

Is the ability of biocontrol fluorescent pseudomonads to produce the antifungal metabolite 2,4-diacetylphloroglucinol really synonymous with higher plant protection?

Fabio Rezzonico^{1,2}, Marcello Zala¹, Christoph Keel^{1,3}, Brion Duffy^{1,4}, Yvan Moënne-Loccoz⁵ and Geneviève Défago¹

¹Phytopathology Group, Institute of Integrative Biology, Swiss Federal Institute of Technology (ETH), Universitätstrasse 2, CH-8092 Zürich, Switzerland;

²SafeCrop Center, c/o Agroscope ACW Changins-Wädenswil, CH-8820 Wädenswil, Switzerland; ³Département de Microbiologie Fondamentale, Université

de Lausanne, CH-1015 Lausanne, Switzerland; ⁴Agroscope ACW Changins-Wädenswil, CH-8820 Wädenswil, Switzerland; ⁵Université de Lyon, Lyon,

F-69003, France; Université Lyon 1, Lyon, F-69003, France; CNRS, UMR5557, Ecologie Microbienne, Villeurbanne, F-69622, France; IFR 41, Villeurbanne,

F-69622, France

Summary

Author for correspondence:

Fabio Rezzonico

Tel: +41 44 7836331

Fax: +41 44 7836305

Email: fabio.rezzonico@acw.admin.ch

Received: 24 June 2006

Accepted: 30 October 2006

- The antifungal compound 2,4-diacetylphloroglucinol (PhI) contributes to biocontrol in pseudomonads, but whether or not PhI⁺ biocontrol pseudomonads display higher plant-protecting activity than PhI⁻ biocontrol pseudomonads remains to be demonstrated. This issue was addressed by assessing 230 biocontrol fluorescent pseudomonads selected from a collection of 3132 bacterial isolates obtained from 63 soils worldwide.
- One-third of the biocontrol pseudomonads were PhI⁺ and almost all PhI⁺ isolates also produced hydrogen cyanide (HCN). The only PhI⁺ HCN⁻ strain did harbor *hcn* genes, but with the deletion of a 134 bp *hcnC* fragment corresponding to an ADP-binding motif.
- Statistical analysis of biocontrol isolate distributions indicated that PhI production ability was associated with superior disease suppression activity in the *Pythium*-cucumber and *Fusarium*-tomato pathosystems, but this was also the case with HCN production ability. However, HCN significance was not as strong, as indicated both by the comparison of PhI⁻ HCN⁺ and PhI⁻ HCN⁻ strains and by correlation analyses.
- This is the first population-level demonstration of the higher plant-protecting activity of PhI⁺ biocontrol pseudomonads in comparison with PhI⁻ biocontrol pseudomonads.

Key words: 2,4-diacetylphloroglucinol (PhI), biocontrol, cucumber, hydrogen cyanide (HCN), *Pseudomonas*, tomato.

New Phytologist (2007) **173**: 861–872

No claim to original Swiss government works.

Journal compilation © *New Phytologist* (2006)

doi: 10.1111/j.1469-8137.2006.01955.x

Introduction

Many biocontrol fluorescent pseudomonads protect plants from soil-borne diseases by the production of antimicrobial secondary metabolite(s) (Haas & Keel, 2003; Morrissey *et al.*, 2004; Haas & Défago, 2005), such as 2,4-diacetylphloroglucinol

(PhI) (Vincent *et al.*, 1991; Fenton *et al.*, 1992; Keel *et al.*, 1992). PhI can inhibit a range of phytopathogens (Keel *et al.*, 1992; Cronin *et al.*, 1997; de Souza *et al.*, 2003), as well as induce a plant systemic response resulting in resistance to pathogens (Iavicoli *et al.*, 2003). The implication of PhI in biocontrol was evidenced for a few strains in plant experiments

where wild-type pseudomonads protected better than mutant derivatives in which Phl production was inactivated (Vincent *et al.*, 1991; Fenton *et al.*, 1992; Keel *et al.*, 1992; Duffy *et al.*, 2004). In addition, acquisition of the ability to produce Phl conferred biocontrol potential to Phl⁻ pseudomonads (Vincent *et al.*, 1991; Fenton *et al.*, 1992; Bakker *et al.*, 2002). Evidence for the implication of Phl in biocontrol originates also from population studies of root-colonizing *Pseudomonas* in disease-suppressive and conducive soils (Raaijmakers & Weller, 1998; Weller *et al.*, 2002), although Phl⁺ strains were also found in conducive soils (Raaijmakers *et al.*, 1997), sometimes in high numbers (Ramette *et al.*, 2003b).

Thus, the implication of Phl in biocontrol interactions is now well established. However, many biocontrol pseudomonads can produce several biocontrol compounds, such as lytic enzymes, surfactants, siderophores, antibiotics, or metabolites (so far unknown) secreted by the type III secretion system (Morrissey *et al.*, 2004; Haas & Défago, 2005; Rezzonico *et al.*, 2005; Cazorla *et al.*, 2006). This applies to Phl⁺ biocontrol pseudomonads, and for instance Phl⁺ pseudomonads produce siderophore(s) and (for most of them) the antifungal compound hydrogen cyanide (Keel *et al.*, 1996; Ramette *et al.*, 2003a). Indeed, *Pseudomonas* strains in which one biocontrol trait was eliminated by mutation often retained some degree of biocontrol activity (Haas & Défago, 2005; Rezzonico *et al.*, 2005; Cazorla *et al.*, 2006). In this context, the relative importance of Phl in biocontrol is difficult to establish, because mutant derivatives in which biocontrol genes are inactivated have been developed for only a few Phl⁺ *Pseudomonas* strains (Vincent *et al.*, 1991; Fenton *et al.*, 1992; Keel *et al.*, 1992), and abolition of one biocontrol trait can modify the expression of other biocontrol traits (Schnider-Keel *et al.*, 2000; Haas & Keel, 2003; Baehler *et al.*, 2006).

Whether or not the ability to produce Phl is associated with higher plant protection in biocontrol pseudomonads has been investigated using collections of plant-protecting strains. Sharifi-Tehrani *et al.* (1998) showed that a majority of the most effective biocontrol strains in two pathosystems were Phl⁺, but the number of strains was rather small (14 Phl⁺ vs seven Phl⁻ pseudomonads); the collection included well-studied Phl⁺ pseudomonads, meaning that there could have been a sampling bias towards the more effective Phl⁺ strains; and correlation data suggested that HCN production ability could have contributed to the finding. In another study, correlation analysis indicated that, again, the ability to produce HCN was an important biocontrol trait for pseudomonads (in a pea-*Pythium* pathosystem), but there Phl production ability was not associated with superior plant protection (Ellis *et al.*, 2000). The reason for the discrepancy between both studies is not known but, once more, in the latter work, a rather limited number of biocontrol pseudomonads were studied ($n = 29$) and the strain collection was assembled by gathering biocontrol agents obtained in different types of screenings and by different laboratories, which means sampling-related statistical bias cannot be discounted.

Overall, it appears that statistically unbiased comparison studies of wild-type biocontrol pseudomonads have yet to be performed to confirm at population level that the ability to produce Phl is indeed associated with superior plant protection. Therefore, the aim of the current work was to assess whether or not Phl⁺ biocontrol pseudomonads display higher plant-protecting activity than Phl⁻ biocontrol pseudomonads. Precautions were taken to be statistically meaningful, as follows: (i) this assessment was done using a much larger collection of biocontrol fluorescent pseudomonads ($n = 230$) compared with previous studies (Sharifi-Tehrani *et al.*, 1998; Ellis *et al.*, 2000); (ii) the biocontrol pseudomonads were obtained following a homogenous screening protocol performed mainly *in planta*, starting from 3132 bacterial isolates collected in six consecutive years from roots of plants grown in 63 soils gathered from all over the world; (iii) biocontrol assessments were carried out in two different pathosystems; and (iv) statistical steps were taken to contrast the relative importance of Phl and HCN production abilities, since almost all Phl⁺ pseudomonads are also HCN⁺ but many HCN⁺ strains are Phl⁻.

Materials and Methods

Bacterial isolation

Topsoil (upper layer 0–20 cm) was collected at 63 locations in 18 countries, representing every arable continent (Table 1), including disease-suppressive soils from Switzerland (Stutz *et al.*, 1986), Italy (Tamiotti *et al.*, 1993) and Ghana (Keel *et al.*, 1996). For each soil sample, one pot containing approx. 200 g of soil was sown with surface-disinfected seeds (Sharifi-Tehrani *et al.*, 1998) of each of the following bait plants: cucumber (*Cucumis sativus* L. cv. 'Chinesische Schlange' or cv. 'sensation'), tomato (*Lycopersicon esculentum* Miller cv. 'Bonny Best' or 'Supermarmande'), wheat (*Triticum aestivum* L. cv. 'Arina'), tobacco (*Nicotiana glutinosa* L.), cotton (*Gossypium hirsutum* L. cv. 'Deltapine'), bean (*Phaseolus vulgaris* L.) and radish (*Raphanus sativus* L.). Bait plants, which served as enrichment for rhizosphere-competent bacteria, were grown at 70% relative humidity (RH) with 16 h of light at $80 \mu\text{E}^{-2} \text{s}^{-1}$ and 22°C followed by 8 h of darkness at 18°C, and watered two to three times per wk using twice-distilled water. After 2–3 wk the plants were harvested. The roots were washed gently with tap water to remove adhering soil, surface-disinfected with 70% ethanol and 5% H₂O₂ and macerated using a mortar and pestle before plating. This was done with the objective of targeting bacteria with an intimate relationship with plant roots. Plating was carried out on the semiselective *Pseudomonas* media S1 (Gould *et al.*, 1985) and King's B (KB; King *et al.*, 1954), and sometimes on 1/10 tryptic soy agar (TSA; Difco, Detroit, MI, USA). After a 2 d incubation at 24°C, at least four colonies were randomly chosen from plates for each pot, yielding a total of 3132 colonies.

Table 1 Biocontrol fluorescent pseudomonads obtained in the study

Year and geographic origin	Candidate biocontrol bacteria tested	Biocontrol fluorescent pseudomonads studied			
		Pseudomonads selected ^a	HCN ⁺ pseudomonads	Phl ⁺ pseudomonads	Published strains
1997	549	43	32 (74%)	13 (30%)	
Switzerland ^b		13	12	2	P97.38, P97.39 (Wang <i>et al.</i> , 2001)
Bhutan		10	6	5	P97.1, P97.2, P97.6, P97.26, P97.27 (Wang <i>et al.</i> , 2001)
USA		5	4	1	P97.20 (Wang <i>et al.</i> , 2001)
Czech Rep.		7	5	5	P97.30, P97.31, P97.32, P97.33, P97.34 (Wang <i>et al.</i> , 2001)
India		4	3	0	
Ecuador		1	0	0	
Hungary		3	2	0	
1996	546	38	25 (66%)	2 (5%)	
Switzerland ^b		5	5	0	
China		8	2	0	
Mexico		5	5	0	F96.26 (Wang <i>et al.</i> , 2001)
Nepal		6	6	0	
Russia		11	4	2	P96.20, P96.25 (Wang <i>et al.</i> , 2001)
Estonia		3	3	0	F96.27 (Wang <i>et al.</i> , 2001)
1995	504	24	14 (58%)	3 (13%)	
Switzerland ^b		9	4	3	K95.34, K95.43, K95.44, K95.45 (Wang <i>et al.</i> , 2001)
China		9	6	0	KD (Sharifi-Tehrani <i>et al.</i> , 1998)
Australia		2	1	0	
Pakistan		4	3	0	CP7 (Sharifi-Tehrani <i>et al.</i> , 1998)
1994	448	41	25 (61%)	6 (15%)	
Switzerland ^b		21	11	4	TM3 (Sharifi-Tehrani <i>et al.</i> , 1998)
					K94.4, K94.5, K94.6, K94.18, K94.30 (Wang <i>et al.</i> , 2001)
					K94.8, K94.23 (Rezzonico <i>et al.</i> , 2004)
Czech Rep.		10	7	2	K94.31, K94.37, K94.38, K94.40, K94.41 (Wang <i>et al.</i> , 2001)
Romania		3	3	0	
India		4	1	0	CSL26 (Sharifi-Tehrani <i>et al.</i> , 1998)
Italy		3	3	0	CA2 (Sharifi-Tehrani <i>et al.</i> , 1998)
					K94.14 (Rezzonico <i>et al.</i> , 2004)
1993	765	47	43 (91%)	39 (83%)	
Switzerland ^b		31	28	27	K93.1 to K93.24, K93.39 (Wang <i>et al.</i> , 2001)
Italy		7	7	7	PITR3, PINR2, PINR3, PITR2, PILH1 (Keel <i>et al.</i> , 1996)
					K93.25, K93.52 (Wang <i>et al.</i> , 2001)
Ghana		8	8	5	PGNR1, PGNR3, PGNR4, PGNR2, PGNL1 (Keel <i>et al.</i> , 1996)
1992	320	37	25 (68%)	13 (35%)	
Switzerland ^b		37	25	13 ^c	TM1A3, TM1B2, C*1A1 (Fuchs & Défago, 1991)
					CΔ*1'B2, TM1'A5, CM1'A2, TM1'A4, TM1A5 (Keel <i>et al.</i> , 1996)
					K92.14, K92.48, K92.53, K92.59 (Wang <i>et al.</i> , 2001)
					K92.46 (Rezzonico <i>et al.</i> , 2004)
Total	3132	230	164 (71%)	76 (33%)	

^aNo *Pseudomonas* isolate with biocontrol potential was found in 1994 from soils originating from Australia and Sri Lanka, and in 1995 from soils originating from Estonia, Ghana and the Czech Republic.

^bSwiss isolates originated from Morens (1992); Morens and Geneva (1993); Morens, Brusio, Valais and Reckenholz (1994); Morens and Vouvy (1995); Evolène (1996); and Morens (1997).

^cAll Phl⁺ *Pseudomonas* but one (K92.53) were also HCN⁺.

Identification of biocontrol isolates in the *Pythium ultimum*–cucumber pathosystem

All 3132 bacterial isolates were screened for biocontrol activity either *in vitro* (in 1992 and 1993) by measuring *P. ultimum* inhibition on KB and on malt agar (Difco), or *in planta* (from 1994 to 1997) by assessing biocontrol of *Pythium* damping-off of cucumber, as described by Sharifi-Tehrani *et al.* (1998). Briefly, each bacterial isolate was added to soil (10^7 colony-forming units (CFU) cm^{-3}) in three pots filled with *c.* 170 cm^3 of steam-pasteurized St Aubin clay loam soil (Canton Fribourg, Switzerland) infested with *P. ultimum* (4 mg millet seed inoculum cm^{-3} of soil). Steam pasteurization of soil was performed because it is a standard practice for commercial production of cucumber. Two days later, four aseptically grown cucumber seedlings were transplanted into each pot. The pots were placed in the growth chamber described earlier for 12 d. The biomass of emerged plants was recorded. Biocontrol activity (%) was calculated as:

$$(1 - ((W_C - W_I)/(W_C - W_P))) \times 100$$

using plant fresh weight obtained in presence of the isolate tested (W_I), in the control with no microorganisms added (W_C) and in the unprotected control with the pathogen alone (W_P). The model biocontrol pseudomonad CHA0 (Stutz *et al.*, 1986) was included as reference in every experiment. Each experiment tested eight to 12 isolates, with a total of *c.* 300 experiments (> 9000 pots) needed to assess all 3132 isolates. Biocontrol bacteria were identified based on at least 5 mm inhibition zone of *P. ultimum* on KB or malt agar (*in vitro* experiments), or significant plant protection, especially in comparison with the biocontrol performance of the model biocontrol strain CHA0 (*in planta* experiments).

All biocontrol isolates identified were assessed for growth on S1 plates and fluorescence under UV light (366 nm) on succinate minimal medium (Meyer & Abdallah, 1978) supplemented with $100 \mu\text{g mL}^{-1}$ ethylenediaminedi[*o*-hydroxyphenylacetic acid] (EDDHA) and on S1 itself. Isolates exhibiting both properties (i.e. putative pseudomonads) were taken for further analysis.

Analysis of biocontrol pseudomonads in two pathosystems

The biocontrol pseudomonads identified in the current work (Table 1) were further tested for biocontrol activity in the *P. ultimum*–cucumber pathosystem described earlier (this time using six pots/replicates instead of three) and also (for the 1993–97 isolates) in the *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL)–tomato pathosystem, using rockwool as substrate (Sharifi-Tehrani *et al.*, 1998) to mimic soilless conditions of glasshouse tomato production. Briefly, each isolate was studied using three rockwool cubes (with 12 tomato

seedlings each) soaked in plant nutrient solution containing 10^6 conidia of FORL and 10^7 CFU of the pseudomonad mL^{-1} . After 14 d of growth, disease severity was assessed (0, healthy seedlings; 1, one or two brown marks on the roots or the crown; 2, several small brown marks or a few large lesions; 3, extensive root necrosis but plant still alive; 4, plant dead) and biocontrol activity (%) computed as:

$$(1 - ((I_C - I_I)/(I_C - I_P))) \times 100$$

in which *I*-values represent disease indices. In both pathosystems, the experiment was carried out twice for each isolate, requiring > 4000 pots or rockwool cubes in total.

Ability of biocontrol pseudomonads to produce PhI and/or HCN

The ability of the biocontrol fluorescent pseudomonads to produce PhI was determined by extraction from 3-d-old liquid KB cultures with 80% acetone followed with HPLC analysis, as has been described (Keel *et al.*, 1992, 1996). Production of HCN was studied qualitatively on KB plates using a modification of the method described by Castric & Castric (1983). Briefly, a piece of Whatman 3 mm chromatography paper, which was soaked for 24 h in a chloroform solution containing (mL^{-1}) 5 mg 4,4'-methylenebis-(*N,N*-dimethylaniline) (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and 5 mg copper(II)-ethylacetoacetate (MP Biochemicals, Eschwege, Germany) and then dried in darkness, was placed in the lid of each plate. A positive reaction resulted in the indicator paper turning blue after overnight incubation of the plates at 28°C.

Statistical analyses

Two main complementary statistical approaches were followed with biocontrol data. In approach I, one-way Kruskal–Wallis tests were done to compare the overall biocontrol performance of pseudomonads able or not to produce PhI or HCN. In approach II, this comparison was based on the distinction between three arbitrarily defined classes of poorly effective, moderately effective and highly effective strains, using 3×2 contingency tables and χ^2 tests. In addition, pairwise comparisons of mean biocontrol performance levels were done using one-tailed Student *t*-tests. Finally, correlation analyses were performed, using Pearson's coefficient and Bonferroni significance test. All statistics were performed at $P < 0.05$ using Systat version 10.0 (Systat Inc., Evanston, IL, USA).

Analysis of *hcnABC* genes

Part (i.e. 587 bp) of the *hcnBC* genes was amplified by PCR using primers ACa (5'-ATCGCCAGGGGCGGATGTGC-3') and ACb (5'-ACGATGTGCTCGGCGTAC-3'), as previously

Table 2 Top six biocontrol isolates in the *Pythium ultimum*–cucumber and in the *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL)–tomato pathosystems

Strains ^a	Origin	Biocontrol <i>P. ultimum</i> –cucumber		Biocontrol FORL–tomato	
		Rank ^b	Biocontrol activity (%)	Rank ^b	Biocontrol activity (%)
Against <i>P. ultimum</i>					
K93.28	Ghana (tobacco)	1	80.8	123	18.2
K93.30	Ghana (tobacco)	2	80.6	73	31.9
K93.52	Italy (tomato)	3	79.5	9	59.7
K93.21	Switzerland (tomato)	4	75.2	60	36.0
K93.48	Italy (wheat)	5	73.4	70	32.2
K94.1	Italy (cucumber)	6	69.1	50	39.6
Against FORL					
K93.37	Switzerland (wheat)	77	42.5	1	73.2
K93.4	Switzerland (tobacco)	57	46.5	2	67.4
K93.26	Italy (tobacco)	8	67.6	3	65.5
K93.53	Italy (tomato)	11	66.9	4	65.2
K93.39	Switzerland (wheat)	10	67.2	5	63.4
P96.9	Estonia (tomato)	156	17.2	6	60.8
Reference					
CHA0	Switzerland (tobacco)	79	41.2	84	29.6

^aAll strains were obtained in this work except CHA0, which was isolated from a Morens soil (Stutz *et al.*, 1986). The first number in strain names indicates the year of isolation. All produce HCN, PhI and siderophore(s) *in vitro*. Strains K93.28 and K93.30 also produce the antimicrobial compound pyoluteorin, as does strain CHA0.

^bOut of 230 (*P. ultimum*–cucumber) and 185 (FORL–tomato) isolates. Strain CHA0 was the external reference for the 230 isolates, and the theoretical ranks displayed for strain CHA0 are the ones that would have been obtained if strain CHA0 had been included in the study.

described (Ramette *et al.*, 2003a). A 571 bp fragment of *hcnAB* was amplified with primers PM2 (5'-TGCGGCATGGG-CGTGTGCCATTGCTGCCTGG-3') and PM7-26R (5'-CCGCTCTTGATCTGCAATTGCAGGCC-3') (M. Svercel, unpublished). Amplicon sequencing was performed using an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and sequence analysis was performed in relation to key HcnBC domains described in Laville *et al.* (1998). The partial *hcnBC* sequence obtained for strain K92.53 is available at NCBI under accession number DQ485347.

Results

Identification of biocontrol pseudomonads

Over the 6 yr survey, as many as 307 isolates effective against *P. ultimum* were identified from the 3132 isolates tested (i.e. 9.8%) (Table 1). Among these 307 biocontrol isolates, 230 (i.e. 74.9%) were identified as fluorescent pseudomonads based on the abilities to grow on S1 plates, and display fluorescence under UV light on EDDHA succinate minimal medium and on S1. They originated from cucumber (110 isolates), tomato (43 isolates), wheat (42 isolates), tobacco (25 isolates), cotton (four isolates) and bean (four isolates), and also from bulk soil (two isolates).

Plant protection ability of biocontrol pseudomonads in two pathosystems

Almost all fluorescent *Pseudomonas* isolates identified as potential biocontrol agents for cucumber did protect cucumber from *P. ultimum* when further biocontrol experiments were performed (Fig. 1). A majority of these biocontrol agents were also tested for protection of tomato from FORL. Certain isolates did not protect (and were even detrimental for 14 of them) in the FORL–tomato pathosystem. The biocontrol performance of the best strain in one pathosystem was much lower in the other pathosystem, and the top six performers in one pathosystem differed from the top six performers in the other (Table 2). These 12 isolates originated from cucumber (one isolate), tomato (four isolates), tobacco (four isolates) or wheat (three isolates) grown in soil from Switzerland (four isolates), Italy (five isolates), Estonia (one isolate) or Ghana (two isolates). Their performance level was much higher than that of reference strain CHA0 (a Swiss isolate from tobacco) in at least one of the pathosystems.

Despite the fact that many strains did not perform well in the two pathosystems, certain strains could protect plants in both, for example, the tomato isolate K93.52 (ranked third against *P. ultimum* and ninth against FORL) and the tobacco isolate K93.26 (ranked eighth against *P. ultimum* and third against FORL) (Table 2). Overall, a significant positive

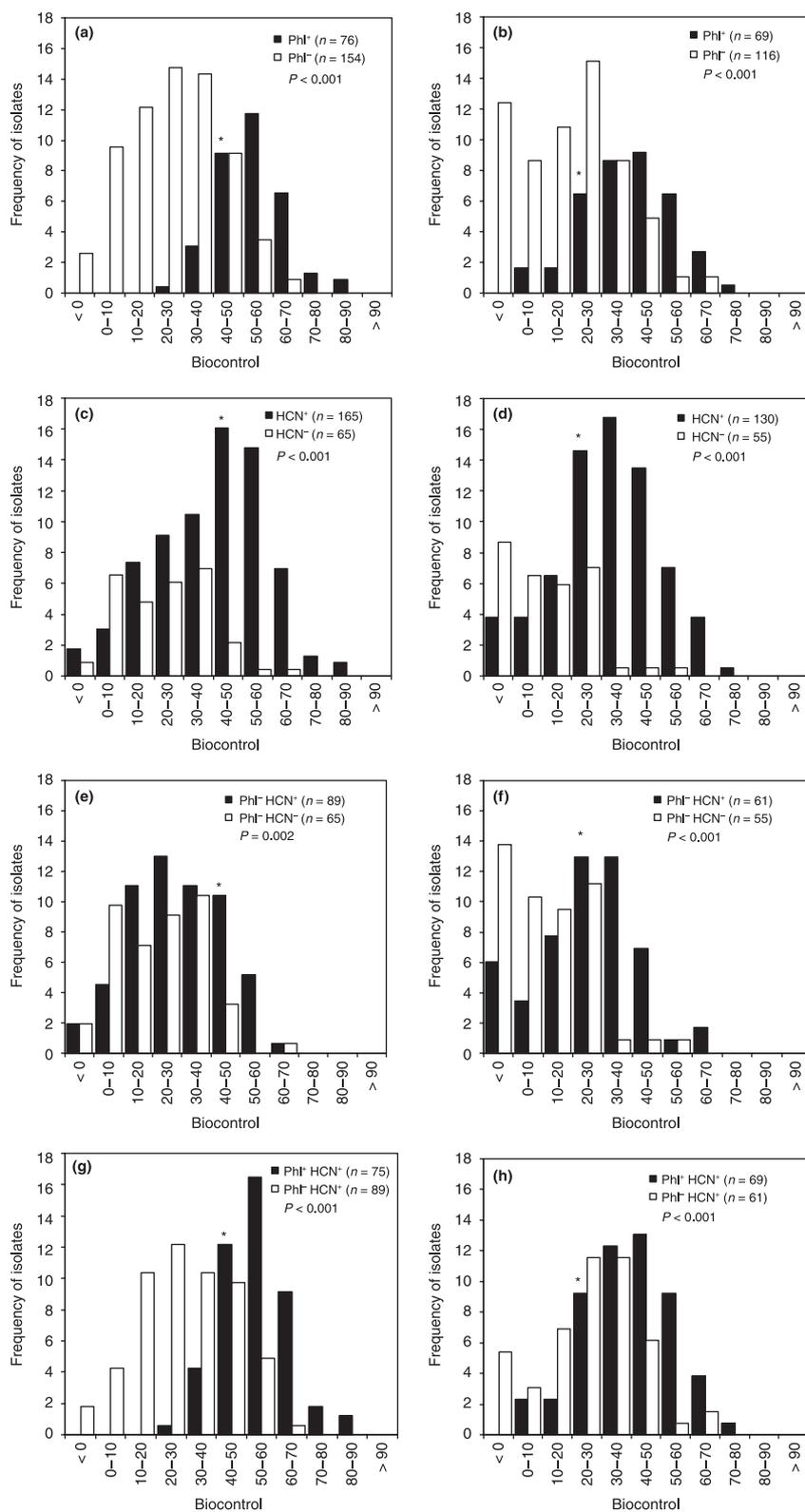


Fig. 1 Distribution of *Pseudomonas* isolates with respect to their biocontrol activity in the *Pythium ultimum*-cucumber (a, c, e, g) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL)-tomato (b, d, f, h) pathosystems. Producers (closed bars) of antimicrobial secondary metabolites 2,4-diacetylphloroglucinol (PhI; a, b) and hydrogen cyanide (HCN; c, d) were compared with the respective nonproducers (open bars) in their ability to protect in each particular pathosystem. The effect of HCN production ability in PhI nonproducers is depicted in (e) and (f), and that of PhI production ability in HCN producers is shown in (g) and (h). The biocontrol activity was calculated based on plant fresh weight (*P. ultimum*-cucumber) or disease index (FORL-tomato) and the asterisk indicates the position of *Pseudomonas* strain CHA0 used as reference biocontrol agent over the 6 yr of the study (41.2% in the *P. ultimum*-cucumber pathosystem and 29.6% in the FORL-tomato pathosystem). The *P*-values at which the two distributions are different in a Kruskal-Wallis one-way analysis (statistical approach I) are shown within each graph.

Table 3 Distribution of poorly effective, moderately effective and highly effective^a biocontrol fluorescent pseudomonads^b among isolates with or without the ability to produce hydrogen cyanide (HCN) and/or 2,4-diacetylphloroglucinol (Phl) (statistical approach II^c)

Analysis		Poorly effective strains		Moderately effective strains		Highly effective strains		P-level
Strain subset	Production ability	Nonproducers	Producers	Nonproducers	Producers	Nonproducers	Producers	
<i>Pythium ultimum</i> –cucumber pathosystem								
All strains	Phl	58.4%	1.3%	40.3%	72.4%	1.3%	26.3%	< 0.001
All strains	HCN	64.6%	29.7%	33.9%	57.6%	1.5%	12.7%	< 0.001
Phl ⁻ strains	HCN	66.1%	52.8%	32.4%	46.1%	1.5%	1.1%	0.228
HCN ⁺ strains	Phl	52.8%	1.3%	46.1%	71.0%	1.1%	26.7%	< 0.001
<i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i> (FORL)–tomato pathosystem								
All strains	Phl	50.9%	8.7%	45.7%	65.8%	3.4%	26.1%	< 0.001
All strains	HCN	70.9%	20.0%	27.3%	63.8%	1.8%	16.2%	< 0.001
Phl ⁻ strains	HCN	70.9%	32.8%	27.3%	62.3%	1.8%	4.9%	< 0.001
HCN ⁺ strains	Phl	32.8%	8.7%	62.3%	65.2%	4.9%	26.1%	< 0.001

^aPoorly effective strains: < 30 and 20% biocontrol activity in the *P. ultimum*–cucumber and FORL–tomato pathosystems, respectively; moderately effective strains: 30–60% and 20–50% biocontrol activity in the *P. ultimum*–cucumber and FORL–tomato pathosystems, respectively; highly effective strains: > 60 and 50% biocontrol activity in the *P. ultimum*–cucumber and FORL–tomato pathosystems, respectively.

^bPercentage data for poorly effective, moderately effective and highly effective pseudomonads were obtained among producers as well as among nonproducers (total = 100% in both cases).

^cFor each strain subset, the statistical distribution of nonproducers and of producers was tested based on the numbers of isolates in the poorly effective, moderately effective and highly effective classes and was performed using 3 × 2 contingency tables followed by χ^2 tests ($P < 0.05$).

Table 4 Ability of fluorescent pseudomonads isolated from different bait plants and unplanted soil to produce hydrogen cyanide (HCN) and 2,4-diacetylphloroglucinol (Phl) and to control pathogens *in planta*

Bait plant or soil	Number of isolates	HCN producers		Phl producers		Ratio Phl ⁺ /HCN ⁺	<i>P. ultimum</i> –cucumber		FORL–tomato	
		Number	% HCN ⁺	Number	% Phl ⁺		Biocontrol %	SE	Biocontrol %	SE
Cucumber	110	79	71.8	23	20.9	0.29	31.9	1.8	25.7	2.1
Tomato	43	39	90.7	27	62.8	0.69	45.1	3.2	31.7	3.7
Wheat	42	23	54.8	9	21.4	0.39	30.8	2.5	22.9	3.4
Tobacco	25	17	68.0	14	56.0	0.82	43.5	4.1	36.8	4.4
Bean	4	3	75.0	1	25.0	0.33	26.6	6.7	26.4	14.7
Cotton	4	4	100	2	50.0	0.50	41.5	4.9	ND ^a	ND
Soil	2	0	0	0	0	–	21.7	9.1	16.2	1.0
Total	230	165	71.7	76	33.0	0.46	35.4	1.3	24.5	1.8

^aNot done.

correlation was even found between biocontrol activity levels in the two pathosystems ($r = 0.48$; $n = 164$, $P < 0.01$), and a majority of biocontrol pseudomonads reached thresholds of at least 30 and 20% biocontrol activity in the *P. ultimum*–cucumber and FORL–tomato pathosystems, respectively (Table 3).

Cucumber isolates represented almost half the biocontrol pseudomonads identified after initial screening in the cucumber-based system, but collectively they did not display higher biocontrol ability in the *P. ultimum*–cucumber pathosystem in comparison with biocontrol isolates from tomato, wheat, or tobacco (Table 4). In fact, the overall biocontrol performance in the *P. ultimum*–cucumber pathosystem was even lower

($P = 0.01$, one-tailed t -test) for cucumber isolates, that is, 31.9 (± 1.8)% (\pm SE), than for noncucumber isolates (from the five other host plants and from soil), that is, 38.7 (± 1.8)%.

In the FORL–tomato pathosystem, tomato isolates protected better ($P < 0.05$, one-tailed t -test) than average (i.e. when comparing the mean for tomato isolates with the mean for all isolates). In addition, when all nontomato isolates were considered together, the overall biocontrol performance for tomato isolates (i.e. 31.7 (± 3.7)%) was higher ($P = 0.03$, one-tailed t -test) than that for nontomato isolates (from the four other host plants and from soil; i.e. 22.9 (± 2.0)%).

Relationship between biocontrol and the ability to produce PhI

PhI production ability was found in 76 of the 230 biocontrol fluorescent pseudomonads (i.e. 33%). In each pathosystem (Fig. 1a,b), it was associated with superior disease suppression ability (statistical approach I). This is also indicated by the fact that as many as 26.3% of PhI⁺ isolates exceeded a 60% biocontrol activity in the *P. ultimum*–cucumber pathosystem (vs 1.3% of PhI⁻ isolates), whereas only 1.3% were below a 30% biocontrol activity (vs 58.4% of PhI⁻ isolates) (statistical approach II; Table 3). A similar situation was observed in the FORL–tomato system (Table 3). Furthermore, the data from cucumber, tomato, tobacco and wheat isolates in the *P. ultimum*–cucumber pathosystem (Table 4) gave a correlation coefficient of 0.99 ($n = 4$; $P < 0.01$) between the percentage of PhI⁺ pseudomonads and their overall biocontrol activity, but the correlation was not significant in the FORL–tomato pathosystem. However, both correlations were significant ($n = 7$, $P < 0.01$) when all data from Table 4 were considered ($r = 0.97$ in the *P. ultimum*–cucumber pathosystem and $r = 0.93$ in the FORL–tomato pathosystem).

Relationship between biocontrol and the ability to produce HCN

HCN production ability was found in as many as 165 of the 230 biocontrol fluorescent pseudomonads (i.e. 72%). When the whole collection was analyzed, HCN production ability was associated with effective disease suppression ability in each pathosystem, regardless of whether statistical approach I

(Fig. 1c,d) or II (Table 3) was followed. However, there was no significant correlation between the percentage of HCN⁺ strains and their overall biocontrol activity when assessing the data from Table 4, regardless of whether correlation was computed with only data from cucumber, tomato, tobacco and wheat isolates ($n = 4$), or with all data ($n = 7$).

When only PhI⁻ strains were considered, it appears that higher biocontrol responses were again obtained with HCN⁺ strains compared with HCN⁻ strains in the FORL–tomato pathosystem (Fig. 1f and Table 3). However, a different situation was found in the *P. ultimum*–cucumber pathosystem, where HCN production ability gave less significant results in approach I ($P = 0.002$ instead of $P < 0.001$; Fig. 1e) and was not statistically significant in approach II (Table 3).

When assessing data for the subset of HCN⁺ isolates, PhI production ability was again associated with superior biocontrol performance, based on the comparison of HCN⁺ PhI⁺ and HCN⁺ PhI⁻ pseudomonads. This was found with statistical approaches I (Fig. 1g,h) and II (Table 3), regardless of the pathosystem studied.

Analysis of the sole HCN⁻ PhI⁺ biocontrol pseudomonad identified

All 76 PhI⁺ isolates but one (K92.53) also produced HCN. This strain and CHA0 gave PCR products of similar length (568 and 571 bp, respectively) and sequence (78.5% identity) when assessing the partial *hcnAB* amplicon obtained with primers PM2 and PM7-26R. In contrast, the *hcnBC* amplicon obtained from K92.53 was smaller than the 587 bp amplicon for HCN⁺ *Pseudomonas* strains (Fig. 2a). Sequencing of the

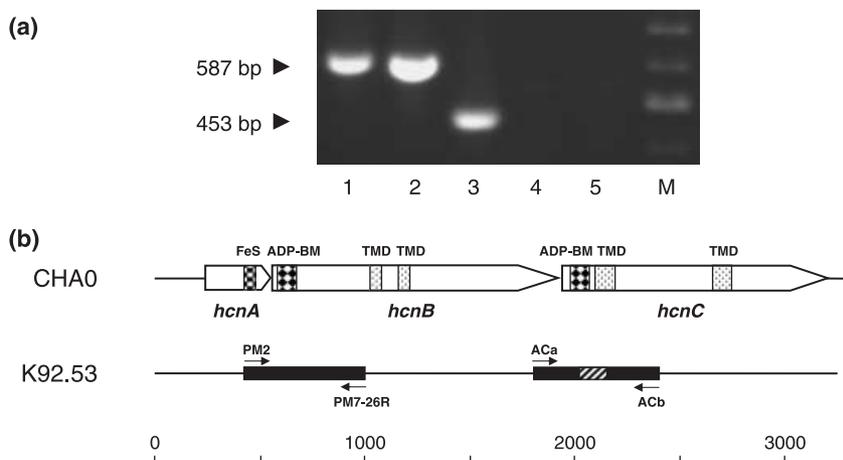


Fig. 2 (a) PCR amplification of *hcnBC* in biocontrol *Pseudomonas* sp. CHA0 (HCN⁺ PhI⁺; lane 1), pathogenic *P. aeruginosa* PAO1 (HCN⁺ PhI⁻; lane 2), and biocontrol *Pseudomonas* sp. K92.53 (HCN⁻ PhI⁺; lane 3) and K94.8 (HCN⁻ PhI⁻; lane 4). Lane 5 is the negative control (H₂O). M, 100 bp ladder (0.2 µg). Amplification of HCN⁺ strains CHA0 and PAO1 yielded a 587 bp *hcnBC* amplicon. The HCN⁻ strain K94.8 produced no amplification, as expected, whereas the HCN⁻ strain K92.53 yielded a curtailed 453 bp amplicon. (b) Alignment of partial *hcnBC* sequences from *Pseudomonas* sp. strains CHA0 and K92.53. The position of the sequences encoding key domains is marked by the different fill effects. FeS, potential binding site for Fe-S cluster; ADP-BM, ADP binding motif; TMD, transmembrane domain. The hatched line within the ACa/ACb amplicon indicates the position of the deletion on *hcnC*.

amplicon in K92.53 revealed a 134 bp deletion in *hcnC* (Fig. 2b), located between positions 2024 and 2157 of the *hcnBC* sequence of strain CHA0 (accession number AF053760). The deleted fragment corresponds to the N-terminal domain of the putative amino-acid oxidase HcnC (Laville *et al.*, 1998). This leads to the complete deletion of the loop forming the ADP-binding $\beta\alpha\beta$ -fold (Wierenga *et al.*, 1986), including the removal of four of the 11 amino acid residues within HcnC that define an ADP-binding motif, and 23 of 26 residues constituting the subsequent transmembrane domain (Laville *et al.*, 1998). The deletion is in accordance with the HCN⁻ phenotype of this strain.

The biocontrol efficacy of strain K92.53 was studied only in the *P. ultimum*-cucumber pathosystem, giving 54.5% protection. The strain was ranked 38th of the 79 Phl⁺ biocontrol pseudomonads (average protection 53.3%), but ranked second of the 66 HCN⁻ biocontrol pseudomonads (average protection 22.3%).

Discussion

In this work, a very large screening was implemented and 230 biocontrol pseudomonads were identified after analysis of 3132 bacterial isolates. More than a third of these 230 pseudomonads have been previously evaluated by restriction analysis of 16S rDNA (Keel *et al.*, 1996) or 16S-23S rDNA amplicons (Sharifi-Tehrani *et al.*, 1998), RAPD analysis (Keel *et al.*, 1996), catabolic profiling (Wang *et al.*, 2001), and/or diversity analysis of *phlD* (Wang *et al.*, 2001), *hcnBC* (Ramette *et al.*, 2003b), *hrcN* and *rrs* (Rezzonico *et al.*, 2004). Results indicated that a majority of isolates were unique strains, even those originating from the same field site. This ensured that we considered a genetically diverse group of biocontrol pseudomonads in the study.

Since biocontrol pseudomonads need to colonize plant roots efficiently, it could be thought that biocontrol strains aimed at a particular pathosystem should ideally be isolated from the corresponding plant, and selected based on successful inhibition of the corresponding pathogen and/or effective biocontrol in that pathosystem. The data for the *P. ultimum*-cucumber pathosystem indicate otherwise. Here, 84 of the 230 biocontrol pseudomonads were selected based on *in vitro* inhibition of cucumber pathogen *P. ultimum* and the 146 others because they protected cucumber from the same pathogen. Yet about half the 230 biocontrol pseudomonads, that is, 26 out of 84 (31%) in the *in vitro* screening and 84 out of 146 (58%) in the *in planta* screening (in steamed soil) originated from a host plant different from cucumber. Furthermore, cucumber isolates did not protect better than noncucumber isolates overall in the *P. ultimum*-cucumber pathosystem, and five of the six best biocontrol pseudomonads originated from tobacco, tomato or wheat (Table 2). This points to a lack of specificity in the *Pseudomonas*-*P. ultimum*-cucumber biocontrol interactions.

The findings were largely different in the FORL-tomato pathosystem. Although four of the six best biocontrol pseudomonads originated from wheat or tobacco (Table 2), and tomato isolates did not protect as well as tobacco isolates, the tomato isolates did display higher phytoprotection when they were collectively compared with all nontomato isolates in the FORL-tomato pathosystem. This may be because FORL has a narrower plant host range than *P. ultimum*. The correlation between biocontrol activity levels in the two pathosystems was statistically significant ($P < 0.01$) but the correlation coefficient was not very high ($r = 0.48$), suggesting that certain but not all strain properties are useful in both pathosystems.

Biocontrol pseudomonads were readily obtained during each of the 7 yr of the study, at a rate of 24–47 yr⁻¹ (Table 1). However, there was more year-to-year fluctuation in the yield of HCN⁺ biocontrol pseudomonads (14–43 yr⁻¹) and especially Phl⁺ biocontrol pseudomonads (2–39 yr⁻¹). Since different soils were used in different years, the year may not be the main factor determining Phl⁺ isolate yield. Many Swiss soils were included in the study and the yield in Phl⁺ biocontrol isolates was particularly high for Swiss soils (especially from different *Thielaviopsis basicola*-suppressive soils from Morens) in 1993 (Table 1), which explains why as many as 43 of the 76 Phl⁺ biocontrol pseudomonads (i.e. 57%) originated from Switzerland.

Data showed that Phl⁺ biocontrol pseudomonads protected better than Phl⁻ biocontrol pseudomonads overall. We focused on Phl because it is involved in biocontrol of a broad spectrum of diseases by many antagonistic strains (Haas & Défago, 2005). These findings are important, because despite the demonstration of the role of Phl in a few individual strains (Vincent *et al.*, 1991; Fenton *et al.*, 1992; Keel *et al.*, 1992), and the quantitative prevalence of Phl⁺ pseudomonads in suppressive soils (Raaijmakers & Weller, 1998; Weller *et al.*, 2002; Moënne-Loccoz & Défago, 2004), this superiority has failed so far to be demonstrated when statistically comparing a range of Phl⁺ and Phl⁻ wild-type biocontrol strains (Ellis *et al.*, 2000). In the current experimental conditions, it appears that Phl production ability was more important than the plant species of origin in determining strain biocontrol potential. This may explain the relatively poor performance of cucumber isolates (low proportion of Phl producers) and the good performance of tomato isolates (high proportion of Phl producers). No correlation was found between the amount of Phl produced *in vitro* by the Phl producers and their biocontrol efficiency *in planta*, regardless of the pathosystem (data not shown). However, this result is of limited biological significance, as the conditions of Phl production *in planta* differ greatly from those *in vitro* (Notz *et al.*, 2001).

Prior studies have suggested that HCN may be a distinctive marker for deleterious soil microorganisms (Schippers *et al.*, 1990; Paszkowski & Dwornikiewicz, 2003; Benizri *et al.*, 2005), and, in fact, HCN-producing bacteria have been exploited for weed biocontrol (Kremer & Souissi, 2001;

Owen & Zdor, 2001). In fact, it is not uncommon for certain plant-beneficial traits to be also found in deleterious bacteria (Blaha *et al.*, 2006). Here, population statistics demonstrated that HCN-producing ability was a positive attribute for plant-associated biocontrol *Pseudomonas* strains, when linked to the ability to also produce Phl. This is in agreement with data obtained from mutant analysis (Voisard *et al.*, 1989), and it confirms and strengthens (because the strain collection was much larger) previous findings from the assessment of strain collections (Sharifi-Tehrani *et al.*, 1998; Ellis *et al.*, 2000; Ramette *et al.*, 2003a). However, they also suggest that the ability to produce HCN is statistically of less significance for biocontrol than that of Phl, in contrast to findings obtained with a smaller strain collection in another *P. ultimum*-based pathosystem (Ellis *et al.*, 2000).

All but one Phl⁺ *Pseudomonas* strains published so far have been found to produce HCN as well, complicating efforts to determine the relative importance of each biocontrol compound in plant protection. Here, molecular analysis of HCN⁻ Phl⁺ biocontrol strain K92.53 showed that *hcn* genes were present in the pseudomonad, but *hcnC* displayed a deletion in a key functional region. The process by which this deletion took place is unknown. This is the first demonstration of a natural mutation occurring in *hcn* genes. Therefore, Phl production ability is restricted to pseudomonads carrying *hcn* genes. Interestingly, the biocontrol efficacy of strain K92.53 was rather ordinary when compared with other Phl producers, but it was outstanding when weighed against other HCN⁻ strains. This suggests again that Phl production is indeed the main factor for biocontrol in the *P. ultimum*-cucumber system.

This work focused on Phl, and to a lesser extent on HCN, but several metabolites other than HCN and Phl are also active in the biological control activity of pseudomonads (Haas & Keel, 2003; Moënne-Loccoz & Défago, 2004; Morrissey *et al.*, 2004), including biocontrol metabolites that have not yet been identified (Rezzonico *et al.*, 2005), and their role should not be discounted. For instance, the HCN⁻ Phl⁻ biocontrol strain K94.8, which ranked first among HCN⁻ strains in the *P. ultimum*-cucumber system, displays type three secretion genes (Rezzonico *et al.*, 2004), and this may contribute to protection in that pathosystem (Rezzonico *et al.*, 2005). Another limitation is that only one *P. ultimum* strain and one FORL strain could be used, considering the scale of the study. For certain fungal pathogens, noticeably *Pythium*, sensitivity to Phl may vary from one strain to the next (de Souza *et al.*, 2003).

The two pathosystems selected were studied under conditions relevant for commercial glasshouse production of cucumber and tomato. However, whether findings can be generalized to situations where crops are grown in nontreated field soil remains to be established, all the more so as biocontrol assessment was carried out in rather short-term experiments, during which certain defense mechanisms of the pathogen may not have time to develop (Fedi *et al.*, 1997). Other key strain features, such as plant colonization ability, are likely to

be of higher importance for *Pseudomonas* inoculants in other pathosystems. For instance, certain genotypes of Phl⁺ pseudomonads are better adapted than others for colonization of pea (Landa *et al.*, 2002). Caution is also needed when considering implications in the case of disease-suppressive soils, as the host plant species has a significant influence on the dynamics, composition and activity of indigenous antagonistic *Pseudomonas* spp. (Weller *et al.*, 2002; Bergsma-Vlami *et al.*, 2005) and the latter display endemism (Weller *et al.*, 2002; Ramette *et al.*, 2006). However, even conducive soils may be a useful source of *Pseudomonas* strains for inoculation purposes (Ramette *et al.*, 2006).

In certain cases, it could be that specific types of biocontrol strains may be needed for specific conditions, for example, when dealing with particular cultivars, soil types, and/or climatic areas. However, when it comes to finding new biocontrol agents, effective strategies to replace the labor-intensive *in planta* screenings are lacking. Our results suggest that Phl and HCN could be used in combination as biocontrol markers for streamlining this process. PCR protocols are available to identify *phl* (McSpadden Gardener *et al.*, 2001; Rezzonico *et al.*, 2003) and *hcn* genes in *Pseudomonas* (Ramette *et al.*, 2003a). Since Phl production is found naturally only in pseudomonads harboring *hcn* genes, and because HCN is much simpler to detect, the search for additional Phl⁺ strains may be preceded by a rapid phenotypic screen for HCN⁺ isolates.

Acknowledgements

FR and MZ contributed equally to this work. We thank Hans-Joachim Kempf (Syngenta, Basel, Switzerland) for technical assistance and helpful discussions, and Patrick Rüggele and Davide Gobbin (ETH) for help with the statistics. Financial support from Novartis (Basel, Switzerland), the SafeCrop Center (funded by Fondo per la Ricerca, Autonomous Province of Trento, Italy) and the French Embassy in Switzerland (France-Switzerland research grant) is gratefully acknowledged.

References

- Baehler E, de Werra P, Wick LY, Pechy-Tarr M, Mathys S, Maurhofer M, Keel C. 2006. Two novel MvaT-like global regulators control exoproduct formation and biocontrol activity in root-associated *Pseudomonas fluorescens* CHA0. *Molecular Plant-Microbe Interact* 19: 313–329.
- Bakker PAHM, Glandorf DCM, Viebahn M, Ouwens TWM, Smit E, Leeftang P, Wernars K, Thomashow LS, Thomas-Oates JE, van Loon LC. 2002. Effects of *Pseudomonas putida* modified to produce phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol on the microflora of field grown wheat. *Antonie Van Leeuwenhoek* 81: 617–624.
- Benizri E, Piutti S, Verger S, Pagès L, Vercambre G, Poessel JL, Michelot P. 2005. Replant diseases: bacterial community structure and diversity in peach rhizosphere as determined by metabolic and genetic fingerprinting. *Soil Biology Biochemistry* 37: 1738–1746.
- Bergsma-Vlami M, Prins ME, Raaijmakers JM. 2005. Influence of plant species on population dynamics, genotypic diversity and antibiotic

- production in the rhizosphere by indigenous *Pseudomonas* spp. *FEMS Microb. Ecology* 52: 59–69.
- Blaha D, Prigent-Combaret C, Mirza MS, Moëgne-Loccoz Y. 2006. Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminase-encoding gene *acdS* in phyto-beneficial and pathogenic *Proteobacteria* and relation with strain biogeography. *FEMS Microbiological Ecology* 56: 455–470.
- Castric KF, Castric PA. 1983. Method for rapid detection of cyanogenic bacteria. *Applied Environmental Microbiology* 61: 3002–3007.
- Cazorla FM, Duckett SB, Bergström ET, Noreen S, Odijk R, Lugtenberg BJJ, Thomas-Oates JE, Bloembergen GV. 2006. Biocontrol of avocado Dematophora root rot by antagonistic *Pseudomonas fluorescens* PCL1606 correlates with the production of 2-hexyl 5-propyl resorcinol. *Molecular Plant-Microbe Interact* 19: 418–428.
- Cronin D, Moëgne-Loccoz Y, Fenton A, Dowling DN, O'Gara F. 1997. Ecological interaction of a biocontrol *Pseudomonas fluorescens* strain producing 2,4-diacetylphloroglucinol with the soft rot potato pathogen *Erwinia carotovora* subsp. *Atroseptica* *FEMS Microbiological Ecology* 23: 95–106.
- Duffy B, Keel C, Défago G. 2004. Potential role of pathogen signaling in multitrophic plant-microbe interactions involved in disease protection. *Applied Environmental Microbiology* 70: 1836–1842.
- Ellis RJ, Timms-Wilson TM, Bailey MJ. 2000. Identification of conserved traits in fluorescent pseudomonads with antifungal activity. *Environmental Microbiology* 2: 274–284.
- Fedi S, Tola E, Moëgne-Loccoz Y, Dowling DN, Smith LM, O'Gara F. 1997. Evidence for signaling between the phytopathogenic fungus *Pythium ultimum* and *Pseudomonas fluorescens* F113: *P. ultimum* represses the expression of genes in *P. fluorescens* F113, resulting in altered ecological fitness. *Applied Environmental Microbiology* 63: 4261–4266.
- Fenton AM, Stephens PM, Crowley J, O'Callaghan M, O'Gara F. 1992. Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. *Applied Environmental Microbiology* 58: 3873–3878.
- Fuchs J, Défago G. 1991. Protection of cucumber plants against black root rot caused by *Phomopsis sclerotoides* with rhizobacteria. *IOBC/WPRS Bulletin* 14: 57–62.
- Gould WD, Hagedorn C, Bardinelli TR, Zablutowicz RM. 1985. New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats. *Applied Environmental Microbiology* 49: 28–32.
- Haas D, Défago G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Review of Microbiology* 3: 307–319.
- Haas D, Keel C. 2003. Regulation of antibiotic production in root colonizing *Pseudomonas* spp. & relevance for biological control of plant disease. *Annual Review of Phytopathology* 41: 117–153.
- Iavicoli A, Boutet E, Buchala A, Métraux J-P. 2003. Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. *Molecular Plant-Microbe Interact* 16: 851–858.
- Keel C, Schnider U, Maurhofer M, Voisard C, Laville J, Burger U, Wirthner P, Haas D, Défago G. 1992. Suppression of root diseases by *Pseudomonas fluorescens* CHA0: Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Molecular Plant-Microbe Interact* 5: 4–13.
- Keel C, Weller DM, Natsch A, Défago G, Cook RJ, Thomashow LS. 1996. Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. *Applied Environmental Microbiology* 62: 552–563.
- King EO, Ward MK, Raney DE. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory Clinical Medy* 44: 301–307.
- Kremer RJ, Souissi T. 2001. Cyanide production by rhizobacteria and potential for suppression of weed seedling growth. *Current Microbiology* 43: 182–186.
- Landa BB, Mavrodi OV, Raaijmakers JM, McSpadden Gardener BB, Thomashow LS, Weller DM. 2002. Differential ability of genotypes of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* strains to colonize the roots of pea plants. *Applied Environmental Microbiology* 68: 3226–3237.
- Laville J, Blumer B, Von Schroetter C, Gaia V, Défago G, Keel C, Haas D. 1998. Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. *Journal of Bacteriology* 180: 3187–3196.
- McSpadden Gardener BB, Mavrodi DV, Thomashow LS, Weller DM. 2001. A rapid polymerase chain reaction-based assay characterizing rhizosphere populations of 2,4-diacetylphloroglucinol-producing bacteria. *Phytopathology* 91: 44–54.
- Meyer JM, Abdallah MA. 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *Journal of General Microbiology* 107: 319–328.
- Moëgne-Loccoz Y, Défago G. 2004. Life as a biocontrol pseudomonad. In: Ramos JL, ed. *Pseudomonas: genomics, life style and molecular architecture*, Vol. 1. New York, NY, USA: Kluwer Academic/Plenum Publishers, 457–476.
- Morrissey JP, Abbas A, Mark L, Cullinane M, O'Gara F. 2004. Biosynthesis of antifungal metabolites by biocontrol strains of *Pseudomonas*. In: Ramos JL, ed. *Pseudomonas: Biosynthesis of macromolecules and molecular metabolism*, Vol. 3. New York, NY, USA: Kluwer Academic/Plenum Publishers, 635–670.
- Notz R, Maurhofer M, Schnider-Keel U, Duffy B, Haas D, Défago G. 2001. Biotic factors affecting expression of the 2,4-diacetylphloroglucinol biosynthesis gene *phlA*. *Pseudomonas fluorescens* biocontrol strain CHA0 in the rhizosphere. *Phytopathology* 91: 873–881.
- Owen A, Zdor R. 2001. Effect of cyanogenic rhizobacteria on the growth of velvetleaf (*Abutilon theophrasti*) and corn (*Zea mays*) in autoclaved soil and the influence of supplemental glycine. *Soil Biology Biochemistry* 33: 801–809.
- Paszkowski WL, Dwornikiewicz J. 2003. Effect of green manure on the incidence of cyanogenic *Pseudomonas* strains in hop garden soils. *Journal of Chemistry Ecology* 29: 1159–1165.
- Raaijmakers JM, Weller DM. 1998. Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. *Molecular Plant-Microbe Interact* 11: 144–152.
- Raaijmakers JM, Weller DM, Thomashow LS. 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Applied Environmental Microbiology* 63: 881–887.
- Ramette A, Frapolli M, Défago G, Moëgne-Loccoz Y. 2003a. Phylogeny of HCN synthase-encoding *hcnBC* genes in biocontrol fluorescent pseudomonads and its relationship with host plant species and HCN synthesis ability. *Molecular Plant-Microbe Interact* 16: 525–535.
- Ramette A, Moëgne-Loccoz Y, Défago G. 2003b. Prevalence of fluorescent pseudomonads producing antifungal phloroglucinols and/or hydrogen cyanide in soils naturally suppressive or conducive to tobacco black root rot. *FEMS Microbiological Ecology* 44: 35–43.
- Ramette A, Moëgne-Loccoz Y, Défago G. 2006. Genetic diversity and biocontrol potential of fluorescent pseudomonads producing phloroglucinols and HCN from Swiss soils naturally suppressive or conducive to *Thielaviopsis basicola*-mediated black root rot of tobacco. *FEMS Microbiological Ecology* 55: 369–381.
- Rezzonico F, Binder C, Défago G, Moëgne-Loccoz Y. 2005. The type III secretion system of biocontrol *Pseudomonas fluorescens* KD targets the phytopathogenic Chromista *Pythium ultimum* and promotes cucumber protection. *Molecular Plant-Microbe Interact* 18: 991–1001.
- Rezzonico F, Défago G, Moëgne-Loccoz Y. 2004. Comparison of ATPase-encoding type III secretion system *hrcN* genes in biocontrol fluorescent pseudomonads and in phytopathogenic proteobacteria. *Applied Environmental Microbiology* 70: 5119–5131.

- Rezzonico F, Moëgne-Loccoz Y, Défago G. 2003. Effect of stress on the ability of a *phlA*-based quantitative competitive PCR assay to monitor biocontrol strain *Pseudomonas fluorescens* CHA0. *Applied Environmental Microbiology* 69: 686–690.
- Schippers B, Bakker AW, Bakker PAHM, van Peer R. 1990. Beneficial and deleterious effects of HCN producing pseudomonads on rhizosphere interactions. *Plant Soil* 129: 75–83.
- Schnider-Keel U, Seematter A, Maurhofer M, Blumer C, Duffy B, Gigot-Bonnefoy C, Reimmann C, Notz R, Défago G, Haas D, Keel C. 2000. Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *Journal of Bacteriology* 182: 1215–1225.
- Sharifi-Tehrani A, Zala M, Natsch A, Moëgne-Loccoz Y, Défago G. 1998. Biocontrol of soilborne fungal plant diseases by 2,4-diacetylphloroglucinol-producing fluorescent pseudomonads with different restriction profiles of amplified 16S rDNA. *European Journal of Plant Pathology* 104: 631–643.
- de Souza JT, Arnould C, Deulvot C, Lemanceau P, Gianinazzi-Pearson V, Raaijmakers JM. 2003. Effect of 2,4-diacetylphloroglucinol on *Pythium*: cellular responses and variation in sensitivity among propagules and species. *Phytopathology* 93: 966–975.
- Stutz E, Défago G, Kern H. 1986. Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. *Phytopathology* 76: 181–185.
- Tamietti G, Ferraris L, Matta A, Abbattista Gentile I. 1993. Physiological responses of tomato plants grown in *Fusarium* suppressive soil. *Journal of Phytopathology* 138: 66–76.
- Vincent MN, Harrison LA, Brackin JM, Kovacevich PA, Murkerji P, Weller DM, Pierson EA. 1991. Genetic analysis of the anti-fungal activity of a soilborne *Pseudomonas aureofaciens* strain. *Applied Environmental Microbiology* 57: 2928–2934.
- Voisard C, Keel C, Haas D, Défago G. 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root of tobacco under gnotobiotic conditions. *EMBO Journal* 8: 351–358.
- Wang C, Ramette A, Punjasarnwong P, Zala M, Natsch A, Moëgne-Loccoz Y, Défago G. 2001. Cosmopolitan distribution of *phlD*-containing dicotyledonous crop-associated biocontrol pseudomonads of worldwide origin. *FEMS Microbiological Ecology* 37: 105–116.
- Weller D, Raaijmakers JM, McSpadden Gardener BB, Thomashow LS. 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annual Review of Phytopathology* 40: 309–348.
- Wierenga RK, Terpstra P, Hol WGJ. 1986. Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$ -fold in proteins, using an amino acid sequence fingerprint. *Journal of Molecular Biology* 187: 101–107.



About New Phytologist

- *New Phytologist* is owned by a non-profit-making **charitable trust** dedicated to the promotion of plant science, facilitating projects from symposia to open access for our Tansley reviews. Complete information is available at www.newphytologist.org.
- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication 'as-ready' via *OnlineEarly* – our average submission to decision time is just 30 days. Online-only colour is **free**, and essential print colour costs will be met if necessary. We also provide 25 offprints as well as a PDF for each article.
- For online summaries and ToC alerts, go to the website and click on 'Journal online'. You can take out a **personal subscription** to the journal for a fraction of the institutional price. Rates start at £131 in Europe/\$244 in the USA & Canada for the online edition (click on 'Subscribe' at the website).
- If you have any questions, do get in touch with Central Office (newphytol@lancaster.ac.uk; tel +44 1524 594691) or, for a local contact in North America, the US Office (newphytol@ornl.gov; tel +1 865 576 5261).