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Pyrroloquinoline Quinone Biosynthesis Gene *pqqC*, a Novel Molecular Marker for Studying the Phylogeny and Diversity of Phosphate-Solubilizing Pseudomonads▽†

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Many root-colonizing pseudomonads are able to promote plant growth by increasing phosphate availability in soil through solubilization of poorly soluble rock phosphates. The major mechanism of phosphate solubilization by pseudomonads is the secretion of gluconic acid, which requires the enzyme glucose dehydrogenase and its cofactor pyrroloquinoline quinone (PQQ). The main aim of this study was to evaluate whether a PQQ biosynthetic gene is suitable to study the phylogeny of phosphate-solubilizing pseudomonads. To this end, two new primers, which specifically amplify the *pqqC* gene of the *Pseudomonas* genus, were designed. *pqqC* fragments were amplified and sequenced from a *Pseudomonas* strain collection and from a natural wheat rhizosphere population using cultivation-dependent and cultivation-independent approaches. Phylogenetic trees based on *pqqC* sequences were compared to trees obtained with the two concatenated housekeeping genes *rpoD* and *gyrB*. For both *pqqC* and *rpoD-gyrB*, similar main phylogenetic clusters were found. However, in the *pqqC* but not in the *rpoD-gyrB* tree, the group of fluorescent pseudomonads producing the antifungal compounds 2,4-diacetylphloroglucinol and pyoluteorin was located outside the *Pseudomonas fluorescens* group. *pqqC* sequences from isolated pseudomonads were differently distributed among the identified phylogenetic groups than *pqqC* sequences derived from the cultivation-independent approach. Comparing *pqqC* phylogeny and phosphate solubilization activity, we identified one phylogenetic group with high solubilization activity. In summary, we demonstrate that the gene *pqqC* is a novel molecular marker that can be used complementary to housekeeping genes for studying the diversity and evolution of plant-beneficial pseudomonads.

Phosphorus (P) is an essential macroelement for plants, and its bioavailability is often limited in soil because it forms highly insoluble iron-aluminum oxide complexes (11). Several bacteria are known to solubilize phosphate from these soil complexes, rendering phosphorus available to plants and thereby improving plant growth. Among P-solubilizing microorganisms, rhizosphere-colonizing pseudomonads are of major interest, as they possess many other plant-beneficial traits, such as the capacity to directly improve growth, induce systemic resistance in plants, and suppress soilborne diseases (15). A recent metagenomic analysis of rhizosphere microbiomes has shown that *Gammaproteobacteria* (with pseudomonads being the most important group of rhizosphere-associated *Gammaproteobacteria*) are enriched in disease-suppressive soils and also in response to attack by fungal pathogens (26). Fluorescent *Pseudomonas* spp. have received special attention because they are efficient root colonizers, and strains belonging to a subgroup that produces the potent antifungal metabolites 2,4-diacetylphloroglucinol (DAPG) and hydrogen cyanide (HCN) are particularly efficient in providing protection against several fungal pathogens of different plant species (15, 16, 18).

Phosphate solubilization by *Pseudomonas* spp. in soil is associated mainly with the production and excretion of gluconic acid, which chelates the cations bound to phosphate, thereby releasing the element (7). The production of this acid implies the direct oxidation of glucose catalyzed by a periplasmic membrane-bound glucose dehydrogenase (GDH), which forms a complex with the cofactor pyrroloquinoline quinone (PQQ).

PQQ also serves as a redox cofactor for various bacterial dehydrogenases other than GDH but is not produced in animals or plants. Several studies with bacterial mutants unable to produce PQQ and gluconic acid have demonstrated the intimate relation of the cofactor to phosphate solubilization processes (7, 17). Besides its relevant role in P solubilization, PQQ is reported to be a potent growth-promoting factor for bacteria and plants, has antioxidant properties (5), and is directly related to the production of antimicrobial substances (7, 14, 36) as well as to the induction of systemic plant defenses (17). Hence, the cofactor PQQ has multiple plant beneficial effects.

The genes responsible for PQQ production have been cloned and sequenced in several bacterial genera, including *Pseudomonas*, *Methylobacterium*, *Acinetobacter*, *Klebsiella*, *Enterobacter*, and *Rahnella* (5, 14, 17, 36, 42). In *P. fluorescens* B16, the PQQ operon is formed by 11 genes, *pqqA*, *-B*, *-C*, *-D*, *-E*, *-F*, *-H*, *-I*, *-J*, *-K*, and *pqqM* (5). The *pqqC* gene encodes the

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pyrroloquinoline quinone synthase C (PqqC), which is the best-characterized enzyme in the pathway and catalyzes the final step of the PQQ biosynthesis, namely, cyclization and oxidation of the intermediate 3a-(2-amino-2-carboxy-ethyl)-4,5-dioxo-4,5,6,7,8,9-hexahydroquinoline-7,9-dicarboxylic acid to PQQ (22).

No studies have yet focused on the evolutionary history and the genetic diversity of PQQ in bacteria, although this would be particularly interesting for the agriculturally important genus *Pseudomonas*. Previous studies on the occurrence, diversity, and evolution of plant-associated *Pseudomonas* spp. have focused mostly on the diversity of the 16S rRNA (4, 31, 33), other housekeeping genes (4, 8, 32), or biocontrol-relevant genes involved in the suppression of various plant pathogenic fungi (6, 8, 9, 18, 31, 33, 40). In addition, little is known about the occurrence of *pqq* genes among *Pseudomonas* species. The main aim of this study was therefore to investigate the phylogeny of the *pqqC* gene and to evaluate whether it could serve as a marker to study the diversity and evolution in pseudomonads. To this end, we designed primers for the amplification of *pqqC* specifically in the genus *Pseudomonas.* The phylogeny of *pqqC* was then inferred from sequences of *Pseudomonas* reference strains and of *Pseudomonas* spp. isolated from wheat roots and compared to that of the two housekeeping genes, *rpoD* and *gyrB*. To capture most of the *pqqC* diversity present in the wheat root samples that we studied, we used cultivation-dependent and cultivation-independent approaches. Finally, we related the *pqqC* phylogeny to the phosphate-solubilizing activity of the pseudomonads investigated.

MATERIALS AND METHODS

Bacterial culture conditions and genomic DNA extraction. *Pseudomonas* strains (Table 1) were routinely cultured at 27°C in King's medium B (KMB) (19) or in the *Pseudomonas* selective medium KMB^{+++} containing 40 μg/ml ampicillin, 13 μg/ml chloramphenicol, and 100 μg/ml cycloheximide (39). All other bacterial genera were cultivated in LB broth (2) at 27°C, except *Escherichia coli* K-12, which was cultured at 37°C. Genomic DNA from bacterial strains used as the template in PCR was obtained by lysing bacterial suspensions for 10 min at 96°C, followed by centrifugation and collection of the supernatants.

Isolation of pseudomonads from a wheat field. Root samples of the Mexican wheat cultivar Bobwhite were taken from three replicate plots (three samples in total, one sample consisting of the roots of 10 plants taken from one replicate plot) of a field experiment performed at the research station Agroscope Reckenholz-Tänikon (ART) in Zurich-Reckenholz in 2009 within the frame of the Swiss National Foundation research program NRP59. Samplings were performed 6 weeks after sowing, when plants were at the tillering stage. Roots were washed with tap water and briefly dried on paper tissues. Each root sample was then placed in a 100-ml Erlenmeyer flask partly filled with 50 ml sterile 0.9% NaCl solution and stored overnight at 4°C. The root suspensions were then shaken for 30 min at 350 rpm. Subsequently, 20 μl of the root suspensions was inoculated into 180 μl liquid KMB^{+++} medium, selective for pseudomonads, and grown overnight with slight agitation at 27°C. The remaining root suspensions, including the roots, were stored at −20°C for total DNA extraction and subsequent PCR and cloning procedures (see below). From the liquid KMB^{+++} cultures, serial dilutions were prepared and plated onto KMB^{+++} agar plates. After incubation for 48 h, 40 to 50 colonies were selected per root sample replicate, resulting in a total of 140 colonies (isolates). The isolates were further used for *hcnAB* and *phlD* amplification, for sequencing of *pqqC*, *rpoD*, and *gyrB* genes, and for phosphate solubilization studies (described below).

Total DNA extraction from root samples. The total DNA from root pieces (0.5 g) and 50 ml root suspension (prepared as described above) was extracted using a Fast DNA spin kit for soil (MP Biomedicals, Irvine, CA). The frozen samples were briefly thawed overnight at 4°C, and root pieces were added to DNA extraction tubes. The remaining root suspension was centrifuged at 3,500 rpm for 20 min, and 50 μl of the resulting pellets was additionally added to the extraction tubes. Total DNA was further extracted according to the manufacturer's recommendations, but reduced volumes of the sodium phosphate and MT buffers were used. DNA was diluted to a concentration of 10 ng/μl. DNA extracts were then subjected to *pqqC* PCR amplification, cloning, and sequencing.

Design of *pqqC* primers pqqCr1 and pqqCf1. Alignment of the *pqqC* regions retrieved from the GenBank database was performed using the multiple sequence alignment program ClustalW 1.8 (41) to determine regions conserved only within the genus *Pseudomonas*. The primers pqqCr1 (5′-CAGGGCTGGG TCGCCAACC-3′) and pqqCf1 (5′-CATGGCATCGAGCATGCTCC-3′), which amplify a 546-bp-long (including primer sequences) *pqqC* fragment, were designed, and their specificity was tested against DNA sequences available in GenBank with Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (41). Furthermore, *pqqC* primer specificity was tested by PCR amplification (see below) on 57 reference *Pseudomonas* strains (i.e., 37 DAPG-producing fluorescent pseudomonads and 20 pseudomonads not producing DAPG, representing the major phylogenetic groups of the *Pseudomonas* genus) and on bacterial strains representative of 12 different non-*Pseudomonas* genera, found mostly in the soil environment (listed in Table 1). For phylogenetic analyses, a subset of 36 *Pseudomonas* reference strains were used as described below.

PCR assays. PCR amplifications of *pqqC* from bacterial DNA lysates were carried out in 20-μl mixtures containing 1× ThermoPol buffer (New England BioLabs, Inc., Beverly, MA), 100 μM (each) deoxynucleoside triphosphate (dNTP), 0.4 μM (each) forward and reverse primer, 0.75 U *Taq* DNA polymerase (5,000 U/ml; New England BioLabs, Inc.), and 2 μl of genomic DNA. The following thermocycling conditions were used: initial denaturation at 96°C for 10 min followed by 30 cycles of 96°C for 30 s, 63°C for 30 s, 72°C for 1 min, and final elongation at 72°C for 10 min. For *pqqC* amplification from roots, 20 ng total DNA extracts, 5% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO), and 5% bovine serum albumin were added to the PCR mix, and thermocycling conditions were slightly modified from those described above, i.e., 35 cycles instead of 30, denaturing and annealing times of 1 min, and an elongation time of 2 min per cycle. The presence of amplified fragments was checked by standard gel electrophoresis and ethidium bromide staining.

The genes involved in the biosynthesis of HCN (*hcnA*, *hcnB*), DAPG (*phlD*), and phenazine (*phzF*) and two housekeeping genes (*gyrB* and *rpoD*) were amplified with primers and the annealing temperatures listed in Table 2, using the same thermocycling conditions as those for the *pqqC* gene.

Prior to ligation and sequencing reactions, all PCR amplicons were purified on a MultiScreen PCR plate (Millipore, Molsheim, France), resuspended in 30 μl of sterile double-distilled water, and quantified using Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE).

***pqqC* cloning.** Purified *pqqC* fragments amplified from DNA extracted from root samples were cloned using the TA cloning vector pJET1.2 (CloneJet PCR cloning kit; Fermentas, Glen Burnie, MD). The constructs were transformed into chemically competent *E. coli* One Shot TOP10 cells (Invitrogen, Carlsbad, CA), and a total of 107 transformants (30 to 40 per root sample replicate) containing the pJET1.2_*pqqC* construct were selected for sequencing.

Sequencing of the *pqqC*, *rpoD*, and *gyrB* genes. Sequencing reactions were performed with 3 to 10 ng of purified PCR product and primers at a final concentration of 0.16 μM, using an ABI PRISM BigDye Terminator version 3.0 cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The obtained products were cleaned by gel filtration through Sephadex G-50 columns (Amersham Biosciences, Uppsala, Sweden) on MultiScreen-HV plates (Millipore). Purified products were sequenced using an ABI Prism 3130 genetic analyzer (Applied Biosystems, Foster City, CA) at the Genetic Diversity Centre of the ETH Zürich. DNA sequences were edited using the Sequencher package (Gene Codes, Ann Arbor, MI).

Phylogenetic analysis. *Pseudomonas* phylogenies shown in Fig. 1 and 2 and Fig. S1, S2, and S3 in the supplemental material were inferred from *pqqC*, *rpoD*, and *gyrB* sequences from a selection of 36 *Pseudomonas* reference strains, including 12 DAPG-producing and 14 non-DAPG-producing fluorescent pseudomonads listed in Table 1 (underlined strains) and GenBank sequences of 10 *Pseudomonas* strains: *P. aeruginosa* UCBPP-PA14 (GenBank database accession number CP000438.1), *P. entomophila* L48 (CT573326.1), *P. mendocina* ymp (CP000680.1), *P. putida* GB-1 (CP000926.1), *P. putida* KT2440 (AE015451.1), *P. putida* W619 (CP000949.1), *P. stutzeri* A1501 (CP000304.1), *P. syringae* pv. phaseolicola 1448A (CP000058.1), *P. syringae* pv. syringae B728a (CP000075.1), and *P. syringae* pv. tomato DC3000 (AE016853.1). The alignment of DNA sequences was performed with ClustalW 1.8 implemented in the MEGA software version 4.0 package (41). Phylogenetic trees were constructed with MEGA software version 4.0 (41), using the neighbor-joining (NJ) method (35) for trees shown in Fig. 1 and 2 and in Fig. S1, S2, and S3 in the supplemental material or with the PhyML 3.0 phylogeny software (12), using the maximum likelihood (ML)

TABLE 1. Bacterial strains and isolates used in this study

Bacterial strain(s)[a]	Description	Reference[b]
DAPG-producing reference pseudomonads		
(A): C*1A1, CM1′A2, K93.3, K94.31, P96.25, P97.30, Q65c-80, Q128-87, S8-151, TM1B2	Biocontrol	8, 18
(B): F113, K93.2, K94.37, P12, Q37-87	Biocontrol	8, 18
(C): Q2-87, Q7-87, Q12-87, Q86-87	Biocontrol	8, 18
(D): C10-186, C10-190, K93.52, PILH1, PITR2	Biocontrol	8, 18
(E): F96.26, P97.1, P97.6, P97.38, F96.27	Biocontrol	8, 18
(F): CHA0, K94.41, PF, Pf-5, PGNL1, PGNR1, S8-62	Biocontrol	8, 18
(−): P97.26	Biocontrol	8, 18
Reference pseudomonads, not producing DAPG		
P. aeruginosa PAO1^T (LMG12228^T)	Human pathogen, biocontrol, type strain	BCCM
P. caricapapayae LMG2152^T	Plant pathogen, type strain	BCCM
P. chlororaphis 30-84	Biocontrol	23
P. chlororaphis DTR133	Soil bacterium	21
P. chlororaphis LMG1245^T	Type strain	BCCM
P. chlororaphis LMG5004^T	Type strain	BCCM
P. corrugata LMG2172^T	Plant pathogen, type strain	BCCM
P. fluorescens 2-79	Biocontrol	23
P. fluorescens DSS73	Biocontrol	C. Keel (UNIL)
P. fluorescens KD	Biocontrol	33
P. fluorescens LMG1794^T	Type strain	BCCM
P. fluorescens MIACH	Soil bacterium	This study
P. fluorescens GUGO	Soil bacterium	This study
P. fluorescens Pf0-1	Soil bacterium	38
P. fluorescens SBW25	Biocontrol	38
P. kilonensis 520-20^T (DSM13647 ^{T})	Type strain	DMSZ
P. plecoglossicida PCPF1	Soil bacterium	3
P. putida LMG2257^T	Type strain	BCCM
P. putida P3	Soil bacterium	C. Keel (UNIL)
P. rhizospherae IH5^T (LMG21640^T)	Type strain	BCCM
Non-*Pseudomonas* bacteria		
Agrobacterium tumefaciens	Plant pathogen	C. Keel (UNIL)
Bacillus mycoides A23	Not documented	10
Burkholderia cepacia	Biocontrol	C. Keel (UNIL)
Cupriavidus necator JMP134 (LMG1197)	Biodegradation	BCCM
Escherichia coli DH5α	Laboratory strain	Invitrogen, Carlsbad, CA
Erwinia carotovora ATTn10	Plant pathogen	24
Photorhabdus luminescens TT01^T (DSM15139^T)	Insect pathogen, type strain	DMSZ
Rhodococcus sp. strain C125 (DSM44236)	Biodegradation	DMSZ
Sphingomonas herbicidovorans MH^T (DSM11019^T)	Biodegradation, type strain	DMSZ
Sphingobium japonicum UT26^T (DSM16413^T)	Biodegradation, type strain	DMSZ
Streptomyces scabies Sy9103	Plant pathogen	C. Beaulieu (UdeS)
Xanthomonas campestris LMG568^T	Plant pathogen, type strain	BCCM
Pseudomonas wheat root isolates		
140 isolates representing 34 OTUs (see Fig. 2) described as RW09-C1.x to RW09-C34.x (see Table S1 in the supplemental material), where x stands for the isolate no.	Soil bacteria	This study

[a] Letters in parentheses indicate the multilocus group of DAPG-producing pseudomonads as defined by Frapolli et al. (8). Underlined strains were included in the phylogenetic analysis presented in Fig. 1 and 2 as well as in Fig. S1 and S2 in the supplemental material. −, not belonging to any of the six multilocus groups.

[b] BCCM, Belgian Coordinated Collections of Microorganisms; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; UNIL, University of Lausanne, Switzerland; UdeS, University of Sherbrooke, Canada.

method for trees additionally included in the Shimodaira-Hasegawa (SH) test (see below). The genetic distances were computed based on the maximum composite likelihood estimated by using the Tamura-Nei model and MEGA software version 4.0 (41). The nucleotide sequences of the *pqqC*, *gyrB*, and *rpoD* genes from *P. aeruginosa* PAO1, *P. aeruginosa* UCBPP-PA14, *P. mendocina* ymp, and *P. stutzeri* A1501 were used as outgroups.

The Shimodaira-Hasegawa (SH) test (37) implemented in the Phylogenetic Analysis by Maximum Likelihood (PAML) software package version 3.14 (44) was performed to compare different ML and NJ trees of the *Pseudomonas* genus (36 reference strains) inferred from single and concatenated loci.

***pqqC* sequence analysis.** The GC content, the diversity index (π), and ratios of nonsynonymous to synonymous substitutions (dN/dS) were calculated for *pqqC* sequences derived from (i) a subset of 19 reference *Pseudomonas* strains representing the main phylogenetic groups as defined by Mulet et al. (29), including six strains of the *P. fluorescens* group 1 (LMG2172, Pf0-1, 30-84, CHA0, 2-79, SBW25), four strains of the *P. syringae* group 2 (LMG2152, DC3000, B728a, 140BA), five strains of the *P. putida* group 3 (P3, GB1, W619, KT2440, L48), and four strains of the *P. aeruginosa* group 4 (PAO1, UCBPP-PA14, ymp, A1501), and (ii) a *Pseudomonas* wheat root population illustrated in Fig. 2B representing 106 different operational taxonomical units (OTUs). The GC content and nucleotide diversity indexes were calculated with the DnaSP program version 5 (34). The diversity index expresses the genetic diversity or polymorphism of a gene in a population, with a π value of 0 meaning no polymorphism and a π value of 1 indicating maximal polymorphism.

TABLE 2. Primers used in this study

Gene product	Primer sequence (5′→3′)	Primer name	Annealing temp (°C)	Product length (bp)	Target gene(s)	Reference
PQQ oxidase	CAGGGCTGGGTCGCCAACC CATGGCATCGAGCATGCTCC	pqqCf1 pqqCr1	63	546	*pqqC*	This study
DNA gyrase subunit B	TTCAGCTGGGACATCCTGGCCAA TCGATCATCTTGCCGACRACCA	gyrBf gyrBr2	65	584–587	*gyrB*	8
RNA polymerase subunit D	ACTTCCCTGGCACGGTTGACCA TCGACATGCGACGGTTGATGTC	rpoDf rpoDr	60	693–696	*rpoD*	8
Phenazine biosynthetic enzyme	ATCTTCACCCCGGTCAACG CCRTAGGCCGGTGAGAAC	Ps_up1 Ps_low1	57	427	*phzF*	23
DAPG type III polyketide synthase	ACCCACCGCAGCATCGTTTATGAGC CCGCCGGTATGGAAGATGAAAAAGTC	B2BF BPR4	60	629	*phlD*	25
Hydrogen cyanide biosynthetic enzymes	TGCGGCATGGGCGTGTGCCATTGCTGCCTGG CCGCTCTTGATCTGCAATTGCAGGCC	PM2 PM7-26R	67	570	*hcnAB*	40

In order to identify the type of selection acting on *pqqC*, *gyrB*, and *rpoD* codons, the fast single-likelihood ancestor (SLAC) counting method was applied. The SLAC method is available in a free public Web implementation (http://www.datamonkey.org) and compares the ratio of nonsynonymous (dN) and synonymous (dS) codon changes of a given population assuming neutral evolution, providing information about the type of selective constraint acting on the proteins (20). A dN/dS value of <1 indicates purifying selection, a dN/dS value of >1 indicates positive selection, and a dN/dS value of ≈1 indicates neutral selection.

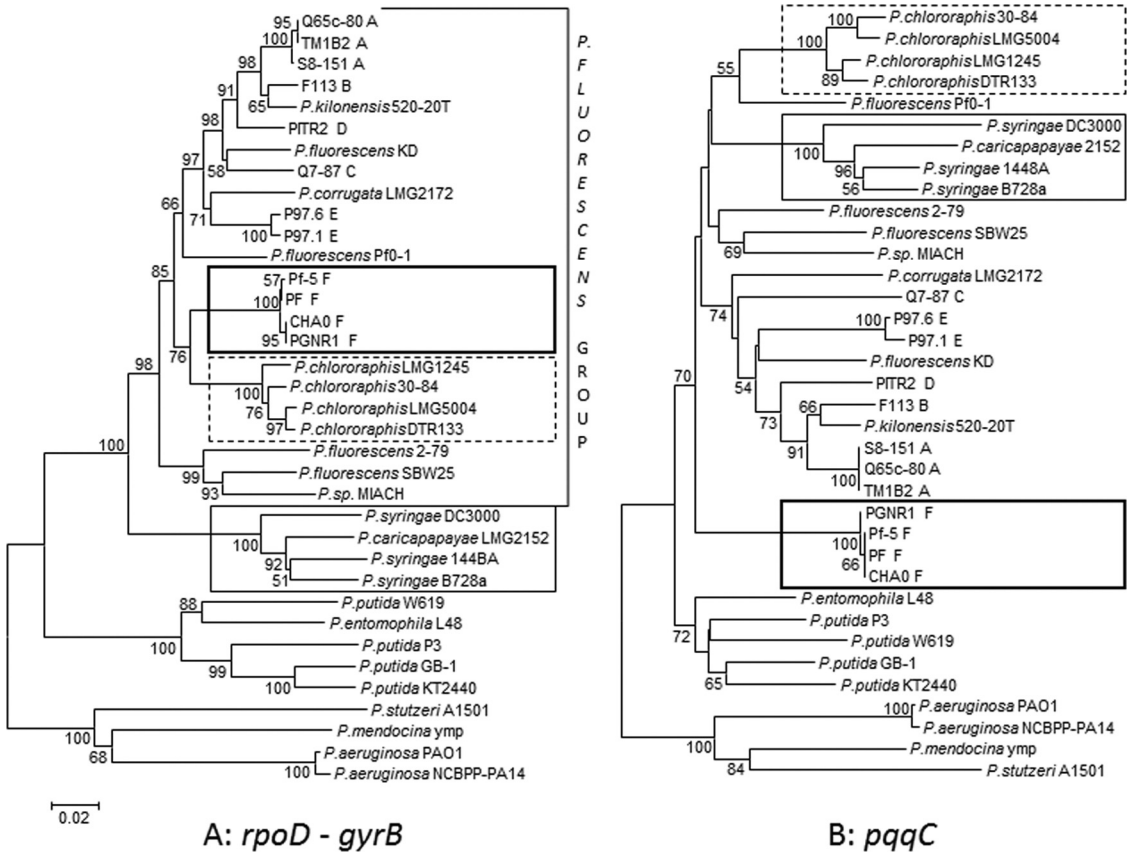

FIG. 1. Phylogenetic relationships among 36 *Pseudomonas* reference strains, including known biocontrol strains described in Table 1. The neighbor-joining (NJ) trees were inferred from concatenated sequences of the two housekeeping genes *rpoD* and *gyrB* (1,130 bp) (A) and from *pqqC* (507 bp) sequences (B). Only bootstrap values greater than 50% are shown. Scale bar = 0.02 substitutions per site. Thin-lined box, *P. syringae* group; dashed-lined box, *P. chlororaphis* group; and thick-lined box, subgroup 1d (containing strains described as *P. protegens* by Ramette et al. [32]). Capital letters following the names of DAPG-producing strains indicate the multilocus group defined by Frapolli et al. (8).

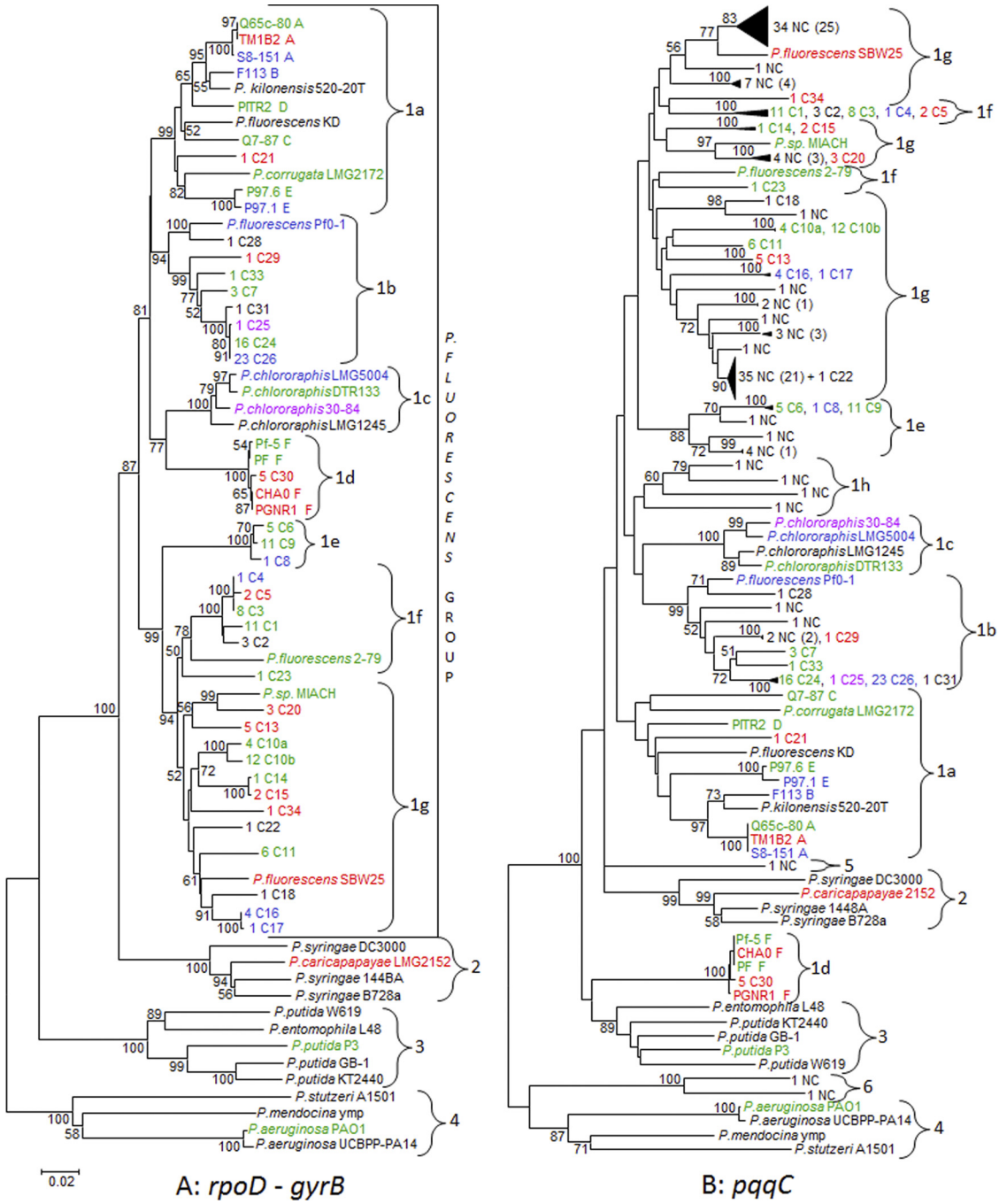

FIG. 2. Phylogenetic relationships among *Pseudomonas* reference strains and pseudomonads isolated from the rhizosphere of field-grown wheat in this study. The neighbor-joining (NJ) trees were inferred from concatenated sequences of the two housekeeping genes *rpoD* and *gyrB* (1,130 bp) (A) and from *pqqC* sequences (507 bp) (B). Only bootstrap values greater than 50% are shown. Scale bar = 0.02 substitutions per site. Both trees include sequences of 36 reference strains and of 136 pseudomonads isolated from wheat roots. Furthermore, the *pqqC* tree (B) includes an additional 107 partial *pqqC* sequences of noncultivated bacteria (cloned sequences). *Pseudomonas* isolates are described as follows: the first and the second number indicate the number of isolates per OTU and the OTU number, respectively, while C designates cultivated bacteria. Noncultivated pseudomonads are described by NC preceded by a figure indicating the number of cloned sequences and followed by a figure in parentheses describing the number of different OTUs identified from these sequences. Cultivated and noncultivated bacteria were obtained from the same wheat roots sampled in a Swiss field. Capital letters following the names of reference strains indicate the multilocus group defined by Frapolli et al. (8). Font colors indicate phosphate solubilization classes on NBRIP medium (based on halo diameter) as described in Materials and Methods and correspond to very low (violet), low (dark blue), medium (green), or strong (red) activity or undetermined activity (black).

Phosphate solubilization activity of *Pseudomonas* isolates. The ability of *Pseudomonas* strains/isolates to solubilize phosphate was measured for 41 pseudomonad reference strains and 29 wheat root isolates, each belonging to a different *rpoD-gyrB* OTU (see Table S1 in the supplemental material) on solid NBRIP medium [5 g/liter $MgCl_2 \cdot 6H_2O$, 0.25 g/liter $MgSO_4 \cdot H_2O$, 0.2 g/liter HCL, 0.1 g/liter $(NH_4)_2SO_4$, 5 g/liter $Ca_3(PO_4)_2$, 10 g/liter glucose] (30) as follows. LB overnight cultures of bacterial strains were used to spot inoculate (5 μl) the NBRIP plates, which were then sealed with parafilm and incubated in the dark at 27°C for 19 days. Solubilization of tricalcium phosphate resulted in the formation of clear halos around the bacterial colonies. The solubilization activity per bacterial strain was evaluated by subtracting the diameter of the colonies from the entire diameter of the halos (7). Strains were divided into four classes according to their P solubilization activity: 1, very low activity (0.0 to 4.4 mm); 2, low activity (4.5 to 5.4 mm); 3, medium activity (5.5 to 6.4 mm); and 4, strong activity (6.5 to 7.5 mm). Three to six replicate plates were prepared for each strain.

P solubilization data were statistically analyzed using Systat 12 (Systat Software, Inc., San Jose, CA) at the probability threshold of 0.05. Data were first analyzed by analysis of variance (ANOVA), and pairwise mean comparisons of different (sub)groups were subsequently done, using Tukey's range test.

Nucleotide sequence accession numbers. Sequences of 507-bp-long *pqqC* fragments (without primer sequences) were submitted to GenBank under accession numbers JN397402 to JN397560 (76 sequences from cloned *pqqC* fragments, 33 *pqqC* sequences amplified from wheat root isolates, and 50 *pqqC* sequences from *Pseudomonas* reference strains). Sequences of 533- to 536-bp-long *gyrB* fragments and of 581- to 587-bp-long *rpoD* fragments from 5 (*gyrB*) and 8 (*rpoD*) reference pseudomonads and from 32 (*gyrB*) and 33 (*rpoD*) wheat root isolates were submitted to GenBank under accession numbers JN397569 to JN397573, JN397607 to JN397638, JN397561 to JN397568, and JN397574 to JN397606, respectively.

RESULTS

Primer specificity and *pqqC* abundance in pseudomonads. The pqqCf1 and pqqCr1 primer pair was specific for the genus *Pseudomonas* and did not amplify any of the 12 other tested genera. The *pqqC* gene could be amplified in all tested pseudomonads, i.e., in 57 reference strains (37 DAPG-producing fluorescent pseudomonads and 20 pseudomonads not producing DAPG belonging to at least nine different species) and in 140 wheat root isolates from a Swiss field site belonging to the *Pseudomonas* genus based on similarity levels determined by a BLAST search of sequenced *gyrB* or *rpoD* fragments. All strains produced a single amplicon, except DAPG-producing strains of the multilocus group E (strains F96.27, P97.38, P97.6, P97.1, and F96.26) (8) and *P. aeruginosa* PAO1, which produced unspecific PCR products.

Phylogenetic comparison between *pqqC* and the two housekeeping genes *rpoD* and *gyrB* within the genus *Pseudomonas*. The phylogeny of the *pqqC* gene was compared to that of the two housekeeping genes *rpoD* and *gyrB* for 36 *Pseudomonas* reference strains, including known biocontrol strains and other strains found in the soil environment. To allow a better resolution of the *Pseudomonas* phylogeny, the *rpoD* and *gyrB* sequences were concatenated as done by Yamamoto et al. before inferring the phylogenetic tree (Fig. 1A) (43). The tree based on *pqqC* sequences (Fig. 1B) showed phylogenetic clusters similar to those of the tree inferred from the concatenated housekeeping genes (Fig. 1A). Nevertheless, there were some emplacement differences. First, in the concatenated *rpoD-gyrB* tree, the *P. syringae* strains (Fig. 1, thin-lined box) were well separated from the *P. fluorescens* main group as defined in Mulet et al. (29), whereas in the *pqqC* tree, they clustered within this group. Second, in the *rpoD-gyrB* tree, the group of DAPG and pyoluteorin (PLT) producers (Fig. 1, thick-lined box) were located within the *P. fluorescens* group and clustered close to the *P. chlororaphis* subgroup (Fig. 1, dashed-lined box), while in the *pqqC* tree, these strains clustered apart from the *P. fluorescens* group.

Single-locus trees often display incongruent topologies compared with one another. Concatenation of two or more loci has been shown to increase the resolution of the inferred phylogenies (43). Assuming that the *gyrB-rpoD* tree (Fig. 1A) represents a good explanation of the species phylogeny of the genus *Pseudomonas* (43), we compared different tree topologies based on single loci (*gyrB*, *rpoD*, and *pqqC*) and concatenated loci (*gyrB-pqqC*, *gyrB-rpoD-pqqC*, and *rpoD-pqqC*) to that reference tree using the SH test. The *pqqC* tree was found to be the only single-locus tree incongruent with the *gyrB-rpoD* reference tree ($P < 0.05$). However, all the concatenated trees containing *pqqC* were congruent (*P* values of 0.09 for *gyrB-pqqC*, 0.25 for *rpoD-pqqC*, and 0.79 for *gyrB-rpoD-pqqC*) with the *gyrB-rpoD* tree.

***pqqC* diversity of a *Pseudomonas* population in the rhizosphere of field-grown wheat.** To explore the diversity of the *pqqC* gene in an agricultural environment, a total of 140 *Pseudomonas* colonies were isolated on *Pseudomonas*-specific medium from the rhizosphere of wheat grown in a conventional Swiss field. The *pqqC*, *gyrB*, and *rpoD* genes of these cultivated strains were sequenced. In addition, a cultivation-independent approach was undertaken, in which total DNA was extracted directly from the same wheat roots used for *Pseudomonas* isolation and used as a template for *pqqC* amplification, cloning, and partial sequencing. Based on partial *pqqC*, *gyrB*, and *rpoD* sequences, the 140 cultivated colonies could be allocated to 34 OTUs, named RW09-C1 to RW09-C34 (Fig. 2; see also Table S1 and Fig. S2 in the supplemental material [in the figures, the designation RW09 is omitted]). For 45 isolates, the *gyrB* (or *rpoD*) sequences showed highest identity to *P. koreensis* (98% to 99% *gyrB* identity). For the other isolates, BLAST identity results for *gyrB* (or *rpoD*) sequences are displayed in Table S1 in the supplemental material. Based on partial *pqqC* sequences from 107 clones derived from total DNA extracted from wheat roots, 76 different OTUs could be identified (Fig. 2B). Interestingly, only two wheat root isolates (RW09-C22 and RW09-C29) shared the same OTU as some cloned *pqqC* fragments. This means a total of 108 different OTUs were detected in the wheat rhizosphere population investigated. The amount of total *pqqC* pseudomonads on the roots of the wheat plants and in the bulk soil was quantified by the most probable number technique (MPN) and resulted in 2.83E8 pseudomonads per gram of root and 1.77E6 pseudomonads per gram of soil, respectively.

Phylogenetic analyses of pseudomonads colonizing the roots of field-grown wheat based on *pqqC* or concatenated *rpoD-gyrB* sequences. Phylogenetic trees were inferred from concatenated *rpoD-gyrB* and from *pqqC* sequences of 36 reference pseudomonads and 136 pseudomonads isolated from wheat roots (Fig. 2A; see also Fig. S2 in the supplemental material). Moreover, a *pqqC* phylogenetic tree was constructed which additionally included 107 cloned *pqqC* sequences obtained from a cultivation-independent method (Fig. 2B). In the *rpoD-gyrB* tree (Fig. 2A), two lineages and four main groups were identified according to Mulet et al. (29), i.e., the *P. fluorescens* lineage containing *P. fluorescens* (group 1), *P. syringae* (group

TABLE 3. Distribution of *pqqC* sequences derived from cultivated and noncultivated (cloned sequences) pseudomonads obtained from wheat roots among the phylogenetic groups defined in Fig. 2 and phosphate solubilization activity of different phylogenetic groups

Main group[a]	Subgroup	Description	Percentage of bacteria per phylogenetic (sub)group[b]		Halo diam (mm) ± SD (no. of *Pseudomonas* isolates tested for P solubilization ability)[c]
			Cultivated	Noncultivated	
1. *P. fluorescens* group	1a	DAPG producers, *P. kilonensis* and *P. corrugata*	1	0	5.80 ± 0.11 (23)
	1b	Containing *P. fluorescens* Pf0-1	34	4	5.52 ± 0.27 (10)
	1c	*P. chlororaphis*	0	0	5.70 ± 0.24 (3)
	1d	DAPG and PLT producers	4	0	6.74 ± 0.17 (7)*
	1e	Wheat isolates from this study	12	6	5.64 ± 0.57 (3)
	1f	Containing *P. fluorescens* 2-79	19	0	5.91 ± 0.37 (6)
	1g	Containing *P. fluorescens* SBW25	29	84	6.02 ± 0.11 (12)
	1h	Noncultivated bacteria from this study	0	4	
2. *P. syringae*			0	0	6.50 ± 0.18 (1)
3. *P. putida*			1	0	5.96 ± 0.18 (5)
4. *P. aeruginosa*, *P. mendocina*, and *P. stutzeri*			0	0	5.80 ± 0.22 (2)
5. Noncultivated bacteria from this study			0	1	
6. Noncultivated bacteria from this study			0	1	

[a] *Pseudomonas* phylogenetic groups as defined in Fig. 2.
[b] One hundred forty *pqqC* sequences from pseudomonads isolated from the wheat rhizosphere and 107 *pqqC* sequences cloned from the wheat rhizosphere were analyzed.
[c] P solubilization activity was defined based on the diameters of halos on NBRIP medium. Averages ± standard errors are shown. *, group with significantly higher solubilization activity compared to all other groups.

2), and *P. putida* (group 3) and the *P. aeruginosa* lineage containing *P. aeruginosa*, *P. mendocina*, and *P. stutzeri* (group 4). The *P. fluorescens* group 1 was further subdivided into seven subgroups: i.e., 1a (DAPG producers, *P. kilonensis* and *P. corrugata*), 1b (containing *P. fluorescens* Pf0-1), 1c (*P. chlororaphis*), 1d (DAPG and PLT producers), 1e (wheat isolates from this study), 1f (containing *P. fluorescens* 2-79), and 1g (containing *P. fluorescens* SBW25).

The *pqqC* trees displayed a topology similar to that of the *rpoD-gyrB* tree; however, some differences in group allocations were detected. Remarkably, in both *pqqC* trees (Fig. 2B; see also Fig. S2B in the supplemental material) including or not including sequences from noncultivated pseudomonads, subgroup 1d (DAPG and PLT producers) clearly clustered away from the *P. fluorescens* group, whereas the *P. syringae* group clustered within the *P. fluorescens* group. In the *pqqC* tree that is based on sequences of cultivated pseudomonads only (see Fig. S2B), subgroup 1c, which contains the *P. chlororaphis* strains, also clustered outside the *P. fluorescens* group and close to subgroup 1d. Three *pqqC* sequences from noncultivated pseudomonads did not fit in any of the four main groups and were thus designated group 5 (one sequence), which clustered close to the *P. syringae* group, and group 6 (two sequences), which clustered close to the *P. aeruginosa* group (Fig. 2B). Finally, four different sequences derived from the *pqqC* clone library clustered within the *P. fluorescens* group 1 but away from any defined subgroup and were thus defined as subgroup 1h.

Phylogenetic distribution of cultivated and noncultivated pseudomonads from wheat roots based on *pqqC*. Nearly all of 140 root-isolated pseudomonads as well as the great majority (98%) of the 107 cloned *pqqC* sequences derived from the cultivation-independent approach were found to cluster within *P. fluorescens* group 1 (Fig. 2B, Table 3). However, their distribution among the subgroups of group 1 was different. The majority of cloned *pqqC* sequences (84%) were located in subgroup 1g. In contrast, most of the cultivated bacteria were distributed within subgroups 1b (34%), 1g (29%), 1f (19%), and 1e (12%). Interestingly, subgroups 1a, 1d, and 1f contained only cultivated bacteria, whereas subgroup 1h contained only noncultivated bacteria.

***pqqC*, *gyrB*, and *rpoD* GC contents and polymorphism.** To gain an insight into the polymorphism and the selective pressure acting on *pqqC*, the GC content, the diversity index (π), and ratios of nonsynonymous to synonymous substitutions (dN/dS) were calculated for the genus *Pseudomonas* and a wheat root population (Table 4). The nucleotide diversity (π) of strains representing the genus *Pseudomonas* was 0.145 for *pqqC*. This π value was situated between the π values of the housekeeping genes *gyrB* (0.125) and *rpoD* (0.236), indicating a polymorphism of *pqqC* greater than *gyrB* but lower than *rpoD*. For all the investigated data sets, dN/dS ratios were significantly below 1, indicating that the selective pressure acting on *pqqC*, *gyrB*, and *rpoD* is purifying (Table 4).

Phosphate solubilization activity and presence of *hcnAB* and *phlD* genes. The relationship between the *pqqC* phylogeny and the P solubilization activity and also the presence of *hcnAB* and *phlD* genes which are known to be involved in antifungal activity of fluorescent pseudomonads was investigated (see Table S1 in the supplemental material). DAPG- and PLT-producing reference strain CHA0 of subgroup 1d and the RW09-C21 isolate of subgroup 1a were the strongest phosphate solubilizers (7.25-mm halo size on NBRIP agar plates). Isolate RW09-C25 of subgroup 1b exhibited the lowest solubilization activity (1.25-mm halo size). When comparing the average solubilization activities of the different phylogenetic groups, the highest

TABLE 4. GC content, π, and dN/dS substitution rates of *pqqC*, *gyrB*, and *rpoD* sequences among the *Pseudomonas* genus and pseudomonads from wheat roots[a]

Group (no. of analyzed sequences)	Gene	% GC	π	dN/dS ratio
Pseudomonas genus (19)	*pqqC*	65.5	0.145	0.036
Pseudomonas genus (19)	*gyrB*	56.3	0.125	0.035
Pseudomonas genus (19)	*rpoD*	62.4	0.236	0.250
Pseudomonads representing a wheat root population (106)	*pqqC*	64.2	0.097	0.044

[a] *Pseudomonas* genus (*pqqC*, *gyrB*, and *rpoD*): six strains of the phylogenetic *P. fluorescens* group 1 (LMG2172, Pf0-1, 30-84, CHA0, 2-79, SBW25), four strains of the *P. syringae* group 2 (LMG2152, DC3000, B728a, 140BA), five strains of the *P. putida* group 3 (P3, GB1, W619, KT2440, L48), and four strains of the *P. aeruginosa* group 4 (PAO1, UCBPP-PA14, ymp, A1501). *Pseudomonas* spp. from wheat roots (only *pqqC*): 30 RW09 isolates and 76 cloned *pqqC* sequences all representing different OTUs. π, nucleotide diversity per site; dS, number of synonymous substitutions per site; dN, number of nonsynonymous substitutions per site.

solubilization activity was found for bacteria of subgroup 1d, with an average halo size of 6.74 mm, which was significantly ($P < 0.05$) higher than that of the other subgroups (Table 3). All other (sub)groups were generally more heterogeneous in their solubilization activity and did not differ significantly from one another. Nevertheless, subgroup 1g (average halo size, 6.02 mm) also contained some isolates with high solubilization properties (see Table S1). Among the bacteria isolated from wheat roots, only six were found to contain the *hcnAB* genes, and five among them also contain the *phlD* gene and are located in subgroup 1d (see Table S1).

DISCUSSION

This work represents the first study on the phylogeny of a gene involved in the pyrroloquinoline quinone (PQQ) biosynthesis in the genus *Pseudomonas*. We furthermore provide data on the frequency and diversity of this gene, *pqqC*, in a natural *Pseudomonas* rhizosphere population. The development of a *Pseudomonas*-specific primer pair targeting *pqqC* allowed us to detect the gene by PCR in all tested species, ranging from human- and plant-pathogenic pseudomonads (*P. aeruginosa*, *P. corrugata*, and *P. syringae*) to plant- and soil-associated nonpathogenic pseudomonads (*P. fluorescens*, *P. kilonensis*, and *P. putida*), and also in all the pseudomonads we have isolated from wheat roots. Overall, this suggests that *pqqC* is ubiquitous in the genus *Pseudomonas*. Although PQQ seems to be present also in a majority of other bacterial genera, there are certain species and strains that live in anaerobic environments and do not use glucose as a carbon source. These bacteria produce PQQ-dependent GDH but not the PQQ cofactor; thus, the enzyme remains inactive (1). In contrast, the majority of the *Pseudomonas* species are strictly aerobic organisms and glucose oxidizers.

So far, mainly 16S rRNA and a few other housekeeping genes were considered to be suitable for studying species phylogeny (43), because they are conserved and ubiquitous among genera. Here, we demonstrate the usefulness of *pqqC*, a gene which is involved in plant beneficial activities for phylogeny studies in the genus *Pseudomonas*. The *pqqC* gene delineated similar phylogenetic groups as the concatenated *rpoD-gyrB* genes (43) when considering reference pseudomonads only (Fig. 1), demonstrating that *pqqC* is conserved. It is worth noting, however, that the emplacement of some strains, such as those from subgroup 1d (with reference strains CHA0 and Pf5) and group 2 (*P. syringae*), in the *pqqC* tree was different from that of those in the *rpoD*, *gyrB*, and *rpoD-gyrB* trees. Similar findings were obtained by de Souza et al. for the topology of the *gacA* gene, which encodes a global regulator of secondary metabolite production (6). Interestingly in the *pqqC*-based trees (Fig. 1B and Fig. 2B), subgroup 1d was always phylogenetically well separated from the *P. fluorescens* main group 1. The phylogenetic incongruence between the *pqqC* tree and the *rpoD-gyrB* tree is in accordance with results obtained by Frapolli et al., which showed that single-locus topologies of different housekeeping genes were mostly incongruent compared with each other (8). In fact, the addition of *pqqC* to the *rpoD-gyrB* data set resulted in a topology congruent to that of the concatenated *rpoD-gyrB* tree (see Fig. S1 in the supplemental material). The clear demarcation, however, of subgroup 1d strains from other DAPG-producing fluorescent pseudomonads observed in other studies based on genetic and phenotypic traits (8, 18, 31) led to a comprehensive taxonomic study on CHA0/Pf-5-like strains, for which the name *Pseudomonas protegens* was suggested (32). In the present study, the phylogenetic analysis of the *pqqC* gene of this species and its greater ability to solubilize phosphate than that of other strains (see below) points to the same conclusion with respect to the particular position of this group within the fluorescent pseudomonads.

Purifying and/or neutral selection is a major selective force acting on housekeeping gene codons (8) to remove alleles that are deleterious or to preserve protein functions, respectively. Here, purifying selection acting on *pqqC* (dN/dS = 0.036) was detected when considering strains belonging to the major phylogenetic groups of the *Pseudomonas* genus described by Mulet et al. (29). Therefore, it can be assumed that *pqqC* plays an important role in the *Pseudomonas* genus. However, since *pqqC* is not constitutively expressed, and its regulation depends on environmental factors (42), it cannot substitute for loci commonly used for phylogenetic studies, such as 16S rRNA or housekeeping genes, but should be considered a complementary molecular marker. Our results revealed that *pqqC* is an excellent marker to study the diversity of phosphate-solubilizing pseudomonads in rhizosphere populations (Fig. 2B; see also Fig. S2B in the supplemental material). In fact, *pqqC* polymorphism found within the *Pseudomonas* genus was high enough ($\pi = 0.145$) to ensure a resolving power similar to that obtained with *rpoD* ($\pi = 0.236$) or *gyrB* ($\pi = 0.125$). Moreover, *pqqC* allowed a clear grouping of DAPG-producing *Pseudomonas* strains into the five multilocus groups A, C, D, E, and F, as defined by Frapolli et al. (8), hence a differentiation at the subspecies level (see Fig. S3 in the supplemental material).

When comparing a cultivation-independent with a cultivation-dependent approach, we found that the first allowed the detection of more OTUs and that the proportions of *pqqC* clones and *Pseudomonas* isolates per phylogenetic (sub)group were different (Table 3). The large majority of noncultivated bacteria was included in subgroup 1g (with reference strain SBW25), whereas the cultivated isolates were more evenly

distributed. Interestingly, only two out of a total of 108 OTUs were detected by both methods. Our data indicate that the two approaches detect different pseudomonads, thereby complementing each other. The cultivation-dependent method probably detects bacteria that are present in low numbers and thus below the detection limit of the cultivation-independent method but that grow very well in liquid culture; these bacteria are selected for by the cultivation step. This finding is supported by other reports indicating that cultivation-dependent methods allow the identification of different genotypes compared to cultivation-independent methods (9, 45). Bobwhite, the variety used in this study, seems to accumulate pseudomonads of subgroup 1g. For wheat, it is known that there is a cultivar-specific preference for certain *Pseudomonas* groups or genotypes. In a previous study, we have shown that different wheat cultivars accumulate different genotypes of DAPG-producing pseudomonads (27).

The facts that all analyzed pseudomonads were able to solubilize phosphate on NBRIP and that the *pqqC* gene was always amplified in these strains suggest the presence of a functional PQQ enzyme. *Pseudomonas* spp. from the subgroup 1d (corresponding to the newly described *P. protegens*) were found to solubilize significantly more phosphate on NBRIP plates than pseudomonads from other (sub)groups. Similarly, Miller et al. (28) identified strains Pf-5 and CHA0 as superior P solubilizers and hypothesized that multiple copies of *pqqA* and *pqqB*, confirmed by BLAST analysis of the sequenced genome of Pf-5 (NC_004129), could lead to increased PQQ and gluconic acid production. Remarkably, besides being strong phosphate solubilizers, the pseudomonads of subgroup 1d are potent biocontrol bacteria producing multiple antimicrobial substances, such as DAPG, HCN, PLT, and pyrrolnitrin. Another subgroup harboring many efficient phosphate-solubilizing bacteria was subgroup 1g (with reference strain SBW25). There are other studies that have identified *Pseudomonas* strains closely related to SBW25 as strong P solubilizers (4, 13). For future studies, it would be interesting to test strong phosphate solubilizers selected from subgroups 1d and 1g for efficacy *in planta* in comparison to strains of other phylogenetic backgrounds.

In conclusion, we provide strong evidence for the ubiquitous presence of the *pqqC* gene in the genus *Pseudomonas.* Our results on *pqqC* diversity indicate that this gene is conserved within the genus *Pseudomonas* and has a high phylogenetic resolving power comparable to that of the widely used *gyrB* and *rpoD* genes. *pqqC* therefore emerges as a novel marker, complementary to the conventionally used housekeeping genes, which is suited for phylogenetic studies on the *Pseudomonas* genus and for the analysis of *Pseudomonas* populations in natural habitats.

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