Phylogeny of HCN Synthase-Encoding *hcnBC* Genes in Biocontrol Fluorescent *Pseudomonas* and Its Relationship with Host Plant Species and HCN Synthesis Ability

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Hydrogen cyanide (HCN) is a broad-spectrum antimicrobial compound involved in biological control of root diseases by many plant-associated fluorescent pseudomonads. The HCN synthase is encoded by three biosynthetic genes (*hcnA*, *hcnB*, and *hcnC*), but little is known about the diversity of these genes in fluorescent *Pseudomonas* spp. and in other bacteria. Here, the partial *hcnBC* sequence was determined for a worldwide collection of biocontrol fluorescent *Pseudomonas* spp. Phylogenies based on *hcnBC* and deduced protein sequences revealed four main bacterial groups, but topological incongruences were found between *hcnBC* and *rrs*-based phylogenies, suggesting past lateral transfer of *hcnBC* among saprophytic root-colonizing pseudomonads. Three of the four groups included isolates from different countries and host plants. Yet, these groups corresponded to distinct, ecologically-adapted populations of HCN-producing biocontrol fluorescent pseudomonads, as indicated by high *hcnBC* distinctness ratio values and the differences in production levels of HCN in vitro found between groups. This is in accordance with previous results on catabolic properties and biocontrol abilities of these strains. HCN synthase gene diversity may thus reflect the adaptive radiation of HCN⁺ biocontrol fluorescent pseudomonads. Positive correlations were found between HCN production in vitro and plant protection in the cucumber/*Pythium ultimum* and tomato/*Fusarium oxysporum* f. sp. *radicis-lycopersici* pathosystems.

Additional keyword: evolution.

The genus *Pseudomonas* is ubiquitous in nature and can be found readily in ecological habitats as diverse as water, soil, and plant surfaces. The interface between soil and plant roots (i.e., the rhizosphere) is a microbial hot spot in which root-colonizing pseudomonads play a prominent role (Bakker and Schippers 1987; Bossis et al. 2000; Dowling and O'Gara

1994). Indeed, many rhizosphere-inhabiting fluorescent pseudomonads exhibit antagonistic effects toward fungal pathogens of plant roots, thereby protecting the plant from disease (Keel and Défago 1997; Schisler et al. 1997; Vincent et al. 1991). Among the different mechanisms involved in disease suppression, the production by fluorescent pseudomonads of antimicrobial secondary metabolites such as hydrogen cyanide (HCN) (Laville et al. 1998; Voisard et al. 1989) or 2,4-diacetylphloroglucinol (Phl) (Fenton et al. 1992; Keel et al. 1992) is recognized to be of significance for effective biocontrol.

Production of HCN by biocontrol fluorescent pseudomonads is implicated in suppression of diseases caused by phytopathogenic fungi, such as *Thielaviopsis basicola* on tobacco (Laville et al. 1998; Voisard et al. 1989), *Septoria tritici*, and *Puccinia recondita* f. sp. *tritici* on wheat (by recombinant HCN-producing *P. putida* strains) (Flaishman et al. 1996). Direct inhibition of the fungi by HCN is thought to be the main mechanism of action (Blumer and Haas 2000), in which case, the effect of the bacterium would be comparable to the HCN-mediated plant defense mechanism (Luckner 1990). HCN inhibits the terminal cytochrome c oxidase in the respiratory chain (Knowles 1976) and binds to metalloenzymes (Blumer and Haas 2000). However, apart from its role in plant protection, a deleterious effect of microbial HCN on several plants has also been reported (Alström and Burns 1989; Bakker and Schippers 1987), and indeed, HCN production appears to represent a virulence factor in *P. aeruginosa* (Gallagher and Manoel 2001). Deleterious HCN-producing strains have even been studied for biological control of weeds (Kremer and Souissi 2001). For plant-associated fluorescent pseudomonads capable of HCN production, the difference between biocontrol and deleterious strains perhaps results from differences in patterns of HCN production on plant surfaces, inside plant tissues, or both, but experimental data are not available to assess this hypothesis.

Among prokaryotes, HCN production seems restricted to Proteobacteria, in which it has been evidenced in several *Pseudomonas* species (*P. fluorescens*, *P. aeruginosa*, *P. chlororaphis*, and *P. aureofaciens*), *Chromobacterium violaceum*, *Rhizobium leguminosarum*, and to certain cyanobacteria (Blumer and Haas 2000). HCN synthase, which catalyses the formation of HCN and CO₂ from glycine, is believed to be a membrane-bound flavoprotein (Wissing and Andersen 1981). This hypothesis was strengthened by protein sequence data (Laville et al. 1998). The systematic name cyanide-forming glycine hydrogenase was proposed for HCN synthase based on

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sequence similarities with known dehydrogenases and oxidases (Blumer and Haas 2000). However, the molecular structure of the enzyme is still unknown. Although three structural genes (*hcnA*, *hcnB*, and *hcnC*) encoding HCN synthase are reported for strain *P. aeruginosa* PAO1 (Pessi and Haas 2000) and the fluorescent *Pseudomonas* sp. strain CHA0 (Laville et al. 1998), little is known about the polymorphism of these genes. Perhaps there is a link between HCN synthase characteristics and HCN production, a possibility raised by the recent comparison of *phlD* polymorphism and Phl production (Ramette et al. 2001). A relationship seems to exist between the ability of biocontrol pseudomonads to produce HCN and their biocontrol capacity (Ellis et al. 2000; Sharifi-Tehrani et al. 1998), but very little has been done to date to compare the HCN-producing ability of pseudomonads and the *hcn* structural genes.

The purpose of this work was to assess the polymorphism of *hcnBC* genes in biocontrol fluorescent pseudomonads, which was achieved by studying the level of DNA diversity and reconstructing the phylogeny of these genes and comparing the latter with *rrs* phylogeny. In addition, the sequence differences observed among *hcnBC* alleles and deduced HcnBC gene products were investigated with regard to strain origin (i.e., geographic location and plant host) and function (i.e., HCN production ability in vitro and biocontrol ability in planta). The study is based on the analysis of partial *hcnBC* sequences derived from a well-characterized collection of HCN-producing biocontrol fluorescent pseudomonads of worldwide origin (Keel et al. 1996; Ramette et al. 2001).

RESULTS

Amplification of *hcnBC* sequences in the *Pseudomonas* spp. collection.

Primers ACa and ACb were designed based on whole *hcnABC* sequences already available in *Pseudomonas* strains

CHA0 and PAO1 (at accession numbers AF053760 and AF208523, respectively). In these two strains, the *hcnBC* fragment amplified (587 nucleotides) (Fig. 1) included 156 bp of 1,404 bp of *hcnB* and 436 bp of 1,251 bp of *hcnC*. With our polymerase chain reaction (PCR) conditions, a single product of about 590 bp in length was obtained for all HCN⁺ strains of the study (Table 1), whereas nothing was obtained from several HCN⁻ pseudomonads (data not shown). Several pairs of primers were also designed upstream of the ACa priming site in *hcnA* and *hcnB*, but when PCR products were obtained, it turned out that amplification was either nonspecific or specific only for some of the strains (e.g., with primers H19a and H9b, shown in Table 2, which are specific only for the group of strains designated group Hcn-4 in the following section of this report).

Phylogenetic analysis based on partial *hcnBC* and deduced protein sequences.

Significant polymorphism was found when comparing *hcnBC* sequences, and the phylogeny inferred from *hcnBC* (Fig. 2A) gave a topology very similar to that derived from *hcnB* or *hcnC* alone (data not shown). Differences between strains were also evidenced when assessing deduced HcnB and HcnC protein sequences (data not shown). Phylogenetic analysis at the protein level was carried out using deduced HcnC (Fig. 2B), as the deduced HcnB sequence available was only about 50 residues long. Phylogeny of the deduced HcnC protein segment was congruent with that of *hcnBC* (Fig. 2), indicating that both types of characters supported the same phylogenetic reconstruction (except for strain 2172, discussed below).

Four main Hcn groups of biocontrol pseudomonads were defined from deduced protein sequences based on about 130 amino acid residues (Fig. 2). These Hcn groups gathered pseudomonads displaying *hcnBC* pairwise distances below 0.01

Fig. 1. Alignment of partial *hcnBC* sequences of *Pseudomonas* strains CHA0 (accession number AF053760) and PAO1 (AF208523). Dots and dashes represent conserved bases and alignment gaps, respectively. The Shine-Dalgarno (SD) sequence and the *hcnC* start codon are underlined. The sites annealing to the polymerase chain reaction primers ACa and ACb are boxed. The thick underline = the TAA stop codon of *hcnB* located within *hcnC* (*hcnB* and *hcnC* overlap).

base substitution per site within each group, with the exception of strains in Hcn-2 group with pairwise distances ranging from 0.04 to 0.09 substitutions per site. At the protein level, the number of amino acid substitutions per site was less than three when pairwise distances within Hcn groups were calculated based on HcnC sequences.

For two of the pseudomonads (i.e., Hcn-2 strains P12 and Q37-87), the phylogeny inferred from *hcnB* resulted in their assignment to a different Hcn group (i.e., Hcn-1) compared with the results of *hcnBC* (or *hcnC*) analysis. The same situation was also found when the phylogeny was inferred from HcnB (based on distance or Poisson correction methods) rather than HcnC.

Apart from the reference strain *P. aeruginosa* PAO1, which was used as the outgroup of the phylogeny, analyses included also the reference strain *P. corrugata* 2172. Phylogenies based on *hcnBC*, *hcnB*, *hcnC*, or deduced HcnB sequences indicated that this strain was found basal to group Hcn-3 (Fig. 2A), but it was placed basal to group Hcn-2 when the phylogeny was inferred from HcnC sequences (Fig. 2B). Both results were strongly supported by parsimony analysis of gene or protein sequences (considering all strains) or maximum-likelihood analysis of *hcnB* or *hcnC* data sets (considering eight strains representative of the different Hcn groups) (data not shown). In addition, Kishino-Hagesawa (KH) tests (Kishino and Hagesawa

1989) performed for the same set of eight strains indicated that, for both *hcnB* and *hcnC* data sets, the two topologies shown in Figure 2 were not significantly different in terms of likelihood scores (although they differed significantly when the *hcnBC* data set was considered, with a worse likelihood score for the topology in Figure 2B). Finally, statistically significant g1 statistics (lower than -0.60 for *hcnB* and for *hcnC*, based on 61 and 149 variable characters, respectively; $P < 0.01$), and permutation tail probability tests ($P < 0.01$ for both data sets) indicated that both genes contained more phylogenetic signal than expected randomly. Therefore, the different positions of strain 2172 in Figure 2 can be interpreted as a lack of resolution of a shorter, yet informative *hcnB* sequence rather than an incongruence between *hcnBC* and HcnC-based phylogenies.

Geographic distribution and plant hosts for Hcn groups.

All pseudomonads in group Hcn-3 (Fig. 2) were isolated from the roots of wheat grown in the state of Washington (Table 1). For each of the three other Hcn groups, strains originated from at least three different countries and three crop plants. For instance, Hcn-4 was comprised of isolates from Switzerland, Italy, Oklahoma, Texas, and Ghana and from host plants as diverse as e.g., wheat, tobacco, and cotton. Moreover, each geographic location or host plant studied yielded pseudo-

Table 1. Origin and biocontrol properties of HCN⁺ *Pseudomonas* strains used in the study

Isolates	Biocontrol activity ^a	Source ^b	Reference
Switzerland			
CHA0	Tobacco (Tb), wheat (Ggt), cucumber (Pu)	Tobacco	Stutz et al. 1986
Pf1	Tobacco (Tb), cucumber (Pu)	Tobacco	Keel et al. 1996
P12	Tobacco (Tb), cucumber (Pu)	Tobacco	Keel et al. 1996
C*1A1, CM1'A2	Cucumber (Pu, Ps), cotton (Rs)	Cucumber	Fuchs and Défago 1991
TM1A3, TM1B2	Cucumber (Pu, Ps), cotton (Rs)	Tomato	Fuchs and Défago 1991
TM1'A4, TM1'A5	Cucumber (Pu, Ps), cotton (Rs)	Tomato	Keel et al. 1996
Washington, U.S.A.			
Q2-87	Wheat (Ggt)	Wheat	Vincent et al. 1991
Q1-87, Q4-87, Q7-87, Q9-87, Q12-87, Q13-87, Q37-87, Q86-87, Q95-87, Q107-87, Q139-87	Wheat (Ggt)	Wheat	Keel et al. 1996
Q65c-80, Q69c-80, Q128-87	Wheat (Ggt)	Wheat	Harrison et al. 1993
Texas, U.S.A.			
PF-5	Cotton (Pu, Rs), cucumber (Pu)	Cotton	Howell and Stipanovic 1979
Oklahoma, U.S.A.			
PF	Wheat (St)	Wheat	Levy et al. 1992
Ireland			
F113	Sugarbeet (Pu), potato (Eca)	Sugar beet	Fenton et al. 1992
Italy			
PINR3	Cucumber (Pu), tomato (FORL)	Tobacco	Keel et al. 1996
PITR2	Cucumber (Pu), tomato (FORL)	Wheat	Keel et al. 1996
PILH1	Cucumber (Pu), tomato (FORL)	Tomato	Keel et al. 1996
CA2	Cucumber (Pu), tomato (FORL)	Cucumber	Sharifi-Tehrani et al. 1998
Ghana			
PGNR1, PGNR2, PGNR3, PGNR4, PGNL1	Cucumber (Pu), tomato (FORL)	Tobacco	Keel et al. 1996
Reference strains			
2172 (<i>P. corrugata</i>)	Not applicable; pith necrosis pathogen	Tomato	Sutra et al. 1997
PAO1 (<i>P. aeruginosa</i>)	Not applicable; human pathogen	Human wound	Holloway 1955

^a Tb = *Thielaviopsis basicola*; Ggt = *Gaeumannomyces graminis* var. *tritici*; Pu = *Pythium ultimum*; Rs = *Rhizoctonia solani*; Ps = *Phomopsis sclerotoides*; St = *Septoria tritici*; Eca = *Erwinia carotovora* subsp. *carotovora*.

^b All biocontrol pseudomonads were isolated from plant roots with the exception of strain PF, which was isolated from wheat leaves. Root samples consisted of either macerated roots or roots previously washed of soil. Strain 2172 was isolated from infected tomato stem.

Table 2. Primers designed in this study

Name ^a	Primer sequence	% GC	Position ^b	Amplification ^c
ACa	5'-ACTGCCAGGGGCGGATGTGC-3'	70	1811	All strains
ACb	5'-ACGATGTGCTCGGCGTAC-3'	61	2397	
H19a	5'-GAACACAACCTTCAGCGACGA-3'	50	1396	Hcn-4 strains
H9b	5'-GGCGATCTTCCTCGTCATAG-3'	55	2378	

^a Forward primers correspond to ACa and H19a and reverse primers to ACb and H9b.

^b Position of the 5' end of the primer within the sequence of strain CHA0 (accession number AF053760).

^c Polymerase chain reaction (PCR) amplification (same PCR conditions used for both primer sets) was successful for the strains indicated.

monads from at least two Hcn groups. For instance, isolates from Washington (all from wheat) belonged to Hcn-1, Hcn-2, or Hcn-3. Likewise, wheat isolates were found in each of the four Hcn groups. Therefore, it appears that the Hcn groups de-

rived from phylogenetic analysis of *hcnBC* or deduced protein sequences are cosmopolitan, as worldwide distributions were clearly evidenced for three of four Hcn groups, even though the number of isolates studied was relatively small.

Fig. 2. Phylogenetic trees for **A**, *hcnBC* sequences generated by the neighbor-joining (NJ) method based on Kimura-two parameter correction of evolutionary distances and **B**, deduced amino acid sequences (139 residues) of HcnC (NJ tree constructed based on the number of differences). Hcn groups are indicated in both trees. For both phylogenies, nodal supports were assessed by bootstrap resampling (1,000 replicates), and only values greater than 50% were retained and indicated. The scale bar represents the number of substitutions per site. Strains for which the *rrs* sequence was already available or determined in this work are underlined.

Fig. 3. Alignment of **A**, partial *hcnB* and *hcnC* sequences and **B**, the deduced N-terminal sequence of HcnC for pseudomonads representing the different Hcn groups. In **A**, the conserved Shine-Dalgarno (SD) sequence and ATG start codon of *hcnC* are underlined. The 15-bp additional sequence for *Pseudomonas corrugata* 2172 is boxed. In **B**, the 11 key residues (underlined) of HcnC correspond to the sequence fingerprint of the ADP-binding βαβ fold (Wierenga et al. 1986), which also includes a variable loop (dotted line). The putative transmembrane region (Laville et al. 1998) is well conserved for all sequences. Dots and dashes represent identical sites and alignment gaps, respectively.

DNA sequence analysis.

For *hcnBC* sequences, 340 (62%) of the 549 sites used for phylogenetic analysis were conserved, and the 156 others (28%) were parsimony informative. Sequence alignment revealed a high conservation of the Shine-Dalgarno motif and the start codon for *hcnC* (Fig. 3A). Only the first base of the Shine-Dalgarno motif was polymorphic, and it consisted of adenine (only for Hcn-4 strains) or a pyrimidine (in the three other groups). Many other sites outside of the Shine-Dalgarno motif presented such fixed mutations (data not shown). The *hcnBC* sequence of *P. corrugata* 2172 was readily aligned with that of the other pseudomonads considered in the study, except for the presence in this strain of a supplementary 15-bp sequence (of unknown significance for cyanogenesis) located a few bases downstream from the start codon (Fig. 3A).

The nucleotide diversity at the entire population level (π_T) of *hcnBC* (0.130 ± 0.011) was not statistically different from that of *hcnC* (0.111 ± 0.011), and both were lower than that of *hcnB* (0.191 ± 0.027), as indicated by statistically significant Z values. Further, the coefficient of nucleotide differentiation (expected proportion of total DNA diversity; N_{ST}) was very similar for *hcnBC* (0.865 ± 0.013), *hcnB* (0.874 ± 0.022), and *hcnC* (0.863 ± 0.018). As a whole, this suggests that *hcnB* and *hcnC* data sets were equally informative in terms of population structure at the nucleotide level.

When DNA diversity within Hcn groups were computed, it appeared that group Hcn-2 had the highest nucleotide diversity (π_w) among the four Hcn groups, for *hcnB* and *hcnC* (Table 3), and for the concatenated *hcnBC* sequences. π_w for the latter sequences amounted to 0.006 ± 0.001 (in Hcn-1), 0.059 ± 0.007 (in Hcn-2), 0.004 ± 0.002 (in Hcn-3), and 0.001 ± 0.001 (in Hcn-4), which is similar to values for *hcnC* (Table 3). For *hcnB*, π_w of the three other Hcn groups were not significantly different from each other. For *hcnC*, however, π_w of Hcn-1 was significantly higher ($P = 0.0077$) than that of Hcn-3 or Hcn-4 ($Z = 2.74$ and $P < 0.01$ for both comparisons). *hcnB* and *hcnC* had similar π_w values, except in Hcn-1, in which π_w was significantly higher for *hcnC* than for *hcnB* ($Z = 2.82$; $P < 0.01$). In summary, strain diversity was higher within Hcn-2 than in the other Hcn groups, as far as *hcnBC* is concerned.

Comparing the number of nonsynonymous substitutions per nonsynonymous sites (dN) and the number of synonymous substitutions per synonymous sites (dS) is useful to detect the kind of selection governing the nature of substitutions. dN and dS were estimated to be 0.535 ± 0.083 and 0.095 ± 0.029 , respectively, for *hcnB* (45 codons) and 0.338 ± 0.040 and 0.052 ± 0.010 , respectively, for *hcnC* (139 codons). Estimated transition/transversion ratios used in the calculations were 1.62 ± 0.82 for *hcnB* and 1.59 ± 0.37 for *hcnC*. For both genes, dS was significantly greater than dN

($P < 0.001$), strongly indicating that these genes were subjected to purifying selection. Interestingly, dN was not statistically different for the two genes, whereas dS for *hcnB* was greater than that for *hcnC* ($Z = 2.13$ and $P = 0.016$, one-tailed test). This suggests that the 3' end of *hcnB* is subjected to less substitutional constraints, compared with the 5' end of *hcnC*.

The distinctness ratio (k), which has been proposed to differentiate different ecologically-adapted bacterial populations (Palys et al. 2000), was greater than two for all pairwise Hcn-group comparisons, for *hcnB* and *hcnC*, except when *hcnB* was compared in Hcn-1 and Hcn-2 ($k = 1.4$) (Table 3). The largest k values were found for pairwise comparisons involving Hcn-4. Therefore, these results suggest that each Hcn group could represent a different ecological population of HCN-producing pseudomonads.

Protein sequence analysis.

Three additional residues (proline, alanine, and leucine), whose effects on the enzyme's structure and function are unknown, were present at the C-terminal end of the deduced HcnB sequence for all strains in Hcn-3. The deduced HcnB sequence was not analyzed in more detail, because only 45 or 48 C-terminal residues were available and no particular function was attributed to this part (Blumer and Haas 2000; Laville et al. 1998).

A total of 139 HcnC residues were identified in all strains, except in *P. corrugata* 2172, in which five additional residues were present in the N-terminal part of HcnC (but the 139 others from strain 2172 aligned readily with those from the other pseudomonads) (Fig. 3B). The putative transmembrane region and the ADP-binding $\beta\alpha\beta$ fold revealed by Laville and associates (1998) were well conserved among the different strains. In all HCN⁺ pseudomonads, the $\beta\alpha\beta$ fold (expected to bind the ADP moiety of the dinucleotide electron carrier FAD or NAD) displayed the 32-residue fingerprint described by Wierenga and associates (1986). This fingerprint is comprised of a core (which contains three glycines and eight other key residues) and a loop (variable in length or composition) between the α helix and the second β strand. These observations (Fig. 3B) were also supported by identical hydropathicity predictions (Kyte and Doolittle 1982) for all strains (data not shown).

Overall, protein sequence analyses point to a similar structure and function for the N-terminal part of HcnC in all HCN⁺ pseudomonads studied. However, nothing is known about the functional role, if any, of the remaining variable sites in the sequence. These sites are mostly responsible for the different clustering of the strains when phylogenetic relationships are inferred from protein sequences (Fig. 2B).

Table 3. Nucleotide diversity (π_w), nucleotide divergence (π_b) and distinctness ratio (k) for the Hcn groups

	Hcn-1	Hcn-2	Hcn-3	Hcn-4
<i>hcnB</i>				
Hcn-1	0.001^a ± 0.001			
Hcn-2	0.057 ^b ± 0.013 (1.4) ^c	0.080 ± 0.015		
Hcn-3	0.161 ± 0.035 (23.6)	0.168 ± 0.031 (3.6)	0.012 ± 0.006	
Hcn-4	0.306 ± 0.059 (160.2)	0.330 ± 0.059 (8.0)	0.428 ± 0.071 (57.0)	0.003 ± 0.003
<i>hcnC</i>				
Hcn-1	0.008 ± 0.002			
Hcn-2	0.072 ± 0.011 (2.5)	0.051 ± 0.008		
Hcn-3	0.125 ± 0.020 (27.5)	0.144 ± 0.020 (5.5)	0.001 ± 0.001	
Hcn-4	0.154 ± 0.022 (33.8)	0.161 ± 0.020 (6.2)	0.203 ± 0.026 (145.2)	0.001 ± 0.001

^a The nucleotide diversity ($\pm$ SE, which was computed by the bootstrap method with 1,000 replicates) for *hcnB* (135 bp) and *hcnC* (391 bp) within Hcn groups is indicated in bold.

^b Nucleotide divergence ($\pm$ SE) between Hcn groups.

^c The distinctness ratio between different Hcn groups is indicated in parenthesis.

Production of HCN in vitro and relationship with biocontrol ability in planta.

For each strain, HCN production was comparable both times the experiment was carried out, except for Hcn-1 strains Q107-87 (coefficient of variation of 55%) and Q128-87 (70%) and Hcn-4 strain Pf-5 (53%), and there was no effect of Hcn groups on the coefficient of variation ($P = 0.642$). Hcn-4 strains produced significantly ($P < 0.001$) less HCN ($940 \pm$ standard error (SE) $70 \text{ nmol OD}_{600}^{-1}$; $n = 12$ strains) compared with strains in groups Hcn-1 ($1550 \pm 130 \text{ nmol OD}_{600}^{-1}$; $n = 6$), Hcn-2 ($1430 \pm 140 \text{ nmol OD}_{600}^{-1}$; $n = 8$), or Hcn-3 ($1410 \pm 100 \text{ nmol OD}_{600}^{-1}$; $n = 10$). The pathogen *P. corrugata* 2172 produced only $440 \text{ nmol of HCN OD}_{600}^{-1}$.

HCN production ability in vitro was compared with previous data (Sharifi-Tehrani et al. 1998) of biocontrol efficacy in planta, and a negative correlation was obtained between HCN production and severity of crown and root rot of tomato exposed to *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) in growth chamber rhizosphere microcosms (Table 4). Similarly, a positive correlation was found between HCN production in vitro and plant weight of cucumber confronted with the damping-off fungus *P. ultimum* (Table 4). The main difference was between Hcn-4 and the other Hcn groups based on *hcnBC* and *HcnBC* analyses (Fig. 2). Hcn-4 strains ($n = 7$) protected less compared with those from the other Hcn groups ($n = 8$) based on Student *t* tests of data taken from Sharifi-Tehrani and associates (1998), whereas pseudomonads in groups Hcn-1 ($n = 3$) and Hcn-3 ($n = 1$) represented the most promising biological control agents, regardless of the pathosystem considered (data not shown). Positive correlations were also found in this work between HCN production in vitro and growth inhibition data (Sharifi-Tehrani et al. 1998) for *P. ultimum* (on malt agar or King's B [KB] agar), but not for FORL (Table 4).

16S rDNA-based comparison of HCN-producing pseudomonads.

16S rDNA sequences derived from HCN⁺ biocontrol strains were compared with *Pseudomonas* type strains (Fig. 4). The

Table 4. Correlation between HCN production in vitro^a and biocontrol ability in vitro and in planta for biocontrol pseudomonads^b

Pathosystem	r ^c	P ^d
Tomato-FORL		
Disease index in vivo (0 to 4) ^e	-0.79	<0.0001
FORL inhibition on KB agar (mm) ^f		n.s.c.
FORL inhibition on malt agar (mm)		n.s.c.
Cucumber- <i>P. ultimum</i>		
Plant fresh weight (g pot ⁻¹) ^g	0.69	0.001
<i>P. ultimum</i> inhibition on KB agar (mm)	0.57	0.014
<i>P. ultimum</i> inhibition on malt agar (mm)	0.59	0.010

^a Expressed as $\text{nmol OD}_{600}^{-1}$.

^b $n = 18$.

^c Pearson's correlation coefficient. The 18 biocontrol pseudomonads included 15 HCN⁺ strains from Hcn-1 (C*1A1, TM1A3, TM1B2), Hcn-2 (Q69c-80, CA2, PITR2, PILH1), Hcn-3 (Q2-87), and Hcn-4 (CHA0, Pf-5, Pf1, PINR3, PF, PGNL1, PGNR2) and three HCN⁻ strains (TM3, CSL26, 2-79).

^d Bonferroni's significance test at $P < 0.05$ (n.s.c. = no significant correlation).

^e Disease index (0-4) was obtained by Sharifi-Tehrani and associates (1998) in growth chamber rhizosphere microcosms (12 plants per rockwool cube; 6 rockwool cubes per strain). The disease index was 0 in the absence of the fungus and 3.41 when only the fungus was added.

^f Inhibition of fungal growth in vitro by individual pseudomonads was studied in triplicate on KB agar and malt agar by Sharifi-Tehrani and associates (1998). Inhibition was quantified by measuring the distance between the edge of bacterial and fungal colonies (mm).

^g Total plant fresh weight per pot (four plants per pot, 12 pots per strain) was measured 12 days after planting by Sharifi-Tehrani and associates (1998). Plant fresh weight was 5.5 g pot^{-1} in the absence of the fungus and 0.04 g pot^{-1} when only the fungus was added.

resulting phylogeny of *Pseudomonas* spp. was in accordance with those obtained by Anzai and associates (2000) and Ramette and associates (2001). Maximum-likelihood phylogenetic reconstruction restricted to the HCN⁺ biocontrol strains resulted in the same topology (data not shown). Strains from groups Hcn-1 to Hcn-3 clustered closely with *P. corrugata* 2172, *P. thivervalensis*, and *P. brassicacearum* in the '*P. fluorescens*' group (Achouak et al. 2000), which is considered to represent the authentic *Pseudomonas* genus (de Vos et al. 1989). More specifically, strain CM1'A2 (Hcn-1) clustered with *P. brassicacearum* (99.6% sequence identity), PITR2 (Hcn-2) with *P. thivervalensis* (99.8%), and F113 (Hcn-1) with *P. corrugata* (99.6%), and therefore, these three HCN⁺ pseudomonads are likely to belong to distinct species. Strains Q2-87 (Hcn-3), Q65c-80 (Hcn-1), and Q37-87 (Hcn-2) clustered apart from the three other HCN⁺ biocontrol strains and might also correspond to a different species. Strains from Hcn-4 were all found in a distantly related cluster basal to the '*P. syringae*' group (Fig. 4). Among them, *rrs* sequences of strains CHA0, PF, and PGNR1 were identical (1,408 bp used) and shared 99.6% identity with that of strain Pf-5. Therefore, based on the 16S rDNA phylogeny, HCN-producing biocontrol pseudomonads may be present in at least five different species.

No apparent relationship was found between the *rrs* clusters and the Hcn groups defined in Figure 2. Indeed, strains from the same Hcn group (e.g., Hcn-1 strains Q65c-80, CM1'A2, and F113) could be present in different *rrs* clusters. Besides, strains from different Hcn groups (e.g., Q2-87 [Hcn-3], Q37-87 [Hcn-2], and Q65c-80 [Hcn-1]) could cluster together in the 16S rDNA tree.

To assess further the level of incongruence between *rrs* and *hcnBC* phylogenies, the likelihood of obtaining i) the *rrs* topology (Fig. 4) given the *hcnB*, *hcnC*, or *hcnBC* data sets and ii) the constrained *hcnBC* topology of Figure 2A given the *rrs* data set were estimated based on sequences of *P. corrugata* 2172 and the seven HCN⁺ biocontrol strains used in Figure 4. With the *hcnB* data set, the two topologies were not significantly different from each other, whereas with the *hcnC* or the concatenated *hcnBC* data sets, the *rrs* topology was significantly worse (KH test, $P < 0.05$) than that of *hcnBC* shown in Figure 2A. The same result was also obtained when strain 2172 was removed from the trees. With the *rrs* data set, the *hcnBC* topology was not statistically different from that of *rrs* but did differ when strain 2172 was not used. Therefore, this result suggests that the presence of strain 2172 could lead to wrong conclusions about both *hcnBC* and *rrs* phylogenies. Yet, without this strain, the congruent phylogenies derived from partial *hcnBC* genes or translated proteins revealed still a decoupled history from the evolution of the strains harboring them (based on 16S rRNA phylogeny).

DISCUSSION

Our work is based on the analysis of a partial *hcnBC* sequence that is essential for cyanogenesis, as Tn1725 insertion there (in the 3' end of *hcnB* or the 5' end of *hcnC*) resulted in a HCN⁻ phenotype in the HCN⁺ strain CHA0 (Laville et al. 1998). Such transposon insertions would probably have the same effect in the other HCN-producing strains, considering the similarity of *hcnBC* and of deduced HcnBC sequences (Fig. 3).

Functional analysis of *hcnBC* partial sequences evidenced a conserved Shine-Dalgarno motif and a same composition and position for the start codon (Fig. 3A), which raises the possibility of identical translation patterns. In addition, results indicate that the two partial sequences corresponding to the two main subunits of the HCN synthase were conserved structurally (and probably functionally), based on conserved features such as the transmembrane region (confirmed by hydrophobicity profiles)

(data not shown) and the essential (Laville et al. 1998) ADP-binding $\beta\alpha\beta$ fold carriers (Fig. 3). No essential function has yet been reported for the C-terminal parts of HcnB and HcnC, whereas it is known that the N-terminal parts of both proteins display identical features important for cyanogenesis (Blumer and Haas 2000; Laville et al. 1998). The latter could account for the lower levels of total nucleotide diversity (τ_T) and the lower number of synonymous substitutions found here for partial *hcnC* compared with partial *hcnB*, and both point to the existence of more substitutional constraints (i.e., less variation) for the essential N-terminal part of HcnC. These extensive structural and functional similarities raise the possibility that a phylogenetic reconstruction based on complete *hcnB* or *hcnC* sequences would lead to the same conclusions as the ones reached in the present study. However, other *hcn* sequences that are implicated in the regulation of HCN synthesis were not

included in this work. For instance, the promoter region of *hcnABC* contains specific regions involved in transcriptional control of *hcnABC*, such as the ANR box and a LuxR recognition site present in strain PAO1 but not in CHA0 (Blumer and Haas 2000). A posttranscriptional level of control (via noncoding RNAs) involves the region around the *hcnA* ribosome binding site in *P. aeruginosa* (Pessi et al. 2001). The level of conservation of these features in HCN⁺ pseudomonads remains to be established.

The 16S rDNA-based phylogeny is often used as reference for pseudomonad classification (Anzai et al. 2000; Moore et al. 1996), and in this work, *hcnBC* and *rrs* phylogenies were compared. Although *rrs* is under stringent functional constraints (Woese 1987), complete or partial replacements can, nevertheless, occur and result in the formation of mosaic sequences, thereby leading to a wrong organismal phylogeny in certain

Fig. 4. Phylogenetic comparison of the 16S rDNA for biocontrol HCN⁺ *Pseudomonas* strains (underlined in the tree) representing the different Hcn groups and several reference pseudomonads. The neighbor-joining (NJ) tree was constructed using the Jukes-Cantor model, and nodal supports of the inferred tree were evaluated by 1,000 bootstrap replicates. The scale bar represents the number of substitutions per site. The “*P. fluorescens*,” “*P. chlororaphis*,” and “*P. syringae*” groups were those defined by Anzai and associates (2000). The pseudomonads studied (with their accession number) were Q2-87 (AJ278813), Q65c-80 (AJ417074), Q37-80 (AJ417069), Pf-5 (AJ417072), CHA0 (AJ278812), PF (AJ417073), PGNR1 (AJ417071), CM1'A2 (AJ417068), PITR2 (AJ417070), F113 (AJ278814), *P. brassicaeaeum* (AF100322), *P. thivervalensis* (AF100323), *P. corrugata* 2172 (= strain ATCC29736) (D84012), *P. migulae* CIP105470 (AF074383), *P. fluorescens* IAM 12022 (D84013), ATCC49642 (AF094732), and DSM50090 (Z76662), *P. marginalis* (AB021401), *P. rhodesiae* (AF064459), *P. orientalis* (AF064457), *P. cedrella* (AF064461), *P. gessardii* (AF074384), *P. fragi* (AB021413), *P. lundensis* (AB021395), *P. chlororaphis* IFO3904 (D86004), ATCC13985 (AF094722), and LMG5004 (Z76657), *P. aureofaciens* (Z76656), *P. savastanoi* (AB021402), *P. meliae* (AB021382), *P. ficuserectae* (AB021378), *P. syringae* LMG1247 (Z76669) and ATCC19310 (D84026), *P. syringae* pv. *phaseolicola* (AB001448) and pv. *tagetis* (AB001449), *P. caricapapayae* (D84010), *P. putida* IAM 1236 (D84020), and *P. aeruginosa* PAO1 (AE004844).

cases (Wang and Zhang 2000; Yap et al. 1999). However, this approach was appropriate in this study, as the *rrs*-based species phylogeny obtained here (Fig. 4) was in accord with the results from several genotypic and phenotypic studies on the taxonomy of the *Pseudomonas* spp. (Achouak et al. 2000; Anzai et al. 2000; Palleroni 1984; Ramette et al. 2001). Phylogenetic analysis of *rrs* revealed that HCN production was present in biocontrol strains belonging to different *Pseudomonas* species (Fig. 4). In addition, this trait was also shared by plant-deleterious pseudomonads and by strains pathogenic to plants or animals, e.g., the opportunistic pathogens *P. corrugata* 2172 and *P. aeruginosa* PAO1, used here as reference HCN⁺ strains. Interestingly, HCN⁺ biocontrol strains clustered in company of phytopathogenic pseudomonads belonging to e.g., *P. syringae*, *P. brassicacearum*, and *P. corrugata* (Fig. 4). This may reflect the partial overlap of their ecological habitats, the incomplete assessment of their biocontrol and pathogenic behaviors on different host plants, or both. Indeed, certain pathogenic *P. brassicacearum*, *P. thivervalensis*, or *P. corrugata* isolates can protect wheat from take-all (Ross et al. 2000; Schmidt et al. 1997) or potato from Fusarium dry rot (Schisler et al. 1997).

Based on the assumption that the *rrs*-based phylogeny is the species phylogeny, the phylogeny derived from *hcnBC* was found partly incongruent with the former. This may be due to genomic rearrangements and recombinations or the occurrence of lateral gene transfer. Lateral gene transfer events can lead to a distorted history between ribosomal and protein-coding genes (Ochman et al. 2000). Among the pseudomonads studied here, the reference strain *P. corrugata* 2172 appeared to be responsible for some topological incongruities between phylogenies inferred from *hcnBC* sequences and their deduced proteins. The atypical sequence features in this strain, such as its supplementary residues (Fig. 3), may result from lateral gene transfer events (Lawrence and Ochman 1997). Yet, for biocontrol pseudomonads that produce both HCN and PhI, cluster analyses of biosynthetic genes *hcnBC* and *phlD* (Ramette et al. 2001; Wang et al. 2001) indicated that their phylogeny is not only congruent with each other but also very similar to the dendrograms based on randomly amplified polymorphic DNA data (Keel et al. 1996) and repetitive sequence-based PCR data (Mavrodi et al. 2001). Therefore, *hcnBC* evolution may parallel whole genome history.

Four Hcn groups were identified based on the analysis of HcnBC (and *hcnBC*) sequences (Fig. 2), and most distinctness ratios (*k*) were over two when Hcn groups were compared two by two, regardless of whether *hcnB* or *hcnC* was considered (Table 3). These *k* values indicate (Palys et al. 2000) that the Hcn groups could correspond to different ecologically adapted populations of HCN-producing pseudomonads, a hypothesis strengthened by the analysis of phenotypic characteristics. First, results showed that Hcn-4 strains produced less HCN overall compared with the others. Interestingly, Hcn-4 strains Pf-5 and PF, which produced the maximal values of their group, clustered separately from the other Hcn-4 strains in Figure 2A when *hcnBC*-based phylogeny was considered. Group Hcn-2 did not differ from Hcn-1 and Hcn-3 in terms of HCN production, but it displayed the highest strain to strain variation in HCN production and the highest DNA diversity for both *hcnB* and *hcnC* data sets (Table 3). Thus, certain differences in *hcnBC* sequences may correlate with quantitative HCN production. However, it must be kept in mind that nothing is known about actual HCN production by these strains in their natural environment (i.e., the rhizosphere), as direct HCN measurement in situ remains a major technical issue.

Second, HCN production is an important trait for biocontrol of phytopathogenic fungi (Ellis et al. 2000; Voisard et al. 1989), and indeed, Sharifi-Tehrani and associates (1998)

showed that HCN⁺ biocontrol pseudomonads were more efficient than their HCN⁻ counterparts against Fusarium crown and root rot of tomato and Pythium damping-off of cucumber. These findings were strengthened and expanded in this work, as positive correlations were found between HCN production in vitro and biocontrol ability of the strains in planta in two pathosystems (Table 4). Hcn-4 strains, which produced less HCN than pseudomonads from the other groups, protected less than the latter in both pathosystems when analyzing previous data from Sharifi-Tehrani and associates (1998).

Third, 20 of the HCN⁺ strains included in the current work have been studied for carbon source utilization (Wang et al. 2001). Hcn-1 (*n* = 7 strains studied), Hcn-2 (*n* = 2), and Hcn-3 (*n* = 1) strains belonged to a same catabolic cluster, with the exception of Hcn-1 strain F113. Hcn-4 strains (*n* = 10) were found in two other clusters, strains Pf-5 and PF clustering separately from the other Hcn-4 pseudomonads (Wang et al. 2001). In the rhizosphere, carbon sources available to rhizobacteria are mainly provided by root exudates (Hale et al. 1978), whose composition differs depending on the plant species. Thus, the ability to utilize particular root exudate compounds is probably an adaptation to rhizosphere habitats.

MATERIALS AND METHODS

Bacterial strains, culture conditions and storage.

The HCN⁺ bacteria used in this study are biocontrol fluorescent pseudomonads (Table 1), except the nonfluorescent opportunistic pathogen *P. corrugata* 2172 (Sutra et al. 1997) and *P. aeruginosa* PAO1 (Holloway 1955) used as reference strains. HCN production was shown using the phenotypic test of Castric and Castric (1983). Almost all efficient biocontrol pseudomonads synthesize more than one biocontrol compound (Dowling and O'Gara 1994; Keel and Défago 1997; Wang et al. 2001), and in the collection studied here, biocontrol-relevant metabolites (other than HCN) produced by the biocontrol pseudomonads included fluorescent siderophores (in all strains), protease activity (in nearly all strains) (Wang et al. 2001; Sharifi-Tehrani et al. 1998), and PhI (in all but strains Q69c-80 and CA2) (Keel et al. 1996). All pseudomonads were routinely grown at 27°C on KB agar (King et al. 1954) and stored at -80°C in 40% glycerol.

PCR primer design, amplification of *hcnBC*, and sequencing.

The *hcnBC* amino acid sequences of *P. aeruginosa* PAO1 (accession number AF208523) and *Pseudomonas* sp. strain CHA0 (AF053760) were aligned using Clustal W (Thompson et al. 1994). The consensus sequence was used to design the 20-mer forward primer ACa (annealing within *hcnB*) and the 18-mer reverse primer ACb (annealing within *hcnC*) (Fig. 1; Table 2). PCR primers were synthesized by MWG Biotech (Münchenstein, Switzerland).

DNA preparation of pseudomonads was done as described by Wang and associates (2001). PCR amplification of *hcnBC* sequences was carried out in 50-μl reaction mixtures containing 10 μl of heat-lysed bacterial suspension, 1× PCR buffer (Amersham Pharmacia, Uppsala, Sweden), bovine serum albumin (0.5 g l⁻¹) (Fluka, Buchs, Switzerland), 5% dimethyl sulfoxide (Fluka), 100 μM each of dATP, dCTP, dGTP, and dTTP (Amersham Pharmacia), 0.40 μM of each primer, and 3.5 U of Taq DNA polymerase (Amersham Pharmacia). Amplifications were performed with a programmable thermal controller 100 (MJ Research Inc., Watertown, MA, U.S.A.). The initial denaturation (2 min 30 s at 94°C) was followed by 30 PCR cycles (94°C for 30 s, 63°C for 30 s, and 72°C for 1 min) and a final

extension at 72°C for 10 min. PCR products were electrophoresed and visualized following standard protocols (Sambrook et al. 1989).

PCR products were purified with the Amicon microcon-PCR centrifugal filter devices (Millipore Corp., Bedford, MA, U.S.A.) and double-strand sequenced (Microsynth, Balgach, Switzerland) using primers ACa and ACb. Consensus sequences were then assembled using the Genetic Computer Group fragment assembly system (GCG version 10; University of Wisconsin, Madison, WI, U.S.A.). Virtual restriction (using *Cfo*I, *Hae*III, *Msp*I, and *Nde*II) of the *hcnBC* sequences obtained in this work and experimental restriction of PCR products gave identical banding patterns (data not shown). *hcnBC* sequences have been deposited with the EMBL database under accession numbers AJ418442 through AJ418477.

Phylogenetic inference.

Multiple *hcnBC* sequences were aligned (CLUSTAL W) and sites presenting gaps or unknown bases were excluded from analysis. The Molecular Evolutionary Genetics Analysis program (MEGA version 2.1, S. Kumar, K. Tamura and M. Nei, Pennsylvania State University, University Park, PA, U.S.A.) was used to calculate evolutionary distances and infer trees based on the neighbor-joining (NJ) method. Programs in the package were also used to perform parsimony analysis, bootstrap resampling, estimations of diversity parameters, and statistical tests for detecting the kind of selection governing substitutions. To estimate the number of nucleotide substitutions per site, Kimura 2-Parameter (K2P) or Jukes-Cantor (JC) formulas were used. Nodal supports of the inferred trees were assessed by bootstrapping (1,000 replicates).

DNA sequences were translated into predicted HcnB and HcnC amino acid sequences by comparison with already known HcnB and HcnC sequences of strain CHA0 (Laville et al. 1998). NJ trees were constructed based on the number of differences or Poisson-corrected distances. Statistical supports of the inferred trees were evaluated by 1,000 bootstrap replicates. Maximum parsimony analyses were performed with the PROTPARS program from the Phylogeny Inference Package (PHYLIP; J. Felsenstein, University of Washington, Seattle, WA, U.S.A.) and with the Phylogenetic Analysis Using Parsimony package (PAUP*; D. L. Swofford, Sinauer Associates, Sunderland, MA, U.S.A.) using heuristic or exhaustive search. The latter package was also used to test for the presence of phylogenetic signal in the actual data set. First, *g*₁ statistics were calculated for 10,000 random trees using the parsimony criterion (Hillis and Huelsenbeck 1992). Second, the permutation tail probability (PTP) test was performed by generating 1,000 random data sets for 10 representative taxa and comparing the frequency at which trees of same size or shorter than the original tree were obtained (Faith 1991). The default DNAML program implemented in the PHYLIP package was used for estimation of the maximum-likelihood topologies on a representative subset of the different groups. The KH test implemented in the PHYLIP package was used to compare topologies inferred from *hcnBC* (or HcnBC) and *rrs* sequences for a same data set. That is, different topologies without constraints on branch lengths were applied to a given data set and their maximum likelihood scores (DNAML default) were compared with the highest scores of the optimal topologies to determine whether the alternative topologies were significantly better to explain the current data set.

DNA and protein sequence analysis.

Different Hcn groups were defined for the biocontrol fluorescent pseudomonads, based on the result of HcnBC comparisons. Then, nucleotide diversity within Hcn groups and diver-

gence between Hcn groups (π_w and π_b , respectively) (Nei and Li 1979) were calculated based on the JC formula, using 135 bp of *hcnB*, 391 bp of *hcnC*, or the 549 bp available in *hcnBC*. The SE of these estimates was determined by 1,000 bootstrap replicates, as suggested by Nei and Kumar (2000). To test differences in nucleotide diversity between groups, Z tests were performed using the variances determined by the bootstrap method at a significance level of $P < 0.05$.

The distinctness ratio (k) between two groups was calculated according to Palys and associates (2000), using the formula $k = \pi_{bij}$ divided by $[0.5 (\pi_{wi} + \pi_{wj})]$, where π_{wi} and π_{wj} are nucleotide diversities within groups i and j , respectively, and π_{bij} the divergence between the two groups. The population structure-independent nucleotide diversity for the whole collection of biocontrol fluorescent pseudomonads (π_T) and the coefficient of nucleotide differentiation (N_{ST}) were also estimated based on the JC formula, and their variances were determined by the bootstrap method.

dN and dS were estimated by the Kumar method, as described by Nei and Kumar (2000). This method incorporates the transition/transversion bias, which was estimated by the program MEGA. Its SE was computed by the bootstrap method (1,000 replicates). Statistical comparisons ($P < 0.05$) of dN and dS were performed with Z tests or Fisher-Exact tests (small-sample tests).

Quantitative analysis of hydrogen cyanide production and correlation with biocontrol ability.

HCN production was measured according to Keel and associates (1989) and Gewitz and associates (1976). Briefly, 100 μ l (about 10^9 colony-forming units ml^{-1}) of an overnight nutrient yeast extract broth (NYB) (Difco Laboratories, Detroit, MI, U.S.A.) shake culture were inoculated into 40 ml of Castirc's medium (Castirc 1975, as modified by Keel and associates 1989) supplemented with 20 μM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 125-ml hermetically-sealed sterile glass flasks (previously washed with 1 M HCl, rinsed with deionized water, and dried). Bacteria were allowed to grow exactly 24 h at 27°C (160 rpm). A standard curve was made, using KCN solutions (1 to 16 μM) prepared in 0.1 M NaOH. Stationary-phase bacterial cultures (diluted 10-fold in 0.1 M NaOH) and KCN solutions were added (200 μ l) to 1 ml of 0.1 M NaOH and were mixed thoroughly. To 600 μ l of these samples, 1 ml of 0.1 M *o*-dinitrobenzene (Sigma Chemicals, St. Louis) dissolved in ethylene glycol monomethyl ether (EGME) (Sigma Chemicals), and 15 s later, 1 ml of 0.2 M 4-nitrobenzaldehyde (Fluka) in EGME were added (at 24°C). The change of color (from colorless to dark brown) that begins after addition of 4-nitrobenzaldehyde is proportional to the amount of KCN (for the reference solutions) or HCN (for the bacterial suspensions), and it was quantified by measuring absorption (578 nm) exactly 30 min after the 4-nitrobenzaldehyde addition.

Three measurements were made for each point of the KCN standard curve. One flask was used for each pseudomonad. HCN production in each flask was measured twice (within 30 min) and the two measurements were similar (for a few strains) or identical (for most strains). The whole experiment was repeated on a separate occasion, again with one flask per pseudomonad. Turbidity ranged from 1.0 to 1.5 OD₆₀₀ units (i.e., in the order of 10^9 colony-forming units ml^{-1}) in modified Castirc medium at the end of the 24-h incubation, and HCN levels were expressed as nmol HCN OD₆₀₀⁻¹. Statistical analysis of HCN production was carried out to compare the four Hcn groups in each run of the experiment, and for individual strains, the mean of repeated measurements was used in the analysis. This was done by analysis of variance followed by Tukey's honestly significantly different tests ($P < 0.05$). The

same statistical results were obtained in each run of the experiment (even when data from one measurement were used instead of the means of repeated measurements), and mean values obtained by pooling data from both experiments are given. Statistical analyses were performed using SYSTAT (version 9; SPSS Science, Chicago, IL, U.S.A.).

In addition, the link between HCN production *in vitro* and biocontrol ability in planta was assessed by correlation analysis using plant protection data obtained by Sharifi-Tehrani and associates (1998) for 15 HCN⁺ representative strains and 3 HCN⁻ reference biocontrol pseudomonads in the cucumber/*Pythium ultimum* and tomato/FORL pathosystems. Disease indices for Fusarium crown and root rot were transformed to arcsines prior to statistical analysis. Correlation analyses were performed using Pearson's correlation coefficient (*r*) followed by Bonferroni's significance test at *P* < 0.05 level.

Amplification and sequencing of 16S rRNA genes.

DNA preparation, PCR conditions, and sequencing of 16S rRNA genes (*rrs*) were done as described elsewhere (Ramette et al. 2001). Briefly, the 16S rRNA gene was amplified with primers 63f (22-mer, 5'-GGAGAGTTAGATCTTGGCTCAG-3') and 153r (20-mer, 5'-AAGGAGGGGATCCAGCCGCA-3') (Normand 1995), using the proof-reading polymerase of the expand high fidelity PCR system (Roche Diagnostics, Mannheim, Germany). PCR conditions consisted of an initial denaturation (2 min at 94°C), followed by 10 PCR cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min, 20 PCR cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min with a 5-s increment for each cycle, and a final extension at 72°C for 7 min. Water was used as a negative control. Since *hcnBC* and *rrs*-based phylogenies were compared in this work, restriction analysis of PCR-amplified *hcnBC* was done concomitantly on the same DNA preparation each time *rrs* was amplified, to confirm the absence of contamination. PCR amplicons were purified with the Amicon microcon-PCR centrifugal filter devices (Millipore) and were sequenced by Genome Express (Grenoble, France). Almost complete 16S rRNA sequences were determined for strains Pf-5 (1,526 bp), PF (1,533 bp), Q37-87 (1,532 bp), Q65c-80 (1,532 bp), CM1'A2 (1,532 bp), PITR2 (1,491 bp), and PGNR1 (1,534 bp), and they are available at the EMBL database at accession numbers listed in Figure 4. For the other pseudomonads studied, these sequences were already available (Fig. 4).

The 16S rRNA sequences were aligned with the most similar sequences of the Ribosomal Database Project database. Only complete sequences that yielded highest similarity scores were kept for subsequent analysis. The sequence alignment was checked manually and corrected. The NJ tree was constructed from evolutionary distances estimated with the model of JC. Nodal supports of the inferred tree were evaluated by 1,000 bootstrap replicates.

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