The EMBO Journal vol.8 no.2 pp.351 - 358, 1989

Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions

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Communicated by M.van Montagu

***Pseudomonas fluorescens* CHA0 suppresses black root rot of tobacco, a disease caused by the fungus *Thielaviopsis basicola*. Strain CHA0 excretes several metabolites with antifungal properties. The importance of one such metabolite, hydrogen cyanide, was tested in a gnotobiotic system containing an artificial, iron-rich soil. A cyanide-negative (*hcn*) mutant, CHA5, constructed by a gene replacement technique, protected the tobacco plant less effectively than did the wild-type CHA0. Complementation of strain CHA5 by the cloned wild-type *hcn*$^+$ genes restored the strain's ability to suppress disease. An artificial transposon carrying the *hcn*$^+$ genes of strain CHA0 (Tn*hcn*) was constructed and inserted into the genome of another *P.fluorescens* strain, P3, which naturally does not produce cyanide and gives poor plant protection. The P3::Tn*hcn* derivative synthesized cyanide and exhibited an improved ability to suppress disease. All bacterial strains colonized the roots similarly and did not influence significantly the survival of *T.basicola* in soil. We conclude that bacterial cyanide is an important but not the only factor involved in suppression of black root rot.**

Key words: black root rot/cyanide/disease suppression/ *Pseudomonas fluorescens*/*Thielaviopsis basicola*

Introduction

Root-colonizing, fluorescent pseudomonads suppress plant diseases caused by soilborne pathogens in greenhouse experiments and in field trials (Schroth and Hancock, 1982; Davison, 1988; Weller, 1988; Défago and Haas, 1989). These plant-beneficial pseudomonads have a common property: they are antagonists of phytopathogenic fungi and bacteria (de Weger *et al.*, 1987). Two major hypotheses have been proposed to explain the suppressive and antagonistic effects of fluorescent pseudomonads. According to one hypothesis, the pathogen is inhibited by competition for iron; in some soils, the concentration of available iron [mainly Fe(III)] is very low, and the fluorescent pseudomonads, which produce high-affinity iron-chelators (e.g. pyoverdine; Neilands and Leong, 1986; Leong, 1986) may compete out harmful microorganisms having less potent siderophores (Kloepper *et al.*, 1980). According to another hypothesis, the fluorescent pseudomonads inhibit pathogens by producing secondary metabolites with antibiotic activity, e.g. phenazines, pyrroles, acetylphloroglucinols, or cyanide (Davison, 1988; Défago and Haas, 1989). Disease suppression is multifactorial, hence these hypotheses are not mutually exclusive, and additional factors such as aggressive root colonization may also be important.

Pseudomonas fluorescens strain CHA0 was isolated from a suppressive soil near Payerne (Switzerland). This strain suppresses black root rot of tobacco, a disease caused by the fungus *Thielaviopsis basicola*. Disease suppression occurs in iron-rich natural and artificial soils containing vermiculite clay (Stutz *et al.*, 1986; Keel *et al.*, 1989). Strain CHA0 also protects wheat from *Gäumannomyces graminis* var. *tritici* in greenhouse and field experiments (Défago *et al.*, 1987). Several products of strain CHA0 may contribute to disease suppression: pyoverdine, antibiotics, and hydrogen cyanide (HCN) (Ahl *et al.*, 1986). To assess the importance of these compounds, we have developed a gnotobiotic test system which consists of an artificial soil, a tobacco plant and known amounts of *T.basicola* endoconidia and *P.fluorescens* cells (Keel *et al.*, 1989). In this system, the suppressive effects of the wild-type and mutant strains of *P.fluorescens* can be measured quantitatively and reproducibly.

About 800 plant species liberate cyanide when they are wounded or attacked by fungi and in this way may inhibit the cyanide-sensitive pathogens (Mansfield, 1983). Some successful fungal pathogens of cyanogenic plants are tolerant to cyanide because they can detoxify it by converting it to formamide (VanEtten and Kistler, 1984). *T.basicola* is sensitive to cyanide at concentrations which are produced by *P.fluorescens* CHA0 growing on nutrient agar or on pieces of cotton roots (Ahl *et al.*, 1986). However, it is not known whether *P.fluorescens* produces cyanide in soil. The question, therefore, is whether the suppressive effect of *P.fluorescens* is due, at least in part, to the production of cyanide *in situ*. Our experiments conducted in the gnotobiotic system indicate that this is the case.

Results

Cyanide production by P.fluorescens

P.fluorescens CHA0 produced cyanide in the chemically defined medium of Castric (1977). Glycine, the precursor of cyanide in *Pseudomonas aeruginosa* (Castric, 1977), and Fe^{3+} ions stimulated cyanide production in strain CHA0 (Table I); cyanogenesis occurred at the end of exponential growth and at the beginning of stationary phase in batch culture (data not shown). Similar results have been obtained previously for other *P.fluorescens* strains and for *P.aeruginosa* (Askeland and Morrison, 1983; Castric, 1977, 1983). Strain CHA0 produced 80 μM HCN after growth on tobacco root pieces (1 g fresh wt in 3 ml buffer) for 2 days.

Complementation of cyanide-nonproducing (Hcn$^-$) mutants of P.fluorescens by recombinant plasmids

Several transposon delivery systems were tried for mutagenesis of *P.fluorescens* CHA0. The most effective

Table I. Cyanide production by *P.fluorescens* CHA0 in batch culture

Addition to culture medium[a]	HCN produced[b] (μM)
$FeCl_3$ (20 μM)	11 ± 3
$FeCl_3$ (20 μM) + glycine (12.5 mM)	73 ± 10
Glycine (12.5 mM) + EDDHA (140 μM)[c]	<5

[a]Complete Castric medium (Materials and methods) but without $FeCl_3$ and glycine.
[b]Mean values of triplicate experiments (±SD) after growth at 30°C for 48 h.
[c]Addition of EDDHA sequesters residual Fe^{3+}.

transposon donor was *Escherichia coli* carrying the IncIα plasmid pLG221 (= ColIb-P9*drd-1*::Tn*5*); this plasmid is transmissible to *Pseudomonas*, where, however, it does not replicate (Boulnois *et al.*, 1985). Kanamycin-resistant (Km^r) transconjugants of strain CHA0, i.e. presumptive Tn*5* insertion mutants, were recovered at 10^{-7} per donor. Among 4700 transconjugants, 0.6% were auxotrophs and three strains (CHA501, CHA503, CHA505) did not produce cyanide. Recombinant plasmids consisting of the broad-host-range cosmid pVK100 (Knauf and Nester, 1982) and genomic DNA of strain CHA0 were screened for complementation of the Hcn^- mutants. To this end, the recombinant plasmids were mobilized by the IncP helper plasmid pME497 (Voisard *et al.*, 1988) from *E.coli* to the *P.fluorescens* Hcn^- mutants. Six plasmids which gave complementation had a common *Hin*dIII insert of 8.4 kb and additional *Hin*dIII fragments of different sizes. The 8.4-kb fragment was subcloned into pVK100, giving pME3013 (Figure 1A). This plasmid phenotypically restored cyanide production in strains CHA501, CHA503, and CHA505 and also enabled the non-cyanogenic strain P3 of *P.fluorescens* to synthesize cyanide (Table II, see below). Thus, the 8.4-kb fragment contains genes (*hcn*) necessary for cyanide synthesis. In cultures of *E.coli* ED8767(pME3013) no cyanide could be detected (Table II), presumably because *E.coli* lacks some functions required for cyanogenesis.

We verified by Southern hybridization with pME3013 that the 8.4-kb fragment originated from strain CHA0 (Figure 1B, lane 1). Unexpectedly, the Hcn^- Tn*5* insertion mutants CHA501, CHA503 and CHA505 also revealed a chromosomal fragment of 8.4 kb, not disrupted by Tn*5* insertion (Figure 1B, lanes 2–4). Hybridization of genomic DNA from the Hcn^- mutants with a Tn*5* probe indicated that in each case a single Tn*5* copy was inserted into the *P.fluorescens* genome outside the 8.4-kb fragment (data not shown). We assume that the Hcn^- phenotype of the three mutants is caused by Tn*5* insertions into some regulatory genes required for secondary metabolism, because all three mutants were pleiotropically defective in antibiotic production (data not shown). If, as we believe, pME3013 carries the structural genes for cyanogenesis, the plasmid might override the control mutations by a copy number effect and confer an Hcn^+ phenotype on the mutants. However, other explanations are also possible, and hence we decided to inactivate the chromosomal *hcn* genes of strain CHA0 by a gene replacement technique.

Insertional inactivation of the chromosomal hcn genes of P.fluorescens CHA0

A system for gene replacement in *P.fluorescens* was set up on the basis of the IncIα transfer functions, since the IncIα plasmid pLG221 had proved an efficient transposon donor. Plasmid ColE1 is mobilized at high frequencies by another derepressed IncIα plasmid, R64*drd-11*, in *E.coli* (Warren

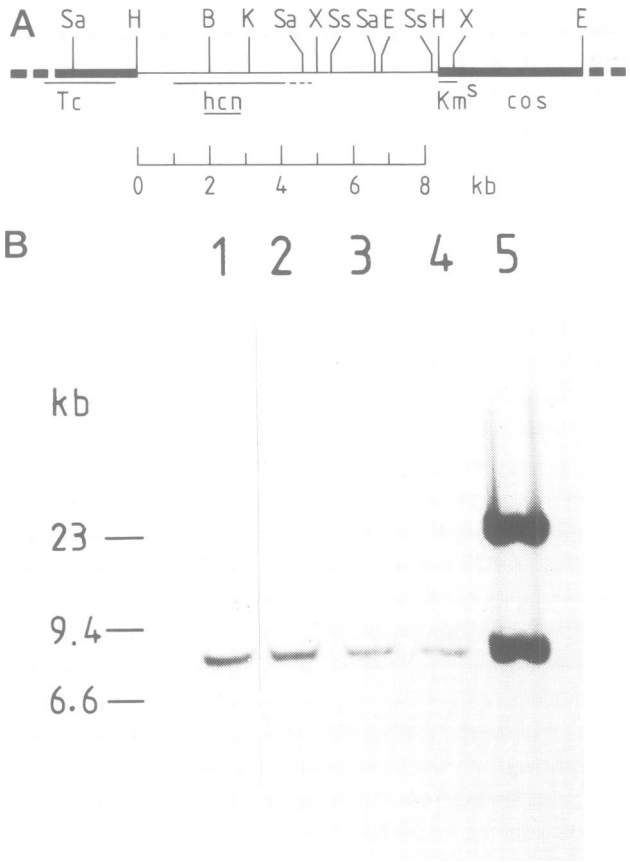

Fig. 1. Cloning of the *hcn* genes from strain CHA0 into the broad-host-range cosmid pVK100. (**A**) Map of pME3013. The extent of the *hcn* genes (for hydrogen cyanide synthesis) is deduced from subcloning experiments, transposon insertions, and *in vitro* deletions (not shown), dashed lines indicate uncertainty. —, CHA0 insert (8.4 kb); ▬ vector (23 kb); B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Sa, *Sal*I; Ss, *Sst*I; X, *Xho*I. (**B**) Hybridization of genomic DNA from *P.fluorescens* CHA strains with pME3013. *Hin*dIII-digested DNA was run on a 0.6% agarose gel at 70 V for 16 h, transferred to a nitrocellulose membrane and hybridized with ^{35}S-labelled pME3013. The 8.4-kb chromosomal bands also hybridized to the isolated 8.4-kb fragment of pME3013 used as a probe (data not shown); **Lane 1**, CHA0; **lane 2**, CHA501; **lane 3**, CHA503; **lane 4**, CHA505; **lane 5**, pME3013.

Table II. Cyanide production by genetically manipulated *P.fluorescens* strains in batch culture

Strain	HCN produced[a] (μM)
P.fluorescens	
CHA0 [wild-type]	73 ± 10
CHA5 [*hcn*::Ω-Hg]	<5
CHA5(pME3013)	327 ± 21
P3 [wild-type]	<5
P3(pME3013)	573 ± 60
P3::Tn*hcn*	122 ± 2
P3::Tn*TpSm*	<5
E.coli	
ED8767 (pME3013)	<5

[a]Mean values of triplicate experiments (±SD) after growth in complete Castric medium at 30°C for 48 h.

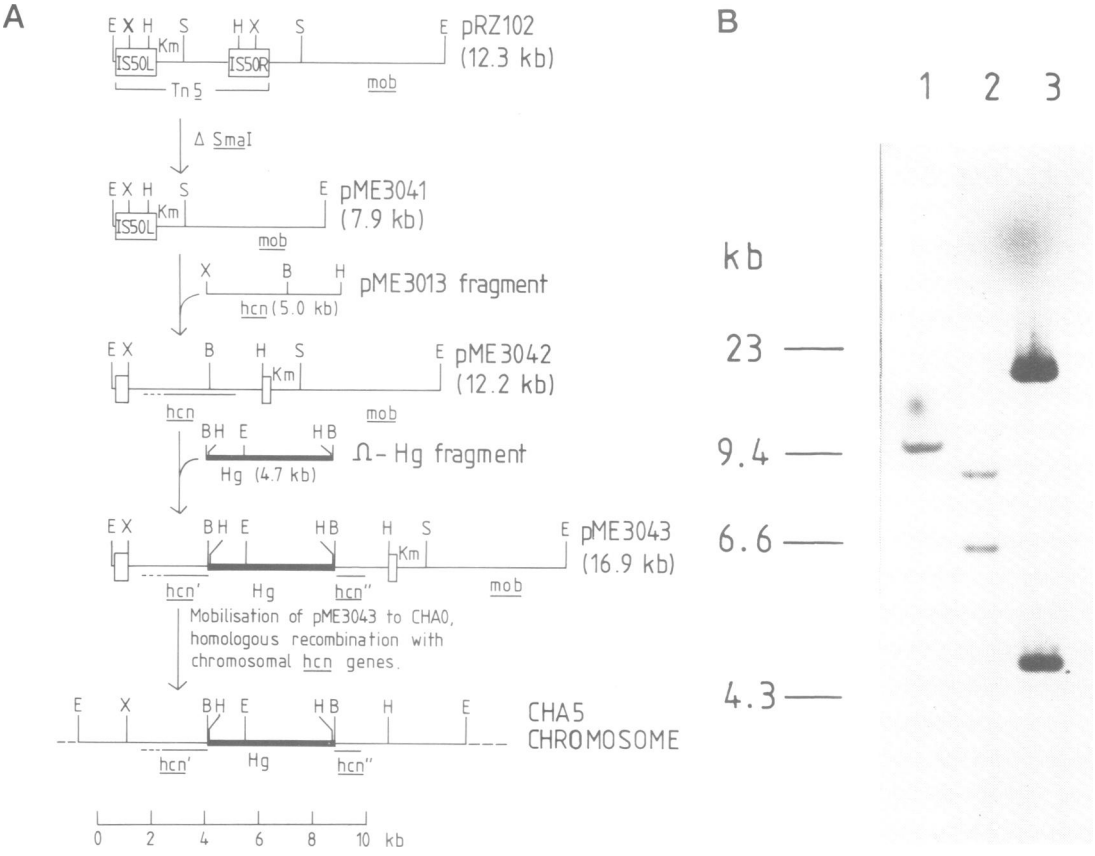

Fig. 2. Site-directed mutation of the chromosomal *hcn* genes of strain CHA0 by insertion of an Ω-Hg fragment. (**A**) Cloning of the *hcn* genes into a mobilizable suicide plasmid (pME3041) and replacement of the chromosomal *hcn*$^+$ genes by inactivated *hcn*::Ω-Hg genes. The first three steps are *in vitro* constructions done in *E.coli*. In the last step, pME3043 was mobilized by R64*drd-11* from *E.coli* SK1592 to *P.fluorescens* CHA0, with selection on nutrient agar containing chloramphenicol (20 μg/ml) and $HgCl_2$ (20 μg/ml). Cross-overs in the *hcn* regions flanking the Ω-Hg insert produced the recombinant strain CHA5 (*hcn*::Ω-Hg). Abbreviations are the same as in Figure 1; plus □, IS*50*; S, *Sma*I. (**B**) Verification of the gene replacement in strain CHA5 by hybridization with pME3043. *Eco*RI-digested DNA was run on a 0.6% agarose gel at 60 V for 16 h, transferred to a nitrocellulose membrane and hybridized with ^{35}S-labelled pME3043. **Lane 1**, CHA0; **lane 2**, CHA5; **lane 3**, pME3043.

et al., 1979). ColE1 plasmids do not replicate in *Pseudomonas* (Simon *et al.*, 1983). We checked that R64*drd-11*-dependent mobilization of pRZ102 (= ColE1::Tn*5*) produced Tn*5* insertion mutants in *P.fluorescens* CHA0 at 10^{-8} per donor. Hence, a derivative of pRZ102, pME3041 (Figure 2A), was chosen as a mobilizable suicide vector.

The *hcn* genes on a 5.0-kb fragment from pME3013 were cloned into pME3041 and inactivated by insertion of the transcription/translation stop element Ω-Hg (Fellay *et al.*, 1987). The resulting plasmid pME3043 (Figure 2A) was transferred from *E.coli* to *P.fluorescens* CHA0. Hg resistant (Hg^r) transconjugants appeared at 10^{-7} per donor. 87% were Hg^{r}Km^r and presumably had integrated the entire pME3043 via a single cross-over; 13% were Hg^{r}Km^s, indicating the loss of the vector by a second cross-over (240 colonies tested). Five Hg^{r}Km^r clones tested were Hcn$^+$; five Hg^{r}Km^s clones were Hcn$^-$, as expected for an *hcn*$^+$ → *hcn*::Ω-Hg replacement (Figure 2A). A representative Hg^{r}Km^s strain, CHA5 (*hcn*::Ω-Hg), was analysed by Southern hybridization with pME3043. In an *Eco*RI digest, bands of ~6.4 kb and 8.3 kb were revealed (Figure 2B, lane 2). The sum of these bands approximately equals the sum of the 9.6-kb chromosomal *Eco*RI fragment carrying the *hcn*$^+$ genes (Figure 2B, lane 1) plus the 4.7-kb Ω-Hg insert (Figure 2A). We conclude that strain CHA5 contains an *hcn*::Ω-Hg insertion, and this result was confirmed by a *Sal*I digest (not shown). In a quantitative cyanide assay, strain CHA5 was totally negative in Castric medium (Table II) and on tobacco roots. The Hcn$^-$ defect of CHA5 was complemented by pME3013. In fact, strain CHA5(pME3013) produced more cyanide than did strain CHA0 (Table II), presumably as a consequence of multiple pME3013 copies. Antibiotic production in strain CHA5 was indistinguishable from that in the wild-type CHA0 (data not shown).

To rule out the possibility that the Hg resistance determinant might confer a selective disadvantage on *P.fluorescens*, we also constructed a derivative of strain CHA0 with a random Hg resistance gene insertion, CHA25. This strain was Hcn$^+$ and contained a copy of Tn*5-259* (Km^{r}Hg^r), which had been inserted into a non-essential region by the use of the transposon donor plasmid pME12 (Voisard *et al.*, 1988). The Hg resistance genes of Ω-Hg and Tn*5-259* are identical (Fellay *et al.*, 1987; Voisard *et al.*, 1988).

Disease suppression by P.fluorescens CHA strains

In the gnotobiotic system, infection with *T.basicola* drastically reduced the weight of plant roots and shoots after incubation for 3 weeks (Figure 3A, columns 1 and 6), and a large proportion of the root surface was covered with chlamydospores (Figure 3B, column 6). The wild-type *P.fluorescens* CHA0 afforded good protection against black root rot: the plant weight was increased ~5-fold and the infected root surface was reduced to half (Figure 3A and

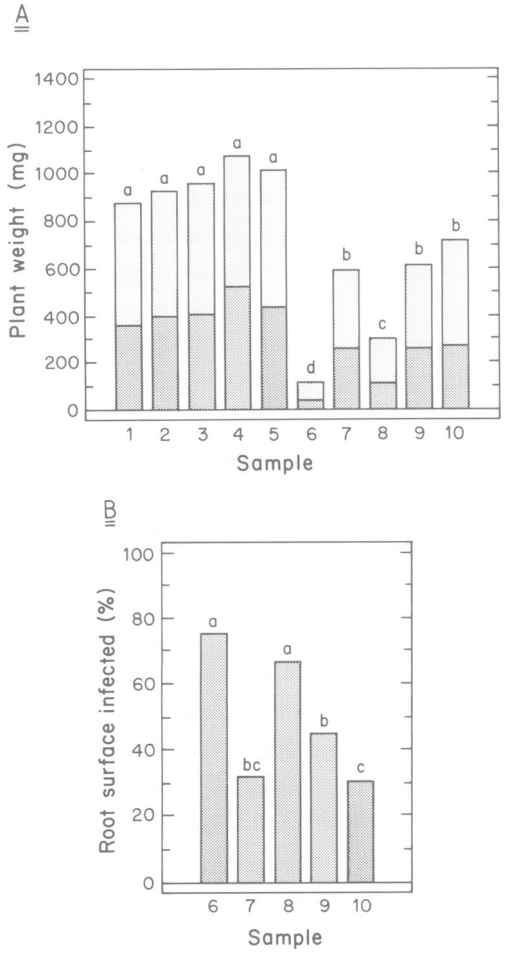

Fig. 3. Suppression of *T.basicola*-induced black root rot of tobacco by strain CHA0 and its derivatives. (**A**) Fresh plant weight is expressed as the sum of root weight (▨) plus shoot weight (▩). (**B**) Root surface infection was calculated according to Stutz *et al.* (1986). Each value is the mean of three repetitions, with 8–10 plants per repetition. Means represented by columns are headed by the same letter when they are not significantly different at $P = 0.05$ according to the multiple t test. **Column 1**, control plant without fungi (*T.basicola* D127) and bacteria (*P.fluorescens* CHA); **2**, + CHA0; **3**, + CHA5; **4**, + CHA5(pME3013); **5**, + CHA25; **6**, + D127; **7**, + D127 + CHA0; **8**, + D127 + CHA5; **9**, + D127 + CHA5(pME3013); **10**, + D127 + CHA25.

B, column 7). In this and a number of similar experiments, the level of protection was ~70%; that is, plants which were infected with *T.basicola* and treated with strain CHA0 reached 70% of the weight of uninfected control plants, grown with or without bacteria (Keel *et al.*, 1989). In the absence of the pathogen, the bacteria did not significantly influence the final plant weight (Figure 3A, columns 1–5).

The Hcn$^-$ mutant CHA5 provided a significantly lower degree of protection and did not reduce the percentage of the infected root surface (Figure 3A and B, column 8). Complementation of the *hcn*::Ω-Hg mutation of strain CHA5 with pME3013 restored the strain's capacity to suppress the disease, in terms of both plant weight and disease severity (Figure 3A and B, column 9). After incubation for 4 weeks in the gnotobiotic system, 60–80% of the bacteria still contained pME3013. The Hg resistance determinant, which was used for inactivation of the *hcn* genes in strain CHA5, *per se* had no adverse effect on disease suppression, as shown by the wild-type suppressive properties of the random Hg^r insertion mutant CHA25 (Figure 3A and B, column 10). All bacterial strains used in this test colonized the roots to a similar extent; $2 \times 10^8 - 3 \times 10^9$ c.f.u. were recovered per g of fresh root weight at the end of the experiments. Strains CHA0, CHA5 and CHA5(pME3013) had no measurable influence on the survival of *T.basicola* in the artificial soil planted with tobacco: the number of propagules reisolated per g of soil after 4 weeks ranged from 2×10^3 to 5×10^3, irrespective of the bacterial strain. In conclusion, mutation of the *hcn* genes abolished part of the suppressive ability of *P.fluorescens* CHA0, but did not change bacterial root colonization.

Transposition of the hcn+ genes into P.fluorescens P3

P.fluorescens P3 is an isolate from barley roots grown in the region of Münstertal (Graubünden, Switzerland). This strain does not produce cyanide naturally and gives poor protection against black root rot in the gnotobiotic system (cf. Figure 6A, column 8). To test whether cyanide production would improve the suppressive ability of strain P3, we introduced pME3013 into this strain. The transconjugant produced cyanide in batch culture (Table II), but could not be tested in the gnotobiotic system because the plasmid was too unstable in the absence of antibiotic selection. Therefore, an artificial transposon containing the *hcn*$^+$ genes (Tn*hcn*) was constructed and transposed into the P3 genome (Figure 4A).

Tn*hcn* (Figure 4A) was bordered by inverted repeats of IS*50* and activated by the transposase of IS*50*R (Berg and Berg, 1983). The transposon also contained selectable trimethoprim (Tp) and streptomycin (Sm)/spectinomycin (Sp) resistance markers (Figure 4A). The delivery plasmid, pME3045, which is based on ColE1, was mobilizable by R64*drd-11*. As a control, a similar transposon without *hcn* genes, Tn*TpSm*, was constructed on pME3047 (Figure 4B).

Transposition of Tn*hcn* and Tn*TpSm* into the *P.fluorescens* P3 genome occurred at ~10^{-8} per *E.coli* donor and was confirmed by Southern hybridization with pME3045. Internal *Bam*HI fragments of 2.8 kb (Tn*TpSm*) or 5.1 kb (Tn*hcn*) plus two larger border fragments of variable sizes were found to hybridize with DNA of strain P3 carrying either transposon (Figure 5, lanes 2 and 3). The insertions of Tn*hcn* and Tn*TpSm* were in different *Bam*HI fragments of the host. The P3 wild-type DNA did not hybridize with the *hcn* genes (Figure 5, lane 4). In batch culture (Table II) and on tobacco roots strain P3::Tn*hcn* produced cyanide in amounts comparable to those made by CHA0; P3::Tn*TpSm* did not synthesize measurable quantities of cyanide.

Disease suppression by P.fluorescens P3 derivatives

Strain P3 gave marginal protection against black root rot in the gnotobiotic system and was unable to reduce the percentage of infected root surface. Similar properties were found for the control strain P3::Tn*TpSm* (Figure 6A and B, columns 8 and 10). In contrast, strain P3::Tn*hcn*, which carries the *hcn*$^+$ genes of strain CHA0, was able to protect tobacco partially against the symptoms of *T.basicola* infection: plant growth was improved ~3-fold, by comparison with the effect of the wild-type P3 (Figure 6A, columns 8 and 9). However, the infected root surface was almost the same as in a control with *T.basicola* alone (Figure 6B, columns 6 and 9). The ability to colonize tobacco roots

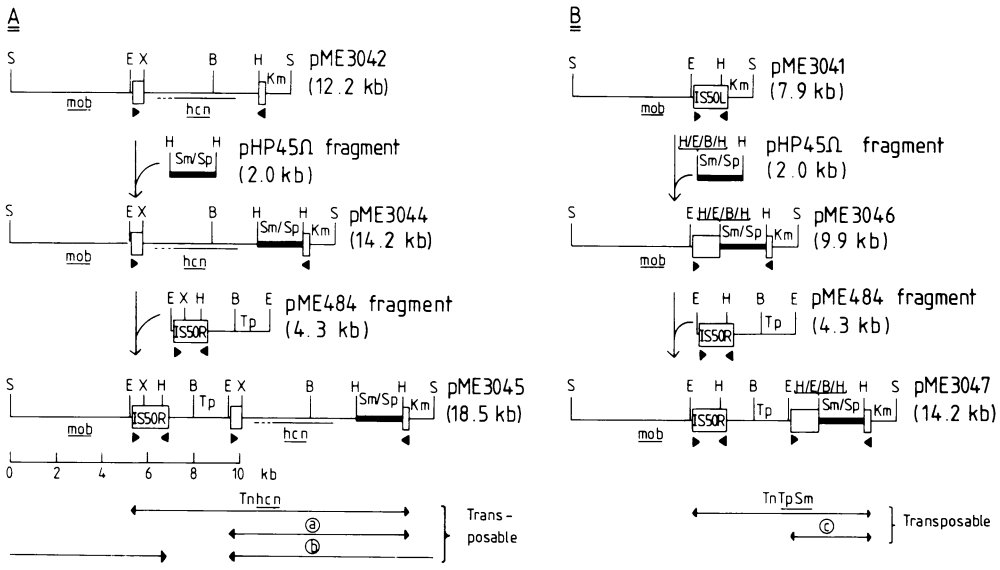

Fig. 4. Construction and transposition of Tn*hcn* and Tn*TpSm*. (**A**) Construction of pME3045 from pME3042; (**B**) construction of pME3047 from pME3041. Abbreviations are the same as in Figures 1 and 2. ◄ and ►, inverted 19-bp repeats of IS*50* (Berg and Berg, 1983). H/E/B/H, polylinker of pHP45Ω. For transposon delivery, *E.coli* SK1592 (R64*drd-11* + pME3045 or pME3047) was mated with *P.fluorescens* P3; selection was made on nutrient agar + chloramphenicol (20 μg/ml) + Sm (200 μg/ml). Among 84 P3 (pME3045) transconjugants, 27% were Tp^r/Sm^r and presumably had Tn*hcn*; 0% were Sm^r (transposition of ⓐ); 3% were Sm^rKm^r (transposition of ⓑ); 70% were $Tp^rSm^rKm^r$ (integration of pME3045). Among 16 P3(pME3047) transconjugants, 12% were Tp^rSm^r and presumably had Tn*TpSm*; 69% were Sm^r (transposition of ⓒ); 19% were $Tp^rSm^rKm^r$ (integration of pME3047).

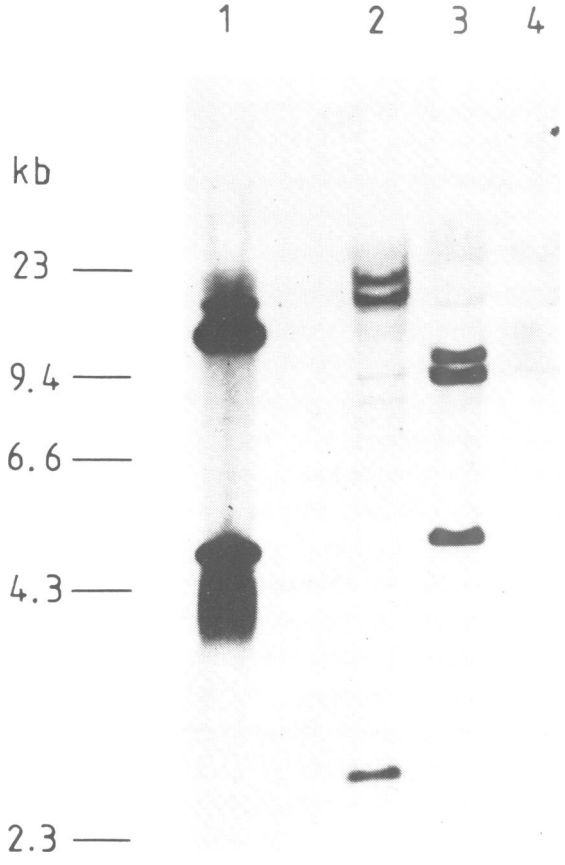

Fig. 5. Verification of transposition of Tn*hcn* and Tn*TpSm* into strain P3. Total DNA was digested with *Bam*HI, separated on a 0.6% agarose gel (60 V, 16 h), transferred to a nitrocellulose membrane and hybridized with ^{35}S-labelled pME3045 (Figure 4A). **Lane 1**, pME3045; **lane 2**, P3::Tn*TpSm*; **lane 3**, P3::Tn*hcn*; **lane 4**, P3.

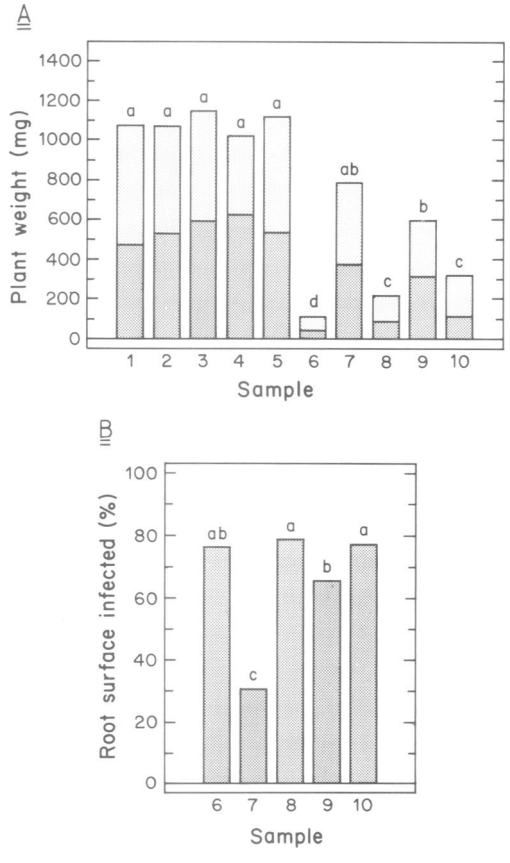

Fig. 6. Suppression of *T.basicola*-induced black root rot of tobacco by *P.fluorescens* P3 and its derivatives. (**A**) Fresh plant weight (roots + shoots); (**B**) root surface infection. Symbols are the same as in Figure 3. **Column 1**, control plant without fungi (D127) or bacteria; **2**, + CHA0; **3**, + P3; **4**, + P3::Tn*hcn*; **5**, + P3::Tn*TpSm*; **6**, + D127; **7**, + D127 + CHA0; **8**, + D127 + P3; **9**, + D127 + P3::Tn*hcn*; **10**, + D127 + P3::Tn*TpSm*.

was similar for all P3 derivatives and strain CHA0 (data not shown). In summary, the addition of the *hcn*$^+$ genes to strain P3 resulted in an improvement of disease suppression, but the suppressive properties of strain CHA0 were superior (Figure 6A and B, column 7).

Discussion

Glycine stimulated cyanide production in *P.fluorescens* CHA0 (Table I). In other *Pseudomonas* strains the same stimulation is observed, and glycine is the precursor of cyanide. The C2 atom of glycine is recovered mostly in HCN, whereas C1 is converted essentially to CO_2 (Castric, 1977; Askeland and Morrison, 1983). The mechanism of glycine oxidation to HCN and CO_2 in bacteria is not known, nor is the number of genes involved (Wissing and Andersen, 1981). The *hcn* genes on pME3013 occupy 3.0−3.8 kb (our unpublished results), corresponding to a coding capacity for ~2−5 polypeptides. If pME3013 does indeed contain the *hcn* structural genes, glycine oxidation may require not more than ~5 polypeptides.

When cyanogenesis is induced in batch cultures of *P.aeruginosa* by conditions of oxygen limitation at the end of exponential growth, an alternate, cyanide-insensitive cytochrome oxidase is produced (Trutko *et al.*, 1979; Matsushita *et al.*, 1983). A cyanide-resistant, alternative respiratory pathway also occurs in roots and leaves of plants (Laties, 1982; Lambers *et al.*, 1983) and in fungi (Garraway and Evans, 1984). Nevertheless, many fungi including *T.basicola* are sensitive to cyanide (Ahl *et al.*, 1986). It seems possible therefore that cyanide production by *P.fluorescens* CHA0 directly antagonizes *T.basicola* on the roots, without damaging the plants. The antagonistic effect on the fungus would be restricted to the vicinity of the root surface because the overall survival of *T.basicola* in the artificial soil of the gnotobiotic system was not reduced measurably by strain CHA0. Since the artificial soil does not contain an added carbon source, the nutrients for the microorganisms are provided by the plant. We expect the metabolic activities of the microorganisms to be strongest in the zone of root exudates. Glycine, which is present in root exudates (Rovira, 1969), may locally stimulate cyanide production by *Pseudomonas* and thus lead to an inhibition of the fungus or to an attenuation of fungal pathogenicity. However, an alternative interpretation is equally plausible. We observed that the cyanide-producer CHA0, but not the Hcn$^-$ mutant CHA5, stimulated root hair formation in tobacco (our unpublished data), and it is conceivable that cyanide stress might also induce some plant defence mechanisms against the pathogen.

The role of bacterial cyanogenesis has not been evident (Castric, 1981). Our experiments now provide evidence for an ecological role of cyanogenesis: it constitutes part of the suppressive ability of *P.fluorescens* strains. The physiological functions of cyanogenesis for the bacteria, in contrast, remain enigmatic. For instance, the mutant CHA5 was not impaired in the utilization of glycine as a nitrogen source.

Bakker and Schippers (1987) have proposed that cyanide-producing pseudomonads reduce potato plant growth and tuber yields in Dutch soils which are cropped with potato in short rotation. These authors hypothesize that non-cyanogenic pseudomonads compete effectively with cyanide-producing, plant-deleterious strains and thus, by reducing cyanide formation, enhance potato growth. In our gnotobiotic system an adverse effect of cyanide production on tobacco growth is not apparent (Figures 3A and 6A). Cyanide-producing *P.fluorescens* strains are superior with respect to disease suppression in this system as well as in other experiments involving autoclaved natural soil, wheat and *G.graminis* var. *tritici* (B.Wüthrich, personal communication). Therefore, it would be premature to make any generalizations regarding the effect of bacterial cyanogenesis on plant growth.

Fe(III) is required for induction of cyanogenesis in *Pseudomonas* (Table I; Castric, 1981; Askeland and Morrison, 1983). Our gnotobiotic system contains vermiculite, which releases ~80 μM iron into soil water in the presence of a tobacco plant (Keel *et al.*, 1989). Thus, conditions of iron sufficiency prevail, and it is unlikely that competition for iron (Kloepper *et al.*, 1980) could account for the suppression of black root rot by *P.fluorescens* CHA0. As we have shown here, in the gnotobiotic system cyanide production is important for disease suppression. This does not exclude the possibility that other metabolites of *P.fluorescens* also contribute significantly to suppression.

Materials and methods

Microorganisms and plasmids

The strains used were *T.basicola* strain ETH D127; *P.fluorescens* CHA0 (Stutz *et al.*, 1986); *P.fluorescens* P3, a barley root isolate from Alp Lü (Münstertal, Switzerland); *E.coli* strains ED8767 (*met hsdS supE supF recA56*; Murray *et al.*, 1977), HB101 (*hsdS20 recA13 proA2 leu-6 thi-1 rpsL20 ara-14 galK2 lacY1 xyl-5 mtl-1 supE44*; Boyer and Roulland-Dussoix, 1969), SK1592 (*endA thi gal hsdR4 hsdM*$^+$ *sbcB15* T1^r; Kushner, 1978) and W3110 (prototrophic; Boulnois *et al.*, 1985). The following plasmids were used: R64*drd-11* (Tc Sm Tra IncIα; Meynell and Datta, 1967); pHP45Ω (Ap Sm/Sp; Fellay *et al.*, 1987); pHP45Ω-Hg (Ap Hg; Fellay *et al.*, 1987); pLG221 (Km Tra Incα, Tn*5* donor; Boulnois *et al.*, 1985); pME12 (Ap Tc Km Hg Tra Rep(ts) IncP, Tn*5-259* donor; Voisard *et al.*, 1988), pME483 (= pBR322ΔTc::Tn*5*, Ap Km; Rella *et al.*, 1985); pME484 (= pBR322Δ Tc::Tn*5-751*, Ap Km Tp; Rella *et al.*, 1985); pME497 (Ap Tra Rep(ts) IncP; Voisard *et al.*, 1988); pRK2013 (Km Tra; Figurski and Helinski, 1979); pRZ102 (= ColE1::Tn*5*, Km; Jorgensen *et al.*, 1979); pVK100 (Tc Km Cos Mob IncP; Knauf and Nester, 1982).

Media and growth conditions

E.coli and *P.fluorescens* were grown in nutrient yeast broth (NYB), on nutrient agar (NA) (Stanisich and Holloway, 1972), minimal medium E (Vogel and Bonner, 1956), or King's medium B (King *et al.*, 1954). For cyanide production, the synthetic medium of Castric (1977) was used. Selective antimetabolite concentrations were, for *E.coli*: ampicillin (Ap, 100 μg/ml); tetracycline hydrochloride (Tc; 10−25 μg/ml); kanamycin sulphate (Km; 25 μg/ml); streptomycin sulphate (Sm; 20 μg/ml); $HgCl_2$ (20 μg/ml); trimethoprim (Tp; 50 μg/ml); for *P.fluorescens* CHA: Tc (125 μg/ml); Km (25 μg/ml); Sm (100−200 μg/ml); $HgCl_2$ (10−40 μg/ml); Tp (1000 μg/ml); chloramphenicol (Cm; 20 μg/ml, intrinsic resistance); for *P.fluorescens* P3 the concentrations were the same except for Tp (500 μg/ml in minimal medium E). *E.coli* was cultivated at 37°C. *P.fluorescens* strains were usually grown at 28−30°C with aeration. *T.basicola* was grown on malt agar at 24°C in the dark (Stutz *et al.*, 1986).

Transposon mutagenesis

Before the mating, *P.fluorescens* CHA0 was grown at 35°C without shaking. This treatment reduces the restriction of *E.coli* DNA about 100-fold (Voisard *et al.*, 1988). Equal amounts of overnight cultures of strain CHA0 and *E.coli* W3110(pLG221) were mixed, concentrated 40-fold and incubated on NA at 30°C for 2−3 h. The bacteria were harvested in 0.85% NaCl and plated on selective medium, usually NA + Km (25 μg/ml) + Cm (20 μg/ml). The Tn*5* insertion mutants were purified on NA + Km. The random Tn*5-259* insertion mutant CHA25 was obtained by using an *E.coli* pME12 donor (Voisard *et al.*, 1988).

DNA manipulation

Minipreparations of plasmid DNA from *E.coli* and *P.fluorescens* were obtained by alkaline lysis followed by phenol–chloroform extraction (Maniatis *et al.*, 1982). The conditions for large-scale isolation of plasmid DNA, restriction, ligation, and transformation have been given (Itoh *et al.*, 1984; Rella *et al.*, 1985). Enzymes were purchased from Boehringer. Southern hybridization and nick translation with [α-^{35}S]dCTP were performed according to Maniatis *et al.* (1982) and the instructions of the supplier (Amersham, kit N5000), respectively.

Cosmid cloning and mobilization

Genomic DNA was isolated from *P.fluorescens* CHA0 by the method of Chesney *et al.* (1979), partially digested with *Hin*dIII, purified by two phenol–chloroform extractions and four ether extractions and fractionated on a 0.5% low melting agarose (Bethesda Research Laboratories) gel in Tris–acetate/EDTA (TAE) buffer pH 7.8 (Maniatis *et al.*, 1982). DNA fragments of 15–30 kb were eluted with TAE buffer from gel pieces (cut out with a scalpel) in a Biotrap apparatus (Schleicher and Schuell), precipitated in 400 μl buffer with two volumes of isopropanol, washed twice with 70% ethanol, dried and finally dissolved in 10 mM Tris–HCl, 1 mM EDTA pH 8. 4 μg of this DNA preparation was ligated at 12°C with 10 μg of cosmid vector (pVK100) DNA, which had been digested with *Hin*dIII and treated with alkaline phosphatase (Boehringer), in a total volume of 20 μl overnight. Aliquots of this ligation mixture containing 3 μg DNA were mixed with 50 μl of a thawing λ packaging mixture prepared according to Maniatis *et al.* (1982) and incubated at 37°C for 1 h. After addition of 1 ml of SM buffer (Maniatis *et al.*, 1982) and 50 μl chloroform, the lysate was centrifuged briefly and used, in 10–50 μl aliquots, to infect maltose-grown *E.coli* HB101 (200 μl). 2000 Tc^r, Km^s clones selected on NA + Tc (25 μg/ml) were stocked in storage medium (Gergen *et al.*, 1979). The inserts in pVK100 were in the size range of 16–25 kb. Recombinant cosmids were mobilized by pME497 to strain CHA0 (Voisard *et al.*, 1988) or by pRK2013 to strain P3; the conditions of these triparental matings were similar to those used for transposon mutagenesis.

Assay of cyanide

P.fluorescens CHA0 transposon insertion mutants were screened for Hcn$^-$ derivatives in microtitre trays; each well contained 100 μl NYB. The wells were covered tightly with an HCN-indicator paper [Whatman 3M, soaked in a solution of Cu(II) ethyl acetoacetate (5 mg; Kodak) + 4,4′-methylene-bis-*N*,*N*-dimethyl-aniline (5 mg; Fluka) per ml chloroform, dried and stored in the dark (Castric and Castric, 1983)]. After incubation at 30°C for 16–24 h, the paper was exposed to air for 15 min; blue spots indicated cyanide production. For quantitative cyanide determinations *P.fluorescens* cultures were grown with shaking in 10 ml Castric medium placed in a 120-ml flask, which was sealed hermetically. At the end of incubation at 30°C, cyanide in solution was determined by the method of Gewitz *et al.* (1976). Roots of 4-week-old tobacco plants grown under sterile conditions were cut into pieces of 7 mm and incubated in 50 mM MOPS buffer pH 6.5 amended with 15 mM sucrose and ~10^8 c.f.u. bacteria (Lundberg *et al.*, 1986). Cyanide in solution was measured after 2 days at 26°C, by the above method.

The gnotobiotic system

This will be described in detail elsewhere (Keel *et al.*, 1989). Briefly, the system contained: quartz sand, quartz powder, and vermiculite (expanded with 30% H_2O_2 at 60°C) at 70:20:10, by weight. After moistening with 10% (w/w) H_2O, the artificial soil (75 g) was placed into a 100 ml flask, which was plugged with cotton, and autoclaved. The soil was inoculated with 6×10^8 c.f.u. of *P.fluorescens* (grown in King B agar at 28°C for 24 h and suspended in 5 ml H_2O) and 6×10^5 endoconidia of *T.basicola* (harvested in 5 ml H_2O from 3-week-old malt agar cultures). Controls contained equivalent amounts of H_2O. After incubation for 1 week in a dark growth chamber, a tobacco plant (*Nicotiana glutinosa* L., with four leaves, grown under sterile conditions) was transferred to each flask, together with 3 ml Knop solution containing 50 μM FeEDDHA [ethylene-diamine-di(*o*-hydroxyphenylacetate); from Ciba-Geigy]. The plants were grown in a growth chamber under the conditions described (Stutz *et al.*, 1986) for 3 weeks, washed with tap water and weighed.

Bacterial root colonization and assay of survival of T.basicola

Washed roots were shaken in 100 ml 0.85% NaCl for 30 min. Appropriate dilutions of the resulting suspension were plated on King B agar. Fluorescent colonies were counted after 48 h at 28°C. The stability of chromosomal insertions and recombinant plasmids was tested by replica plating onto medium containing $HgCl_2$, Km, Sm or Tc. For the re-isolation of *T.basicola* propagules, the soil which remained after removal of the plant was mixed thoroughly. One g of soil was suspended in 50 ml H_2O and shaken vigorously for 30 min. 100 μl of a 10^{-1} dilution was plated on malt agar containing 156 μg Tc/ml. Colonies were counted after 7–9 days at 24°C.

Acknowledgements

We thank R.Gälli and L.Meile for providing the λ packaging mix, L.Hollenstein and J.Laville for localizing the *hcn* genes on pME3013, H.Strahm for technical assistance and U.Rosenberger for typing the manuscript. This work was supported by the 'Schweizerischer Nationalfonds' (project 3.133-0.85).

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Received on October 7, 1988; revised on November 11, 1988