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ORIGINAL ARTICLE

Comparison of rhizobacterial community composition in soil suppressive or conducive to tobacco black root rot disease

Martina Kyselková[1,2,3], Jan Kopecký[4], Michele Frapolli[5], Geneviève Défago[5], Markéta Ságová-Marečková[4], Geneviève L Grundmann[1,2,3] and Yvan Moënne-Loccoz[1,2,3]
[1]*Université de Lyon, Lyon, France;* [2]*Université Lyon 1, Villeurbanne, France;* [3]*CNRS, UMR5557, Ecologie Microbienne, Villeurbanne, France;* [4]*Crop Research Institute, Praha-Ruzyně, Czech Republic and* [5]*Plant Pathology Group, Institute of Integrative Biology, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland*

Work on soils suppressive to *Thielaviopsis basicola*-mediated tobacco black root rot has focused on antagonistic pseudomonads to date. The role of non-*Pseudomonas* rhizosphere populations has been neglected, and whether they differ in black root rot-suppressive versus -conducive soils is unknown. To assess this possibility, tobacco was grown in a suppressive and a conducive soil of similar physicochemical properties, and rhizobacterial community composition was compared using a 16S rRNA taxonomic microarray. The microarray contains 1033 probes and targets 19 bacterial phyla. Among them, 398 probes were designed for *Proteobacteria*, *Firmicutes*, *Actinomycetes*, *Cyanobacteria* and *Bacteroidetes* genera/species known to include strains relevant for plant protection or plant growth promotion. Hierarchical clustering as well as principal component analysis of microarray data discriminated clearly between black root rot-suppressive and -conducive soils. In contrast, *T. basicola* inoculation had no impact on rhizobacterial community composition. In addition to fluorescent *Pseudomonas*, the taxa *Azospirillum*, *Gluconacetobacter*, *Burkholderia*, *Comamonas* and *Sphingomonadaceae*, which are known to comprise strains with plant-beneficial properties, were more prevalent in the suppressive soil. *Mycobacterium*, *Bradyrhizobium*, *Rhodobacteraceae*, *Rhodospirillum* and others were more prevalent in the conducive soil. For selected taxa, microarray results were largely corroborated by quantitative PCR and cloning/sequencing. In conclusion, this work identified novel bacterial taxa that could serve as indicators of disease suppressiveness in soil-quality assessments, and it extends the range of bacterial taxa hypothesized to participate in black root rot suppression.
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Introduction

Soil microorganisms have a major role in soil functioning and health, including the ability to control pathogens of plants (van Elsas *et al.*, 2007). In disease-suppressive soils, some of the indigenous microorganisms protect susceptible crops from certain phytopathogens, whereas disease-conducive soils do not provide protection and permit spread of the pathogen (Alabouvette *et al.*, 1996; Weller *et al.*, 2002; Garbeva *et al.*, 2004). Despite being an important component of soil quality (van Bruggen and Semenov, 2000), disease suppressiveness is not well understood. In certain cases, suppression is induced by crop monoculture and materializes as a decline of the disease, which follows earlier outbreaks. Induced disease suppression is well documented with wheat take-all, a disease mediated by *Gaeumannomyces graminis* var. *tritici* (Weller *et al.*, 2002; Lebreton *et al.*, 2004), and take-all decline involves enrichment effects of antagonistic root-colonizing *Pseudomonas* bacteria (Sarniguet and Lucas, 1992; Weller, 2007). In other cases,

Correspondence: Y Moënne-Loccoz, UMR CNRS 5557 Ecologie Microbienne, University Lyon 1, 43 bd du 11 Novembre, Villeurbanne 69622, France.
E-mail: moenne@biomserv.univ-lyon1.fr
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disease suppression is a natural property of the soil and is compatible with crop rotation (Stutz *et al.*, 1986), even though the extent of disease suppression may change during crop rotation (Ramette *et al.*, 2003). Natural/long-standing suppression is known for several diseases, such as *Fusarium oxysporum*-mediated Fusarium wilt (Alabouvette *et al.*, 1996) and black root rot caused by the fungus *Thielaviopsis basicola* (Stutz *et al.*, 1986).

Soils naturally suppressive to *T. basicola*-mediated black root rot of tobacco have been described at Morens (Switzerland), on sandstone overlaid by shallow morainic material (Stutz *et al.*, 1985, 1989). Disease-conducive soils occur nearby on sandstone sediments. The comparison of up to 96 Morens fields indicated that both types of soils are cambisols, with a largely similar soil chemistry (Stutz *et al.*, 1985, 1986; Ramette *et al.*, 2003). Indeed, the only consistent difference is clay mineralogy, as vermiculite predominates in suppressive soils and illite in conducive soils (Stutz *et al.*, 1989). None of the other characteristics correlates with soil suppressiveness (Stutz *et al.*, 1985; Ramette *et al.*, 2003).

Disease suppressiveness is a property conferred by soil microbial community. This is indicated by the facts that (i) soil pasteurization or sterilization abolished disease-control capacity in Morens suppressive soil, and (ii) the transfer of suppressiveness occurred when conducive soil received a small amount of suppressive soil (Stutz *et al.*, 1985, 1986). Soil pasteurization/sterilization data (and results from artificial vermiculitic soils; Ramette *et al.*, 2006) also showed that the presence of vermiculite did not impede the ability of *T. basicola* to infect tobacco. This is consistent with previous observations that the suppressive status of the soils did not influence the number of indigenous, virulent *T. basicola* propagules (Gasser and Défago, 1981) or the survival of inoculated *T. basicola* (Berling *et al.*, 1984).

The initial studies attributed disease suppression to fluorescent pseudomonads (Stutz *et al.*, 1986). Indeed, many *Pseudomonas* isolates from these soils (including the well-established model strain CHA0) (i) produced the antifungal compounds 2,4-diacetylphloroglucinol (Phl) and/or hydrogen cyanide (HCN), (ii) were antagonistic to *T. basicola in vitro* and (iii) protected tobacco from black root rot (Stutz *et al.*, 1986; Ramette *et al.*, 2003). However, in contrast to the situation of the take-all decline soils, HCN+ Phl+ *Pseudomonas* were also isolated in high numbers from black root rot-conducive soils in the same Morens area (Ramette *et al.*, 2003; Frapolli *et al.*, 2008). Furthermore, they could also protect tobacco from *T. basicola* when used as inoculants (Ramette *et al.*, 2006). Therefore, disease suppressiveness of Morens soils to black root rot is unlikely to result only from enrichment effects of HCN+ Phl+ pseudomonads.

This study is based on the rationale that natural disease suppressiveness of soil may involve far more than the extensively studied biocontrol contribution of antagonistic fluorescent pseudomonads (Ramette *et al.*, 2006). Indeed, the rhizosphere microbial community is highly diverse (van Elsas *et al.*, 2007), and it is conceivable that several other microbial taxa could have an important part in disease suppression at Morens. They may act by protecting the plant directly, for example, through the release of pathogen inhibitors (Raaijmakers *et al.*, 2008), or indirectly by promoting plant growth (Bally and Elmerich, 2005) or enhancing rhizosphere functioning of antagonistic pseudomonads (Lemanceau and Alabouvette, 1991). Community-level assessment of taxa more prevalent in suppressive soils was advocated to identify candidate microorganisms that may contribute to disease suppressiveness (Borneman and Becker, 2007), but this has rarely been performed with naturally suppressive soils (Rimé *et al.*, 2003; Hjort *et al.*, 2007) and never at Morens.

Therefore, the objective of this work was to compare the composition of the tobacco rhizobacterial community in black root rot-suppressive versus -conducive soils from Morens, and to identify non-*Pseudomonas* taxa more prevalent in black root rot-suppressive soil. To this end, a taxonomic microarray based on the 16S rRNA gene *rrs* and validated for rhizosphere assessments was further developed from earlier prototypes (Sanguin *et al.*, 2006a, b, 2008; Demanèche *et al.*, 2008; Kyselková *et al.*, 2008) and was used to assess tobacco at an early stage of plant development that is, at the time when biocontrol interactions are of particular relevance. In addition, the possible effect of the pathogen on rhizobacterial community composition was addressed by studying both non-inoculated tobacco as well as tobacco seedlings inoculated with *T. basicola*.

Materials and methods

Soil harvesting and growth chamber experiment

Soils were collected (5–30 cm depth) from fields MS8 (disease-suppressive cambisol; sandy loam, pH 7.8, CEC 5.7 cmol kg^{-1} with ammonium acetate method, 1.3% organic matter, 0.13% total N) and MC112 (disease-conducive cambisol; sandy loam, pH 6.8, CEC 8.5 cmol kg^{-1}, 2.2% organic matter, 0.13% total N) near Morens, Switzerland, in June 2006 (Frapolli *et al.*, 2008). Preparation of soil, tobacco seedlings (*Nicotiana glutinosa* L.) and endoconidia of *T. basicola* Ferraris strain ETH D127 was performed as described by Ramette *et al.* (2003). Soil received 10^3 endoconidia per cm^3 soil (or sterile water in the controls) when transplanting 4-week-old tobacco plants in soil. The number of pots was eight per treatment. Soil water content was adjusted to 70% of water retention capacity. Plants were cultivated in a growth chamber at 22 °C (day, 16 h) and 18 °C (night, 8 h) at 70% relative humidity.

Disease severity was recorded for each plant at 3 weeks after inoculation, as the percentage of root surface covered by *T. basicola* chlamydospores (Stutz *et al.*, 1986), confirming that MS8 was suppressive (disease index 9.2% ± 1.8% after *T. basicola* inoculation) and MC112 conducive (41.7% ± 2.8% after inoculation). No disease was observed without inoculation. Rhizosphere soil, that is soil tightly adherent to roots (Frapolli *et al.*, 2008), was used (250 mg) for DNA extraction with PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA).

PCR amplification of rrs genes and transcription labeling

The universal eubacterial primers *T7*-pA (forward: *TAATACGACTCACTATAG*AGAGTTTGATCCTGGCT CAG) and pH (reverse: AAGGAGGTGATCCAGCCG CA) (Bruce *et al.*, 1992) were used to amplify *rrs* from DNA extracts obtained from pure strains (Supplementary Table 1) or rhizosphere soil. Primer *T7*-pA includes at the 5′-end of the sequence of T7 promoter (in italics above), which enabled a subsequent T7 RNA polymerase-mediated *in vitro* transcription. The PCR conditions were as described by Sanguin *et al.* (2008). PCR products were purified with MinElute PCR purification kit (Qiagen, Courtaboeuf, France). DNA concentration was determined spectrophotometrically and was adjusted to 50 ng μl^{-1}. Fluorescence labeling by *in vitro* transcription (Stralis-Pavese *et al.*, 2004), RNA purification and fragmentation was carried out as described by Sanguin *et al.* (2008).

Design of probes and microarray manufacturing

Probes were designed using ARB (Technical University Munich, Munich, Germany) and its *rrs* database (ssu_jan04_corr_opt.arb; http://www.arb-home.de; Ludwig *et al.*, 2004), and were verified using the Silva-94 database (http://www.arb-silva.de; Pruesse *et al.*, 2007). The parameters of the Probe Design function were chosen according to Sanguin *et al.* (2006a, b), especially a weighted mismatch (WMM) value below 2 with the targeted taxa and more than 2 with non-targets. The WMM value is computed using ARB (Ludwig *et al.*, 2004), and is 0 in the absence of any mismatch. The probes were further tested *in silico*, according to Sanguin *et al.* (2006a, b). Probes were custom synthesized (Eurogentec, Seraing, Belgium) with a 5′-NH_2-C6 group for covalent attachment onto aldehyde slides AL (Schott Nexterion AG, Mainz, Germany). Spotting and treatment of slides were performed as described previously (Sanguin *et al.*, 2008). Each probe was repeated four times per slide.

Hybridization protocol

Two slides were hybridized per sample. Hybridization (overnight at 57 °C) was carried out in a custom-tailored aluminum block used as an insert for a temperature-controlled Belly Dancer (Stovall Life Sciences, Greensboro, NC, USA) set at maximum bending (Bodrossy *et al.*, 2003), as described (Sanguin *et al.*, 2008). Slide washing and handling were carried out as described by Sanguin *et al.* (2008).

Scanning, image analysis, filtration and normalization of microarray data

The slides were scanned at 532 nm with 10 μm resolution, using a GeneTac LS IV scanner (GenomicSolutions, Huntingdon, UK). Images were analyzed with the GenePix 4.01 software (Axon, Union City, CA, USA). Spot quality was always visually checked, and spots of poor quality (presence of dust) were excluded from further analyses, as described previously (Sanguin *et al.*, 2006b).

Data filtration was conducted with the R 2.2.0 statistical computing environment (http://www.r-project.org). A given spot was considered hybridized when 80% of the spot pixels had an intensity higher than the median local background pixel intensity plus twice the s.d. of the local background. The intensity signals (median of signal minus background) were replaced by their square root value and the intensity of each spot was then expressed as a fraction of the total intensity signal of the basic pattern it belongs to (Sanguin *et al.*, 2006b). Finally, a given feature probe was considered positive when (i) hybridization signals were superior to the mean signal of the negative controls and (ii) at least three of four replicate spots were hybridized.

Microarray validation by cloning/sequencing

Validation of microarray data was sought for important probes by cloning/sequencing, after amplification with specific primer pairs (Supplementary Table 2). When possible, the probe was used as one of the two primers (with if necessary a modification in 3′ to improve primer specificity). The new primers were designed using the ARB software (Ludwig *et al.*, 2004). For each PCR, 20 ng of purified *rrs* PCR product (see above) obtained from one non-inoculated plant in soil MC112 or one non-inoculated plant in soil MS8 was used as template (see above for PCR conditions and purification of PCR products). Annealing temperature for each primer pair is indicated in Supplementary Table 2.

Purified PCR products were cloned into the plasmid vector pGEM-T (pGEMs-T Easy Vector System kit; Promega, Charbonnieres, France) according to the manufacturer's protocol. Five to six clones were sequenced on both strands (CoGenics, Meylan, France). Sequences were checked and edited with BioEdit version 5.0.9 (Ibis Therapeutics, Carlsbad, CA, USA; http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Chimeric 16S rRNA gene sequences were identified using the chimera detection program Pintail version 0.33 from

Bioinformatics Toolkit (University of Cardiff, Cardiff, UK; http://www.bioinformatics-toolkit.org/; Ashelford *et al*., 2002), and putative chimeric clones were discarded. Sequence affiliation of non-chimeric sequences was performed using algorithm BlastN with default parameters at NCBI Blast (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequences are available at GenBank (accession numbers FJ447917–FJ447984).

Quantitative PCR

Relative amounts of *rrs* sequences of selected bacterial taxa (that is *Sphingobacteriaceae*, *Mycobacterium* and *Bradyrhizobium*) in soil MC112 versus MS8 were assessed with a quantitative PCR (qPCR) approach. The primers from the cloning/sequencing approach were used for *Sphingomonadaceae* and *Mycobacterium*, whereas a new primer was needed for *Bradyrhizobium* (Supplementary Table 2). PCRs (20 µl) were carried out in 96-well microplates. Each reaction contained 5 µl of PCR grade water, 2 µl of each primer (a final concentration of 0.5 µM), 10 µl of LightCycler-DNA Master SYBER Green I master mix (Roche Applied Science, Meylan, France) and 30 pg of soil DNA template. Thermal cycling was carried out with Light Cycler 480 (Roche Applied Science), with an initial denaturation step at 95 °C for 10 min, followed by 55 cycles with 30 s denaturation at 94 °C, annealing (*Sphingobacteriaceae* 30 s, *Mycobacterium* 20 s, *Bradyrhizobium* 20 s; annealing temperatures in Supplementary Table 2) and 30 s of elongation at 72 °C. Each sample was amplified in three replicates. The presence of a single specific PCR product was checked by assessing occurrence of (i) a single band after DNA migration in a 1.5% (w/v) agarose gel and (ii) a single peak in product melting curves provided by Light Cycler 480.

Three non-inoculated rhizosphere samples from each soil were studied. Relative quantities of *rrs* genes were assessed according to Pfaffl (2001), based on the ratio $R_{(MS8/MC112)}$ of *rrs* copies in MS8 versus MC112, which was computed as $R_{(MS8/MC112)} = E^{\Delta Cp(MC112-MS8)}$, where E means PCR efficiency and ΔCp (MC112-MS8) is computed for each MS8 sample as the difference between the mean crossing point for the three MC112 samples and the crossing point for the MS8 sample. PCR efficiency was calculated from dilution series curves (5, 10, 20, 40 and 80 pg of input DNA, each in three replicates) according to the equation $E = 10^{(-1/slope)}$, the slope being determined by plotting of crossing points to the log value of DNA quantity input.

For the validation of qPCR data by cloning sequencing, qPCR products obtained for each primer pair, using one plant from each soil, were purified with MinElute kit (Qiagen) and reamplified by PCR, as described above. The resulting amplicons were purified (MinElute kit; Qiagen), cloned and eight clones from each were sequenced, as described above. The sequences are available at GenBank (accession numbers FJ890751-FJ890797).

Statistical analysis

Microarray data were treated by hierarchical clustering as well as principal component analysis. Hierarchical clustering was performed with CLUSFAVOR version 6.0 (Baylor College of Medicine, Houston, TX, USA; Peterson, 2002) using the Unweighted Pair Group with Mathematical Average method, based on Euclidean distance matrix calculated for all 199 positive probes. The output is displayed along with a Heatmap image (obtained using CLUSFAVOR 6.0) showing hybridization levels for the 76 positive probes that differed significantly in their signal intensities between treatments based on Fisher's least significant difference tests (see below). Principal component analysis of hybridization data was performed with ADE-4 (Thioulouse *et al*., 1997) in R environment (http://www.r-project.org), based on the correlation matrix. This was followed by a comparison of the treatments by two-factor (that is, soil × *T. basicola* inoculation) analysis of variance and Fisher's least significant difference tests (using DSAASTAT for Excel, version 1.0192; available at http://www.unipg.it/~onofri/DSAASTAT/DSAASTAT.htm; Onofri, 2006) along each of the first two axes, that is based on PC1 coordinates as well as PC2 coordinates ($P<0.05$). Two-factor analysis of variance was also used to compare treatments probe by probe.

Results

Extension of probe set and validation with pure strains

In this study, 468 new 16S rRNA probes were designed based on the criteria established for the previous 684 *rrs* probes of Sanguin *et al*. (2006a, b, 2008), Kyselková *et al*. (2008) and Demanèche *et al*. (2008). When the whole probe set (1152 probes) was hybridized to *rrs* genes amplified from 23 bacterial strains (Supplementary Table 1) and one *Mycobacterium* environmental clone, aberrant results (that is, high signal levels despite high WMM values) were found for 70 of the 468 new probes (that is, about 15%), 1 of the 113 probes from Kyselková *et al*. (2008) and 48 of the 571 probes (that is, about 7%) from Demanèche *et al*. (2008). Rarefaction analysis of excluded probe number with increasing number of hybridized strains showed that the number of strains used was sufficient to reveal aberrant probes (not shown).

Once the 119 aberrant probes were excluded, the signals obtained for the 1033 probes left (398 new probes and 634 published probes; Table 1, Supplementary Table 3) decreased with the WMM values, and a steep dropout was observed at WMM = 1.5 (Supplementary Figure 1). Above a WMM of 1.6, the median signal was close to zero and WMM 1.5 was taken as a threshold for expected hybridization. For

Table 1 Coverage of bacterial phyla by rRNA gene probes

Phylum	*Number of RDP-10 entries*	*Number of probes*[a]	*Number of genera covered specifically*[b]
Acidobacteria	22 860	9	2
Actinobacteria	53 771	207	82
Aquificae	1267	0	0
Bacteroidetes	72 984	41	19
BRC1	82	0	0
Chlamydiae	634	1	0
Chlorobi	713	1	1
Chloroflexi	5127	1	0
Chrysiogenetes	4	0	0
Cyanobacteria	18 906	24	14
Deferribacteres	433	4	2
Dehalococcoides	194	0	0
Deinococcus–Thermus	1232	1	1
Dictyoglomi	22	0	0
Fibrobacteres	357	1	1
Firmicutes	171 252	166	21
Fusobacteria	1824	1	0
Gemmatimonadetes	1489	0	0
Lentisphaerae	220	0	0
Nitrospira	1883	5	3
OD1	181	0	0
OP10	345	0	0
OP11	131	4	0
Planctomycetes	5914	13	2
Proteobacteria	236 226	526	144
Alphaproteobacteria	60 987	142	49
Betaproteobacteria	45 490	91	24
Gammaproteobacteria	98 283	197	46
Deltaproteobacteria	18 282	73	20
Epsilonproteobacteria	7025	23	5
Spirochaetes	4026	0	0
SR1	55	0	0
Tenericutes	2414	9	3
Thermodesulfobacteria	124	1	2
Thermomicrobia	30	0	0
Thermotogae	396	2	2
TM7	841	0	0
Verrucomicrobia	6645	2	0
WS3	181	0	0
Total	643 916	1119	299

[a]Probes at any taxonomic level between phylum and species.
[b]Probes at genus or species level.

WMM = 0, the signal intensity was always above 0.025 normalized intensity units (Supplementary Figure 2, indicated in orange or red). On the basis of the WMM threshold of 1.5, only 2.3% of all 24 792 hybridizations (1033 probes × 24 strains) were unexpected (that is, false positive). In addition, 1.7% of the expected hybridizations (corresponding to less than 0.02% of all hybridizations) were false negative, that is a probe did not give any signal despite expectation (and it was always at WMM between 1.2 and 1.5).

The final list of probes with probe characteristics will be available at the ProbeBase site (http://www.microbial-ecology.net/probebase/). Generally, the probes are 17–26-mer oligonucleotides, the majority being 20-mers, with a G + C content between 35% and 70% (average 53%). The melting temperature (T_m) of the probes is between 49.7 and 76.7 °C, and 84% of the probes have a T_m between 60 and 70 °C. On the basis of nomenclatural taxonomy presented at RDP II (http://rdp.cme.msu.edu/; Garrity *et al.*, 2007), the probe set targets 19 of 34 bacterial phyla with at least one probe (Table 1). In addition, some phyla, noticeably the *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes,* are covered with probes at lower taxonomical levels, that is class, family, genus and/or species (Supplementary Table 3). Overall, the microarray covers, at the genus and/or species level, (i) most bacterial taxa (that is, about 90 species in 50 genera) known to include potentially plant-beneficial bacteria (based on *rrs* sequences available in databases) and (ii) a wide range of other bacteria known to be present in soil, including some human pathogens (Berg *et al.*, 2005).

Comparison of rhizobacterial community in black root rot-suppressive and -conducive soils

When rhizosphere samples from both *T. basicola*-inoculated and -non-inoculated tobacco plants were

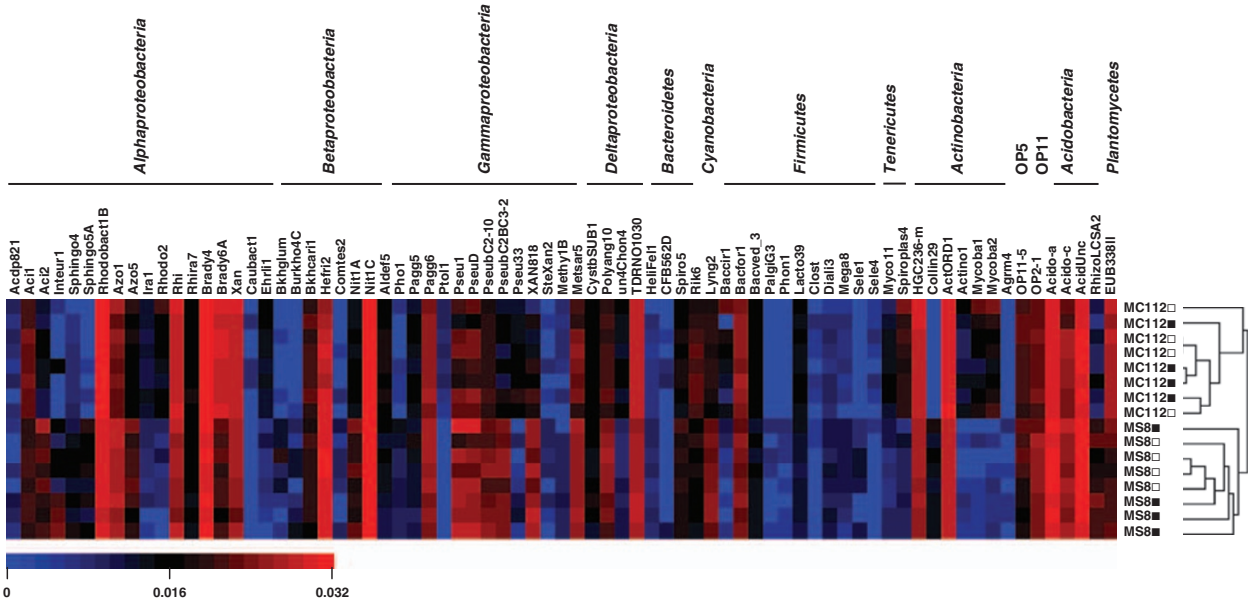

Figure 1 Comparison of suppressive soil MS8 and conducive soil MC112 based on clustering of microarray hybridization data for the 199 positive bacterial rRNA gene probes (using Euclidean distance matrix and UPGMA algorithm). Plants inoculated with the pathogen *T. basicola* are indicated with black squares and non-inoculated plants with open squares. The heatmap shows hybridization levels only for the 76 probes that gave statistically different signals between the treatments (that is, the four soil × inoculation combinations). The taxonomic significance of probes is given in Table 2. UPGMA, Unweighted Pair Group with Mathematical Average.

analyzed with the microarray, the number of positive probes was 144–152 per sample in soil MS8 and 137–153 in soil MC112. Hierarchical clustering performed on microarray data distinguished clearly between soils MS8 and MC112 (Figure 1). Inoculation with the phytopathogen *T. basicola* had no impact, regardless of whether soil MS8 or MC112 was considered. Concordant results were obtained with principal component analysis, where soils were clearly separated along the first principal axis (confirmed by analysis of variance of treatment replicates), whereas no separation was observed between inoculated and non-inoculated samples for any of the two soils (not shown).

The differences between the two soils were based mainly on variations in signal intensity rather than on the presence/absence of signals. As many as 69 of all 199 positive probes gave statistically different hybridization levels between the two soils, 34 of them higher for MS8 and the 35 others lower (Table 2). Twelve probes yielded significantly different signals as a consequence of *T. basicola* inoculation, and these differences were insufficient to make the whole community statistically different. In addition, the interaction between soil and *T. basicola* inoculation was significant for 13 probes.

Analysis of probes discriminating between black root rot-suppressive and -conducive soils

The *Pseudomonas* probes contributed substantially to the discrimination between the two soils (Figure 1). One probe for the *Pseudomonas* genus (that is, Pseu1) and two probes (that is, PseubC2BC3-2 and PseubC2-10) targeting fluorescent pseudomonads from clusters C3, C4 and C5 (Sanguin *et al.*, 2008) gave significantly higher signals with suppressive soil (Table 2). In contrast, the probe Pseu33 targeting *Pseudomonas citronellolis*/*P. nitroreducens* displayed a higher signal with conducive soil.

Besides *Pseudomonas*, probes for various bacteria also contributed significantly to separation of soils, MS8 and MC112, according to principal component analysis (not shown) and analysis of variance (Figure 1 and Table 2). Probes giving higher signals in suppressive soil targeted noticeably (Table 2) (i) the *Alphaproteobacteria* family *Sphingomonadaceae*, genera *Gluconacetobacter* and *Azospirillum*, and species *Azospirillum lipoferum*, (ii) the *Betaproteobacteria* genera *Nitrosospira*/*Nitrosovibrio* and *Comamonas*, and species *Burkholderia glathei*/*B. multivorans*/*B. cepacia*/*B. andropogonis*, *B. glumae* and *Herbaspirillum seropedicae*, (iii) the *Gammaproteobacteria* family *Xanthomonadaceae*, and genera *Stenotrophomonas*/*Xanthomonas*, *Photorhabdus*, *Methylosarcina* and *Methylomonas*, (iv) the *Deltaproteobacteria* family *Polyangiaceae*, (v) the *Actinobacteria* genera *Agromyces* and *Collinsella*, (vi) the *Firmicutes* species *Paenibacillus alginolyticus* (and closely related species), (vii) the *Cyanobacteria* genus *Lyngbia* and (viii) *Acidobacteria*. Probes giving higher signals in conducive soil included in particular those targeting (i) the *Alphaproteobacteria* family *Rhodobacteraceae*, genera *Rhodospirillum*, *Bradyrhizobium*, *Xanthobacter*, *Ehrlichia* and *Azospirillum irakense*/*Rhodocista* spp., (ii) the *Gammaproteobacteria* species *Pantoea*

Table 2 Statistical results for the 76 individual bacterial rRNA gene probes that displayed significant ($P<0.05$) treatment effect(s) in two-factor ANOVA

Target range		*Probe*	*Statistical results*[a]		
Taxonomic affiliation of the target	*Target*		*Soil*	Thielaviopsis basicola	*Soil* × Thielaviopsis basicola
Alphaproteobacteria					
Acetobacteraceae	*Acidiphillium*	Acdp821			*
	Acidiphillium	Aci1	MC112**		
	Acidiphillium/Acidocella	Aci2	MS8**		
	Gluconacetobacter	Inteur1	MS8**	I*	
Sphingomonadaceae	*Sphingomonadaceae*	Sphingo4	MS8**		
	Sphingomonadaceae	Sphingo5A	MS8**		
Rhodobacteraceae	*Rhodobacteraceae*	Rhodobact1B	MC112**	N*	
Rhodospirillaceae	*Azospirillum* and relatives	Azo1	MS8**		
	Azospirillum and relatives	Azo5	MS8*		
	Azospirillum irakense/Rhodocista spp.	Ira1	MC112**		
	Rhodospirillum	Rhodo2	MC112**		
Rhizobiaceae	*Rhizobium/Sinorhizobium/Ensifer*	Rhi	MC112**		
	Agrobacterium tumefaciens	Rhira7	MC112**		
Bradyrhizobiaceae	*Bradyrhizobium*	Brady4	MC112**		
	Bradyrhizobium	Brady6A	MC112**	I*	
Xanthobacteraceae	*Xanthobacter*	Xan	MC112**		
Caulobacteraceae	*Caulobacter*	Caubact1	MC112*	N*	*
Anaplasmataceae	*Ehrlichia*	Ehrli1	MC112**		
Betaproteobacteria					
Burkholderiaceae	*Burkholderia glumae*	Bkhglum	MS8*		
	Burkholderia glathei, multivorans, cepacia, andropogonis	Burkho4C	MS8*		
	Burkholderia caribensis, hospita	Bkhcari1			**
Oxalobacteraceae	*Herbaspirillum seropediceae*	Hefri2	MS8*		
Comamonadaceae	*Comamonas*	Comtes2	MS8*		
Nitrosomonadaceae	*Nitrosospira/Nitrosovibrio*	Nit1A	MS8**		
	Nitrosospira/Nitrosovibrio	Nit1C		I*	
Alcaligenaceae	*Castellaniella*	Aldef5	MC112**		
Gammaproteobacteria					
Enterobacteriaceae	*Photorhabdus*	Pho1	MS8**		
	Pantoea agglomerans	Pagg6			*
	Pantoea agglomerans	Pagg5	MC112*		
	Pantoea toletana	Ptol1	MC112**(#)		
Pseudomonadaceae	*Pseudomonas*	Pseu1	MS8*		**
	Pseudomonas	PseuD			**
	Pseudomonas clusters C3–C5	PseubC2-10	MS8**		
	Pseudomonas clusters C3–C5	PseubC2BC3-2	MS8**		
	Pseudomonas citronellolis, *nitroreducens*	Pseu33	MC112**	I*	
Xanthomonadaceae	*Xanthomonadaceae*	XAN818	MS8**	I*	
	Stenotrophomonas/Xanthomonas	SteXan2	MS8*		
Methylococcaceae	*Methylomonas*	Methy1B	MS8**		
	Methylosarcina	Metsar5	MS8**		
Deltaproteobacteria					
Cystobacterineae	*Cystobacterineae*	CystbSUB1	MC112		
Polyangiaceae	*Polyangiaceae*	Polyang10	MS8**		*
	Uncultured *Chondromyces*	un4Chon4	MC112**		
Syntrophobacteraceae	*Thermodesulforhabdus*	TDRNO1030	MC112**		
Epsilonproteobacteria					
Helicobacteraceae	*Helicobacter heilmanni* and relatives	HeliFel1	MS8*		
Bacteroidetes	*Bacteroidetes*	CFB562C		I*	
Flexibacteraceae	*Spirosoma*	Spiro5	MS8*		
Rikenellaceae	*Rikenella*	Rik6	MC112**		
Cyanobacteria					
Unclassified	*Lyngbia*	Lyng2	MS8**		
Firmicutes					
Bacillaceae	*Bacillus circulans*	Baccir1	MC112**		
	Bacillus fortis and relatives	Bacfor1	MC112*		
	Bacillus vedderi	Bacved_3	MC112**		

Table 2 (Continued)

Target range		*Probe*	*Statistical results*[a]		
Taxonomic affiliation of the target	*Target*		*Soil*	Thielaviopsis basicola	*Soil* × Thielaviopsis basicola
Paenibacillaceae	*Paenibacillus alginolyticus* and relatives	PalgiG3	MS8*(#)		
	Cohnella	Phon1	MS8*(#)		
Lactobacillaceae	*Lactobacillus crispatus*	Lacto39	MC112**		
Clostridiaceae	*Clostridium*	Clost	MC112*		
Veillonellaceae	*Dialister*	Diali3	MS8**		
	Megasphaera	Mega8	MS8**		*
	Selenomonas noxia, flueggei, infelix	Sele1	MS8*(#)	N*	*
	Uncultured *Selenomonas*	Sele4	MC112*		
Tenericutes					
Mycoplasmataceae	*Mycoplasma*	Myco11	MC112**	I*	
Spiroplasmataceae	*Spiroplasma*	Spiroplas4	MC112**		
Actinobacteria	*Actinobacteria*	HGC236-m	MC112**		
Cardiobacteriaceae	*Collinsella*	Collin29	MS8**(#)		
Actinomycetales	*Actinomycetales*	ActORD1	MC112**		
Streptosporangineae	*Streptosporangineae*	Actino1	MC112**		
Corynebacteriaceae	*Mycobacterium*	Mycoba1	MC112**	N**	**
	Mycobacterium	Mycoba2	MC112**	N**	**
Microbacteriaceae	*Agromyces*	Agrm4	MS8*(#)		
Candidate phylum OP2	OP2	OP2-1	MS8*		*
OP11	OP11	OP11-5	MC112*		
Acidobacteria					
Acidobacteriaceae	Uncultured *Acidobacteria* group 4	Acido-c			*
	Uncultured *Acidobacteria* group 6	Acido-a	MS8**		
	Uncultured *Acidobacteria* group 6	AcidUnc	MS8**		
	Maize rhizosphere clones affiliated to *Acidobacteria* group 7	RhizoLCSA2	MS8**		
Planctomycetes	*Planctomycetes*	EUB338II	MC112**		

Abbreviation: ANOVA, analysis of variance.
[a]Significant results for soil factor, *T. basicola* factor and soil × *T. basicola* interaction are indicated with *($P<0.05$) or **($P<0.01$). When soil factor was significant, the soil giving higher hybridization signal is shown, and the symbol (#) is added when the other soil did not yield any signal. When *T. basicola* factor was significant, the treatment (I for inoculated and N for non-inoculated) giving higher hybridization signal is indicated.

(ex *Erwinia*) *toletana* and *Pantoea agglomerans*, (iii) the *Deltaproteobacteria* genus *Thermodesulforhabdus*, (iv) the *Mollicutes* genera *Spiroplasma* and *Mycoplasma*, (v) the *Actinobacteria* in general and the *Actinobacteria* genus *Mycobacterium* and (vi) the *Firmicutes* species *Bacillus vedderi*, *Bacillus fortis* (and closely related species) and *Bacillus circulans*.

Validation of selected microarray results by cloning/sequencing of PCR products and/or qPCR

The presence of selected taxa, for which the corresponding probes (i) gave positive signals both with soils MS8 and MC112, but (ii) contributed significantly to soil discrimination, was verified by cloning/sequencing (Supplementary Table 4). The genera *Azospirillum*, *Burkholderia*, *Bradyrhizobium*, *Nitrosospira*/*Nitrosovibrio*, *Mycobacterium* and genera within the *Sphingomonadaceae* family were successfully evidenced in both soils. The presence of *Herbaspirillum* (rather than the species *H. seropedicae* targeted by probe Hefri2) was confirmed only in soil MS8, as only clones of its close relative *Janthinobacterium* were found in soil MC112. Overall, with the exception of *Herbaspirillum* primers, the majority of clones were indeed affiliated to the expected taxonomic group, indicating that probe specificity was reliable.

qPCR procedures were developed and their specificity validated by cloning–sequencing (Supplementary Table 4) for a few selected taxa. qPCR results (Table 3) confirmed microarray findings that bacteria affiliated to *Sphingomonadaceae* were more prevalent in the tobacco rhizosphere in suppressive soil and *Mycobacterium* in conducive soil. For two plants grown in MS8, bacteria affiliated to *Bradyrhizobium* were less prevalent than in conducive soil, as expected from microarray results, but for the third plant in MS8, the abundance was comparable with that in the rhizosphere of plants grown in MC112. For all the taxa tested, the between-plant variability within a soil was higher with qPCR than with microarray.

Table 3 Comparison of microarray and qPCR results for *Bradyrhizobium*, *Mycobacterium* and *Sphingomonadaceae*

	Bradyrhizobium	Mycobacterium	Sphingomonadaceae
Microarray	0.62 ± 0.02	0.03 ± 0.03	5.47 ± 0.64
qPCR	0.41 ± 0.52	0.35 ± 0.34	15.8 ± 9.9

Abbreviation: qPCR, quantitative PCR.
Microarray results are shown as the ratio (mean ± s.d.) of signal intensities between soil MS8 and MC112 for probes Brady4 (*Bradyrhizobium*), Mycoba2 (*Mycobacterium*) and Sphingo4 (*Sphingomonadaceae*), which for each plant grown in MS8 was obtained by dividing signal intensity by the mean signal intensity obtained with the three plants from MC112. Quantitative PCR data represent the ratio (mean ± s.d.) of *rrs* copies between soil MS8 and MC112 using *Bradyrhizobium*-, *Mycobacterium*- and *Sphingomonadaceae*-specific primers (Supplementary Table 2).

Discussion

The majority of studies on disease-suppressive soils have been restricted to one effective microbial population, omitting the rest of the microbial communities (Mazzola, 2002; Nel *et al.*, 2006; Weller, 2007; Frapolli *et al.*, 2008). In the rhizosphere, however, the microbial community is diverse, which means that different types of microbial populations might protect the plant. In addition, complex interactions take place between microorganisms and roots, and the effective plant-protecting populations may be influenced by accompanying microbiota (Duijff *et al.*, 1999; Raaijmakers *et al.*, 2008). This is especially relevant in the case of black root rot-suppressive soils from Morens, in which the antagonistic *Pseudomonas* populations do not differ extensively from those in conducive soils (Ramette *et al.*, 2003; Frapolli *et al.*, 2008).

The new 16S rRNA gene-based taxonomic microarray developed here combines previous prototypes, which provided an extensive coverage of bacterial diversity at higher taxonomical units (19 of 34 bacterial phyla can be detected) and have been validated for analysis of soil/rhizosphere samples (Sanguin *et al.*, 2006a, b, 2008; Demanèche *et al.*, 2008; Kyselková *et al.*, 2008). To access rhizobacterial populations potentially relevant in suppressive soils, however, the microarray had to be significantly expanded (Table 1). This was achieved by designing several 100 additional probes, to target low-taxonomic-level taxa (that is, genus/species) known to include at least certain strains involved in beneficial interactions with plants and/or biocontrol of plant pathogens (Rodriguez and Fraga, 1999; Raaijmakers *et al.*, 2002, 2008; Kennedy *et al.*, 2004; Bally and Elmerich, 2005). The level of unexpected hybridizations with pure strains (2.3%, often corresponding to weak nonspecific signals) was well in line with that in other microarray studies (Bodrossy *et al.*, 2003; Loy *et al.*, 2005; Sanguin *et al.*, 2006a; Kyselková *et al.*, 2008).

To identify taxa indicative of soil suppressive status, we compared tobacco rhizobacterial communities from a suppressive and a conducive soil. Data showed that they differed rather in terms of probe signal intensity than in the number of positive probes. This is in accordance with the fact that suppressive and neighboring conducive soils near Morens are largely similar in physicochemical properties (except clay mineralogy; Stutz *et al.*, 1989) and subjected to the same type of crop rotation and farming practices (Stutz *et al.*, 1985; Ramette *et al.*, 2003). Indeed, only a few taxa (corresponding to six probes) were detected in one soil only, and when so, probe signals were weak (often close to detection limit). It was the case for *P. toletana* (described as a secondary invader of olive knots; Rojas *et al.*, 2004) found only in conducive soil, and *Agromyces* (rather common soil actinomycete), *Collinsella* (human intestine bacterium; Zoetendal *et al.*, 2004), *Selenomonas noxia*/*S. flueggei*/*S. infelix* (anaerobes associated with periodontal pockets; Moore *et al.*, 1987), *Paenibacillus alginolyticus*/*P. chondroitinus* and *Cohnella* (all three are phytopolymer degraders from the *Paenibacillaceae* family; Nakamura, 1987; Yoon *et al.*, 2007) detected only in suppressive soil. Similarly, the presence/absence of signals from one plant to the next within a soil concerned only weakly hybridized probes.

As many as 69 of the 199 probes positive with both soils (that is, 35%) gave statistically significant differences in signal intensities between suppressive and conducive soil. Variability in signal intensity of a probe may be due to a different quantity of target molecules, and/or weak nonspecific binding of non-targets (especially in the absence of target molecules), as shown with pure strains. Therefore, for selected probes that discriminated between the two soils, we (i) verified that the targets were actually present in both soils (by cloning/sequencing) and/or (ii) confirmed the higher prevalence of the corresponding taxa in one of the soils (by qPCR). A good agreement between microarray and cloning/sequencing results was already found with partial probe sets (Sanguin *et al.*, 2006a, b, 2008; Kyselková *et al.*, 2008). Therefore, the differences of signal intensities between soils MS8 and MC112 may be generally interpreted as differences in taxa abundance.

For about half of the probes discriminating between Morens suppressive and conducive soils, the mean signal intensity was 2–60 times higher or lower in one of the soils (Figure 1), pointing to important quantitative differences in community composition. Comparable results were obtained in two studies assessing whole rhizobacterial communities (i) of clubroot suppressive soil after amendments with, for example, chitin (Hjort *et al.*, 2007) or (ii) in neighboring soils suppressive or not to ectoparasitic nematodes (where rhizobacterial community structure differed despite a similar soil composition; Rimé *et al.*, 2003). In the two studies, however, the fingerprinting methodology did not enable direct identification of the corresponding taxa.

Though a function cannot be directly attributed to bacteria identified with *rrs* microarray, it is interesting to note that several taxa associated with suppressive soil MS8 are known to include strains with biocontrol capacity (Raaijmakers *et al.*, 2008). Besides fluorescent pseudomonads, for which Phl+ strains have already been studied in Morens (for example, Stutz *et al.*, 1986; Ramette *et al.*, 2003; Frapolli *et al.*, 2008), it is the case for the *Burkholderia* spp., for which certain strains produce various antifungal compounds and can antagonize fungal phytopathogens (Hwang *et al.*, 2002; Compant *et al.*, 2008). It applies also to taxa for which biocontrol ability has been less studied, noticeably *Azospirillum* spp. (Bashan and de-Bashan, 2002; Russo *et al.*, 2008) and *Comamonas* (Thompson *et al.*, 1998). In addition, probes for several taxa extensively studied for direct stimulation of plant growth (Baldani *et al.*, 1986; Mehnaz and Lazarovits, 2006) also gave higher signals in the suppressive soil. It is the case for the *Azospirillum* spp. well known for associative nitrogen fixation, auxin production and/or ACC deaminase activity (Dobbelaere *et al.*, 1999; Blaha *et al.*, 2006), as well as certain strains from genera *Herbaspirillum* (Baldani *et al.*, 1986), *Sphingomonas* (Adhikari *et al.*, 2001) and *Gluconacetobacter* (Kennedy *et al.*, 2004) studied for associative nitrogen fixation. This can be relevant for plant health because an enhanced plant vigor is important for resistance to pathogen attacks (Agrios, 1997). These taxa are of interest as bioindicators of disease suppressiveness, and they would merit an assessment of their potential functional role in suppressive soils. Comparably fewer taxa known to include bacteria with plant-beneficial properties were associated with the conducive soil MC112, for example *Bradyrhizobium* and *P. agglomerans*, which contain biocontrol strains (Siddiqui and Shaukat, 2002; Stockwell *et al.*, 2002).

Root necrosis causes leakage of organic root constituents, which leads to distinct ecological conditions of rhizosphere colonization (Yang *et al.*, 2001; Chapon *et al.*, 2002). The shifts in various rhizosphere populations that can take place due to higher disease levels in conducive soil (Sarniguet and Lucas, 1992; McSpadden Gardener and Weller, 2001) may represent a bias when comparing suppressive and conducive soils (Ramette *et al.*, 2003). In this work, however, root damage by added *T. basicola* did not affect rhizobacterial community composition, even in conducive soil. This result strengthens and expands the previous observation derived from the sole analysis of *Pseudomonas* populations (Frapolli *et al.*, 2008). Perhaps lytic activity of *T. basicola* was not very strong when infecting tobacco roots (Hood and Shew, 1997), which means that root damage could have taken place without extensive nutrient leakage from diseased roots.

In conclusion, microarray comparison of a suppressive and conducive soil from Morens revealed previously unseen differences in the predominance of a large number of bacterial taxa between the two soils. We suppose that such differences in rhizobacterial community composition may be found for different types of suppressive soils and some of the taxa identified in this study could prove useful as disease suppressiveness indicators. In addition, many of these taxa are known to include strains with biocontrol or plant growth-promoting properties, and they represent key new targets whose potential functional role in disease suppression will need to be assessed.

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