New BIOTECHNOLOGY 70 (2022) 116–128

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com/locate/nbt

From the bacterial citrus microbiome to the selection of potentially host-beneficial microbes

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ARTICLE INFO

Keywords:
Citrus microbiomes
Metataxonomics
Culturomics
Rhizospheric bacteria
Beneficial bacteria
Agricultural probiotics

ABSTRACT

Citrus is the most cultivated fruit crop worldwide. The modern citrus industry needs new bioproducts to overcome phytopathological threats, tolerate stresses and increase yield and quality. Mutualistic microbes from roots significantly impact host physiology and health and are a potentially beneficial resource. The bacterial microbiome can be surveyed to select potentially host-beneficial microbes. To achieve this goal, a prevalent "core-citrus" bacterial microbiome was obtained by picking those operational taxonomic units (OTUs) shared among samples within and across two *Citrus* rootstock genotypes grown in the same soil for more than 20 years. A subselection of main OTUs from the defined "core-citrus" microbiome was made based on abundance, host-enriched versus bulk soil, and rhizosphere-indicator species. In parallel, an extensive census of the cultivable microbiota was performed to collect a large number of bacterial citrus isolates. Metataxonomic data were linked to cultured microbes, matching 16S rRNA gene sequences from bacterial isolates with those counterpart OTU reference sequences from the selected bacterial "core-citrus" microbiome. This approach allowed selection of potentially host-beneficial bacteria to mine for agricultural probiotics in future biotechnological applications required for the citrus industry.

Introduction

Citrus are widely consumed worldwide as juice or as fresh fruit. Citrus fruits are commonly considered a good source of vitamin C, but they provide much more than this and like most other whole foods, citrus fruits also contain various essential nutrients and health-promoting compounds [1]. Citrus is the most cultivated fruit worldwide, with an estimated global production of over 143 M metric tons in 2019 (FAOSTAT 2019). Commercial citrus trees are usually grafted rootstocks which are responsible for absorption of water and nutrients and are the hub of the most important horticultural and pathological traits. Mutualistic microbes present in root-associated communities provide a potential source of various benefits for their hosts, including improved growth, nutrition, and protection from biotic and abiotic stresses [2,3]. The agricultural industry needs new bioproducts to overcome 21st-century challenges and unraveling plant microbiomes might help achieve this goal, paving the way for plant microbiomes and agricultural probiotics to enter the spotlight [4–7].

Mutualistic microbes associated with plants have enormous economic potential and are accepted in sustainable agriculture [3]. Nevertheless, only a few commercial products are available, and there is a need for innovative bioproducts required for safe, environmentally sustainable, and eco-friendly citrus production. Exploring citrus microbiota to identify potentially beneficial microbes is the first step in developing future biotechnological applications for the citrus industry [4–7]. Based on potential conclusions from comparison of "core-citrus" and "genotype-specific" members from the assembled microbiomes obtained from two citrus rootstock genotypes grown in one soil (Fig. 1; [8]), we have applied this conceptual possibility to an important crop to generate a collection of bacteria that is potentially beneficial for citrus.

Abbreviations: OUT, operational taxonomic unit; IVIA, Valencian Institute for Agricultural Research.

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https://doi.org/10.1016/j.nbt.2022.06.002
Received 31 January 2022; Received in revised form 9 June 2022; Accepted 11 June 2022
Available online 15 June 2022

To fully harness the benefits provided by the bacterial communities associated with roots, it is crucial to discover, to the extent possible, the full diversity of the citrus microbiota. This work analyzed bacterial microbiomes using a non-cultured broad-sense metagenomic approach (high-throughput 16S rRNA gene sequencing). Specifically, the bacterial rhizospheric microbiomes were determined for two citrus rootstock genotypes plants grown in the same soil. Plants from the same origin (from the IVIA germplasm bank, Valencia, Spain) and nursery production system were established under the same edaphic and climatic conditions and cultivated under standard agricultural practices for more than 20 years. A prevalent "core-citrus" microbiome was then obtained, defined as the suite of members shared among microbial consortia from all analyzed trees of two citrus rootstock genotypes grown in one soil. Those shared operational taxonomic units (OTUs) would thus represent potentially beneficial bacteria for citrus [8,9] (Fig. 1). To capture as much of the bacterial diversity as possible, the cultivable microbiota from the same rhizosphere samples were also isolated (cultured approach). Metataxonomic data were linked to microbe selection by matching 16S rRNA gene sequences from bacterial isolates with those counterpart OTU sequences from the defined and selected "core-citrus" microbiome. This approach allowed the creation of a citrus-adapted and host-selected bacterial collection. Thus, the bacterial collection generated is a source from which to mine for plant-beneficial microbes for future biotechnological applications needed in the citrus industry [7].

Materials & methods

Experimental field plot design

The field plot contained ten blocks with two citrus rootstock genotypes (grafted with Nules clementine). The citrus genotypes were randomly distributed and planted when the plants were one year old in May 1996 in an orchard located at the "Comunidad Valenciana" (39°56'37.5" N 0°08'03.7" W). The soil of the selected experimental field plot is representative of the main citrus growing region in Spain (Valencia region), this being the most important citrus growing region in the Mediterranean basin. The citrus rootstock genotypes evaluated were Carrizo citrange [*Citrus sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.], the rootstock most commercially used in Spain, and Forner–Alcaide 5 [*Citrus reshni* Hort ex Tan x *Poncirus trifoliata* (L.) Raf.] [10], released by the citrus rootstock breeding program from the Valencian Institute for Agricultural Research (IVIA, Valencia, Spain). These two citrus rootstock genotypes represent more than 90 % of planted trees in Spain. Other citrus rootstock genotypes are marginal. All plants had the same origin and were produced under the same nursery procedures at the germplasm bank at IVIA. The citrus trees were cultivated for about 20 years following standard cultural practices with drip irrigation and chemical weed control.

Collection of rhizosphere and soil samples

In April 2015, fibrous roots (6 g of fresh weight) were washed in 100 ml of 0.25X Ringer solution containing 0.05 % Tween 20 and shaken for 45 min at 10 g [11]. Soil attached to and affected by roots and the root surface where bacteria reside (rhizosphere soil plus rhizoplane – ectorhizosphere) comprised the sampled rhizospheric bacterial community. One gram of non-rhizospheric soil from 5 to 20 cm depth constituted the bulk soil samples. To assemble the citrus microbiomes, four randomly selected 20-year-old trees of each citrus rootstock genotype, and their corresponding pooled samples were analyzed.

DNA extraction and quality control

Twenty ml of rhizospheric wash solutions were centrifuged at 10,000xg for 10 min at 4ºC. DNA was extracted from the rhizospheric attached soil or bulk soil (100 mg) and bacterial pellets using the PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA). DNA was cleaned and concentrated by the QIAamp DNA blood Mini kit (QIAGEN, Hilden, Germany). DNA quality was measured using a nanodrop 2000 series (Thermo-Fisher, Waltham, Massachusetts, USA). DNA quality parameters of the samples used had the following ranges: [DNA]= 18–28 ng/µl; $A_{260/280}$ = 1.8–1.9 and $A_{260/230}$ = 1.2–1.8.

Library preparation and deep sequencing

PCR-based bacterial 16 S rRNA gene capture was performed using primers to amplify the V3-V4 region [12]. Samples were quantified with QuanIT Picogreen and pooled equimolarly after an ampure XP (Beckman, Indianapolis, IN, USA) cleaning of small PCR fragments from each sample. Paired-end next-generation sequencing was performed using MiSeq platform following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA) and performed by Life Sequencing S.L. (Valencia, Spain).

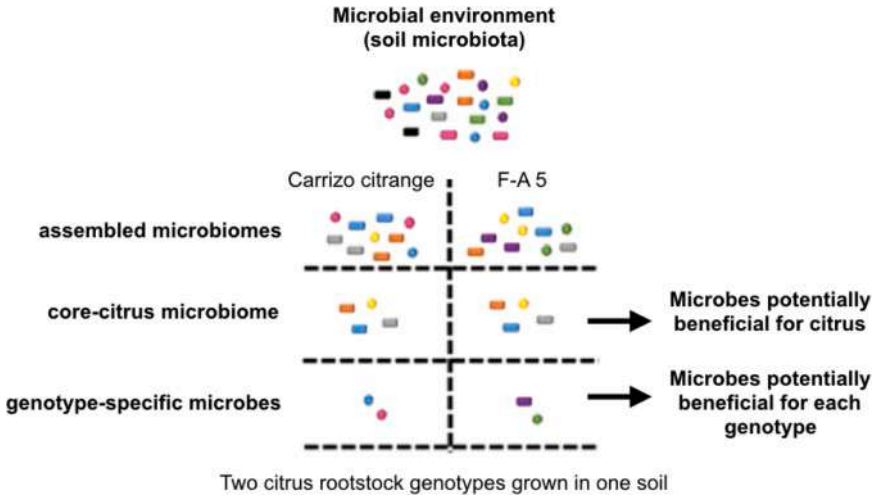

Fig. 1. Conceptual diagram comparing "core" and "specific" citrus rootstock genotype bacterial microbiomes. Microbes potentially beneficial for *Citrus* that might be inferred from "core" and "specific" members when comparing communities from two citrus rootstock genotype plants grown in one soil.
Adapted from [8].

Processing of 16S rRNA gene sequences and taxonomical profiling of OTUs

The 16S rRNA raw sequences were analyzed following the recommendations of the Brazilian Microbiome Project [13]. The OTU table was built using the UPARSE pipeline [14]. The reads were truncated at 380 bp and quality filtered using a maximum expected error of 0.5 (i.e. on average, one nucleotide in every two sequences is incorrect). Filtered reads were dereplicated, and singletons were removed. The sequences were clustered into OTUs at a 97 % similarity cutoff, and chimeras were checked to obtain representative sequences for each phylotype. Taxonomic classification was carried out in QIIME [15] based on the UCLUST method against the Greengenes 13.5 database [16] with a confidence threshold of 80 %. The sampling effort was estimated using Good's coverage [17]. The observed OTU richness and the Shannon diversity index were calculated and plotted using the "phyloseq" package [18].

Alpha and beta variability between assembled microbiomes

Principal coordinates plots (PCoA) of the microbial communities from the two populations of citrus rootstocks genotypes were analyzed by Bray Curtis distance metrics, which accounts for differences in the relative abundance or for the presence/absence of taxonomic unities. The strength and statistical significance of groups were verified by Multivariate ANOVA based on dissimilarities using the Adonis function with the vegan package [19]. The homogeneity of multivariate dispersions within citrus populations and the pairwise difference between groups were analyzed using the parametric Tukey's HSD test. Both datasets were rarefied to the same number of sequences before measurements (13,000 reads). The alpha diversity measurements of the microbial communities were analyzed using Welch's Two Sample t-test.

Rhizosphere-indicator species concept

Rhizosphere-indicator species, meaning those OTUs that are characteristic of the roots versus soil by abundance and prevalence in samples, were identified by the indicator species analysis (ISA) performed with the "indicspecies" package for R [20]. The relative abundances of OTUs in each sample were used to calculate the Indicator Value (IndVal) and its significance [21].

Bacterial isolation

Cultivable bacteria from each rhizosphere sample were isolated by direct plating and enrichment procedures. Seven culture conditions were applied for bacterial isolation. *i-iii*) Bacteria isolated on general media: serial dilutions of rhizosphere washing solutions were placed on three rich general media; King's medium B (KB) [22], MG/L [23], and YPGA [24], and incubated at 26 ºC. A total of 20 bacterial isolates, representing all morphotypes found in any combination of sample (8) x medium (3) (n = 24), were purified. *iv*) Diazotrophic bacteria: serial dilutions were plated on AB minimal medium supplemented with mannitol but depleted nitrogen source (without NH_4Cl) (ABM-N_2). Thirty-five representative bacteria were purified per sample. (*v*) ACC deaminase-producing bacteria: samples were plated on ABM-N_2 supplemented with 1-aminocyclopropane-1-carboxylic acid (acc) as a nitrogen source [25]. Twenty-five isolates were purified from each sample. *vi*) Sporulated bacteria: a *Bacillus*-type selective enrichment by heating rhizosphere washing solutions at 80 ºC was also performed [26]. Seventeen isolates were purified per sample. *vii*) Thermophiles bacteria: samples were inoculated on KB medium and incubated at 50ºC. Ten representative isolates were purified per sampled tree.

Sequencing of bacterial 16S rRNA genes from cultivable microbes

Genomic DNA from liquid cultures was obtained by the GenElute™ Bacterial Genomic Kit (Sigma-Aldrich, St Louis, MN, USA) following the manufacturer's instructions. Alternatively, a CTAB-based protocol was also used [27]. 16S rRNA genes from representative isolates from each bacterial morphotype found in any combination (rootstock genotype, plant, and culture condition) were doubled-strand sequenced after two independent amplifications with primers 16 SF1: 5′-GASTTTGATCCTGGCTYAG-3′ and 16 SR1:5′-GACGGGCGGTGWGTRCA-3′. Subsequently, both PCR products were sequenced by the Sanger technology at Secugen S.L. (Madrid, Spain), and assembled using Unipro UGENE and CLUSTAL tools. The closest relative of each sequenced bacterial isolate was identified by BLASTn to NCBI's Reference 16S rRNA bacterial database.

Selecting potentially beneficial bacteria for Citrus

A database was created with all 16S gene sequences from isolates of a given bacterial genus used as a "subject" in a local BLAST search [28]. Each OTU reference sequence assigned to the same genus from the defined core-citrus microbiome comprised the "query" sequences. A given bacterial isolate was selected for the collection when it showed > 98 % similarity to the corresponding OTU reference sequence.

Fragment recruitment analysis of selected isolate sequences from environmental samples

The initial high-throughput raw data were filtered by quality Phred Score and then collapsed using the flash algorithm [29]. The recruitment of each 16S sequence from each selected isolate was then evaluated using blastn [28]. Finally, the reads with more than 99 % of homology, considering a Phred Quality Score of 20, 100 nucleotides of alignment length, and a minimum of 10 reads, were deemed to be present in the environmental samples. This last step was performed using a custom script in Perl.

Results

DNA high-throughput sequencing

A total of 180,286 high-quality 16S rRNA amplicon sequences were generated from eight rhizospheric samples covering four replicate trees of two citrus rootstock genotypes grown in the same soil, plus two additional pooled samples for each rootstock (Carrizo citrange "C" and Forner–Alcaide 5 "F"). The numbers of high-quality sequences obtained in C and F citrus rootstock genotypes were 85,910 and 94,376, respectively. A total of 3035 OTUs (97 % nucleotide identity [ID]) were identified for further taxonomical profiling (Supplementary Table S1). Raw high-throughput sequencing data from 16S rRNA amplicon libraries for rhizosphere samples are deposited at the European Nucleotide Archive from EMBL (ENA; http://www.ebi.ac.uk/ena) under accession numbers ERR5243817 to ERR5243824.

Structure, variation, and assembly of the rhizospheric bacterial citrus microbiome

The ten most abundant phylotypes represented between 23.5 % and 37.3 % of the total rhizospheric bacterial community associated with citrus trees, independently of the rootstock genotype (Fig. 2). The same most abundant phylotypes were found in all plants from both citrus rootstock genotypes. The citrus microbiota was dominated by either the genera *Pseudomonas* (ranging from 4.0 % to 20.3 %), by the yet uncultured candidate orders *WD2101* (2.2–6.4 %) and *iii1–15* (2.4–6.1 %), or

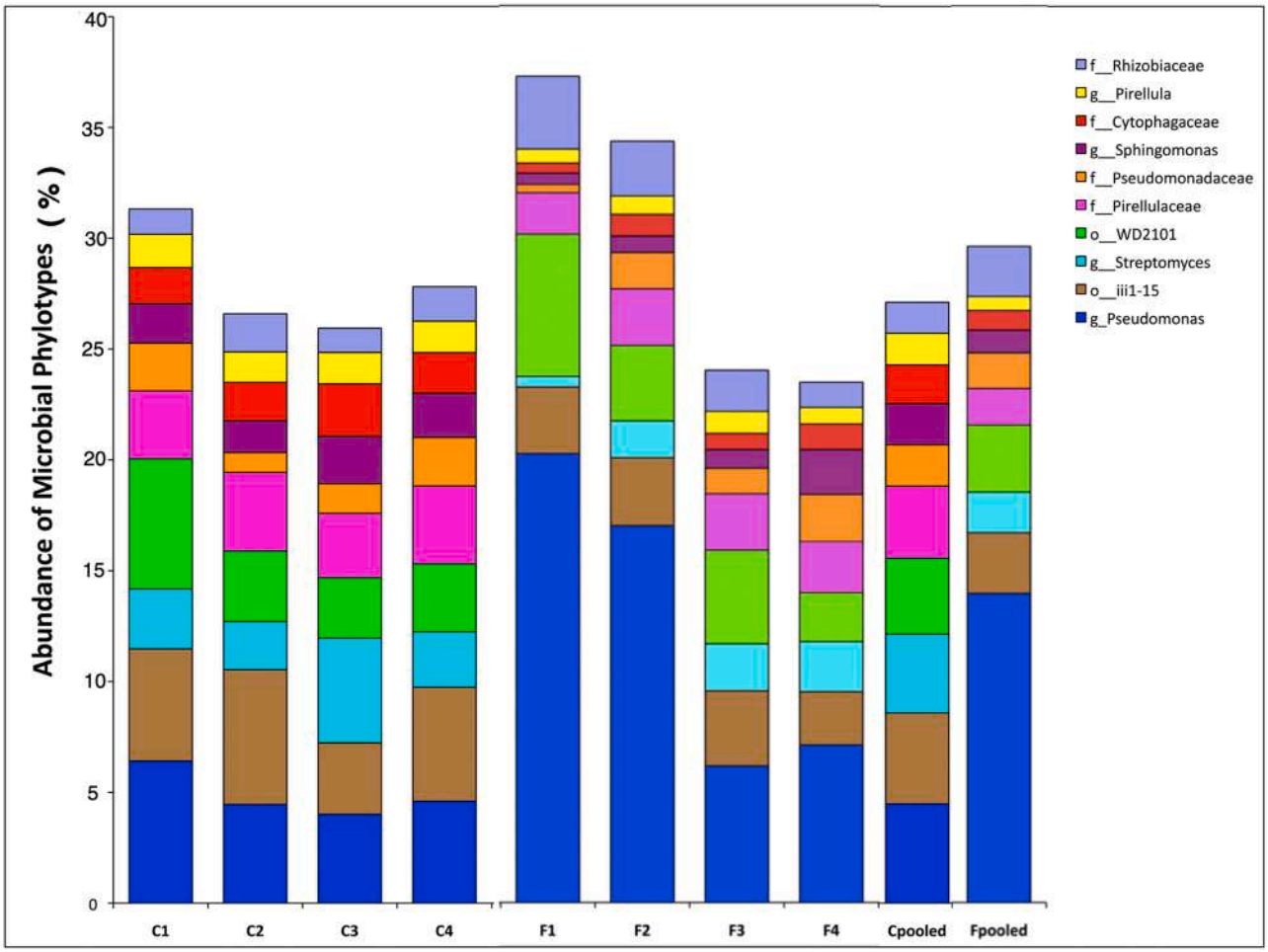

Fig. 2. The ten most abundant bacterial phylotypes found in citrus microbiomes represent ca. 30 % of the total rhizospheric bacterial community associated with citrus trees, the genus *Pseudomonas* being the most abundant phylotype. Samples labeled "C" correspond to Carrizo citrange and those labeled "F" to Forner-Alcaide 5 citrus rootstock genotypes, respectively. Cpooled and Fpooled are the corresponding pooled samples from each citrus rootstock genotype.

by the genera *Streptomyces* (0.5–4.7 %) (Fig. 2). The next most abundant phylotypes were from the families *Pirellulaceae* (1.9–3.5 %) and *Rhizobiaceae* (1.1–3.3 %). After these phylotypes, the families *Cytophagaceae* and *Pseudomadaceae* and the genera *Sphingomonas* and *Pirellula* represented from 0.4 % to 2.4 % of the total rhizospheric bacterial community associated with citrus trees. Carrizo citrange "C" microbiota was dominated by the phylotypes *Pseudomonas*, *iii1–15* or *Streptomyces*, while all samples from Forner-Alcaide 5 "F" citrus trees were dominated by *Pseudomonas* (Fig. 2).

Citrus rootstock genotype comparisons

Twenty-one differentially abundant phylotypes at the taxonomic level of genera were found among rootstock genotypes ($P < 0.05$) (Fig. 3). Seven affiliated genera showed significantly higher proportions in C rhizospheres, the genus *Pirellula* being the most significantly abundant (Fig. 3). All differentially abundant microbial phylotypes were present in both plant genotypes. All bacterial phylotypes were in higher proportions in the Carrizo citrange citrus rootstock genotype (Fig. 3). The bacterial abundance rather than presence or absence explains this difference. Accordingly, significant differences were found between the assembled microbiomes from both rootstocks when comparing for the abundance of OTUs ($P = 0.03$) (Supplementary Fig. S1a). However, this significant difference was not observed between rootstock genotypes when comparing the presence/absence of OTUs ($P = 0.37$) (Fig. 3 and Supplementary Fig. S1b). The phylotype distribution within plants from the Citrange carrizo rootstock was significantly more homogenous than the assembled microbiomes from the Forner-Alcaide 5 citrus rootstock ($P = 0.03$), which varied considerably between plants (Fig. 2 and Supplementary Fig. S1c). Alpha-diversity analyses showed that assembled rhizospheric citrus microbiomes from Carrizo citrange plants present slightly higher diversity than those from the Forner-Alcaide 5. However, no significant differences were found between citrus rootstock genotypes (Observed $P = 0.10$ and Shannon $P = 0.06$) (Supplementary Fig. S2).

Defining a prevalent core-citrus microbiome

Both citrus rootstock genotypes shared 76.3 % of the citrus-associated bacterial community comprising 2,478 OTUs (Fig. 4a), allowing for further identification of citrus-adapted microbes. To identify prevalent "core-citrus" microbiome members, the OTUs shared among plants within and across genotypes were selected, thus reflecting high host adaptation [9]. Only 544 out of the total shared OTUs were found in all replicate plants from both citrus rootstock genotypes ($n = 8$) (Fig. 4b). Taxonomical profiling of those prevalent citrus adapted OTUs classified them into 197 phylotypes at the species level (Supplementary Table S2). Of these phylotypes, 72 were assigned to a given genus (36.5 %), comprising 144 out of the 544 OTUs of the "core-citrus" microbiome (26.5 %) (Supplementary Table S2). As shown in Fig. 5**a,b**, the core-citrus microbiome members belong mainly to the phyla *Proteobacteria* (45.30 % of the core microbiome; 150 OTUs); *Actinobacteria*

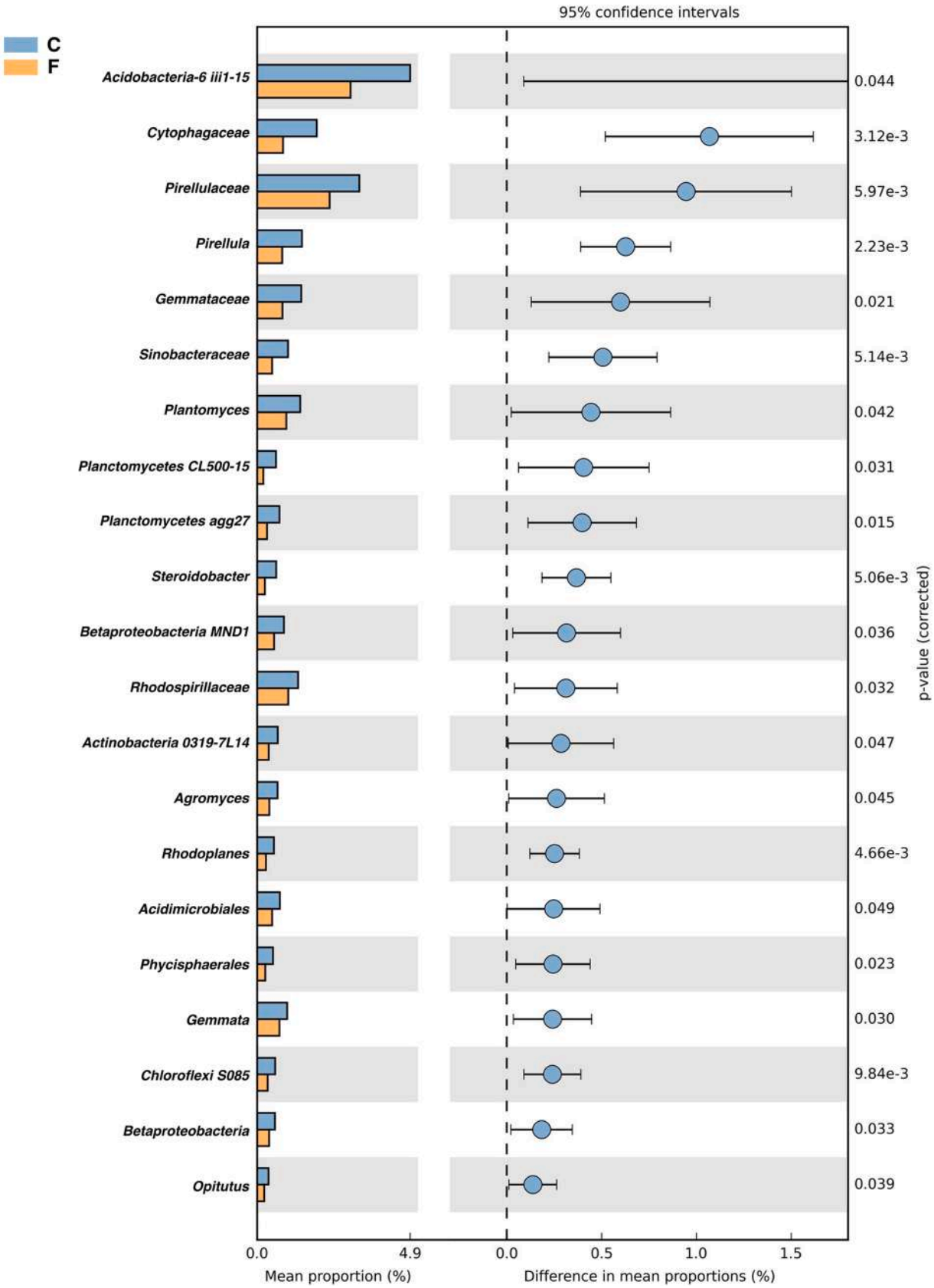

Fig. 3. The significant differential OTUs found between both citrus rootstock genotypes are based on their abundance, rather than in their presence or absence.

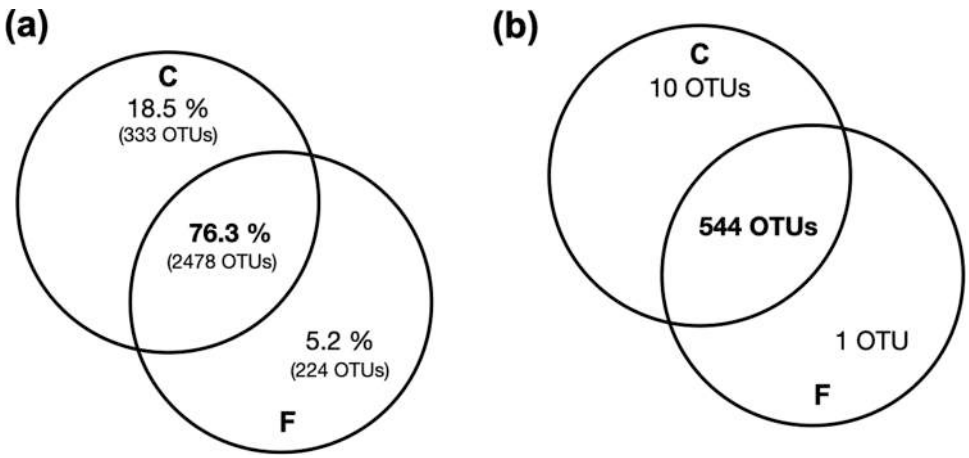

Fig. 4. Sharing ("core-citrus" microbiome) and unique ("genotype-specific" microbiomes) bacterial communities obtained from the comparison of the assembled citrus microbiomes from the two citrus rootstock genotypes grown in one soil. Prior to Venn diagram analyses, sequencing depth was rarefied to 13,000 reads among samples and singleton OTUs were discarded. The resulting data set consisted of 3,035 OTUs (Supplementary Table S1). Carrizo citrange (C) and Forner-Alcaide 5 (F) rootstock genotypes. (a) Venn diagram according to membership: shared OTU occurrences across communities. (b) Venn diagram according to prevalence: shared OTUs among plants within and across genotypes (n = 8) [9].

(13.94 %; 81 OTUs); *Planctomycetes* (10.84 %; 96 OTUs); *Acidobacteria* (9.49 %; 69 OTUs); *Bacteroidetes* (6.00 %; 36 OTUs); *Cloroflexi* (3.37 %; 38 OTUs); *Verrucomicrobia* (3.18 %; 23 OTUs); *Firmicutes* (2.57 %; 10 OTUs); TM7 (2.51 %; 13 OTUs); Gemmatimonadetes (1.06 %; 11 OTUs); Nitrospirae (1.00 %; 7 OTUs); and others minor phyla (0.73 %; 10 OTUs) (Fig. 5a,b). Within the most abundant "core-citrus" phyla, proteobacteria members belong to the classes *Gammaproteobacteria* (18.76 % of the core microbiome; 25 OTUs); *Alphaproteobacteria* (18.75 %; 75 OTUs); *Betaproteobacteria* (6.6 %; 31 OTUs); and *Deltaproteobacteria* (1.19 %; 19 OTUs) (Figure 5ab). The ten major affiliated families (>1 % of relative abundance) represented 42.17 % of the "core-citrus" bacterial community and comprised 106 OTUs (Table 1). Only the *Pseudomnonadaceae* family represents almost 14 % of this "core-citrus" microbiome (5 OTUs), followed by the *Sphingomonadaceae* (6.56 %) and *Pirellulaceae* (4.08 %) of the bacterial community (Table 1). Surprisingly, 40 OTUs were identified in this last major family. The ten major affiliated genera represented 27.55 % of the "core-citrus" bacterial community (36 OTUs). Again, the *Pseudomonas* genus alone represents almost 12 % of this core microbiome, followed by *Streptomyces* (3,17 %) and *Sphingomonas* (1.94 %). The 17 major abundant OTUs (> 1.0 %) contributed to 30.83 % of the "core-citrus" rhizospheric community (Table 1). Most of the OTU lineages at each highest taxonomic level linked them to the ten major families and genera described above. Only three major OTUs representing 3.37 % of the "core-citrus" microbiome were affiliated with families beyond the major ones listed above, such as *Nitrosomonadaceae*, *Comamonadaceae,* and *Oxalobacteraceae* (Table 1).

In contrast, 18.5 % of the Carrizo citrange-associated bacterial community was exclusive of this genotype, comprising 333 OTUs (Fig. 4a). To identify prevalent "genotype-specific" microbiome members, the OTUs shared among all replicate plants within this genotype

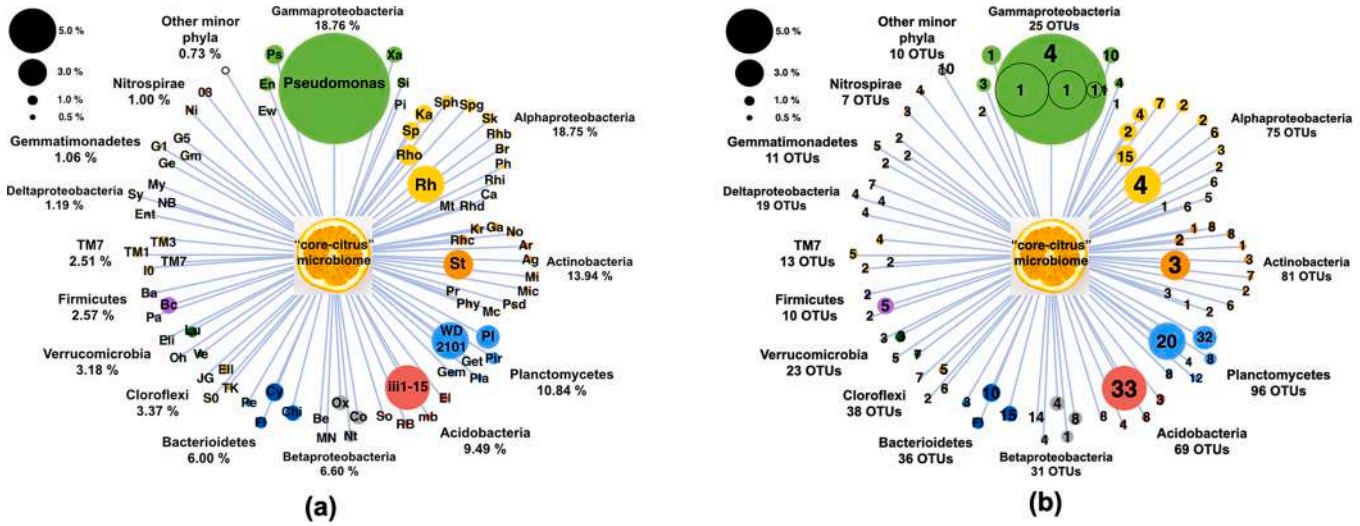

Fig. 5. "Core-citrus" bacterial microbiome members. (a) Percentage after the phylum/class indicates the respective contribution to the core microbiome. Node labels indicate the taxonomical profiling of identified phylotypes. The diameter of each node is proportional to the abundance of corresponding taxa in the core microbiome according to the scale. For phylum: Gm, Gemmatimonadetes; for class: Be, Betaproteobacteria; Ell, Ellin6529; G1, Gemm-1; G5, Gemm-5; Ge, Gemmatimonadetes; TM1, TM7–1; TM3, TM7–3; TK, TK17; for order: I0, I025; JG, JG30-KF-CM45; MN, MND1; My, Myxococcales; NB, NB1; RB, RB41; Rhi, Rhizobiales; Rho, Rhodospirillales; S0, S0208; So, Solibacterales; Sph, Sphingomonadales; for family: 03, 0319–6A21; Ba, Bacillaceae; Br, Bradyrhizobiaceae; Ca, Caulobacteraceae; Chi, Chitinophasaceae; Co, Comamonadaceae; Cy, Cytophagaceae; El, Ellin6075; Eli, Ellin517; En, Enterobacteriaceae; Ent, Entotheonellaceae; Ga, Gaiellaceae; Get, Gemmataceae; mb, mb2424; Mc, Micrococcaceae; Mi, Micromonosporaceae; Mt, mitochondria; No, Nocardioidaceae; Nt, Nitrosomonadaceae; Oh, Ohthoniobacteraceae; Ox, Oxalobacteraceae; Ph, Phyllobacteriaceae; Pl, Pirellulaceae; Pi, Piscirickettsiaceae; Pr, Propionibacteriaceae; Ps, Pseudomonadaceae; Psd, Pseudonocardiaceae; Rh, Rhizobiaceae; Rhb, Rhodobacteraceae; Si, Sinobacteraceae; Sph, Sphingomonadaceae; Sy, Syntrophobacteraceae; Ve, Verrucomicrobiceae; Xa, Xhantomonadaceae; for genus: Ag, Agromyces; Ar, Arthrobacter; Bc, Bacillus; Ew, Erwinia; Fl, Flavobacterium; Gem, Gemmata; Ka, Kaistobacter; Kr, Kribbella; Lu, Luteolibacter; Mic, Microbacterium; Ni, Nitrospira; Pa, Paenibacillus; Pe, Pedobacter; Phy, Phycicoccus; Pir, Pirellula; Pla, Planctomyces; Rhc, Rhodococcus; Rhd, Rhodoplanes; Sk, Skermanella; Sp, Sphingomonas; Spg, Sphingobium; St, Streptomyces. (b) Numbers inside the nodes indicate the number of OTUs assigned to each identified phylotypes of the "core-citrus" microbiome according to the same panel a.

Table 1
Major (>1 % of relative abundance) affiliated families, genus and OTUs lineages identified in the defined bacterial "core-citrus" microbiome.

	% relative abundance	Number of OTUs
Bacterial family		
Pseudomonadaceae	13,97	5
Sphingomonadaceae	6,56	11
Pirellulaceae	4,08	40
Rhizobiaceae	3,99	4
Streptomycetaceae	3,17	3
Bacillaceae	2,42	7
Rhodospirillaceae	2,23	11
Enterobacteriaceae	1,96	5
Cytophagaceae	1,91	10
Verrucomicrobiaceae	1,88	10
Total	**42.05**	**106**
Bacterial genus		
Pseudomonas	11,94	4
Streptomyces	3,17	3
Sphingomonas	1,94	2
Kaistobacter	1,93	4
Bacillus	1.80	5
Agrobacterium/Rhizobium	1.60	3
Skermanella	1,45	2
Pirellula	1,36	8
Sphingobium	1,30	2
Flavobacterium	1,26	3
Total	**27.55**	**36**
Major single OTUs		**Phylotype**
1	5,84	g_Pseudomonas
2	4,29	"
3	2,39	f_Rhizobiaceae
4	2,03	f_Pseudomonadaceae
5	1,95	g_Streptomyces
6	1,71	g_Sphingomonas
7	1,68	g_Pseudomonas
8	1,32	f_Nitrosomonadaceae
9	1,18	g_Bacillus
10	1,14	g_Skermanella
11	1,14	g_Flavobacterium
12	1,07	g_Kaistobacter
13	1,05	f_Comamonadaceae
14	1,03	g_Sphingobium
15	1,02	g_Streptomyces
16	1,00	o_WD2101
17	1,00	f_Oxalobacteraceae
Total	**30.84**	

were again selected [9]. As a result, only 10 out of the total shared OTUs were found in the four replicate plants of the Carrizo citrange genotype (Fig. 4b). Taxonomical profiling of those ten unique Carrizo citrange-specific OTUs classified them into nine phylotypes at the species level, where only four of them have representative cultured bacteria. Moreover, no OTU was affiliated with a defined genus (Supplementary Table S2).

As for Forner-Alcaide 5 genotype plants, only 5.2 % of the associated bacterial community was unique to this genotype, comprising 224 OTUs (Fig. 4a). Again, to identify "genotype-specific" microbiome members, we select those OTUs shared among all replicate plants within the genotype [9]. As a result, only one OTU was unique and present in the four replicate plants of Forner-Alcaide 5 citrus rootstock genotype (Fig. 4b). This OTU was assigned to the genus *Rhodoccocus* (Supplementary Table S2).

Main OTUs

A sub-selection of main OTUs from the defined core-citrus bacterial microbiome was made based on three important traits that could further select putatively beneficial microbes: relative abundance, root-enriched vs. bulk soil (host selection), and the rhizosphere-indicator species ecological concept.

i) *Major OTUs (> 1 %)*. Eleven out of the 17 most abundant OTUs from the "core-citrus" microbiome were linked to a defined genus (Table 1 and Supplementary Table S3). The most abundant assigned genus was *Pseudomonas,* with 3 OTUs representing 11.81 % of the core-citrus microbiome, followed by the genera *Streptomyces* (2 OTUs; 2.97 %) and *Sphingomonas* (1 OTU; 1.71 %) (Table 1).

ii) *Root-enriched OTUs (host selection)*. Since some of the OTUs from the core-citrus bacterial microbiome could not be selected by the host, and since some were from soil particles not affected by root exudates but present in all rhizosphere samples [30], the bacterial microbiome of the bulk soil samples was analyzed to determine which OTUs from the "core-citrus" microbiome were affected by the roots by selecting the OTUs that were significantly more abundant in the roots than in the bulk soil (root-enriched).

A total of 304,556 high-quality 16S rRNA gene sequences were generated from eight bulk soil samples. The average number of analyzed sequences per sample was 38,070 ± 16,029. A total of 3402 OTUs (97 % nucleotide identity) were identified for further taxonomical profiling (Supplementary Table S1). Raw high-throughput sequencing data from 16S rRNA amplicon libraries for bulk soil samples are deposited at the European Nucleotide Archive from EMBL (ENA; http://www.ebi.ac.uk/ena) under accession numbers ERR1654710 to ERR1654719 (Project **PRJEB15329** and Study **ERP017048**). The abundance of each selected core-citrus OTU was then compared for the rhizosphere microbiomes vs. bulk soil. A total of 30 out of the 544 core-citrus OTUs showed a significantly higher abundance in the rhizosphere than in the soil (rhizosphere-enriched) ($P < 0.001$). Out of these 30 OTUs, 11 were assigned to a given genus (Supplementary Table S3).

iii) *Rhizosphere-indicator species*. "Indicator species" is an ecological parameter based on abundance and prevalence in samples of a given environment (see Materials & Methods). Sixty-two OTUs of the 544 "core-citrus" OTUs were identified as "rhizosphere indicator species" by their abundance in the rhizosphere vs. bulk soil and prevalence in the rhizosphere compartment ($P < 0.001$). Of these 62 OTUs, 27 were assigned to a given genus (Supplementary Table S3).

Overall, a sub-set of 70 OTUs of the 544 "core-citrus" OTUs were classified as main OTUs by any of the three criteria described above (Supplementary Table S3). As might be expected, the distribution of those main OTUs according to their relative abundance shows that they are slanted towards the most abundant (Fig. 6a). Nevertheless, a few main OTUs were found in very low abundance. A Venn diagram of the main OTUs shows that nine major OTUs were also host-enriched and behave as rhizosphere-indicator species (Fig. 6b). All host-enriched OTUs were also classified as rhizosphere-indicator species. Moreover, 9 OTUs from the "core-citrus" microbiome fulfill all three criteria to classify them as main OTUs (Fig. 6b). Of those nine main OTUs, five were assigned to the following defined genera: *Pseudomonas* (2 OTUs); *Sphingobium* (1); *Sphingomonas* (1), and *Streptomyces* (1) (Fig. 6b).

Generating an extensive census of the cultivable citrus-rhizosphere microbiota

An extensive bacterial culturing approach was carried out from the same rhizospheric washing solutions used to assemble the citrus microbiomes. Multiple culture conditions were applied to generate many rhizospheric citrus isolates (see Material & Methods for details). A total of 1188 bacterial isolates (599 and 589 from C and F rootstock genotypes, respectively) were collected. Of these bacterial isolates, *i*) 486 bacterial isolates were purified from three general media; *ii*) 281 bacterial isolates were purified from a nitrogen-depleted medium; *iii*) 199 bacterial isolates were purified from a medium containing acc as a

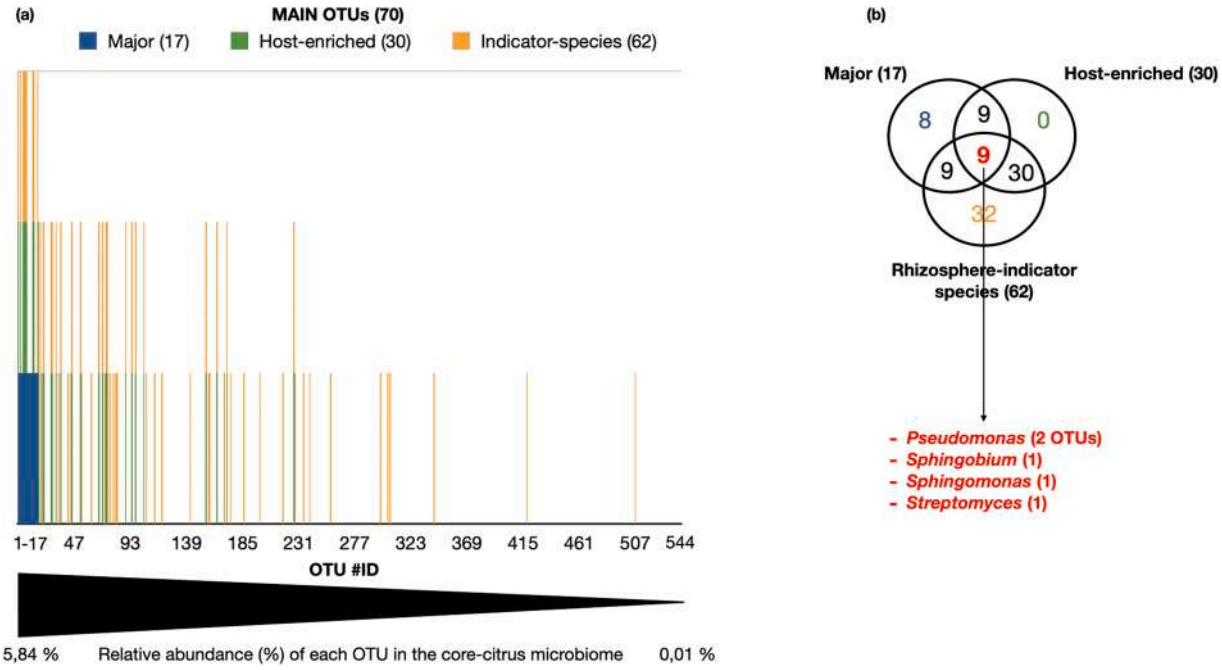

Fig. 6. A total of 70 OTUs from the defined core-citrus microbiome were classified as main OTUs based on abundance, host-enriched vs. bulk soil and rhizosphere-indicator species concept. (a) Distribution of main OTUs in the core-citrus bacterial microbiome. (b) Venn diagram showing the overlap of OTUs between the three traits used to classified them as main OTUs.

nitrogen source; *iv*) 140 bacterial isolates were purified after a temperature enrichment for sporulated bacteria; and *v*) 82 bacterial isolates were purified after growth at 50 ºC. 482 bacterial isolates were identified by double-strand sequencing of the almost complete 16S rRNA gene, including several representatives of any bacterial morphotype found in any combination for the bacterial isolation [plant sample (8) x culture condition (7) (n = 56)]. The cultivable citrus microbiota isolated belong to 57 bacterial genera (Table 2). Among these genera, 21 are represented in the defined core-citrus microbiome by OTUs assigned to the corresponding genus. As a result, one can now compare both sequences (isolates vs. OTUs) and associate real microbes with selected OTUs.

Linking metataxonomic data to microbes selection: providing citrus-adapted and host-selected bacteria potentially beneficial for Citrus (rCitrusBBC collection)

To provide a subset of microbes highly adapted to and selected by the two rootstock citrus genotypes after 20 years of cultivation in the same soil, the sequence of each OTU from the selected core-citrus microbiome linked to a given genus was compared with the 16S rRNA gene sequences from bacterial isolates belonging to the same genus as described in Material & Methods. A total of 147 bacterial isolates were closely associated with 25 OTUs from the selected core-citrus microbiome assigned to the related genus (Table 3), thus generating a collection of rhizospheric microbes potentially beneficial for Citrus (Fig. 1). Of the 25 associated-isolate OTUs, 16 were classified as main OTUs (Table 3). Bacterial isolates in the generated *r*hizospheric *Citrus B*eneficial *B*acterial *C*ollection (r*Citrus*BBC) belong to the genera *Pseudomonas* (49 isolates), *Bacillus* (29), *Rhizobium* (*Agrobacterium*) (13), *Microbacterium* (11), *Arthrobacter* (10), *Rhodococcus* (6), *Erwinia* (*Pantoea*) (5), *Paracoccus* (5), *Stenotrophomonas* (5), *Pseudoxanthomonas* (4), *Nocardia* (3), *Pedobacter* (2), *Agromyces* (1), *Flavobacterium* (1), *Janthinobacterium* (1), *Kribbella* (1) and *Terribacillus* (1) (Table 3). 16S rRNA gene sequences from these selected bacterial isolates are deposited at Genebank from NCBI under accession number OK298500 to OK298946.

Since the bacterial isolates of the r*Citrus*BBC collection were selected by comparison with OTU reference sequences, and each OTU represents a population of different sequences, it was of interest to determine whether a particular isolate sequence was present in the environmental samples without any OTU clustering (raw data). Each isolate sequence was thus recruited from environmental NGS sequences as indicated in Material & Methods. Out of the 147 r*Citrus*BBC bacterial isolate sequences, 95 (64.2 %) were recruited from some of the rhizospheric samples, demonstrating that a given isolate sequence was present in the environmental samples (Table 3). Furthermore, no specific bacteria potentially beneficial for the Carrizo citrange (C) genotype were selected since any of the ten specific OTUs was assigned to a defined genus (Supplementary Table S2). However, one isolate belonging to the genus *Rhodococcus* was closely associated with the unique selected OTU specific for the Forner–Alcaide 5 (F) genotype (Supplementary Table S2), and is thus a potentially beneficial microbe for this genotype. The closest relative in databases for this F3–101n(a) isolate is *R. cercidiphylli* (98.9 %).

Discussion

The citrus industry needs new bioproducts to mitigate the agricultural challenges of the 21st century. The first step in developing future biotechnological applications is to explore the citrus microbiota for beneficial microbes [4–7]. Mutualistic bacteria can boost plant growth, control pathogens, or alleviate abiotic stresses in plants, including citrus [31]. Bacterial microbiome analyses can help to select potentially host-beneficial microbes ([8]; Fig. 1), but it is crucial to capture as much bacterial diversity as possible. With this purpose in mind, both a high-throughput 16S rRNA gene sequencing (non-cultured) and an extensive census of bacterial isolation by culturomics (cultured) approaches were used to uncover the broad diversity of plant-associated communities. Here, a detailed characterization is presented of the bacterial ectorhizosphere-associated microbiome of two citrus rootstock genotypes grown in the same soil for over 20 years based on deep sequencing of 16 S gene amplicon libraries and isolation of cultivable bacteria in different culture conditions, with the ultimate purpose of generating a bacterial collection theoretically enriched in microbes beneficial for *Citrus* ([8]; Fig. 1).

Table 2
Bacterial genera and number of isolates identified by 16S gene sequencing from the cultivable citrus-rhizosphere microbiota.

Genus[a]	number of isolates
Ensifer (Sinorhizobium)	91
Bacillus	87
Pseudomonas	75
Microbacterium	21
Thermoactinomyces	16
Staphylococcus	15
Arthrobacter	14
Stenotrophomonas	14
Rhizobium (Agrobacterium)	13
Variovorax	12
Cupriavidus	10
Rhodococcus	9
Brevibacillus	7
Flavobacterium	6
Agromyces	5
Cellulosimicrobium	5
Erwinia / Pantoea	5
Paracoccus	5
Pseudoxanthomonas	5
Lysinibacillus	4
Nocardia	4
Paenarthrobacter	4
Pedobacter	4
Streptomyces	4
Aminobacter / Carbophilus	3
Pseudosphingobacterium	3
Shinella	3
Achromobacter	2
Acinetobacter	2
Buttiauxella	2
Enterobacter / Leclercia	2
Leclercia / Kluyvera	2
Klebsiella	2
Neisseria	2
Paenibacillus	2
Amycolatopsis	1
Brachybacterium	1
Chryseobacterium	1
Enterobacter	1
Janthinobacterium	1
Krasilnikoviella	1
Kribbella	1
Leclercia / Pantoea	1
Leclercia / Serratia	1
Lelliottia	1
Leucobacter	1
Micromonospora	1
Nocardioides	1
Ochrobactrum	1
Pantoea / Leclercia	1
Rahnella	1
Ralstonia	1
Serratia	1
Siccibacter	1
Sphingobium	1
Terribacillus	1
Ureibacillus	1
Total number of 16 S gene sequenced isolates	**482**
Total number of identified genera	**57**

[a] Genera written in bold characters are represented in the defined core-citrus microbiome by OTUs assigned to the corresponding genus.

The bacterial citrus microbiomes were dominated by the genera *Pseudomonas* and *Streptomyces,* and two candidate phyla. Since two out of the ten most representative phyla in the assembled citrus microbiomes are composed exclusively of uncultured representatives (*iii1–15* and *WD2101*), unravelling the metabolic and ecological functions of significant bacterial diversity for citrus plants should represent a grand challenge for future scientific projects [6,32]. Other than *Arabidopsis*, few studies have explored the magnitude of the effect of host genotype-dependent variation on bacterial rhizosphere microbiota profiles [33]. In agricultural systems, the impact of the host genotype on the composition of rhizosphere microbiota is much smaller than that of soil type. However, both the plant species and the cultivar can affect the composition of the rhizosphere microbiota [34]. Rhizosphere communities and other microbes could have a particular affinity for specific plant genotypes [2]. Both citrus rootstock genotype plants analyzed in the current study had the same origin and were grown in the same orchad (soil microbiota) for over 20 years. This allowed an exploration of whether a genotype-dependent fine-tuning of the bacterial community exists after such a long period of host and genotype selection. The assembled microbiomes from the two citrus rootstock genotypes differ in OTU abundance but not in their presence or absence. The assembled microbiomes from the Citrange carrizo rootstock were more homogenous than those found from the Forner-Alcaide 5 citrus plants. A significantly genotype-dependent fine-tuning of the bacterial communities was not found, since neither citrus rootstock genotype selected a specific bacterial community.

Nevertheless, all differentially abundant OTUs found were in higher proportions in Carrizo citrange, suggesting that this citrus rootstock genotype has a higher affinity for certain bacteria. Even though both rootstocks are genetically and agronomically distinct [35,36], they likely do not differ in traits shaping the rhizosphere, a “growth chamber” for the root microbiota, the second driving factor after the soil [37]. One of the main traits driving the rhizosphere community is the root exudate composition (rhizodeposition), which could not differ considerably between rootstock genotypes.

Other core citrus microbiomes were defined elsewhere with phytopathological purposes related to the infection and control treatments of the Huanglongbing (greening) citrus disease [38–41]. Since the soil is thought to be the primary factor driving rhizosphere microbiomes, a “seed bank” for root microbiota [37], and the fact that plant microbiomes are also influenced by the plant fraction and plant genotypes [42], it is difficult to obtain sound conclusions from the comparison of the present data with the bacterial microbiomes of the control (healthy) plants from those previous studies. Furthermore, the definition of the core microbiome is not unique and can be based on different shared parameters, such as prevalence (presence/absence) and not only membership [9]. Moreover, the differential composition of the assembled microbiomes depends on the abundance testing method applied [43]. However, the structure of a global citrus rhizosphere microbiome has been established, where *Proteobacteria*, *Actinobacteria*, *Acidobacteria,* and *Bacteriodetes* are predominant taxa [44]. These taxa are also dominant in the core-citrus microbiome described here, representing 73.54 % of the bacterial community (Fig. 5a). While the taxa *Planctomycetes* is not predominant in the global citrus rhizosphere microbiome (< 3 %), it represents 10.84 % of the bacterial core-citrus microbiome in this study (Fig. 5a). The global citrus rhizosphere microbiome comprises mainly *Pseudomonas*, *Rhizobium (Agrobacterium)*, *Cupriavidus*, *Bradyrhizobium*, *Mesorhizobium*, *Burkholderia*, *Cellvibrio*, *Sphingomonas*, *Variovorax,* and *Paraburkholderia* [44]. The genera *Pseudomonas, Rhizobium* (*Agrobacterium*), and *Sphingomonas* are also predominant in the present core-citrus microbiome representing 15.48 % of the bacterial community (Table 1).

Plant microbial composition is usually remarkably robust compared to the complex and dynamic microbial environments (mainly soil microbiota) from which they are formed [34], suggesting finely tuned discrimination by the plant host. If so, it would be expected that a subset of soil microbes, including root colonizers enriched in plant growth-promoting bacteria and related functional genes, were selected by the citrus plants over the 20 years of cultivation [45]. By comparing "core" and "specific" members of the two citrus rootstock genotypes grown in one soil, potentially beneficial microbes were identified for *Citrus* and some genotype-specific microbes after more than 20 years of host selection [8]. The rhizospheric citrus microbiomes from both rootstock genotypes exhibited more overlap than distinct microbial

Table 3
Selected bacteria potentially beneficial for citrus (r*Citrus*BBC collection).

				Main OTUs[a]				
r*Citrus*BBC #ID	Strain name	NCBI accession number[b]	The closest relative (%)[c]	OTU[d]	Major	Host selection	Rhizosfere-indicator species	Fragment recruitment analysis of isolate sequences[e]
1	**C3–177a**	OK298710	**Pseudomonas** baetica (99.3)	**OTU_1**	**X**	**X**	**X**	2/8
2	**F1–3**		" (99.2)	"				7/8
3	**F3–64(b)**	OK298708	P. brassicacearum (99.7)	"				5/8
4	**F1–26**	OK298698	P. entomophila (99.5)	"				4/8
5	**F4–7**	OK298691	" (99.6)	"				"
6	**C2–34**		P. helmanticensis (99.4)	"				0
7	**C1–73**		P. jessenii (99.3)	"				7/8
8	**F1–18**	OK298696	" (99.2)	"				"
9	**F1–19**	OK298719	" (99.4)	"				2/8
10	**F1–60**	OK298704	" (99.1)	"				7/8
11	**F2–19**		" (98.6)	"				0
12	**F2–25**		" (99.0)	"				7/8
13	**F2–29**	OK298685	" (98.4)	"				0
14	**F2–36**	OK298718	" (99.4)	"				"
15	**F3–280a**	OK298716	" (99.1)	"				7/8
16	**C3–7**	OK298687	P. monteilli (98.9)	"				4/8
17	**F2–74**		P. plecoglossicida (99.8)	"				"
18	**C1–75**		P. putida (99.5)	"				"
19	**C4–35**		" (99.4)	"				5/8
20	**C4–54**		" (99.4)	"				"
21	**F1–2**		" (99.3)	"				4/8
22	**F1–14**		" (99.9)	"				5/8
23	**F1–56**	OK298683	" (100)	"				"
24	**F3–62**	OK298707	" (99.5)	"				"
25	**F4–50**		" (99.5)	"				"
26	**F4–150n**	OK298689	" (99.8)	"				"
27	**F1–6**	OK298713	P. silesiensis (100)	"				1/8
28	**F1–21**	OK298714	" (99.9)	"				2/8
29	**F1–68**	OK298699	" (100)	"				5/8
30	**C2–213a**		P. taiwanensis (99.6)	"				"
31	**C3–131 ng**	OK298709	" (97.9)	"				4/8
32	**F1–20**	OK298697	" (99.1)	"				"
33	**F2–236a**	OK298703	" (99.6)	"				"
34	**C1–55a**	OK298700	P. frederiksbergensis (99.5)	**OTU_7**	**X**	**X**	**X**	4/8
35	**C2–20**		" (99.5)	"				"
36	**C1–28**		P. helmanticensis (98.9)	"				"
37	**F1–4**	OK298680	" (98.7)	"				"
38	**F2–72**	OK298717	" (98.8)	"				"
39	**F1–47**		P. lini (99.6)	"				"
40	**F1–55**		" (99.7)	"				"
41	**F2–50**	OK298686	P. japonica (98.7)	**OTU_2**	**X**			2/8
42	**C4–30**	OK298690	P. oryzihabitans (99.4)	"				"
43	**F1–30**	OK298715	" (97.4)	"				"
44	**F3–26**		" (99.5)	"				4/8
45	**F3–39**	OK298705	" (99.5)	"				"
46	**F4–26**	OK298692	" (99.4)	"				2/8
47	**F4–72**	OK298693	" (98.4)	"				"
48	**C2–25**		P. plecoglossicida (99.6)	"				4/8
49	**C2–12**		P. putida (99.1)	"				2/8
50	**F4–195n**	OK298851	**Flavobacterium** frigidimaris (98.9)	**OTU_11**	**X**			1/8
51	**C3–169a**	OK298931	**Kribbella** swartbergensis (99.1)	**OTU_20**		**X**	**X**	3/8
52	**F4–206a**	OK298807	**Rhizobium** herbae (99.0)	**OTU_28**		**X**	**X**	0
53	**F4–169 ng**	OK298800	R. giardinii (99.0)	"				"
54	**F4–204ag**	OK298887	**Nocardia** globerula (99.8)	**OTU_36**		**X**	**X**	1/8
55	**F4–229a**	OK298885	" (99.8)	"				"
56	**C2–173n**	OK298841	**Rhodococcus** koreensis (99.6)	"				1/8
57	**C2–212a**	OK298839	" (99.1)	"				"
58	**F2–194n**	OK298838	" (99.5)	"				"
59	**C3–4**	OK298805	**Agrobacterium** tumefaciens (99.8)	**OTU_21**			**X**	1/8
60	**C3–54**	OK298806	" (99.8)	"				"
61	**C3–136 ng**	OK298801	" (99.8)	"				"
62	**C3–176a**	OK298809	" (99.8)	"				"
63	**F1–34**	OK298810	" (99.7)	"				"
64	**F1–37**	OK298798	" (100)	"				"
65	**F1–43**	OK298808	" (99.7)	"				"
66	**F1–124 ng**	OK298802	" (99.7)	"				"

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Table 3 (*continued*)

			Main OTUs[a]			
67	**F1–131n**	OK298803	" (99.8)	"		"
68	**F2–201n**	OK298804	" (99.8)	"		"
69	**F4–167 ng (a)**	OK298799	" (99.4)	"		"
70	**F3–20**	OK298626	**Bacillus** cereus (99.6)	**OTU_42**	**X**	2/8
71	**C1–69**	OK298640	B. mobilis (99.8)	"		"
72	**F3–35**	OK298627	" (99.6)	"		"
73	**C1–89b**	OK298593	B. mojavensis (99.8)	"		"
74	**F3–90b**	OK298662	B. paramycoides (98.4)	"		"
75	**F2–99b**	OK298633	B. toyonensis (99.6)	"		"
76	**C1–84b**	OK298638	B. wiedmannii (99.8)	"		"
77	**F1–85b**	OK298675	" (99.8)	"	**X**	"
78	**C2–87b**	OK298648	" (99.2)	"		"
79	**C2–88b**	OK298649	" (99.7)	"		"
80	**C2–98b**	OK298631	" (99.9)	"		"
81	**C2–104b**	OK298651	" (99.8)	"		"
82	**C4–94b**	OK298665	" (99.8)	"		"
83	**C4–95b**	OK298604	" (99.6)	"		"
84	**C4–98b**	OK298666	" (99.9)	"		"
85	**C4–100b**	OK298606	" (99.6)	"		"
86	**C4–101b**	OK298607	" (99.9)	"		"
87	**C4–194a**	OK298673	" (99.9)	"		"
88	**F2–93b**	OK298653	" (100)	"		"
89	**F2–104b**	OK298634	" (99.8)	"		"
90	**F3–89b**	OK298624	" (99.4)	"		"
91	**F4–87b**	OK298609	" (97.2)	"		"
92	**F4–89b**	OK298610	" (99.9)	"		"
93	**F4–92b**	OK298668	" (99.9)	"		"
94	**F4–94b**	OK298612	" (99.2)	"		"
95	**F4–95b**	OK298613	" (99.8)	"		"
96	**F4–97b**	OK298669	" (100)	"		"
97	**F4–106b**	OK298671	" (99.9)	"		"
98	**F1–52**	OK298728	**Microbacterium** paraoxydans (99.7)	**OTU_44**	**X**	0
99	**C1–33**	OK298720	M. trichothecenolyticum (98.2)	"		"
100	**C1–44**	OK298730	" (98.6)	"		"
101	**C1–48**	OK298734	" (98.5)	"		"
102	**C2–211a**	OK298731	" (98.5)	"		"
103	**C3–107n**	OK298738	" (98.4)	"		"
104	**F2–213a**	OK298733	" (99.1)	"		"
105	**C4–168n**	OK298724	M. yannicii (98.7)	"		"
106	**F3–7**	OK298735	" (99.0)	"		"
107	**C1–106 ng**	OK298892	**Pedobacter** steynii (98.9)	**OTU_72**	**X**	0
108	**C2–58**	OK298893	Pd. weterhofensis (97.6)	"		"
109	**C3–6**	OK298877	**Pseudoxanthomonas** japonensis (99.7)	**OTU_79**	**X**	0
110	**C3–129n (a)**	OK298878	" (99.6)	"		"
111	**C4–144n**	OK298875	" (96.2)	"		"
112	**C4–144n (c)**	OK298879	" (99.7)	"		"
113	**F4–40b**	OK298929	**Janthinobacterium** lividum (99.2)	**OTU_82**	**X**	0
114	**C3–34**	OK298945	**Terribacillus** goriensis (99,7)	**OTU_142**	**X**	0
115	**C1–36**	OK298870	**Paracoccus** litorisediminis (99.8)	**OTU_170**	**X**	0
116	**C2–166n**	OK298872	" (99.8)	"		"
117	**C3–132 ng**	OK298871	" (99.5)	"		"
118	**C4–198a**	OK298874	" (99.5)	"		"
119	**F4–153 ng (b)**	OK298873	" (99.6)	"		"
120	**C3–37**	OK298790	**Stenotrophomonas** maltophilia (99.4)	**OTU_298**	**X**	0
121	**C4–33**	OK298793	" (98.7)	"		"
122	**C4–175 ng (a)**	OK298795	" (98.9)	"		"
123	**F4–171 ng(b)**		" (98.5)	"		"
124	**F4–183 ng**	OK298794	" (98.9)	"		"
125	**C2–228a**	OK298778	**Arthrobacter** cupressi (97.9)	**OTU_24**		0
126	**C1–126n**	OK298779	A. globiformis (98.4)	"		"
127	**C3–74**	OK298782	" (99.5)	"		"
128	**C3–141n**	OK298784	" (98.3)	"		"
129	**F2–197n**	OK298777	" (98.6)	"		"

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Table 3 (*continued*)

			Main OTUs[a]		
130	**F3–10**	OK298783	" (98.2)	"	"
131	**F4–211a**	OK298785	" (98.5)	"	"
132	**F2–73**	OK298775	A. humicola (98.4)	"	"
133	**F3–13**	OK298776	A. luteolus (99.2)	"	"
134	**C1–40**	OK298772	A. pascens (99.8)	"	"
135	**C3–118 ng**	OK298836	**Rhodococcus** wratislaviensis (99.2)	**OTU_31**	2/8
136	**C3–174a**	OK298835	R. wratislaviensis (99.1)	"	"
137	**F3–33**	OK298834	" (99.1)	"	"
138	**F3–37**	OK298939	**Pantoea** rodasii (96.3)	**OTU_35**	0
139	**C1–7**	OK298639	**Bacillus** litoralis (98,45 %)	**OTU_40**	2/8
140	**C1–130n**	OK298867	**Erwinia** endophytica (98.9)	**OTU_63**	0
141	**C1–74**	OK298865	E. tasmaniensis (99.2)		"
142	**C4–73**	OK298855	**Agromyces** fucosus (99.5)	**OTU_123**	0
143	**C1–19**	OK298866	**Erwinia** billingiae (99.4)	**OTU_159**	0
144	**F1–70**	OK298869	" (99.2)	"	"
145	**C3–44**	OK298739	**Microbacterium** profundi (98.9)	**OTU_280**	0
146	**C2–4**	OK298721	" (99.0)	"	"
147	**C3–173a**	OK298884	**Nocardia** salmonicida (99.2)	**OTU_541**	0

[a] Main OTUs from the selected core-citrus microbiome were stablished according to relative abundance (major >1 %), root-enriched vs. bulk soil (host selection) and rhizosfere-indicator species as described in M&M (Supplementary Table S3).

[b] 16 S rRNA gene sequences from bacterial isolates are deposited at Genebank from NCBI under accession number OK298500 to OK298946.

[c] % of similarity in the Reference 16S rRNA type_strains database (NCBI) at 15th of April 2020.

[d] 16 S rRNA gene sequence from the isolate shows > 98 % similarity with the OTU assigned to the corresponding genus. OTUs are named by order of relative abundance in the core-citrus microbiome from OTU_1 (5.837 %) to OTU-544 (0014 %) (Supplementary Table S2).

[e] Fragment Recruitment Analysis of each bacterial isolate sequence from environmental samples. Proportion of rhizosphere samples (n = 8) were a given bacterial isolate 16 S rRNA sequence was found in the raw sequencing data without any clustering at > 99 % of similarity.

communities, and the corresponding "core" microbiome was determined. Subsequently, a further prevalent "core-citrus" microbiome was established (OTUs detected consistently across replicates). To link metataxonomic data to actual microbes, many bacterial citrus isolates from the same rhizospheric samples were collected to develop an extensive census of the cultivable microbiome. 16 S rRNA gene sequences from 482 bacterial isolates allocated them to 57 genera, many of which had never previously been isolated from *Citrus* [46,47]. Since many metataxonomic-detected genera were not isolated and vice versa, this study is another clear example that shows that both approaches are complementary in defining a broad picture of plant microbiomes, as has been observed in many other studies. To generate a powerful citrus-adapted and host-selected bacterial collection, 16S rRNA isolate sequences were correlated with the counterpart OTU reference sequences from the defined "core-citrus" microbiome. A total of 147 isolate sequences were closely associated with the OTU reference sequences assigned to the related genus, allowing a bacterial collection to be generated theoretically enriched in beneficial microbes for *Citrus* ([8]; Fig. 1*)*.

Despite the limited number of commercialized bacterial bioproducts used in agriculture, as expected, these selected bacteria were dominated by genera that are already commercially available as agricultural probiotics, such as *Pseudomonas*, *Bacillus*, *Rhizobium* (*Agrobacterium*), *Erwinia* (*Pantoea*) and *Flavobacterium* [48,49]. Some other potentially host-beneficial isolates were not selected since they do not have a counterpart OTU assigned to the same genus in the defined "core-citrus" microbiome, likely the genera *Ensifer* (*Sinorhizobium*) or *Variovorax,* which were associated with OTUs assigned to the family level, which also includes other genera. It is known that adding other locations with the same experimental design and criteria would generate a "core-citrus" microbiome with a reduced but more restrictive and consistent number of selected OTUs. However, this aspect does not invalidate the bacterial selection made. There is a concern that there is insufficient evidence that the selected bacteria are beneficial in this study. However, the first step in developing new bioproducts is the selection of potentially beneficial microbes. Subsequently, since the ability of a given bacterium to function as a real plant-growth-promoting organism in the field is determined at the strain rather than the species level, a second step would be to explore each bacterium (alone or in combinations (SynComs)) directly *in planta* to select those new agricultural probiotics for the citrus crop.

In summary, the first bacterial culture collection has been generated from a woody fruit crop selected by coupling metataxonomic and culturomic analyses of rhizospheric microbiomes and theoretically enriched in host-beneficial bacteria [8,50]. The untapped diversity of rhizospheric citrus microbiomes has provided a citrus-adapted and citrus-selected bacteria that is a source to mine for beneficial bacteria [7]. Further *in planta* research will determine the potential of the selected bacteria as agricultural probiotics for future biotechnological applications in the citrus industry.

Conflict of Interest

No conflict of interest exist.

Acknowledgments

We thank all those undergraduate students, especially the pioneer Laura Camacho, for their contribution to the large bacterial isolation, and to Felix Morán for technical support in the bacterial sequences analysis. This work was supported by grants RTA2015–00087-C2–01, AGL2017–91803-EXP and RTI2018–098379-R-I00 from Spanish Agency of Science (AEI, Spain) and co-funded by the European Social Fund to E. Marco-Noales, R. Penyalver and M.A. Forner-Giner, respectively. Part of this work was also conducted during a visiting stay of R. Penyalver at the Universidade Federal do Pampa (Unipampa), Sao Gabriel, RS. Brazil funded by the "Programa Movilidad" from AEI (Spain) PRX15/00253. We especially thank Antonio de Vicente and Carlos Pedrós-Alió for support and suggestions; and Alice Mockford and Ansley Scudder Evans for English editing.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2022.06.002.

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