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Diversity of *phlD* alleles in the rhizosphere of wheat cropped under annual rice–wheat rotation in fields of the Indo-Gangetic plains: influence of cultivation conditions

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The antibiotic 2,4-diacetyphloroglucinol is a major determinant in the biocontrol of plant growth promoting rhizobacteria associated with crops of agronomic relevance. The *phlD* gene is a useful marker of genetic and phenotypic diversity of 2,4-DAPG-producing rhizobacteria. A two-step amplification procedure was developed in order to assess directly the presence of *phlD* in environmental DNA, avoiding the tedious procedure of *phlD*-positive strain screening and isolation. We found a predominance of one or two *phlD* alleles in wheat fields cultivated in rice–wheat rotations for twenty years, suggesting that continuous rice–wheat cropping would lead to an enrichment of particular *phlD* genotypes. We also recovered new sequences with no close relative among known *phlD* sequences, indicating that part of the *phlD* allelic diversity might have been missed using standard media culture conditions.

Keywords: 2,4-DAPG, wheat rhizosphere, rice–wheat, *phlD*.

THE limited incidence of soil-borne pathogens in the rice–wheat systems, is probably due to the repeated transitions

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from anaerobic (rice season) to aerobic conditions (wheat season). However, survival of pathogens during the rice phase as well as their proliferation on crop residues[1] may reduce the yield up to 20%, if their propagation occurs before leaf emergence[2]. In the rice–wheat cropping fields of the Indo-Gangetic plains region, adoption of the raised beds system allows the upper part of the bed to remain in oxic conditions even during the rice season. The use of this technique optimizes water use efficiency, improves weed management and opens up investment opportunities by diminishing the production costs[1,3]. Nonetheless, raised bed system, by combining reduced-tillage and a permanent oxic zone, could favour re-emergence of pests and diseases kept at low levels during the rice season. The biological approaches that are currently being developed to control a variety of phytopathogenic agents include the use of beneficial free-living bacteria, usually referred to as plant growth promoting rhizobacteria (PGPR)[4]. PGPR are involved in disease management by different mechanisms that include niche exclusion through microbial competition, stimulation of plant defence or production of antibiotics[4,5].

The bacterial antibiotic 2,4-diacetyphloroglucinol (2,4-DAPG) has antifungal, antibacterial, anthelminthic and phytotoxic properties[6]. Moreover, the 2,4-DAPG producing fluorescent pseudomonads from various crops have been shown to share the same biosynthetic locus[7]. The *phlD* gene is located in the *phl*ACBD operon coding for the production of 2,4-DAPG[8]. In addition, the *phlD* gene was shown to be a useful marker of genetic and phenotypic diversity of 2,4-DAPG-producing rhizobacteria[9,10]. This diversity has been studied in relation to biological control, root colonization and soil suppressiveness[11]. To date, diversity studies of 2,4-DAPG producers are mostly based on screening approaches targetting pseudomonad isolates. Several diversity studies of functional bacterial groups showed that only a small fraction of the diversity is revealed by cultivable strains. For example, Hamelin *et al.*[12] demonstrated that the most dominant *nifH* cluster included only environmental clones and there were no sequences related to cultivable diazotrophs in the rhizosphere of *Molinia coerulea*. We can then expect that some key 2,4-DAPG-producing organisms may be ignored by culture-based experiments. Therefore, in order to avoid a cultivation bias, the investigation was aimed at assessing the allelic diversity of the *phlD* gene from DNA extracted directly from the root environment in fields under annual rice–wheat rotation. The diversity of the *phlD* sequence pools was compared in root and root-adhering soil with respect to (i) raised beds versus traditional plain fields, (ii) plain fields with the same practice but with different grain yields.

Trials were carried out in two experimental sites located in Uttar Pradesh, India. The first one was located in Bhavanipur village (Badaun district lat. 28°02′N, long. 79°10′E) and the second near Ghaziabad (Ghaziabad district, lat. 28°40′N, long. 77°28′E). Both sites have a history of twenty years of rice–wheat rotation and the same wheat cultivar UP 2338 (provided by the GB Pant University of Agriculture and Technology, Pantnagar, Uttaranchal, India) was grown for five years. Two conventionally tilled fields of 4000 m^2 were selected on the Bhavanipur site. They were fertilized with 50 kg ha^{-1} urea at their preparation stage and differed mainly by their wheat grain yields[13,14]. They were named low input low yield (LL) and low input high yield (LH). In Ghaziabad, two fields that differed only in agricultural practices were selected: one being a conventionally tilled plain field (PF) and the other a three-year-old practiced raised bed field (RB). The two Ghaziabad fields were fertilized with 120 kg ha^{-1} urea, 40 kg ha^{-1} diammonium phosphate, 40 kg ha^{-1} potash mureate and 25 kg ha^{-1} zinc.

Sampling was performed at the tillering stage (45 d) at Bhavanipur, and at the late tillering stage (60 d) at Ghaziabad, according to Roesti *et al.*[14]. Briefly, three to four wheat plants with roots and soil cores were sampled under semi-sterile conditions. The bulk soil was separated from the adhering soil by shaking the plants. A fraction of the root systems with adhering soil was put into sterile 0.1 M sodium phosphate buffer (pH 7.0) and stirred to separate the rhizosphere soil (RS) from the roots. The washed roots were removed and rinsed with sterile deionized water and constituted the rhizoplane–endorhizosphere (RE) fraction. DNA was extracted from RS and RE fractions by a bead-beater technique (Fast Prep FP120, SAVANT, BIO101, Carlsbad, USA) using a FastDNA Spin Kit (BIO101), according to the manufacturer's protocol. The extracted DNA was further purified with a GENECLEAN® II kit (BIO101) and stored at –20°C in Tris-EDTA (pH 8.0) buffer.

A two-step amplification procedure was designed to increase the *phlD* fragment yield from environmental DNA samples. The first amplification was carried out using phl2a and phl2b[15] primers (Table 1). The amplification reaction mix was composed of 1X Taq DNA polymerase reaction buffer (Promega, Madison, USA), 3 mM $MgCl_2$ (Promega), 0.2 mM of each dNTP (Gibco, Cheshire, UK), 0.25 µM of each primer (Microsynth, Balgach, Switzerland) and 0.05 U $µl^{-1}$ Taq DNA polymerase in buffer B (Promega). A final concentration of 0.1–1 ng $µl^{-1}$ of crude extracted DNA was used as a template for a 20 µl reaction. Amplification was carried out in a PTC-200 Thermocycler (MJ Research Inc., Reno, USA), with an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 40 s, 59°C for 30 s, and 74°C for 45 s, and completed by a final elongation step at 74°C for 10 min. The products of the first amplification were diluted tenfold in sterile, deionized water before being used for nested amplification reaction. Primers phl310f and phl310chf were mixed (50 : 50) prior to use in the PCR mix composed of 1X Taq DNA polymerase reaction buffer (Promega), 3 mM $MgCl_2$ (Promega), 0.2 mM of each dNTP (Gibco), 0.25 µM of the mix containing phl310f and phl310chf (Microsynth),

Table 1. List of primers used in the present study. The relative nucleotide positions were set according to the position of primer phl2b developed by Raaijmakers *et al.*[15] on the *phlD* gene. Annealing temperatures were defined experimentally

Designation	5′-3′ sequence	Size (bp)	Position	T_m (°C)	Reference
phl2a	GAGGACGTCGAAGACCACCA	20	728–747	59.5	15
phl2b	ACCGCAGCATCGTGTATGAG	21	1–20	57.5	15
phl310f	CTCTGCTATCAACCMCA	17	310–326	51.0	This study
phl310chf	CTGTGCTACCAGCCGGA	17	310–326	53.0	This study
phl536r	TTRATGGAGTTCATSAC	17	520–536	47.0	This study

0.25 µM of primer phl536r (Microsynth) and 0.05 U μl^{-1} Taq DNA polymerase in buffer B (Promega). The nested amplification programme consisted of an initial denaturation at 95°C for 4½min, followed by 34 cycles at 94°C for 30 s, 52°C for 1 min and at 74°C for 30 s, and completed by a final elongation step at 74°C for 10 min. A DNA fragment of 227 bp in size was generated after the nested PCR. The two-step PCR assay was first validated on the 2,4-DAPG-producing strains CHA0 (ref. 16), PGNL1 (ref. 7), PGNR1 (ref. 7), PITR2 (ref. 7), PILH1 (ref. 7), TM1'A4 (ref. 7) and TM1A3 (ref. 17). It was further tested on environmental DNA from tobacco rhizosphere planted in vineyard soils enabling to retrieve five main *phlD* clusters (F. Poly, unpublished). No PCR product was obtained from *phlD*$^-$ reference strains *Escherichia coli* (Neuchâtel collection of microorganisms Neu 1006), *Pseudomonas aeruginosa* (ATCC 10145), *Alcaligenes faecalis* (Neu 1033), *Aquaspirillum autotrophicum* (ATCC 29984) and *Micrococcus luteus* (Neu 1013).

PCR products were purified employing a *NUCLEO TRAP-CR kit* (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The ligation was carried out in pGEM®-T Vector System (Promega), following manufacturer's protocol. Transformation was performed by electroporation using the *Bio-rad Gene Pulser XCell* and *PC module* into *E. coli* XLI-Blue. The transformed colonies were plated onto Luria–Bertani (LB) agar containing ampicillin (150 µg ml^{-1}), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (0.1 mM) and IPTG (isopropyl-β-D-thiogalactopyranoside) (0.2 mM). Plasmids were recovered from white colonies using a NucleoSpin Plasmid kit (Macherey–Nagel) according to the manufacturer's protocol. The inserts were sequenced by Synergen (Schlieren, Switzerland). *phlD* sequences were aligned using the ClustalX software[18] and trees were constructed using the neighbour-joining method[19] on 227 bp sequences with the NJplot software (ftp://pbil.univ-lyon1.fr/pub/mol_phylogeny/njplot)[20]. The topology of the distance tree was tested by resampling data with 100 bootstraps[21] to provide confidence estimates for tree topologies.

The amplicons obtained from environmental DNA were cloned and sequenced. All the products were related to *phlD* sequences (sequence identity > 93%). Sequencing of the 227 bp *phlD* fragment was more discriminating than RFLP analysis performed on a bigger *phlD* fragment by Ramette *et al.*[10]. Figures 1 and 2 represent the phylogenetic position of the 227 bp *phlD* sequences compared to *phlD* sequences for known DAPG producers, for Ghaziabad and Bhavanipur sites, constructed according to previously defined clusters by Ramette *et al.*[10]. Sequences with a high level of similarity were grouped into clusters. For Ghaziabad environmental sequences, two main clusters A and B were identified (Figure 1). Cluster A included three of the seven clones of the RE fraction of wheat plant cropped under the raised beds agricultural practice and the *phlD* sequence of the *Pseudomonas* sp. biocontrol strain *M196*. Cluster B included sequences retrieved from RS and RE of both plain field and raised bed practices, and were not related to any reference sequences. The discrepancy between the *phlD* sequences related to plain field and raised bed practice would have been triggered by the recent introduction of the latter. This observation is consistent with several other studies, suggesting that modifications in the agronomic practices influence the bacterial community structure in the rhizosphere[22]. The environmental *phlD* sequences from Bhavanipur fields were grouped into three different clusters, according to the field or the origin of the root fraction (Figure 2), i.e. cluster A and B (cf. the sequences from Ghaziabad fields) and cluster C, which included CHA0 reference strain. Cluster A contained exclusively sequences of the LH field (RS and RE fractions), whereas cluster B contained sequences from the RE fraction for both LL and LH fields; cluster C contained sequences from the RS fraction of LL. In case of the LL field, a particular allele was selected with respect to the vicinity of the root. Similarly, Picard *et al.*[23] observed a spatial selection gradient for DAPG producers, which most probably resulted from the selective influence of root exudates.

Investigations carried out on pseudomonad populations suggested that strains with different *phlD* alleles may coexist in the rhizosphere of the same dicot at a particular geographic location[24,25]. Our field studies revealed that there was a predominance of one or two *phlD* alleles within the same field condition and rhizosphere fraction. Possibly, flooded field condition during the rice season could lower the level of *Pseudomonas* spp. population, affecting *phlD* diversity. Moreover, Mavrodi *et al.*[9] reported that in different take-all decline fields that were subjected to wheat monoculture, up to four different *phlD* alleles

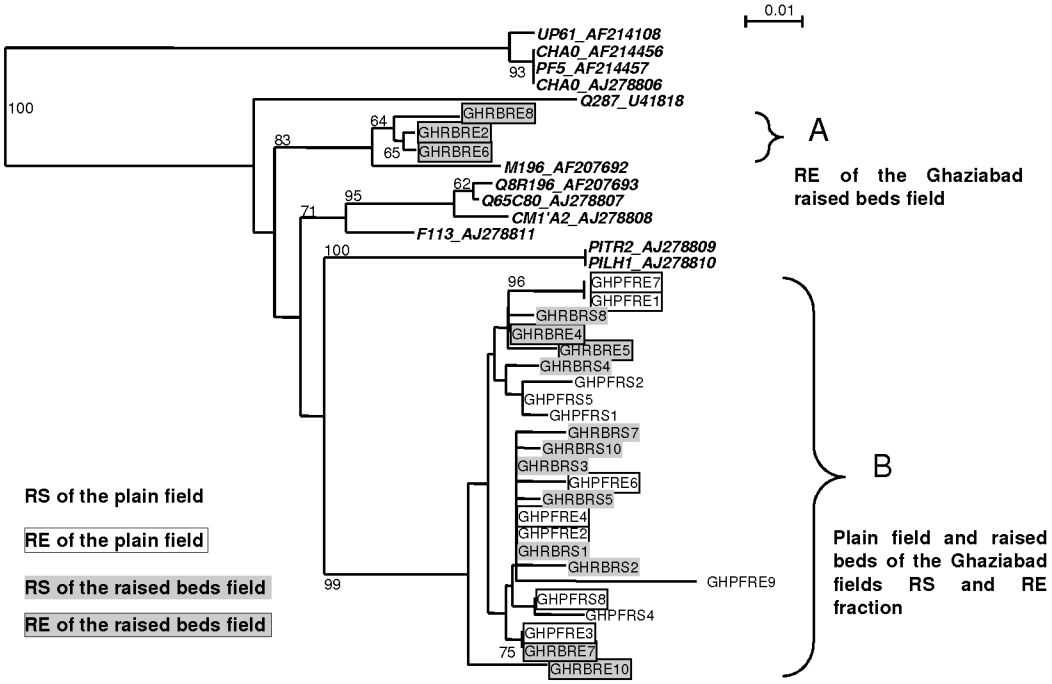

Figure 1. Phylogeny to *phlD* nucleotide sequences using partial *phlD* gene sequences from the GenBank database (bold, italics) and sequences obtained from cloning of the 227 bp amplicons from DNA extracted from the soil and root fractions of the wheat rhizosphere, on the basis of Ghaziabad raised beds and plain fields samples. The tree was constructed by neighbour-joining method[19] with Njplot[20]. Robustness of the inferred tree was assessed by 100 bootstrap replicates. Nodal support is indicated when higher than 50%. Scale bar indicates the number of substitutions per site.

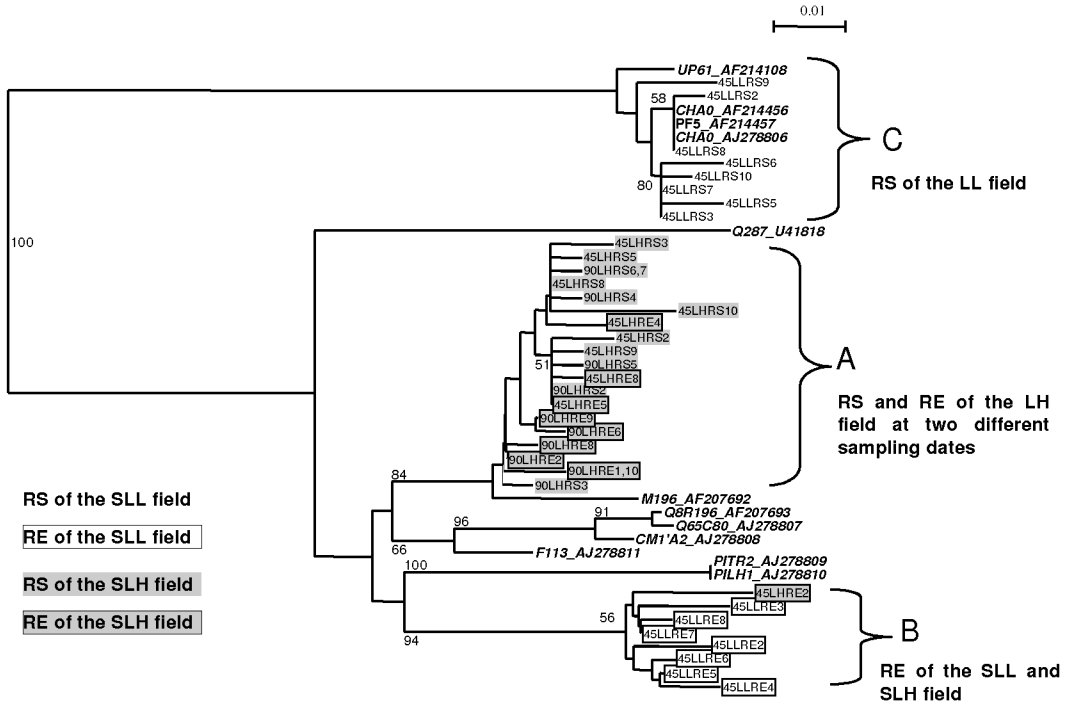

Figure 2. Phylogeny to *phlD* nucleotide sequences using partial *phlD* gene sequences from the GenBank database (bold, italics) and sequences obtained from cloning of the 227 bp amplicons from DNA extracted from the soil and root fractions of the wheat rhizosphere, on the basis of Bhavanipur samples. The tree was constructed by neighbour-joining method[19] with Njplot[20]. Robustness of the inferred tree was assessed by 100 bootstrap replicates. Nodal support is indicated when higher than 50%. Scale bar indicates the number of substitutions per site.

could be found and that a single *phlD*-based genotypic group dominated in the rhizosphere at each location studied. Their data indicated that monoculture conditions maintained for several decades might promote an enrichment of certain genotypes of DAPG-producing *Pseudomonas* spp. The continuous rice–wheat rotation for twenty years and the use of the same wheat cultivar in our studied fields might have created homogenous conditions, similar to monoculture practices.

Studies on *phlD*-harbouring bacteria have until now focused on the cultivable fluorescent pseudomonad group[11]. However, Picard *et al.*[23] isolated *phlD*$^+$ strains not only related to the fluorescent pseudomonads group, but also to the family Enterobacteriaceae. Interestingly, our molecular tools applied to environmental DNA allowed to recover new sequences with no close relative among known *phlD* sequences (cluster B). Part of the *phlD* allelic diversity might therefore have been missed using standard culture media conditions. Moreover, it is possible that non-cultivable (or not-yet cultured) bacteria could play a significant role in the biocontrol of cultivated plants.

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