Induction of Systemic Resistance of Tobacco to Tobacco Necrosis Virus by the Root-Colonizing *Pseudomonas fluorescens* Strain CHA0: Influence of the *gacA* Gene and of Pyoverdine Production

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ABSTRACT

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Pseudomonas fluorescens strain CHA0, which suppresses various plant diseases caused by soilborne pathogens, also can restrict leaf disease. Plants of *Nicotiana glutinosa* and of two cultivars of *N. tabacum* were grown in autoclaved natural soil previously inoculated with strain CHA0. After 6 wk, all the plants tested showed resistance in leaves to infection with tobacco necrosis virus (TNV) to the same extent as plants previously immunized with TNV (induced resistance control). Polyacrylamide gel electrophoresis and enzyme assays showed that the same amount of PR proteins (PR-1 group proteins, β-1,3-glucanases, and endochitinases) was induced in the intercellular fluid of leaves of plants grown in the presence of strain CHA0 as in the intercellular fluid of leaves of plants immunized by a previous TNV inoculation on a lower leaf. Strain CHA0 was reisolated from the roots but could not be detected in stems or leaves. Strain CHA96, a *gacA* (global activator)-negative mutant of strain CHA0 defective in the production of antibiotics and in the suppression of black root rot of tobacco, had the same capacity to induce PR proteins and resistance against TNV as did the wild-type strain. CHA400, a pyoverdine-negative mutant of strain CHA0 with the same capacity to suppress black root rot of tobacco and take-all of wheat as the wild-type strain, was able to induce PR proteins but only partial resistance against TNV. P3, another *P. fluorescens* wild-type strain, does not suppress diseases caused by soilborne pathogens and induced neither resistance nor PR proteins in tobacco leaves. Root colonization of tobacco plants with strain CHA0 and its derivatives as well as leaf infection with TNV caused an increase in salicylic acid in leaves. These results show that colonization of tobacco roots by strain CHA0 reduces TNV leaf necrosis and induces physiological changes in the plant to the same extent as does induction of systemic resistance by leaf inoculation with TNV. We further conclude that the bacterial *gacA* gene, which is important in the protection of the roots, has no influence on the induction of leaf resistance and that the bacterial production of the siderophore pyoverdine, which has no role in the protection of the roots, is involved in the induction of resistance in the leaves.

Root-colonizing bacteria, especially fluorescent pseudomonads, can efficiently control diseases caused by soilborne pathogens (3,7,9,19,31,37). Such beneficial rhizobacteria are included among plant growth-promoting rhizobacteria (PGPR). Recent studies show that some PGPR are also able to induce systemic resistance in plants (2,34,36).

Pseudomonas fluorescens strain CHA0 is an effective biocontrol agent of take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* in the field (38) and of other diseases caused by soilborne pathogens in greenhouse experiments (8). This strain produces the fluorescent siderophore pyoverdine (Pvd) (8), salicylic acid (27), indoleacetate (8), and several toxic metabolites, notably hydrogen cyanide (HCN) (8), 2,4-diacetylphloroglucinol (Phl) (8), and pyoluteorin (Plt) (8). Important roles have been demonstrated in a standardized gnotobiotic system for HCN (35) and Phl (15,17) in the suppression of black root rot of tobacco and for Phl in the suppression of take-all of wheat (14,15). Pyoverdine, however, does not significantly affect disease suppression (8,12,13,16). Strain CHA400, a pyoverdine-negative transposon insertion mutant of strain CHA0, suppresses black root rot of tobacco and take-all of wheat as efficiently as does the wild-type strain (13,16). In strain CHA0, a gene of 0.8 kb named *gacA* (global activator), which regulates the expression of secondary metabolites, has been identified and sequenced (21). Mutations in the *gacA* gene pleiotropically block the production of HCN, Phl, and Plt (21) and lead in strain CHA0 to a loss of the capacity to protect tobacco from black root rot caused by *Thielaviopsis basicola* (21).

There is circumstantial evidence for an effect of strain CHA0 on plant metabolism in the absence of a pathogen. In the presence of strain CHA0, tobacco roots show an increase in root hair formation compared with plants grown in the absence of bacteria (8). Because strain CHA0 also can be found in the root cortex (4), metabolites might be delivered into the plant and perhaps result in enhanced stress for the plant. It is known that stress can induce defense mechanisms against pathogens. Such stress might be caused by Plt and Phl, two phytotoxic compounds secreted by strain CHA0 (15,24). Another candidate that might trigger systemic induced resistance is the salicylate produced by strain CHA0 (27). Salicylic acid, delivered into leaves, may also be an endogenous plant signal that moves from the infection site to other parts of the plant and thereby induces resistance systemically (22,25).

The purpose of this study was to determine whether strain CHA0 is able to induce resistance against leaf necrosis caused by tobacco necrosis virus (TNV) in tobacco plants. Of further interest was whether the *gacA* gene, which is involved in the protection of tobacco roots against black root rot, is also involved in the induction of resistance in tobacco leaves and whether the pyoverdine-negative strain CHA400, which is still protecting roots, is also able to induce leaf resistance.

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MATERIALS AND METHODS

Microorganisms, plants, and culture conditions. The fluorescent pseudomonads used in the experiments (Table 1) were cultivated in media described previously (16,35). Strain CHA96 contains a *gacA'::'lacZ* translational fusion introduced into the CHA0 chromosome by gene replacement (21). The mutation in the *gacA* gene pleiotropically blocks the production of the antimicrobial metabolites Phl, Plt, and HCN and of other secondary metabolites (21). Strain CHA400 was obtained by insertion of the transposon Tn*1733* (containing kanamycin resistance) into strain CHA0 (16). For plant colonization experiments, natural rifampicin-resistant mutants of strain CHA0 and the *gacA*-negative strain CHA96 were used with the same disease-suppressive capacities as the original strains. Cultures of the rifampicin-resistant strains had 100 μg of rifampicin and cultures of the pyoverdine-negative strain CHA400 had 25 μl of kanamycin added per milliliter of medium. For soil inoculation, single colonies were transferred into 8 ml of nutrient yeast broth (32) and grown overnight at 27 C with shaking at 150 rpm. The next day, 200 μl of all suspensions were plated on King's medium B agar (18), and the plates were incubated for 48 h at 27 C. The bacteria were scraped from the plates with 20 ml of sterile bidistilled water; suspensions were centrifuged, and the pellet was resuspended in 20 ml of sterile double distilled water for immediate soil inoculation.

TNV (Forschungsanstalt für Pflanzenbau, Reckenholz, Zurich, Switzerland) was stored on dried infected tobacco leaves at 4 C. TNV strain 152 was multiplied on *Nicotiana tabacum* L. 'Xanthi nc' (Institute du Tabac, Bergerac, France) or on *N. glutinosa* L. and strain 149 on *N. tabacum* L. 'Burley 63' (Centre Suisse de Recherches sur le Tabac, Payerne, Switzerland) as follows: 0.05 g of dried tobacco leaves with TNV lesions was ground with 2 ml of a 20 mM phosphate buffer (pH 7.6). Tobacco leaves were inoculated by using a gauze pad to rub Carborundum-dusted leaves with 200 μl of the virus suspension. Plants were placed in a growth chamber with 70% relative humidity and incubated at 22 C with 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light for 16 h followed by an 8-h dark period at 15 C. After 7 days, 0.1 g of fresh leaves densely covered with TNV lesions was ground with 2 ml of the phosphate buffer and used for inoculation of plants.

Biocontrol of TNV. A tobacco-TNV assay was used for testing *P. fluorescens* strain CHA0 and its derivatives CHA96 and CHA400 as inducers of systemic resistance. As controls, all experiments also were conducted with P3, another *P. fluorescens* wild-type strain, which has no disease suppressive capacity (35). Cultivars Xanthi nc and Burley 63 of *N. tabacum* as well as *N. glutinosa* plants were grown from seeds on autoclaved quartz sand supplied with modified Knop solution (16) in a greenhouse for 4 wk. Plastic pots (300 cm^3) were surface disinfected with 70% ethanol and filled with 300 g of natural soil from Eschikon, Switzerland, which had been mixed with 1/4 volume of quartz sand and autoclaved twice; there was a 2-day interval between autoclavings. The bacterial suspension was added at 10^7 colony-forming units (cfu) per gram of soil to each pot, and the soil was then mixed with a sterile spatula. No bacteria were added to the pots of the TNV control and the induced resistance control. The induced resistance control consisted of plants that were each induced in the classical way with a first TNV inoculation on a lower leaf and then challenge inoculated with a second TNV inoculation on an upper leaf. Two days after the addition of bacteria, a tobacco plant, grown as described above, was transplanted into each pot. Plants were transferred to a growth chamber with the same conditions as described above. After 6 wk of growth in soil inoculated with bacteria, all plants were inoculated with TNV (challenge inoculation), except for three plants of each treatment that were used for enzyme assays and detection of PR proteins and salicylic acid. Plants of the induced resistance control had been induced with a first TNV inoculation 7 days before challenge inoculation. For inducing and challenge inoculations, the youngest fully developed leaf (*N. tabacum*) or the three youngest fully developed leaves (*N. glutinosa*) of each plant were dusted with Carborundum and, with a gauze pad, inoculated with four drops of 50 μl of a diluted (10× [Xanthi nc] or 100× [Burley 63]) or undiluted (*N. glutinosa*) virus suspension prepared as described above. After 7 days, the challenge-inoculated leaves were assessed for number and size of lesions. The total necrotic area for each leaf was calculated by adding the areas of all the lesions on the leaf. Area of one lesion = $(\text{lesion diameter}/2)^2 \times 3.1416$.

The experiments were repeated seven times (Xanthi nc), nine times (Burley 63), or four times (*N. glutinosa*) over a 2-yr period. Each experiment consisted of six to 10 replicates per treatment and one plant with one (*N. tabacum*) or three (*N. glutinosa*) challenged leaves per replicate.

Plant colonization. Root colonization and possible systemic colonization of the plants were examined with strain CHA400 and rifampicin-resistant mutants of strains CHA0 and CHA96. At the time of challenge inoculation, after 6 wk of growth in soil inoculated with bacteria as described above, three plants of each treatment were assessed for root colonization and possible systemic colonization. To quantify root colonization, roots of three plants were pooled, washed with double distilled water, and shaken in a 100-ml Erlenmeyer flask with 50 ml of autoclaved water at 330 rpm for 1 h. Serial dilutions were plated on King's medium B agar and incubated at 27 C for 48 h. The bacterial colonies were transferred by replica plating on King's medium B agar supplemented with 100 μg of rifampicin (CHA0, CHA96) or 25 μg of kanamycin (CHA400) per milliliter of medium. After 2 days at 27 C, the colonies were counted, and the mean number of colony-forming units per gram of root (fresh weight) was calculated. The experiment was repeated four times with three replicates per treatment and roots of three plants per replicate. To determine possible systemic colonization of the plants, stems and leaves of plants that had been assessed for root colonization were surface disinfected first with 70% ethanol for 30 s, then with 5% H_2O_2 for 10 min, and finally rinsed three times with sterile double distilled water. Afterward, stems and leaves were ground with a sterile mortar and pestle in 5 ml of a 0.1 M phosphate buffer (pH 7). Serial dilutions were plated on King's medium B agar. After 2 days at 27 C, the bacterial colonies were transferred onto King's medium B agar containing either rifampicin (CHA0, CHA96) or kanamycin (CHA400) as described above. The experiment was repeated four times with three replicates per treatment and stem and leaves of one plant per replicate.

Collection of intercellular fluids. Intercellular fluids from intact leaves of test plants grown for 6 wk in bacterized or nonbacterized soil or from plants grown in nonbacterized soil that had been infected previously with TNV (induced resistance control) were collected by vacuum infiltration at 4 C with a buffer containing 25 mM Tris (pH 7.8), 500 mM sucrose, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM mer-

TABLE 1. *Pseudomonas fluorescens* strains used in the experiments

Strain	Genotype	Phenotype[z]	Suppression of black root rot of tobacco	References
CHA0	Wild type	HCN^+, Phl^+, Plt^+, Pvd^+, Sal^+	+	1,15,27,33
CHA96	*gacA'::'lacZ* derivative of CHA0	HCN^-, Phl^-, Plt^-, Pvd^+, Sal^+	−	21
CHA400	*pvd*, obtained by Tn*1733* insertion in CHA0	HCN^+, Phl^+, Plt^+, Pvd^-, Sal^+, Km^R	+	16
P3	Wild type	HCN^-, Phl^-, Plt^-, fluorescence$^+$	−	35

[z] HCN = hydrogen cyanide; Phl = 2,4-diacetylphloroglucinol; Plt = pyoluteorin; Pvd = pyoverdine; Sal = salicylic acid; and Km = kanamycin.

captoethanol (30). Excess fluid was removed with a paper tissue, and the intercellular fluids were recovered by centrifuging the leaves at 1,000 *g* for 30 min.

Characterization of PR proteins in the intercellular fluid. Proteins in intercellular fluid samples were separated by polyacrylamide gel electrophoresis on a 10% resolving gel with a 4% stacking gel (mini-Protean-II slab gel, Bio-Rad Laboratories, Richmond, CA) at 180 V with an electrophoresis buffer containing 25 mM Tris (pH 8.3) and 192 mM glycine. Proteins were stained with silver nitrate according to the method of Blum et al (5).

Detection of the PR proteins 1a, 1b, 1c, P, and Q on gels. Proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane (0.2 μm, Bio-Rad) in 0.7% acetic acid (pH 2.8, migration toward cathode) at 20 V during 90 min (1a, 1b, and 1c) or in a buffer containing 25 mM Tris and 192 mM glycine (pH 8.3) at 30 V during 12 h (P and Q). The PR proteins 1a, 1b, 1c, or P and Q on the nitrocellulose membrane were detected as described by Geoffroy et al (11) with rabbit anti-tobacco PR-Q/P antibodies, kindly provided by M. Legrand, CNRS, Strasbourg, France, or with rabbit anti-tobacco PR-1 antibodies, kindly provided by H. Kessmann, CIBA-GEIGY, Basel, Switzerland.

Detection of β-1,3-glucanases on gels. The β-1,3-glucanases were stained on polyacrylamide gels with the method of Pan et al (29).

Enzyme activities. β-1,3-Glucanase activity was determined by a colorimetric measurement. Laminarin was used as substrate, and the reducing sugars were determined by the method of Nelson (28). Units of activity were defined as the amount of enzyme releasing 1 μg of glucose equivalents per milliliter of reaction mixture (10 μl of intercellular fluid in 300 μl of 50 mM sodium acetate, pH 5, 0.5% laminarin) in 1 min at 37 C.

Chitinase activity was measured by the radiochemical assay of Boller et al (6) with [^{3}H]chitin as substrate. The reaction mixture contained 50 μl of intercellular fluid, 1.5 mg of [^{3}H]chitin, and 200 μl of a 67 mM phosphate buffer, pH 6.6. The reaction was stopped after 20 min by the addition of 250 μl of 1 M trichloroacetic acid. The radioactivity in 300 μl of the supernatant was determined after centrifugation. The unit of measurement was the katal, defined as the amount of enzyme degrading 1 mol of chitin in 1 s at 37 C.

Enzyme activity was measured in four independent experiments with three replicates per treatment and one enzyme assay per replicate.

Extraction of salicylic acid. Salicylic acid was extracted from tobacco leaves and analyzed by high-performance liquid chromatography according to the method of Meuwly and Métraux (26). The values presented are the means of measurements in three plants from three different experiments per treatment.

Statistics. *Biocontrol of TNV.* Experiments were first analyzed separately (Table 2) with the Duncan multiple range test after a significant *F* test ($P = 0.05$). Then the experiments of 2 yr were analyzed together (Table 3) to show the reproducibility of biocontrol of TNV over time. Each value presented in Table 3 is the mean of the means of the individual experiments (data were not pooled). Each mean in Table 3 was compared with all other means by the Student *t* test; one independent experiment was considered a repetition.

Enzyme assays. Means of four experiments are presented. Each mean was compared with all other means by the Student *t* test; one independent experiment was considered a repetition.

TABLE 2. Effect of soil treatment with *Pseudomonas fluorescens* strain CHA0 and its *gacA*-negative derivative CHA96 and its pyoverdine-negative derivative CHA400 on leaf necrosis of *Nicotiana tabacum* 'Xanthi nc,' *N. tabacum* 'Burley 63,' and *N. glutinosa* caused by tobacco necrosis virus (TNV)

	Experiment 1		Experiment 2	
Treatment[w]	Number of lesions per leaf[x]	Lesion diameter[y] (mm)	Number of lesions per leaf	Lesion diameter (mm)
N. tabacum 'Xanthi nc'				
Tobacco necrosis virus control	145 a	1.0 a	139 a	1.8 a
Standard induced resistance control	30 c	0.5 b	10 c	0.8 b
Roots colonized with				
P. fluorescens CHA0	29 c	0.7 b	21 c	1.0 b
P. fluorescens CHA96	31 c	0.7 b	25 c	1.0 b
P. fluorescens CHA400	105 b	0.9 a	90 b	1.7 a
P. fluorescens P3	82 b	1.0 a	110 ab	1.6 a
N. tabacum 'Burley 63'				
Tobacco necrosis virus control	19 a	1.3 b	5 a	1.3 a
Standard induced resistance control	6 b	0.7 c	1 b	0.6 b
Roots colonized with				
P. fluorescens CHA0	8 b	0.8 c	1 b	0.8 b
P. fluorescens CHA96	9 b	0.8 c	1 b	0.6 b
P. fluorescens CHA400	22 a	1.8 a	4 a	1.3 a
N. glutinosa				
Tobacco necrosis virus control	10 a	1.1 ab	27 a	1.4 a
Standard induced resistance control	3 b	0.8 b	2 c	0.6 c
Roots colonized with				
P. fluorescens CHA0	3 b	0.9 b	4 c	0.7 c
P. fluorescens CHA96	4 b	1.0 ab	3 c	0.7 c
P. fluorescens CHA400	14 a	1.1 a	17 b	1.1 b
P. fluorescens P3	ND[z]	ND	26 a	1.3 a

[w] CHA0 = wild-type strain that suppresses black root rot; P3 = wild-type strain that does not suppress black root rot; CHA96 = *gacA* mutant of CHA0 (hydrogen cyanide negative, 2,4-diacetylphloroglucinol negative, pyoluteorin negative); CHA400 = pyoverdine-negative transposon insertion mutant of CHA0. The soil was inoculated with bacteria (except the two control treatments) 2 days before planting. Plants were grown for 6 wk and then challenge inoculated with TNV (*N. glutinosa* and *N. tabacum* 'Xanthi nc' with TNV 152, *N. tabacum* 'Burley 63' with TNV 149). Plants of the standard induced resistance control were induced with a TNV inoculation on a lower leaf 7 days before challenge inoculation of the upper leaf.

[x] Analysis of variance showed that the treatment effect was highly significant ($P < 0.0001$) for lesion number in all the experiments and for lesion diameter in all experiments except experiment 1 of *N. glutinosa* ($P = 0.08$). Values in the same column for the same cultivar followed by the same letter do not differ at $P = 0.05$ according to Duncan's multiple range test.

[y] Mean lesion diameter per leaf. Values in the same column for the same cultivar followed by the same letter do not differ at $P = 0.05$ according to Duncan's multiple range test.

[z] Not determined.

RESULTS

Biocontrol of TNV. Two representative experiments of each tobacco species and cultivar tested are presented in Table 2. The means of all experiments repeated during a period of 2 yr are presented in Table 3. Growth of *N. tabacum* 'Xanthi nc' and 'Burley 63' and of *N. glutinosa* in soil previously inoculated with strain CHA0 resulted in a significant reduction in lesion number, diameter, and area after infection with both TNV strains compared with the TNV control (Tables 2 and 3, Fig. 1). The total necrotic area of leaves from plants grown in the presence of strain CHA0 did not differ significantly from the total necrotic area developed on leaves from plants previously immunized with TNV (Table 3). The *gacA*-negative strain CHA96 was able to reduce leaf necrosis of Xanthi nc and Burley 63 to the same extent as was the wild-type strain (Tables 2 and 3, Fig. 2). The total necrotic area of plants grown in the presence of the pyoverdine-negative mutant CHA400, however, was significantly greater than that of plants grown with strain CHA0 (Table 3, Fig. 2). Soil inoculation with *P. fluorescens* strain P3 had no significant influence on TNV leaf necrosis (Tables 2 and 3, Fig. 1). Experiments with rifampicin-resistant mutants of strains CHA0 and CHA96 gave the same results (data not shown).

Plant colonization. Rifampicin-resistant mutants of strains CHA0 and CHA96 and of strain CHA400 were recovered from tobacco roots at mean population densities of 4.8–5.4 log cfu (Xanthi nc), 4.3–4.5 log cfu (Burley 63), and 4.8–5.7 log cfu (*N. glutinosa*) per gram of root (fresh weight) 42 days after planting. Total bacteria were recovered from the roots of all the treatments at mean population densities of 7.6–8.2 log cfu (Xanthi nc), 6.7–7.0 log cfu (Burley 63), and 7.7–8.3 log cfu (*N. glutinosa*) per gram of root (fresh weight). No rifampicin-resistant or kanamycin-resistant bacteria could be detected in stem or leaf samples of Xanthi nc, Burley 63, or *N. glutinosa* plants 42 days after planting. In two of seven experiments with Xanthi nc and in two of nine experiments with Burley 63, 10–100 kanamycin-sensitive and rifampicin-sensitive colony-forming units per gram of leaf (fresh weight) could be isolated from leaf samples of all the treatments.

Induction of PR proteins. Several clearly visible protein bands appeared on the polyacrylamide gel done with intercellular fluids of intact leaves of Xanthi nc plants grown in the presence of strain CHA0 and its derivatives and of plants previously immunized with TNV (Fig. 3). These protein bands were undetectable or of low intensity in the intercellular fluid of leaves of untreated control plants or of plants grown in the presence of strain P3. Eight of these proteins could be identified as known PR proteins by the use of immunoblotting or glucanase staining. The PR proteins 1a, 1b, and 1c were identified together with the β-1,3-glucanases 2, N, and O and the endochitinases P and Q (Fig. 3).

β-1,3-Glucanase activity. Compared with the treatments with-

TABLE 3. Influence of the *gacA* gene and of pyoverdine production in *Pseudomonas fluorescens* strain CHA0 on its ability to induce PR proteins and resistance to tobacco necrosis virus (TNV) in *Nicotiana tabacum* 'Xanthi nc' and 'Burley 63' and in *N. glutinosa*

Treatment[t]	Total necrotic area per leaf[u] (%)	Production of PR-1 proteins[v]	β-1,3-Glucanase activity[u,v]	Chitinase activity[u,v]
N. tabacum 'Xanthi nc'				
Tobacco necrosis virus control	100 a[w]	–[x]	1.0 a	1.0 a
Standard induced resistance control	17 c	+	14.0 c	5.1 c
Roots colonized with				
P. fluorescens CHA0	20 c	+	6.6 c	3.8 bc
P. fluorescens CHA96	23 c	+	5.2 bc	5.5 c
P. fluorescens CHA400	54 b	+	3.4 bc	2.5 bc
P. fluorescens P3	85 ab	(+)	1.4 ab	1.3 ab
N. tabacum 'Burley 63'				
Tobacco necrosis virus control	100 a	–	1.0 a	1.0 a
Standard induced resistance control	9 b	+	2.1 a	3.2 a
Roots colonized with				
P. fluorescens CHA0	21 b	+	2.1 a	2.3 a
P. fluorescens CHA96	9 b	+	2.0 a	4.4 a
P. fluorescens CHA400	66 a	+	2.7 a	3.4 a
P. fluorescens P3	89 a	(+)	1.0 a	1.6 a
N. glutinosa				
Tobacco necrosis virus control	100 a	ND[y]	1.0 a	1.0 a
Standard induced resistance control	10 c	ND	5.8 b	2.3 a
Roots colonized with				
P. fluorescens CHA0	18 c	ND	4.9 b	2.5 a
P. fluorescens CHA96	32 bc	ND	3.2 b	2.6 a
P. fluorescens CHA400	69 ab	ND	8.3 b	3.2 a
P. fluorescens P3	84[z]	ND	0.6[z]	1.0[z]

[t] CHA0 = wild-type strain that suppresses black root rot; P3 = wild-type strain that does not suppress black root rot; CHA96 = *gacA* mutant of CHA0 (hydrogen cyanide negative, 2,4-diacetylphloroglucinol negative, pyoluteorin negative); CHA400 = pyoverdine-negative transposon insertion mutant of CHA0. The soil was inoculated with bacteria (except the two control treatments) 2 days before planting. Plants were grown for 6 wk and then challenge inoculated with TNV (*N. glutinosa* and *N. tabacum* 'Xanthi nc' with TNV 152, *N. tabacum* 'Burley 63' with TNV 149) or used for enzyme assays or detection of PR-1. Plants of the standard induced resistance control were induced with a TNV inoculation on a lower leaf 7 days before challenge inoculation of the upper leaf.

[u] Values are given in percentage of (total necrotic area) or in ratio to (enzyme activities) the values of the TNV control. Total necrotic area: 100% = 200 mm^2 (Xanthi nc), 95 mm^2 (Burley 63), and 42 mm^2 (*N. glutinosa*). β-1,3-Glucanase activity: 1 = 8 units per gram of leaf (Xanthi nc), 6 units per gram of leaf (Burley 63), and 10 units per gram of leaf (*N. glutinosa*). Chitinase activity: 1 = 28 pkat per gram of leaf (Xanthi nc), 33 pkat per gram of leaf (Burley 63), and 234 pkat per gram of leaf (*N. glutinosa*).

[v] PR-1 proteins and enzyme activities were measured in the intercellular fluid of unchallenged plants.

[w] Means for the same cultivar within the same column followed by the same letter are not significantly different at $P = 0.05$ according to the Student *t* test comparing each treatment with each other treatment; one independent experiment is considered a repetition. Total necrotic area: each value is the mean of seven (Xanthi nc), nine (Burley 63), or four (*N. glutinosa*) independent experiments with six to 10 replicates and one pot with one plant per replicate. All other values are the means of four independent experiments with three replicates and one enzyme assay per replicate. The experiments were repeated within a 2-yr period.

[x] – = No PR-1 proteins detected with immunoblotting; + = PR-1 proteins detected with immunoblotting in all experiments; (+) = PR-1 proteins detected with immunoblotting in two of five experiments.

[y] Not determined.

[z] Mean of one experiment.

out added bacteria, soil inoculation with the strains CHA0, CHA96, and CHA400 increased β-1,3-glucanase activity in the intercellular fluid of Xanthi nc plants by seven, five, and three times, respectively (Table 3). Inoculation with TNV on the lower leaves resulted in a 14-fold increase in β-1,3-glucanase activity measured in the intercellular fluid of upper leaves (Table 3). Strain P3 had no influence on the enzyme activity in the plant (Table 3). Inoculating Burley 63 with TNV and growing the plants in the presence of strain CHA0 and its derivatives resulted in a twofold increase in β-1,3-glucanase activity in the intercellular fluid compared with control plants (Table 3). This increase, however, was not significant. Plants treated with strain P3 produced the same amount of enzyme as did control plants (Table 3). Leaf infection with TNV or soil inoculation with the strains CHA0, CHA96, or CHA400 resulted in enzyme activity that was three to eight times higher in the intercellular fluid of *N. glutinosa* plants compared with control plants grown without bacteria, whereas soil treatment with P3 had no influence on the amount of enzyme measured (Table 3).

Chitinase activity. Immunizing Xanthi nc with TNV and growing the plants with strain CHA0 or its derivatives increased the chitinase activity in the intercellular fluid two and one-half to five times compared with nontreated plants (Table 2). P3 did not increase the enzyme activity (Table 3). Infection with TNV or soil treatment with strain CHA0 or its derivatives increased the chitinase activity in Burley 63 about twofold, but the difference between these plants and the control plants was not significant (Table 3). Treating the soil with P3 did not increase the chitinase

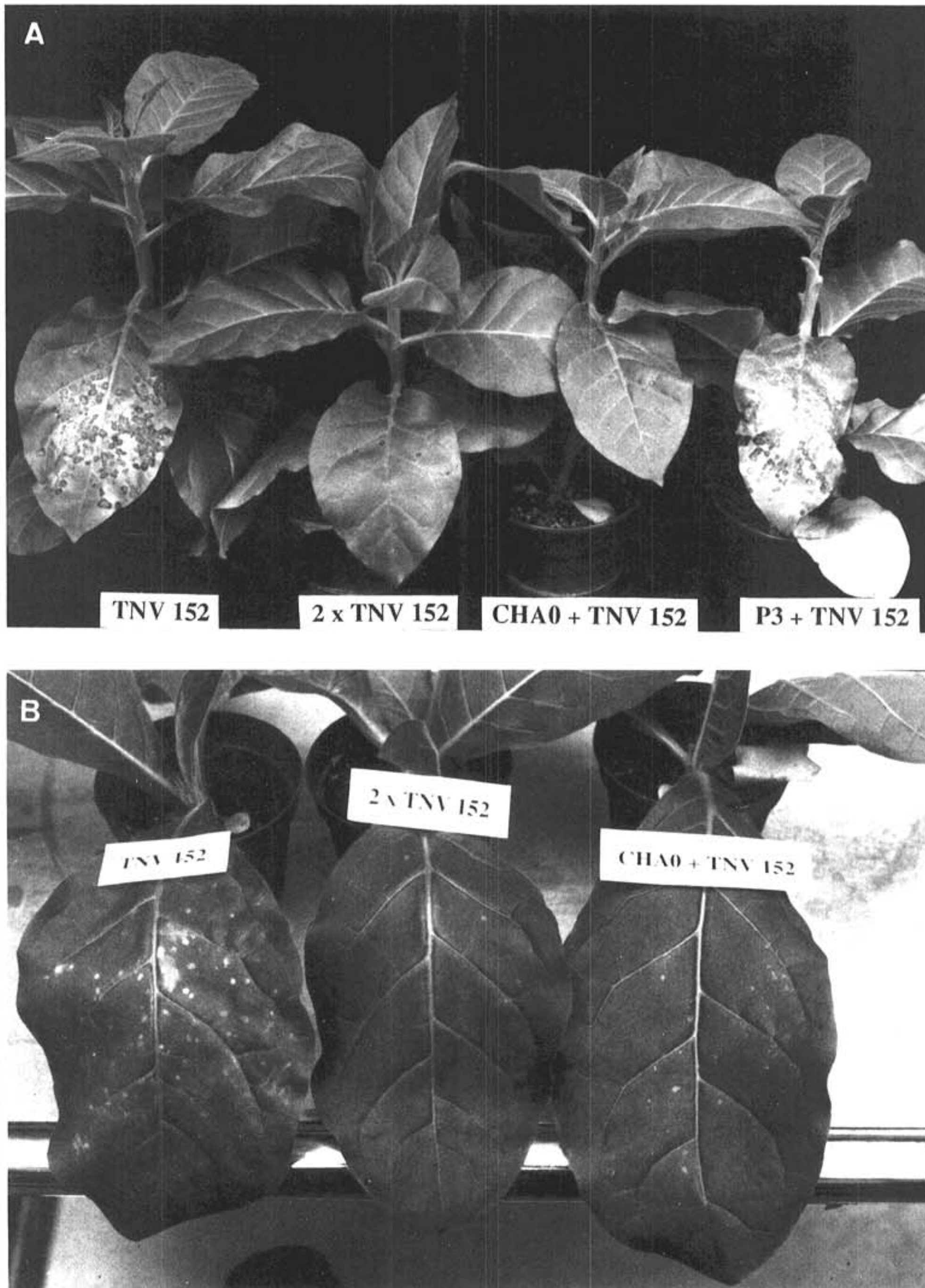

Fig. 1. Suppression of necrosis caused by tobacco necrosis virus (TNV) on *Nicotiana tabacum* **A,** cultivar Xanthi nc and **B,** cultivar Burley 63 after soil inoculation with *Pseudomonas fluorescens* strain CHA0. From left to right: TNV 152 = TNV control (nonbacterized and noninduced); 2 × TNV = standard induced resistance control (nonbacterized, induced with a prior inoculation of a lower leaf and challenge inoculated 7 days later on an upper leaf); CHA0 + TNV 152 and P3 + TNV 152 = bacterized by soil inoculation with strain CHA0 (biocontrol agent) and strain P3 (no biocontrol agent), respectively, 6 wk before challenge inoculation with TNV.

activity in Burley 63 (Table 3). Leaf infection with TNV or soil inoculation with any of the bacteria tested (except strain P3) resulted in chitinase activity that was two to three times higher in *N. glutinosa* plants compared with the activity in control plants, but the difference between these plants and the control plants was not significant (Table 3).

Free salicylic acid. In the upper leaves of mock-inoculated Xanthi nc plants, 15.7 ng of free salicylic acid per gram of leaves (fresh weight) was measured. Infection of a lower leaf with TNV resulted in five and one-half times more salicylic acid 7 days later in the upper leaves than in the mock-inoculated plants. Growing Xanthi nc plants with strains CHA400 or CHA96 resulted in the same increase in salicylic acid as did infection with TNV. However, in the presence of strain CHA0, the amount of salicylic acid in the leaves was only 40% more than that in control plants. Strain P3 had no influence on the amount of salicylic acid in leaves.

DISCUSSION

Our finding that colonization of tobacco roots by *P. fluorescens* strain CHA0 resulted in a reduction of TNV leaf necrosis (Tables 2 and 3, Fig. 1) confirms the results of van Peer et al (34), Wei et al (36), and Alström (2). Wei and coworkers used the classic system of cucumber and anthracnose (20) to demonstrate the ability of different PGPR strains to induce resistance against *Colletotrichum orbiculare* (36). Alström (2) demonstrated the induction of resistance in bean plants against halo blight by the application of *P. fluorescens* strain S 97 to seeds, whereas van Peer et al could protect carnation against Fusarium wilt by root bacterization with *Pseudomonas* spp. strain WCS417r (34). These authors could not detect the PGPR strains in stems or leaves (34,36); these findings correspond with our results. This spatial separation of beneficial and pathogenic microorganisms suggests that the protection against leaf and stem diseases by PGPR strains is due to systemically induced resistance. However, the possibility cannot be excluded that bacterial metabolites are taken up by the plant and transported to the site of infection where they may interact directly with the pathogen or may induce local resistance. van Peer (34) and coworkers investigated the production of phytoalexins in protected and unprotected plants in an effort to confirm the hypothesis that PGPR strains activate plant defense mechanisms. After challenge inoculation, they found an increased amount of phytoalexins in plants treated with strain WCS417r compared with nonbacterized plants. Zdor and Anderson (39) showed that some *Pseudomonas* strains, when colonizing bean roots, induce the mRNA of the PR protein 1a in the leaves. However, Zdor and Anderson did not investigate whether this physiological change in the plant was correlated with a reduction in leaf diseases.

Our work is the first attempt to compare physiological changes in leaves due to systemic resistance induced by the classical method of leaf infection with a pathogen with physiological changes in leaves due to resistance induced by PGPR application to the root system. Strain CHA0 induced PR proteins and resistance to TNV to the same extent as did leaf inoculation with TNV (Tables 2 and 3, Figs. 1 and 3). P3, another wild-type strain of *P. fluorescens*, which does not suppress black root rot of

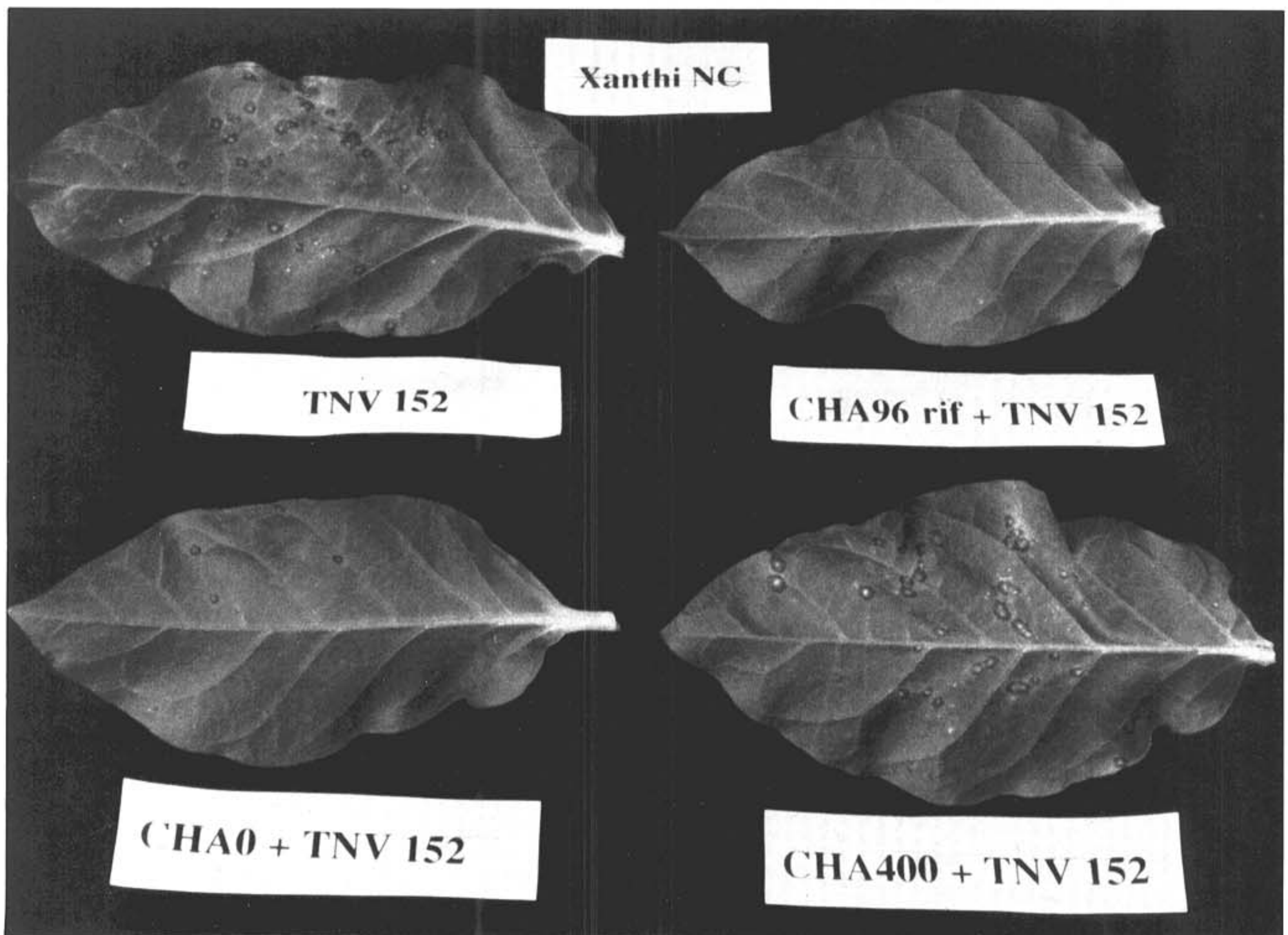

Fig. 2. Influence of pyoverdine production and the *gacA* gene in *Pseudomonas fluorescens* strain CHA0 on its capacity to suppress necrosis of *Nicotiana tabacum* 'Xanthi nc' caused by tobacco necrosis virus (TNV) when colonizing roots. TNV 152 = TNV control (nonbacterized and inoculated with TNV); CHA0 + TNV 152, CHA400 + TNV 152, and CHA96 rif + TNV 152 = bacterized by soil inoculation with strain CHA0 (wild type), strain CHA400 (pyoverdine negative), and a rifampicin-resistant mutant of strain CHA96 (*gacA* negative), respectively, 6 wk before leaf inoculation with TNV.

tobacco, also does not have the capacity to induce resistance against TNV and induces PR proteins only by small amounts (Tables 2 and 3, Figs. 1 and 3). The correlation between the induction of PR proteins and the reduction of leaf necrosis strengthens the hypothesis that the resistance induced by strain CHA0 is systemic, although this correlation may not be causal.

The *gacA*-negative mutant CHA96, which does not suppress black root rot of tobacco (21), controlled TNV leaf necrosis to the same extent as did the wild-type strain CHA0 (Tables 2 and 3, Fig. 2). The pyoverdine-negative mutant CHA400, which does protect the roots as effectively as does strain CHA0 (16), was significantly less able to protect the leaves (Tables 2 and 3, Fig. 2). These results indicate that not all mechanisms that are responsible for the induction of systemic resistance in the leaves are also of importance in the protection of the roots and vice versa. We suggest that the antibiotic metabolites controlled by the *gacA* gene are involved in the suppression of root diseases of tobacco but not in the induction of resistance. However, there is a possibility that strain CHA96 overproduces other metabolites that could be involved in the induction of resistance and is therefore still as effective as the wild-type strain. On King's medium B agar and in succinate minimal medium, strain CHA96 is much more fluorescent than is strain CHA0 (M. Maurhofer, *unpublished data*), indicating an overproduction of pyoverdine that could be responsible for the capacity of strain CHA96 to induce resistance. If so, this correlation would be compatible with the observation that the pyoverdine-negative strain CHA400 does not protect the leaves against TNV as efficiently as does the wild-type strain. However, the transposon insertion in CHA400 is not localized, and it is not clear whether the pyoverdine mutation is the only mutation in strain CHA400. Therefore, it is not yet possible to attribute the inability to induce resistance to the loss of pyoverdine production. Other experiments would be necessary to investigate the exact role of pyoverdine in the induction of resistance by strain CHA0.

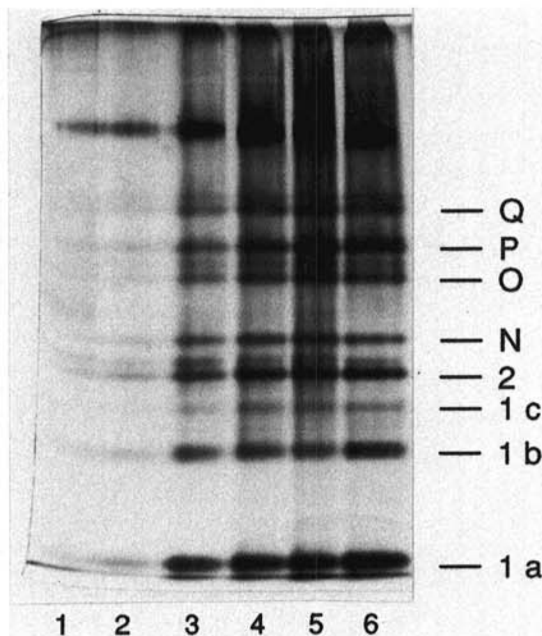

Fig. 3. Comparison of electrophoretic patterns of intercellular fluids from *Nicotiana tabacum* 'Xanthi nc' plants infected with tobacco necrosis virus (TNV) or grown in the presence of *Pseudomonas fluorescens* strain CHA0 (biocontrol agent) and its derivatives, the *gacA*-negative strain CHA96, the pyoverdine-negative strain CHA400, and *P. fluorescens* strain P3 (no biocontrol agent). The intercellular fluid of 10-wk-old plants was collected 7 days after mock inoculation (lane 1), 7 days after inoculation with TNV (lane 3), or 6 wk after root inoculation with *P. fluorescens* strains CHA0 (lane 4), CHA96 (lane 5), CHA400 (lane 6), or P3 (lane 2). Eight bands were identified as PR proteins by immunoblotting or glucanase staining on the gel: the PR-1 group proteins 1a, 1b, and 1c; the β-1,3-glucanases 2, N, and O; and the endochitinases P and Q.

Strain CHA400 induces PR proteins in the plant to the same extent as do the wild-type strain and strain CHA96 or immunization with TNV, but the level of resistance induced by strain CHA400 is much lower (Tables 2 and 3, Figs. 2 and 3). There are other reports of no correlation between induction of systemic resistance and induction of PR proteins (10,23). The role of PR proteins is, therefore, still unclear with respect to induction of systemic resistance by strain CHA0 and its derivatives. However, the possibility that the reduction of leaf necrosis is due to mechanisms other than systemic resistance cannot be excluded.

Our finding that infection of a lower leaf with TNV results in an increase of salicylic acid in the upper leaf confirms the work of Malamy et al (22) and Métraux et al (25). Growing Xanthi nc plants in the presence of strain CHA0 slightly increased salicylic acid, and in the presence of strains CHA96 and CHA400, the increase was the same as that of leaf infection with TNV. At the time chosen here for leaf extraction, there was no correlation between the amount of free salicylic acid and the degree of resistance against TNV necrosis. However, a correlation might exist at the time when resistance is induced, and later some salicylic acid might be bound as *o*-glucoside, which cannot be extracted by the method used here. Our findings indicate that it is possible to cause an increase in salicylic acid in leaves by growing tobacco plants in the presence of root-colonizing bacteria. However, it is not clear whether the bacteria induce the production of salicylic acid in the plant or whether the plant takes up bacterial salicylic acid and translocates it to the leaves.

In conclusion, colonization of tobacco roots by strain CHA0 induces resistance and physiological changes in the leaves to the same extent as does leaf inoculation with TNV. We further conclude that the bacterial *gacA* gene, which is of importance in the protection of the roots, has no influence on the induction of leaf resistance and that the bacterial production of the siderophore pyoverdine, which has no role in the protection of the roots, is involved in the induction of resistance in the leaves. For practical application of strain CHA0 as a resistance-inducing agent, we would need to investigate the longevity of the effect in the field.

LITERATURE CITED

1. Ahl, P., Voisard, C., and Défago, G. 1986. Iron-bound siderophores, cyanic acid, and antibiotics involved in suppression of *Thielaviopsis basicola* by a *Pseudomonas fluorescens* strain. J. Phytopathol. 166:121-134.
2. Alström, S. 1991. Induction of disease resistance in common bean susceptible to halo blight bacterial pathogen after seed bacterization with rhizosphere pseudomonads. J. Gen. Appl. Microbiol. 37:495-501.
3. Baker, R. 1985. Biological control of plant pathogens: Definitions. Pages 25-39 in: Biological Control in Agricultural IPM Systems. M. A. Hoy and D. C. Herzog, eds. Academic Press, New York.
4. Berling, C.-H. 1991. Anwendung der Immunofluoreszenz-Mikroskopie zur Lokalisation eines krankheitsunterdrückenden *Pseudomonas fluorescens*-Stammes auf Tabakwurzeln. Ph.D. thesis 9474. Swiss Federal Institute of Technology, Zurich.
5. Blum, H., Beier, H., and Gross, H. J. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93-99.
6. Boller, T., Gehri, A., Mauch, F., and Vögeli, U. 1983. Chitinase in bean leaves: Induction by ethylene, purification, properties and possible function. Planta 157:22-31.
7. Bruehl, G. W., ed. 1987. Soilborne Plant Pathogens. Macmillan, New York.
8. Défago, G., Berling, C.-H., Burger, U., Haas, D., Kahr, G., Keel, C., Voisard, C., Wirthner, P., and Wüthrich, B. 1990. Suppression of black root rot of tobacco and other root diseases by strains of *Pseudomonas fluorescens:* Potential applications and mechanisms. Pages 93-108 in: Biological Control of Soilborne Plant Pathogens. D. Hornby, R. J. Cook, Y. Henis, W. H. Ko, A. D. Rovira, B. Schippers, and P. R. Scott, eds. C.A.B. International Mycological Insitute, Oxon, England.
9. Défago, G., and Haas, D. 1990. Pseudomonads as antagonists of soilborne plant pathogens: Mode of actions and genetic analysis. Pages

249-291 in: Soil Biochemistry. Vol. 6. J. M. Bollag and G. Stotzky, eds. Marcel Dekker, New York.

10. Fraser, R. S. S. 1982. Are 'pathogenesis-related' proteins involved in acquired systemic resistance of tobacco plants to tobacco mosaic virus? J. Gen. Virol. 58:305-313.
11. Geoffroy, P., Legrand, M., and Fritig, B. 1990. Isolation and characterization of a proteinaceous inhibitor of microbial proteinases induced during the hypersensitive reaction of tobacco to tobacco mosaic virus. Mol. Plant-Microbe Interact. 3:327-333.
12. Haas, D., Keel, C., Laville, J., Maurhofer, M., Oberhänsli, T., Schnider, U., Voisard, C., Wüthrich, B., and Défago, G. 1991. Secondary metabolites of *Pseudomonas fluorescens* strain CHA0 involved in the suppression of root diseases. Pages 450-456 in: Advances in Molecular Genetics of Plant-Microbe Interactions. Vol. 1. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.
13. Keel, C., and Défago, G. 1991. The fluorescent siderophore of *Pseudomonas fluorescens* strain CHA0 has no effect on the suppression of root diseases of wheat. Pages 136-142 in: Plant Growth-Promoting Rhizobacteria: Progress and Prospects. C. Keel, B. Koller, and G. Défago, eds. Int. Org. Biol. Control Noxious Anim. Plants/West Palaearctic Reg. Sec. Bull. 14/8.
14. Keel, C., Maurhofer, M., Oberhänsli, Th., Voisard, C., Haas, D., and Défago, G. 1991. Role of 2,4-diacetylphloroglucinol in the suppression of take-all of wheat by a strain of *Pseudomonas fluorescens*. Pages 335-338 in: Biotic Interactions and Soilborne Diseases. A. B. R. Beemster, G. J. Bollen, M. Gerlagh, M. A. Ruissen, B. Schippers, and A. Tempel, eds. Elsevier, Amsterdam.
15. Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D., and Défago G. 1992. Suppression of root diseases by *Pseudomonas fluorescens* CHA0: Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Mol. Plant-Microbe Interact. 5:4-13.
16. Keel, C., Voisard, C., Berling, C. H., Kahr, G., and Défago, G. 1989. Iron sufficiency, a prerequisite for suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. Phytopathology 79:584-589.
17. Keel, C., Wirthner, Ph., Oberhänsli, Th., Voisard, C., Burger, U., Haas, D., and Défago, G. 1990. Pseudomonads as antagonists of plant pathogens in the rhizosphere: Role of the antibiotic 2,4-diacetylphloroglucinol in the suppression of black root rot of tobacco. Symbiosis 9:327-341.
18. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.
19. Kloepper, J. W., Lifshitz, R., and Zablotowicz, R. M. 1989. Free living bacterial inocula for enhancing crop productivity. Trends Biotechnol. 7:39-44.
20. Kuc, J., Shockley, G., and Kearny, K. 1975. Protection of cucumber against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. Physiol. Plant. Pathol. 7:195-199.
21. Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G., and Haas, D. 1992. Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proc. Natl. Acad. Sci. USA 89:1562-1566.
22. Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. 1990. Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. Science 250:1002-1004.
23. Matta, A. 1991. The role of cell injury in induced resistance. Petria 1:85-98.
24. Maurhofer, M., Keel, C., Schnider, U., Voisard, C., Haas, D., and Défago, G. 1992. Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. Phytopathology 82:190-195.
25. Métraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science 250:1004-1006.
26. Meuwly, P., and Métraux, J.-P. Ortho-anisic acid as internal standard for the simultaneous quantification of salicylic acid and its putative biosynthetic precursors in cucumber leaves. Anal. Biochem. (In press.)
27. Meyer, J.-M., Azelvandre, P., and Georges, C. 1992. Iron metabolism in *Pseudomonas:* Salicylic acid, a siderophore of *Pseudomonas fluorescens* CHA0. BioFactors 4:23-27.
28. Nelson, N. 1944. A photometric adaption of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:275-280.
29. Pan, S. Q., Ye, X. S., and Kuc, J. 1989. Direct detection of β-1,3-glucanase isoenzymes on polyacrylamide electrophoresis and isoelectrofocusing gels. Anal. Biochem. 182:136-140.
30. Parent, J.-G., and Asselin, A. 1984. Detection of pathogenesis-related proteins (PR or b) and of other proteins in the intercellular fluid of hypersensitive plants infected with tobacco mosaic virus. Can. J. Bot. 62:564-569.
31. Schippers, B. 1988. Biological control of pathogens with rhizobacteria. Philos. Trans. R. Soc. Lond. B 318:283-293.
32. Stanisich, V. A., and Holloway, B. W. 1972. A mutant sex factor of *Pseudomonas aeruginosa*. Genet. Res. 19:91-108.
33. Stutz, E., Défago, G., and Kern, H. 1986. Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology 76:181-185.
34. van Peer, R., Niemann, G. J., and Schippers, B. 1991. Induced resistance and phytoalexin accumulation in biological control of Fusarium wilt of carnation by *Pseudomonas* sp. strain WCS417r. Phytopathology 81:728-734.
35. Voisard, C., Keel, C., Haas, D., and Défago, G. 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J. 8:351-358.
36. Wei, G., Kloepper, J. W., and Tuzun, S. 1991. Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. Phytopathology 81:1508-1512.
37. Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26:379-407.
38. Wüthrich, B. 1991. Biologische Bekämpfung der Schwarzbeinigkeit des Weizens durch Einsatz von *Pseudomonas fluorescens* Stamm CHA0 im Feld und Mechanismen der Krankheitsunterdrückung. Ph.D. thesis 9447. Swiss Federal Institute of Technology, Zurich.
39. Zdor, R. E., and Anderson, A. J. 1992. Influence of root colonizing bacteria on the defense responses of bean. Plant Soil 140:99-107.