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Deciphering the Rhizosphere Microbiome for Disease-Suppressive Bacteria

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TFP-mediated ATP generation and to facilitate viral replication.

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Supporting Online Material

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Materials and Methods
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References

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Deciphering the Rhizosphere Microbiome for Disease-Suppressive Bacteria

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Disease-suppressive soils are exceptional ecosystems in which crop plants suffer less from specific soil-borne pathogens than expected owing to the activities of other soil microorganisms. For most disease-suppressive soils, the microbes and mechanisms involved in pathogen control are unknown. By coupling PhyloChip-based metagenomics of the rhizosphere microbiome with culture-dependent functional analyses, we identified key bacterial taxa and genes involved in suppression of a fungal root pathogen. More than 33,000 bacterial and archaeal species were detected, with Proteobacteria, Firmicutes, and Actinobacteria consistently associated with disease suppression. Members of the γ-Proteobacteria were shown to have disease-suppressive activity governed by nonribosomal peptide synthetases. Our data indicate that upon attack by a fungal root pathogen, plants can exploit microbial consortia from soil for protection against infections.

Similar to other eukaryotes, plants and their microbiomes can be viewed as "superorganisms" in which the plant relies, in part, on the soil microbiota for specific functions and traits. In return, plants exude up to 21% of their photosynthetically fixed carbon in the root-soil interface (*1*), i.e., the rhizosphere, thereby feeding the microbial communities and influencing their activity and diversity. For decades, studies about the interplay between plants and rhizosphere microorganisms have focused on pathogens, symbiotic rhizobia, and mycorrhizal fungi, yet there is evidence that other groups of soil microorganisms can affect plant growth and health (*2*). It even has been postulated that plants actively recruit beneficial soil microorganisms in their rhizospheres to counteract pathogen assault (*3*). One well-known phenomenon is the occurrence of disease-suppressive soils, a property conferred by the resident microbiota via as yet unknown mechanisms (*4*, *5*). Hence, the aim of this study is to decipher the rhizosphere microbiome to identify such disease-suppressive microbes and to unravel the mechanisms by which they protect plants against root diseases.

We used a high-density 16*S* ribosomal DNA (rDNA) oligonucleotide microarray, referred to as the PhyloChip (*6*, *7*), to identify key bacterial and archaeal community members in the rhizosphere of plants grown in a disease-suppressive soil. We subsequently targeted and isolated specific bacterial taxa to elucidate the biosynthetic genes and pathways underlying pathogen control.

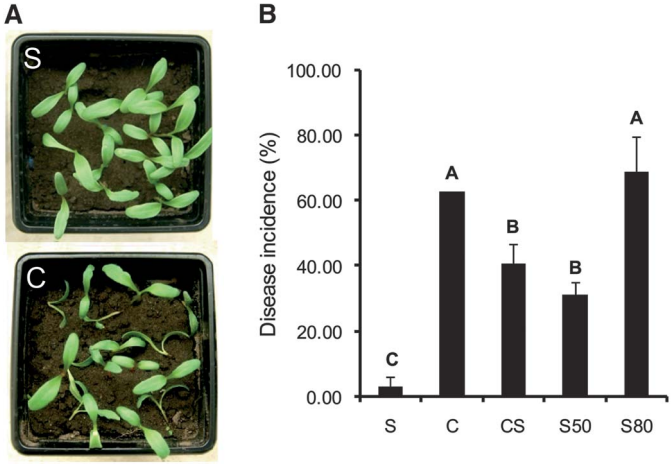

Fig. 1. (**A**) Effect of *R. solani* infection on growth of sugar beet seedlings in disease-suppressive (S) and disease-conducive (C) soils. (**B**) Percentage (mean ± SEM, $N = 4$) of seedlings with damping-off symptoms in suppressive soil (S), conducive soil (C), conducive soil amended with 10% (w/w) of suppressive soil (CS), or suppressive soil heat-treated at 50°C (S50) or 80°C (S80). Different letters above the bars indicate statistically significant differences ($P < 0.05$, Student-Newman-Keuls).

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The soil we investigated is suppressive to *Rhizoctonia solani*, an economically important fungal pathogen of many crops including sugar beet, potato, and rice. This soil was identified in field surveys in the Netherlands conducted by the Institute of Sugar Beet Research in 2004. In the years before its discovery, sugar beet plants grown in this field were severely affected by *R. solani*, suggesting that, similar to other suppressive soils, a disease outbreak is required for the onset of suppressiveness (*4*, *5*). When tested under greenhouse conditions with sugar beet as the host plant, this soil maintained its exceptional disease-suppressive activity toward *R. solani*, whereas a soil with similar physical-chemical properties (table S1), obtained from the margin of the same field, showed a high disease incidence (disease-conducive) (Fig. 1). In the absence of the fungal pathogen, no significant differences in plant growth and health were observed between the suppressive and conducive soils (table S2).

Most suppressive soils lose their disease-suppressive activity when pasteurized (*4*, *5*). When the *Rhizoctonia*-suppressive soil was heat treated at 50°C, disease suppressiveness was partially lost; treatment at 80°C resulted in a complete loss of suppressiveness, i.e., disease incidence increased to a level similar to that of the disease-conducive soil (Fig. 1B and fig. S1). Gamma irradiation too resulted in loss of suppressiveness (fig. S2). Soil-transfer experiments, in which small amounts (1 to 10% w/w) of suppressive soil were mixed with conducive soil before plant cultivation, showed that in a 1:9 (w/w) ratio of the *Rhizoctonia*-suppressive soil to the conducive soil, disease suppressiveness was partially transferred (Fig. 1 and fig. S1). Collectively, these results indicated that disease suppressiveness toward *R. solani* was microbiological in nature.

Metagenomic DNA was isolated from the rhizosphere microbiota of sugar beet plants grown in soils that exhibited different levels of disease suppressiveness: (i) suppressive soil; (ii) conducive soil; (iii) conducive soil amended with 10% (w/w) suppressive soil; (iv) suppressive soil heat-treated at 50°C; (v) suppressive soil heat-treated at 80°C; and (vi) suppressive soil inoculated with *R. solani* to identify any bacterial and archaeal taxa that responded to the presence of the fungal pathogen (fig. S3).

A total of 33,346 bacterial and archaeal operational taxonomic units (OTUs) were detected in the rhizosphere microbiome (Fig. 2A), a richness that surpasses that described in other studies (*7*, *8*). The overall distribution of the predominant bacterial phyla ranged from 1% for the Chloroflexi and Cyanobacteria to 20% and 39% for the Firmicutes and Proteobacteria, respectively; unclassified bacterial phyla represented a relatively large group (16%) (fig. S4).

When comparing the six soil treatments with different levels of disease suppressiveness, no significant differences were found in the number of detected bacterial taxa (Fig. 2A). However, when the abundance of the detected taxa was taken into account, we found six clusters of samples that corresponded to the six soil treatments (Fig. 2B and fig. S5). These results suggest that the relative abundance of several bacterial taxa is a more important indicator of disease suppression than the exclusive presence of specific bacterial taxa.

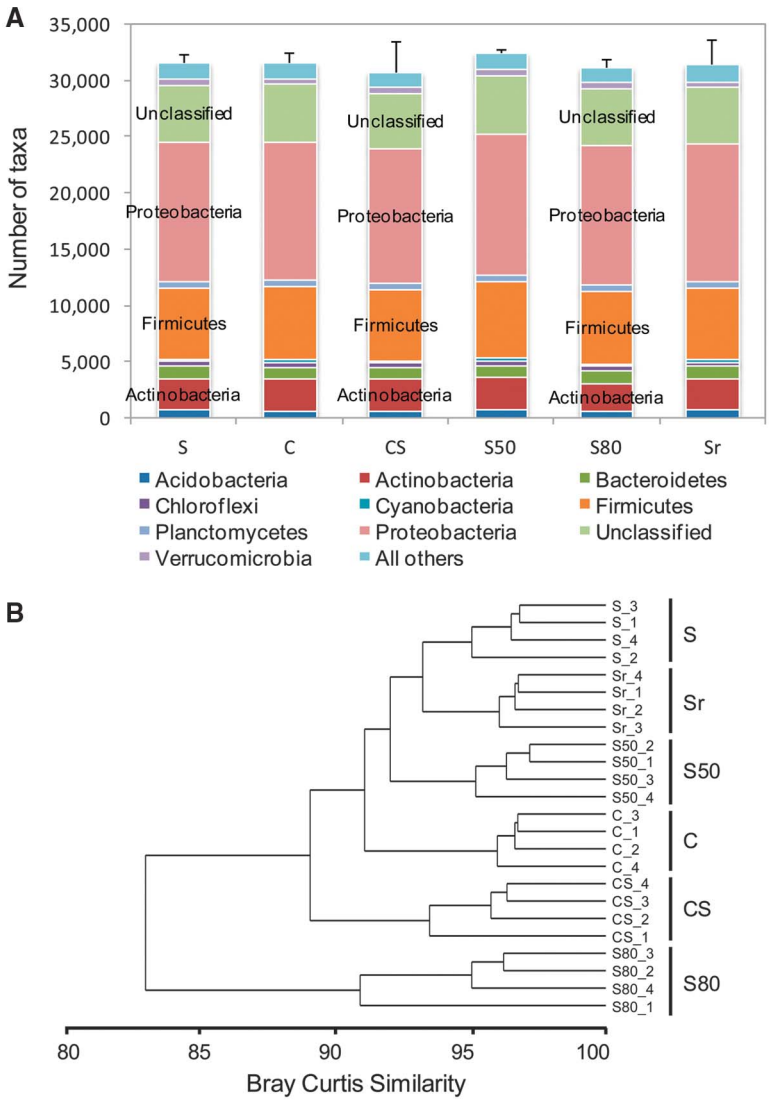

Fig. 2. (**A**) Number of OTUs passing stage 1 PhyCA (mean ± SEM, $N = 4$). (**B**) Cluster analysis (Bray-Curtis) of the rhizosphere microbiomes of sugar beet seedlings grown in soils with different levels of disease suppressiveness. Sr, suppressive soil amended with *R. solani*; other abbreviations as in Fig. 1. In (B), numbers 1 to 4 refer to the replicates of each treatment.

The γ- and β-Proteobacteria (Pseudomonadaceae, Burkholderiaceae, Xanthomonadales) and the Firmicutes (Lactobacillaceae) were identified as the most dynamic taxa associated with disease suppression: These were all more abundant in suppressive soil than in conducive soil; more abundant in the transplantation soil (conducive soil + 10% suppressive soil) than in the conducive soil; and more abundant in the suppressive soil when *R. solani* was present (Fig. 3 and table S3). Separate clustering analyses confirmed their association with disease suppressiveness (fig. S6). The Actinobacteria too were more abundant in suppressive than in conducive soil and were the most dynamic taxa in the suppressive soil amended with the fungal pathogen (Fig. 3).

Culture-based approaches for identifying functional groups involved in disease suppressiveness of soils have focused on bacterial taxa that are easy to grow and amenable to genetic and genomic analyses (*4*). For example, Pseudomonadaceae have been found to contribute to the natural suppressiveness of soils against the fungal pathogens *Fusarium oxysporum* and *Gaeumannomyces graminis* (*4*, *5*). Notably, the culture-independent PhyloChip analysis we conducted also pointed to a prominent role for γ-Proteobacteria, especially Pseudomonadaceae, in soil suppressiveness against *R. solani*. Hence, we focused subsequent studies on this group of bacteria.

Cultures of rhizosphere suspensions of sugar beet plants grown in disease-suppressive or

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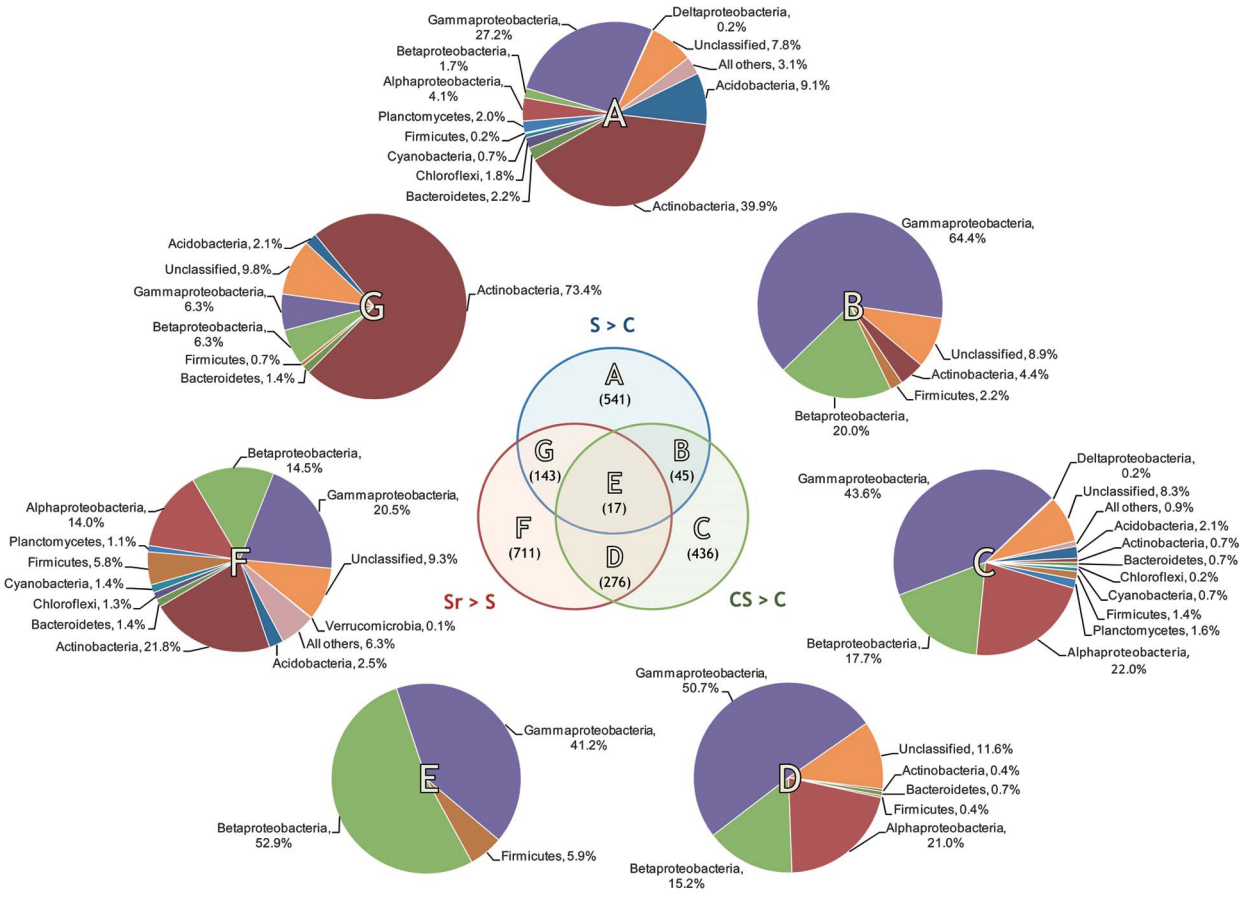

Fig. 3. Bacterial and archaeal taxa associated with disease suppressiveness. Shown are taxa that are more abundant in (i) suppressive (S) than in conducive soil (C) (pie A), (ii) "transplantation soil" (C+10%S) than in C (pie C), and (iii) S amended with *R. solani* (Sr) than in S (pie F). Pairwise comparisons (N = 4) depict the compositions of the top 10% of most dynamic taxa. Numbers of taxa in each subset are in parentheses. In pie charts A to G, names of taxonomic groups are followed by their frequency. The top 10% of most dynamic taxa that meet all three criteria are shown in pie E and in table S3.

disease-conducive soils were randomly selected, purified, and tested for activity against *R. solani* (table S4). Most of the antagonistic bacterial isolates from the suppressive soil (i.e., 104 out of 111) were obtained from the growth medium that is semiselective for the Pseudomonadaceae. DNA fingerprinting by BOX–polymerase chain reaction (PCR) grouped the antagonistic isolates from the suppressive soil into 10 haplotypes (SH-A to SH-J). 16*S* rDNA sequencing confirmed that these isolates belonged to the Pseudomonadaceae (Fig. 4A). Alignments and BLAST searches in the PhyloChip database, using the 16*S* rDNA sequences, verified that these haplotypes were closely related (94 to 98% identity) to the five most dynamic Pseudomonadaceae (table S3) and were more abundant in suppressive than in conducive soil (Fig. 4B).

Haplotypes SH-A (38 isolates), SH-B (21 isolates), and SH-C (37 isolates) constituted 90% of the antagonistic bacterial isolates from the disease-suppressive soil and were selected for further functional analyses. Plant bioassays with representative isolates of each of these three haplotypes showed that only strain SH-C52

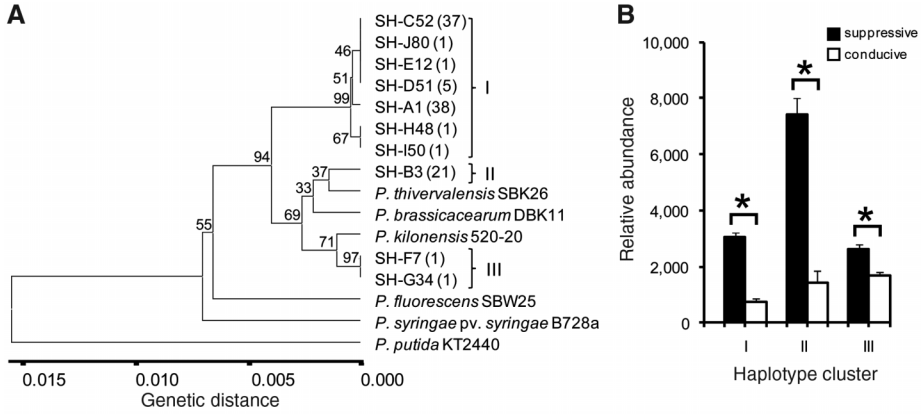

Fig. 4. (**A**) Hierarchical clustering of 16*S* rDNA genes of bacterial strains isolated from the rhizosphere of sugar beet seedlings grown in disease-suppressive soil. Different *Pseudomonas* species and type strains were used as references. Among the isolates that inhibit growth of *R. solani*, 10 haplotypes (SH-A to SH-J) were identified by BOX-PCR. Numbers of isolates of each haplotype are indicated in parentheses, and three haplotype clusters (I to III) were designated. (**B**) Relative abundance of haplotype clusters I to III in suppressive and conducive soils on the basis of PhyloChip analysis. 16*S* rDNA sequences of haplotypes SH-A to SH-J were used in BLAST searches in the PhyloChip database, and the best hits (table S5) were used to calculate the relative abundance of haplotype clusters I to III (mean ± SEM, N = 4). An asterisk indicates a statistically significant difference (P < 0.01, Student's t test) between suppressive and conducive soils.

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protected sugar beet seedlings from infection by *R. solani* (fig. S7). Random transposon mutagenesis generated two mutants of strain SH-C52 with no in vitro activity against *R. solani*. The single transposon insertions were mapped to a nonribosomal peptide synthetase (NRPS) gene with 69% sequence identity to *syrE*, the gene of the syringomycin-syringopeptin (*syr-syp*) biosynthetic pathway in *Pseudomonas syringae* pv. *syringae* (*9*). NRPS-mutant O33 colonized the rhizosphere to the same extent as its parental strain SH-C52, but did not protect sugar beet seedlings from fungal infection (fig. S7). Subsequent genetic analyses revealed that the putative biosynthetic pathway consisted of two gene clusters, designated *thaAB* and *thaC1C2D*, which were predicted to encode a nine–amino acid chlorinated lipopeptide (fig. S8).

The multifaceted approach adopted in this study, linking culture-independent and culture-dependent analyses, shows that plants, like mammals and insects (*10*–*12*), can rely on specific constituents of the microbial community for protection against pathogen infections. We showed that the γ-Proteobacteria, and specifically members of the Pseudomonadaceae, protect plants from fungal infection through the production of a putative chlorinated lipopeptide encoded by NRPS genes. Functional analysis further revealed a significant difference in plant disease suppression between haplotypes SH-A and SH-C (fig. S7), suggesting that in situ antifungal activity is governed by individual members of this bacterial taxon. Next to the Pseudomonadaceae, several other bacterial taxa were found in this study to be associated with disease suppressiveness (Fig. 3). Some of these taxa, including the Burkholderiaceae, Xanthomonadales, and Actinobacteria, harbor genera and species with activity against plant pathogenic fungi, including *R. solani* (*13*). These findings suggest that the complex phenomenon of disease suppressiveness of soils cannot simply be ascribed to a single bacterial taxon or group, but is most likely governed by microbial consortia. The observation that bacterial strains, which lack activity against pathogens when tested alone, can act synergistically when part of microbial consortia (*14*) further exemplifies the complexity of adopting Koch's postulates for identification of microorganisms involved in disease suppressiveness of soils. The bacteria and biosynthetic pathway identified here provide a set of microbial and genetic markers to elucidate whether and how plants recruit beneficial soil microorganisms for protection against infections.

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Supporting Online Material

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Differences Between Tight and Loose Cultures: A 33-Nation Study

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With data from 33 nations, we illustrate the differences between cultures that are tight (have many strong norms and a low tolerance of deviant behavior) versus loose (have weak social norms and a high tolerance of deviant behavior). Tightness-looseness is part of a complex, loosely integrated multilevel system that comprises distal ecological and historical threats (e.g., high population density, resource scarcity, a history of territorial conflict, and disease and environmental threats), broad versus narrow socialization in societal institutions (e.g., autocracy, media regulations), the strength of everyday recurring situations, and micro-level psychological affordances (e.g., prevention self-guides, high regulatory strength, need for structure). This research advances knowledge that can foster cross-cultural understanding in a world of increasing global interdependence and has implications for modeling cultural change.

How "other" cultures differ from one's own has piqued the curiosity of scholars and laypeople across the centuries. As long ago as 400 B.C.E., Herodotus documented a wide variety of cultural practices that he observed in his travels in *The Histories* (*1*). Only in the past few decades have scientists begun to move beyond descriptive accounts of cultural differences to empirically assess ways in which national cultures vary. We examine a neglected source of cultural variation that is dominating the geo-political landscape and has the potential to be a major source of cultural conflict: the difference between nations that are "tight"—have strong norms and a low tolerance of deviant behavior—and those that are "loose"—have weak norms and a high tolerance of deviant behavior.

Early anthropological research showed the promise of this distinction. In his study of 21 traditional societies, Pelto (*2*) documented wide variation in the expression of and adherence to social norms. The Hutterites, Hanno, and Lubara were among the tightest societies, with very strong norms and severe sanctions for norm violation, whereas the Kung Bushman, Cubeo, and the Skolt Lapps were among the loosest societies, with ambiguous norms and greater permissiveness for norm violation. Pelto speculated that these societies may have different ecologies, with tight societies having a higher population per square mile and a higher dependence on crops as compared to loose societies. Later research indeed showed that agricultural societies (e.g., the Temne of Sierra Leone), which require strong norms to foster the coordination necessary to grow crops for survival, had strict child-rearing practices and children who were high

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