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# Rhizosphere bacterial communities associated with disease suppressiveness stages of take-all decline in wheat monoculture

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## Summary

• The decline of take-all disease (*Gaeumannomyces graminis* var. *tritici*), which may take place during wheat monocropping, involves plant-protecting, root-colonizing microorganisms. So far, however, most work has focused on antagonistic fluorescent pseudomonads. Our objective was to assess the changes in rhizobacterial community composition during take-all decline of field-grown wheat.

• The study was based on the development and utilization of a taxonomic 16S rRNA-based microarray of 575 probes, coupled with cloning–sequencing and quantitative PCR. Plots from one experimental field grown with wheat for 1 yr (low level of disease), 5 yr (high level of disease) or 10 yr (low level of disease, suppressiveness reached) were used.

• Microarray data discriminated between the three stages. The outbreak stage (5 yr) was mainly characterized by the prevalence of *Proteobacteria*, notably *Pseudomonas* (*Gammaproteobacteria*), *Nitrosospira* (*Betaproteobacteria*), *Rhizobacteriaceae*, *Sphingomonadaceae*, *Phyllobacteriaceae* (*Alphaproteobacteria*), as well as *Bacteroidetes* and *Verrucomicrobia*. By contrast, suppressiveness (10 yr) correlated with the prevalence of a broader range of taxa, which belonged mainly to *Acidobacteria*, *Planctomycetes*, *Nitrospira*, *Chloroflexi*, *Alphaproteobacteria* (notably *Azospirillum*) and *Firmicutes* (notably *Thermoanaerobacter*).

• In conclusion, take-all decline correlated with multiple changes in rhizobacterial community composition, far beyond the sole case of pseudomonads.

## Introduction

One of the soil microbial ecology challenges is to relate bacterial diversity to the biological status of the soil in terms of biotransformation potential and/or plant growth and health. The latter may involve plant-beneficial interactions, resulting in plant protection from phytopathogens. The bacterial biocontrol of phytopathogens by soil-inhabiting populations has been demonstrated in disease-suppressive soils, based on the study of specific bacterial groups (Mazzola, 2002; Cook, 2003; Raaijmakers *et al.*, 2009). However, very little has been done to implement systematic studies at the scale of the bacterial community (Weller *et al.*, 2002; Yin *et al.*, 2003).

Take-all of wheat (*Triticum aestivum*) is an important disease caused by the soil-borne fungus *Gaeumannomyces graminis* (Sacc.) Arx and Olivier var. *tritici* Walker (*Ggt*) (Hornby *et al.*, 1998). Control of the disease may be achieved using rotation crops (Cook, 2003), delayed sowing (Colbach *et al.*, 1997) and ammoniac fertilizers (Sarniguet *et al.*, 1992a,b). Soil may become suppressive to take-all in the case of long-term wheat monoculture (Hornby, 1979; Weller *et al.*, 2002). Indeed, repeated cropping of wheat leads to increased disease severity during the first years, but,

afterwards, take-all decline (TAD) naturally takes place and low symptom levels are then recorded as long as wheat monoculture continues (Cook, 2003). Lebreton *et al.* (2004) showed that *Ggt* population structure changed during continuous wheat cropping, and aggressiveness differed between *Ggt* populations. A linear relationship was found between *Ggt* genotype frequencies and disease incidence (Lebreton *et al.*, 2007). Therefore, TAD may involve *Ggt* inhibition and/or enrichment of less aggressive genotypes within the *Ggt* complex.

In the case of TAD, disease suppressiveness is attributed to the microbial component of soil, based on the observations that suppressiveness can be transferred to a conducive soil by inoculation, is eliminated by steam pasteurization of soil and develops only in the presence of the pathogen *Ggt* (Mazzola, 2002; Weller *et al.*, 2002). So far, the analysis of microbial populations associated with TAD has focused mainly on antagonistic fluorescent *Pseudomonas* spp. colonizing wheat roots (Sarniguet & Lucas, 1992; Raaijmakers & Weller, 1998), especially those producing antimicrobial compounds such as phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol and pyrrolnitrin (Mazzola *et al.*, 1992; Weller *et al.*, 2007). Differences in rhizosphere *Pseudomonas* populations in relation to TAD were evidenced by 16S rRNA-based microarray analysis in a field experiment (Sanguin *et al.*, 2008). In addition, *Pseudomonas* isolates producing antimicrobial compounds have been used successfully as wheat inoculant for the biological control of the pathogen *Ggt* (Thomashow & Weller, 1988; Keel *et al.*, 1992; Raaijmakers & Weller, 1998). However, relatively little has been done so far to assess the role played by non-*Pseudomonas* bacterial populations in TAD (Andrade *et al.*, 1994; McSpadden Gardener & Weller, 2001), and whether these bacteria can contribute to TAD remains to be established. Many non-*Pseudomonas* bacteria protect plants from soil-borne pathogenic fungi (Dunne *et al.*, 1997; van Dijk & Nelson, 1998; Hebbar *et al.*, 1998; Ryder *et al.*, 1998; Bally & Elmerich, 2007; Raaijmakers *et al.*, 2009) and some may be involved in soil disease suppressiveness (Andrade *et al.*, 1994). Several studies have shown the potential of bacteria belonging to *Actinobacteria* and *Bacillus* to inhibit the growth of the pathogen *Ggt* (Kim *et al.*, 1997; Coombs *et al.*, 2004). Other bacterial groups have been studied using fingerprinting and sequencing methods (McSpadden Gardener & Weller, 2001). One of the main drawbacks of fingerprinting methods is that the identification of bacterial community members is restricted. This limitation can be overcome with the use of microarray technology, which furthermore enables high-throughput analysis (Stralis-Pavese *et al.*, 2004; Sanguin *et al.*, 2006a). A prototype taxonomic microarray targeting the 16S rRNA gene has been developed and been proven to be useful for monitoring the diversity of bacterial populations or subpopulations in complex ecosystems, such as soil or the rhizosphere (Sanguin *et al.*, 2006a,b, 2008).

The objective of this work was to assess whether the composition of the rhizobacterial community changes during TAD and to identify bacterial populations associated with the main TAD stages, using one single experimental field site in which TAD epidemics, *Pseudomonas* and *Ggt* populations were extensively monitored (Lebreton *et al.*, 2004, 2007; Sanguin *et al.*, 2008). Rhizosphere samples were collected from plots grown with wheat for 1 yr (low level of take-all disease, PI), 5 yr (high level of disease, PV) or 10 yr (low level of disease; suppressiveness reached, PX). The changes in bacterial community were assessed with a 16S rRNA-based microarray, which was further developed from a previous microarray (reaching 575 probes in all) to extend its coverage, and with cloning–sequencing.

## Materials and Methods

### Field site and environmental samples

A long-term field experiment established on a luvisol soil (La Gruche, France) and showing all the steps of wheat TAD (Lebreton *et al.*, 2007; Sanguin *et al.*, 2008) was used. Nine plots (6 × 9 m) corresponding to a first (stage PI; three plots), fifth (PV; three plots) and tenth (PX; three plots) year of monoculture with wheat (*Triticum aestivum* L.) cv. Caphorn were studied. First, take-all disease [*Gaeumannomyces graminis* (Sacc.) Arx and Olivier var. *tritici* Walker (*Ggt*)] was assessed at flowering (i.e. GS 65 according to Zadocks' scale; Zadocks *et al.*, 1974) using 20 plants per plot. This was based on the assessment of take-all incidence (i.e. the ratio between the number of infected roots and the number of total roots) and disease severity (i.e. the percentage of root length diseased) on each plant (Lebreton *et al.*, 2007), using roots taken in the silty surface horizon (depth, 10 cm) (1.7% organic matter, pH 5.7).

Second, molecular analyses were carried out using one plant per plot that was representative of the mean health status in the plot (i.e. a total of nine plants: I2, I4 and I5 from PI; V2, V4 and V5 from PV; X2, X4, and X5 from PX). The root systems of wheat plants [from the surface horizon (depth, 10 cm)] were vigorously shaken to dislodge loosely adhering soil, which was discarded. Each root system still held 10–20 g of rhizosphere soil (i.e. tightly adhering soil) from which 0.5 g of rhizosphere soil was used for DNA extraction.

### DNA extraction and PCR amplification of 16S rRNA gene for microarray experiments

Genomic DNA was extracted from pure bacterial cultures (listed in Table 1) with a DNeasy Tissue Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's

**Table 1** Strains used in this study

| Species | Strain | Source or reference |
|---|---|---|
| *Gammaproteobacteria* | | |
| *Pseudomonas* group ARDRA 1 | CHA0 | Keel *et al.* (1992) |
| | Pf-5 | Howell & Stipanovic (1979) |
| *P. chlororaphis* | DSM6698$^T$ | DSMZ |
| *'P. fluorescens'* complex | F113 | Sharifi-Tehrani *et al.* (1998) |
| | PITR2 | Sharifi-Tehrani *et al.* (1998) |
| | P97.26 | Sharifi-Tehrani *et al.* (1998) |
| | CM1'A2 | Sharifi-Tehrani *et al.* (1998) |
| | Q2-87 | Vincent *et al.* (1991) |
| | KD | Sharifi-Tehrani *et al.* (1998) |
| | K94.31 | Blaha *et al.* (2006) |
| | P97.30 | Blaha *et al.* (2006) |
| | TM1A3 | Sharifi-Tehrani *et al.* (1998) |
| *P. syringae* pv. *pisi* | 203 | Taylor *et al.* (1989) |
| *P. stutzeri* | ATCC11607 | Van Niel & Allen (1952) |
| *P. putida* | KT2442 | Huijberts *et al.* (1992) |
| *P. aeruginosa* | PAO1 | Stover *et al.* (2000) |
| *Betaproteobacteria* | | |
| *Burkholderia cenocepacia* | J2315 | Palfreyman *et al.* (1997) |
| *Burkholderia xenovorans* | LB400 | Goris *et al.* (2004) |
| *Ralstonia solanacearum* | GMI1000 | Blaha *et al.* (2006) |
| *Achromobacter xylosoxidans* | Cm4 | Belimov *et al.* (2001) |
| *Alphaproteobacteria* | | |
| *Azospirillum lipoferum* | 4B | Blaha *et al.* (2006) |
| *Azospirillum brasilense* | Sp245 | Blaha *et al.* (2006) |
| *Sphingobium francense* | Sp+ | Cérémonie *et al.* (2006) |
| *Rhizobium etli* | CFN42 | Quinto *et al.* (1985) |
| *Agrobacterium* genomic group 1 | TT111 | Mougel *et al.* (2002) |
| *Agrobacterium* genomic group 8 | C58 | Mougel *et al.* (2002) |
| *Nitrobacter hamburgensis* | X14 | Orso *et al.* (1994) |
| *Wolbachia pipientis* | wRi | Mavingui *et al.* (2005) |
| *Actinobacteria* | | |
| *Rhodococcus* sp. | Fp2 | Belimov *et al.* (2001) |
| *Frankia* sp. | ACN14a | Simonet *et al.* (1985) |
| *Firmicutes* | | |
| *Bacillus* sp. | Bac1 | Sanguin *et al.* (2006b) |
| *Bacillus megaterium* | CIP102542 | CIP |
| *Bacillus cereus* | LMG6923$^T$ | Achouak *et al.* (1999) |
| *Bacillus flexus* | CIP106928 | CIP |
| *Bacillus subtilis* | LMG7135$^T$ | Achouak *et al.* (1999) |
| *Paenibacillus amylolyticus* | LMG11153$^T$ | Achouak *et al.* (1999) |
| *Paenibacillus lautus* | LMG11157$^T$ | Achouak *et al.* (1999) |
| *Paenibacillus glucanolyticus* | LMG12239$^T$ | Achouak *et al.* (1999) |
| *Paenibacillus peoriae* | LMG14832$^T$ | Achouak *et al.* (1999) |
| *Paenibacillus azotofixans* | ATCC35681$^T$ | Achouak *et al.* (1999) |

instructions, or by thermic shock, to allow PCR amplification. DNA extraction of the nine wheat rhizosphere samples was performed following the protocol of Griffiths *et al.* (2000).

The 16S rRNA genes from strains and environmental samples were amplified using universal primers pA and pH′ (Bruce *et al.*, 1992), giving a 1.5 kb amplicon. The forward primer contained a T7 promoter site (5′-TAATACG-ACTCACTATAG-3′) at the 5′end, which enabled T7 RNA polymerase-mediated *in vitro* transcription using the PCR products as templates. The PCR mixture (50 μl) contained 1 × reaction buffer, each primer at 0.5 μM, 1.5 mM $MgCl_2$, 50 μM of each dNTP and 15 ng of environmental DNA or 50 ng of strain genomic DNA. *Taq* Expand High Fidelity (1.25 U; Roche Applied Science, Meylan, France) and T4 Gene 32 (0.025 mg $ml^{-1}$; Roche Applied Science) were used for environmental samples, and *Taq* DNA polymerase (5 U; Invitrogen, Cergy Pontoise, France) for strain DNA. Thermal cycling was carried out with a denaturation step of 94°C for 3 min, 35 cycles of 45 s denaturation at 94°C, 45 s annealing at 55°C, 90 s elongation at 72°C, and a final elongation step for 7 min at 72°C.

PCR products were purified through a QIAquick PCR purification column (Qiagen) according to the

manufacturer's instructions. The DNA concentration was determined spectrophotometrically by measuring the optical density at 260 nm.

### Oligonucleotide probe design, microarray assays and experimental validation

Fluorescence labelling of 16S rRNA PCR products from the nine wheat rhizosphere samples, manufacturing, processing, hybridization and scanning of the 16S rRNA gene-based microarrays were performed as described in Sanguin *et al.* (2008).

Probes were designed according to Sanguin *et al.* (2008) using the phylogenetic software package ARB (Ludwig *et al.*, 2004; http://www.arb-home.de/). Bacteria targeted by each probe, with up to 3.5 weighted mismatches (WMMs), were listed with the Probe Match function. The Probe Match output files are available on request and the probe set characteristics are listed in Table S1.

Probe set specificity was evaluated using 15 *Pseudomonas* and 24 non-*Pseudomonas* strains belonging to the *Alphaproteobacteria*, *Betaproteobacteria*, *Firmicutes* and *Actinobacteria*. The relation between hybridization intensity (to be described later) and WMM value was analysed to determine the RNA/DNA hybridization threshold of the probe set, as described previously by Sanguin *et al.* (2006a). In addition, the hybridization data corresponding to the 39 strains hybridized were compared with the expected probe/target WMM patterns.

### Microarray data analysis

Data filtration, normalization and statistical analyses were performed as described in Sanguin *et al.* (2008). Hybridization intensity values were expressed as relative fluorescent units (RFUs) corresponding to the normalized intensity value, as described in Sanguin *et al.* (2008).

Data from strain hybridizations were subjected to cluster analysis using CLUSFAVOR version 6.0 (http://condor.bcm.tmc.edu/genepi/clusfavor.html) via the unweighted pair group with mathematical average (UPGMA) method and the correlation distance function (DeSantis *et al.*, 2005). Analysis of microarray data from rhizosphere samples was performed by principal component analysis (PCA), as described previously (Sanguin *et al.*, 2006a), and the significance of the differences between treatments derived from PCA was evaluated using analysis of variance (ANOVA) tests. For each probe, the comparison between treatments PI, PV and PX was also assessed using ANOVA, followed (as ANOVA was significant) by Tukey's honestly significant different (HSD) tests. Statistics were performed at $P < 0.05$ using the *R* statistical computing environment (http://www.r-project.org).

### Quantitative polymerase chain reaction (qPCR) for the monitoring of the total bacterial community and pseudomonads

The abundances of the total bacterial community and of *Pseudomonas*-related populations were estimated by qPCR targeting 16S rRNA genes. The Com1 (*Escherichia coli* position 519–536)/Com2 (*E. coli* position 907–926) primers (Schwieger & Tebbe, 1998) were used to target the total bacterial community, and pA (*E. coli* position 8–27)/Pseu1r (*E. coli* position 226–245) primers (Bruce *et al.*, 1992; Sanguin *et al.*, 2008) for *Pseudomonas*-related populations in independent runs employing an ABI Prism 770 HT (Applied Biosystems, Courtaboeuf, France). The conditions were as follows: 10.5 μl $H_2O$, 12.5 μl Mastermix Taq-SybrGreen 2× (Kit Quick Gold Star; Eurogentec, Angers, France), 0.5 μl of each 12.5 μM primer and 1 μl of DNA (from wheat rhizosphere sample; 0.5 g). The amplification conditions were as follows: 3 min at 95°C, followed by 40 cycles with two steps of 15 s at 95°C and 1 min at 60°C. The purity of the amplified products was checked with the dissociation analysis based on 15 s at 95°C, 20 s at 60°C and a progressive temperature upgrade to 95°C during 19 min 59 s. The DNA quantity was calculated using the reference qPCR amplification with Com1/Com2 and pA/Pseu1r primers conducted on 1 μl of a standard corresponding to 0.007–1.4 ng of 16S rRNA PCR products obtained previously from wheat rhizosphere DNA using pA/pH' primers.

qPCR experiments were conducted on the nine wheat rhizosphere samples already used for microarray analysis, and two or three technical replicates for the total bacterial community and *Pseudomonas*-related populations, respectively. Data were expressed as the number of gene targets per gram of soil and were statistically compared using ANOVA and a Newman–Keuls test at $P < 0.05$.

### Cloning and sequencing of 16S rRNA environmental clones

PCR products obtained after qPCR from V2 and X2 rhizosphere samples (as mentioned in the previous section) were cloned using a TA cloning kit (Invitrogen) in *Escherichia coli* TOP F10 (Kit OneShot; Invitrogen), according to the manufacturer's recommendations. For cloning, DNA products obtained from qPCR were re-amplified separately with standard conditions using the same Com1/com2 and pA/Pseu1r primer pairs. Sequencing (Macrogen, Seoul, Korea; http://www.dna.macrogen.com) was performed for 47 clones from sample V2 and 52 clones from sample X2 for the total bacterial amplicon (Com1/Com 2), as well as 45 clones from sample V2 and 42 clones from sample X2 for the *Pseudomonas*-related amplicon (pA/Pseu1r).

The detection of potential chimeric 16S rRNA gene sequences was evaluated by comparing the sequence affiliation results obtained using the NAST alignment tool (DeSantis *et al.*, 2006b) and the Classify tool (http://greengenes.lbl.gov/cgi-bin/nph-classify.cgi) from the online ribosomal RNA database Greengenes (DeSantis *et al.*, 2006a), and the SINA webaligner tool (http://www.arb-silva.de/aligner/) from the online ribosomal RNA database SILVA (Pruesse *et al.*, 2007). Taxonomy proposed by three independent curators (Ribosomal Database Project (RDP), Cole *et al.*, 2005; Hugenholtz, 2002; NCBI, http://www.ncbi.nlm.nih.gov/) was followed. Fifteen 16S rRNA sequences (total bacterial amplicons) were excluded because of noncongruent results between the classifications obtained by the different curators or nonreliable affiliation using the SINA webaligner tool. All the sequences obtained in this work have been submitted to the EMBL database under accession numbers FN295956 to FN296039 (total bacterial amplicons) and FN298516 to FN298602 (*Pseudomonas*-related amplicons).

### Phylogenetic analyses of 16S rRNA environmental clones

The 16S rRNA relatives from Greengenes and SILVA databases (Table S2) were used for phylogenetic analyses of the 16S rRNA environmental clones from the total bacterial amplicon. The phylogenetic tree was constructed using the multiple alignment software CLUSTAL X (Thompson *et al.*, 1997), and phylogenetic analyses were inferred using MEGA version 4.0.1 (Kumar *et al.*, 2001). Distances between sequence pairs, the deduced phylogenetic tree and bootstrap values were all computed using the global gap removal option. The neighbour-joining method (Saitou & Nei, 1987) and Kimura 2-parameter were used. The nodal robustness of the tree was assessed using 500 bootstrap replicates and an interior branch test (*t*-test). Affiliation of 16S rRNA environmental clones corresponding to the *Pseudomonas*-related amplicon was performed using Greengenes classify tools (Table S3).

## Results

### Extension and validation of the probe set

In addition to the 183 probes already available (Sanguin *et al.*, 2006a), 474 new 16S rRNA probes were designed, resulting in a total of 657 probes. When probe hybridization intensities and WMM values were compared for the 39 strains studied, a sharp decrease in hybridization intensity for WMM values above 1.5 (Fig. S1) was observed. Thus 1.5 WMM was taken as the hybridization threshold for the probe set in the current conditions. Based on this threshold, 82 of 657 probes (i.e. 12%) were discarded after pure strain hybridizations because of unreliable results in our hybridization conditions, resulting in a final set of 575 probes (Table S1). The final probe set targets bacteria belonging to 21 phyla (*Eubacteria*), including the *Proteobacteria* (56.4% of the probe set), *Firmicutes* (12.9%), *Actinobacteria* (9.7%), *Bacteroidetes* (5.6%), *Cyanobacteria* (4.9%), *Planctomycetes* (2.4%), *Acidobacteria* (1.6%), *Nitrospira* (0.9%), *Deferribacteres* (0.7%), *Verrucomicrobia* (0.3%), *Thermotogae* (0.3%), *Fibrobacteres/Gemmatimonadetes* (0.2%), *Chloroflexi* (0.2%), *Fusobacteria* (0.2%), *Chlorobi* (0.2%), *Chlamydiae* (0.2%), *Deinococcus-Thermus* (0.2%), *Thermodesulfobacteria* (0.2%) and recently established divisions OP11 (0.7%) and OP2 (0.2%), as well as *Euryarchaetoa* (*Archaea*; 0.7%).

Comparison of experimental and expected hybridization patterns (Fig. 1) on the final probe set, according to the hybridization threshold of 1.5 WMM, revealed 0.75% of false positives among the 22 425 individual probe × target hybridizations. Considering only hybridizations yielding moderate or high intensity values (> 0.017 RFU, third quartile), false positives dropped to 0.20%, showing that high intensities correspond to very reliable hybridizations. The main cross-hybridizations (Fig. 1) were obtained for *Betaproteobacteria*-probe BONE23Am with nontargets belonging to *Pseudomonas* and *Burkholderia* (WMM > 1.5), as well as *Leginonellales* probe Legiob (at low hybridization intensity levels) with some *Pseudomonas* and *Frankia* strains (WMM > 1.5). Other probes, such as *Betaproteobacteria* probe Beta4, *Burkholderia* probe Burkho3 and *Alphaproteobacteria* probe Rgal157, displayed a broader range of taxonomic groups detected under the hybridization conditions used (WMM < 1.5) compared with those initially targeted (perfect match; WMM = 0). Consequently, positive hybridizations of these probes should be carefully interpreted in complex samples. Cluster analysis of strain hybridization data indicated that the microarray had the potential to discriminate between bacterial strains at different taxonomic levels (Fig. 2). The results were in agreement with the phylogenetic relationship for 37 of the 39 strains studied, as only two strains inside the *Bacillus/Paenibacillus* group were misplaced.

### Field experiment and taxa identified

The take-all incidence (i.e. the ratio between the amount of infected roots and the amount of total roots) was 89 ± 16 for PV, **vs** only 14 ± 16 for PI and 30 ± 26 for PX. In addition, the disease severity (i.e. percentage of infected roots) was 69 ± 21% for PV **vs** 14 ± 16% for PI and 24 ± 25% for PX. Thus, plant health status in the plots studied fits with the main stages of the TAD process, and the results are in accordance with the findings of Lebreton *et al.* (2007) for the whole experimental field.

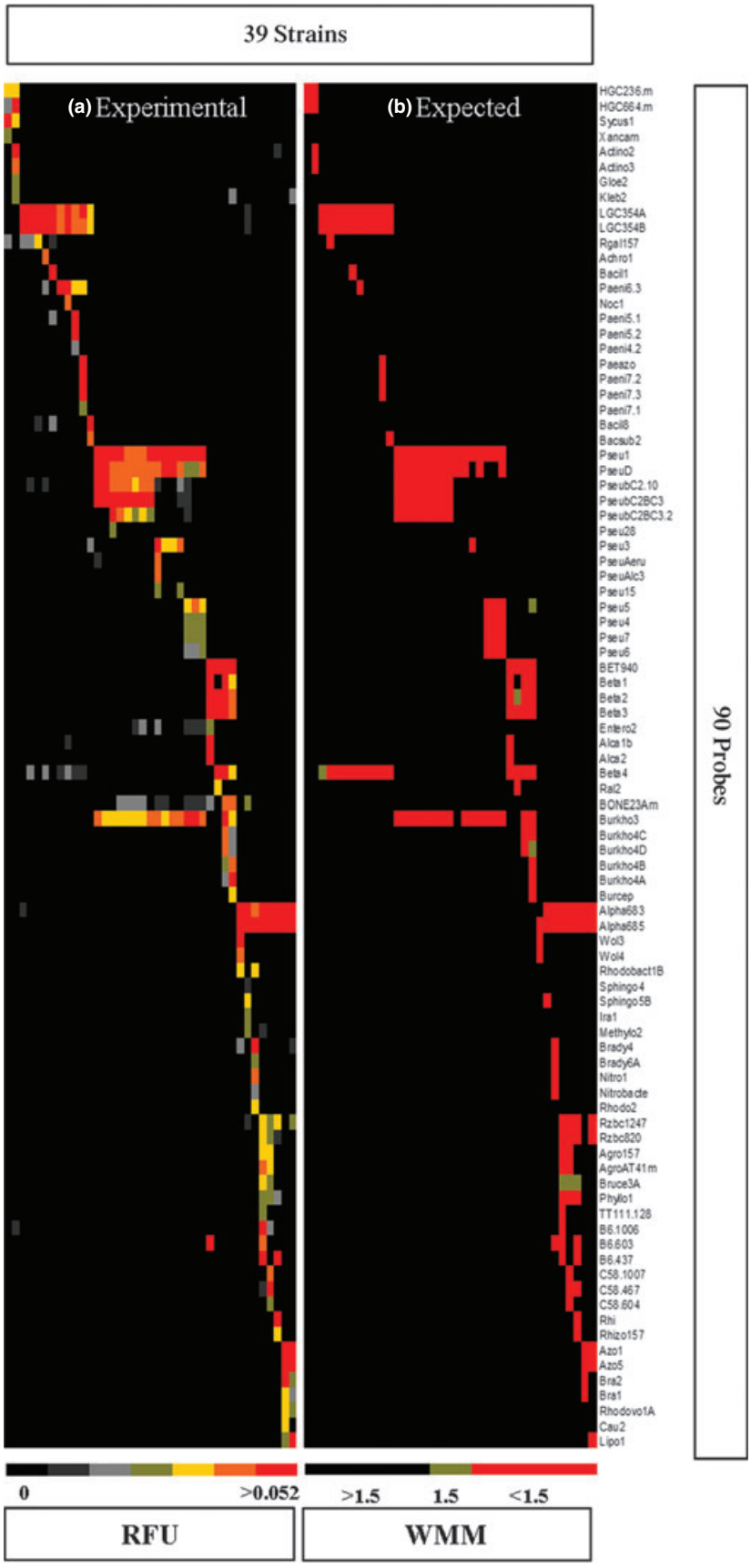



**Fig. 1** Comparison of experimental (a) and expected (b) hybridization patterns for 39 strains based on the hybridization threshold of 1.5 weighted mismatches (WMMs). The colour scale (a) represents the range of hybridization intensity values for whole strain hybridizations (first quartile, 0.006; second quartile, 0.028; third quartile, 0.052). Colour gradations are from black to green to red, denoting no intensity to moderate intensity to high intensity, respectively. RFU, relative fluorescent unit. The colour scale (b) represents the range of WMM based on the hybridization threshold of 1.5 WMM. Colour gradations are from black to green to red, denoting WMM values superior to 1.5, equal to 1.5 and inferior to 1.5 WMM, respectively. Black indicates nonexpected hybridization and red indicates expected hybridization. Green indicates expected hybridization to be considered with care because the WMM value corresponds to the hybridization threshold. Only the probes that yielded moderate intensity values for at least one strain are represented.

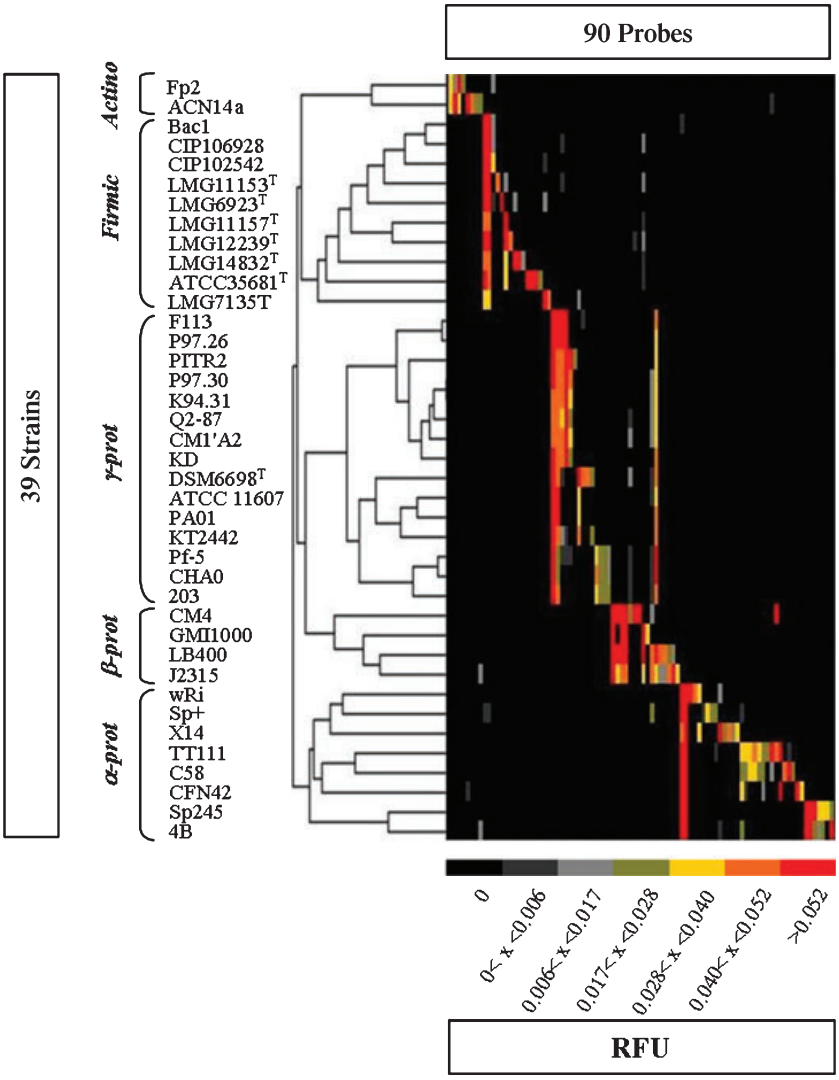



**Fig. 2** Hybridization patterns of 39 strains belonging to *Proteobacteria* (*Gammaproteobacteria*, *Betaproteobacteria* and *Alphaproteobacteria*), *Actinobacteria* and *Firmicutes*. Analysis of strain hybridization data was performed using CLUSFAVOR version 6.0, employing the unweighted pair group with mathematical average (UPGMA) method and the correlation distance function. The colour scale represents the range of hybridization intensity values for whole strain hybridizations (first quartile, 0.006; second quartile, 0.028; third quartile, 0.052). Colour gradations are from black to green to red, denoting no intensity to moderate intensity to high intensity, respectively. Only the probes that yielded moderate intensity values for at least one strain are represented. RFU, relative fluorescent unit. *Actino*, *Actinobacteria*; *Firmic*, *Firmicutes*; *γ-prot*, *Gammaproteobacteria*; *β-prot*, *Betaproteobacteria*; *α-prot*, *Alphaproteobacteria*.

In the field experiment, 16S rRNA environmental libraries were constructed from two wheat rhizosphere samples (V2 and X2) for the identification of prevalent taxa. Eighty-four 16S rRNA clones were analysed, revealing 63% affiliation to *Proteobacteria* or *Acidobacteria*, whereas the others corresponded to *Actinobacteria*, *Chloroflexi*, *Nitrospira*, *Verrucomicrobia*, *Planctomycetes*, *Bacteroidetes*, *Fibrobacteres* and the newly described *Gemmatimonadetes* (Fig. 3). No *Pseudomonas* clones were found. All the phyla identified by cloning–sequencing were also detected by microarray (Fig. 3), except for *Fibrobacteres/Gemmatimonadetes*, probably as a result of the poor representation of these taxa in the current probe set. By contrast, positive hybridization signals were observed for probes targeting *Epsilonproteobacteria*, *Firmicutes* and *Cyanobacteria*, which were not detected by cloning–sequencing. At the lower taxonomic level, congruent results between the two approaches were observed for the majority of *Alphaproteobacteria* and *Betaproteobacteria* families (i.e. *Rhizobiaceae*, *Rhodospirillaceace*, *Phyllobacteriaceace*, *Acetobacteraceae*, *Burkholderiaceae* and *Commamonadaceae*), as well as *Acidobacteria* (Groups 6/4/1). In the case of *Gammaproteobacteria*, the taxa detected by the two approaches were different, that is *Xanthomonadaceae* by cloning–sequencing and *Pseudomonadaceae/Enterobacteriaceae* by microarray. No reliable comparison could be performed for the other taxa at low taxonomic levels, mainly because of the lack of taxonomic information for the 16S rRNA environmental clones.

## Dynamics of the total bacterial community during TAD

The range of hybridization intensity levels (Fig. S2), the number of positive probes and the taxonomic groups identified were rather similar for the three TAD stages, but the latter differed in terms of the probe hybridization intensity

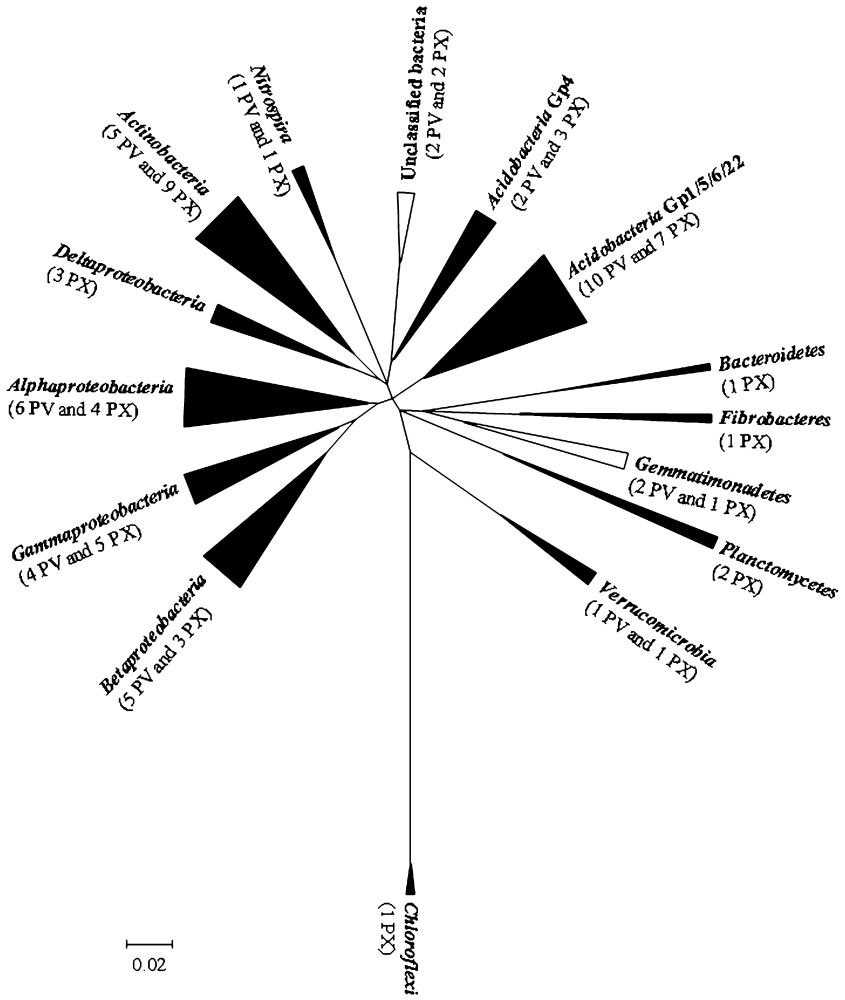
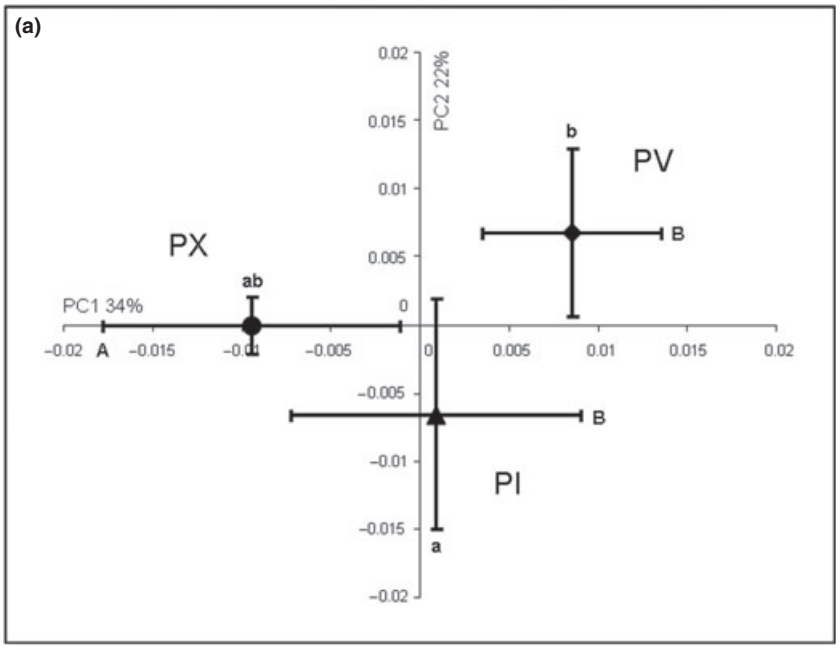



**Fig. 3** Comparison of bacterial community structure from wheat (*Triticum aestivum*) rhizospheres by microarray and cloning–sequencing approaches. The neighbour-joining phylogenetic tree for 16S rRNA genes is based on 84 environmental clones and 114 sequences from SILVA and Greengenes databases. All the phyla detected by both cloning–sequencing and microarray (black), or only by cloning–sequencing (white), are indicated. The nodal robustness of the bacterial groups was supported by the interior branch test (*t*-test; $P < 0.05$). The numbers of 16S rRNA environmental clones for stages PV and PX are indicated in brackets.

levels (Fig. S3). Three classes of hybridization intensity levels were arbitrarily defined based on the range of hybridization intensity (second quartile, 0.003; third quartile, 0.009) obtained for rhizosphere samples (Fig. S2), that is low (< 0.003 RFU), moderate ($0.003 < x < 0.009$) and high (> 0.009) intensity. PCA of the microarray results for the total bacterial community of the wheat rhizosphere strongly separated (with $P < 0.001$; ANOVA and Tukey HSD test) between samples from the disease stage (PV) and the suppressive stage (PX) along the first axis (34% of the total variability) (Fig. 4a). Stages PI and PX (both with low level disease) were also separated along the first axis (but only at $P < 0.05$), whereas stages PI and PV were not. Along the second axis (22% of the total variability), significant separation was obtained between stages PI and PV ($P < 0.001$).

Significance analysis of probe hybridization intensity for the different TAD stages (Table S4) revealed differences for a large number of probes (52% of the positive probes), mainly between stages PV and PX (34% of the positive probes), corresponding to an important bacterial taxonomic diversity. The suppressive stage (PX) was characterized by the prevalence ($P < 0.01$), compared with the disease stage (PV), of taxa belonging to *Planctomycetes*, *Nitrospira*, *Acidobacteria* and *Chloroflexi*, as well as, at a finer taxonomic level, to *Azospirillum* and *Acidocella/Acidiphillium* (*Alphaproteobacteria*), *Burkholderia* and *Methylophilus* (*Betaproteobacteria*), *Thermoanaerobacter* and *Lactobacillus* (*Firmicutes*), *Geobacter* (*Deltaproteobacteria*), *Campylobacter* (*Epsilonproteobacteria*) and *Lyngbya* (*Cyanobacteria*) (Fig. 4b; Table S4). Except for probes targeting *Methylophilus*, *Lactobacillus*, *Geobacter* and *Lyngbya*, all significant probes corresponded to moderate to high hybridization intensities (> 0.003 RFU; Fig. S3).

In comparison, the disease stage (PV) was characterized by the prevalence of well-known soil- and root-associated proteobacterial taxa, i.e. *Pseudomonas* (*Gammaproteobacteria*), *Sphingomonadaceae*, *Rhizobiaceae*, *Phyllobacteriaceae* and *Methylobacterium* (*Alphaproteobacteria*), *Nitrosospira* and *Variovorax* (*Betaproteobacteria*). *Bacteroidetes* and *Verrucomicrobia* phyla, as well as a subgroup of *Acidobacteria* (related to 16S rRNA clones retrieved from maize agricultural soil; Sanguin *et al.*, 2006a), also appeared as



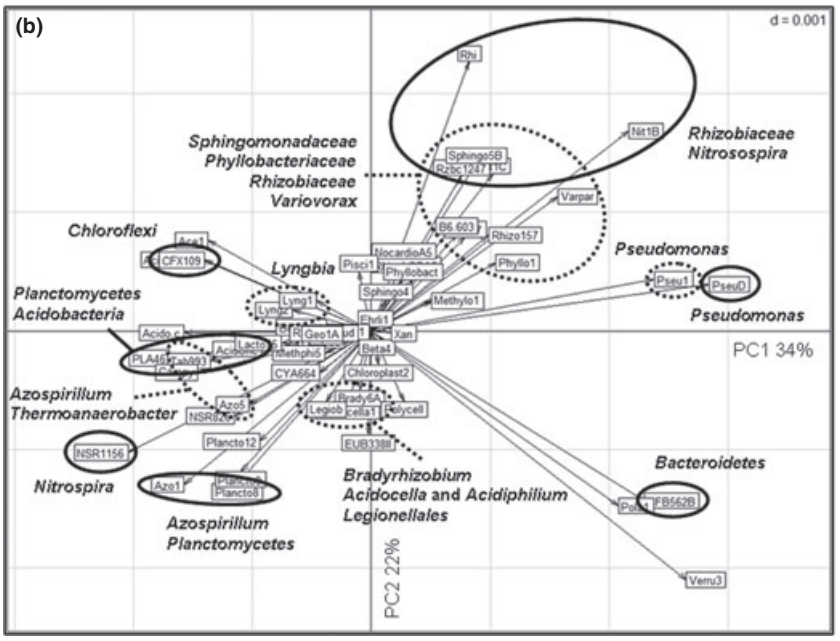




**Fig. 4** Hybridization pattern analysis of the total bacterial community from stages PI (low disease level; triangles), PV (high severity; diamonds) and PX (suppressiveness; circles). For each stage, the mean and standard deviation of hybridization patterns are represented. The samples are shown in (a) and the statistics are indicated along axes PC1 (by letters A and B) and PC2 (by letters a and b). The positive probes contributing significantly ($P < 0.05$; Table S4) to the separation between take-all decline (TAD) stages are shown in (b). For probes giving significant differences at $P < 0.001$ (Table S4), the name(s) of the taxa targeted are indicated by circles with dashed lines (when the signal levels were moderate) or full lines (high signals).

significantly prevalent in the disease stage (PV) compared with the suppressive stage (PX; Fig. 4b; Table S4). Most significant probes displayed moderate to high hybridization intensities (> 0.003 RFU), except for a few probes targeting the *Proteobacteria* and the subgroup of *Acidobacteria* (Fig. S3).

Concerning stage PI (low disease level; initial stage), proteobacterial groups affiliated to *Rhizobiaceae* and *Phyllobacteriaceae* (*Alphaproteobacteria*), *Polyangium* (*Deltaproteobacteria*) as well as *Bacteroidetes* and *Verrucomicrobia* were significantly prevalent ($P < 0.01$) compared with stage PX (suppressiveness) (Fig 4b; Table S4). By contrast, a lower prevalence was observed for proteobacterial groups affiliated to *Bradyrhizobiaceae* and *Acetobacteraceae* (*Alphaproteobacteria*), *Burkholderia* and *Legionellales* (*Betaproteobacteria*), *Geobacter* (*Deltaproteobacteria*), as well as *Planctomycetes*, *Chloroflexi* and *Lyngbya* (*Cyanobacteria*) ($P < 0.01$; Fig 4b; Table S4). Compared with stage PV (diseases), PI was characterized by a significant prevalence ($P < 0.01$) of bacterial groups affiliated to *Azospirillum*, *Bradyrhizobium* and *Acidocella/Acidiphillium* (*Alphaproteobacteria*), *Legionellales* (*Betaproteobacteria*), *Polyangium* (*Deltaproteobacteria*), *Thermoanaerobacter* and *Lactobacillus* (*Firmicutes*), as well as *Cyanobacteria* and specific subgroups of *Planctomycetes* and

*Nitrospira*. By contrast, a lower prevalence was observed for a large range of *Proteobacteria*, i.e. *Sphingomonadaceae*, *Rhizobiaceae* and *Phyllobacteriaceae* (*Alphaproteobacteria*), *Nitrosospira* and *Variovorax* (*Betaproteobacteria*), *Pseudomonas* (*Gammaproteobacteria*), as well as a subgroup of *Acidobacteria* (related to 16S rRNA clones retrieved from maize agricultural soil; Sanguin *et al.*, 2006a).

### qPCR analysis

qPCR analysis was carried out to complement the previous results obtained on the diversity of *Pseudomonas* populations in the same experiment (Sanguin *et al.*, 2008), that is to assess the prevalence of *Pseudomonas* in PV compared with PX. The specificity of the *Pseudomonas*-related qPCR approach was evaluated by the cloning–sequencing of two amplicons (obtained from plants V2 and X2) and, indeed, all 16S rRNA sequences belonged to the *Gammaproteobacteria*. As much as 70% of the sequences originated from *Pseudomonas*, and the others mainly from the *Xanthomonadaceae* and *Enterobacteriaceae* (Table S3). qPCR indicated that *Pseudomonas* and close relatives were prevalent in the disease stage (PV) compared with the other stages (Fig. 5), which confirmed the microarray results obtained for *Pseudomonas* (Fig. 4).

The relative abundance of *Pseudomonas* in the total bacterial community was assessed for the three TAD stages, as the size of the total community was also measured by qPCR. The results showed that the proportion of pseudomonads and close relatives in the total bacterial community increased significantly from stages PI to PV (Fig. S4).

## Discussion

The decline of take-all disease and the establishment of disease suppressiveness are attributed to the microbial component of soil (Mazzola, 2002). TAD soils are of particular interest when studying microbial communities defining soil biological status, as a given soil can be suppressive or conducive depending on monoculture length. Antagonistic pseudomonads, including strains producing 2,4-diacetylphloroglucinol and strains unable to produce this compound, are thought to play an important role in TAD (Mazzola *et al.*, 1992; Chapon *et al.*, 2002; Weller *et al.*, 2007). They may act by several mechanisms, including 2,4-diacetylphloroglucinol-mediated antibiosis, selection of less aggressive *Ggt* genotypes and perhaps also induced systemic resistance (Weller *et al.*, 2002; Lebreton *et al.*, 2004, 2007). In the current field experiment, changes in specific *Pseudomonas* subgroups were found between treatments using 16S rRNA probes targeting taxa containing antagonistic strains (Sanguin *et al.*, 2008).

In addition to antagonistic pseudomonads, earlier work has raised the possibility that non-*Pseudomonas* populations may also be involved in TAD (Weller *et al.*, 2002), at least by interacting with the former. However, no thorough appraisal of the role of non-*Pseudomonas* populations in TAD has been carried out. Before such an assessment may be undertaken, it would be useful to determine first whether community composition differs between the main TAD stages, which has not been studied so far. If community composition differs in relation to TAD, it would lead to the identification of taxonomic bioindicators of soil health status, which has been a long-lasting goal in soil microbial ecology and extends beyond the sole issue of plant health.

The microarray technology used for this purpose is based on probe specificity, and increasing the number of probes did not enhance the number of false positives (0.75% **vs** 0.91% in Sanguin *et al.*, 2006a), which means that the probe set was reliable. Some probes targeting high taxonomic levels, such as *Alpha-* and *Betaproteobacteria*, *Planctomycetes* and *Actinobacteria*, and displaying strong hybridization intensities in the different stages, were not significantly different between stages, probably because of the broad range of taxa targeted, which did not reflect the

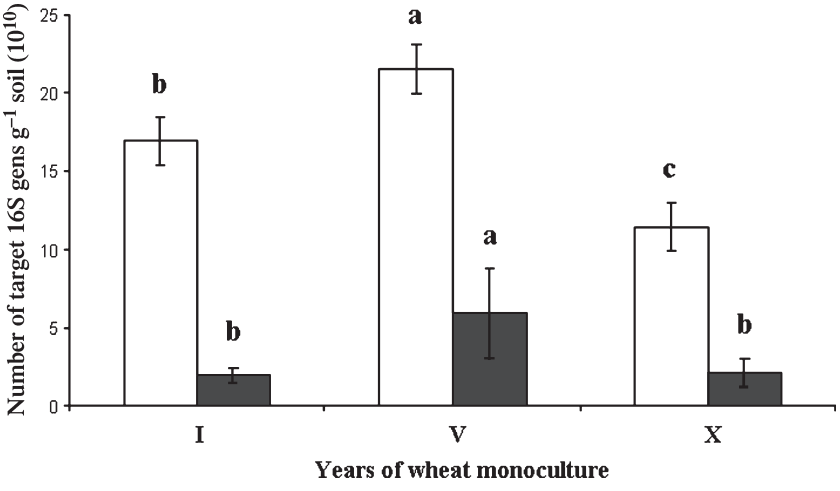



**Fig. 5** Quantification of the total bacterial community and *Pseudomonas*-related populations during the take-all decline (TAD) stages. Quantification was achieved by quantitative polymerase chain reaction (qPCR) on soil extracts from 1-, 5- and 10-yr-old wheat (*Triticum aestivum*) monocultures (I, V, X). The total bacterial community (in white; statistics shown using letters a–c) and *Pseudomonas*-related populations (in black; statistics shown using letters a and b) were monitored with Com1/Com2 and pA/Pseu1r primers, respectively.

changes in populations at lower taxonomic levels. For probes targeting low taxonomic levels, the biological interpretation of probe hybridization data must be carried out carefully when close to the detection threshold. In addition, positive hybridization for some probes, e.g. Legiob (*Legionellales*), should be carefully interpreted because of potential cross-hybridizations with nontargeted taxa, such as *Pseudomonas* (as shown by strain hybridization results).

When screening the total bacterial diversity at different taxonomic levels in the rhizosphere of wheat, we found several taxa characterizing the disease or suppressive stages, but it is not known whether the occurrence of a given population has any functional implication for plant health. Indeed, a population prevalent in the disease stage may be functionally implicated in the transition to the suppressive stage, or merely incidental because it is adapted to the colonization of diseased roots, whereas those prevalent in the suppressive stage may protect the plant or take advantage of their ability to colonize healthy roots. Therefore, the link between the direct involvement of taxa in TAD and its abundance cannot be established with our correlative approach. For example, TAD correlates with changes in specific *Pseudomonas* subgroups (Sanguin *et al.*, 2008), and at least certain pseudomonads are known to play a functional role in TAD (Weller *et al.*, 2002). However, when considered together, the total pseudomonads (and relatives) were more abundant at the higher disease level based on microarray and qPCR data, probably because of their higher colonization rate of damaged roots (Chapon *et al.*, 2002).

We chose a sampling scheme with a single sampling date on plots from a single field, with relevant previous climatic history and farming practices to represent low disease (stage PI), disease (stage PV) and suppressiveness (stage PX). This approach presented the advantage of limiting the effects of soil composition and climate on bacterial community structure and, in future work, the applicability of the current findings will need to be verified at other field locations. In spite of the complex conditions in the field, the community profiles identified were characteristic of each of the three TAD stages. It should be noted that PI was representative of the initial stage at the time of sampling, but perhaps the initial conditions in the plots then in stages PV or PX were different, as they occurred 4 and 9 yr earlier, respectively. Although the main bacterial community changes were observed between the disease and suppressive stages, the comparison with the initial stage is essential to fully understand the dynamics of bacterial changes during TAD. Indeed, it is likely that the final community composition in PX results from a succession of previous interactions in time, following a progressive shift from a community structure characteristic of one status to the next. For instance, the relative abundance of *Bacteroidetes* and *Verrucomicrobia* was similar between the initial and disease stages, followed by a significant decrease when suppressiveness was reached, whereas an important increase was observed for proteobacterial groups in the disease stage compared with the initial stage, followed by a decrease in the suppressive stage.

The suppressive stage (PX) was characterized by the prevalence of major phyla, especially *Acidobacteria*, *Planctomycetes*, *Nitrospira* and a range of *Proteobacteria* (noticeably *Azospirillum*), and, to a lesser extent, *Chloroflexi* and certain *Firmicutes* and *Cyanobacteria*. Many of these taxa have been identified in wheat fields (Smit *et al.*, 2001) and, more generally, in bulk soil and the rhizosphere (Buckley & Schmidt, 2003; Gremion *et al.*, 2003; Filion *et al.*, 2004; Sanguin *et al.*, 2006a; Vandenkoornhuyse *et al.*, 2007; Deangelis *et al.*, 2008; Lee *et al.*, 2008). The higher prevalence of *Azospirillum* in the suppressive phase is interesting, because many of these bacteria promote the growth of wheat (Pothier *et al.*, 2007) and their biocontrol activity is starting to be recognized (Russo *et al.*, 2008). In our pathosystem, they might promote wheat health during suppressiveness via a direct effect on plant physiology and growth. Interestingly, *Azospirillum lipoferum* was detected in the two stages with low-level disease. Other taxa prevalent in the suppressive stage include biocontrol strains, as in the case of the betaproteobacterial genus *Burkholderia* (Burkhead *et al.*, 1994) and the *Firmicutes* (including against *Ggt*; Kim *et al.*, 1997; Coombs *et al.*, 2004).

## Conclusion

The 16S rRNA-based microarray approach, combined with cloning–sequencing, was sufficiently powerful to demonstrate differences in rhizobacterial community composition in relation to TAD. These differences concerned a wide range of different taxa, including taxa not yet documented in relation to TAD and taxa including biocontrol strains, and whose possible functional role needs to be evaluated. The findings raise the hypothesis that TAD may be a complex community-based phenomenon, rather than a population-level mechanism based solely on the contribution of antagonistic fluorescent pseudomonads, indicating that other bacterial populations might also be implicated in the TAD process.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Box plot representation of the distribution of normalized hybridization intensity values for 39 strains according to seven arbitrarily defined classes of weighted mismatch (WMM) values.

**Fig. S2** Box plot representation of the distribution of normalized hybridization intensity values for the take-all decline (TAD) stages studied (PI, PV and PX).

**Fig. S3** Microarray hybridization patterns for rhizosphere samples. *α-prot, Alphaproteobacteria; β-prot, Betaproteobacteria; γ-prot, Gammaproteobacteria; ε-prot, Epsilonproteobacteria; δ-prot, Deltaproteobacteria; Actino, Actinobacteria; Acido, Acidobacteria; Bac, Bacteroidetes; Flav, Flavobacteria; Sphi, Sphingobacteria; Firm, Firmicutes; Planc, Planctomycetes; Verru, Verrucomicrobia; Nit, Nitrospira; Chlo, Chloroflexi; Cya, Cyanobacteria; Flex, Flexistipes*.

**Fig. S4** Estimation of the proportion between the total bacterial community and *Pseudomonas*-related populations, by quantitative PCR, in the three take-all decline (TAD) stages.

**Table S1** List of 16 rRNA targeted oligonucleotide probes designed in this work [and added to the probe set of Sanguin *et al.* (2006a) and Sanguin et al. (2008)].

**Table S2** 16S rRNA sequences used for phylogenetic analysis, which originate from the wheat rhizosphere (i.e. PV and PX clones; this study) or Greengenes and SILVA databases.

**Table S3** Affiliation of *Pseudomonas*-related 16S rRNA clones using the Greengenes Classify tool.

**Table S4** Significance analysis of microarray probe hybridization data from PI, PV and PX stages.

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