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Isolation and Characterization of Beneficial Bacteria Associated with Citrus Roots in Florida

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Abstract Cultivable diversity of bacteria associated with citrus was investigated as part of a larger study to understand the roles of beneficial bacteria and utilize them to increase the productive capacity and sustainability of agro-ecosystems. Citrus roots from Huanglongbing (HLB) diseased symptomatic and asymptomatic citrus were used in this study. A total of 227 and 125 morphologically distinct colonies were isolated and characterized from HLB asymptomatic and symptomatic trees, respectively. We observed that the frequency of bacterial isolates possessing various plant beneficial properties was significantly higher in the asymptomatic samples. A total of 39 bacterial isolates showing a minimum of five beneficial traits related to mineral nutrition [phosphate (P) solubilization, siderophore production, nitrogen (N) fixation], development [indole acetic acid (IAA) synthesis], health [production of antibiotic and lytic enzymes (chitinase)], induction of systemic resistance [salicylic acid (SA) production], stress relief [production of 1-amino-cyclopropane-1-carboxylate deaminase] and production of quorum sensing [*N*-acyl homoserine lactones] signals were characterized. A bioassay using ethidium monoazide (EMA)-qPCR was developed to select bacteria antagonistic to *Candidatus Liberibacter asiaticus*. Using the modified EMA-qPCR assay, we found six bacterial isolates showing maximum similarity to *Paenibacillus validus*, *Lysinibacillus fusiformis*, *Bacillus licheniformis*, *Pseudomonas putida*, *Microbacterium oleivorans*, and *Serratia plymutica* could significantly reduce the population of viable *Ca*. L. asiaticus in HLB symptomatic leaf samples. In conclusion, we have isolated and characterized multiple beneficial bacterial strains from citrus roots which have the potential to enhance plant growth and suppress diseases.

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Introduction

Plants provide a nutrient rich niche for the growth and development of various groups of microorganisms, especially bacteria. Mutualistic interactions between host plant and associated bacteria have emerged as a result of clear positive selection exerted on these associations [15, 27]. Bacteria profit from plants because of the enhanced availability of nutrients, and plants, in turn, benefit from the bacterial associations by growth enhancement, stress reduction, or protection from pathogens [9, 32, 39]. In addition, plant-associated bacterial communities also play key roles in ecosystem processes such as nutrient cycling and conservation of soil structure [31]. Much of the basic information regarding the community structure of plant-associated bacteria, their principal functions, their relative ecological stability, and the organizing forces that govern their continuity is still lacking. Understanding the diversity of plant–associated bacteria and their roles in plant development is crucial if these associations are to be manipulated to increase productivity and sustainability of agro-ecosystems.

The structure and composition of plant-associated bacterial community change in response to various factors which include nature of host plant, management practices,

and interaction with other organisms [2, 38, 52, 58]. Using citrus and Huanglongbing (HLB, citrus greening) as a host-disease model, we have described the fluctuations in the bacterial community in response to phytopathogen infection [40, 52]. HLB is the most devastating diseases of citrus [17] and is linked to a fastidious, gram-negative, phloem-limited bacterium (*Candidatus Liberibacter* spp.) [5], and recent attempts had limited success in culturing the organism [42]. The disease is widespread in most areas of Asian countries that grow citrus, Africa, Brazil, and most recently, Florida [5]. HLB was found in Florida in 2005 and recently discovered in the Yucatan, the western states of Mexico, Belize, and multiple countries in the Caribbean as reviewed by Gottwald [17]. In Florida, *Candidatus Liberibacter asiaticus* is the causal agent of HLB [40] and is transmitted by psyllid vector *Diaphorina citri*. The HLB-associated bacteria can infect most citrus cultivars, and the disease can debilitate the productive capacity of citrus trees with reported losses of 30–100% [17]. HLB not only affects plant health but also restructures the bacterial community associated with citrus roots [52]. Molecular analysis of HLB symptomatic and asymptomatic citrus has revealed profound effect on the structure and composition of the root-associated bacterial community [52]. HLB infection caused significant reduction in the number of various bacterial phylotypes known to possess beneficial traits related to plant growth promotion while facilitating the increase of a few groups which are known to induce disease symptoms or establishment in other citrus diseases [2, 52]. As the diversity and stability of the plant-associated bacterial communities heavily influence soil and plant quality and ecosystem processes [15, 31], erosion of bacterial diversity could have serious implications on the agro-ecosystem sustainability.

Plant-associated bacteria which improve the fertility status of soil and contribute in augmenting overall plant growth and health are receiving increased attention for use as microbial inoculants in agriculture [9, 11, 23, 27, 28]. The mechanisms by which these bacteria support plant health and growth include nutrient solubilization and fixation, production of plant hormones, stress relief, and suppression of plant pathogens by induction of plant defenses, production of antibiotics, and/or out-competition of pathogens [18, 21, 39]. To remove the erratic performance of microbial inoculation, selection of native bacteria acclimatized to a particular set of environmental conditions has been advocated [34]. This favors efficient colonization and manifestation of beneficial traits in bacteria and does not affect the preexisting balance among indigenous populations.

The utilization of plant-associated bacteria in agriculture production depends on our knowledge of bacteria–plant interaction and our ability to maintain, manipulate, and modify beneficial bacterial populations under field conditions [18, 22]. An important requirement for the success of such applications requires characterization and selection of suitable bacteria in candidate plants appropriate for various biotechnological applications. The current knowledge of the structure of plant-associated bacteria community in different plant species is based on both cultivation-dependent and cultivation-independent methods [8, 19, 40, 43, 52, 53, 60]. While cultivation-independent studies give an estimate of the extent of community diversity, it warrants that the organisms are brought to cultivation to facilitate their exploitation in agriculture or fermentation-based application [39, 49]. Isolation and characterization of bacterial strains is also required for the development of effective biocontrol agents and to ensure that they are effective and do not carry pathogenicity risks. However, we lack the knowledge of the cultivable bacteria associated with citrus in Florida and elsewhere. Identification of beneficial bacteria associated with citrus roots in Florida will offer many opportunities to improve plant nutrition, yields, and disease management, while improving the sustainability of agro-ecosystem. The objective of this study was to isolate and characterize beneficial bacteria that are intimately associated with citrus roots. We specifically focused on the delineation of the cultivated plant-associated bacterial isolates and on the characterization of their salient metabolic features. The diversity and putative identities of the cultivated bacteria were determined by genomic DNA fingerprinting using 16S rRNA gene analysis. We used a detailed approach for screening novel plant growth promoting (PGP) isolates by conducting qualitative, quantitative, and PCR-based assays for traits related to mineral nutrition [phosphate (P) solubilization, siderophore production, nitrogen (N) fixation], development [indole acetic acid (IAA) synthesis], health [production of antibiotic and lytic enzymes (chitinase)], induction of systemic resistance [salicylic acid (SA) production], stress relief [production of 1-amino-cyclopropane-1-carboxylate (ACC) deaminase], and production of quorum sensing [*N*-acyl homoserine lactones (AHL)] signals. A novel bioassay using ethidium monoazide (EMA)-qPCR was also developed to select bacteria antagonistic to HLB pathogen.

Material and Methods

Isolation of Root-Associated Bacteria

Root samples were collected from either HLB symptomatic or asymptomatic Valencia orange (*Citrus sinensis*) trees (three each) in a heavily infected grove at Fort Pierce, Florida. Root segments were collected from the stem base with a shovel at a depth of 5 to 15 cm. The samples were washed with tap water to remove attached soil. Subsequently, the roots were

immersed in 70% ethanol for 3 min, washed with fresh sodium hypochloride solution (2.5% available Cl^-) for 5 min, rinsed in 70% ethanol for 30 s and finally, washed five times with sterile distilled water. As a sterility check, a 100-μl sample of the 5th rinse water was plated out onto nutrient agar (NA) medium. The samples were verified for *Ca*. L. asiaticus infection using conventional (using primers A2-J5) and quantitative PCR (using primer/probe combination CQULA04F-CQULAP10-CQULA04R) as described previously [51, 52]. For the isolation of plant-associated bacteria, 1.9 g of root material was macerated in 20 ml of 1 mM $MgSO_4$ using sterile mortar and pestle. The homogenized samples were then used to make serial dilutions and 100 μl of the 10^3–10^8 cfu ml^{-1} dilutions were plated onto NA, tryptone yeast (TY) extract agar, actinomycetes isolation (AI) agar, and King's B (KB) agar. The media were chosen based on their ability to support the growth of fastidious bacteria (NA and TY), actinomycetes, and other slow growing bacteria (AI) and bacteria belonging to genus *Pseudomonas* and *Burkholderia* (KB). The plates were incubated for 3–5 days (5–10 days for AI) at 20°C, 25°C, and 28°C in order to assay for a wide spectrum of bacteria. Bacterial colonies were counted and expressed as colony-forming units (cfu) per gram fresh weight. Bacterial isolates were obtained from agar plates presenting between 30 and 150 colonies. Random sample of 30 bacterial colonies differentiated by their morphology, pigmentation, and growth rates were selected and transferred to the respective medium from which they were isolated. Fourteen isolates proved difficult to be maintained on media and were removed from further analysis on the grounds that an isolate with potential for biotechnological application need to be readily cultured. All the remaining isolates were purified three times and then stored at −80°C in medium broth containing 15% of glycerol for further use. Isolated bacteria were encoded by a combination of numbers and letters indicating sample type (A=Asymptomatic; S=Symptomatic), medium of isolation (NA, TY, AI, or KB) and consecutive number of isolate within each sample.

DNA Extraction, 16S rRNA Gene Amplification, Sequencing, and Strain Identification

Individual colonies were grown in the broth of the respective medium from which they were initially isolated and grown overnight at 28°C on a rotator shaker at 150 rpm. DNA was extracted using the Wizard genomic DNA purification Kit (Promega Corp., Madison, WI) following the manufacturer's instruction. The extracted DNA was dried in a Vacufuge (Eppendorf, Westbury, NY) for 15 min and was then dissolved in 100 μl of DNA rehydration solution (Promega). Amplification of 16S rRNA gene was performed in 25 μl of reaction mixture that contained 0.25 mM of primers 27f (5′-AGAGTTTGATC(A/C)TGGCTCAG-3′) and 1492r (5′-GG(C/T)TACCTTGTTACGACTT-3′) [26], 0.2 mM of each deoxyribonucleodite triphosphate, 1.5 mM $MgCl_2$, 0.1 mg ml^{-1} of bovine serum albumin, 2.5 μl of *Taq* buffer, and 1 U *Taq* DNA polymerase (Invitrogen, USA). PCR amplification was performed on 1 μl of DNA template (0.5–10.0 ng) in a DNAEngine Peltier thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with an initial denaturation (95°C for 5 min) followed by 30 cycles of amplification (94°C for 1 min, 55°C for 1 min, 72°C for 2 min) and a single extension step (72°C for 10 min). The resulting PCR product (5 μl) was then checked by gel electrophoresis at 100 V for 45 min on a 1% agarose gel. Remaining PCR products were cleaned using Wizard SV Gel and DNA purification system (Promega, Cat-A9280) and quantified using nanodrop spectrophotometer and sent to Eton Bioscience Inc. (Research Triangle Park, NC, USA) for sequencing. Sequences were examined and edited using BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Similarity analysis was conducted using the Basic Local Alignment Tool at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequences were deposited in GenBank under accession numbers HQ219832-HQ220183.

Screening for PGP and Biocontrol Abilities

Qualitative estimation of P solubilization and chitinase production was done by observing a halo around the bacterial colony growing in Pikovskaya medium (containing insoluble P) and chitin medium (containing colloidal chitin), respectively [50]. Production of siderophore was estimated on chrome-azurol S-agar medium [41] by observing the development of orange color around the bacterial colony. Qualitative estimation of the production of IAA was determined by the development of pink color in the culture supernatant after the addition of o-phosphoric acid (H_3PO_4) [50]. Bacterial isolates were screened for N-fixation ability by observing the growth on N-free semi-solid BAz minimal medium [12]. AHL production was tested by well diffusion assays using *Agrobacterium tumefaciens* NT1 or *Chromobacterium violaceum* CV026 as indicator strains [37]. To determine the presence of ACC deaminase, the ability of the isolates to use ACC as N source was checked by growing them onto DF salts minimal agar medium supplemented with 3.0 mM ACC instead of $(NH_4)_2SO_4$ as N source [44]. Specific genes for antibiotic synthesis (*phlD*) and nitrogen fixation (*nifH*) were searched using primers and conditions as described previously [14, 57]. The bacterial isolates possessing at least five PGP and biocontrol properties were selected, and the beneficial traits of the promising isolates were further quantified using various standard methods as described by Trivedi et al. [50; P solubilization,

IAA production, siderophore production], Nagarajkumar et al. [30; SA and chitinase production], and Shah et al. [45, ACC deaminase production]. All the experiments were done in triplicate and repeated three times.

Accessing the Antagonistic Properties of Bacterial Isolates against *Ca*. L. asiaticus

We have earlier developed EMA-qPCR based method for the discrimination between live-dead cells of *Ca*. L. asiaticus [51]. This method was further optimized for screening antagonistic ability of bacterial isolates against *Ca*. L. asiaticus. Infected leaf samples were collected, and midribs were separated. The midribs were crushed in sterile water (1:10) and 1 ml of the solution was added to 0.2 ml of overnight grown bacterial suspension diluted approximately to 10^5 cfu ml^{-1}. All the selected bacterial isolates (possessing a minimum of five PGP or biocontrol properties) as described in the previous section along with *Curtobacterium* sp. were tested. The mixture was then incubated at 28°C for 2 days on a rotary shaker at 150 rpm. EMA treatment was done as described previously [51]. The genome equivalent of *Ca*. L. asiaticus in the samples was determined by qPCR using primer probe combination CQULA04F-CQULAP10-CQULA04R [56] and standard equation developed earlier [51]. EMA-treated samples with addition of sterile media without bacterial inoculation served as control. Population reduction in the viable *Ca*. L. asiaticus was determined using the formula $(N_1 - N_2/N_1) \times 100$, where N_1 and N_2 represent the genome equivalent of *Ca*. L. asiaticus in control and inoculated samples after EMA treatment, respectively. The experiments were done in triplicate and repeated three times.

Statistical Analysis

Analysis of the data was carried out using the SAS software package with. Bacterial population data were transformed using $\log_{10}$ of $X+1$ and subsequently analyzed. The data was subjected to one-way analysis of variance and the significance of treatments was computed using Duncan's multiple range test at $P \leq 0.05$.

Results

Detection of *Ca*. L. asiaticus

Conventional PCR using primers A2 and J5, which target the 16S rRNA gene of *Ca*. L. asiaticus, showed a band of approximately 703 bp in the infected root samples, which was not detected in uninfected samples (Fig. 1a). This result was further confirmed using qPCR with primer/probe combination CQULA04F-CQULAP10-CQULA04R targeting beta-operon region of *Ca*. L. asiaticus. qPCR results showed the absence of *Ca*. L. asiaticus in the root samples of uninfected citrus trees (Fig. 1b). The number of *Ca*. L. asiaticus organisms in infected trees ranged from 1.43×10^5 to 3.78×10^5 genome equivalents μg^{-1} of total DNA.

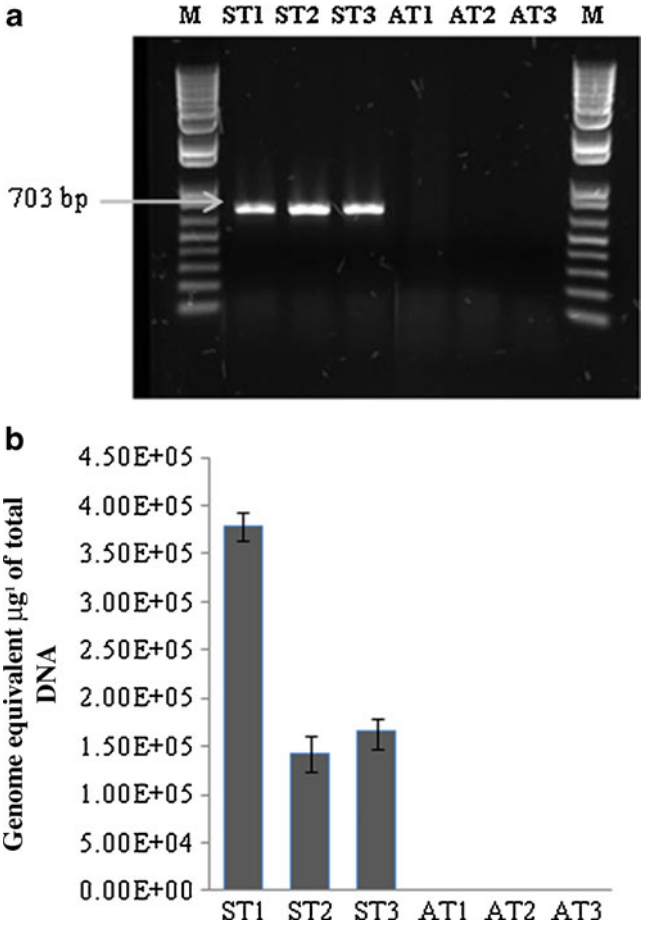

Figure 1 Detection of *Ca*. L. asiaticus in HLB symptomatic and asymptomatic citrus root samples. **a** Agarose gel electrophoresis of PCR products amplified using primers A2-J5 targeting 16S rRNA gene of *Ca*. L. asiaticus. **b** Quantification of *Ca*. L. asiaticus (genome equivalents μg^{-1} of total DNA) by qPCR using primer–probe combination CQULA04FCQULAP10-CQULA04R targeting the β-operon region of *Ca*. L. asiaticus. *ST1–ST3* symptomatic citrus roots, *AT1–AT3* asymptomatic citrus roots, *M* DNA molecular weight size markers

Isolation and Characterization of Root-Associated Bacteria from HLB Symptomatic and Asymptomatic Citrus

Population densities of bacteria isolated under aerobic conditions from the surface sterilized roots of symptomatic and asymptomatic citrus on different sets of medium is presented in Table 1. The population densities were highest on TY and NA, respectively, and there was no significant difference in the bacterial population recovered from symptomatic or asymptomatic trees in these two media. In

Table 1 Frequency of bacteria from the HLB symptomatic and asymptomatic citrus root samples on various media

Sample	Bacterial counts (log cfu g^{-1} FW)			
	Nutrient agar	Tryptone yeast extract agar	Actinomycetes isolation agar	King's B agar
Asymptomatic	6.21±0.12a (88)	6.56±0.24a (82)	3.56±0.17a (20)	4.23±0.34a (37)
Symptomatic	5.98±0.21a (64)	6.60±0.16a (48)	1.22±0.11b (7)	1.29±0.39b (6)

Values in parenthesis represent the number of randomly selected isolates. Column values followed by same letters are not significantly different at $P \leq 0.05$

contrast, asymptomatic samples had a significantly higher population of bacteria growing on AI and KB media. Although the population of total bacteria was similar on NA and TYA, the number of morphologically distinct bacteria were significantly higher in asymptomatic as compared to symptomatic samples. These isolates were then sub-cultured three times to ensure purity and stability. A total of 227 and 125 morphologically distinct colonies were isolated individually from asymptomatic and symptomatic trees, respectively, and were stored adequately for further analysis.

Total genomic DNA of the isolates was extracted and 16S rRNA gene was amplified and sequenced for species identification. A detailed breakdown of the cultured bacterial community from symptomatic and asymptomatic citrus roots is presented in Fig. 2. The bacterial isolates obtained from both sets of samples could be assigned to different genera. There were significant variations in the nature of cultivable bacterial populations from symptomatic and asymptomatic trees. The majority of the isolates from the asymptomatic samples belonged to Gammaproteobacteria (37.8%), with *Pseudomonas* sp., *Pseudomonas fulva*, *Pseudomonas putida*, *Pseudomonas maltophila*, *Pantoea agglomerans*, *Serratia* sp., and *Serratia plymuthica* being the most frequently found. Actinobacteria (22.0%) and Firmicutes (19.8%) represented other dominant groups of the bacterial isolates from the asymptomatic citrus root samples. Among these groups, *Bacillus* sp., *Bacillus subtilis*, *Microbacterium* sp., and *Microbacterium oleivorans* were the most frequently isolated genera. The isolates showing highest similarity to *Curtobacterium* spp. were the most frequently recovered genera from the asymptomatic samples. The strains belonging to Alphaproteabacteria (6.1%) and Betaproteobacteria (14.0%) were also recovered at a higher frequency from asymptomatic samples. Majority of these isolates belonged to family Methylobacteriaceae and Burkholderiaceae.

The majority of the isolates in the HLB symptomatic samples were related to Alphaproteobacteria (52.0%). The genera *Methylobacterium*, *Rhizobium*, and *Brevundimonas* belonging to this group were also isolated from asymptomatic samples but their isolation frequency was much greater in the symptomatic samples. Five isolates belonging to Bacteroidetes, and showing highest similarity to *Chryseobacterium daecheongense*, were only isolated from symptomatic samples. Gammaproteobacteria was the second most abundant class (22.4%) in the culture collection of bacterial isolates from symptomatic samples but the actual number of isolates belonging to genera *Pseudomonas* and *Serratia* were lower as compared with the asymptomatic samples. The number of bacterial isolates representing Actinobacteria was also significantly lower in symptomatic as compared to asymptomatic samples. This phylum was represented by ten genera in asymptomatic samples while only four genera were isolated from the symptomatic samples. Only a single isolate representing Betaproteobacteria and belonging to *Burkholderia* was present in the culture collection of symptomatic samples. Firmicutes comprised 15.2% of the isolates from the symptomatic culture collection. Strains resembling *Burkholderia licheniformis*, *Lysinibacillus fusiformis*, *Paenibacillus validus*, *Paenibacillus glycanilyticus*, *Brevibacillus* sp., and *Brevibacillus parabrevis* frequently recovered from asymptomatic samples were not present in the symptomatic samples, while strains showing highest similarity to *Staphylococcus* sp. and *Staphylococcus pasteuri* were four times greater in symptomatic samples.

Screening for Potential PGP and Biocontrol Abilities of Bacterial Isolates

All the bacterial isolates were screened for their PGP and biocontrol abilities using various qualitative and PCR-based assays. We observed that the frequency of the bacterial isolates possessing various beneficial traits was significantly higher in the asymptomatic samples (Fig. 3). The frequency of bacteria found positive for the production of IAA and ACC deaminase and possessing *nifH* and *phlD* genes were four times greater in asymptomatic as compared to symptomatic samples. The number of bacterial isolates producing AHL signal molecules were 14 (6.2%) and two (1.6%) in the asymptomatic and symptomatic samples, respectively. The frequency of endophytic bacteria able to solubilize insoluble P was higher in comparison to other traits in both samples but asymptomatic samples have higher number of P solubilizing bacteria (45.0%) as compared to symptomatic samples (15.0%). The frequency of isolates producing chitinase and salicylic acid were nearly ninefold greater in asymptomatic samples.

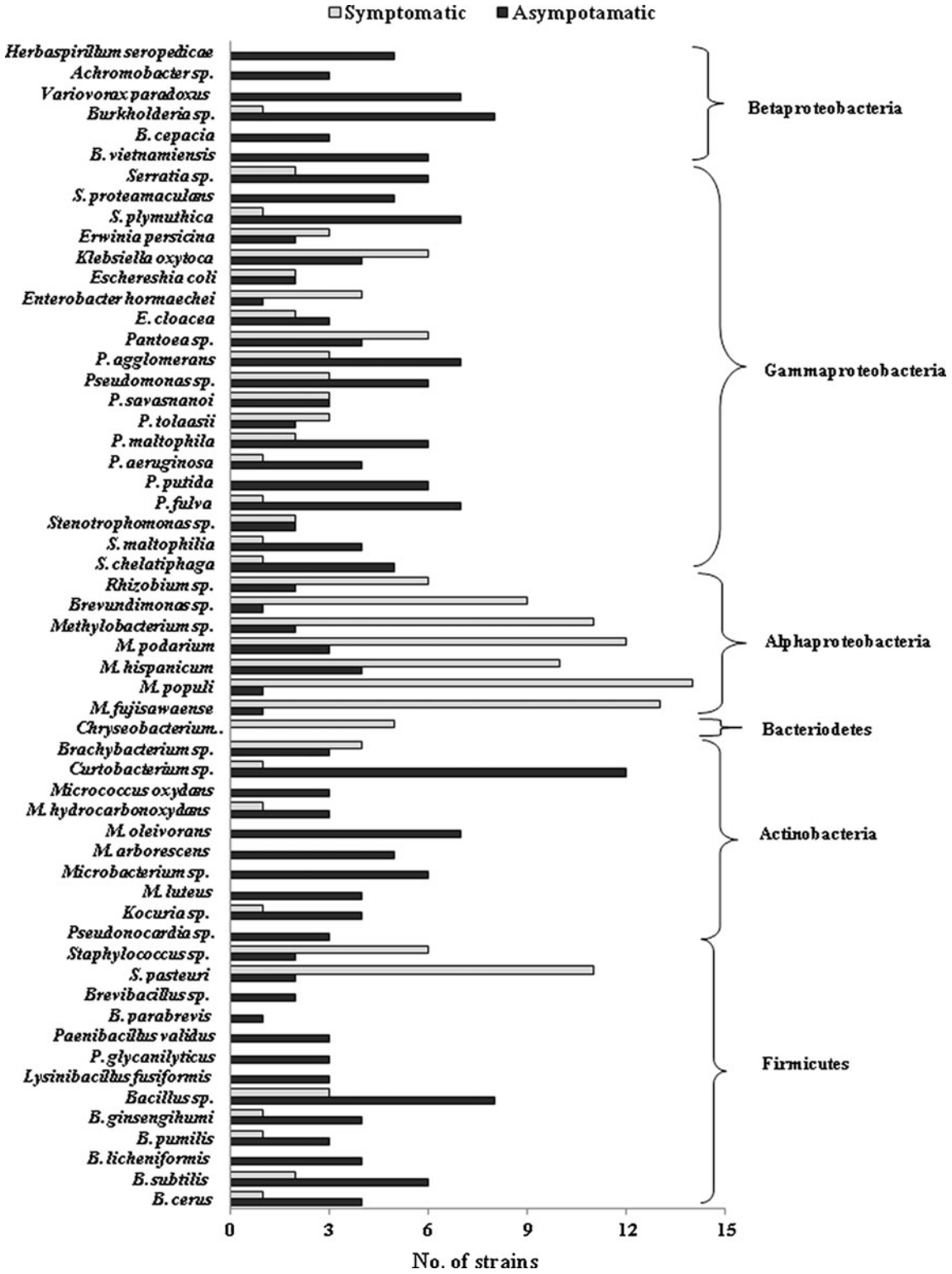

Figure 2 Taxonomic breakdown of 16S rRNA gene sequences of the plant-associated bacterial isolates from HLB symptomatic and asymptomatic citrus roots. Taxonomic classifications were determined according to Wang et al. [55]

A total of 39 bacterial isolates showing a minimum of five beneficial traits were selected and quantitative estimation of various PGP activities were tested. The selected isolates belong to 16 different genera (Table 2). Only five of these isolates were recovered from the symptomatic samples. All the strains possessed the ability to solubilize P and produce IAA at different efficiencies. Highest levels of P solubilization (390.89 μg ml^{-1}) and IAA production

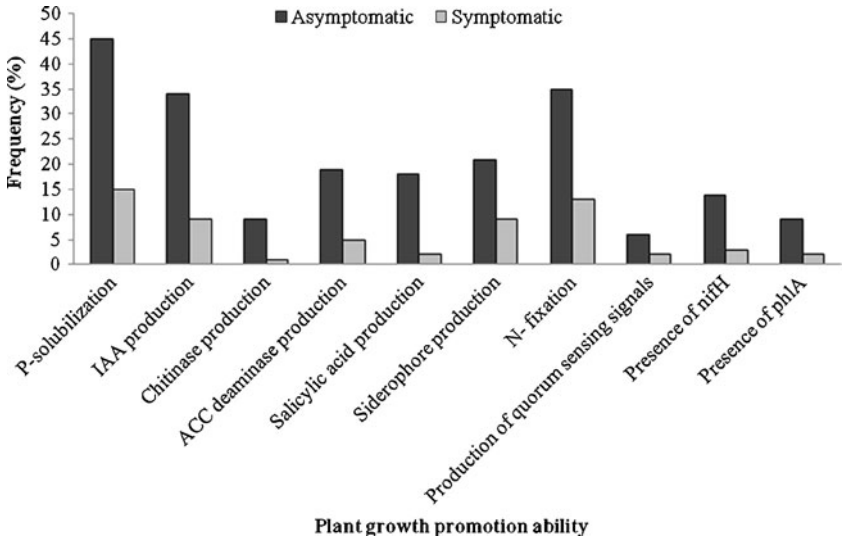

Figure 3 Isolation frequency of bacterial strains possessing various plant growth promotion and biocontrol abilities isolated from HLB symptomatic and asymptomatic citrus roots. The results between the symptomatic and asymptomatic samples were significantly different for all the traits at $P \leq 0.05$

(43.29 μg ml^{-1}) were observed for *Serratia plymutica* strain ANA33 and *Herbaspirillum seropedicae* strain ANA25, respectively. Chitinase production was observed for 30 bacterial isolates with *Burkholderia vietnamiensis* strain AKB9 (9.77 mU ml^{-1}) showing the highest activity. With the exception of Gram positive isolates, most of the other strains produced ACC deaminase with activity ranging from 1.23 nmol mg^{-1} protein h^{-1} (*Serratia* sp. strain ANA37) to 23.23 nmol mg^{-1} protein h^{-1} (*Methylobacterium fujisawaense* strain SNA1). Thirty-five strains produced SA and 36 were found positive for siderophore production. *Pseudomonas stutzeri* strain ATY70 produced exceptionally high amount of siderophore (22.12 μg ml^{-1}) in broth assay. In N-free medium, all the selected isolates except for *Microbacterium arborescens* strain ANA42 showed growth indicative of their N-fixation ability while *nifH* gene was detected in only 20 of these isolates. *Methylobacterium* sp. strain ANA87, *Methylobacterium populi* strain SNA7, *M. fujisawaense* strain SNA1, *Pseudomonas* sp. ANA69, *P. putida* strain AKB28, and all the selected strains belonging to *Burkholderia* produced AHL in presence of both indicator strains. AHL production in *Pseudomonas savastanoi* strain AKB25 and *Rhizobium* sp. strain ATY52 was observed only when indicator strain *C. violaceum* CV026 was used. Strains belonging to *Pseudomonas* and *Burkholderia* also showed the presence of *phlD* gene.

Accessing the Antagonistic Properties of Bacterial Isolates Against *Ca*. L. asiaticus

The number of live *Ca*. L. asiaticus in control (with the addition of sterilized medium) was 5.6×10^4 genome equivalents μg^{-1} of DNA. Using the modified EMA-qPCR assay, we found six bacterial isolates that can significantly reduce the population of viable *Ca*. L. asiaticus in HLB symptomatic leaf samples (Fig. 4). These isolates showed maximum similarity to *P. validus*, *L. fusiformis*, *B. licheniformis*, *P. putida*, *M. oleivorans*, and *S. plymutica*. All of these isolates were initially isolated from asymptomatic samples. Maximum reduction in the viable cells of *Ca*. L. asiaticus was observed when the samples were inoculated with *P. validus* strain ATY16 and *L. fusiformis* strain ANA81, where the reduction in the viable *Ca*. L. asiaticus was nearly 55% as compared to control. No significant reduction in viable cells was observed when *Curtobacterium* sp. was tested (data not shown).

Discussion

Plant–microbe interactions are critical to the integrity, function, and long-term sustainability of agro-ecosystems [31]. Plant-associated bacterial community sustain many vital ecosystem processes, such as nutrient cycling, decomposition of organic matter and waste, nutrient availability, degradation of pesticides, soil structure, and plant growth and health. An understanding of the plant-associated bacterial community is important taking into account their potential significance in plant growth promotion, protection against biotic and abiotic stresses, source of novel biomolecules, and agents in bioremediation and determinants of soil and environmental health [9, 27, 32, 43].

Various bacterial genera possessing multiple plant beneficial traits were found to be associated with citrus roots. Screening for the isolates possessing multiple PGP traits revealed that majority of them belonged to genera *Burkholderia*, *Pantoea*, *Pseudomonas*, *Bacilli*, *Painibacillus*, and *Serratia*. The representative species of these genera have been isolated from various plant species and studied in detail for various plant growth promotion and biocontrol abilities

Table 2 Plant growth promotion and biocontrol activities of selected citrus root-associated bacteria

Strain code	Close relative from Genbank (accession no.)	Genbank no. (% identity)	P sol. (μg ml^{-1})	IAA (μg ml^{-1})	Chitinase (mU ml^{-1})	ACC deaminase	Salicylic acid (μg ml^{-1})	Siderophore (μg ml^{-1})	N-fixation	AHL production		*nifH*	*phlD*
										CV026	NT1		
ANA4	*Bacillus cereus* (EF035137)	HQ219835(97)	212.23	3.23	3.66	−	4.56	–	+	−	−	+	−
ANA13	*Bacillus ginsengihumi* (AB245378)	HQ219844(96)	126.72	21.22	5.66	−	4.97	5.45	+	−	−	−	−
ANA77	*Bacillus licheniformis* (HQ005269)	HQ219908(97)	341.21	12.22	4.52	−	3.90	3.45	+	−	−	+	−
ANA81	*Lysinibacillus fusiformis* (HM480312)	HQ219912(99)	198.22	32.12	3.22	−	4.33	3.49	+	−	−	−	−
ATY11	*Paenibacillus glycanilyticus* (NR_024759)	HQ219930(97)	98.76	4.97	4.56	−	6.43	–	+	−	−	+	−
ATY16	*Paenibacillus validus* (GU191921)	HQ219935(95)	89.90	6.23	5.62	−	7.23	5.67	+	−	−	−	−
ANA84	*Brevibacillus parabrevis* (HM590702)	HQ219915(97)	78.23	5.99	6.72	−	–	–	+	−	−	+	−
ANA42	*Microbacterium arborescens* (NR_029262)	HQ219873(97)	87.23	18.56	5.43	−	–	6.78	−	−	−	−	−
ANA50	*Microbacterium oleivorans* (AJ698725)	HQ219881(96)	211.89	11.23	4.56	−	2.12	7.72	+	−	−	−	−
ANA87	*Methylobacterium* sp. (EU741082)	HQ219918(97)	342.34	19.23	4.22	7.56	3.45	8.22	+	+	+	+	−
SNA7	*M. populi* (NR_029082)	HQ220065(99)	231.23	14.56	6.88	1.89	2.15	3.34	+	+	+	+	−
SNA1	*M. fujisawaense* (AB558142)	HQ220059(98)	198.22	32.12	−	23.23	2.34	12.20	+	+	+	+	−
ATY52	*Rhizobium* sp. (FJ430076)	HQ219971(96)	344.21	12.23	4.56	5.67	−	7.98	+	+	−	+	−
ATY53	*Stenotrophomonas sp.* (AY689032)	HQ219972(99)	197.34	29.90	−	21.22	3.89	16.66	+	−	−	−	−
ATY55	*S. chelatiphaga* (FJ493060)	HQ219974(98)	345.23	9.45	2.22	4.34	4.78	1.22	+	−	−	−	−
ATY60	*S. malthophila* (AY581129)	HQ219979(97)	212.34	22.23	−	19.90	8.23	2.89	+	−	−	−	−
AKB20	*Pseudomonas* sp. (EU373356)	HQ220021(97)	231.29	12.34	2.34	12.29	2.12	5.67	+	−	−	+	+
ANA69	*Pseudomonas* sp. (GU138382)	HQ219900(96)	231.23	14.56	5.68	12.89	2.15	3.34	+	+	+	+	+
SKB2	*P. fulva* (AY855189)	HQ220172(98)	289.23	29.90	4.58	10.98	3.23	7.88	+	−	−	+	+
AKB28	*P. putida* (HQ007288)	HQ220029(97)	312.23	22.12	−	13.32	4.56	9.20	+	+	+	+	−
SKB3	*P. aeruginosa* (HQ023428)	HQ220173(100)	212.98	13.14	−	17.69	3.11	10.96	+	−	−	+	−
ATY70	*P. stutzeri* (HM137032)	HQ219989(99)	289.23	4.12	−	11.09	−	22.12	+	−	−	−	+
ATY65	*P. tolaasii* (AY842149)	HQ219984(97)	318.12	9.88	−	2.34	2.22	11.34	+	−	−	−	+
AKB25	*P. savasnanoi* (AM265392)	HQ220026(99)	239.26	19.56	3.22	3.45	4.55	5.69	+	+	−	−	+
ATY75	*Pantoea* sp. (AB552921)	HQ219994(97)	233.34	11.23	2.34	5.62	5.90	7.22	+	−	−	−	−
ATY82	*Pantoea agglomerans* (AY941841)	HQ220001(97)	123.78	12.21	4.56	4.89	5.78	8.72	+	−	−	−	−
STY27	*P. agglomerans* (AY941838)	HQ220149 (97)	129.90	11.23	3.23	3.34	2.98	3.67	+	−	−	−	−

Table 2 (continued)

Strain code	Close relative from Genbank (accession no.)	Genbank no. (% identity)	P sol. ($\mu g\ ml^{-1}$)	IAA ($\mu g\ ml^{-1}$)	Chitinase ($mUml^{-1}$)	ACC deaminase	Salicylic acid ($\mu g\ ml^{-1}$)	Siderophore ($\mu g\ ml^{-1}$)	N-fixation	AHL production		*nifH*	*phlD*
										CV026	NT1		
ATY78	*P. agglomerans* (AY941838)	HQ219997(98)	290.90	8.55	1.34	3.23	3.89	4.89	+	−	−	−	−
ANA74	*Enterobacter cloacae* (HM162426)	HQ219905(97)	212.89	2.34	8.90	4.23	3.88	4.34	+	−	−	−	−
ANA37	*Serratia* sp. (FJ786079)	HQ219868(97)	212.34	10.20	3.45	1.23	3.78	4.59	+	−	−	−	−
ANA33	*S. plymutica* (GU201850)	HQ219864(98)	390.89	3.44	5.69	2.22	4.78	1.21	+	−	−	−	−
ATY19	*S. protamaculans* (FJ811862)	HQ219938(99)	321.33	4.56	8.72	2.35	4.92	3.45	+	−	−	−	−
AKB3	*Burkholderia* sp. (HM624043)	HQ220004(97)	342.34	5.67	4.44	4.34	3.23	2.22	+	+	+	+	+
AKB7	*Burkholderia* sp. (HM161871)	HQ220008(98)	348.22	3.45	6.78	3.45	3.78	8.92	+	+	+	+	+
AKB9	*B. vietnamiensis* (EU563934)	HQ220010(100)	347.78	7.8	9.77	4.55	5.34	6.34	+	+	+	+	+
AKB16	*B. cepacia* (HM582873)	HQ220017(98)	219.90	8.9	4.56	4.44	4.55	8.34	+	+	+	+	+
ATY30	*Achromobacter* sp. (GU086442)	HQ219949(98)	212.34	7.4	3.33	26.67	8.33	4.45	+	−	−	+	−
ATY18	*Variovorax paradoxus* (FJ527675)	HQ219937(97)	189.23	37.88	−	6.12	3.87	10.9	+	−	−	+	−
ANA25	*Herbaspirillum seropedicae* (H485595)	HQ219856(97)	212.12	43.29	−	16.24	2.34	9.5	+	−	−	+	−

+ presence of trait, − absence of trait

The ACC deaminase activity measured spectrophotometrically at 590 nm and expressed in nmol mg^{-1} protein h^{-1}. AHL production was tested by well diffusion assay using indicator strains *Agrobacterium tumefaciens* NT1 or *Chromobacterium violaceum* CV026

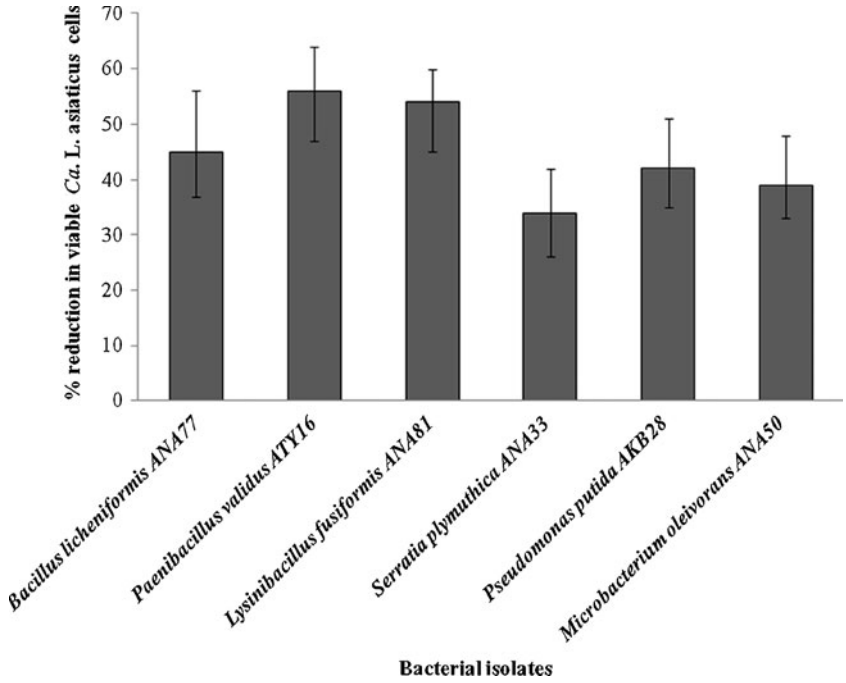

Figure 4 Inhibition assays of selected bacterial isolates against *Ca*. L. asiaticus. The antagonistic properties of bacterial isolates against *Ca*. L. asiaticus were represented with % reduction in the number of live *Ca*. L. asiaticus cells after inoculation with selected bacterial isolates as determined by novel EMA-qPCR-based bioassay. *Error bars* are the standard errors of the mean for the three replicates

[4, 7, 11, 12, 22, 27, 39]. Some understudied species belonging to genus *Microbacterium*, *Lysinibacillus*, *Brevibacillus*, and *Variovorax* were also isolated. Similarity below 98.7–99% on the 16S rDNA gene sequences of two bacterial strains is sufficient to consider them belonging to different species [47]. Several promising strains in our culture collection represent the first isolates of bacterial groups that have only been detected based on culture-independent methods. So far, there have been only a few reports on the effect of beneficial bacteria on citrus growth [1, 16, 24]. The present research will provide the basis for further understanding the community structure and functional properties of citrus-associated bacterial community. This will open the possibility to manage the indigenous antagonistic potential to control pathogens and to optimize biological approaches for citrus growth promotion using a consortium of beneficial bacteria.

Plant-associated bacteria encompass various groups of bacteria that exert a positive effect by various mechanisms ranging from direct to indirect effect. Our results showed that the HLB symptomatic and asymptomatic trees although growing on the same site, do seem to host different bacterial populations, in terms of diversity, abundance, and PGP activities. Earlier reports have also suggested disruption of indigenous bacterial populations possessing plant beneficial traits in response to phytopathogen infection [15, 29, 52]. We have not yet been able to demonstrate whether the bacterial communities associated with healthy roots suppress HLB disease or whether they resulted from the absence of *Ca*. L. asiaticus. Further study is needed to address this perspective. The difference in bacterial communities associated with healthy and infected roots might result from the special characters of the HLB pathogen. *Ca*. L. asiaticus causes blockage of citrus phloem, thus the roots were depleted of starch [13, 20]. Consequently, quantitative and qualitative changes in root exudation patterns might contribute to the shift in microbial diversity associated with HLB diseased and healthy roots. The frequency of chitinase and SA-producing bacteria was significantly higher in asymptomatic plants. It has been reported that the production of cell wall-degrading enzymes and SA by plant beneficial bacteria can induce complex defense responses against pests and pathogen invaders in host plant, a phenomenon termed as systemic acquired resistance [54]. Such resistance reactions involve induction of several host pathogenesis-related (*PR*) genes and can last for several weeks after activation so that the plant is resistant to future invaders [9, 48, 54]. In the present study, strains belonging to genus *Pseudomonas*, *Serratia*, *Burkholderia*, and *Pantoea* were found to produce AHL signals. For many plant beneficial bacteria, it was shown that expression of genes related to disease suppression and growth promotion is regulated in response to quorum sensing [10, 36]. AHL(s) also have a wide range of function in plants, including defense and stress responses, transcriptional regulation, protein processing, responses to plant hormones, as well as primary and secondary metabolism [3, 9]. The systemic transmission of responses to AHL-QS signals could be an important aspect of integrating a plant's overall preparation for dealing with bacterial assault or establishment of a mutualistic relationship [3].

Plant-associated bacteria must compete with plant cells for iron supply, and therefore, siderophore production may be highly important for growth in host [19]. Additionally, the production of siderophores has been reported to be one of the mechanisms to outcompete pathogens [33] by beneficial bacteria. Various groups of bacteria from the asymptomatic samples showed the production of siderophores. In symptomatic samples, only the strains belonging to *Methylobacterium* showed the production of siderophore. Interestingly, *Xylella fastidiosa* (Xf, causal agent of citrus variegated chlorosis) can utilize siderophores produced by *Methylobacterium* spp. to reduce the competition of iron and facilitate its survival inside the xylem of citrus [25, 46]. In our earlier studies, we have observed an increase abundance of genus *Methylobacterium* in HLB infected root and leaf samples of citrus [40, 52]. It is likely that *Ca*. L. asiaticus utilizes heterologous siderophore from *Methylobacterium* spp. during its establishment in host plants. Most of the efficient P solubilizing and ACC deaminase-producing bacteria were isolated from asymptomatic samples. Solubilization of insoluble phosphates by PGP bacteria could enhance the availability of limiting nutrient phosphorus to the host [7, 14, 22, 23]. In accordance with previous studies, our results confirm the wide distribution of ACC deaminase activity in different bacterial genera [44, 45]. It has been postulated that sufficient buildup of ACC deaminase possessing bacterial populations in plant root establish a sink for ACC thereby lowering the endogeneous ethylene levels resulting in stress tolerance and enhanced root elongation [44]. IAA like compound could be detected from the culture supernatant of various bacterial genera in the culture collection. IAA is a plant hormone which stimulates the development of root system and also has been speculated to improve the fitness of plant–microbe interactions [6, 35]. Various bacterial isolates were found positive for nitrogen fixation. However, the bacteria revealed a discrepancy between grown isolates in nitrogen free medium and positive isolates for *nifH* gene. This may be explained by the gene diversity of *nifH* gene [59] or by residual bacterial growth [22].

The trends presented here are in accordance with earlier study using clone library and qPCR analysis [52]. Both the studies pointed towards a decrease in the diversity and the numbers of various beneficial bacterial in response to phytopathogen infection. Differences were however observed in the frequency and recovery of few groups of bacteria when culturable diversity of *Ca*. L. asiaticus infected and uninfected citrus roots were compared with clone library analysis. A significantly higher proportion of bacteria belonging to Firmicutes and Actinobacteria were isolated in culture based analysis. Some of the members of these groups are recalcitrant to cell lysis [53] and therefore, they might be underrepresented in a clone library. However, the selectivity of cultivation as well as preferential amplification of certain bacterial groups with the eubacterial primers could also cause the differences in abundances. A disparity in the representation of different bacterial classes, genera, and species between the isolate collection and clone library had also been observed in previous studies [8, 19, 53]. Therefore, the combination of culturing methods and cloning analysis is needed to give a complete picture of bacterial communities particularly in woody plants.

Development of a proper in vitro screening system that provides repeatable and reliable results in shorter periods of time is an important step for isolation of efficient bacterial antagonists [21]. The widely used dual culture technique could not be applied to screen bacteria antagonistic to *Ca*. L. asiaticus due to our inability to culture the bacterium. We have developed a method to quantify viable *Ca*. L. asiaticus with the aid of EMA which can differentiate live from dead cells [51]. This diagnostic DNA-based method combines the use of a live-dead discriminating dye with the speed and sensitivity of qPCR. The EMA-qPCR assay was optimized for screening potential biocontrol bacteria effective against *Ca*. L. asiaticus. Antagonistic bacterial isolates will cause a decrease in the number of viable *Ca*. L. asiaticus cells, which can then be detected by EMA-qPCR method. The bactericidal activity of the isolates against *Ca*. L. asiaticus could be attributed to the competition for nutrients and/or production of secondary metabolites such as antibiotics, bacteriocins, or volatiles. From the culture collection, six bacterial isolates were found to reduce the number of viable *Ca*. L. asiaticus cells by EMA-qPCR based method. Two of these strains belonged to genus *Bacillus* and *Pseudomonas* which have previously been reported to possess antagonistic activities against some other members of family *Rhizobiaceae* (to which *Ca*. L. asiaticus belongs) [7]. The selected novel isolates will be further tested *in planta* and in field conditions to determine whether they could be used in management of HLB.

In the present study, we have developed a culture collection of bacterial isolates that are cultivable, non-pathogenic, plant specific, possess multiple PGP traits and located within the plant compartment most suitable for biocontrol and plant growth promotion. The selected isolates could be used to develop bacterial formulations for rhizosphere engineering to maintain balance of beneficial bacteria in citrus producing agro-ecosystems. The application of these strains will not bring risks to the environment since they are isolated from Florida citrus groves. Modified EMA-qPCR method will be helpful in understanding the interaction of *Ca*. L. asiaticus with other bacterial groups. The selected isolates could provide a multitude of possibilities to control citrus disease especially HLB such as direct antagonism, priming of tissue culture raised plants, secretion of useful products directly in plant

system, or rhizosphere engineering to maintain balance of beneficial bacteria in citrus rhizosphere. The identification of several new geno-species associated with citrus supports the concept of endemic bacteria and reiterates a need to conserve local resources for future ecosystem stability and as a valuable genetic resource. Enhanced knowledge regarding the beneficial interactions of citrus with associated bacterial communities will be of special importance for sustainable agriculture that relies on biological processes and resources, rather than on the use of agrochemicals for maintaining soil fertility and plant health.

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