Iron Sufficiency, a Prerequisite for the Suppression of Tobacco Black Root Rot by *Pseudomonas fluorescens* Strain CHA0 under Gnotobiotic Conditions

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ABSTRACT

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Strain CHA0 of *Pseudomonas fluorescens* suppressed black root rot of tobacco, caused by *Thielaviopsis basicola*, under gnotobiotic conditions in an artificial soil containing vermiculite as clay mineral. When vermiculite was replaced by illite, *P. fluorescens* provided poor protection. Better protection was obtained by the addition of $FeCl_3$ to illite. When Fe^{3+} chelated with ethylenediaminedi(*o*-hydroxyphenylacetic acid) (FeEDDHA) was added to illite, strain CHA0 gave good protection. Strain CHA400, a nonfluorescent, pyoverdine-negative mutant obtained by transposon insertion, protected the plant to the same extent as did the wild-type strain CHA0 in the systems containing vermiculite, illite, or illite amended with $FeCl_3$. However, in the system containing illite and FeEDDHA, strain CHA400 gave poor protection, in contrast to strain CHA0. No differences were observed between CHA400 and CHA0 with respect to the intensity of root colonization. The survival of *T. basicola* was similar in both the vermiculite and the illite systems. The availability of iron in vermiculite clay was higher than in illite, as indicated by the amount of iron in soil water solution and the amount of HCN produced by *P. fluorescens* in minimal medium amended with the clay minerals. From these experiments, it appears that the suppression of black root rot by strain CHA0 requires sufficient iron, and iron competition is not a suppressive mechanism in this system. We hypothesize that HCN production has a role in disease suppression.

Some native suppressive soils and soils rendered suppressive by the addition of fluorescent strains of *Pseudomonas* lose their ability to suppress disease when the availability of iron to the pathogens is increased (15,17,23–25,36). This phenomenon has been explained by the fact that fluorescent pseudomonads under low-iron conditions produce fluorescent siderophores that sequester iron (15,20,26) and inhibit the growth of fungal and bacterial pathogens through a deprivation of iron (3,4,14,15,21). This mechanism has been implicated in biological control of diseases and in the enhancement of plant growth in experiments conducted on the West Coast of the United States and in sea sediments of the Netherlands (8). In these regions iron availability in the soil appears to be lower than in most areas of western Europe (35).

In the Morens region of Switzerland, suppressive soil contains more iron than neighboring conducive soil (29). A strain of *P. fluorescens* (Trevisan) Migula (strain CHA0) isolated from the suppressive soil promoted wheat growth in field trials (7) and suppressed black root rot of tobacco, caused by *Thielaviopsis basicola* (Berk. & Br.) Ferraris, when added to different conducive soil samples (29). Partially purified, iron-free pyoverdine of *P. fluorescens* CHA0 does not inhibit the growth of wild-type *T. basicola* in vitro. However, high concentrations of iron-bound siderophores reduce the percentage of germination and the growth of *T. basicola* (1).

To test the importance of iron availability in soil, we developed a gnotobiotic system that contains an artificial soil composed of quartz grains and different types of clay minerals. In this system, the plant, the pathogen, and strains of *P. fluorescens* (wild type and siderophore-negative mutant) are grown without microorganisms, and disease suppression can be tested reproducibly.

MATERIALS AND METHODS

Media and growth conditions. Strain CHA0 of *P. fluorescens* (wild type) was grown in shake cultures (110 rpm; 2.0-cm radius) in nutrient yeast broth (NYB: 25 g of Difco nutrient broth, 5 g of Difco yeast extract, and 1 L of double-distilled water; Difco Laboratories, Detroit, MI) at 28 C for 16 hr in the dark. For the production of bacteria for soil, 200 μl of NYB cultures was plated on King B agar (KBA) (13) and grown at 28 C for 24 hr. Bacteria were removed from the plates with sterile double-distilled water, and the resultant suspensions were adjusted to the desired concentration and immediately added to the test soils. *P. fluorescens* CHA400 (*pvd-400*::Tn*1733*), the siderophore-negative mutant, resistant to kanamycin, was cultured similarly, except for the addition of 25 μg of kanamycin sulfate (Sigma Chemical, St. Louis, MO) per milliliter of NYB and KBA.

For HCN production, the following medium (CME) was used: 20 mM L-glutamic acid (Fluka, Buchs, Switzerland); 12.5 mM glycine (Fluka); 5 mM DL-methionine (Fluka); 50 mM tris HCl buffer, pH 7; 5 mM K_2HPO_4; 5 mM $Na_2HPO_4 \cdot H_2O$; and 2 mM $MgSO_4 \cdot 7H_2O$ (5). The medium was filter-sterilized and supplemented with either 1 mg of illite per milliliter, 1 mg of vermiculite per milliliter, 20 μM $FeCl_3 \cdot 6H_2O$, or 20 μM Fe^{3+} chelated with ethylenediaminedi(*o*-hydroxyphenylacetic acid) (FeEDDHA; Sequestren 138, Ciba-Geigy, Basel, Switzerland) and 4 μM EDDHA (Sigma); EDDHA was added to complete the iron chelation. NYB bacterial culture (60 μl), diluted 10^4-fold (1×10^5 colony-forming units [cfu] per milliliter), was transferred to 10 ml of the medium in 20-ml universal bottles (Wheaton, United Glass Ltd., Great Britain) washed with 1 N HCl before use. The bottles were shaken at 28 C for 24 hr.

Strain ETH D 127 of *T. basicola* was grown on malt agar (15 g of Difco malt extract, 15 g of Difco agar, and 1 L of double-distilled water) at 24 C for 3 wk in the dark. Endoconidia that developed were suspended in sterile double-distilled water, separated from chlamydospores and mycelia by filtration through glass wool, and immediately added to the test soils (29).

Nicotiana glutinosa L. was grown in a growth chamber with 16 hr of light (80 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) at 22 C and an 8-hr dark period at 15 C and 70% relative humidity. Seeds were surface-disinfested in

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70% ethanol for 1 min and in 5% H_2O_2 for 10 min and then rinsed with sterile double-distilled water. After 1 wk on 1.7% water agar (Difco), seedlings with similar root length were transferred to Knop nutrient solution (1.00 g of $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 g of KCl, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.25 g of KH_2PO_4, 20.0 mg of FeEDDHA, 1 ml of Hoagland solution, and 1 L of double-distilled water) (38) solidified with 0.8% agar. Four weeks later, when four leaves had developed, plants were removed, roots were washed in sterile double-distilled water, and the seedlings transplanted into the gnotobiotic system.

The gnotobiotic system. Vermiculite (Vermica AG, Bözen, Switzerland) is a native clay mineral from the Toc mine, South Africa, used worldwide for painting and house insulation. To conserve its characteristics (Table 1), this clay mineral was not exfoliated at 1,000 C (12,30); however, it was expanded with H_2O_2: 170-g samples (grain size less than 0.25 mm) were suspended in 200 ml of 30% H_2O_2 (w/w) and heated in a 2,000-ml beaker. When the mixture had expanded to 1,000 ml, 300 ml of distilled water was added. The mixture was comminuted with a blender 1 hr later and left at room temperature for 2 hr. The vermiculite was dried at 60 C for 2 days in glass dishes. Finally, it was ground twice in an ultracentrifugal mill (Schieritz & Hauenstein AG, Arlesheim, Switzerland) to obtain grain sizes smaller than 120 μm.

Illitic clay (illite) of 85% purity grade (Table 1) was taken from a sediment in the Massif Central, France (30). Quartz sand (99.5% SiO_2, 0.24% Al_2O_3, 0.05% Fe_2O_3 + TiO_2, 0.07% CaO + MgO, 0.04% K_2O/Na_2O, and 0.1% moisture) of different grain sizes (10% 1.5–2.0 mm, 9.3% 0.8–1.2 mm, 9.3% 0.5–0.75 mm, 28.6% 0.10–0.50 mm, and 42.8% 0.08–0.20 mm) was mixed with quartz powder (55% of which was of grain sizes less than 40 μm) and clay in the ratio 70:20:10 (w/w) and moistened before use with double-distilled water (10%, v/w, for vermiculite; 8%, v/w, for illite). Wet soil (75 g) was placed in 100-ml flat-bottomed flasks with an opening 3 cm in diameter. The flasks were sealed with cotton wool stoppers and autoclaved at 121 C for 30 min. One day later, 5 ml of bacterial suspension (1.2×10^8 cfu/ml) and, if necessary, either 2.4 mM FeEDDHA or $FeCl_3 \cdot 6H_2O$ (9.9 μg of Fe^{3+} per gram of dry soil) were added to each flask. The bacterial suspension was injected into the soil with a sterile syringe. One day later, 5 ml of fungal suspension (1.2×10^5 endoconidia per milliliter) was added similarly. Control flasks received 10 ml of sterile double-distilled water. The final pH ($CaCl_2$) of the soil water solution was 7.0, and no difference was observed between the different soil types. The flasks were incubated in randomized complete blocks in the growth chamber (under the same conditions described earlier) in the dark for 8 days. Then a tobacco seedling was transplanted into each flask, and 3 ml of Knop nutrient solution was added near the roots. No further watering was necessary. The soil structure did not change during the experiments. Plants were removed from the soil 3 wk later by flooding with tap water. Roots were separated from adhering soil by washing, briefly dried between sheets of blotting paper, and weighed. Black root rot severity was estimated for each plant by assessing the percentage of root surface infected and darkened by the presence of chlamydospores (29,30).

TABLE 1. Characteristics of the pure vermiculitic and illitic clays

	Vermiculite[w]	Illite[w]
Chemical composition (%)		
SiO_2	47	52
Al_2O_3	15	23
Fe_2O_3	7	3
FeO	<1	1
MgO	20	5
CaO	1	1[x]
K_2O	1	9
Na	1	<1[x]
OH residual	7	7
Surface charge density per formula unit	0.66	0.8
Surface (m^2/g)	150[y]	120[z]

[w] Mineral formula: vermiculite, $[Si_{2.95}Al_{1.05}][Al_{0.29}Fe^{3+}_{0.1}Mg_{2.61}]O_{10}(OH)_2Mg_{0.33}$; illite, $[Si_{3.54}Al_{0.46}][Al_{1.38}Fe^{3+}_{0.14}Fe^{2+}_{0.03}Mg_{0.48}]O_{10}(OH)_2Ca_{0.4}$.
[x] Exchangeable.
[y] Mostly internal surface.
[z] Mostly external surface.

Root colonization. After being weighed, roots from each treatment were added to a flask containing 100 ml of isotonic water (0.85% NaCl) and placed on a rotary shaker (150 rpm) for 30 min. Appropriate dilutions of the resultant suspension were plated on KBA, supplemented with kanamycin sulfate (25 μg/ml) for strain CHA400. Colonies that fluoresced under UV light (strain CHA0) or were kanamycin-resistant (strain CHA400) were counted after a 48-hr incubation at 28 C.

Survival of *T. basicola*. One and 8 days after infestation, before the seedlings were transplanted, the contents of each flask were thoroughly mixed with a sterile spoon, and approximately 1 g of soil was withdrawn and suspended in 50 ml of sterile double-distilled water. The soil suspension was weighed and shaken for 30 min on a rotary shaker (250 rpm), and appropriate dilutions were plated on malt agar containing 156 μg of oxytetracyline hydrochloride per milliliter (Pfizer AG, Zürich, Switzerland). Colonies were counted after 7–9 days of incubation at 24 C in the dark. The soil suspension was oven-dried at 80 C for determining soil dry weight.

Iron content of the soil water. The whole plant and soil were removed from the flask with tap water, and the roots washed; soil and water were collected in a plastic beaker. The eight to 10 replicate flasks of a single treatment yielded approximately 600 g of soil and 3 L of water. The suspension was vigorously mixed and left to settle for a few minutes, and a 50-ml sample was centrifuged, filtered, and acidified with 1 ml of 37% HCl (w/v). The total iron concentration of this sample was analyzed with a plasma emission spectrometer, and the iron content of the total amount of water in the gnotobiotic system was calculated.

Measurement of HCN. The bacterial suspension was diluted 10-fold with 0.1 N NaOH. KCN at concentrations of 0.5–16 μM in 0.1 N NaOH was used as a reference. Then 0.2 ml of the dilution or of the references was added to 1.0 ml of 0.088 N NaOH and mixed; 0.6 ml of this suspension was added to a mixture of 1.0 ml of 0.1 M *o*-dinitrobenzene (Sigma) in ethylene glycol monomethyl ether (Fluka) and 1.0 ml of 0.2 M 4-nitrobenzaldehyde (Fluka) in ethylene glycol monomethyl ether. The sample was incubated at 20 C for exactly 30 min without mixing. Extinction was measured at 578 nm (11).

Statistics. Each experiment was repeated at different times; means of at least three experiments are presented. Each mean was compared with all other means with the Student *t*-test (multiple *t*-test).

Transposon mutagenesis. *Escherichia coli* MM294 carrying the plasmid pRU672 (pME305::Tn*1733*) (31) was used for insertion mutagenesis as described (33). Mutants carrying a Tn*1733* insertion were resistant to kanamycin (25 μg/ml) and sensitive to tetracycline (125 μg/ml) on nutrient agar (28), indicating the loss of the vector plasmid. The prototrophic mutant CHA400 was unable to grow on minimal medium (MME) (32) containing 100 μg of EDDHA per milliliter and did not fluoresce on KBA, MME, or CME under UV light (λ = 366 nm).

RESULTS

Disease suppression. Tobacco plants grew well in both vermiculite and illite soils in the absence of microorganisms (Table 2). In the presence of *T. basicola*, final root and shoot weight was significantly reduced, compared with that of uninoculated controls, and roots were covered with chlamydospores. No significant differences in root and shoot weights and disease incidence were observed between the two soils (Table 2). *P. fluorescens* CHA0 had no significant effect on the final weight of plants growing in the two soils in the absence of *T. basicola*. Strain CHA0 provided good protection against symptoms induced by *T. basicola* in the vermiculite system; the root and shoot weights were increased six and three and a half times, respectively, and the percentage of the root surface covered with chlamydospores was

reduced by half (Fig. 1 and Table 2). In the illite soil, CHA0 provided less protection (Fig. 1 and Table 2); however, the bacterium colonized the roots to the same extent in both soils (Table 2).

The addition of Fe^{3+} in the form of $FeCl_3$ or FeEDDHA to vermiculite did not affect plant growth in the presence or absence of either *T. basicola* or *P. fluorescens*. Added Fe^{3+} affected neither the percentage of the root surface infected with *T. basicola* nor the intensity of root colonization by *P. fluorescens* (Table 2). In contrast, the addition of Fe^{3+} to illite increased the capacity of *P. fluorescens* to suppress disease (Fig. 1). In the illite + $FeCl_3$ system, suppression was less than in vermiculite; in illite + FeEDDHA, it was as great as in vermiculite. The addition of iron to illite had no significant influence on the growth of plants inoculated or not with *T. basicola* or *P. fluorescens* alone. The percentage of the root surface covered with chlamydospores in the control infested with *T. basicola* and the intensity of root colonization by *P. fluorescens* were not influenced when different iron supplements were added to the soil (Table 2).

The transposon-insertion siderophore-negative mutant CHA400 suppressed symptoms caused by *T. basicola* to the same extent as did the wild-type strain CHA0 in the vermiculite, illite, and illite + $FeCl_3$ systems (Fig. 1 and Table 3). No significant differences in root and shoot fresh weights and disease incidence were observed between strain CHA0 and strain CHA400 in these systems when the bacteria were added to soil infested with *T. basicola*. However, in illite + FeEDDHA system, strain CHA400 gave less protection than strain CHA0. No difference was observed in the extent of root colonization by the two strains.

Survival of *T. basicola*. *T. basicola* survived well in the artificial soil in the absence of tobacco (Table 4). The number of propagules reisolated 1 and 8 days after infestation was about 60% of that of the added endoconidia. No significant differences were observed between the vermiculite and the illite systems with or without *P. fluorescens* CHA0 (Table 4).

Iron in soil water solution. The soil water solution of the vermiculite soil contained about 86 μM iron at the end of the experiment; that of the illite soil could not be determined accurately because of the small amount of iron (less than 1.6 μM). The presence of microorganisms had no significant effect on the iron content of the vermiculite and the illite soils.

Production of HCN. The production of HCN by *P. fluorescens* CHA0 was very low in the iron-deficient CME medium. After the addition of Fe^{3+}, HCN production was 60 to 70 μM (Fig. 2). The addition of vermiculite had the same effect as the addition of Fe^{3+}. Illite had little effect on cyanogenesis. The siderophore-negative mutant CHA400 produced the same amount of HCN as the wild-type strain in CME medium amended or not with illite or $FeCl_3$. When FeEDDHA + EDDHA was added, HCN production was low with strain CHA400 and high with strain CHA0. The two strains differed slightly in the amount of HCN produced in the medium amended with vermiculite. No differences were observed between the two strains with respect to their growth in the different media. The capacity of strains CHA0 and CHA400 to protect tobacco effectively in the different artificial soils was correlated with their capacity to produce large amounts of HCN in MME supplemented with the clay minerals or the iron form used in the artificial soils.

DISCUSSION

Competition for iron is supposed to be the mechanism by which fluorescent pseudomonads suppress diseases caused by *Pythium* spp., *Fusarium oxysporum*, and *Gaeumannomyces graminis* var. *tritici* (15–17,23,25,36). This theory is supported by several findings: Siderophore-negative mutants suppressed diseases less

TABLE 2. Suppression of tobacco black root rot, caused by *Thielaviopsis basicola* (strain D 127), by the addition of strain CHA0 of *Pseudomonas fluorescens* to artificial soil containing 10% clays and different iron supplements under gnotobiotic conditions

Clay	Iron supplement[v]	Microorganisms added[w] CHA0	Microorganisms added[w] D 127	Shoot fresh weight[x] (mg)	Root fresh weight[x] (mg)	Root surface infected[x,y] (%)	Fluorescent pseudomonads[x] (cfu/g)[z]
Vermiculite	None	−	−	518 ab	328 ab	0 a	0 a
		+	−	528 a	438 a	0 a	4×10^8 bc
		−	+	83 e	37 e	70 e	0 a
		+	+	288 c	220 bc	35 bc	2×10^9 b
Illite	None	−	−	422 b	293 ab	0 a	0 a
		+	−	467 ab	314 ab	0 a	4×10^8 bc
		−	+	80 e	35 e	72 e	0 a
		+	+	175 d	111 d	55 d	1×10^9 b
Vermiculite	$FeCl_3$	−	−	598 a	429 a	0 a	0 a
		+	−	542 a	563 a	0 a	7×10^7 c
		−	+	70 e	31 e	71 e	0 a
		+	+	279 c	214 bc	43 bcd	8×10^8 b
Vermiculite	FeEDDHA	−	−	528 a	291 b	0 a	0 a
		+	−	558 a	357 ab	0 a	5×10^8 bc
		−	+	112 e	38 e	79 e	0 a
		+	+	284 c	176 bc	44 cd	2×10^9 b
Illite	$FeCl_3$	−	−	523 ab	332 ab	0 a	0 a
		+	−	447 ab	268 b	0 a	8×10^8 b
		−	+	68 e	26 e	73 e	0 a
		+	+	264 c	146 cd	49 d	2×10^9 b
Illite	FeEDDHA	−	−	432 b	234 b	0 a	0 a
		+	−	330 bc	286 b	0 a	5×10^8 bc
		−	+	89 e	59 de	71 e	0 a
		+	+	311 bc	259 b	15 b	8×10^8 b

[v] 9.9 μg of Fe^{3+} per gram of dry soil as $FeCl_3 \cdot 6H_2O$ or chelated with ethylenediaminedi(*o*-hydroxyphenylacetic acid) (FeEDDHA).

[w] D 127 = *T. basicola*, and CHA0 = wild-type strain of *P. fluorescens*, added, respectively, at 6×10^3 and 6×10^6 colony-forming units per gram of soil, 8 and 7 days, respectively, before planting.

[x] Means within columns followed by the same letter are not significantly different at $P = 0.05$ according to the multiple *t*-test. Each value is the mean of three experiments, with eight to 10 replicates per experiment and one flask per replicate. Mean separations of root surface infection were calculated from data transformed to arc sines.

[y] Percentage of root surface darkened by the presence of chlamydospores of *T. basicola*.

[z] Colony-forming units per gram of roots.

effectively than wild-type strains (3,16,18); disease suppression occurred only in soil with low iron availability (15,23–25,36); the addition of iron in a form assimilable by the pathogen ($FeCl_3$ or chelated ethylenediaminetetraacetic acid, FeEDTA) reduced the capacity of the bacteria to suppress disease (15,23,25,36); purified iron-free siderophores inhibited fungal growth in vitro and suppressed disease when added to soil (15,21). However, many facts indicate that competition for iron is not the mechanism of suppression of tobacco black root rot, caused by *T. basicola*, in the gnotobiotic system: The siderophore-negative mutant CHA400 suppressed disease as effectively as the wild-type strain CHA0 in the soils containing vermiculite, illite, or illite amended with $FeCl_3$; strain CHA0 suppressed disease more effectively in the iron-rich soil (vermiculite) than in the iron-poor soil (illite); the addition of $FeCl_3$ to vermiculite did not reduce the capacity of the bacteria to suppress disease; the addition of $FeCl_3$ to illite increased it; iron-free siderophores did not inhibit the growth of *T. basicola* in vitro (1).

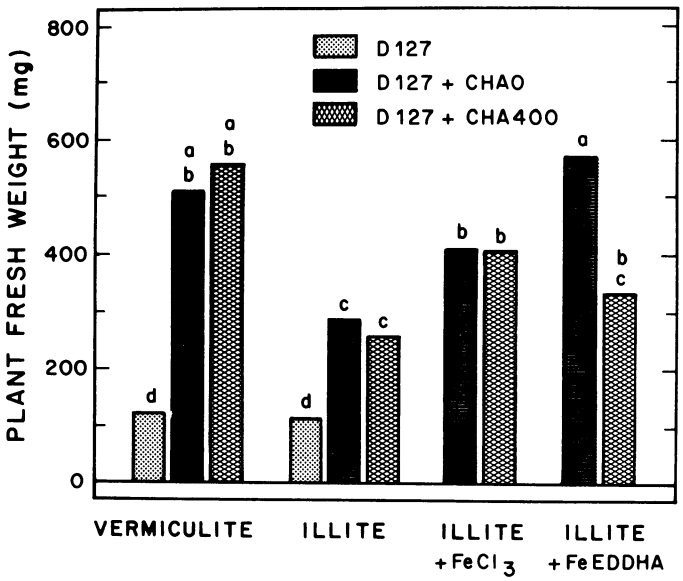

Fig. 1. Influence of iron and clay minerals on the suppression of tobacco black root rot, caused by *Thielaviopsis basicola* (strain D 127), by the addition of strains CHA0 and CHA400 (siderophore-negative mutant) of *Pseudomonas fluorescens*. Disease suppression is evaluated in terms of total plant fresh weight. Bars with the same letter are not significantly different at $P = 0.05$ according to the multiple *t*-test. Each value is the mean of three experiments, with eight to 10 replicates per experiment and one flask per replicate. For the weights of the uninoculated plants, see Table 2.

The fact that iron competition is not the mechanism of black root rot suppression is not surprising. The endoconidia of *T. basicola* may contain enough endogenous iron to initiate germination and infection. Chlamydospores of *F. oxysporum* contain little iron and are profoundly affected by limiting iron conditions, whereas chlamydospores of *F. solani*, with higher iron content, are not affected (9).

The addition of $FeCl_3$ to illite increased the capacity of strain CHA0 and its siderophore-negative mutant CHA400 to suppress disease. This indicates that the bacteria need sufficient iron to suppress disease. The mutant CHA400 was selected for its inability to use Fe^{3+} chelated with EDDHA. The addition of FeEDDHA to illite increased the capacity of strain CHA0, but not of strain CHA400, to suppress disease. This indicates that iron acts on the bacteria. The capacity of strain CHA0 to suppress disease was higher when Fe^{3+} was added as FeEDDHA than when it was added as $FeCl_3$. At pH 7, the pH of the soil water, the FeEDDHA remains in solution, whereas part of the Fe^{3+} added as $FeCl_3$ flocculates as tiny particles of Fe oxides. Some of them are adsorbed by the bacteria, which reduce the iron to make it available (27). The probability of a bacterium's meeting a molecule of iron given as $FeCl_3$ is less than that of meeting a molecule of iron given as FeEDDHA.

The siderophore-negative mutant CHA400, which was obtained after the insertion of transposon Tn*1733*, needs to be characterized further. However, the fact that its capacity to colonize roots and to suppress disease is similar to that of the wild-type strain, except in the presence of FeEDDHA, indicates that the mutant probably contains no gross defect other than the siderophore mutation.

TABLE 3. Comparison between the wild-type strain CHA0 of *Pseudomonas fluorescens* and its transposon-insertion siderophore-negative mutant, CHA400, for the suppression of black root rot of tobacco, caused by *Thielaviopsis basicola* (strain D 127) in two artificial soils containing different clay minerals and iron supplements

Clay	Iron supplement[v]	Microorganisms added[w] CHA0	CHA400	D 127	Shoot fresh weight[x] (mg)	Root fresh weight[x] (mg)	Root surface infected[x,y] (%)	Fluorescent pseudomonads[x] (cfu/g)[z]
Vermiculite	None	+	−	−	528 a	438 a	0 a	4×10^8 a
		−	+	−	534 a	424 ab	0 a	3×10^8 a
		+	−	+	290 bc	220 c	36 c	2×10^9 a
		−	+	+	314 b	241 c	39 bc	2×10^9 a
	$FeCl_3$	+	−	−	542 a	563 a	0 a	7×10^7 a
		−	+	−	544 a	488 a	0 a	1×10^8 a
		+	−	+	279 bc	214 c	43 bc	8×10^8 a
		−	+	+	282 bc	230 c	44 b	6×10^8 a
Illite	None	+	−	+	175 d	111 e	56 b	1×10^9 a
		−	+	+	154 d	100 e	64 b	2×10^9 a
	$FeCl_3$	+	−	+	264 c	146 cd	49 b	2×10^9 a
		−	+	+	248 c	159 cd	45 b	8×10^8 a
	FeEDDHA	+	−	+	311 b	259 bc	15 c	8×10^8 a
		−	+	+	216 cd	116 de	57 b	1×10^9 a

[v] 9.9 μg of Fe^{3+} per gram of dry soil as $FeCl_3 \cdot 6H_2O$ or chelated with ethylenediaminedi(*o*-hydroxyphenylacetic acid) (FeEDDHA).
[w] D 127 = *T. basicola*, CHA0 = wild-type strain of *P. fluorescens*, and CHA400 = transposon-insertion siderophore-negative mutant of strain CHA0, added, respectively, at 6×10^3, 6×10^6, and 6×10^6 colony-forming units per gram of soil, 8, 7, and 7 days, respectively, before planting. For the weights of uninoculated plants and plants inoculated with strain D 127 of *T. basicola* alone, see Table 2.
[x] Means within columns followed by the same letter are not significantly different at $P = 0.05$ according to the multiple *t*-test. Each value is the mean of three experiments, with eight to 10 replicates per experiment and one flask per replicate. Mean separations of root surface infection were calculated from data transformed to arc sines.
[y] Percentage of root surface darkened by the presence of chlamydospores of *T. basicola*.
[z] Colony-forming units per gram of roots.

TABLE 4. Influence of vermiculite, illite, and *Pseudomonas fluorescens* strain CHA0 on the survival of *Thielaviopsis basicola* under gnotobiotic conditions in artificial unplanted soil

Days after infestation[y]	Number of propagules reisolated per gram of soil ($\times 10^3$)[z]			
	Vermiculite − CHA0	Illite − CHA0	Vermiculite + CHA0	Illite + CHA0
1	5.1 ± 1.2	5.0 ± 3.0	5.8 ± 2.5	6.1 ± 1.9
8	6.7 ± 1.4	7.0 ± 2.3	4.7 ± 2.9	4.4 ± 2.0

[y] At day 0, 8.9×10^3 endoconidia were added to the soil.
[z] Mean and standard deviation of the mean of four experiments, with 10 replicates per experiment and one flask per replicate.

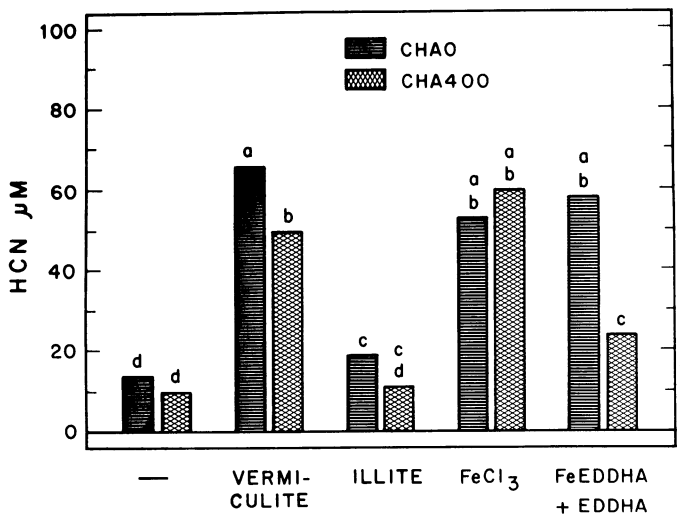

Fig. 2. Production of HCN by strain CHA0 of *Pseudomonas fluorescens* and its transposon-insertion siderophore-negative mutant CHA400 after 24 hr in minimal medium (CME) supplemented or not with 1 mg of vermiculite per milliliter, 1 mg of illite per milliliter, 20 μM $FeCl_3 \cdot 6H_2O$, or 20 μM Fe^{3+} chelated with ethylenediaminedi(*o*-hydroxyphenylacetic acid) (FeEDDHA) and 4 μM EDDHA at 28 C. Bars with the same letter are not significantly different at $P = 0.05$ according to the multiple *t*-test. Each value is the mean of four experiments, with three replicates per experiment and one bottle per replicate.

Sufficient iron is necessary for the bacteria to suppress disease effectively. As the iron supplement did not modify the capacity of the bacteria to colonize the roots, it seems likely that iron modifies bacterial metabolism. Strains CHA0 and CHA400, like other pseudomonads, need sufficient iron for the production of HCN (2,5,6). The capacity of strains CHA0 and CHA400 to protect plants effectively in the different gnotobiotic systems is correlated with their capacity to produce large amounts of HCN in MME supplemented with the clay mineral or the iron form used in the gnotobiotic system. This correlation and the toxicity of HCN for *T. basicola* (1) suggest a role for HCN in disease suppression. We are using genetically engineered bacteria to test this hypothesis (34).

Strain CHA0 suppressed black root rot more effectively in the vermiculite soil than in the illite soil. Vermiculite contains more Mg and Fe than illite. Mg is known to increase the resistance of tobacco to *T. basicola* (10). However, when the illite soil was supplemented with Mg, the capacity of *P. fluorescens* CHA0 to suppress disease did not increase (results not shown). When the illite soil was supplemented with Fe, the capacity of strain CHA0 to suppress disease was the same as in vermiculite with or without an iron supplement. Therefore iron seems to be the important factor in explaining the difference in disease-suppressive capacity between vermiculite and illite soils.

This hypothesis is sustained by two other facts: Vermiculite gives up more iron than illite through weathering, as shown by the iron content of the soil water solution at the end of the experiments; vermiculite gives up more iron to strain CHA0, as shown by the large amount of HCN produced in CME amended with vermiculite and the small amount in CME amended with illite. Cyanogenesis is regulated by iron, phosphate, and organic substances (2,5,6). Only iron is present in the clay minerals, and thus only it can be responsible for the increase in HCN production. Clay minerals give up iron by different mechanisms, for example, after reduction by *Pseudomonas* and other microorganisms (22,37) or after chelation by organic substances (19,22). The vermiculite used in our experiments was derived directly from rocks by weathering, whereas the illite was formed by neogenesis. This explains, in part, the difference in the iron content of the clays and the difference in the availability of iron in them. Vermiculite has a higher surface and exchange capacity than illite. However, when we increased the illite content of the soil, the capacity of the bacteria to colonize roots and suppress disease decreased (30).

The fact that strain CHA0 suppressed black root rot effectively in vermiculite but poorly in illite soil indicates the importance of the nature of the clay mineral in disease suppression.

Our results obtained in the gnotobiotic system using pure clay minerals and quartz grains seem to be relevant to natural conditions. Strain CHA0 was isolated from an iron-rich soil, and it suppressed disease in field trials and pot experiments more effectively in iron-rich soil than in iron-poor soil (7,29). When the conducive soil VC1 with its natural microflora was used in pot experiments (29), strain CHA400 gave the same degree of protection as strain CHA0 (results not shown), as observed in the artificial soil containing vermiculite or illite clay minerals. This indicates that iron competition is also not the mechanism of black root rot suppression under natural conditions.

The gnotobiotic system developed here has a number of advantages. No contaminating microorganisms interfere with the results; the chemical composition and structure of the soil can be standardized and analyzed; and no watering is necessary.

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