Salicylic Acid Biosynthetic Genes Expressed in *Pseudomonas fluorescens* Strain P3 Improve the Induction of Systemic Resistance in Tobacco Against Tobacco Necrosis Virus

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ABSTRACT

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Application of salicylic acid induces systemic acquired resistance in tobacco. *pchA* and *pchB*, which encode for the biosynthesis of salicylic acid in *Pseudomonas aeruginosa*, were cloned into two expression vectors, and these constructs were introduced into two root-colonizing strains of *P. fluorescens*. Introduction of *pchBA* into strain P3, which does not produce salicylic acid, rendered this strain capable of salicylic acid produc-

tion in vitro and significantly improved its ability to induce systemic resistance in tobacco against tobacco necrosis virus. Strain CHA0 is a well-described biocontrol agent that naturally produces salicylic acid under conditions of iron limitation. Introduction of *pchBA* into CHA0 increased the production of salicylic acid in vitro and in the rhizosphere of tobacco, but did not improve the ability of CHA0 to induce systemic resistance in tobacco. In addition, these genes did not improve significantly the capacity of strains P3 and CHA0 to suppress black root rot of tobacco in a gnotobiotic system.

Additional keywords: *Nicotiana glutinosa*, *N. tabacum*, plant growth-promoting rhizobacteria, strain improvement, *Thielaviopsis basicola*.

Plant growth-promoting rhizobacteria (PGPR) suppress a variety of root and vascular diseases caused by soilborne pathogens (7,43). PGPR suppress disease by antagonizing pathogens via different mechanisms such as antibiosis, competition for iron or carbon, and production of lytic enzymes. PGPR also activate plant defense resulting in systemic protection against different fungal, bacterial, and viral pathogens (1,9,25,29,32,38,42), a phenomenon termed induced systemic resistance (ISR) (19).

The O-antigenic sidechain of outer membrane lipopolysaccharide appears to be essential for the induction of systemic resistance by *Pseudomonas fluorescens* strains WCS417r and WCS374 in radish against Fusarium wilt (23,24). Press and Kloepper (30) reported that cell wall constituents and membrane fractions of *P. fluorescens* 89B-61 caused ISR against anthracnose in cucumber plants.

PGPR-mediated ISR may be affected by iron availability. For example, the ability of *Serratia marcescens* 90-166 to induce resistance in cucumber decreased with increasing iron concentration in the fertilizer (31). Some *Pseudomonas* strains reduced Fusarium wilt of radish more effectively when iron availability in the nutrient solution was low (23). *P. aeruginosa* 7NSK2 triggered ISR against *Botrytis cinerea* in bean when the bacterium was cultivated on iron-poor, but not on iron-rich, media (9). These findings suggest that bacterial siderophores, which in general are produced under conditions of iron limitation, might be involved in ISR.

PGPR of the genus *Pseudomonas* can produce several siderophores such as pyoverdine (pseudobactin), pyochelin, and salicylic acid (SA) (9,23,27). Purified pyoverdine from *P. fluorescens* WCS374 induced resistance in radish against Fusarium wilt (23).

In an earlier study, we found that a pyoverdine-negative mutant of *P. fluorescens* strain CHA0 lost part of its ability to induce systemic resistance in tobacco against tobacco necrosis virus (TNV) (25).

SA injected into stems, sprayed onto the entire plant, or applied to the roots is a potent inducer of systemic resistance and pathogenesis-related (PR) proteins (10,23,41,44). Therefore, SA produced by PGPR in the rhizosphere may be involved in ISR. De Meyer and Höfte (9) found that SA produced by *P. aeruginosa* 7NSK2 was important in the induction of systemic resistance against *B. cinerea* in bean.

The aim of this study was to determine whether the introduction of the SA biosynthetic genes *pchBA* from *P. aeruginosa* PAO1 into *P. fluorescens* strains P3 and CHA0 could improve their ability to induce systemic resistance in tobacco against TNV and suppress black root rot of tobacco. *pchBA* were placed under control of a constitutive promoter allowing iron-independent production of SA. *pchBA* are part of the *pchDCBA* operon required for pyochelin biosynthesis in *P. aeruginosa* PAO1 (36). The *pchA* gene product is an isochorismate synthase converting chorismate to isochorismate, and the *pchB* gene product is an isochorismate-pyruvate lyase catalyzing the formation of SA from isochorismate (35; C. Gaille and D. Haas, unpublished data). *P. fluorescens* P3 is SA-negative and a poor biocontrol agent (25,40), whereas *P. fluorescens* CHA0 produces SA under the condition of iron limitation, is a good biocontrol agent of various soilborne pathogens (6), and induces systemic resistance in tobacco against TNV (25). In addition to the siderophores pyoverdine, pyochelin, and SA, strain CHA0 produces hydrogen cyanide and the antibiotics 2,4-diacetylphloroglucinol and pyoluteorin (6,27).

MATERIALS AND METHODS

Organisms, plasmids, and culture conditions. Wild-type strains of *P. fluorescens* CHA0 and P3 (37,40) were used throughout this study, except for experiments conducted in autoclaved Eschikon

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soil when rifampicin-resistant mutants were used. Plasmids pVK100 (20) and pBBR1MCS (22) served as cloning vectors in *Escherichia coli* DH5 α (33). Bacteria were routinely grown in nutrient yeast broth [Difco nutrient broth [Difco Laboratories, Detroit], 25 g; Difco yeast extract [Difco Laboratories], 5 g; and double-distilled H₂O, 1 liter) and on nutrient agar plates (Oxoid blood base agar [Oxoid Ltd., Basingstoke, Great Britain], 40 g; Difco yeast extract, 5 g; and double-distilled H₂O, 1 liter). For disease suppression assays, bacteria were grown on King's B agar (18); bacteria added to soil were prepared as described earlier (17,25). Antibiotics, when required, were added to the growth media at the following concentrations: tetracycline, 25 μ g/ml for *E. coli* and 125 μ g/ml for *P. fluorescens*; chloramphenicol, 50 μ g/ml for *E. coli*; kanamycin, 25 μ g/ml for *E. coli*; and rifampicin, 100 μ g/ml for *P. fluorescens*.

Thielaviopsis basicola (Berk. & Broome) Ferraris strain ETH D127 was grown on malt agar (Oxoid malt extract [Oxoid Ltd.], 15 g; Oxoid bacteriological agar [Oxoid Ltd.], 12 g; and double-distilled H₂O, 1 liter) at 24°C for 3 weeks in the dark. Endoconidia that developed were suspended in sterile double-distilled water, separated from the chlamydospores and mycelium by filtration through sterile glass wool, and immediately added to the soil to give a concentration of 2×10^4 endoconidia per g of soil.

TNV (strain 152; Eidgenössische Forschungsanstalt für Agrarökologie und Landbau, Zürich-Reckenholz, Switzerland) was stored on dried infected tobacco leaves at 4°C. TNV was multiplied on *Nicotiana tabacum* L. cultivar Xanthi nc (Institute du Tabac, Bergerac, France) as described earlier (25).

Seeds of *N. glutinosa* L. were surface-disinfected and germinated on 1.2% (wt/vol) water agar (Oxoid Ltd.) in a growth chamber (70% relative humidity, 22°C with light at 80 μ E m⁻² s⁻¹ for 16 h, and 18°C for 8 h in darkness) as described by Keel et al. (17). After 7 days, seedlings were transferred to modified Knop nutrient solution (17) solidified with 0.6% (wt/vol) agar (Oxoid Ltd.). The 5-week-old seedlings were transplanted into the gnotobiotic system as previously described (17).

For the tobacco-TNV assay, *N. tabacum* cultivar Xanthi nc was grown from seeds on autoclaved quartz sand supplemented with modified Knop solution in a greenhouse for 4 weeks. The seedlings were then transplanted into autoclaved natural soil from Eschikon, Switzerland, amended with the bacterial strains.

DNA manipulations and cloning procedures. Small-scale preparations of plasmid DNA were carried out by the cetyltrimethylammonium bromide method (8), and large-scale preparations were performed using Qiagen-tips 100 (Qiagen AG, Basel, Switzerland). Restriction enzyme digestion, ligation, and agarose gel electrophoresis were performed by standard procedures (33). Restriction fragments were purified from agarose gels using the GeneClean II kit (Bio 101, Inc., Vista, CA). Transformation of *E. coli* and *P. fluorescens* was carried out by the classical CaCl₂ procedure (33) or by electroporation (11).

Construction of the SA expression plasmids pME3377 and pME6127. A 3.3-kb *SalI* fragment carrying *pchCBA* of *P. aeruginosa* (35,36) was inserted into the *XhoI* site of the broad-host-range vector pVK100 (20) such that expression of the SA biosynthetic genes was controlled by the constitutive kanamycin promoter of the vector (Fig. 1A). A 54-bp *SmaI* DNA linker (5'-CCCGGGGATCCGGTGATTGATTGAGCAAGCTTGCTCAATCAATCACCGGATCCCGGG-3') with translation stop signals in all three reading frames was inserted into the unique *SmaI* site of the *pchC* gene to prevent the expression of this protein. The resulting plasmid, pME3377 (Fig. 1A), allowed iron-independent, constitutive expression of *pchBA*.

A second expression plasmid, pME6127, was constructed using pME6000 (Fig. 1B). This new cloning vector was obtained by replacing a 0.3-kb *DraI* fragment in the chloramphenicol resistance gene of the wide-host-range plasmid pBBR1MCS (22) with a T4 DNA polymerase-treated 2.8-kb *HindIII-StuI* fragment of

pVK100 specifying a tetracycline resistance *tetR tetA*. To obtain pME6127, a 5.65-kb *SphI-ClaI* fragment carrying the *pchBA* genes under the control of the constitutive kanamycin promoter was excised from pME3377 and cloned into pME6000 (Fig. 1B).

The vector pVK100, which was used for the construction of pME3377, is a derivative of RK2, a plasmid probably identical with RP1 (3,20). The copy number of RP1 per chromosome equivalent has been estimated to be 3 in *P. aeruginosa* (16) and may be similar in *P. fluorescens*. For the plasmid pME6000, the direct precursor of pME6127, a copy number of 18 was determined per *P. fluorescens* chromosome equivalent (S. Heeb and D. Haas, unpublished data).

Extraction, detection, and quantification of siderophores and antibiotics. Single bacterial colonies were transferred to 20-ml flasks containing 10 ml of glycerol-casamino acids medium (GCM) (Difco casamino acids [Difco Laboratories], 5 g; glycerol, 20 g; K₂HPO₄ · 3 H₂O, 0.75 g; MgSO₄ · 7 H₂O, 0.75 g; and double-distilled H₂O, 1 liter) (C. Keel, unpublished data). After shaking the flasks on a rotary shaker (Lab-Shaker LSR, 110 rpm, 2.5-cm radius; Adolf Kühner AG, Basel, Switzerland) at 27°C for 12 h, 10 μ l of the cultures was used to inoculate 20 ml of GCM medium with or without 10 μ M FeCl₃ in 100-ml Erlenmeyer flasks. The flasks were incubated at 27°C and shaken (rotary shaker; 110 rpm, 2.5-cm radius) for 72 h. The cultures were then acidified to pH 2 with 1 M HCl and extracted with one volume of ethyl acetate. After evaporation of the organic phase, the dry residue was dissolved in 1 ml of methanol and analyzed and then quantified by high-performance liquid chromatography (HPLC) as described before (26). SA was detected by UV absorption at 300 nm, pyochelin at 254 nm, 2,4-diacetylphloroglucinol at 270 nm, and pyoluteorin at 313 nm. The detection limit was 90 ng for SA, 350 ng for pyochelin, 20 ng for 2,4-diacetylphloroglucinol, and 50 ng for pyoluteorin. The metabolites produced were quantified according to standard curves prepared by injecting known amounts of commercial SA (Fluka AG, Buchs, Switzerland), pyochelin (28) purified from *P. aeruginosa* strain PAO1, 2,4-diacetylphloroglucinol synthesized according to Campbell and Coppinger (4), and pyoluteorin synthesized according to Cue et al. (5). The experiment was designed as a 2 $\times$ 3 factorial. The factors were iron and the different bacterial strains (wild type and two recombinants carrying plasmid pME3377 or pME6127). The experiment had five replications blocked over time.

Extraction of SA from the rhizosphere of tobacco grown in artificial soil. Tobacco plants were cultivated in sterile plant tissue culture containers (Wyss Samen und Pflanzen AG, Zuchwil, Solothurn, Switzerland) filled with 80 g of sterile artificial soil containing quartz sand of different sizes, quartz powder, and pure vermiculite clay (four plants per box) (17). Seven days before planting 5-week-old seedlings, bacteria were mixed with the soil to give a final concentration of 10⁷ cells per g of soil. Containers were arranged in a randomized complete block design with 39 replicates (one replicate = one container with four plants) per treatment. After 3 weeks of incubation in a growth chamber with the same conditions as described above, plants were removed and loosely adhering soil was shaken gently from the roots. Roots of 20 plants with closely adhering soil were vigorously shaken for 30 min in 200 ml of ethyl acetate (acidified to pH 2 with 2 M HCl). Roots and soil were removed by filtering the extract through filter paper. The extracts of all replicates of a treatment were then pooled and brought to dryness in vacuo. The residues were redissolved in methanol and analyzed by HPLC as described before (26), except that a Nucleosil 120-5 C18 column (4 $\times$ 250 mm; Macherey und Nagel, Düren, Switzerland) was used. The samples were eluted with a gradient of methanol from 20 to 42% (0 to 8 min), 42 to 45% (8 to 14 min), and 45 to 100% (14 to 25 min) in 0.43% (vol/vol) *o*-phosphoric acid. The retention time of synthetic SA was 13.8 min. The recovery of SA was estimated to be 45% based on results for artificial soil with added synthetic SA. The experiment was conducted three times.

Extraction of SA from the rhizosphere of tobacco grown in autoclaved Eschikon soil. At the end of the last two induced-resistance experiments (experiments 3 and 4), all plants were removed from the pots. Loosely adhering soil was removed from the roots by shaking the plants gently by hand. Roots with closely adhering soil were extracted following the method of Bonsall et al. (2). Treatments (12 plants per treatment) were divided in subsamples of four plants, which were extracted together. After extraction with ethyl acetate, extracts of the subsamples were pooled and brought to dryness in vacuo. Residues were dissolved in methanol and analyzed by HPLC as described for the extraction from artificial soil.

Biocontrol of TNV. The tobacco-TNV assay described by Maurhofer et al. (25) was used for testing the ability of the different strains to induce systemic resistance. Surface-disinfected plastic pots were filled with 300 g of autoclaved soil from Eschikon mixed with $\frac{1}{4}$ volume of quartz sand. Bacteria were added to the mix to give a concentration of 10^7 cells per g of soil. Two days later, a 4-week-old tobacco plant was transplanted into each pot. The pots were arranged in a randomized complete block design with 8 to 12 replicates, and one plants with one challenged leaf served as a replicate. After 6 weeks of incubation in a growth chamber with the same conditions as described above, all plants were challenge-inoculated on the youngest fully developed leaf. Plants of the systemic acquired resistance (SAR) control were induced with an initial TNV inoculation on a lower leaf 7 days prior to challenge inoculation. For TNV inoculations, the leaves were dusted with Carborundum, and four 50- μ l drops of a virus suspension were applied and gently rubbed on the leaf with a gauze pad. Virus suspensions were prepared from a tobacco leaf

densely covered with TNV lesions. A 0.1-g piece was taken and ground with 2 ml of a 20 mM phosphate buffer (pH 7.6) and then diluted 20 times using the same buffer. After 7 days, the number and size of lesions were assessed on challenge-inoculated leaves. The total necrotic area for each leaf was calculated by adding up the area of all the lesions on the leaf.

Root colonization and possible systemic colonization of the plants were examined as described by Maurhofer et al. (25). Population sizes of total aerobic bacteria were determined on tryptic soy agar plates (Difco tryptic soy agar [Difco Laboratories], 40 g; and double-distilled H_2O , 1 liter), and populations of introduced bacteria were determined on King's B agar plates containing rifampicin. To test whether bacteria isolated from the roots still contained plasmid pME3377 or pME6127, the colonies were transferred by replica plating to King's B agar plates containing tetracycline. The experiment was conducted four times over a 4-month period.

Biocontrol of *T. basicola*. Suppression of black root rot of tobacco was tested in a gnotobiotic system as described by Keel et al. (17). Artificial soil was prepared by mixing quartz sand of different sizes, quartz powder, and pure vermiculite clay (17). Sixty grams of artificial soil was placed into 100-ml flasks that were then sealed with cotton plugs and autoclaved at 121°C for 30 min. Five milliliters of *P. fluorescens* suspensions was added to flasks to give 10^7 cells of per g of artificial soil. Control flasks were watered with the same volume of sterile double-distilled water. One day later, 5 ml of a *T. basicola* suspension was mixed into the artificial soil with a sterile spatula to give a concentration of 2×10^4 endoconidia per g of soil. Seven days after the addition of the pathogen, one sterile-grown 5-week-old tobacco plant with four

Fig. 1. Physical maps of the salicylic acid (SA) expression plasmids pME3377, pME6127, and their precursor vectors. *tetR tetA*, tetracycline resistance genes; *kan*, kanamycin resistance gene; and *cat*, chloramphenicol resistance gene. The triangle represents the constitutive kanamycin promoter driving the expression of the SA biosynthetic genes *pchBA* in pME3377 and pME6127. Details for plasmid constructions are given in Materials and Methods. **A**, Plasmid pME3377 is a derivative of pVK100. **B**, Plasmid pME6127 was constructed on the basis of the new cloning vector pME6000, which is derived from pBBR1MCS.

leaves was transplanted into each flask and watered with 3 ml of modified Knop nutrient solution. The flasks were arranged in a randomized complete block design, with eight replicates per treatment and one flask with one plant per replicate. Plants were grown for 3 weeks in a growth chamber under the conditions described above. Plants were removed from flasks by flooding with tap water and then washed to remove the rest of the soil. Plants were then weighed and assessed for disease severity and root colonization as described before (17). The experiment was conducted three times.

TABLE 1. Production of salicylic acid (SA) by *Pseudomonas fluorescens* strains P3 and CHA0 carrying the plasmids pME3377 and pME6127 in GCM^v medium

Strain ^w	Iron added	SA production (µg/10 ⁹ CFU)	ANOVA ^x	
			F value	P
P3	–	<0.02 c ^y		
P3/pME3377	–	0.8 b		
P3/pME6127	–	2.9 a		
P3	+	<0.02 c		
P3/pME3377	+	0.6 b		
P3/pME6127	+	2.8 a		
ANOVA source				
Strain P3			104.7	0.000
Iron			0.201	0.658
Strain P3 × iron			0.053	0.948
CHA0	–	11.2 a		
CHA0/pME3377	–	10.2 a		
CHA0/pME6127	–	12.3 a		
CHA0	+	<0.02 c ^z		
CHA0/pME3377	+	1.3 b ^z		
CHA0/pME6127	+	5.3 a ^z		
ANOVA source				
Strain CHA0			12.0	0.000
Iron			231.2	0.000
Strain CHA0 × iron			4.074	0.030

^v GCM = glycerol-casamino acids.

^w Bacteria were grown in GCM medium with or without the addition of 10 µM FeCl₃ at 27°C for 72 h. SA was extracted from the culture medium with ethyl acetate.

^x ANOVA = analysis of variance.

^y Each value is the mean of five repetitions. Values in the same column followed by the same letter are not significantly different at $P = 0.05$ according to Fisher's protected least significant difference test. For P3 and its derivatives, all means in the column were compared. For CHA0 and its derivatives, means of treatments with iron and means of treatments without iron were compared separately because of the significant strain × iron interaction.

^z These means were compared with each other.

Statistics. For production of SA in vitro (Table 1), the values presented in Table 1 are the means of five replications. Because this experiment was a 2×3 factorial, data were first analyzed by a two-way analysis of variance (Systat version 7.0; Systat Inc., Evanston, IL) and then by Fisher's least significant difference (LSD) test (Systat Inc.). For biocontrol of TNV (Table 2) and *T. basicola* (Table 3), experiments were analyzed separately using Fisher's LSD test (Systat Inc.) after a significant F test ($P \leq 0.05$) (Systat Inc.). Values of the total necrotic area (Table 2) were log normally distributed and, therefore, log-transformed prior to statistical analysis. For black root rot severity (percentage of root surface darkened by chlamydospores) (Table 3), values were transformed to arcsines prior to statistical analysis.

RESULTS

Production of SA, pyochelin, and antibiotics by *Pseudomonas* spp. expressing the *pchBA* genes. Recombinant pseudomonads producing SA constitutively were constructed using the vectors pVK100 and pME6000 (Fig. 1). The production of SA, pyochelin, and antibiotics by strains CHA0 and P3 transformed with either pME3377 (pVK100*pchBA*⁺) or pME6127 (pME6000*pchBA*⁺) was compared with the production of these metabolites by the plasmid-free strains in GCM. Introduction of either plasmid rendered the SA-negative strain P3 capable of producing SA (Table 1). Similar amounts of SA were obtained with and without added iron, confirming iron-independent expression of *pchBA* by both expression plasmids. Strain P3/pME6127 produced significantly more SA than did strain P3/pME3377, possibly reflecting the different plasmid copy numbers. However, SA production of P3/pME6127 remained about four times lower than SA production of strain CHA0 under iron-limited conditions (Table 1). For strain CHA0 and its derivatives carrying *pchBA*, the addition of iron to the growth medium had a significant influence on SA production (Table 1). Under iron-limited conditions, the high amount of SA produced by CHA0 was not increased by the introduction of pME3377 or pME6127. In the presence of added iron, SA production was repressed in CHA0, but was detectable in CHA0/pME3377 and CHA0/pME6127 (Table 1). The amount of SA produced by CHA0/pME6127 in the presence of added iron was greater than that produced by CHA0/pME3377, but still about two times smaller than the amount of SA produced by the wild type under iron-limited conditions.

The plasmids did not influence the amounts of pyochelin produced by strain CHA0. In the absence of iron for strain CHA0 and its derivatives carrying *pchBA*, which produced 9.8 to 12.9 µg of

TABLE 2. Induction of systemic resistance in tobacco against tobacco necrosis virus (TNV) by *Pseudomonas fluorescens* strains P3 and CHA0 carrying the salicylic acid (SA) biosynthetic genes *pchBA*

Treatment ^x	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	TNA ^y	LD ^y	TNA	LD	TNA	LD	TNA	LD
TNV control	465 a ^z	2.5 a	172 a	2.1 a	56 a	2.0 a	39 a	1.9 a
SAR control	55 de	1.0 d	5 d	0.6 e	1 d	0.7 d	3 c	0.6 e
Roots colonized with:								
P3	288 ab	2.5 a	82 ab	1.8 b	20 b	1.5 b	11 b	1.7 ab
P3/pME3377	40 e	2.5 a	53 b	1.6 bc	13 b	1.3 bc	2 c	0.9 de
P3/pME6127	48 de	2.0 bc	24 d	1.2 d	8 c	1.1 c	2 c	1.2 cd
CHA0	93 cd	2.0 bc	55 b	1.5 c	23 b	1.4 b	11 b	1.3 bcd
CHA0/pME3377	150 bc	1.9 c	35 c	1.5 c	21 b	1.3 bc	20 ab	1.8 a
CHA0/pME6127	262 bc	2.3 ab	67 ab	1.4 cd	15 b	1.3 bc	10 b	1.5 abc

^x 'Xanthi nc' plants were grown for 6 weeks and then challenge-inoculated with TNV. Plants of the TNV control were not induced prior to challenge inoculation with TNV. Plants of the systemic acquired resistance (SAR) control were induced with a TNV inoculation on a lower leaf 7 days prior to challenge inoculation. P3 = SA-negative wild-type strain; CHA0 = SA-producing wild-type strain; P3/pME3377, P3/pME6127, CHA0/pME3377, and CHA0/pME6127 = transgenic strains carrying the SA biosynthetic genes *pchBA*.

^y TNA = Total necrotic area per leaf (mm²), and LD = lesion diameter (mm).

^z Values are the mean of 8 (experiments 1 and 2) or 12 (experiments 3 and 4) plants per treatment. Analysis of variance showed that the treatment effect was highly significant ($P \leq 0.001$) in all experiments. Values in the same column followed by the same letter are not significantly different according to Fisher's protected least significant difference test ($P = 0.05$). TNA values were log-transformed prior to statistical analysis.

pyochelin per log 9 CFU, the addition of 10 μM FeCl_3 to the medium resulted in much lower pyochelin production (0.2 to 0.3 μg /log 9 CFU). Strain P3 and its derivatives carrying *pchBA* did not produce any pyochelin. Introduction of *pchBA* into strain CHA0 had no influence on the production of the antibiotics 2,4-diacetylphloroglucinol and pyoluteorin (data not shown). The addition of 10 μM FeCl_3 , however, enhanced the production of 2,4-diacetylphloroglucinol by strain CHA0 from 0.5 to 8.8 μg /log 9 CFU and pyoluteorin from an undetectable level to 0.5 μg /log 9 CFU. Strain P3 and its derivatives carrying *pchBA* did not produce any 2,4-diacetylphloroglucinol or pyoluteorin. Introduction of the plasmids did not influence the growth rates of strains P3 and CHA0. At the time of extraction, the cell densities of the two wild-type strains and their derivatives carrying *pchBA* were between log 8.8 and 9.0 CFU/ml in the absence of added iron and between log 9.4 and 9.5 CFU/ml in the presence of added iron.

Production of SA in the rhizosphere of tobacco. After 3 weeks of growth in artificial soil inoculated with strain CHA0 or CHA0/pME3377, roots with adhering soil were extracted with ethyl acetate. The wild-type strain CHA0 produced 8.5 ± 7.5 ng of SA per g of roots with adhering rhizosphere soil and 45.3 ± 20.4 ng of SA per plant. Plasmid pME3377 enhanced SA production by CHA0 in the rhizosphere by a factor of three. Strain CHA0/pME3377 produced 30.7 ± 7.3 ng of SA per g of roots with adhering rhizosphere soil and 145.1 ± 19.9 ng of SA per plant.

After 7 weeks of growth in Eschikon soil inoculated with strains CHA0, P3, and their derivatives carrying *pchBA*, roots from tobacco plants with adhering rhizosphere soil were extracted with acetone and ethyl acetate. No SA could be detected in the rhizosphere of plants grown in soil inoculated with either strain.

Protection of tobacco against TNV. The influence of the plasmids pME3377 and pME6127 in strains P3 and CHA0 on ISR in tobacco against TNV was tested using 'Xanthi nc' plants grown in autoclaved Eschikon soil that had been inoculated with these bacterial strains. Plants induced by an initial TNV inoculation on a lower leaf (SAR control) were nearly completely resistant to the challenge inoculation in all experiments (Table 2). Strain P3 gave partial protection against TNV and reduced total necrotic leaf area by 38 to 73% (significant in experiments 3 and 4). Plasmid pME6127 significantly improved the ability of strain P3 to induce resistance in all four experiments. Compared with plants colonized by strain P3, the total necrotic area of plants colonized by strain P3/pME6127 was reduced by 60 to 83%, and the lesion diameter was reduced by 20 to 33% (Table 2). Plants protected by strain P3/pME3377 showed a significantly (83%) reduced total necrotic leaf area compared with plants protected by the wild type in experiments 1 and 4. Strain CHA0 induced resistance against the

virus in all experiments and reduced the total necrotic area by 59 to 80% and lesion diameter by 20 to 32%. Strains CHA0/pME3377 and CHA0/pME6127 did not differ from the parental strain in their ability to induce resistance (Table 2). Neither the two wild-type strains P3 and CHA0 nor their derivatives carrying *pchBA* reduced the size of the lesions to the extent found in the SAR control plants. After 7 weeks, mean population densities of all bacterial strains ranged from log 5.1 to 5.3 CFU per g of tobacco roots, and 83 to 90% of the bacteria still contained plasmid pME3377 or pME6127. Population sizes of total aerobic bacteria ranged from log 7.8 to 8.2 CFU/g of tobacco roots in all treatments. None of the strains inoculated was found inside the stems or leaves.

Suppression of black root rot under gnotobiotic conditions. Strains P3 and CHA0 and their transformants carrying *pchBA* were tested for the ability to suppress black root rot of tobacco in a gnotobiotic system. In the absence of the pathogen, neither the two wild-type strains P3 and CHA0 nor their derivatives carrying *pchBA* influenced plant growth. Fresh weights of plants grown in the presence of the bacterial strains alone ranged from 592 to 720 mg. Strain P3 gave in one experiment out of three a significant protection against *Thielaviopsis*, but only in terms of plant fresh weight (Table 3). In all experiments, the root infection of plants inoculated with strain P3 was as severe as that of the control. In contrast to plants grown in the presence of the wild-type strain P3, in all three experiments, plants grown in presence of strains P3/pME3377 and P3/pME6127 had significantly higher fresh weights than did control plants. The difference in fresh weight between plants protected by the wild type and those protected by the recombinant strains, however, was only significant in one case (strain P3/pME6127, experiment 3). The root surface infection of plants grown in the presence of strains P3/pME3377, P3/pME6127, or the parental strain was similar (Table 3). Plants protected by strain CHA0 showed 170 to 280% higher fresh weights and 68 to 78% lower root infection compared with the control (Table 3). Neither plasmid pME3377 nor plasmid pME6127 significantly improved the disease suppressiveness of strain CHA0.

At the end of the experiments, strain CHA0 and its derivatives were recovered from the roots at population densities ranging from log 8.2 to 8.5 CFU/g of root fresh weight; strain P3 and its derivatives were recovered at densities ranging from log 8.4 to 8.9 CFU/g of root fresh weight. In all treatments, 70 to 80% of the bacteria still contained the plasmid pME3377 or pME6127.

DISCUSSION

Expression of the SA biosynthetic genes *pchBA* in the SA-negative, root-colonizing strain P3 significantly improved its abil-

TABLE 3. Suppression of black root rot of tobacco by *Pseudomonas fluorescens* strains P3 and CHA0 carrying the salicylic acid (SA) biosynthetic genes *pchBA*

Treatment ^x	Experiment 1		Experiment 2		Experiment 3	
	Plant fresh weight (mg)	Root infection (%) ^y	Plant fresh weight (mg)	Root infection (%)	Plant fresh weight (mg)	Root infection (%)
Healthy control	679 a ^z	—	664 a	—	676 a	—
Disease control	151 e	100.0 a	147 d	100.0 a	108 e	100.0 a
Plants protected by:						
P3	190 de	100.0 a	248 cd	98.8 a	186 d	98.8 a
P3/pME3377	256 d	98.1 a	268 c	98.1 a	189 d	98.1 a
P3/pME6127	253 d	98.1 a	260 c	98.1 a	258 c	98.1 a
CHA0	401 bc	32.2 b	447 b	23.4 b	411 b	23.4 bc
CHA0/pME3377	458 b	20.9 b	472 b	18.8 b	458 b	16.3 b
CHA0/pME6127	354 c	37.2 b	492 b	22.5 b	324 c	33.8 c

^x *Nicotiana glutinosa* plants were grown in artificial soil for 3 weeks under gnotobiotic conditions. The soil was amended with bacteria (10^7 cells per g) 8 days, and with *Thielaviopsis basicola* (2×10^4 endoconidia per g) 7 days, before addition of the plant. P3 = SA-negative wild-type strain; CHA0 = SA-producing wild-type strain; P3/pME3377, P3/pME6127, CHA0/pME3377, and CHA0/pME6127 = transgenic strains carrying the SA biosynthetic genes *pchBA*. In the healthy control, the soil was not amended with bacteria or *T. basicola*. In the disease control the soil was amended only with *T. basicola*.

^y Percentage of root surface darkened by chlamydospores.

^z Mean of eight replicates with one flask containing one plant per replicate. Analysis of variance showed that the treatment effect was highly significant ($P \leq 0.001$) in all experiments. Values in the same column followed by the same letter are not significantly different according to Fisher's protected least significant difference test ($P = 0.05$). Values of the root infection were arcsin-transformed prior to statistical analysis.

ity to induce systemic resistance against TNV in tobacco. In an earlier study (40), we demonstrated that the suppression of black root rot by strain P3 can be improved by inserting into it the hydrogen cyanide biosynthetic genes of strain CHA0. The biocontrol activity of other pseudomonads has been improved by the introduction of genes required for the production of antifungal antibiotics or chitinase (12,13,21,26,34). However, transgenic bacteria do not always outperform the parental strains. For instance, introducing *pchBA* into strain P3 had little or no effect on the suppression of black root rot of tobacco, suggesting that bacterial SA production in the rhizosphere is not a major factor in the suppression of this disease. Derivatives of strain CHA0 carrying either plasmid pME3377 or pME6127 showed iron-independent SA production in vitro and an enhanced SA production (CHA0/pME3377) in the rhizosphere of tobacco grown in a gnotobiotic system. However, protection of roots against black root rot was not improved by SA overexpression in the tobacco rhizosphere, and derivatives of strain CHA0 carrying *pchBA* did not show improved protection against TNV. This finding does not exclude the possibility that SA production plays a role in disease suppression by strain CHA0. We suggest that the amount of SA produced by strain CHA0 in the rhizosphere is already sufficient to induce systemic resistance and that increasing this amount does not increase the level of ISR. However, we could not detect the presence of SA in the rhizosphere of tobacco plants grown in Eschikon soil, probably due to adsorbance of SA to organic matter or the clay minerals in this soil. Leeman et al. (23) showed that treatment of radish root tips with SA-induced systemic resistance against *Fusarium* wilt independently of the SA concentration (100 fg to 100 µg per plant) used. In an earlier study (25), we found that strain CHA400, a pyoverdine-negative mutant of strain CHA0, lost part of its ability to induce resistance in tobacco against TNV. The remaining ISR capacity could be due to SA production or to other, as yet unidentified, bacterial traits.

The newly constructed cloning vector pME6000 (Fig. 1B) used here is a derivative of pBR1MCS (22), which replicates stably in Gram-negative bacteria including *Vibrio*, *Escherichia*, and *Rhizobium*. We found that pME6000 is maintained in *P. fluorescens* CHA0 for up to 100 generations without antibiotic selection in vitro (S. Heeb and D. Haas, unpublished data), making this vector suitable for environmental studies. The fact that pME6000 has about 18 copies per chromosome equivalent in *P. fluorescens* can be an advantage whenever high gene expression is required in recombinant strains.

In several systems, evidence is now accumulating for a role of bacterial SA in ISR. Our experiments have shown that *P. fluorescens* strain P3 engineered to produce SA had an improved ability to induce systemic resistance against TNV in tobacco. In addition, De Meyer and Höfte (9) found that SA-negative mutants of *P. aeruginosa* 7NSK2 lost their ability to induce systemic resistance against *B. cinerea* in bean. Finally, Leeman et al. (23) attributed the capacity of O-antigenic sidechain-negative mutants of *P. fluorescens* strains WCS374 and WCS417 to induce systemic resistance against *Fusarium* wilt of radish at low, but not at high, iron availability to the production of SA. In contrast to these examples, Press et al. (31) suggested that SA production by *S. marcescens* 90-166 was not important in the induction of systemic resistance in cucumber and tobacco, because SA-negative mutants of strain 90-166 induced the same level of resistance in cucumber, wild-type tobacco, and NahG-tobacco (the NahG gene encodes the enzyme salicylate hydroxylase that degrades SA) expressing salicylate hydroxylase as the wild type. A role of SA in PGPR-mediated ISR seems to depend on the biocontrol strain and maybe also on the plant species. Van Wees et al. (39) showed that the capacity of PGPR strains to induce resistance can be specific not only for certain plant species, but even for ecotypes. A mutant lacking the O-antigenic sidechain of lipopolysaccharide lost its ability to induce resistance in one plant species, but still elicited

ISR in another (39). Moreover, some PGPR strains including CHA0 induce PR proteins in plants (15,25,45), while others do not, although plants can exhibit systemic resistance (14,29). ISR mediated by root-colonizing bacteria appears to involve multiple mechanisms, depending on the biocontrol strains and host plants.

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