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A Plant Growth-Promoting *Pseudomonas fluorescens* GL20: Mechanism for Disease Suppression, Outer Membrane Receptors for Ferric Siderophore, and Genetic Improvement for Increased Biocontrol Efficacy

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Abstract *Pseudomonas fluorescens* GL20 is a plant growth-promoting rhizobacterium that produces a large amount of hydroxamate siderophore under iron-limited conditions. The strain GL20 considerably inhibited the spore germination and hyphal growth of a plant pathogenic fungus, *Fusarium solani*, when iron was limited, significantly suppressed the root-rot disease on beans caused by *F. solani*, and enhanced the plant growth. The mechanism for the beneficial effect of strain GL20 on the disease suppression was due to the siderophore production, evidenced by mutant strains derived from the strain. Analysis of the outer membrane protein profile revealed that the growth of strain GL20 induced the synthesis of specific iron-regulated outer membrane proteins with molecular masses of 85- and 90 kDa as the high-affinity receptors for the ferric siderophore. In addition, a cross-feeding assay revealed the presence of multiple inducible receptors for heterologous siderophores in the strain. In order to induce increased efficacy and potential in biological control of plant disease, a siderophore-overproducing mutant, GL20-S207, was prepared by NTG mutagenesis. The mutant GL20-S207 produced nearly 2.3 times more siderophore than the parent strain. In pot trials of beans with *F. solani*, the mutant increased plant growth up to 1.5 times compared with that of the parent strain. These results suggest that the plant growth-promoting *P. fluorescens* GL20 and the genetically bred *P. fluorescens* GL20-S207 can play an important role in the biological control of soil-borne plant diseases in the rhizosphere.

Key words: *Pseudomonas fluorescens* GL20, siderophore, biological control, *Fusarium solani*, outer membrane receptor

As a cofactor for a variety of functional proteins and enzymes, iron is an essential trace element for microbial growth and multiplication. Despite being one of the most abundant elements in the soil environment, iron is often a limiting factor for microbial growth because it forms highly insoluble ferric hydroxide polymers (K_s=$10^{-38.7}$, [Fe^{3+}]=10^{-17} M) under aerobic conditions at neutral pH [3, 24, 45], thus severely restricting the biological availability of the ionic form. Therefore, many microorganisms usually possess specialized high-affinity iron-uptake systems for obtaining sufficient amounts of this element [35]. Under iron-limiting growth conditions, they synthesize and secrete siderophores, low-molecular weight, high-affinity Fe(III)-chelating agents, that specifically bind ferric iron and consequently transport the iron complex into the cell [29, 30, 35]. The uptake of these complexes is mediated by specific iron-regulated outer membrane protein (IROMP) receptors [34].

Fluorescent pseudomonads have been used as biocontrol agents in several rhizosphere studies, where their inhibitory activity has been attributed to the production of secondary metabolites, such as antibiotics [13, 15, 40, 43, 46], hydrogen cyanide [47], or iron-chelating siderophores [2, 25, 41, 44, 49, 50].

In soils, the capacity to utilize siderophores is important to the growth of bacteria in the rhizosphere and on plant surfaces. *Pseudomonas fluorescens* and *Pseudomonas putida* are prominent members of the microflora in the rhizosphere of many plants. These fluorescent pseudomonads, generally termed plant growth-promoting rhizobacteria (PGPR), rapidly colonize plant roots of a variety of crops, and significantly increase yield by suppressing soil-borne fungal diseases [18]. The capacity of these pseudomonads to suppress some pathogens is partially due to pyoverdines, a class of siderophores with a very high affinity for Fe(III) [29]. It makes iron nutritionally unavailable to deleterious fungi in the rhizosphere, thereby preventing the pathogens from access to the already limited pool of soluble iron. The nutritional competition for this essential element by the beneficial pseudomonads may be a major

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factor to modulate plant root colonization and disease suppression.

Pseudomonas spp. strains can utilize ferric complexes of siderophores produced by other strains of *Pseudomonas* spp. [19], and also ferric complexes of different siderophores produced by diverse species of bacteria and fungi [16, 26, 32]. This iron-acquisition system is due to the presence of multiple outer membrane receptors that recognize heterologous siderophores. The large variety of ferric siderophore uptake systems reflects the importance of iron competition in the rhizosphere environments.

The plant growth-promoting pseudomonads have quite extensively been studied for their ability to control plant disease and enhance plant growth. However, these studies have mainly been applied only to the biological control of fusarium wilt of cucumber, radish, flax, or tobacco caused by *Fusarium oxysporum* [9, 21, 22, 41, 44], damping-off of cucumber, tomato, or cotton and root-rot of wheat by *Pythium ultimum* [2, 6, 25, 37], take-all of wheat by *Gaeumannomyces graminis* var. *tritici* [12, 39, 49], and seed piece decay of potato by *Erwinia carotovora* [50].

Fusarium solani is one of the most destructive plant pathogenic fungi, causing root-rot disease of a wide variety of economically important crops. The disease is characterized by reddish-brown lesions that develop on the hypocotyl and lateral roots. In spite of the economic seriousness of the disease, the contribution of siderophore production in the disease suppression is not clear and the antifungal mechanism is not well established.

Pseudomonas fluorescens GL20 is a plant growth-promoting rhizobacterium, originally isolated from a ginseng rhizosphere that produces a yellow green-fluorescent siderophore under iron-limited conditions [23]. The strain GL20 has previously been shown to significantly suppress the root-rot disease on beans (*Phaseolus vulgaris* L.) caused by *F. solani* [23].

The basic hypothesis of this study is that the siderophore production of rhizosphere-inhabiting *P. fluorescens* GL20 may play a crucial role in the biological control of plant disease. The present work suggests that this siderophore was largely responsible for the ability of strain GL20 to effectively control *F. solani*-induced root-rot disease. In order to clarify the role of siderophore in the disease suppression, the antifungal efficacy of strain GL20 on the spore germination or hyphal growth of *F. solani* under iron limited condition was evaluated. Subsequently, the relationship between the production of siderophore *in vitro* and the degree of disease suppression by mutant strains derived from strain GL20 was investigated. In addition, the iron-regulated outer membrane proteins of strain GL20 were compared with those of the mutant strains, and the probable specific receptor proteins of ferric siderophore were identified.

Since the ability of *P. fluorescens* GL20 to colonize plant roots and suppress disease may be affected by its capacity to utilize siderophores produced by other microorganisms as well as the efficacy of its own siderophore, its capacity to utilize heterologous siderophores to compete for iron in the rhizosphere was also investigated. Mutants overexpressing the biosynthetic genes, and hence overproducing the siderophore, are likely to have increased efficacy and potential in biological control, therefore, the abilities of siderophore-overproducing mutants derived from *P. fluorescens* GL20 as powerful biocontrol agents, were compared with that of the parent strain.

MATERIALS AND METHODS

Microorganisms and Growth Conditions

The siderophore-producing bacterial strains used in this study were grown at 28°C in King's B (KB) medium [17]. For growth in iron-restricted conditions, proteose peptone No. 3 (Difco Lab., Detroit, U.S.A.) was deferrated by extraction with 3% (w/w) 8-hydroxyquinoline (Sigma Chemical Co., St. Louis, U.S.A.) in chloroform solution for 2 days, as described by Waring and Werkman [48].

The plant pathogenic fungus *Fusarium solani* was kindly provided by the Korean Ginseng and Tobacco Research Institute, and grown at 28°C on potato dextrose agar (PDA; Difco Lab., Detroit, U.S.A.). A spore suspension of *F. solani* was prepared by growing it in potato dextrose broth (PDB; Difco Lab., Detroit, U.S.A.) at 28°C for 7 days. The culture was sieved aseptically through 10 layers of sterile cheesecloth, centrifuged, and washed five times with sterile 0.1 M $MgSO_4$ solution. The spore suspension was air-dried, and stored at 4°C.

All strains were maintained in 30% glycerol at −70°C for long-term storage. All glasswares were carefully made iron-free with 6 N HCl, rinsed once in distilled water, and finally rinsed in deionized, double-distilled water obtained from a Milli Q water purification system (Millipore, CA, U.S.A.). All media and solutions were prepared with deionized, double-distilled water.

Siderophore Assays

Siderophore in a cell-free culture supernatant fluid was determined using the chrome azurol S (CAS; Fluka Chemical Corp., NY, U.S.A.) colorimetric liquid assay of Schwyn and Neilands [42]. This assay utilizes a dye complex of CAS and hexadecyltrimethylammonium bromide (HDTMA; Fluka Chemical Corp., NY, U.S.A.) that has a high affinity for iron. The iron dye complex is blue with an absorption maximum at 630 nm. When a strong chelator such as siderophore removes the iron from the dye, its color turns from blue to orange. Siderophore produced was estimated by measuring the decrease in the absorbance after incubating it in the CAS assay solution for 3 h. A standard solution was prepared with deferoxamine mesylate (Sigma Chemical

Co., St. Louis, U.S.A.). The assay mixture consisted of 0.5 ml of a CAS assay solution and 0.5 ml of a culture supernatant fluid.

The CAS assay is a universal chemical assay used for the detection of siderophores. It reacts with any siderophore structure. The presence of catechol-type siderophores in a culture supernatant fluid was detected at 510 nm by the method of Arnow [1]. The assay standard was prepared with 2,3-dihydroxybenzoic acid (2,3-DHBA; Sigma Chemical Co., St. Louis, U.S.A.). The presence of hydroxamate-type siderophores was determined at 543 nm by the modified method of Csaky [8] with hydroxylamine HCl (Sigma Chemical Co., St. Louis, U.S.A.) as the standard. The assay was modified as follows: The acid hydrolysis step was conducted by autoclaving at 120°C for 4 h. A 2.5% sodium thiosulfate solution, instead of 2% sodium arsenite solution, was used as an iodine decolorizing agent. A 0.05% N-(1-naphthyl) ethylenediamine solution, instead of 0.3% α-naphthylamine solution, was used as a coupling agent.

Fungal Inhibition Assays

To evaluate their ability to inhibit fungal growth by siderophore-producing bacterial strains on agar plates, bacterial cells were spotted on the center of iron-deficient KB agar. After 24 h of incubation, these agar plates were lightly oversprayed with a suspension (10^8 cfu/ml) of *F. solani*. The plates were incubated at 28°C for 4 days. The inhibition zone of *F. solani* was measured as the distance between the edge of the bacterial colony and the fungal mycelium. Alternatively, bacterial cells were inoculated at 2 cm from the edge of iron-deficient KB agar. After 24 h of incubation, agar disks (5 mm in diameter) of the inoculum from the leading edge of *F. solani* mycelium grown for 3 days on PDA were placed on the center of the KB plates. After 3 days of incubation at 28°C, the distance of the inhibition zone was measured.

The antifungal activities of siderophore-producing strains against *F. solani* were investigated in dual liquid cultures as follows: To evaluate inhibitory activity on the mycelial growth, bacterial cells were added to an iron-deficient KB culture grown for 3 days with a spore suspension (2×10^8 cfu/ml) of *F. solani*. After 4 days of incubation at 28°C, the fungal mycelia were collected on an oven-dried, pre-weighed filter paper, dried at 105°C, and their dry weights were recorded. The inhibition ratio was expressed relative to a control with water. To evaluate inhibitory activity on the spore germination, bacterial cells were grown in an iron-deficient KB broth with the spore suspension, and dry weights of the cultures were determined after 5 days of incubation at 28°C.

Cross-Feeding Bioassay for Siderophore Utilization

Utilization of heterologous siderophores was detected by a cross-feeding assay. The assay was based on reversal of iron-restricted growth induced by ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDHA; Sigma Chemical Co., St. Louis, U.S.A.), a synthetic iron-chelator. The concentration of EDDHA was determined by an optimal minimal concentration which completely inhibited bacterial growth. EDDHA concentrations required for each test strains were: 6 mM for GL7, GL17, GL19, and GL20; 1 mM for GL14. The surface of the KB agar with optimal minimal concentration of EDDHA was seeded with each of the strains (approximately 10^6 cfu/ml). The KB agar plates (24-h old) were used to ensure full complexation of contaminating iron by EDDHA. Sterilized filter paper disks (6 mm diameter) were impregnated with 20 µl each of filter-sterilized, cell-free culture supernatant fluids of the strains, and placed on the surface of the KB agar. The bacterial growth around the paper disks was observed after 24 to 48 h of incubation at 28°C.

Isolation of Mutants Related to Siderophore Synthesis

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis was performed as described by Miller [33]. Bacterial cells were grown at 28°C in the KB medium to produce a density of approximately 5×10^8 cfu/ml, and centrifuged for 30 min at 6,000 ×g. The pellets were washed with sterile 0.1 M sodium citrate buffer at pH 5.5, and resuspended in a small volume of the same buffer. A freshly prepared solution of NTG in sterile distilled water (1 mg/ml) was added to the cell suspension with 25 µg/ml portions to give final NTG concentrations ranging from 25 to 200 µg/ml. The NTG-treated cells were then incubated at 28°C for 30 min in a water bath without shaking, and then centrifuged for 20 min at 12,000 ×g. The pellets were washed with sterile 0.1 M sodium phosphate buffer at pH 7.0, and the washed pellets were resuspended in a KB broth. After 2 h of incubation at 28°C in a water bath, appropriately diluted cell suspensions were plated on the KB agar and incubated at 28°C for 2 days.

Siderophore-positive/-negative mutants were initially identified by the fluorescence surrounding their colonies under long-wave (365 nm) UV irradiation. These mutants were finally identified by observing orange halos surrounding their colonies on CAS blue agar, according to the method of Schwyn & Neilands [42]. The mutants were further tested for siderophore production and antifungal activity, as described previously.

Outer Membrane Protein Preparation

Bacteria containing approximately 3×10^{10} cells were harvested by centrifugation at 10,000 ×g for 20 min at 4°C, washed twice with ice-cold 10 mM sodium phosphate buffer (pH 7.2), and resuspended in 1 ml of 30 mM Tris-HCl buffer (pH 8.0). Cell envelopes were disrupted by a French press, and bacterial debris were removed by centrifugation (20 min, 10,000 ×g, 4°C). Sarkosyl (*N*-lauroyl-sarcosine, sodium salt;

Sigma Chemical Co., St. Louis, U.S.A.) was added to the supernatant to a final concentration of 2% (w/v) [10], and the mixtures were incubated at room temperature for 30 min and then centrifuged at 36,000 ×g for 1 h at 4°C. The membrane pellets were washed with 30 mM Tris HCl (pH 8.0), resuspended in a small volume of the same buffer, and stored at -70°C. Membrane preparations were resolved by SDS-PAGE [20] with 10% separating gels and 5% stacking gels. The resulting proteins in gels were made visible by staining with Coomassie brilliant blue R250. The molecular weight standards used were purchased from Sigma (St. Louis, U.S.A.).

In Vivo Biocontrol Assay

Suppression of *F. solani*-induced root-rot disease by siderophore-producing bacteria and mutant strains was tested in pots with beans (*Phaseolus vulgaris* L.). Seeds of beans were surface-sterilized twice with immersion in 1% NaOCl, and then washed in sterile distilled water. The seeds were left to germinate in the dark for 3 days between sterile moist cheesecloth at 28°C.

Plastic pots (50×50×50 mm) were filled up to two-thirds with air-dried vermiculite soil, and 3-day-old seedlings were transplanted into each pot. A seed cover layer (one-third of the pot's depth) was infested with a spore suspension (3×10^9 cfu per g of dry soil) of *F. solani* and mixed with 5 ml of bacterial suspension (approximately 1×10^9 cfu/ml). The plants were incubated in a growth chamber at 22°C with 65% relative humidity (RH) and 16 h light-8 h dark cycle. The plants were watered daily with 100 ml of plant nutrient solution [5]. The solution was prepared by adding 2 ml of the following stock solutions to 1 l of water: 0.5 M KNO_3, 0.5 M $Ca(NO_3)_2$, 0.2 M $MgSO_4$, and 0.1 M K_2HPO_4. The plants were harvested after 14 days of transplanting. The bean seedlings were examined for the emergence of root-rots and their symptoms. The number of healthy plants was recorded for disease incidence. The weights of whole plants were recorded to measure growth promotion.

RESULTS AND DISCUSSION

Antifungal Mechanism and Inhibitory Activity

It was previously found that *P. fluorescens* GL20 produced a large amount of hydoxamate siderophore and considerably inhibited the growth of plant pathogenic fungus *F. solani* under iron-limited conditions (Table 1) [23], demonstrating that the fungal inhibition was partially due to the siderophore production [23].

To precisely define the mechanism responsible for *in vitro* antagonism, the inhibitory activity of siderophore-producing *P. fluorescens* GL20 on hyphal growth or spore germination of *F. solani* was investigated in a dual culture. Under a phase-contrast microscope, decrease of the fungal mycelial mass could be observed from the first day after the bacterial cells were added to an iron-deficient KB medium which had previously been grown for 3 days with *F. solani*. After 4 days of culture, strain GL20 remarkably inhibited the fungal growth by 81%, showing a considerable decrease of the mycelial mass (Fig. 1). In addition, strain GL20 greately suppressed the spore germination in an iron-deficient KB culture which had been grown for 5 days with a spore suspension (2×10^8 cfu/ml) of *F. solani* (Fig. 1). The germ tube elongation was almost unobservable under a phase-contrast microscope: *F. solani* did not grow

Table 1. Characterization of siderophore-producing bacteria.

Strain	Siderophore[a]		Fluorescent pigment[d]	Antibiotic[e]	Chitinase[f]	Antifungal activity[g]
	Hydoxamate[b]	Catechol[c]				
GL7	25.73	0	+	-	-	+
GL14	0	26.36	-	+	-	+
GL17	18.75	0	+	-	-	+
GL19	7.59	0	+	-	-	+
GL20	28.54	0	+	-	-	+

[a]All strains were grown at 28°C for 40 h in iron-deficient KB medium.
[b]The presence of hydroxamate-type siderophores was detected at 543 nm by the method of Csaky [8]. The values are µg of hydroxylamine HCl equivalents per ml.
[c]The presence of catechol-type siderophores was detected at 510 nm by the method of Arnow [1]. The values are µg of 2,3-dihydroxybenzoic acid equivalents per ml.
[d]Diffusible fluorescent pigment was tested on iron-deficient KB agar plates.
[e]Antibiotic production was tested on PDA agar. The bacterial cells were spotted on the center of the plates. After 24 h of incubation, the plates were lightly oversprayed with a suspension (10^8 cfu/ml) of *F. solani*. The production of an inhibition zone around the bacterial colony suggested the presence of an antibiotic.
[f]Chitinase production was tested on chitin minimal agar. The production of a clear zone around the bacterial colony suggested the presence of a chitinase.
[g]The bacterial cells were spotted on the center of iron-deficient KB agar plates. After 24 h of incubation, the plates were lightly oversprayed with a suspension (10^8 cfu/ml) of *F. solani*. After 4 days of incubation, the inhibition zone of *F. solani* was determined.
All values are means of three replicates.

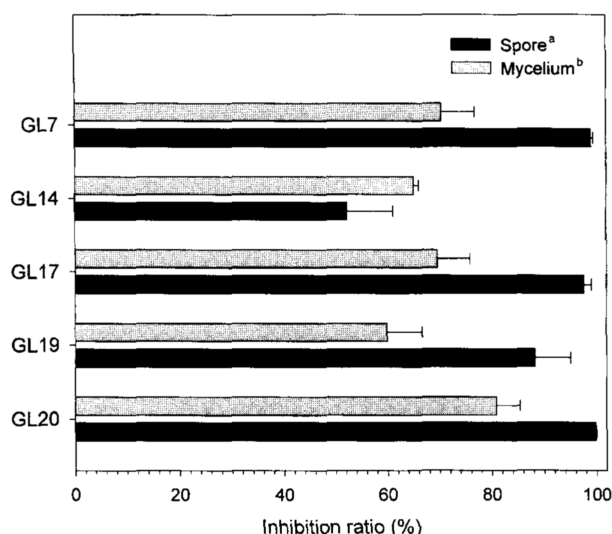

Fig. 1. Antifungal activities of siderophore-producing bacteria on the spore germination and mycelial growth of *F. solani* in dual cultures.
[a]Bacterial cells were grown in an iron-deficient KB broth with a spore suspension (2×10^8 cfu/ml) of *F. solani*. Dry weights of the cultures were determined after 5 days of incubation at 28°C. Inhibition ratio (%) was expressed relative to a control with water. [b]Bacterial cells were added to an iron-deficient KB culture grown for 3 days with *F. solani*. Dry weights of the cultures were determined after 4 days of incubation. Each horizontal bar represents the mean of three replications and the error bar represents SD.

in the culture at all. These results suggested that the complete growth inhibition was due to the siderophore's sequestration of the iron required for spore germination and hyphal growth of *F. solani*, when iron was limited.

Utilization of Siderophores by Different Strains

To determine whether *P. fluorescens* GL20 could utilize ferric siderophores from different strains for efficient iron-sequestrating, a cross-feeding assay was performed in iron-restricted conditions. As illustrated in Table 2, the strain GL20 was able to utilize ferric complexes of siderophores produced by *P. fluorescens* strains of GL7, GL17, and GL19, *B. subtilis* GL14, and *P. aeruginosa* ATCC 15692, evidenced by growth-intensive zones around filter paper disks. On the other hand, strains of GL14 and GL19 did not produce cross-feeding zones with other strains tested, showing clear zones around filter paper disks (Table 2). A culture supernatant fluid of strain GL14 was able to promote the growth of GL7, GL17, and GL20 strains (Table 2). Although these three strains did not produce catechol-type siderophores, they were able to use the ferric siderophore produced by strain GL14. These results suggested that *P. fluorescens* GL20 had the capacity to utilize a variety of siderophores of heterologous origin for iron aquisition. The iron-aquisition system is generally considered to induce the synthesis of specific outer membrane proteins in the strain, which suggests the presence of multiple inducible receptors for ferric siderophores. This ability is thought to enhance the capacity of strain GL20 to compete for iron in the rhizosphere environments.

Characteristics of Mutants Related to Siderophore Synthesis

Mutant strains (Sid^-) defective in siderophore production were obtained from *P. fluorescens* GL20 by NTG mutagenesis. Mutants GL20-S101 and GL20-S111 barely produced siderophores in iron-deficient KB cultures (Table 3). These mutants were nonfluorescent on the KB agar and unable to produce orange halos on CAS blue agar (data not shown), indicating a defect in the biosynthesis of siderophore.

The mutant GL20-S111 exhibited siderophore-dependent growth on an iron-deficient KB medium containing the synthetic iron chelator EDDHA. The mutant did not grow on the medium unless supplemented with wild-type siderophore. Interestingly, the growth of mutant GL20-S101 was severely restricted under low-iron conditions. The mutant showed markedly reduced growth on the EDDHA-containing KB

Table 2. Utilization of siderophores by various strains under iron-restricted conditions.

Strain	Growth stimulated by iron-restricted culture supernatant[a]					
	GL7	GL14	GL17	GL19	GL20	*P. aeruginosa*
GL7	+	+	−	−	−	+
GL14	−	+	−	−	−	−
GL17	−	+	+	−	−	+
GL19	−	−	−	+	−	−
GL20	+	+	+	+	+	+

[a]Growth around filter paper disks (6 mm diam) impregnated with 20 μl of cell-free culture supernatant fluids on iron-restricted KB agar plates containing appropriate concentrations of EDDHA seeded with 10^6 cfu per ml of test strains. For GL14, 1 mM EDDHA was used; for other strains, 6 mM EDDHA was used. "+", bacterial growth; "−", no bacterial growth. All values are means of three replicates.

Table 3. Characterization of siderophore-positive/-negative mutants.

Strain	CAS	Hydroxamate	
	% reduction in A_{630}[a]	A_{543}[b]	[μg/(mg cell)][c]
GL20	91.2	1.360	14.69
GL20-S101	2.7	0.079	0.94
GL20-S111	1.4	0.145	1.48
GL20-S203	93.3	1.712	18.33
GL20-S207	96.2	3.116	33.77

All strains were grown for 40 h at 28°C in iron-deficient KB medium.
[a]Percent values of 1−absorbance in cell-free culture supernatant fluid/absorbance of reference in non-inoculated KB medium at 630 nm.
[b]Values of absorbance in cell-free supernatant fluid per mg cell protein present per ml of a culture.
[c]The values are μg of hydroxylamine HCl equivalents per ml.
All values are means of three replicates.

medium supplemented with wild-type siderophore, compared with that of the parent strain. As a result, *P. fluorescens* GL20 grew on an iron-deficient KB medium containing EDDHA up to 5 mM, the highest concentration tested (data not shown). The mutant GL20-S111 grew on the medium containing the same concentration of EDDHA, whereas mutant GL20-S101 did not grow on the medium when it contained more than 0.1 mM EDDHA (data not shown). This result indicated that mutant GL20-S101 appeared to be defective in the ferric siderophore uptake system as well as in the biosynthesis of siderophore.

To confirm the role of siderophore in inhibiting the growth of *F. solani*, the antifungal activity of siderophore-deficient mutant GL20-S101 was investigated. As seen in Fig. 2, the mutant failed to inhibit the fungal growth on an iron-deficient KB agar, thus supporting the hypothesis that the production of siderophore by *P. fluorescens* GL20 was largely responsible for the potential ability of the strain to inhibit the growth of *F. solani*.

To induce greater biocontrol efficacy for plant disease than the parent strain through the overproduction of siderophore, *P. fluorescens* GL20 was mutated by NTG. Consequently, mutants GL20-S203 and GL20-S207 that produced larger orange halos than the parent strain on CAS agar were obtained, indicating that they were siderophore overproducers (Sid^{+}). The mutant GL20-S207 produced nearly 2.3 times more siderophore in an iron-deficient KB culture than the parent strain (Table 3). Moreover, the siderophore-overproducing GL20-S207 inhibited the growth of *F. solani* significantly more than its parent strain on the KB agar (Fig. 2).

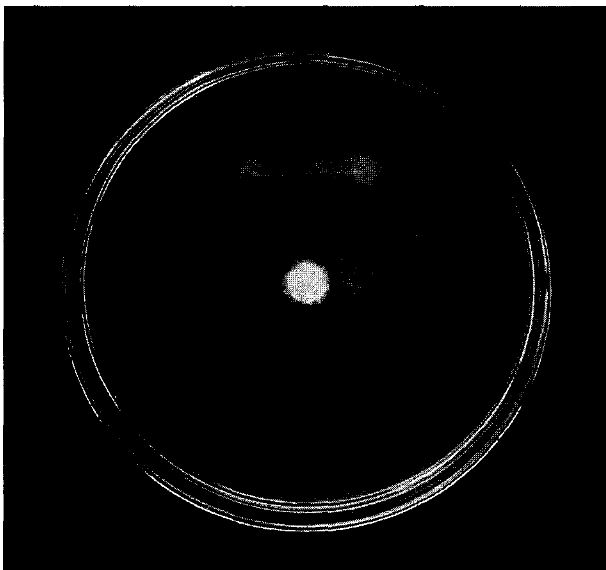

Fig. 2. Antifungal inhibition of siderophore-producing *P. fluorescens* GL20 and its mutants against *F. solani*.
All strains were grown at 28°C for 3 days on iron-deficient KB agar. Top, *P. fluorescens* GL20; left, *P. fluorescens* GL20-S207 (Sid^{+}); right, P. fluorescens GL20-S101 (Sid^{-}); bottom, *P. fluorescens* GL20-S203 (Sid^{+}); center, *F. solani*.

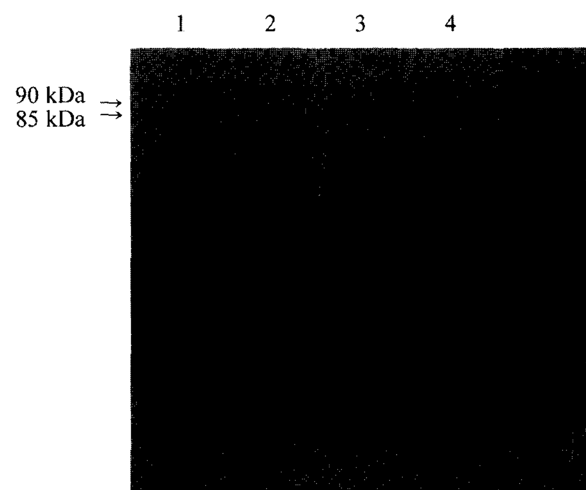

Fig. 3. Outer membrane protein profiles of *P. fluorescens* GL20 and its mutants grown in KB medium with (lane 2) or without (lanes 1, 3, and 4) 100 μM $FeCl_3$.
Lanes 1 and 2, *P. fluorescens* GL20; lane 3, *P. fluorescens* GL20-S207 (Sid^{+}); lane 4, *P. fluorescens* GL20-S101 (Sid^{-}); right side, molecular weight standard proteins of 97.4, 66.2, 55, 42.7, 40, and 31 kDa.

Outer Membrane Receptors for Ferric Siderophore

In order to identify and characterize the receptors involved in siderophore-mediated iron transport in *P. fluorescens* GL20, outer membrane protein (OMP) profiles were examined with bacterial cultures under iron-deficient and -rich KB media (Fig. 3, Lanes 1 and 2). When grown in iron-limited conditions, strain GL20 produced two OMPs with approximate molecular masses of 85- and 90-kDa (Lane 1). These two proteins were not expressed under conditions of excess iron (Lane 2). Comparision of OMP profiles revealed that two iron-regulated outer membrane proteins (IROMPs) were induced by iron deficiency in *P. fluorescens* GL20.

OMP patterns from mutants GL20-S207 and GL20-S101 of *P. fluorescens* GL20 are shown in Lanes 3 and 4, respectively. The siderophore-deficient GL20-S101 specifically lacked the major 85-kDa IROMP and the expression of the 90-kDa IROMP was slightly increased compared with that in the parent strain (Lanes 1 and 4). The OMP pattern of siderophore-overproducing GL20-S207 was characterized by a protein band with apparent 90-kDa, heavy compared with that in the parent strain, indicating an increase of the expression of the 90-kDa IROMP (Lanes 1 and 3).

These results on the analysis of the OMP profiles led to the conclusion that growth of *P. fluorescens* GL20 induced the formation of specific IROMPs with molecular masses of 85- and 90-kDa, relatively large for a siderophore-mediated high-affinity iron-uptake system under iron-limited conditions. Several groups have reported IROMPs in the

range of 70- to 90-kDa that are associated with ferric siderophore uptake in different *Pseudomonas* strains. The receptors for ferric pyoverdines have been reported to be 80-kDa [7, 31], 85-kDa [4, 11, 14, 27], or 90-kDa [14, 28, 36, 38] OMP. Therefore, it is suggested that the 85- and 90-kDa IROMPs are high-affinity receptors for ferric pyoverdin in *P. fluorescens* GL20.

Plant Growth-Promoting Activity and Disease Control

To define more precisely the role of siderophore in suppression of root-rot disease caused by *F. solani*, the efficacy of *P. fluorescens* GL20 to control the disease on beans (*Phaseolus vulgaris* L.) was compared with that of its mutant strains. In a pot trial of beans with *F. solani* alone, the incidence of the disease reached up to 72% after 14 days of transplanting (Fig. 4). Treatment with strain GL20 remarkably reduced the incidence of the disease down to 4% (Fig. 4). In addition to the disease control, strain GL20 had a plant growth-promoting effect by increasing the plant weights up to 1.6 times compared with that of the control (Table 4).

The siderophore-deficient mutants GL20-S101 and GL20-S111 from *P. fluorescens* GL20 were much less effective than the parent strain in suppressing the plant disease (Fig. 4). The plants grown from seeds treated with these mutants had considerably more root-rot, as indicated by the number of reddish-brown lesions at the root-stem interface. The plant weights were much lower than the parent strain and

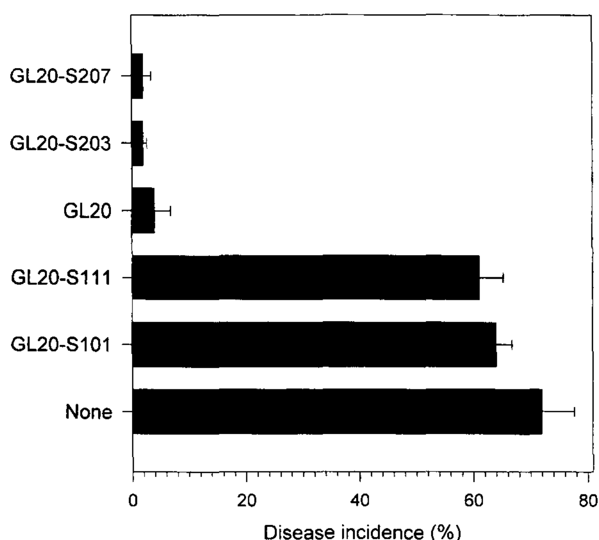

Fig. 4. Suppression of *F. solani*-induced root-rot disease by *P. fluorescens* GL20 and its mutants.

Plastic pots were filled up to two-third with a vermiculite soil, and a 3-day-old seedling of bean was transplanted into each pot. A seed cover layer (one-third of the pot's depth) was infested with a preparation (3×10^9 cfu per g of dry soil) of *F. solani* and mixed with 5 ml of bacterial suspension (approx. 1×10^9 cfu/ml). Each horizontal bar represents the mean of three replications of 50 seedlings each and the error bar represents SD.

Table 4. Enhanced plant growth by wild-type and siderophore-overproducing mutants of *P. fluorescens* GL20.

Strain	Average weight per plant (g)	Increase compared to control (%)
Control	6.3	100
GL20	10.1*	160
GL20-S101 (Sid^-)	6.7	106
GL20-S111 (Sid^-)	6.9	109
GL20-S203 (Sid^+)	11.2*	177
GL20-S207 (Sid^+)	15.2*	241

Bean-seedlings were infested with a preparation (3×10^9 cfu per g of dry soil) of *F. solani* and mixed with 5 ml of the bacterial suspension (approx 1×10^9 cfu/ml). Control seedlings were untreated with *F. solani* and bacteria. The plants were harvested 14 days after transplanting, and the weights of whole plant were recorded. Each value is the average of three replications of 50 seedlings.

*Indicates significant increase compared to control (P=0.05).

similar to that of the control (Table 4). A siderophore-overproducing mutant GL20-S207 increased plant weights up to about 2.4 times compared with that of the control (Table 4), showing a significant suppression of the disease down to 2% (Fig. 4). These results suggested that the ability of *P. fluorescens* GL20 to reduce the incidence of root-rot disease and promote plant growth in the pot trials was due to the siderophore produced by the strain.

The results described above led us to suggest that the plant growth-promoting *P. fluorescens* GL20 and the genetically bred *P. fluorescens* GL20-S207 (Sid^+) may have a major role in the biological control of plant diseases.

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