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Suppression of Root Diseases by *Pseudomonas fluorescens* CHA0: Importance of the Bacterial Secondary Metabolite 2,4-Diacetylphloroglucinol

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***Pseudomonas fluorescens* strain CHA0 suppresses *Thielaviopsis basicola*-induced black root rot of tobacco and *Gaeumannomyces graminis* var. *tritici*-induced take-all of wheat. Strain CHA0 produces 2,4-diacetylphloroglucinol, a metabolite with antifungal, antibacterial, and phytotoxic activity. The role of this compound in disease suppression was tested under gnotobiotic conditions. A *P. fluorescens* mutant, obtained by Tn*5* insertion, did not produce 2,4-diacetylphloroglucinol, showed diminished inhibition of *T. basicola* and of *G. g.* var. *tritici in vitro*, and had a reduced suppressive effect on tobacco black root rot and on take-all of wheat, compared with wild-type CHA0. Complementation of the mutant with an 11-kb DNA fragment from a genomic library of wild-type CHA0 largely restored production of the metabolite, inhibition of the fungal pathogens *in vitro* and disease suppression. The Tn*5* insertion was physically mapped using a 5.8-kb complementing fragment as a probe. 2,4-Diacetylphloroglucinol was shown to be produced in the rhizosphere of wheat by strain CHA0 and by the complemented mutant, but not by the mutant defective in 2,4-diacetylphloroglucinol synthesis. These results support the importance of 2,4-diacetylphloroglucinol production by strain CHA0 in the suppression of soilborne plant pathogens in the rhizosphere.**

Additional keywords: antibiotics, biological control, Tn*5* mutagenesis.

Suppression of soilborne plant pathogens by fluorescent pseudomonads (Baker 1985; Burr and Caesar 1984; Défago and Haas 1990; Kloepper *et al.* 1989; Schippers 1988; Weller 1988) depends on complex interactions between the pseudomonads and their biotic and abiotic environments. To function effectively as biocontrol agents, the fluorescent pseudomonads should have the ability to colonize the roots (Parke 1990; Weller 1988) and to produce certain secondary metabolites (Défago and Haas 1990; Kiprianova and Smirnov 1981; Leisinger and Margraff 1979). Evidence has been obtained for an involvement of two groups of metabolites, siderophores and antibiotic compounds, in pathogen suppression (reviewed by Défago and Haas 1990; Fravel 1988; Keel *et al.* 1990; Leong 1986; Loper and Buyer 1991; Weller 1988; Weller and Thomashow 1990).

Support for a role of bacterial antibiotics in the biological control of soilborne pathogens has come from a correlation of antibiotic production and pathogen inhibition *in vitro* and disease suppression *in vivo* (Ahl *et al.* 1986; Homma *et al.* 1989; Howell and Stipanovic 1979, 1980; Lambert *et al.* 1987). When purified antibiotics, e.g., pyoluteorin or pyrrolnitrin, isolated from the biocontrol agents, were applied to seeds (Homma and Suzui 1989; Howell and Stipanovic 1979, 1980) or fruits (Janisiewicz and Roitman 1988) disease symptoms were suppresssed. Recent approaches to demonstrating the role of antibiotic metabolites in disease suppression have been described in studies on chemically or genetically generated mutants that were defective in the production of specific compounds, e.g., oomycin A, pyrrolnitrin, pyoluteorin, phenazines, or cyanide, and these mutants were tested for disease suppression (Gutterson 1990; Gutterson *et al.* 1990; Homma and Suzui 1989; Kraus and Loper 1989, 1990; Thomashow and Weller 1988; Voisard *et al.* 1989). Thomashow and Weller (1988) have shown that Tn*5* insertion mutants of *Pseudomonas fluorescens* strain 2-79 defective in phenazine-1-carboxylic acid (PCA) production have a significantly reduced capacity to suppress take-all of wheat, a disease induced by *Gaeumannomyces graminis* var. *tritici*. PCA production and disease suppression are restored in the mutants by complementation with cloned PCA biosynthetic genes. Furthermore, PCA could be detected in the rhizosphere of wheat, i.e., at the site of take-all suppression (Thomashow *et al.* 1990). Although earlier work had indicated that the fluorescent siderophores (pyoverdine) produced by *P. fluorescens* strains contribute to the suppression of some root diseases (Leong 1986; Weller and Cook 1986) no or little activity could be ascribed to pyoverdine in biological control of take-all (Thomashow and Weller 1990).

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Our studies on *P. fluorescens* strain CHA0 are in line with the concept that secondary metabolites have a role in pathogen suppression. This strain was isolated from a Swiss soil that is naturally suppressive to tobacco black root rot caused by *Thielaviopsis basicola* (Stutz *et al.* 1986). Suppression of this disease by strain CHA0 occurs in iron-sufficient soils and depends on the nature of clay minerals (Keel *et al.* 1989; Stutz *et al.* 1985, 1986, 1989). Strain CHA0 also protects wheat from *G. g.* var. *tritici* in greenhouse and field experiments (Défago *et al.* 1990) and is active against a variety of other root diseases (Défago *et al.* 1990). Pyoverdine and several secondary metabolites with antibiotic properties, e.g., hydrogen cyanide, pyoluteorin, and 2,4-diacetylphloroglucinol (Phl) are produced by strain CHA0 (Ahl *et al.* 1986; Défago *et al.* 1990; Voisard *et al.* 1989). In a standardized gnotobiotic system (Keel *et al.* 1989) cyanide has been shown to be involved in the suppression of black root rot of tobacco (Voisard *et al.* 1989), whereas no significant effect on disease suppression was found for pyoverdine (Défago *et al.* 1990; Haas *et al.* 1991; Keel *et al.* 1989).

Furthermore, preliminary experiments have demonstrated that a Tn*5* insertion mutant of strain CHA0 having a defect in Phl synthesis is partially impaired in the suppression of black root rot of tobacco and take-all of wheat (Haas *et al.* 1991; Keel *et al.* 1990, 1991). We have now physically mapped the Tn*5* insertion in the Phl^- mutant; an 11-kb genomic fragment from the wild-type strain complements the mutation and essentially restores plant protection. We also report the detection of Phl in the rhizosphere of wheat colonized by strain CHA0 and we describe the inhibitory effects of Phl on bacteria, fungi, and plants.

MATERIALS AND METHODS

Organisms and culture conditions. *Pseudomonas fluorescens* (Trevisan) Migula wild-type strain CHA0 (Stutz *et al.* 1986) was cultivated in nutrient yeast broth (NYB; Stanisich and Holloway 1972), on nutrient agar (NA; Stanisich and Holloway 1972), King's medium B agar (KBA; King *et al.* 1954), or on malt agar (15 g of malt extract, 17 g of agar, Difco Laboratories, Detroit, MI, and 1 L of double-distilled water) and added to artificial soil (see below) as described earlier (Keel *et al.* 1989). *P. fluorescens* strains CHA625 (*phl-625*::Tn*5*; a Phl^- mutant; Keel *et al.* 1990) and CHA625/pME3128 (the complemented mutant, see below) were cultivated similarly, except for the addition of 25 μg/ml of kanamycin sulfate (Sigma Chemical, St. Louis, MO) and 125 μg/ml of tetracycline hydrochloride (Sigma), respectively, to NYB, NA, or KBA.

For recombinant DNA work, *Escherichia coli* ED8767 (*met hsdS recA56 supE supF*; Murray *et al.* 1977) was used as the host.

Thielaviopsis basicola (Berk. & Br.) Ferraris strain ETH D127 was cultivated on malt agar and added to artificial soil as described elsewhere (Keel *et al.* 1989). *Pythium debaryanum* Drechsler strain ETH D19 and *P. ultimum* Trow strain ETH 71 were grown on malt agar at 20° C. *Fusarium oxysporum* Schlecht f. sp. *lycopersici* (Sacc.) Snyd. & Hans strain FOL 15 (obtained from C. Alabouvette, INRA, Dijon, France), *F. o.* f. sp. *lini* (Bolley) Snyd. & Hans. strain CBS216.49, *Gaeumannomyces graminis* Arx & Olivier var. *tritici* Walker strain ETH 1000, and *Rhizoctonia solani* Kühn strain 160 (obtained from Ciba Geigy, Basel, Switzerland) were cultivated on potato-dextrose agar (PDA; Difco) at 24° C, unless otherwise specified. The following millet-seed inoculum of *G. g.* var. *tritici* was used to infest artificial soil: 25 g of millet seeds (1.2 mm diameter; Biofarm, Kleindietwil, Switzerland) was added to 300-ml flasks and autoclaved twice at 121° C. Three 0.6-cm plugs from the 14-day-old fungal culture were transferred to each flask and incubated at 24° C for 21 days.

Surface-disinfestation of tobacco seeds (*Nicotiana glutinosa* L.) and cultivation of seedlings on modified Knop nutrient solution agar were described previously (Keel *et al.* 1989). Seeds of wheat (*Triticum aestivum* L. 'Arina') were surface-disinfested in 5% sodium hypochlorite for 5 min, rinsed with sterile double-distilled water and then germinated for 2 days on 0.85% water agar (Difco). Plants were grown in growth chambers containing 70% relative humidity; for tobacco, incubation was at 22° C with light (80 $\mu E\ m^{-2}\ sec^{-1}$) for 16 hr, followed by an 8-hr dark period at 18° C; for wheat, incubation was at 18° C with light (160 $\mu E\ m^{-2}\ sec^{-1}$) for 16 hr, followed by an 8-hr dark period at 13° C.

Identification of secondary metabolites produced by *P. fluorescens* strain CHA0. Strain CHA0 was incubated on malt agar for 7 days at 27° C. The content of 10 petri dishes was extracted with 80% aqueous acetone (100 ml). The filtered solution was reduced *in vacuo* to one tenth of its initial volume and centrifuged at 6,000 × *g*. Ten milliliters of 5% aqueous NaCl was added to the supernatant. The mixture was extracted three times with ethyl acetate whereupon the organic solvent was removed at 45° C under vacuum. Preparative thin-layer chromatography (TLC) of the remaining oil on silica gel (Merck 60 F_{254}) with $CHCl_3$MeOH (100:1, v/v) gave two crude antibiotic fractions at $R_f = 0.08$ and $R_f = 0.18$ according to bioautographic monitoring with *Bacillus subtilis*. Repetition of preparative TLC with the more polar eluent mixture $CHCl_3$MeOH (19:1, v/v) gave 35 mg of the antibiotic A ($R_f = 0.2$) and 11 mg of B ($R_f = 0.5$). Analytical samples of high purity were obtained by final preparative high-performance liquid chromatography (HPLC) (Kontron MSI-660-T liquid chromatograph) on a Merck Lichrosorb Si60 column ($CHCl_3$MeOH 9:1, v/v) with UV detection at 254 nm. Compound A is optically inactive. It gave a positive Gibbs test for phenols and was shown by ^{1}H-NMR, ^{13}C-NMR, IR and mass spectrometry to be identical with an authentic sample of 2,4-diacetyl-1,3,5-trihydroxybenzene (2,4-diacetylphloroglucinol) prepared according to Campbell and Coppinger (1951). NMR-spectra were obtained on a Varian FT-NMR spectrometer XL-200 operating at 4.7 tesla. Mass spectra were recorded on a Finnigan-4023 spectrometer. Synthetic Phl (purity grade >97%) was obtained as slightly orange crystals of mp 166–167° C (according to Campbell and Coppinger 1951: 168° C). ^{1}H-NMR (CD_3OD, 200 MHz): 5.81(s,H-C(6),1H), 2.62(s,Ac,6H). ^{13}C-NMR (CD_3OD, 50 MHz): 205.1(CO of Ac), 172.5(C(3)OH), 170.1(C(1)OH/C(5)OH), 104.8(C(2)/C(4)), 95.59(C(6)H), 32.94(CH_3 of Ac). Mass

spectrum (electron impact 70 eV) m/z (rel. intensity): 210(51,M+), 195(100), 177(61), 149(17), 69(77), 67(35), 57(38), and 55(35). Compound B is also optically inactive and it gave a positive Gibbs test for phenols, too. High-resolution mass spectrometry suggested the molecular formula $C_{11}H_7Cl_2NO_3$. The compound was shown by IR, ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry to be identical with an authentic sample of 4,5-dichloro-2-(2′,6′-dihydroxybenzoyl)-pyrrole (pyoluteorin; purity grade 87%) synthesized according to Cue *et al.* (1981).

Extraction and analytical detection of metabolites. Bacterial cultures grown on malt agar (for Phl production) or King's B agar (for pyoluteorin production) at 27° C or 18° C for 3 days were extracted with 80% aqueous acetone according to Howell and Stipanovic (1979). The extracts were condensed by removal of the acetone *in vacuo*. The aqueous concentrates were acidified to pH 2 with 2 M HCl and extracted with ethyl acetate. The ethyl acetate extracts were reduced to dryness *in vacuo*. The residue was dissolved in 1 ml of 65% (v/v) methanol; 50-μl samples were chromatographed on TLC plates (Silica gel 60 F_{254}, Merck) with toluene-acetone (4:1, v/v) and sprayed with 1% (w/v) vanillin in sulfuric acid (95-97%). R_f values were 0.41 for Phl and 0.31 for pyoluteorin. For analytical HPLC, 5–10 μl of the extracts was analyzed by a Hewlett Packard 1090 liquid chromatograph, using a reverse-phase column (4 × 100 mm) packed with Nucleosil 120-5-C18 (Macherey-Nagel, Oensingen, Switzerland), which was thermostatically controlled at 45° C. The samples were eluted with a three-step linear gradient of methanol from 18 to 23% (0–5 min), 23 to 53% (5–6 min), and 53 to 68% (5–15 min) in 0.43% (v/v) *o*-phosphoric acid (pH 2.8), with a flow rate of 1 ml/min. Specific components were detected by an UV diode-array-detector at 270 nm for Phl and at 313 nm for pyoluteorin. The retention times of authentic samples of Phl and pyoluteorin were about 12.2 and 9.6 min, respectively. Peak areas were converted to micrograms of the metabolites from a calibration curve prepared by injecting known amounts of the synthetic compounds. The detection limit was 5 ng for Phl and 50 ng for pyoluteorin with a 10-μl-injection volume.

Fungal inhibition assay. Inhibition of *T. basicola, F. o.* f. sp. *lycopersici*, and *G. g.* var. *tritici* by strain CHA0 and its derivatives *in vitro* was assayed on malt agar plates as described by Thomashow and Weller (1988). Samples (5 μl containing approximately 5 × 10^6 cfu) from overnight bacterial cultures in NYB were spotted on plates at 1 cm from the edge. One day later a 0.6-cm plug from the fungal culture grown at 24°C for 14 days was placed in the center of the plate. Plates were incubated at 27° C and fungal inhibition was assessed after 14 days by measuring the distance between the edges of the bacterial colony and the fungal mycelium. Inhibition was expressed as the percentage of inhibition obtained with the wild-type CHA0, which had been spotted on the same plate.

Toxicity of 2,4-diacetylphloroglucinol. Synthetic Phl was dissolved in EtOH and added to the media at concentrations ranging from 0 to 1,024 μg/ml. All media including controls without the compound contained 0.1% (v/v) EtOH. In each test, a freshly prepared Phl solution was used.

Toxicity to fungi. Each 0.6-cm plug of 3-wk-old malt agar or PDA fungal cultures was transferred to the center of a malt agar plate and grown at 27° C in the dark. After incubation for 7 days the radial mycelial growth was measured. For endoconidia germination of *T. basicola* a suspension was prepared (Keel *et al.* 1989) and immediately spread on malt agar. The malt agar plates were incubated at 24° C in the dark for 3 hr and assessed for the percentage of germinated endoconidia.

Toxicity to bacteria. Samples (5 μl) of 10^{-4} diluted overnight NYB cultures of the bacterial strains (Table 1; obtained from Department of Microbiology, ETH, Zürich, Switzerland) were inoculated into 150 μl of NYB in microtiter plates. The medium was checked for turbidity after incubation at 32° C for 72 hr.

Toxicity to seed germination. Untreated seeds of cress (*Lepidium sativum* L.) and flax (*Linum grandiflorum* L.) and surface-sterilized seeds of cotton (*Gossypium hirsutum* L.; pentachloronitrobenzene treated), cucumber (*Cucumis sativa* L.), and tomato (*Lycopersicon esculentum* Mill.) (1% sodium hypochlorite for 30 min), tobacco and wheat were spread on 0.85% water agar (Difco). Incubation was in a growth chamber under the conditions described for the cultivation of tobacco. After 3–7 days, the percentage of germinated seeds was recorded.

Toxicity to plant growth. Seedlings with similar root length of cucumber, flax, corn (*Zea mays* L.), and sweet corn (*Zea mays* L. convar. *saccharata*) (4 days old), of tobacco and tomato (7 days old), and of wheat (2 days old) grown on 0.85% water agar (Difco) were transferred into sterile plant tissue culture containers (Flow Laboratories, McLean, VA), or in the case of tobacco to petri dishes, each containing modified Knop nutrient solution agar (Keel *et al.* 1989). Cress was not pregerminated. Corn, cress, cucumber, sweet corn, and wheat were grown for 7 days, flax, tobacco, and tomato for 18, 21, and 14 days, respectively, in the growth chamber, washed, and weighed.

Recombinant DNA techniques. Plasmid isolation by alkaline lysis, restriction, DNA fragment isolation from low-melting agarose, ligation, transformation of *E. coli*, and Southern blots were all done by standard methods (Maniatis *et al.* 1982). Chromosomal DNA was isolated by the procedure of Lewington *et al.* (1987). A genomic library of strain CHA0 has previously been constructed in the cosmid pVK100 (Voisard *et al.* 1989). Recombinant cosmids were mobilized from *E. coli* to *P. fluorescens* strain CHA625 (*phl*::Tn*5*) with the helper plasmid pME497 (Voisard *et al.* 1988); selection was on NA with tetracycline (125 μg/ml) and kanamycin (25 μg/ml). The transconjugants thus obtained were tested for complementation of the *phl* mutation by monitoring growth inhibition of *F. oxysporum* on malt agar plates as described above.

Suppression of black root rot and take-all in the gnotobiotic system. The system for testing suppression of tobacco black root rot has been previously described in detail (Keel *et al.* 1989); it was slightly modified. Artificial soil was made up from pure vermiculite clay (expanded with 30% H_2O_2), quartz sand, and quartz powder (10/70/20, w/w) and moistened with 10% (w/w) double-distilled water (Keel *et al.* 1989). For tobacco 60 cm^3 of artificial soil was filled per 100-ml flask. For wheat 400 cm^3 of

artificial soil was placed per 1-L flat-bottomed flask with an opening of 5 cm in diameter. The flasks were sealed with cotton wool stoppers and autoclaved at 121° C for 30 min. Bacterial supensions were added to flasks to give 10^7 cfu of *P. fluorescens* per cubic centimeter of artificial soil. Control flasks were watered with the same volume (3 ml for tobacco and 12 ml for wheat) of sterile double-distilled water. One and seven days later, respectively, *T. basicola* (5×10^3 endoconidia per cubic centimeter of artificial soil; Keel *et al.* 1989) and *G. g.* var. *tritici* (1.25 mg of millet-seed inoculum per cubic centimeter of artificial soil) was mixed into the artificial soil. Control flasks were amended with the same amount of sterile millet seed. Seven days after the addition of the pathogen, one sterile-grown tobacco plant (5 wk old, four leaves) or five sterile-grown wheat seedlings (2 days old) were transplanted into each flask and supplemented, respectively, with 3 and 15 ml of modified Knop nutrient solution (Keel *et al.* 1989). No further watering was necessary. The flasks were incubated in randomized complete blocks in growth chambers under the same conditions described earlier. After 3 wk plants were washed, weighed, and assessed for bacterial root colonization (Keel *et al.* 1989). Black root rot severity was evaluated as described before (Keel *et al.* 1989; Stutz *et al.* 1986). Take-all incidence was rated on a 0 to 4 scale (Weller and Cook 1983, modified; 0 = no disease, 1 = less than 25% of the roots with lesions, 2 = 25–100% of the roots with lesions, 3 = lesions at the base of the tillers, 4 = plants dead).

Extraction of 2,4-diacetylphloroglucinol from the rhizosphere of wheat. After 3 wk of growth in the take-all suppression gnotobiotic system (see above), plants were removed by pushing out the contents of the flasks. Excess and loosely adhering artificial soil was shaken gently from the roots. Portions (200 g) of wheat roots with closely adhering artificial soil from each treatment were vigorously shaken for 30 min in 500-ml volumes of ethyl acetate (acidified to pH 2.0 with 2 M HCl). The extracts of each treatment were pooled and reduced to dryness *in vacuo*. The efficiency of recovery by this procedure was estimated to be 40–50% on the basis of samples in which 50 μg of synthetic Phl was added per gram of artificial vermiculite soil (data not shown). The residues were redissolved in methanol and analyzed by HPLC as described above with the exception of using a Nucleosil 120-5-C8 column (4 × 250 mm) and eluting the samples with a modified gradient of methanol from 20 to 42% (0–8 min), 42 to 45% (8–14 min) and 45 to 100% (14–25 min). The retention time was 20.6 min for Phl.

Statistics. Each plant protection experiment in the gnotobiotic system was repeated at different times; means of three to six independent experiments are presented. Each mean was compared with all other means by Student's *t* test (multiple *t* test; Keel *et al.* 1989), if experiments were not significantly different in an *F* test.

RESULTS

Identification and characterization of 2,4-diacetylphloroglucinol. *P. fluorescens* strain CHA0 grown on malt agar produced two antibiotics that could be identified in ethyl acetate extracts as Phl and pyoluteorin by standard spectroscopic and chromatographic means. Whereas malt agar proved suitable for the production of Phl, subsequent experiments showed that better yields of pyoluteorin were obtained when King's medium B plates were used instead of malt agar. Phl was synthesized chemically. Synthetic Phl exhibited moderate antibiotic activity against several soilborne fungal pathogens: *G. g.* var. *tritici* appeared to be the most sensitive fungus (Table 1). The compound showed high antibiotic activity against some species of bacteria, especially *Bacillus subtilis, B. thuringiensis, Micrococcus luteus, Pseudomonas syringae* pv. *phaseolicola, P. s.* pv. *tabaci*, and *Staphylococcus aureus* (Table 2). In contrast, strains of the *P. fluorescens-putida* group, including strain CHA0 and its Phl^- mutant CHA625, and *P. aeruginosa* were quite insensitive to the drug (Table 2). The effect of Phl on plants was then tested. In general, Phl was more toxic to dicotyledonous than to monocotyledonous plants in terms of growth and germination, cucumber being an exception (Table 3). Cress turned out to be sensitive to Phl at a concentration that

Table 1. Antifungal activity of 2,4-diacetylphloroglucinol (Phl)

Test fungus	MICs (μg/ml)[a] I_{50}	I_{100}
Fusarium oxysporum f. sp. *lycopersici*	16	128
Fusarium oxysporum f. sp. *lini*	32	128
Gaeumannomyces graminis var. *tritici*	16–32	64
Pythium debaryanum	64	128
Pythium ultimum	64	128
Rhizoctonia solani	32–64	128
Thielaviopsis basicola[b]	32–64	128

[a] MICs are defined as the minimal amount of Phl causing 50% (I_{50}) or total (I_{100}) inhibition of fungal growth on malt agar within 7 days. Concentrations of Phl were varied by twofold dilutions.
[b] In addition, germination of endoconidia of *T. basicola* was completely inhibited at 256 μg/ml on malt agar.

Table 2. Antibacterial activity of 2,4-diacetylphloroglucinol (Phl)

Test bacterium	MIC (μg/ml)[a]
Bacillus subtilis[b]	5
Bacillus thuringiensis[b]	5
Enterococcus faecalis[b]	50
Erwinia carotovora pv. *carotovora*[b]	250
Escherichia coli[b]	500
Klebsiella aerogenes[b]	500
Micrococcus luteus[b]	10
Pseudomonas aeruginosa[b]	>1,000
Pseudomonas fluorescens strain CHA0 (Phl^+)	>1,000
Pseudomonas fluorescens strain CHA625 (Phl^-)	1,000
Pseudomonas fluorescens strain P3[c]	500
Pseudomonas putida[b]	>1,000
Pseudomonas syringae pv. *phaseolicola*[b]	5
Pseudomonas syringae pv. *tabaci*[b]	5
Staphylococcus aureus[b]	5
Streptomyces echinatus[b]	250
Streptomyces lividans[b]	5

[a] MIC is defined as the minimal amount of Phl causing total inhibition of bacterial growth at 32°C in NYB, within 72 hr. Concentrations of Phl tested were: 5, 10, 50, 100, 250, 500, and 1,000 μg/ml.
[b] These strains were obtained from the collection of the Department of Microbiology, ETH, Zürich, Switzerland.
[c] Voisard *et al.* (1989).

was only partially inhibitory to most fungi tested (Table 3). These results are in accordance with earlier data that demonstrated Phl to be more toxic to growth and germination of tobacco than to growth and endoconidia germination of *T. basicola*, whereas the compound was less inhibitory to growth of wheat than to that of *G. g.* var. *tritici* (Keel *et al.* 1990, 1991).

Physical mapping of a *phl*::Tn*5* mutation and complementation by a recombinant cosmid. In a previous study (Keel *et al.* 1990) we described the isolation of a *P. fluorescens* mutant defective in the production of Phl. This mutant (CHA625) was obtained by Tn*5* mutagenesis and found by screening 1,800 transposon insertion mutants of strain CHA0 for reduced growth inhibition of *F. o.* f. sp. *lycopersici* on malt agar. Strain CHA625 also showed reduced inhibition of *T. basicola* and *G. g.* var. *tritici* on malt agar (Table 4) but was still able to produce other antimicrobial compounds such as HCN and pyoluteorin. Moreover, strain CHA625 gave wild-type growth rates in NYB batch cultures and synthesized pyoverdine normally (data not shown). Genomic DNA from strain CHA625 was digested with *Eco*RI and hybridized to a Tn*5*-internal *Hpa*I fragment. Only one signal was obtained, indicating that this strain carried a single Tn*5* insertion (data not shown).

Mobilization of a genomic library (established in the broad-host-range cosmid pVK100) to strain CHA625 led to the isolation of pME3101, which restored Phl production in the mutant (Keel *et al.* 1990). Plasmid pME3101 contained a 22-kb insert of genomic DNA from strain CHA0. Partial digestion of pME3101 with *Hin*dIII produced pME3128, which had an 11-kb genomic insert on a single *Hin*dIII fragment (Fig. 1). Plasmid pME3128 brought back Phl production to wild-type levels on malt agar at 27° C after 2 days, but only partially restored fungal growth inhibition on the same medium (Table 4). In the fungal inhibition test, incubation on malt agar was for 7 days and after this time about 10% of the CHA625/pME3128 transconjugants had lost the plasmid. This plasmid instability might be a reason for the incomplete restoration of inhibition. Accumulation of Phl on malt agar was also measured at 18° C (Table 4). This temperature cor-

Table 3. Phytotoxic activity of 2,4-diacetylphloroglucinol (Phl)

Plant tested	MICs (μg/ml)[a] I_{50}	I_{100}
On plant growth		
Corn	16–32	>256
Cress	8–16	64
Cucumber	32	>256
Flax	32	256
Sweet corn	16–32	>256
Tobacco	8	128
Tomato	16	256
Wheat	32–64	>1,024
On seed germination		
Cotton	256–512	512
Cress	16	64
Cucumber	128–256	>256
Flax	16	32
Tobacco	8–16	32
Tomato	8–16	64
Wheat	>1,024	>1,024

[a]MICs are defined as the minimal amount of Phl causing 50% (I_{50}) or total (I_{100}) inhibition of plant growth on Knop nutrient solution agar and of seed germination on 0.85% water agar, respectively, as described in the text. In each experiment, Phl concentrations were varied by twofold dilutions.

Table 4. Production of 2,4-diacetylphloroglucinol (Phl) and inhibition of *Thielaviopsis basicola, Gaeumannomyces graminis* var. *tritici* and *Fusarium oxysporum* f. sp. *lycopersici* by *P. fluorescens* strain CHA0 and its derivatives on malt agar

Strain	Production of Phl[a] (μg/ml) 27° C	18° C	Relative inhibition[b] of: *T. basicola*	*G. graminis*	*F. oxysporum*
CHA0	6.6 a	2.0 a	1.00 a	1.00 a	1.00 a
CHA625	<0.02	<0.02	0.67 c	0.12 c	0.43 c
CHA625/pME3128	7.4 a	0.4 b	0.75 b	0.75 b	0.79 b

[a]The amount of Phl produced was determined for cultures grown for 48 hr on malt agar and extracted as described in the text. Each value is the mean of three independent experiments with one plate per experiment.

[b]Inhibition of fungal growth on malt agar at 27° C was measured after 7 days as described in the text and is expressed relative to the inhibition by wild-type CHA0. Each value is the mean from four independent experiments with three plates per experiment. Means within columns followed by the same letter are not signficantly different at $P = 0.05$ according to Student's *t* test.

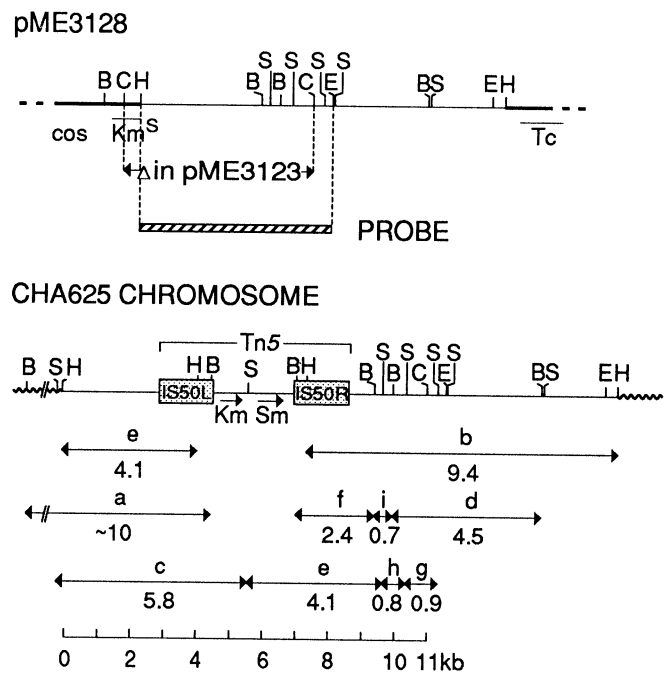

Fig. 1. Physical mapping of the *phl*::Tn*5* insertion of strain CHA625. The recombinant cosmid pME3128 complemented strain CHA625 for Phl synthesis. The 5.7-kb *Cla*I fragment of pME3128 was deleted, giving pME3123; this plasmid was Phl⁻. The 5.8-kb *Hin*dIII-*Eco*RI fragment marked "probe" was purified from low-melting agarose, labeled with 40 μCi[α-^{32}P]dCTP in the random priming reaction kit RPN.1600Y (Amersham, Arlington Heights, IL) and used in the hybridization experiment shown in Figure 2. —, *phl* region of strain CHA0; —, vector pVK100 having a *cos* site; ∿∿, genomic DNA of strain CHA0 outside the *phl* region. Restriction sites are: B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I. Resistance determinants are Km, kanamycin, Tc, tetracycline; Sm, streptomycin. Letters above arrows indicate restriction fragments visualized in Figure 2. Fragments sizes are in kilobases.

responded to that used in plant experiments, whereas 27° C was optimal for Phl production *in vitro*.

Plasmid pME3128 was digested with *Cla*I and religated. The resulting construct pME3123 (Fig. 1) was unable to complement strain CHA625 for Phl synthesis. This indicated that at least part of the *phl* gene region was located in the left half of the pME3128 insert and, therefore, a 5.8-kb *Hin*dIII-*Eco*RI fragment covering this region (Fig. 1) was chosen as a hybridization probe to locate the Tn*5* insertion in strain CHA625. A Southern blot (Fig. 2) showed that pME3128 carried authentic CHA0 DNA (lanes 1/7, 3/8, and 5/9) and that the Tn*5* insertion in strain CHA625 had occurred in the genomic region homologous to the probe. The site of the Tn*5* insertion could be mapped to position 2.9 kb from the left *Hin*dIII site, as indicated by the diagnostic fragments e, f, and c (Fig. 2, lanes 2, 4, and 6, respectively). These results show that the Phl$^-$ phenotype of strain CHA625 is caused by this Tn*5* insertion.

Role of 2,4-diacetylphloroglucinol in disease suppression. Preliminary experiments have indicated that strain CHA625 is less effective than the wild-type strain CHA0 in protecting tobacco from black root rot and wheat from take-all (Keel *et al.* 1990, 1991). We have now carried out a detailed quantitative analysis of the effect which the *phl*::Tn*5* mutation has on disease suppression under gnotobiotic conditions. In the presence of *T. basicola* or *G. g.* var. *tritici*, the final plant and root weights were drastically reduced, compared with those of uninoculated controls, and plants were heavily infected (Tables 5 and 6). The wild-type CHA0 provided good protection against symptoms induced by the pathogens: Plant and root weights were not significantly different from those of uninoculated controls, and disease incidence was more than four times lower (Tables 5 and 6). The Phl$^-$ mutant CHA625 afforded a significantly lower degree of protection. In the case of tobacco the infected root surface was as extensive as in the control (*T. basicola* infection without bacteria), and plant and root weights were three and four times lower, respectively, as compared to plants treated with wild-type CHA0 (Table 5). In wheat, plant and root weights were about 20% lower than those of plants protected by strain CHA0 (Table 6). This suggests that the protective effect of Phl is higher for tobacco than for wheat. In strain CHA625 carrying the recombinant cosmid pME3128 (*phl*$^+$) the suppressive capacity was restored (Tables 5 and 6) in terms of both plant weight and disease severity, although not to the full extent. Incomplete restoration of suppressive capacity and of fungal inhibition *in vitro* correlated (Tables 4–6). In the gnotobiotic system the complementing plasmid pME3128 was not entirely stable, i.e., as no antibiotic selection was possible. After incubation for 4 wk in the tobacco system or 5 wk in the wheat system, 12–18% of the CHA625 transconjugants had lost the plasmid as indicated by the loss of the tetracycline resistance phenotype.

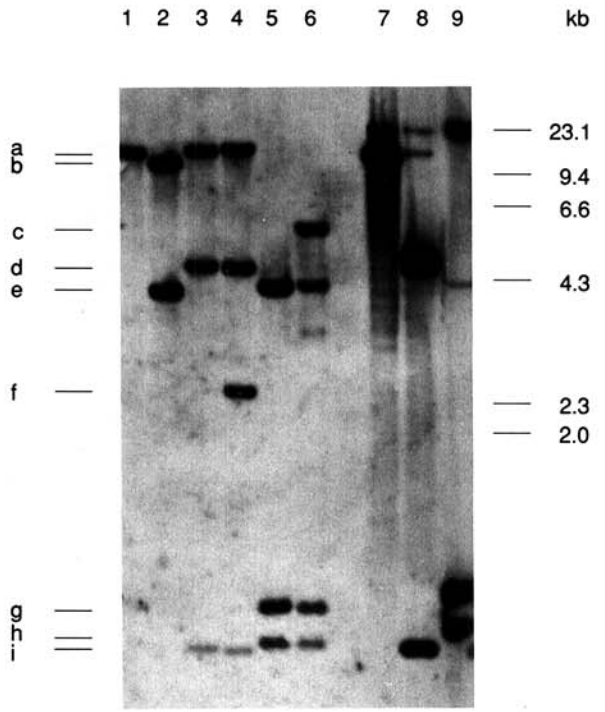

Fig. 2. Southern hybridization of *phl* genomic region. Chromosomal DNA (~3 μg) from strains CHA0 and CHA625 and DNA of the recombinant cosmid pME3128 was digested with *Hin*dIII, *Bgl*II, or *Sal*I, electrophoresed in a 0.7% agarose gel (45 V, 16 hr), transferred to a nitrocellulose membrane, and hybridized to the probe (the 5.8-kb *Hin*dIII-*Eco*RI fragment) shown in Figure 1 according to the procedure of Maniatis *et al.* (1982). Lane 1, CHA0/*Hin*dIII; lane 2, CHA625/*Hin*dIII; lane 3, CHA0/*Bgl*II; lane 4, CHA625/*Bgl*II; lane 5, CHA0/*Sal*I; lane 6, CHA625/*Sal*I; lane 7, pME3128/*Hin*dIII; lane 8, pME3128/*Bgl*II; lane 9, pME3128/*Sal*I. Bands a to i correspond to fragments a to i in Figure 1.

Because Phl has herbicidal effects on tobacco and, to a lesser extent, on wheat (Table 3), it was of interest to see whether the wild-type strain CHA0 affects plant growth in the absence of a pathogen. The controls performed without *T. basicola* or *G. g.* var. *tritici* show that the fresh weights of tobacco and wheat were not influenced by bacterial inocula (CHA0, CHA625, CHA625/pME3128) (Tables 5 and 6); these findings are in agreement with earlier results (Voisard *et al.* 1989; Keel *et al.* 1991). We conclude that the amount of Phl produced in the rhizosphere was insufficient to cause significant inhibition of plant growth.

The colonization ability of the bacterial strains was also checked. In the tobacco and in the wheat system all bacterial strains colonized the roots to the same extent (1.2–5.8 × 10^8 cfu and 0.7–1.4 × 10^8 cfu, respectively, per gram fresh weight at the end of the experiment; Tables 5 and 6).

Production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. After growth in the presence of strain CHA0, wheat roots with the closely adhering artificial soil were extracted with ethyl acetate. In the extract a compound was found that comigrated with synthetic Phl after separation by TLC (data not shown) and HPLC (Fig. 3A). Comparison of spectra (Fig. 3B) and peak purity analysis (data not shown) showed the compound to be homogeneous and indistinguishable from synthetic Phl; this was confirmed by coinjection of extracted and synthetic Phl. Phl was produced by strain CHA0 at concentrations of 0.94–1.36 μg per gram of roots (Table 6). The presence of *G. g.* var. *tritici* had no significant influence on the recovery of Phl. No antibiotic was detected in rhizosphere extracts of wheat grown in the presence of the Phl$^-$ mutant CHA625 or without addition of bacteria. Distinctly less antibiotic was produced by strain CHA625 carrying the

plasmid pME3128, which complements the strain for Phl production *in vitro* (Table 6). Suppression of take-all of wheat was correlated with Phl production by strain CHA0 in the rhizosphere of wheat: Roots from which the antibiotic was recovered had significantly higher weights and less disease than roots from which the antibiotic could not be detected (Table 6).

DISCUSSION

The results reported here provide further evidence that, in addition to cyanide (Keel *et al.* 1989; Voisard *et al.* 1989), Phl is another factor involved in disease suppression by *P. fluorescens* strain CHA0. In accordance with preliminary findings (Keel *et al.* 1990, 1991), strain CHA625, the Phl^- mutant, was impaired in plant protection (Tables 5 and 6). Pot experiments with natural field soil confirmed the reduced antagonistic activity of strain CHA625 against take-all of wheat (Wüthrich 1991). The reduced suppressiveness of the Phl^- mutant cannot be attributed to its reduced ability to maintain effective populations in the rhizosphere since the mutant and the parental strain did not differ significantly in root colonization (Tables 5 and 6; Wüthrich 1991). This is in agreement with studies on other biocontrol rhizobacteria where mutants deficient in antibiotic or siderophore production were not affected in their root colonization capacity (Bakker *et al.* 1987; Loper 1988; Thomashow and Weller 1988). However, Thomashow and Pierson (1991) have obtained evidence that bacterial phenazine antibiotic production does contribute to the long-term survival of *P. fluorescens* strains 2-79 and 30-84 in soil habitats.

In our study Phl production *in vitro* and in the rhizosphere of wheat, pathogen inhibition *in vitro* and plant protection were coordinately restored when CHA625 carried the cosmid pME3128, although not to the wild-type extent (Tables 4–6). Molecular evidence for a key role of antibiosis in disease suppression has also come from recent studies on Tn*5* mutants of *P. fluorescens* 2-79 and *P. aureofaciens* 30-84, which are deficient in the synthesis of a phenazine antibiotic and provide significantly less protection against take-all disease of wheat than do their

Table 5. Influence of 2,4-diacetylphloroglucinol (Phl) production by *Pseudomonas fluorescens* strain CHA0 on the suppression of *Thielaviopsis basicola*-induced black root rot of tobacco under gnotobiotic conditions

Microorganisms added[a]		Plant fresh weight[b] (mg)	Root fresh weight[b] (mg)	Root surface infected[b,c] (%)	Fluorescent pseudomonads[b] (10^8 cfu per g of root)
P. fluorescens	*T. basicola*				
None	−	782 a	398 ab	0 c	0 b
	+	114 c	23 d	86 a	0 b
CHA0 (Phl^+)	−	754 a	418 a	0 c	1.2 a
	+	732 a	391 ab	17 b	3.0 a
CHA625 (Phl^-)	−	725 a	385 ab	0 c	1.8 a
	+	242 b	95 c	71 a	5.8 a
CHA625/pME3128 (Phl^+)	−	748 a	417 ab	0 c	1.4 a
	+	560 a	267 b	31 b	3.1 a

[a] *T. basicola*, CHA0 (= wild-type strain of *P. fluorescens*), and its derivatives CHA625 (= Tn*5*-insertion Phl-negative mutant), and CHA625/pME3128 (= transconjugant of CHA625, restored in Phl-production), were added, respectively, at 5×10^3 endoconidia, and 10^7 cfu/cm^3 of artificial soil, 6 and 7 days, respectively, before planting.

[b] Means within columns followed by the same letter are not significantly different at $P = 0.05$ according to Student's t test. Each value is the mean of three independent experiments with 10 replicates per experiment and one flask (one plant) per replicate (see text).

[c] Percentage of root surface darkened by the presence of chlamydospores of *T. basicola* (Keel *et al.* 1989; Stutz *et al.* 1986).

Table 6. Production of 2,4-diacetylphloroglucinol (Phl) by *Pseudomonas fluorescens* strain CHA0 and its derivatives in the rhizosphere of wheat grown under gnotobiotic conditions and relationship between antibiotic production and suppression of *Gaeumannomyces graminis* var. *tritici*-induced take-all by the bacteria

Microorganisms added[a]		µg of Phl[b] per g		Plant fresh weight[d] (mg)	Root fresh weight[d] (mg)	Disease rating[d,e]	Fluorescent pseudomonads[d] (10^8 cfu per g of root)
P. fluorescens	*G. graminis*	Rhizosphere[c]	Root				
None	−	<0.001	<0.01	598 ab	320 a	0 e	0 b
	+	<0.001	<0.01	318 d	156 c	3.1 a	0 b
CHA0 (Phl^+)	−	0.04 ± 0.02	0.94 ± 0.48	638 a	332 a	0 e	1.1 a
	+	0.10 ± 0.05	1.36 ± 0.16	606 ab	323 a	0.7 d	1.2 a
CHA625 (Phl^-)	−	<0.001	<0.01	609 ab	320 a	0 e	0.7 a
	+	<0.001	<0.01	496 c	249 b	1.9 b	1.1 a
CHA625/pME3128 (Phl^+)	−	0.01 ± 0.008	0.26 ± 0.14	631 a	335 a	0 e	1.3 a
	+	0.01 ± 0.003	0.19 ± 0.05	540 ab	294 a	1.3 c	1.4 a

[a] CHA0 (= wild-type strain of *P. fluorescens*), and its derivatives CHA625 (= Tn*5*-insertion Phl-negative mutant), and CHA625/pME3128 (= transconjugant of CHA625, restored in Phl production) and *G. graminis* var. *tritici* were added, respectively, at 10^7 cfu and as 1.25 mg of colonized millet seed per cm^3 of artificial soil, 14 and 7 days, respectively, before planting.

[b] Phl was extracted from the roots and the adhering artificial soil of 125 plants per treatment. Each value is the mean of three experiments.

[c] Rhizosphere weight included fresh weight of the roots with the adhering rhizosphere soil.

[d] Means within columns followed by the same letter are not significantly different at $P = 0.05$ according to Student's t test. Each value is the mean of six independent experiments with three or 25 replicates (for Phl extraction from rhizosphere) per experiment and one flask (five plants) per replicate (see text).

[e] Disease severity was rated on a 0–4 scale (0 = no disease; 4 = plants dead; Weller and Cook 1983, modified).

parental strains (Thomashow and Weller 1988; Thomashow *et al.* 1990). Disease suppression capacity of the strains was correlated with the presence of the antibiotic in the rhizosphere of wheat (Thomashow *et al.* 1990). Similarly, the antifungal antibiotic oomycin A produced by *P. fluorescens* Hv37a accounts for about half of the reduction of *Pythium* infection of cotton; this was demonstrated with the help of chemically induced mutants lacking this metabolite. A reporter gene approach with an *afuE-lux* transcriptional fusion was used to measure indirectly as bioluminiscence the expression of *afuE*, a biosynthetic gene required for the production of oomycin A, in the rhizosphere (reviewed in Gutterson 1990; Gutterson *et al.* 1990).

A number of *Pseudomonas* strains have been shown to produce Phl (Broadbent *et al.* 1976; Garagulya *et al.* 1974; Kiprianova and Smirnov 1981; Kiprianova *et al.* 1985; Reddi and Borovkov 1970) but, to our knowledge, the only previous report of a possible involvement of Phl in the biocontrol of soilborne pathogens comes from studies on a *P. aurantiaca* strain, which produces Phl, inhibits *Fusarium oxysporum in vitro* and protects wheat from the attack by the pathogen (Garagulya *et al.* 1974; Pidoplichko and Garagulya 1974). In contrast, the broad toxic activity of Phl is well documented. Interestingly, this compound is not only toxic to fungi and bacteria (Broadbent *et al.* 1976; Garagulya *et al.* 1987; Reddi and Borovkov 1970; Strunz *et al.* 1978) but also exerts herbicidal activity resembling that of 2,4-dichlorophenoxyacetate (2,4-D) (Kataryan and Torgashova 1976; Reddi *et al.* 1969) and furthermore has anthelmintic (Bowden *et al.* 1965) and antiviral (Tada *et al.* 1990) properties. Our results provide additional evidence for the antibiotic and phytotoxic activity of Phl (Tables 1–3). Addition of 40 μg of pure, synthetic Phl per gram of artificial soil was clearly herbicidal to tobacco since the final plant weight in the absence of the pathogen was drastically reduced (Keel *et al.* 1990). However, the present study demonstrates that the amount of Phl produced by strain CHA0 in the rhizosphere was clearly too low to give a visible herbicidal effect since plant growth was not affected by the bacteria in the absence of the pathogens. The mode of action of Phl is largely unclear. Phl produced by strain CHA0 in the rhizosphere might locally antagonize the pathogen on the root; massive killing of the pathogen could not be detected. It has been shown that addition of 40 μg of synthetic Phl per gram of artificial soil drastically reduces the severity of tobacco black root rot (Keel *et al.* 1990). An interesting parallel to this hypothesis is the finding by Tomás-Lorente *et al.* (1989) that certain plants produce antibiotic phloroglucinols, possibly as a biochemical defense against fungi. Alternatively, subinhibitory quantities of Phl produced by *P. fluorescens* in the rhizosphere might induce plant defense mechanisms against the pathogen. It is known that some other herbicides can induce resistance in plants (Altman and Campbell 1977; Cohen *et al.* 1986). However, little is known about the modes of antibiotic action of Phl. Yoneyama *et al.* (1990) observed that substituted phloroglucinols are potent photosystem II inhibitors.

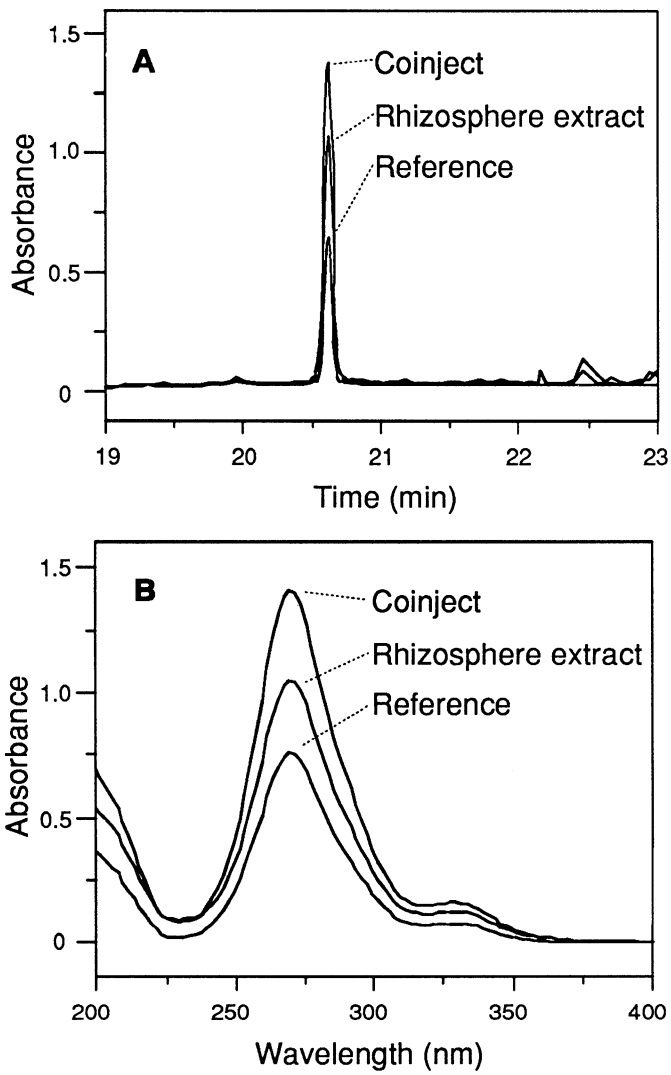

Fig. 3. Identification of 2,4-diacetylphloroglucinol in extracts from wheat roots with adhering artificial soil. **A,** HPLC chromatogram of synthetic Phl (Reference), of an extract of roots with adherent rhizosphere soil (Rhizosphere extract) and of a mixture of both samples (Coinject). Each sample contained a major peak at about 20.6 min. **B,** Comparison of spectra of the samples of panel A. Spectra were measured at the peak apex in each elution profile.

Phl$^-$ and cyanide-negative mutants of *P. fluorescens* CHA0 still exhibit some plant protection in the gnotobiotic system (Tables 5 and 6; Voisard *et al.* 1989). Because studies on a pyoverdine-negative mutant of strain CHA0 gave no evidence for a role of the fluorescent siderophore in disease suppression (Haas *et al.* 1991; Keel *et al.* 1989; Défago *et al.* 1990), other mechanisms such as competition for nutrients (Weller 1988) or detoxification of fungal virulence factors (Toyoda *et al.* 1988), may be responsible for the residual suppressiveness. In conclusion, our present data and the results obtained by other groups (Homma and Suzui 1989; Kraus and Loper 1989, 1990; Thomashow *et al.* 1990) suggest that suppression of root diseases by rhizosphere pseudomonads is multifactorial with bacterial secondary metabolites having a key role.

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