested for their siderophore producing capabilities by CAS-shuttle assay. Among these, *P. fluorescens* 2112 showed the highest siderophore activity, having as much as 12.1 units, even though all the end-points for complete decolorization of the ferric-CAS complex were shorter than 20 min (Table 1). The siderophore specific activity of *P. fluorescens* 2112 was calculated as 27.5 units/mg of cell protein, which was much higher than previously reported [18]. This implies that *P. fluorescens* 2112 is the most promising antagonistic bacterium among siderophore-producing isolates.

Most of the siderophores produced by *P. fluorescens* 2112 were identified as the hydroxamate type of siderophore.

Table 1. Siderophore activities of strains using CAS-shuttle assay.

Strain	Total siderophore activity[a] (U)	Total siderophore specific activity[b] (U)	Endpoint[c] (min)
1011	9.7	10.6	6-8
1402	0.6	0.4	4-6
1507	0.4	0.2	5-7
1912	11.6	18.2	15-20
2112	12.1	27.5	11-20

All strains were grown at 28°C for 40 h in iron-deficient King's B medium. The CAS assay of the culture supernatant was conducted at 37°C. All values are means of three replicates.

[a]Siderophore activity was calculated from the initial rate of decolorization, where one unit was defined as the amount causing a decrease of 0.001 of A_{630} per sec.

[b]Siderophore specific activity was claculated as the siderophore activity per mg of cell protein.

[c]End-point of the reaction was determined as the time at which no further decolorization of the CAS complex was observed.

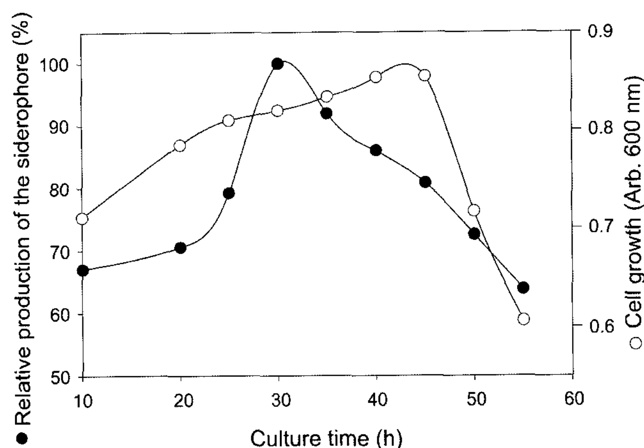


Fig. 1. Time course for the production of siderophore by *P. fluorescens* 2112.

P. fluorescens 2112 was grown in King's B broth at 30°C and the siderophore activity was assayed for hydroxamates by the Csaky method. ○: Cell growth; ●: Siderophore production.

Optimal Conditions for Siderophore Production

For maximal siderophore production, the optimal culture conditions for *P. fluorescens* 2112 were examined. The fermentative profile of this strain in KB broth at 30°C showed that maximal siderophore production was achieved after 30-h cultivation when midexponential phase was reached, even though the cell growth continued until 45-h cultivation (Fig. 1).

The optimal culture temperature for the production of siderophore was also investigated from 20°C to 45°C. The highest siderophore production was observed at 25°C, whereas the cell growth was best at 30°C (Fig. 2A). This result showed that this strain prefers lower temperatures for the production of siderophore.

When the amount of the siderophore produced was checked by varying the initial pH of the KB broth, it was observed that the highest production of siderophore was achieved at pH 6.5, as shown in Fig. 2B. Generally, the soil pH is known to be the most important factor for microbial acquisition of ferric ion in rhizosphere. Iron exists in soluble ion form in acidic soil (conductive soil), but insoluble ferric-oxyhydroxide polymer form in alkaline soil (suppressive soil) [21]. Thus, most soil microorganisms can easily utilize ferric ion in acidic conditions, resulting in the acceleration of plant rot by phytopathogenic fungi. From this point of view, *P. fluorescens* 2112 was proved to be a suitable strain for the biocontrol of phytopathogenic fungi, because more siderophore production and better cell growth were obtained in weak acidic or neutral pH conditions.

The effect of ferric ion concentration in culture broth on siderophore production by *P. fluorescens* 2112 was also examined. The induction of siderophore biosynthesis by ferric ions was observed, and the siderophore production gradually increased with the supplementation of ferric ions

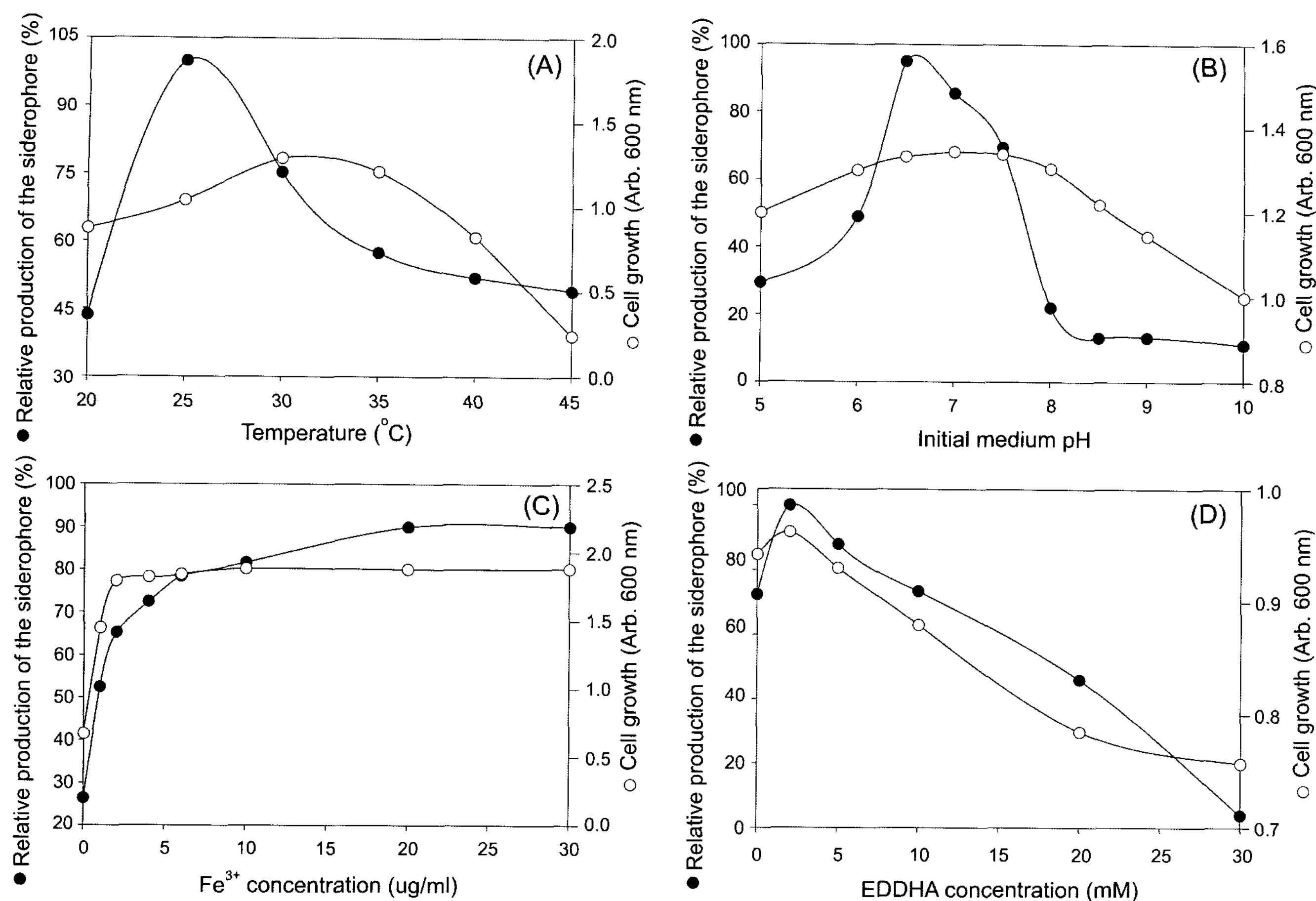


Fig. 2. Effect of culture temperature, initial pH, iron concentration, and EDDHA, a synthetic iron chelator, on the production of siderophore by *P. fluorescens* 2112.

A. *P. fluorescens* 2112 was grown in King's B broth at each temperature for 30 h. B. *P. fluorescens* 2112 was inoculated in King's B broth adjusted to an initial pH between 5 and 10, and cultured at 30°C for 30 h. C. *P. fluorescens* 2112 was inoculated in King's B broth, supplemented with different concentrations of ferric chloride, and cultured at 30°C for 30 h. D. *P. fluorescens* 2112 was inoculated in King's B broth containing different concentrations of ethylenediamine di-(-0-hydroxyphenylacetic acid) (EDDHA), and cultured at 30°C for 30 h. The siderophore activity was assayed for hydroxamates by the Csaky method. ○: Cell growth; ●: Siderophore production.

up to 20 μg/ml of broth, as seen in Fig. 2C. On the other hand, the addition of higher than 2 mM EDDHA, a synthetic iron chelator, decreased siderophore production and cell growth (Fig. 2D). However, this strain had still half the amount of siderophore productivity even in the presence of 20 mM EDDHA. This suggests that *P. fluorescens* 2112 could be used as an effective biocontrol measure against phytopathogenic fungi in the rhizosphere. Furthermore, siderophore production was strongly promoted by ferric ions and maintained even in the high concentration of iron chelator.

Purification and Characterization of Siderophore

Because iron-free siderophore was reported to be more unstable than ferric siderophore forms [18], the isolation of ferric siderophore from antagonistic *P. fluorescens* 2112 was attempted. Even though *P. fluorescens* 2112 produced yellow-green and fluorescent iron-free siderophore in deferrated KB broth, the addition of ferric chloride (100 mg/l) into culture supernatant turned it to a dark reddish-brown color of ferric siderophore. After extraction from chloroform-phenol (1:1, v/w), the ferric siderophore was purified twice by CM-Sephadex C-25 cation exchange column chromatography, to give rise to a single reddish-brown peak. The iron-free siderophore of yellow-green color was prepared by a Sephadex G-25 gel filtration column. The purity of the siderophore was measured, as seen in Fig. 3.

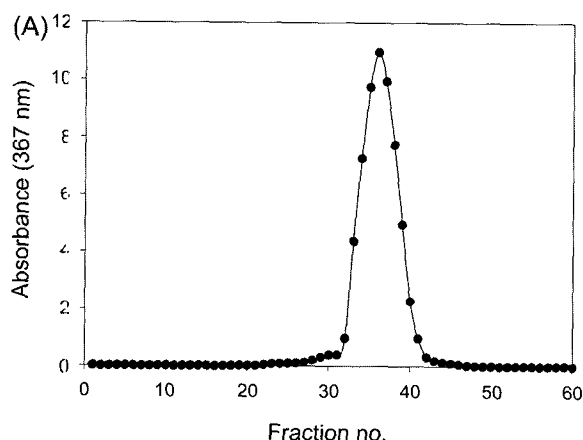
The molecular weight of the iron-free siderophore was determined to be 1,958 Da by FAB mass spectrometer (Fig. 7). The UV spectra for ferric and iron-free siderophore showed maximal absorption at 400 nm and 370 nm, respectively (Fig. 8), which are similar to the result for pseudobactin and pyoverdin [4, 22]. The amino acid

Table 2. The amino acid composition of the pyoverdin$_{2112}$ produced from *P. fluorescens* 2112.

Amino acid	Mole %	Component ratio (%)
Glycine	31.7	3
Threonine	21.2	2
Serine	12.3	1
Glutamic acid	14.0	1
Alanine	11.1	1
Lysine	9.8	1

The pyoverdin sample was hydrolyzed with 6 N HCl for 24 h at 110°C *in vacuo*.

composition of the iron-free siderophore revealed glycine-threonine-serine-glutamic acid-alanine-lysine with the molar ratio of 3:2:1:1:1:1. Teinze *et al.* [28] reported that the presence of DL-threo-β-hydroxyaspartic acid was confirmed as being eluted in the front of the aspartic acid peak and N^{δ}-hydroxyornithine was found in 47% hydroiodic acid hydrolysate, which is one of the essential constituents of pyoverdin [18]. Thus, it could be concluded that the purified siderophore from *P. fluorescens* 2112 belongs to the pyoverdin class among the known *Pseudomonas* siderophores, which include pyochelin, pseudobactin, ferribactin, and pyoverdin [5, 6, 8, 9].



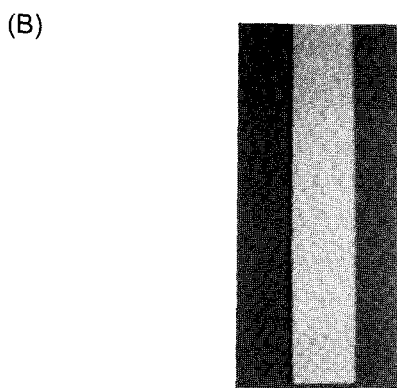


Fig. 3. Chromatograms of the purified siderophore.
(A) Elution profile of siderophore produced by *P. fluorescens* 2112 through Sephadex G-25 gel filtration column. The fraction were collected and absorbance monitored at 367 nm. (B) Thin layer chromatogram of the purified siderophore. The purified ferric siderophore was run on Silica gel $60F_{254}$ plates, developed with chloroform/acetic acid/ethanol (90:5:5, v/v/v), and visualized with iodine vapor.

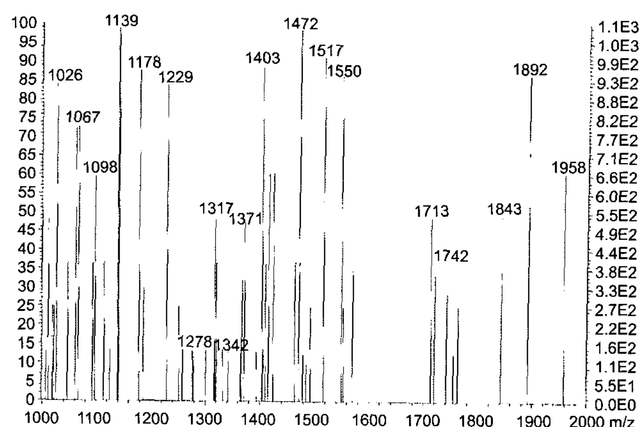


Fig. 4. Fab-mass of pyoverdin$_{2112}$ produced from *P. fluorescens* 2112.
The molecular weight of siderophore was determined using a fast atom bombardment (FAM) mass spectrometer (JMS-AX505H, Jeol) using a mixture of glycerol and thioglycerol (1:1) as the FAM matrix.

In Vitro and *In Vivo* Test of Siderophore

The antifungal activity of this purified siderophore was evaluated in an *in vitro* antifungal test and an *in vivo* blight-suppressive test. In the *in vitro* test, the purified siderophore (pyoverdin$_{2112}$) from *P. fluorescens* 2112 showed good antifungal activity against the phytopathogenic fungi,

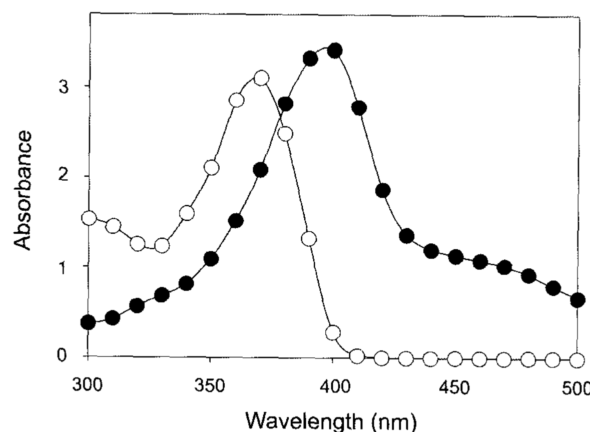


Fig. 5. Absorption spectra of ferric and iron-free siderophore purified from the culture broth of *P. fluorescens* 2112.
The purified siderophores were dissolved in an aqueous solution, and its absorption spectra were recorded from 300 to 500 nm. ●: ferric siderophore; ○: iron-free siderophore.

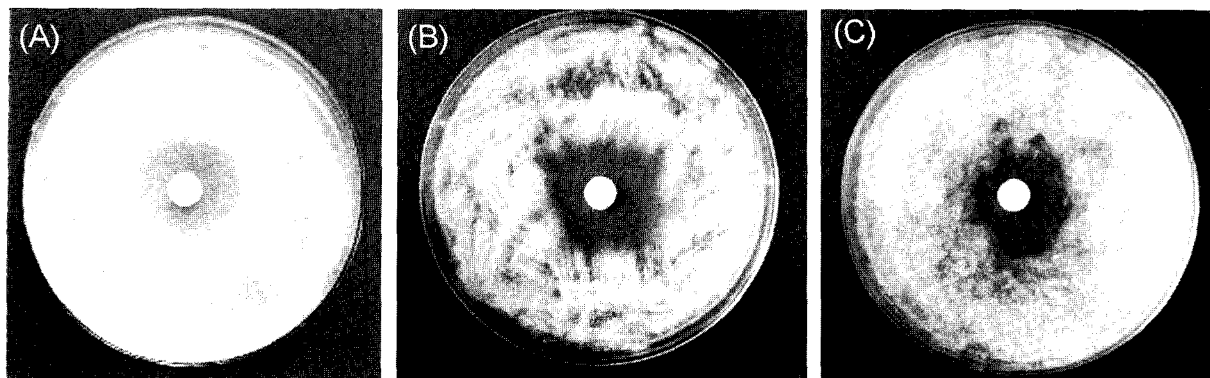


Fig. 6. *In vitro* antagonistic test of the purified siderophore from *P. fluorescens* 2112 on phytopathogenic fungi.
After 50 mg/ml of the purified siderophore was applied on the disc-paper, it was placed on the centers of potato dextrose agar plates, on which spores of *F. oxysporum* (A), spores of *F. solani* (B), or zoospores of *P. capsici* (C) were already scraped.

P. capsici, *F. solani*, and *F. oxysporum*, on a PDA plate, as shown in Fig. 6. These results are consistent with Lim's report [18], but slightly different from others [3, 11] in which this purified siderophore showed no antifungal activity during an *in vitro* test. In order to ascertain its applicability as a biocontrol measure against red-pepper rot in the rhizosphere, the purified siderophore (pyoverdin$_{2112}$) was directly spread on the red-pepper plant *C. annum* infected with phytopathogenic *P. capsici*. When more than 25 mg of siderophore was treated on red-pepper, *C. annum*, the incidence of red-pepper rot caused by *P. capsici* was significantly reduced (Fig. 7). In particular, higher than 70% protective effect was observed by treatment with 37.5 mg of siderophore. These observations led us to conclude that the siderophore of the isolated antagonistic bacterium *P. fluorescens* 2112 showed biocontrol activity against phytopathogenic fungi in the rhizosphere.

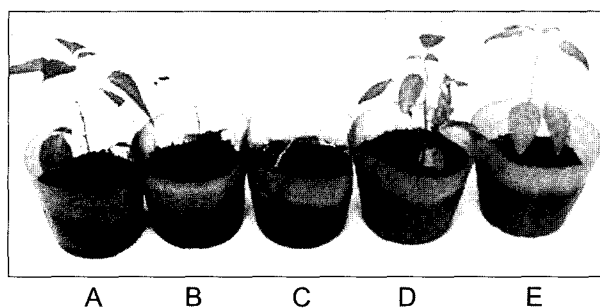


Fig. 7. *In vivo* antagonistic test of the purified siderophore from *P. fluorescens* 2112 on phytopathogenic fungi.
After zoospores of *P. capsici* (5.32×10^2 spores/ml) were inoculated at the fourth leaves of red-pepper *C. annum* grown in pots, plants were grown at 28°C under 70% humidity for 2 days, and the purified siderophore (25 mg/ml) was spread onto the same pot. (A) Red-pepper not infected by *P. capsici*; (B) red-pepper infected by *P. capsici*; (C) red-pepper infected by *P. capsici* and treated with 0.5 ml of purified siderophore; (D) red-pepper infected by *P. capsici* and treated with 1.0 ml of purified siderophore; (E) red-pepper infected by *P. capsici* and treated with 1.5 ml of purified siderophore.

Acknowledgment

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