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Comparison of ATPase-Encoding Type III Secretion System *hrcN* Genes in Biocontrol Fluorescent Pseudomonads and in Phytopathogenic Proteobacteria

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Type III protein secretion systems play a key role in the virulence of many pathogenic proteobacteria, but they also occur in nonpathogenic, plant-associated bacteria. Certain type III protein secretion genes (e.g., *hrcC*) have been found in *Pseudomonas* sp. strain SBW25 (and other biocontrol pseudomonads), but other type III protein secretion genes, such as the ATPase-encoding gene *hrcN*, have not been found. Using both colony hybridization and a PCR approach, we show here that *hrcN* is nevertheless present in many biocontrol fluorescent pseudomonads. The phylogeny of biocontrol *Pseudomonas* strains based on partial *hrcN* sequences was largely congruent with the phylogenies derived from analyses of *rrs* (encoding 16S rRNA) and, to a lesser extent, biocontrol genes, such as *phlD* (for 2,4-diacetylphloroglucinol production) and *hcnBC* (for HCN production). Most biocontrol pseudomonads clustered separately from phytopathogenic proteobacteria, including pathogenic pseudomonads, in the *hrcN* tree. The exception was strain KD, which clustered with phytopathogenic pseudomonads, such as *Pseudomonas syringae*, suggesting that *hrcN* was acquired from the latter species. Indeed, strain KD (unlike strain SBW25) displayed the same organization of the *hrpJ* operon, which contains *hrcN*, as *P. syringae*. These results indicate that the occurrence of *hrcN* in most biocontrol pseudomonads is not the result of recent horizontal gene transfer from phytopathogenic bacteria, although such transfer might have occurred for a minority of biocontrol strains.

Type III protein secretion systems (TTSS) are widely distributed among proteobacterial pathogens of plants (belonging to the genera *Pseudomonas*, *Erwinia*, *Xanthomonas*, and *Ralstonia*), animals, and humans (27). TTSS are specialized machineries for introduction of proteinaceous virulence factors directly into eukaryotic host cells, and mutations in the TTSS genes result in a loss of pathogenicity (22). The basic mechanism of protein secretion is the same even when bacterial species as divergent as the human plague-causing agents *Yersinia* spp. (18) and the plant pathogen *Pseudomonas syringae* (35) are compared. However, the proteins secreted can differ from one pathogen to the next (27). TTSS genes are often thought to have evolved from the genes encoding the flagellar export mechanism (17, 37, 42), but recent findings suggest instead that the two types of genes have a common ancestor (19).

In plant pathogens, functional TTSS are essential for induction of disease in susceptible host plants (3) and are encoded by *hrc* or *hrp* genes (17). In contrast, resistant plants develop a defense reaction called the hypersensitive response in the presence of a phytopathogen with a functional TTSS. The hypersensitive response is a local tissue necrosis, accompanied by the production of antimicrobial substances, which is aimed at preventing further spread of the infecting bacterium in the plant (32, 36).

In addition to phytopathogens, the existence of TTSS has also been demonstrated in plant endosymbionts, such as the nitrogen-fixing bacterium *Rhizobium* (15, 20, 39). TTSS mutants of *Rhizobium* are affected in nodulation ability and display altered host specificity (62). Therefore, it appears that TTSS can also be involved in beneficial prokaryote-eukaryote interactions. Recently, genes coding for a functional TTSS (e.g., *hrcC*) have been found in *Pseudomonas* strains capable of protecting a plant from disease (47), which raises the possibility that TTSS could also play a role in biocontrol interactions. The origin of TTSS genes in plant-beneficial bacteria is unknown, because so far the work has focused on pathogens (19). This issue is important, because (i) the molecular differences between biocontrol and phytopathogenic pseudomonads are poorly understood and (ii) until the work of Preston et al. (47) the presence of a TTSS was one criterion pointing to a pathogenic status. The organization and sequences of TTSS genes in biocontrol fluorescent *Pseudomonas* sp. strain SBW25 were similar to those in strain 61 of *P. syringae* (47), a taxon in which *hrp* genes are thought to be ancestral (55). This suggests that a horizontal gene transfer(s) took place from pathogenic to biocontrol pseudomonads. However, certain TTSS genes present in the pathogen *P. syringae* 61 were not present in the biocontrol strain SBW25; these genes include *hrcN*, which encodes a conserved peripheral membrane ATPase (46). Therefore, if the horizontal gene transfer hypothesis described above is valid, this finding could perhaps be explained by (i) incomplete gene transfer from a pathogenic pseudomonad or (ii) subsequent gene loss once the entire TTSS gene set was acquired by a biocontrol pseudomonad.

In this paper, we report the existence of TTSS sequences in a wide range of biocontrol pseudomonads. TTSS sequences were analyzed to determine the phylogenetic relationships be-

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tween biocontrol fluorescent pseudomonads and plant pathogens, with the objective of assessing the likelihood of past transfer of a TTSS gene(s) between these plant-associated bacteria. Previous work on the occurrence of TTSS genes in nonpathogenic *Pseudomonas* has focused on strain SBW25 and *hrcC* (47), but as highlighted above, the range of TTSS genes in *Pseudomonas* sp. strain SBW25 is incomplete. Since our preliminary observations indicated that TTSS genes that were absent from strain SBW25 (e.g., *hrcN*) could be present in other biocontrol *Pseudomonas* strains along with *hrcC*, *hrcN* was preferred to the latter gene in the analysis. This choice was based on the assumption that biocontrol strains targeted in this way would display an organization of TTSS genes more comparable to that in their pathogenic counterparts. The phylogenetic analysis was done by using partial nucleotide sequences of the *hrcN* genes in a well-characterized collection of biocontrol fluorescent pseudomonads having worldwide origins (30, 51) and reference phytopathogenic proteobacteria (Table 1). In addition, the phylogenetic relationship between *hrcN* and structural genes for synthesis of the biocontrol compounds 2,4-diacetylphloroglucinol (Phl) and hydrogen cyanide (HCN) in biocontrol pseudomonads was assessed. Finally, the organization of key TTSS genes in one $hrcN^+$ biocontrol strain was compared to that in the biocontrol strain SBW25 and the pathogen *P. syringae*.

MATERIALS AND METHODS

PCR amplification and sequencing of *hrcN*. The *hrcN* sequences of *P. syringae* pv. tomato DC3000 (accession number AF232004) and *Erwinia amylovora* strain CNPB136 (accession number L25828) were aligned by using LAlign software (26). The consensus sequence was used to design the degenerate 20-mer primers hrcN-5rR (forward) and hrcN-4r (reverse) (Table 2 and Fig. 1A). Primers were synthesized by MWG Biotech (Münchenstein, Switzerland).

The bacteria used in this study (Table 1) were grown at 27°C on King's B agar (31) or Luria-Bertani (LB) agar (54). PCR amplification was carried out in 20-μl reaction mixtures containing 5 μl of cell lysate (for pseudomonads), which was obtained by heating the cultures for 10 min at 99°C with 95 μl of lysis buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 0.1% Tween 20) in a PTC-100 thermal cycler (MJ Research, Waltham, Mass.), or 200 pg of genomic DNA of *E. amylovora* strain 22716, 22770, or 23482 (DNA kindly provided by E. Holliger, FAW Wädenswil, Wädenswil, Switzerland). Each PCR was performed in 1× PCR buffer containing 100 μM dATP, 100 μM dCTP, 100 μM dGTP, 100 μM dTTP, 0.07 U of *Taq* polymerase (Amersham Pharmacia Biotech, Piscataway, N.J.) per μl, and each primer at a concentration of 0.20 μM by using an initial denaturation step consisting of 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C and then a final elongation for 10 min at 72°C. The sizes of PCR products were checked by electrophoresis in 1.5% agarose.

PCR amplicons were purified from a PCR mixture by two washes with 100 μl of double-distilled water on a MultiScreen PCR plate (Millipore, Molsheim, France), resuspended in 30 μl of double-distilled water, and visually quantified by using an agarose gel. The cycle sequencing reaction was performed with 3 to 10 ng of purified PCR product by using an ABI PRISM BigDye Terminator v3.0 cycle sequencing kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions and primers hrcN-4r and hrcN-5rR, each at a final concentration of 0.16 μM. Cycle sequencing products were cleaned by passage through water-swelled Sephadex G-50 columns (Amersham Biosciences, Uppsala, Sweden) on MultiScreen HV plates (Millipore) and were sequenced with an ABI PRISM 3100 genetic analyzer. The identities of the sequenced fragments were determined by BLASTN comparison with known sequences.

PCR amplification and sequencing of 16S rDNA. PCR amplification of approximately 1.5 kb of the 16S ribosomal DNA (rDNA) gene (*rrs*) was performed by using the universal 20-mer primers PH-16S and PA-16S (12) (Table 2). PCR was done by using the conditions described above but with initial denaturation for 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 45 s at 72°C and a final elongation for 7 min at 72°C. Purification and sequencing of *rrs* amplicons were performed as described above for *hrcN*, except that an additional primer, Intern 2a (derived in this work from Intern 2 proposed by Johnsen et al. [28]) (Table 2), was used when it was needed to obtain complete double-stranded coverage of *rrs*. Sequences were obtained for 18 strains.

Phylogenetic inference. DNA sequences were assembled by using the freeware Chromas, version 1.45 (http://www.technelysium.com.au/chromas.html; Technelysium Pty. Ltd., Helensvale, Australia), and were aligned with ClustalW (60). Sites with alignment gaps were excluded from the analysis. The Molecular Evolutionary Genetics Analysis (MEGA) program, version 2.1 (33), was used to calculate evolutionary distances and to infer trees based on the neighbor-joining (NJ) and maximum-parsimony (MP) methods. The maximum-likelihood (ML) tree for *hrcN* was calculated with the Phylogenetic Analysis Using Parsimony package (PAUP*, version 4.0; D. L. Swofford, Sinauer Associates, Sunderland, Mass.) by using a heuristic search. The pathogen *Xanthomonas campestris* was used as the outgroup in the *rrs* phylogenetic tree (NJ method), and the flagellar ATPase gene *fliI* of *Pseudomonas putida* KT2440 was used as the outgroup in the *hrcN* trees. The best evolutionary model was inferred by calculating log likelihood scores with the program ModelTest (45) implemented in PAUP*, version 4.0, and on this basis the number of nucleotide substitutions per site was estimated with the help of the Jukes-Cantor (JC) formula with the gamma parameter (29) (implemented in MEGA). The nodal robustness of the inferred trees was assessed by using 200 (ML method) or 1,000 (NJ and MP methods) bootstrap replicates.

DNA sequences were translated into predicted HrcN amino acid sequences, and an NJ tree was constructed with MEGA based on the number of differences or Poisson-corrected distances. MP analysis of protein sequences was also performed with MEGA by using a close-neighbor-interchange approach. FliI of *P. putida* KT2440 was used as the outgroup. Statistical support of the inferred trees was assessed by using 1,000 bootstrap replicates.

Detection of TTSS by hybridization. Detection of the TTSS gene *hrcV* in *Pseudomonas* strains KD, K92.46, K94.14, K94.23, K93.37, CHA0, and P3 was carried out by hybridization to a digoxigenin-labeled probe which corresponded to positions 1376 to 2132 of *hrcV* in *E. amylovora* CNPB136 (accession number L25828) and was obtained by amplifying a 757-bp fragment from plasmid pCPP1103 (7) with primers HRCV-L and HRCV-R (Table 2), as described by Stuber et al. (58). Genomic DNA was purified by the Triton-Prep method, which consisted essentially of lysis of pelleted cells by 90 s of boiling in STET buffer (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl, 50 mM EDTA; pH 8.0) containing 1 mg of lysozyme ml^{-1} and 0.1 mg of RNase ml^{-1}, followed by phenol-chloroform extraction and precipitation of the DNA with 0.4 M lithium chloride. Purified DNA was digested for 2 h with 1.5 U of PstI and separated on 1.5% agarose. Southern blotting was done (54) by alkaline transfer of DNA onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech) by using an LCB 2016 Vacu Gene vacuum blotting unit (Pharmacia LKB Biotechnology AB, Bromma, Sweden). DNA was cross-linked on the membrane by UV irradiation. After overnight hybridization at 60°C, the blots were washed twice for 5 min at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate) containing 0.1% sodium dodecyl sulfate. Signals were detected by chemiluminescence by using a DIG luminescent detection kit for nucleic acids (Roche Diagnostic Corporation, Mannheim, Germany), in which disodium 3-(4-methoxyspiro{l,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.$1^{3,7}$]decan}-4-yl)phenyl phosphate was used as the substrate, and blots were exposed to Kodak Biomax MS film (Eastman Kodak Company, Rochester, N.Y.) and developed according to the manufacturer's instructions.

Analysis of *hrcN* by dot blot DNA hybridization was performed for all biocontrol strains for which PCR amplification of *hrcN* failed. The probe consisted of the 249-bp *hrcN* fragment that was amplified from strain KD by using primers hrcN-4r and hrcN-5rR and was labeled by using the ECL direct nucleic acid labeling and detection system (Amersham Biosciences). Detection was performed according to the manufacturer's instructions. The biocontrol pseudomonads PILH1, Q2-87, TM1′A4, K94.23, F113, P97.38, 2-79, and KD, the pathogenic *Pseudomonas* strain VS01, and *E. amylovora* 22770 were used as positive controls, and *Escherichia coli* strains XL10-Gold (Stratagene, Cedar Creek, Tex.) and DH5α (Invitrogen, Paisley, United Kingdom) were used as negative controls. All controls gave the expected results. All positive results were confirmed by a second hybridization with the *hrcV* probe described above.

Cloning of a 1.7-kb *hrcV* fragment from strain KD into plasmid pUK21. A 1.7-kb PstI fragment from strain KD that hybridized with the *hrcV* probe was cloned in plasmid pUK21 by using the following procedure. Genomic DNA of strain KD was digested with PstI and electrophoresed on a 1.5% agarose, resulting in a smear. With the help of a 1-kb DNA ladder and a 0.16- to 1.77-kb RNA ladder (Invitrogen), the region containing fragments around 1.7 kb was identified and excised from the gel. The fragments were purified by using a QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and ligated overnight

TABLE 1. Bacterial strains used in the study

Strain(s)	Biocontrol or pathogenic properties[a]	Plant origin[b]	Geographic origin	*hrcN* analysis		Strain reference
				PCR	Hybridization	
Biocontrol *Pseudomonas* spp. strains						
C*1A1, CM1′A2	Cucumber (*P. ultimum, P. sclerotioides*), cotton (*R. solani*)	Cucumber	Switzerland	+		16
K92.46, K94.08	Cucumber (*P. ultimum*)	Cucumber	Switzerland	+		This study
P97.38	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Cucumber	Switzerland	+	+	64
CHA0	Tobacco (*T. basicola*), wheat, (*G. graminis*), cucumber (*P. ultimum*)	Tobacco	Switzerland	−	−	59
K93.2	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Tobacco	Switzerland	−	−	64
P12	Tobacco (*T. basicola*), cucumber (*P. ultimum*)	Tobacco	Switzerland	+		30
Pf1	Cucumber (*P. ultimum*), tobacco (*T. basicola*)	Tobacco	Switzerland	−	+	30
K94.3	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Tomato	Switzerland	−	−	This study
TM1A3, TM1B2	Cucumber (*P. ultimum, P. sclerotioides*), cotton (*R. solani*)	Tomato	Switzerland	+		16
TM1′A4	Cucumber (*P. ultimum, P. sclerotioides*), cotton (*R. solani*)	Tomato	Switzerland	+	+	30
TM1′A5	Cucumber (*P. ultimum, P. sclerotioides*), cotton (*R. solani*)	Tomato	Switzerland	+		30
K93.37	Cucumber (*P. ultimum*)	Wheat	Switzerland	−	−	This study
K94.23	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Wheat	Switzerland	+	+	This study
K94.31	Tomato (*F. oxysporum*)	Cucumber	Czech Republic	+		64
K94.35	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Cucumber	Czech Republic	−	−	This study
K94.37	Tomato (*F. oxysporum*)	Cucumber	Czech Republic	−	−	64
P97.30	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Wheat	Czech Republic	+		64
DR54	Sugar beet (*P. ultimum*)	Sugar beet	Denmark	−	+	43
F96.27	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Cucumber	Estonia	−	−	64
K94.2	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Cucumber	Italy	−	−	This study
K94.14	Cucumber, (*P. ultimum*), tomato (*F. oxysporum*)	Cucumber	Italy	+		This study
PINR3	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Tobacco	Italy	−	−	30
PILH1	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Tomato	Italy	+	+	30
PITR2	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Wheat	Italy	+		30
F113	Sugar beet (*P. ultimum*), potato (*E. carotovora*)	Sugar beet	Ireland	+	+	14
K94.56	Cucumber (*P. ultimum*)	Cucumber	Romania	−	−	This study
SBW25	Pea (*P. ultimum*)	Sugar beet	United Kingdom	−	−	49
P97.26	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Tomato	Bhutan	−	−	64
KD	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Wheat	China	+	+	56
K94.26	Cucumber (*P. ultimum*)	Cucumber	India	−	−	This study
K95.7	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Cucumber	Pakistan	−	−	This study
PGNR1, PGNR2, PGNR3, PGNR4, PGNL1	Cucumber (*P. ultimum*), tomato (*F. oxysporum*)	Tobacco	Ghana	−	−	30
PF	Wheat (*S. tritici*)	Wheat	Texas	−	−	34
Pf-5	Cotton (*P. ultimum, R. solani*), cucumber (*P. ultimum*)	Cotton	Texas	−	−	25
2-79	Wheat (*G. graminis*), Kentucky bluegrass (*M. poae*)	Wheat	Washington state	+	+	65
Q1-87, Q4-87, Q7-87, Q9-87, Q12-87, Q13-87, Q37-87, Q86-87, Q95-87, Q107-87, Q139-87	Wheat (*G. graminis*)	Wheat	Washington state	+		30
Q2-87	Wheat (*G. graminis*)	Wheat	Washington state	+	+	61
Q65c-80, Q128-87	Wheat (*G. graminis*)	Wheat	Washington state	+		21
Q69c-80	Wheat (*G. graminis*)	Wheat	Washington state	−	−	21
Pathogenic *Pseudomonas* spp. strains						
P. caricapapayae LMG 2152	Papaya (not documented)	Papaya	Brazil	+		53
P. syringae ATCC 19310	Unknown	Common lilac	United Kingdom	+		57
P. syringae pv. tomato DC3000	Tomato and *Arabidopsis* (bacterial speck)	Tomato	Unknown	+		10
P. syringae pv. *phaseolicola*	Bean (halo blight)	Bean	Unknown	+		9
Other *Pseudomonas* spp. strains						
VS01	Isolated from disease lesion of apple fruit	Apple	Switzerland	+	+	This study
VS02	Isolated from disease lesion of apple fruit	Apple	Switzerland	+		This study
P3	Saprophytic, no biocontrol ability documented	Barley	Switzerland	+		63
BE07, BE08	Isolated from disease lesion of sugar beet leaf	Sugar beet	Switzerland	+		This study
Pathogens other than pseudomonads						
E. herbicola pv. gypsophilae	*Gypsophila* (crown gall)	*Gypsophila*	Unknown	+		8
E. amylovora CNPB136	Unknown	Unknown	Unknown	+		6
E. amylovora 22716	Apple (fire blight)	Apple	Switzerland	+		This study
E. amylovora 22770	Pear (fire blight)	Pear	Switzerland	+	+	This study
E. amylovora 23482	Pyracantha (fire blight)	Firethorn	Switzerland	+		This study
X. campestris ATCC 33913	Rutabaga (not documented)	Rutabaga	United States	+		44

[a] The corresponding pathogens are *Erwinia carotovora* subsp. *carotovora* (*E. carotovora*), *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F. oxysporum*), *Gaeumannomyces graminis* var. *tritici* (*G. graminis*), *Magnaporthe poae* (*M. poae*), *Phomopsis sclerotioides* (*P. sclerotioides*), *Pythium ultimum* (*P. ultimum*), *Rhizoctonia solani* (*R. solani*), *Septoria tritici* (*S. tritici*), and *Thielaviopsis basicola* (*T. basicola*).

[b] All biocontrol pseudomonads were isolated from macerated roots or roots that were previously washed to remove the soil.

TABLE 2. Primers used in this work

Primer	Sequence (5′-3′)	Use	Reference
hrcV and neighboring genes			
HRCV-L	CCGGAATTCTGCG	Construction of *hrcV* probe	58
HRCV-R	ATTGTCATGTCGAT	Construction of *hrcV* probe	58
T3f2	GGTTTAGCAGGTCGATAATC	Synthesis of KD-j	This study
prottss-1r	GTCGAGCTGACGAAGGAGAG	Synthesis of KD-j	This study
T3f5	CTCGATCACTTATCCGGCTC	Synthesis of KD-l	This study
T3r8	GGCCTTCATGATGACTTCCA	Synthesis of KD-l	This study
pKD2cV-1r	GCAATCGCCTAGTGGTGAAC	Synthesis of KD-vq and KD-vn	This study
hrpQ-1rR	CCGKTCAGTACGCGTAATTCA	Synthesis of KD-vq	This study
hrcN-1r	CTGGGCWGCGTGCTGGAYGG	Synthesis of KD-n	This study
hrcN-3rR	GGCAGCAGGGTGTAMACCGA	Synthesis of KD-vn	This study
hrcN-4r	CGAGCAGGAYTCGATGAACG	Partial *hrcN* sequencing	This study
hrcN-5rR	CCGGWYTGGTATTCACCCAG	Synthesis of KD-n, partial *hrcN* sequencing	This study
16S rDNA gene			
PH-16S	AAGGAGGTGATCCAGCCGCA	Sequencing of 16S rDNA gene	12
PA-16S	AGAGTTTGATCCTGGCTCAG	Sequencing of 16S rDNA gene	12
Intern 2a	GATGATCAGCCACAC	Sequencing of 16S rDNA gene	This study

at 4°C by using T4 DNA ligase (Stratagene) into the multiple cloning site of plasmid pUK19 (previously digested with PstI and dephosphorylated). The ligation reaction mixture was used to transform MAX Efficiency DH5α competent cells (Invitrogen) according to the manufacturer's instructions. White-blue screening was performed on LB agar containing kanamycin (50 μg ml^{-1}), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (40 μg ml^{-1}), and isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) to identify colonies transformed with a plasmid containing an insert.

White colonies were subcultured overnight in LB agar containing kanamycin (50 μg ml^{-1}) on 96-well microtiter plates. Ten microliters of each culture was lysed with 190 μl of 0.4 M NaOH–0.01 mM EDTA by heating at 95°C in a thermal cycler for 10 min. The samples were dot blotted onto a Hybond-N^{+} nylon membrane and hybridized with the *hrcV* probe, as described above. For positive colonies the plasmid was extracted by using the Wizard Plus SV Minipreps DNA purification system (Promega, Madison, Wis.). The insert was analyzed by restriction with PstI and by sequencing, which was performed by Microsynth GmbH (Balgach, Switzerland).

Partial sequencing of TTSS genes in the biocontrol organism *Pseudomonas* sp. strain KD. Partial sequencing of the *hrcJ* operon of the biocontrol organism *Pseudomonas* sp. strain KD was carried out as follows. The nucleotide sequences of the *hrpJ* operons of *P. syringae* pv. tomato DC3000 (accession number AF232004) and *E. amylovora* CNPB136 (accession number L25828) were aligned by using LAlign software (26), and primers with low degeneracy were designed manually based on conserved nucleotide sequences. Primer pKD2cV-1r was designed based on sequencing results for the PstI fragment cloned into pUK18. PCRs were performed by using combinations of the different forward and reverse primers and a TGradient thermal gradient cycler (Biometra, Göttingen, Germany). Twelve different PCRs at different annealing temperatures were done to test each set of primers.

PCRs were performed directly with cell lysates of strain KD, which were prepared as described by Rezzonico et al. (52). Briefly, 5 μl of an overnight LB medium culture was mixed with 95 μl of lysis buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 0.1% Tween 20) and heated for 10 min at 99°C in a PTC-100 thermal cycler (MJ Research). PCR amplification was carried out in 20-μl reaction mixtures by using 5 μl of cell lysate and 1× PCR buffer (described above).

After an initial denaturation step consisting of 10 min at 95°C, there were 35 cycles consisting of 1 min at 95°C, 1 min of annealing (12 different temperatures ranging from 52 to 66°C were tested), and 3 min at 72°C, followed by final elongation for 10 min at 72°C. DNA fragments of the expected size were excised from 1.5% agarose electrophoresis gels, purified with a QIAquick gel extraction kit (QIAGEN), and sent to Microsynth GmbH for sequencing with the primers used for amplification of the corresponding PCR fragments. The sequences were analyzed by BLASTN and BLASTP (4).

Nucleotide sequence accession numbers. Nucleotide sequence data reported here are available at the National Center for Biotechnology Information database under accession numbers AY456697 to AY456712, AY622219, and AY622220 for *rrs*, AY456994 to AY457036 for *hrcN*, and AY463491 for *hrpL*, *hrpJ*, *hrcV*, *hrpQ*, and part of *hrcN* in strain KD.

RESULTS

Distribution of *hrcN* sequences in biocontrol *Pseudomonas* spp. Degenerate primers hrcN-4r and hrcN-5rR (Table 2) were designed from a comparison of the *hrcN* sequences of *E. amylovora* CNPB136 and *P. syringae* pv. tomato DC3000. They amplified a fragment that was about 250 bp long, as predicted, both in the biocontrol agent *Pseudomonas* sp. strain KD and the plant pathogens *E. amylovora* and *P. syringae* (Fig. 1A), which are known to have a TTSS. The following PCR conditions were the best compromise between reaction specificity and target detection for biocontrol pseudomonads: after an initial denaturation step of 10 min at 95°C, 30 cycles consisting of 30 s at 95°C, 30 s of annealing at 60°C, and 1 min at 72°C, followed by a final elongation for 10 min at 72°C.

In addition to strain KD, PCR amplification was also successful for 33 of 57 biocontrol fluorescent pseudomonads (Fig. 2 and Table 1). The identities of the amplified fragments were confirmed by sequencing followed by database comparison by using BLASTN. *hrcN* amplicons were also obtained with nonbiocontrol *Pseudomonas* strains P3, BE07, BE08, VS01, and VS02. All fragments of biocontrol pseudomonads analyzed showed the highest levels of similarity with previously sequenced *hrcN* genes, like the gene found in *P. syringae* pv. tomato DC3000 (the levels of nucleotide identity were between 72.5% for K94.08 and 85.7% for KD), and only moderate levels of similarity (less than 60% identity) with other ATPase genes, such as the flagellum-associated ATPase gene *fliI* of *P. putida* KT2440. BLASTP analysis of the deduced 81-amino-acid HrcN sequence in biocontrol pseudomonads revealed the presence (in the first 46 residues) of a conserved ATP synthase α/β family nucleotide-binding domain (CDD accession number pfam00006), which included one α helix and two β sheets (Fig. 1B). This family includes the α and β subunits of the flagellum-associated ATP synthase.

The use of PCR enhancers, such as bovine serum albumin (25 μg ml^{-1}) and dimethyl sulfoxide (5.5 mg ml^{-1}), had no effect on PCR results in most cases. The exception was strain P97.26, which yielded an amplicon of the expected size only when both bovine serum albumin and dimethyl sulfoxide were

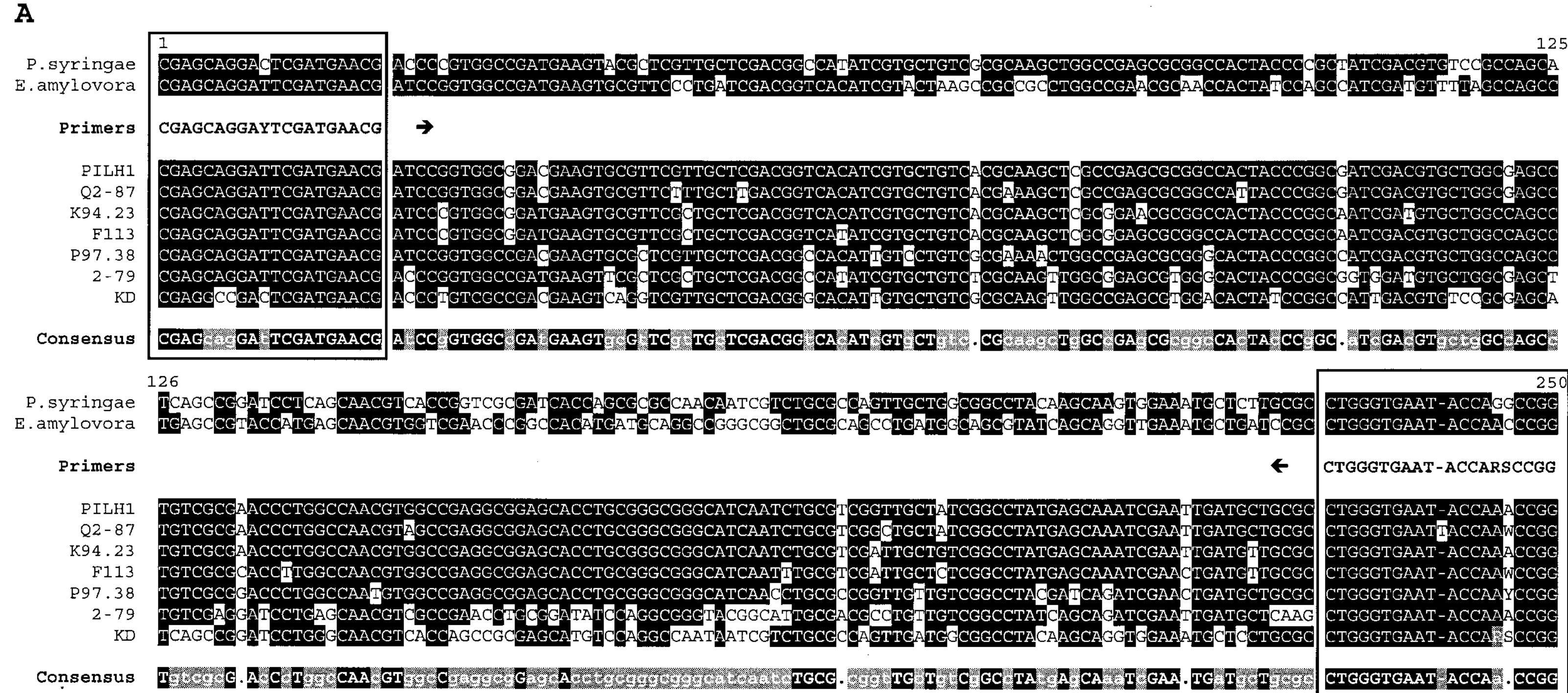
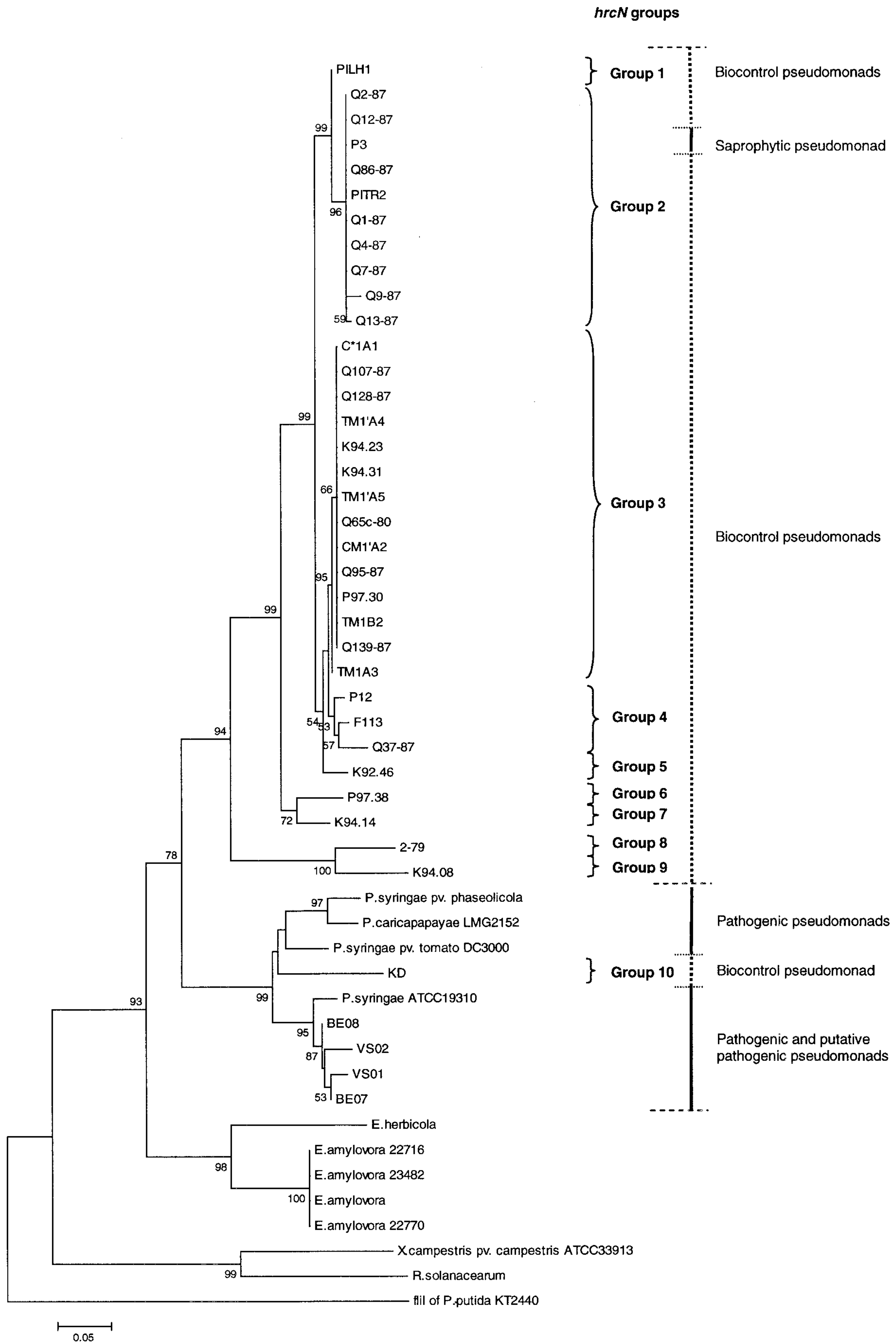


FIG. 1. Alignment of partial *hrcN* nucleotide (A) and deduced HrcN amino acid (B) sequences of *P. syringae* pv. tomato DC3000, *E. amylovora* CNPB136, and selected biocontrol pseudomonads, including PILH1 (*hrcN* group 1), Q2-87 (*hrcN* group 2), K94.23 (*hrcN* group 3), F113 (*hrcN* group 4), P97.38 (*hrcN* group 6), 2-79 (*hrcN* group 8), and KD (*hrcN* group 10). The sites annealing to the PCR primers hrcN-4r (reverse) and hrcN-5rR (forward) are enclosed in boxes. The *hrcN* consensus sequence is indicated by uppercase letters (>90% identity), lowercase letters (between 50 and 90% identity), or a dot (<50% identity). The HrcN consensus sequence is indicated by a black background (100% identity) or a grey background (<100% identity but 100% similarity). Similarity was calculated by using a BLOSUM62 matrix (23) implemented in LALIGN (26). For each strain, amino acids are indicated by a black background if they are identical to the amino acids in the consensus sequence or by a grey background in case of homologous amino acids. The deduced FliI sequence of *P. putida* KT2440 is shown for reference.



hrcN groups
PILH1
Q2-87
Q12-87
P3
Q86-87
PITR2
Q1-87
Q4-87
Q7-87
Q9-87
Q13-87
C*1A1
Q107-87
Q128-87
TM1'A4
K94.23
K94.31
TM1'A5
Q65c-80
CM1'A2
Q95-87
P97.30
TM1B2
Q139-87
TM1A3
P12
F113
Q37-87
K92.46
P97.38
K94.14
2-79
K94.08
P.syringae pv. phaseolicola
P.caricapapayae LMG2152
P.syringae pv. tomato DC3000
KD
P.syringae ATCC19310
BE08
VS02
VS01
BE07
E.herbicola
E.amylovora 22716
E.amylovora 23482
E.amylovora
E.amylovora 22770
X.campestris pv. campestris ATCC33913
R.solanacearum
fliI of P.putida KT2440
0.05
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Biocontrol pseudomonads
Saprophytic pseudomonad
Biocontrol pseudomonads
Pathogenic pseudomonads
Biocontrol pseudomonad
Pathogenic and putative pathogenic pseudomonads

added, but sequencing of this fragment failed. Several other primers were designed based on whole *hrcN* sequences, but when PCR products were obtained, it turned out that amplification was either nonspecific or specific only for some strains. Of the 24 strains that were *hrcN* negative by PCR, only 2 (DR54 and Pf1) hybridized to an *hrcN* probe. Both of these strains reacted to also an *hrcV* hybridization probe, but they did not yield any TTSS-related PCR amplicon.

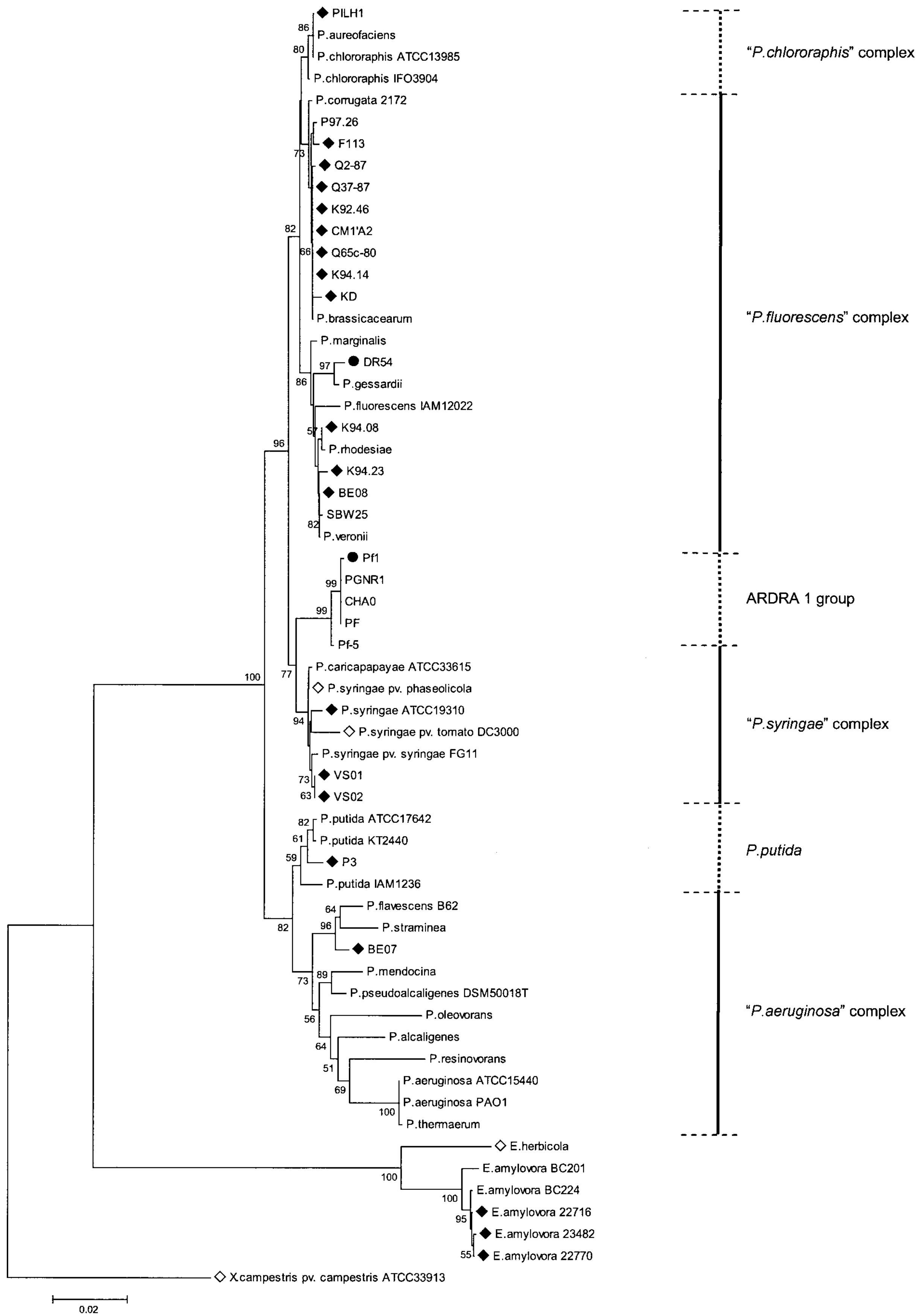
There was no particular relationship between the occurrence of *hrcN* and the geographic origin of biocontrol pseudomonads, as the gene was found in strains from different continents. Similarly, the occurrence of *hrcN* was not linked with the type of plant host, as the gene was identified in biocontrol pseudomonads isolated from wheat, tobacco, cucumber, tomato, and sugar beet. *hrcN* was found in almost all groups of biocontrol pseudomonads described previously (30, 50, 64) based on the phylogeny of *hcnBC*, *phlD*, or *rrs* or the results of amplified 16S rDNA restriction analysis (ARDRA) or random amplified polymorphic DNA (RAPD) analysis. No amplification was obtained with strains belonging to the group containing strain CHA0, which can be distinguished from other biocontrol pseudomonads based on the analysis of RAPD markers (RAPD group 1) (30) or particular genes, including *rrs* (ARDRA group 1) (30), *phlD* (group PhlD1) (64), and *hcnBC* (group Hcn-4) (50), but as indicated above, *hrcN* hybridization was successful for one RAPD-1 ARDRA-1 strain (strain Pf1).

Phylogenetic analysis of *hrcN* in biocontrol *Pseudomonas* spp. The *hrcN* alleles identified in biocontrol pseudomonads were similar to those in pathogenic pseudomonads (as determined by BLASTN analysis), yet most of them clustered separately from *hrcN* sequences of *E. amylovora* and pathogenic *Pseudomonas* strains in NJ phylogenetic comparisons (Fig. 2). Nevertheless, four strains (BE07, BE08, P3, and the biocontrol pseudomonad strain KD) had incongruent phylogenetic positions when *hrcN* and *rrs* trees were compared. Two strains belonging to the *Pseudomonas fluorescens* complex based on *rrs* properties (Fig. 3), i.e., strain KD (closely related to *Pseudomonas corrugata* and *Pseudomonas brassicacearum*) and strain BE08, as well as strain BE07 (belonging to the *Pseudomonas aeruginosa* complex), clustered with *P. syringae* when *hrcN* was considered (Fig. 2). *P. putida* P3 clustered with bacteria belonging to the *P. fluorescens* complex in the *hrcN* tree. The same findings were obtained when the MP or ML methods were used to construct the *hrcN* tree and when trees derived from deduced protein sequences were analyzed (data not shown). However, the internal position of KD within the *P. syringae* cluster in the *hrcN* and HrcN trees varied depending on the inference method (Fig. 4).

hrcN groups were arbitrarily defined for biocontrol pseudomonads based on the topology of the NJ *hrcN* tree (Fig. 2). The *hrcN* pairwise distances were less than 0.018 base substitution per site within each group, with the exception of strains in group 4, which had pairwise distances ranging from 0.022 to 0.040 substitution per site. The same groups could be identified by using deduced protein sequences, and the numbers of amino acid substitutions per site were less than 0.049 within the groups. Two main *hrcN* groups (groups 2 and 3) were identified. Group 2 contained mostly biocontrol pseudomonads isolated from wheat in Washington state, along with a wheat isolate from Italy (strain PITR2) and another monocot strain (barley isolate P3, which has no biocontrol activity). Group 3 included biocontrol strains from wheat, cucumber, and tomato, which originated from Washington state, Switzerland, and the Czech Republic. Several groups were comprised of a single strain. These results were strongly supported by MP or ML analysis of *hrcN* and analysis of deduced HrcN sequences (by the NJ or MP method).

Certain strains used in this work can produce the biocontrol metabolite HCN and/or the biocontrol metabolite Phl and have been compared previously based on *hcnBC* or *phlD* properties (50, 51, 64). Here, a relationship was found between *hrcN* groups and previous *Pseudomonas* biocontrol groups derived from analysis of *hcnBC*, *phlD*, or RAPD markers (Table 3). For instance, *hrcN* group 2 contained strains belonging to groups PhlD3/Hcn-3 and PhlD7/Hcn-2, whereas *hrcN* group 3 included strains belonging to groups PhlD4 (or PhlD2) and Hcn-1. This relationship was not perfect; for example, certain strains belonging to *hrcN* groups 1, 2, and 4 belonged to a single *hcnBC* group (group Hcn-2). When *hrcN* groups and *rrs* properties were compared, it appeared that *hrcN* group 1 strains were found only in the *rrs*-defined *Pseudomonas chlororaphis* complex (Fig. 2 and 3). Within the *P. fluorescens* complex, *hrcN* groups 2, 4, 5, 7 and 10 were associated with the *P. corrugata*-*P. brassicacearum* cluster, and *hrcN* group 9 was

FIG. 2. Phylogenetic relationships based on partial *hrcN* sequences of biocontrol fluorescent pseudomonads and phytopathogenic bacteria belonging to the genera *Pseudomonas*, *Erwinia*, *Xanthomonas*, and *Ralstonia*. The distance tree was generated by the NJ method with the JC formula by using the flagellar ATPase gene *fliI* of *P. putida* KT2440 (accession number AE016790) as the outgroup. Nodal support was assessed by using 1,000 bootstrap replicates. Only bootstrap values greater than 50% are shown. Scale bar = 0.05 substitution per site. *hrcN* groups arbitrarily defined for biocontrol pseudomonads based on the topology of the *hrcN* tree are indicated on the right. The phytopathogenic bacteria included *P. syringae* pv. phaseolicola (accession number AJ430232), *P. syringae* pv. tomato DC3000 (AE016860), *Erwinia herbicola* (X99768), *E. amylovora* (L25828), *X. campestris* pv. campestris ATCC 33913 (AE012222), and *Ralstonia solanacearum* (AJ245811). Sequences were obtained in this study for *Pseudomonas* strains KD (AY456994), VS01 (AY456998), VS02 (AY456999), BE07 (AY457000), BE08 (AY457001), K94.23 (AY457002), C*1A1 (AY457003), TM1′A4 (AY457004), PITR2 (AY457005), K94.31 (AY457006), Q65c-80 (AY457007), CM1′A2 (AY457008), TM1′A5 (AY457009), Q1-87 (AY457010), TM1B2 (AY457011), Q7-87 (AY457012), Q86-87 (AY457013), Q9-87 (AY457014), Q12-87 (AY457015), Q128-87 (AY457016), P97.30 (AY457017), Q139-87 (AY457018), TM1A3 (AY457019), P97.38 (AY457020), F113 (AY457021), Q95-87 (AY457022), Q37-87 (AY457023), and 2-79 (AY457024), *Pseudomonas caricapapayae* LGM2152 (AY457025), *Pseudomonas* strains Q4-87 (AY457026), Q2-87 (AY457027), P12 (AY457028), P3 (AY457029), K92.46 (AY457030), K94.14 (AY457031), Q107-87 (AY457032), Q13-87 (AY457033), K94.08 (AY457034), and PILH1 (AY457035), *P. syringae* ATCC 19310 (AY457036), and *E. amylovora* 22716 (AY456995), 22770 (AY456996), and 23482 (AY456997). The G+C contents of the *hrcN* fragment studied were 58.3% ± 2.5% for the *Erwinia* cluster ($n = 5$), 60.4% ± 2.0% for the cluster comprised of established and putative pathogenic pseudomonads and strain KD ($n = 9$) (the G+C content of KD was 60.6%), and 61.7% ± 1.0% for the cluster containing all biocontrol pseudomonads except strain KD and the saprophytic pseudomonad P3 ($n = 33$). The values for the latter two clusters were significantly different ($P < 0.05$, as determined by the Mann-Whitney test).


PILH1
86
P.aureofaciens
80
P.chlororaphis ATCC13985
P.chlororaphis IFO3904
P.corrugata 2172
P97.26
F113
73
Q2-87
Q37-87
K92.46
82
CM1'A2
66
Q65c-80
K94.14
KD
P.brassicacearum
P.marginalis
97
DR54
86
P.gessardii
P.fluorescens IAM12022
57
K94.08
96
P.rhodesiae
K94.23
BE08
SBW25
82
P.veronii
Pf1
99
PGNR1
CHA0
99
PF
Pf-5
P.caricapapayae ATCC33615
100
77
P.syringae pv. phaseolicola
P.syringae ATCC19310
94
P.syringae pv. tomato DC3000
P.syringae pv. syringae FG11
73
VS01
63
VS02
82
P.putida ATCC17642
61
P.putida KT2440
59
P3
P.putida IAM1236
64
P.flavescens B62
96
P.straminea
82
BE07
89
P.mendocina
73
P.pseudoalcaligenes DSM50018T
P.oleovorans
56
P.alcaligenes
64
P.resinovorans
51
P.aeruginosa ATCC15440
69
P.aeruginosa PAO1
100
P.thermaerum
E.herbicola
E.amylovora BC201
100
E.amylovora BC224
100
E.amylovora 22716
95
E.amylovora 23482
55
E.amylovora 22770
X.campestris pv. campestris ATCC33913
0.02
"P.chlororaphis" complex
"P.fluorescens" complex
ARDRA 1 group
"P.syringae" complex
P.putida
"P.aeruginosa" complex

associated with the *Pseudomonas marginalis-P. fluorescens* cluster, but *hrcN* group 3 was found in both clusters.

Organization of TTSS genes in the biocontrol organism *Pseudomonas* sp. strain KD. *Pseudomonas* sp. strain KD was the only biocontrol strain for which incongruent data were observed when the *hrcN* and *rrs* trees were compared. If the horizontal gene transfer hypothesis is valid for this strain, it can be anticipated that in addition to having a similar *hrcN* allele, strain KD would display the same organization of TTSS genes as *P. syringae*. Therefore, the organization of TTSS genes was investigated in *Pseudomonas* sp. strain KD. This was done by using an *hrcV* probe that was derived from *E. amylovora* CNPB136 (as described by Stuber et al. [58]) and, as expected, hybridized with *E. amylovora* CNPB136 and *Erwinia chrysanthemi* (data not shown). A positive response was observed with strain KD (and with the *hrcN*$^+$ biocontrol pseudomonads K92.46 and K94.14).

In strain KD, the *hrcV* probe hybridized with a PstI fragment that was approximately 1.7 kb long, which was subsequently cloned in plasmid pUK21 and sequenced. The insert was verified by digestion with PstI and dot blot hybridization to the *hrcV* probe, and its size was determined to be 1,718 bp based on sequencing results. The insert corresponded to a region that included the last 219 bp of *hrpJ* and the first 1,503 bp of *hrcV*, with the two genes overlapping by 4 bp (Fig. 5). The plasmid was designated pCBTypeIII.

Several degenerate PCR primer sets were designed (Table 2) based on sequence alignment of TTSS genes from *P. syringae* pv. tomato DC3000 and *E. amylovora* CNPB136, and they were tested in strain KD at 12 different annealing temperatures between 52 and 66°C. Fragment KD-vq (990 bp) (Fig. 5) was obtained with primers pKD2cV-1r and hrpQ-1rR by using an annealing temperature of 60°C. Fragment KD-n (902 bp) was obtained with primers hrcN-1r and hrcN-5rR at an annealing temperature of 62°C, and fragment KD-vn (2,816 bp) was obtained with primers pKD2cV-1r and hrcN-3rR at an annealing temperature of 58°C. Assembled together, the three PCR amplicons and the 1,718-bp PstI fragment from pCBTypeIII yielded a 4,499-bp sequence spanning from *hrpJ* to *hrcN*, which was homologous to the corresponding region in the *hrpJ* operon of *P. syringae* pv. tomato DC3000 (Fig. 5). This *hrpJ* operon is a 5,651-bp regulation unit, which includes *hrpJ*, *hrcV*, *hrpQ*, and *hrcN*.

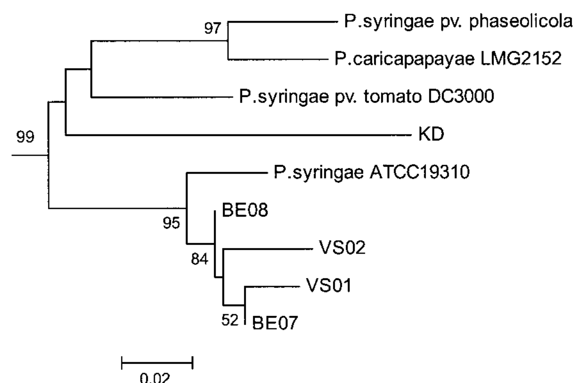
Other primers (Table 2) were used to sequence upstream of the PstI fragment and recover the remaining 897 bp of *hrpJ*. Fragment KD-j (1,446 bp) was obtained with primers prottss-1r and T3f2 at an annealing temperature of 62°C, and fragment KD-l (1,242 bp) was obtained with primers T3r8 and T3f5 at an annealing temperature of 63°C (Fig. 5E). The presence of an open reading frame homologous (61.5% nucleotide identity) to the *hrpL* gene of DC3000 was demonstrated. *hrpL* encodes a TTSS-specific sigma factor necessary for recognition of *hrp* boxes and transcription of the corresponding TTSS genes.

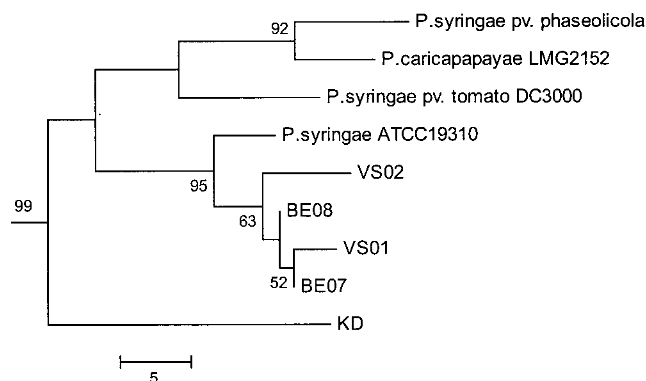
Overall, it appears that the TTSS region studied in *Pseudomonas* sp. strain KD is organized in a similar manner in *P. syringae* pv. tomato DC3000 and in *Pseudomonas* sp. strains SBW25 and KD, except that in SBW25 part of the *hrpJ* operon is absent (Fig. 5A). For both DNA and deduced protein sequences, the levels of identity between strains KD and DC3000 varied according to the TTSS gene considered (Fig. 5D). With strain SBW25, comparisons could only be made for *hrpL*, and the levels of identity between KD and DC3000 (61.5% for nucleotides, 46.0% for amino acids) were higher than the levels of identity between KD and SBW25 (56.2% for nucleotides, 40.5% for amino acids). In contrast, the genes or sequences downstream of *hrpL* were different in the three strains. This position is often occupied by effector genes, which are usually poorly conserved (2, 11).

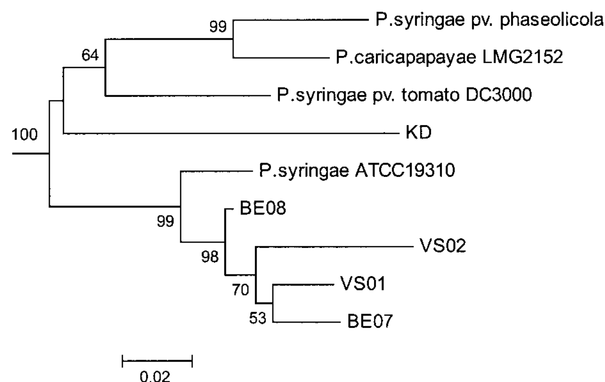
DISCUSSION

In this work, *hrcN* was found in about 60% of the biocontrol pseudomonads studied. This gene is not present in *Pseudomonas* sp. strain SBW25 (which was confirmed here), a strain that nevertheless contains other TTSS genes (47). One prominent group of biocontrol pseudomonads includes strains that produce HCN, Phl, and Plt (pyoluteorin) (previously designated ARDRA group 1 [30]), and 9 of the 10 strains from this group studied were *hrcN* negative. PCR with these 10 strains also failed when alternative PCR primers were used or when other TTSS genes were targeted. This indicates that for the most part a TTSS is absent from this phylogenetic group.

FIG. 3. Phylogenetic relationships based on 16S rDNA sequences for biocontrol fluorescent pseudomonads and reference bacteria belonging to the genera *Pseudomonas* and *Erwinia*. The distance tree was generated by the NJ method with the JC formula by using *X. campestris* pv. campestris ATCC 33913 (accession number AE012540) as the outgroup. Nodal support was assessed by using 1,000 bootstrap replicates. Only bootstrap values greater than 50% are shown. Scale bar = 0.02 substitution per site. The *P. chlororaphis*, *P. fluorescens*, *P. syringae*, and *P. aeruginosa* complexes were those defined in Anzai et al. (5). Strains for which the *hrcN* sequence is available are indicated by a solid diamond for new sequences or an open diamond for *hrcN* sequences published previously (National Center for Biotechnology Information). The two strains positive by *hrcN* hybridization but *hrcN* negative as determined by PCR are indicated by solid circles. Sequences were obtained in this work for *Pseudomonas* strains KD (accession number AY456697), VS01 (AY456698), VS02 (AY456699), BE07 (AY456700), BE08 (AY456701), K92.46 (AY456702), K94.08 (AY456703), K94.14 (AY456704), K94.23 (AY456705), P3 (AY456706), P97.26 (AY456707), SBW25 (AY456712), and PILH1 (AY456708) and for *E. amylovora* 22716 (AY456709), 22770 (AY456710), and 23482 (AY456711). The other sequences used were the sequences for pseudomonad strains CHA0 (AJ278812), Q2-87 (AJ278813), F113 (AJ278814), CM1′A2 (AJ417068), Q37-87 (AJ417069), PGNR1 (AJ417071), Pf-5 (AJ417072), PF (AJ417073), Q65c-80 (AJ417074), DR54 (AY622219), and Pf1 (AY622220), *P. chlororaphis* ATCC 13985 (AF094722) and IFO3904 (D86004), *P. aureofaciens* (Z76656), *P. corrugata* 2172 (= ATCC 29736) (D84012), *P. brassicacearum* (AF100322), *P. marginalis* (AB021401), *P. fluorescens* IAM12022 (D84013), *P. gessardii* (AF074384), *P. rhodesiae* (AF064459), *P. veronii* (AB056120), *P. caricapapayae* ATCC 33615 (D84010), *P. syringae* pv. phaseolicola (AB001448) and ATCC 19310 (D84026), *P. syringae* pv. tomato DC3000 (AE016875), *P. syringae* pv. syringae FG11 (AY242068), *P. putida* KT2440 (AE016775), ATCC 17642 (AF094744), and IAM1236 (D84020), *P. flavescens* B62 (V01916), *P. straminea* (D084023), *P. mendocina* (D84016), *P. pseudoalcaligenes* (Z76675), *P. oleovorans* (D84018), *P. alcaligenes* (Z76653), *P. resinovorans* (Z76668), *P. aeruginosa* PAO1 (AE004844) and ATCC 15442 (AF094718), *P. thermaerum* (AB088116), *Erwinia herbicola* (U80202), and *E. amylovora* BC201 (AF141892) and BC224 (AF140339).







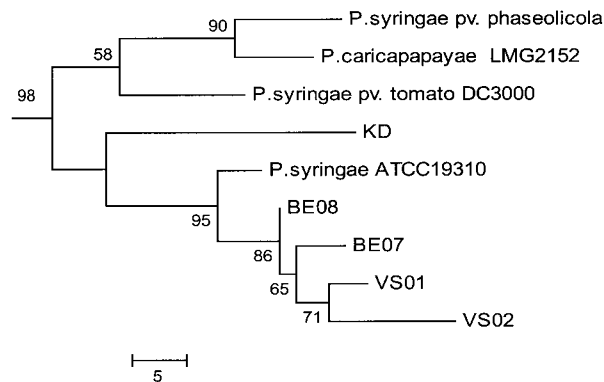


FIG. 4. Relationship between the biocontrol organism *Pseudomonas* sp. strain KD and pathogenic (or putatively pathogenic) pseudomonads in the *P. syringae* cluster obtained by phylogenetic analysis of TTSS sequences. All trees were constructed by using the complete collection of strains shown in Fig. 2, but only the *P. syringae* cluster is shown. Trees were obtained for partial *hrcN* sequences by using the NJ (A) or MP (B) method and the flagellar ATPase gene *fliI* of *P. putida* KT2440 as the outgroup, as well as for deduced HrcN sequences by using the NJ (C) or MP (D) method and FliI of *P. putida* KT2440 as the outgroup. The JC formula was used for nucleotide sequences, and the Poisson correction was used for deduced amino acid sequences. Nodal support was assessed by using 1,000 bootstrap replicates, and only bootstrap values greater than 50% are shown. The scale bars indicate the number of substitutions per site (NJ trees) or the number of changes (MP trees).

For a majority of $hrcN^+$ biocontrol strains, the *hrcN* and *rrs* trees were largely congruent, which means that *hrcN* evolved in parallel with *rrs* and diverged sometime in the past in the corresponding taxa, rather than resulting from recent gene transfer. In this context, the *hrcN* alleles of most biocontrol pseudomonads differed clearly from those found in their phytopathogenic counterparts, in which, at least for *P. syringae* pathovars, the *hrp* gene cluster is considered to be ancestral (55). Two main groups of nonpathogenic pseudomonads were defined based on the *hrcN* tree (Fig. 2). Considering the geographic origins of the strains in each of these two groups, it appears that both groups have a cosmopolitan distribution worldwide. One of them was comprised only of monocot isolates, which might have ecological implications in terms of adaptation to the plant, whereas the other contained pseudomonads from monocots and dicots.

The ability to produce biocontrol metabolites (e.g., HCN and Phl) is widespread in biocontrol pseudomonads, and many strains included in this study have also been analyzed based on *hcnBC* and *phlD* sequences (50, 51). In the present work, there was some relationship between the *hrcN* phylogeny and the phylogenies based on *phlD* or *hcnBC*. The existence of this relationship can be explained by the fact that the *phlD*, *hcnBC*, and *rrs* phylogenies were highly congruent, which is also illustrated by the relationship among the *hrcN*, *phlD*, *hcnBC*, and RAPD groups (Table 3).

In *Pseudomonas* spp., the 16S rDNA-based phylogeny can be considered the species phylogeny (5, 40). Based on this assumption, incongruent results were obtained for four strains (BE07, BE08, P3, and KD) when the *hrcV* and *rrs* trees were compared, which raises the possibility of lateral gene transfer. This did not come as a surprise, as it has been established that TTSS genes are often present in pathogenicity islands, which are prone to lateral gene transfer (38). Strains BE07 and BE08 clustered with *P. syringae* based on *hrcN* phylogeny. Strain BE07 is probably a pathogen since it was isolated from a sugar

TABLE 3. Relationship among *hrcN, phlD, hcnBC*, and RAPD groups for HCN$^+$ Phl$^+$ biocontrol pseudomonads[a]

hrcN group	Strain(s)	*phlD* group	*hcnBC* group	RAPD group
1	PILH1	PhlD7	Hcn-2	5
2	P1TR2	PhlD7	Hcn-2	5
	Q1-87, Q4-87, Q7-87, Q9-87, Q12-87, Q13-87, Q86-87[b]	ND[d]	Hcn-3	4
	Q2-87	PhlD3	Hcn-3	4
3	K94.31	PhlD2	Hcn-1	ND
	Q65c-80, Q95-87, Q107-87, Q128-87, Q139-87[c]	ND	Hcn-1	3
	TM1A3, TM1′A4, TM1′A5, TM1B2, C*1A1, CM1′A2	PhlD4	Hcn-1	3
4	P12	ND	Hcn-2	8
	Q37-87	ND	Hcn-2	6
	F113	PhlD6	Hcn-1	7
6	P97.38	PhlD8	Hcn-3	ND

[a] *phlD, hcnBC*, and RAPD groups were determined by Wang et al. (64), Ramette et al. (51), and Keel et al. (30), respectively.
[b] These strains produced the same *phlD* restriction pattern (HaeIII) as group PhlD3 strain Q2-87, but *phlD* was not sequenced (unlike Q2-87).
[c] These strains produced the same *phlD* restriction pattern (HaeIII) as group PhlD4 strains TM1A3, TM1B2, and C*1A1, but *phlD* was not sequenced (unlike TM1A3, TM1B2, and C*1A1).
[d] ND, not determined.

beet disease lesion and displays 98.4% *rrs* identity with the walnut blight canker isolate *Pseudomonas flavescens* B62 (24) (accession number U01916), which is distinct from *P. syringae*. Therefore, this finding points to lateral transfer of TTSS genes between different pathogenic *Pseudomonas* species. The status of strain BE08 is less clear because the closest relatives of this sugar beet disease lesion isolate are bioremediation strains belonging to *Pseudomonas veronii* (1) (99.5% *rrs* identity; accession number AB056120) and biocontrol pseudomonads (including the hypersensitive response-inducing strain SBW25), both of which belong to the *P. fluorescens* complex. *P. putida* P3, which also contains *hrcV* (data not shown), clustered with biocontrol strains belonging to the *P. fluorescens* group based on *hrcN*, but this bacterium has no biocontrol capacity.

The case of *Pseudomonas* sp. strain KD is of particular interest since this strain was the only biocontrol strain that clustered among *P. syringae* pathogens in the *hrcN* tree. Strain KD belongs to the *P. fluorescens* complex. It produces HCN and a siderophore(s) but not Phl or Plt, and it efficiently suppresses *Pythium ultimum* on cucumber and *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato (56). Although it can be

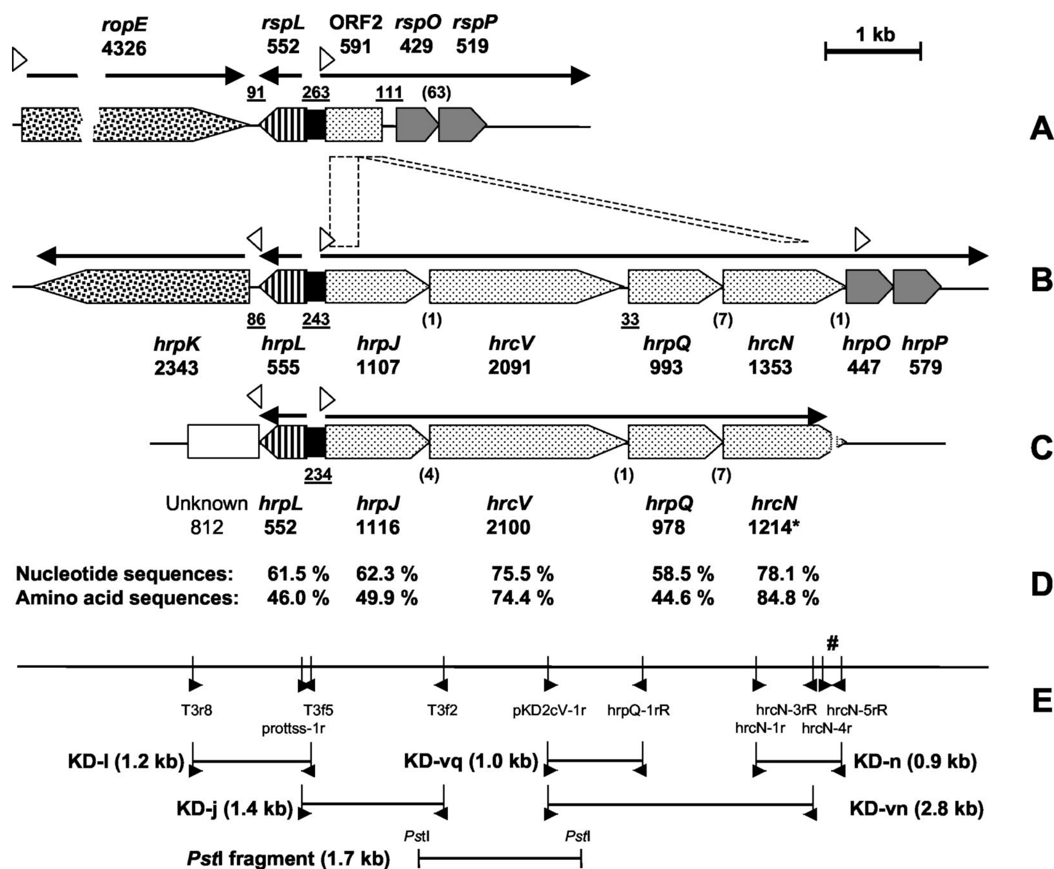


FIG. 5. (A to C) Comparison of the organization of TTSS genes in the biocontrol organism *Pseudomonas* sp. strain SBW25 (A), the phytopathogen *P. syringae* pv. tomato DC3000 (B), and the biocontrol organism *Pseudomonas* sp. strain KD (accession number AY463491) (C). (D) Levels of nucleotide and amino acid sequence identity between strains DC3000 and KD. (E) Positions of PCR primers used for sequencing. In panels A, B and C, the length of each gene (in base pairs) is indicated below its designation; one gene (*hrcN*) was sequenced only partially (indicated by an asterisk). The lengths of noncoding, intergenic gaps are underlined. The numbers of bases shared by overlapping genes are indicated in parentheses. The arrows above the genes represent the operons and the direction in which they are transcribed in DC3000. The open triangles indicate the positions and orientations of the *hrp* transcription boxes. In panel E, the positions and directions of primers used for sequencing are indicated by solid triangles. The number sign indicates the position of the hrcN-4r–hrcN-5rR fragment used for phylogenetic analysis.

hypothesized that this biocontrol bacterium acquired a TTSS gene(s) from *P. syringae*, no plasmid was found in strain KD when alkaline lysis was used (54), when the Wizard Plus SV Minipreps DNA purification system (Promega) was used, or when QIAGEN plasmid maxi and mega kits (QIAGEN) were used, indicating that *hrcN* is chromosomal. Despite the presence of a *P. syringae*-like TTSS gene(s), this strain did not elicit a hypersensitive response in tobacco or cucumber (data not shown) and is not known to cause any plant disease. When additional TTSS genes in strain KD were sought, it appeared that unlike *Pseudomonas* sp. strain SBW25, (i) other TTSS genes, such as *hrpQ*, were present and (ii) the organization of the *hrpJ* operon was the same as that in *P. syringae*. Thus, it is conceivable that KD acquired several TTSS genes, or even a whole pathogenicity island, from *P. syringae*. The position of KD within the *P. syringae* cluster differed somewhat in the MP *hrcN* tree compared with the two other *hrcN* trees and the HrcN trees, which might correspond to a long-branch attraction artifact (13).

This work was based on the assumption that gene transfer took place from phytopathogenic to biocontrol bacteria rather than the other way around, because individual pathogens were never found in biocontrol clusters in the TTSS trees, despite the fact that all available *hrcN* sequences from phytopathogens were considered. In contrast, one biocontrol pseudomonad (strain KD) clustered with its pathogenic counterparts. However, it must be kept in mind that very little has been done to date to analyze the organization and role of TTSS in plant-beneficial bacteria, and this area deserves further work. For instance, when the numbers of nonsynonymous substitutions per nonsynonymous site (dN) and the numbers of synonymous substitutions per synonymous site (dS) for *hrcN* were estimated by using the method of Nei and Kumar (41) and MEGA, it appeared that dS was significantly greater (as determined by Fisher exact tests) than dN, both for pathogenic pseudomonads (0.314 ± 0.070 versus 0.022 ± 0.008) and for biocontrol pseudomonads (0.218 ± 0.039 versus 0.031 ± 0.008 for *hrcN* groups 1 to 9 [i.e., without KD]; and 0.250 ± 0.043 versus 0.045 ± 0.009 for *hrcN* groups 1 to 10 [when KD was included]). For comparison, the dS and dN values for the five *Erwinia* strains studied were 0.246 ± 0.077 and 0.042 ± 0.014, respectively. These results indicate that *hrcN* is subjected to purifying selection in both types of pseudomonads, indicating that this gene has an important ecological role in biocontrol pseudomonads, as in pathogenic strains. Accordingly, the TTSS gene *rscC*/*hrcC* is expressed in *Pseudomonas* sp. strain SBW25 in the rhizosphere (48), and preliminary results obtained by using transcriptional fusions indicate that the TTSS genes *hrpJ* (from the same operon as *hrcN*) and *hrpL* are transcribed in *Pseudomonas* sp. strain KD under in vitro conditions (unpublished data). This suggests that TTSS genes are functional in biocontrol pseudomonads.

In conclusion, it appears that the presence of *hrcN* in biocontrol pseudomonads is ancient in most cases, but in the biocontrol strain *Pseudomonas* sp. strain KD a set of TTSS genes was probably acquired more recently from phytopathogenic *P. syringae*. Nothing is known about the possible role of TTSS in biocontrol, and it will be of interest to determine whether TTSS genes contribute to or interfere with the biocontrol activity of strain KD and other biocontrol pseudomonads.

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