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

KEEL, Christoph, et al.

**Abstract**

*Pseudomonas fluorescens* strain CHAO suppresses *Thielaviopsis basicola*-induced black root rot of tobacco and *Gaeumannomyces graminis* var. *tritici*-induced take-all of wheat. Strain CHAO 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 Tn5 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 CHAO. Complementation of the mutant with an 11-kb DNA fragment from a genomic library of wild-type CHAO largely restored production of the metabolite, inhibition of the fungal pathogens in vitro and disease suppression. The Tn5 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 CHAO and by the complemented mutant, but not by the mutant defective [...]
Suppression of Root Diseases by *Pseudomonas fluorescens* CHA0: Importance of the Bacterial Secondary Metabolite 2,4-Diacetylphloroglucinol

Christoph Keel¹, Ursula Schneider², Monika Maurhofer¹, Christophe Voisard², Jacques Laville², Ulrich Burger¹, Philippe Wirthner³, Dieter Haas², and Geneviève Défago¹

Departments of ¹Plant Sciences/Phytomedicine and ²Microbiology, Swiss Federal Institute of Technology (ETH) of Zurich, and ³Department of Organic Chemistry, University of Geneva, Switzerland.

Received 17 May 1991. Accepted 1 August 1991.

*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 Tn5 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.

Additional keywords: antibiotics, biological control, Tn5 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, siderophores and antibiotic compounds, 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.

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 Tn5 insertion was physically mapped using a 5.5-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.
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* root rot caused by strain CHA0 (Ahl et al. 1989; Defago 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; Defago 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 (Defago et al. 1990; Haas et al. 1991; Keel et al. 1989).

Furthermore, preliminary experiments have demonstrated that a Tn5 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 Tn5 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::Tn5; 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 recA36 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. ulitimum* 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. ʻArina) 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 µE 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 µE 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 F₂₅₄) with CHCl₃:MeOH (100:1, v/v) gave two crude antibiotic fractions at Rₐ = 0.08 and Rₐ = 0.18 according to bioautographic monitoring with *Bacillus subtilis*. Repetition of preparative TLC with the more polar eluent mixture CHCl₃:MeOH (19:1, v/v) gave 35 mg of the antibiotic A (Rₐ = 0.2) and 11 mg of B (Rₐ = 0.5). Analytical samples of high purity were obtained by final preparative high-performance liquid chromatography (HPLC) on a Merck Lichrosorb Si60 column (CHCl₃: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 ¹H-NMR, ¹³C-NMR, IR and mass spectrometry to be identical with an authentic sample of 2,4-diacetyl-1,3,5-tri-hydroxybenzene (2,4-diacetylphloroglucinol) prepared according to Campbell and Copinger (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 Copinger 1951: 168°C). ¹H-NMR (CD₂OD, 200 MHz): 5.81(s,H-C(6),1H), 2.62(s,Ac,6H). ¹³C-NMR (CD₂OD, 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(CH₃) of Ac. Mass...
spectral (electron impact 70 eV) m/z (rel. intensity): 210(51, M+), 195(100), 177(61), 149(17), 67(77), 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_{91}H_{12}Cl_{2}NO_{3}. The compound was shown by IR, 'H-NMR, '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 Phi 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 F254; 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 Phi 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 Phi and at 313 nm for pyoluteorin. The retention times of authentic samples of Phi and pyoluteorin were about 12.2 and 9.6 min, respectively. Peak areas were converted to micrograms 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. *sacharata*) (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 CHAO has previously been constructed in the cosmid pVK100 (Voisard et al. 1989). Recombinant cosmids were mobilized from *E. coli* to *P. fluorescens* strain CHA625 (*phi*: Tn5) with the helper plasmid pME497 (Voisard et al. 1989); 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_{2}O_{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 cm³ of artificial soil was filled per 100-ml flask. For wheat 400 cm³ of 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.
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 suspensions were added to flasks to give one and seven days later, respectively, with cotton wool stoppers and autoclaved at 121° C for soil. Control flasks were watered with the same volume of distilled water. 30 min. Bacterial suspensions were added to flasks to give an opening of 5 cm in diameter. The flasks were sealed and loosely adhering artificial soil was shaken gently from all suppression gnotobiotic system (see above), plants were taken all at the same time. Five 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 artificial soil; Keel (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-diacylphloroglucinol 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 Phi 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 Phi.

Identification and characterization of 2,4-diacylphloroglucinol. *P. fluorescens* strain CHA0 grown on malt agar produced two antibiotics that could be identified in ethyl acetate extracts as Phi and pyoluteorin by standard spectroscopic and chromatographic means. Whereas malt agar proved suitable for the production of Phi, subsequent experiments showed that better yields of pyoluteorin were obtained when King's medium B plates were used instead of malt agar. Phi was synthesized chemically. Synthetic Phi 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 Phi- mutant CHA625, and *P. aeruginosa* were quite insensitive to the drug (Table 2). The effect of Phi on plants was then tested. In general, Phi 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 Phi at a concentration that

| Table 1. Antifungal activity of 2,4-diacylphloroglucinol (Phi) |
|-------------------|-----|-----|
| **Test fungus**    | **MICs (µg/ml)** |
|                    | **I₀** | **I₁₀** |
| 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<sup>b</sup> | 32–64 | 128 |

*MCIs are defined as the minimal amount of Phi causing 50% (I₀) or total (I₁₀) inhibition of fungal growth on malt agar within 7 days. Concentrations of Phi were varied by twofold dilutions.

*In addition, germination of endoconidia of *T. basicola* was completely inhibited at 256 µg/ml on malt agar.

### RESULTS

**Identification and characterization of 2,4-diacylphloroglucinol.** *P. fluorescens* strain CHA0 grown on malt agar produced two antibiotics that could be identified in ethyl acetate extracts as Phi and pyoluteorin by standard spectroscopic and chromatographic means. Whereas malt agar proved suitable for the production of Phi, subsequent experiments showed that better yields of pyoluteorin were obtained when King's medium B plates were used instead of malt agar. Phi was synthesized chemically. Synthetic Phi 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 Phi- mutant CHA625, and *P. aeruginosa* were quite insensitive to the drug (Table 2). The effect of Phi on plants was then tested. In general, Phi 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 Phi at a concentration that

**Table 2. Antibacterial activity of 2,4-diacylphloroglucinol (Phi)**

<table>
<thead>
<tr>
<th>Test bacterium</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50</td>
</tr>
<tr>
<td><em>Erwinia carotovora</em> pv. <em>carotovora</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>250</td>
</tr>
<tr>
<td><em>Escherichia coli</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>500</td>
</tr>
<tr>
<td><em>Klebsiella aerogenes</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>500</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> strain CHA0 (Phi&lt;sup&gt;&lt;sup&gt;+&lt;/sup&gt;&lt;/sup&gt;)</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> strain CHA625 (Phi&lt;sup&gt;&lt;sup&gt;-&lt;/sup&gt;&lt;/sup&gt;)</td>
<td>1,000</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> strain P3&lt;sup&gt;3&lt;/sup&gt;</td>
<td>500</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td><em>Pseudomonas syringae</em> pv. <em>phaseolicola</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td><em>Pseudomonas syringae</em> pv. <em>tabaci</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>5</td>
</tr>
<tr>
<td><em>Streptomyces echinatus</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>250</td>
</tr>
<tr>
<td><em>Streptomyces lividans</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
</tr>
</tbody>
</table>

*MCIs are defined as the minimal amount of Phi causing total inhibition of bacterial growth at 32° C in NYB, within 7 hr. Concentrations of Phi tested were: 5, 10, 50, 100, 250, 500, and 1,000 µg/ml.

*These strains were obtained from the collection of the Department of Microbiology, ETH, Zürich, Switzerland.

*Voisard et al. (1989).*
was only partially inhibitory to most fungi tested (Table 3). These results are in accordance with earlier data that demonstrated Phi to be more toxic to growth and 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 phi::Tn5 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 Phi. This mutant (CHA625) was obtained by Tn5 mutagenesis and found by screening 1,800 transposon insertion mutants of strain CHA0 for reduced growth inhibition of *F. a. 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 EcoRI and hybridized to a Tn5-internal HpaI fragment. Only one signal was obtained, indicating that this strain carried a single Tn5 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 Phi 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 *HindIII* produced pME3128, which had an 11-kb genomic insert on a single *HindIII* fragment (Fig. 1). Plasmid pME3128 brought back Phi 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 Phi on malt agar was also measured at 18° C (Table 4). This temperature cor-
responded to that used in plant experiments, whereas 27°C was optimal for Phi production in vitro.

Plasmid pME3128 was digested with Clai and religated. The resulting construct pME3123 (Fig. 1) was unable to complement strain CHA625 for Phi synthesis. This indicates that at least part of the phi gene region was located in the left half of the pME3128 insert and, therefore, a 5.8-kb HindIII-EcoRI fragment covering this region (Fig. 1) was chosen as a hybridization probe to locate the Tn5 insertion in strain CHA625. A Southern blot (Fig. 2) that pME3128 carried authentic CHAO DNA (lanes 1/7, 3/8, and 5/9) and that the Tn5 insertion in strain CHA625 had occurred in the genomic region homologous to the probe. The site of the Tn5 insertion could be mapped to position 2.9 kb from the left HindIII site, as indicated by the diagnostic fragments e, f, and c (Fig. 2, lanes 2, 4, and 6, respectively). These results show that the phi phenotype of strain CHA625 is caused by this Tn5 insertion.

Role of 2,4-diacetylphloroglucinol in disease suppression. Preliminary experiments have indicated that strain CHA625 is less effective than the wild-type strain CHAO 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 phi::Tn5 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 CHAO 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 Phi' 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 CHAO (Table 5). In wheat, plant and root weights were about 20% lower than those of those plants protected by strain CHAO (Table 6). This suggests that the protective effect of Phi is higher for tobacco than for wheat. In strain CHA625 carrying the recombinant cosmid pME3128 (phi') 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.

Because Phi 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 CHAO 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 (CHAO, 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 Phi 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 CHAO, wheat roots with the closely adhering artificial soil were extracted with ethyl acetate. In the extract a compound was found that comigrated with synthetic Phi 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 Phi; this was confirmed by coinjection of extracted and synthetic Phi. Phi was produced by strain CHAO 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 Phi. No antibiotic was detected in rhizosphere extracts of wheat grown in the presence of the Phi' mutant CHA625 or without addition of bacteria. Distinctly less antibiotic was produced by strain CHA625 carrying the
plasmid pME3128, which complements the strain for Phi production in vitro (Table 6). Suppression of take-all of wheat was correlated with Phi 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), Phi 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 Phi− 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 Phi− 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 Phi 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 antibiotics in disease suppression has also come from recent studies on Tn5 mutants of *P. fluorescens* strains 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 (Phi) production by *Pseudomonas fluorescens* strain CHA0 on the suppression of *Thielaviopsis basicola*-induced black root rot of tobacco under gnotobiotic conditions

<table>
<thead>
<tr>
<th>Microorganisms added*</th>
<th>Plant fresh weightb (mg)</th>
<th>Root fresh weightb (mg)</th>
<th>Root surface infectedc (%)</th>
<th>Fluorescent pseudomonadsb (10^6 cfu g of root)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
<td>0 c</td>
<td>0 b</td>
</tr>
<tr>
<td>CHA0 (Phi+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CHA625 (Phi−)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CHA625/pME3128 (Phi+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*T. basicola, CHA0 (= wild-type strain of *P. fluorescens*), and its derivatives CHA625 (= Tn5-insertion Phi-negative mutant), and CHA625/pME3128 (= transconjugant of CHA625, restored in Phi-production), were added, respectively, at 5 x 10^4 endoconidia, and 10^6 cfu/cm^2 of artificial soil, 6 and 7 days, respectively, before planting.

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).

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

---

**Table 6.** Production of 2,4-diacetylphloroglucinol (Phi) 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

<table>
<thead>
<tr>
<th>Microorganisms added*</th>
<th>µg of Phib per g</th>
<th>Plant fresh weightb (mg)</th>
<th>Root fresh weightb (mg)</th>
<th>Root surface infected (%)</th>
<th>Disease ratingc,d</th>
<th>Fluorescent pseudomonadsb (10^6 cfu g of root)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>598 ab</td>
<td>320 a</td>
<td>0 e</td>
<td>0 b</td>
<td>-</td>
</tr>
<tr>
<td>CHA0 (Phi+)</td>
<td>+</td>
<td>318 d</td>
<td>332 b</td>
<td>0 e</td>
<td>0 b</td>
<td>-</td>
</tr>
<tr>
<td>CHA625 (Phi−)</td>
<td>-</td>
<td>638 a</td>
<td>323 a</td>
<td>0.7 d</td>
<td>1.1 a</td>
<td>-</td>
</tr>
<tr>
<td>CHA625/pME3128 (Phi+)</td>
<td>+</td>
<td>609 ab</td>
<td>320 a</td>
<td>0 e</td>
<td>0.7 a</td>
<td>-</td>
</tr>
</tbody>
</table>

*CHA0 (= wild-type strain of *P. fluorescens*), and its derivatives CHA625 (= Tn5-insertion Phi-negative mutant), and CHA625/pME3128 (= transconjugant of CHA625, restored in Phi production) and *G. graminis* var. tritici were added, respectively, at 10^7 cfu and as 1.25 mg of colonized millet seed per cm^2 of artificial soil, 14 and 7 days, respectively, before planting.

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

*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 Phi extraction from rhizosphere) per experiment and one flask (five plants) per replicate (see text).

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 oomyctin 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 oomyctin 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 antihelminthic (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.

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.

**ACKNOWLEDGMENTS**

We would like to thank Helen Kuhn for technical assistance. This work was supported by grants from the Zentenarfonds and the Swiss

---

**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.
National Science Foundation 31-25321.88 and 31-28570.90 projects.

**LITERATURE CITED**


**Toyoda, H., Hashimoto, H., Utsumi, R., Kobayashi, H., and Ouchi, S. 1988.** Detoxification of fusaric acid by a fusaric acid-resistant mutant of *Pseudomonas solanacearum* and its application to biological control of Fusarium wilt of tomato. Phytopathology 78:1307-1311.


