

Characterization of cucumber rhizosphere bacterial community with high-throughput amplicon sequencing

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ABSTRACT

Cucumber rhizosphere and bulk soils were sampled from a greenhouse, which has been continuously monocropped with cucumber. Bacterial community composition was analyzed by high-throughput sequencing of 16S rRNA genes on an Illumina Miseq platform. A total of 211,027 quality sequences were obtained and were classified into more than 1,800 Operational taxonomic units at the 97% sequence similarity. Cucumber rhizosphere soil sample had lower bacterial alpha diversity than bulk soil sample. Bacterial community composition also differed between the rhizosphere and bulk soils. Rhizosphere soil sample was dominated by *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Bacteroidetes* and *Actinobacteria* phyla and classes of *Gammaproteobacteria*, *Alphaproteobacteria*, *Cyanobacteria*, *Bacilli* and *Actinobacteria*. Compared with the bulk soil, cucumber rhizosphere soil had higher relative abundances of *Bacillus*, *Lysobacter*, *Streptomyces*, *Microbacterium* and *Rhizobium* spp. and lower relative abundances of *Clostridium sensu stricto 1*, *Sphingomonas* and *Microvirga* spp. ($P < 0.05$).

Key words: Bacterial community, *Cucumis sativus* L., Illumina sequencing, rhizosphere soil

INTRODUCTION

Many important economic crops [wheat (10), cucumber (53) and soybean (27)] have allelopathy potential. The allelochemicals are released through root exudation, leaching, volatilization, or residue decomposition (16,22,33). The root exudates and decomposing residues of several crops are autotoxic, which is one of the main causes of soil sickness (9,15,21,55).

Agricultural productivity relies on wide range of ecosystem services provided by soil microorganisms (6,17,31,36). Soil microorganisms are critical to many processes [nutrients cycling, organic matter turnover, soil structure formation and suppression of soil-borne diseases] (8,29,40). The rhizosphere is hot spot of plant-microbe interactions, as the species, distribution and structure of microorganisms are governed by the root exudates (3). Previous studies have shown that exogenously applied root exudates components, such as phenolic compounds with allelopathic potentials, changes the compositions of soil microbial community (14,42,44,55). Rhizosphere microbial communities of several plant species, including *Arabidopsis thaliana*, rice and maize, have been characterized. These earlier studies have shown that there were overlaps and differences in microorganisms in rhizosphere and non-rhizosphere soils and the rhizosphere microbes have strong dependence on plant species (4,12,25,30).

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Cucumber is popular vegetable and its production is usually threatened by soil sickness (48,53,54). Autotoxicity of compounds from the root exudates is one of the main causes of soil sickness in cucumber (47,50,51,55). Limited information is available on how root exudates regulate the cucumber rhizosphere microbial communities (1,18). Therefore, this study aimed to compare the rhizosphere and bulk soils bacterial communities using the high-throughput sequencing technology.

MATERIALS AND METHODS

Greenhouse experiment

The greenhouse experiment was done in 2017 in the Experimental Station, Northeast Agricultural University, Harbin, China (45°41'N, 126°37'E, mean height above sea level: 127.95 m, annual precipitation: 524.5 mm, maximum and minimum temperature: 36.7°C, -37.7°C). The greenhouse had been used for continuous monocropping of cucumber since 2006. The soil was black (Mollisol) with sandy loam texture and contained sand (326 g kg⁻¹), silt (384 g kg⁻¹), clay (270 g kg⁻¹), organic matter (3.51%), available N (NH₄⁺-N and NO₃⁻-N), 146.60 mg kg⁻¹; Olsen P (284.20 mg kg⁻¹) available K (341.80 mg kg⁻¹); EC (1:2.5, w/v), 0.43 mS cm⁻¹; and pH (1:2.5, w/v), 7.64 (48).

There were three plots (6-m long, 2.4-m wide), each plot contained four rows; the central 2-rows were used for soil sampling. Raised beds (height 15 cm, width 40 cm, distance between beds 15 cm) were made 5-days before transplanting of cucumber seedlings. Before transplanting of cucumber seedlings, the basal dose of decomposed manure (containing N 225.0 kg ha⁻¹, P 225.0 kg ha⁻¹ and K 180.0 kg ha⁻¹) at 45,000 kg ha⁻¹ and diammonium hydrogen phosphate (containing N 36.0 kg ha⁻¹ and P 40.1 kg ha⁻¹) at 200 kg ha⁻¹ were applied. Cucumber seedlings (cv. Jinlv 3) with two leaves were transplanted in greenhouse on April 25, 2017. The plant density was 6 plants per m². Flooding irrigation with groundwater was given when necessary. Weeds were manually removed.

Soil Sampling and DNA Extraction

Bulk soil and cucumber rhizosphere soil samples were collected on 30th day after transplanting as described before (48,55). For bulk soil sampling, ten soil cores (8 cms dia, 20 cms deep) were randomly collected from the inner two rows of cucumber in each plot and were mixed to make a composite sample. For rhizosphere soil sampling, samples from five plants in each plot were pooled to make a composite sample. After sieving (2 mm), soil samples were stored at -70°C.

Total soil DNA was extracted with the Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) as per the manufacturer's instructions. Each soil sample was extracted in triplicate and the extracted DNA solutions were pooled. In total, there were six composite DNA samples.

High-throughput Amplicon Sequencing and Data Processing

Total soil bacterial community compositions were estimated with high-throughput sequencing on an Illumina MiSeq platform. Primer sets of F515/R907 were used to amplify

V4-V5 regions of the bacterial 16S rRNA gene as described before (49,53). There were three technically replicated PCR reactions for each DNA sample, which were subsequently pooled and purified using an Agarose Gel DNA purification kit (TaKaRa, China). Then, the purified amplicons were quantified by a TBS-380 micro fluorometer with Picogreen reagent (Invitrogen, USA) and mixed accordingly to achieve equal concentration in the final mixture. The mixture was then paired-end sequenced (2×300) on an Illumina Miseq platform at Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China.

Raw sequence reads were de-multiplexed, quality-filtered and processed using FLASH (26) as described before (47). Operational taxonomic units (OTUs) were delineated at 97% sequence similarity with USEARCH using an agglomerative clustering algorithm (11). Then, a representative sequence of each OTU was taxonomically classified through BLAST in the SILVA databases (34). Chimeric sequences were identified and removed using USEARCH 6.1 in QIIME (5). To avoid potential bias caused by sequencing depth, a random subsampling of 20,769 16S rRNA gene sequences per sample was done for further analysis.

Statistical Analysis

The defined OTUs were used to calculate taxon accumulation curves with the ‘vegan’ package in ‘R’ (Version 3.3.1). Alpha diversity indices, Shannon index and inverse Simpson index were calculated with the ‘vegan’ package in ‘R’ (Version 3.3.1) (23). For beta diversity analysis, Bray-Curtis distances were calculated and principal coordinates analysis (PCoA) was conducted to visualize the community similarity with the ‘vegan’ package in ‘R’ (Version 3.3.1). Differences in alpha diversity indices and relative abundances of microbial taxa between treatments were analyzed using Welch’s *t* test in ‘R’ (Version 3.3.1).

RESULTS AND DISCUSSION

Illumina Miseq sequencing data

In total, we obtained 211,027 quality bacterial 16S rRNA gene sequences from all samples. The average read length of these sequences was 396 bp. Across all samples, 1,837 OTUs were observed at the 97% sequence similarity (data not shown). Rarefaction curves of OTUs of all samples tended to approach the saturation plateau (Figure 1a) and the Good’s coverage was larger than 98.5% for each sample (Figure 1b). Therefore, the number of sequences was sufficient to assess the diversity of cucumber rhizosphere bacterial communities.

Compared with bulk soil samples, cucumber rhizosphere soil samples had lower bacterial community richness [number of observed (OTUs)] and diversity (Shannon and Inverse Simpson indices) indices ($P < 0.05$) (Fig1b). PCoA analysis at the OTU level showed clear separation of cucumber rhizosphere soil samples and bulk soil samples (Fig 2a). It has been suggested that the competition among the bacterial populations for nutrition from the roots, changes some taxa and in turn decreases the rhizosphere microbial diversity (3). Our results are in agreement with previous studies which reported that the

rhizosphere had lower diversity in microbial community in some plant species [soybean, potato and *Arabidopsis thaliana*] (7,10,25,27).

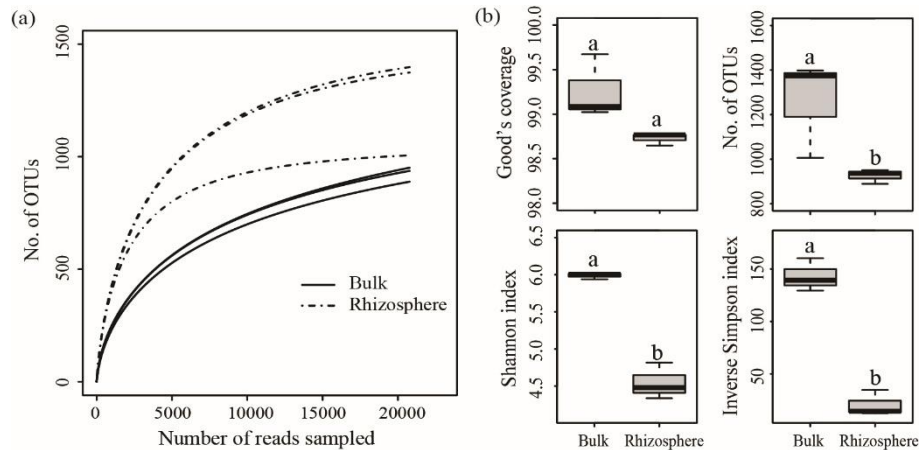


Figure 1. (a) Rarefaction curves of the number of OTUs, (b) The Good's coverage and diversity and richness indices of bacterial communities. Different letters indicate significant differences between treatments ($P < 0.05$).

Taxonomical classification at the phylum level revealed that 33 bacterial phyla were detected in total (Data not shown) and 1.14% bacterial sequences were unclassified at this level. The dominant phyla (relative abundance >5%) across all soil samples were *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Bacteroidetes* and *Actinobacteria*, which accounted for more than 90% of the total bacterial sequences (Figure 2b). Compared with bulk soil samples, cucumber rhizosphere soil samples had lower relative abundances of *Acidobacteria*, *Chloroflexi*, *Gemmatimonadetes*, *Nitrospirae*, *Planctomycetes*, *Proteobacteria* and *Saccharibacteria* ($P < 0.05$).

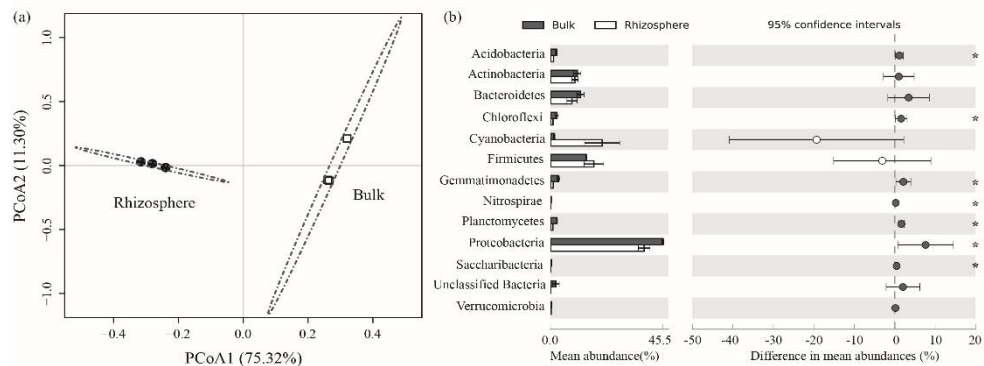


Figure 2. (a) Beta diversities of bacterial communities, (b) Relative abundances of main bacterial phyla. Circles represent 95 % confidence intervals. *: Significant differences between treatments ($P < 0.05$).

Plant roots can select the specific microorganisms to colonize their rhizosphere, which providing an energy source of carbon-containing compounds (9). The main phyla of bacteria identified earlier in the rhizosphere are *Proteobacteria* (39), followed by *Actinobacteria*, *Firmicutes* and *Bacteroidetes*, which was consistent with our results. Previous studies showed that there is higher abundance of copiotrophic bacteria of *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* and higher abundance of oligotrophic bacteria such as *Acidobacteria* in the rhizosphere (41,46). *Proteobacteria* phylum is the most common phylum with a higher relative abundance than other phyla. *Acidobacterium*, which can degrade polysaccharide widely exists in the rhizosphere of plants (43). In this study, both *Acidobacteria* and *Proteobacteria* had lower abundances in rhizosphere soil, but the relative abundance of *Proteobacteria* was higher than *Acidobacteria*, suggesting that the rhizosphere usually inhabited by r-strategy organisms, which usually exhibit high growth rates, when resource are abundant (32). The rhizosphere usually has very high concentrations of easily degradable carbon sources (9), hence, the root exudates secreted by plants decreased the growth of *Chloroflexi* and *Planctomycetes*, which are usually present in less fertile soils with limited nutrients availability (6).

Table 1. Relative abundances (%) of main Bacterial classes in bulk and cucumber rhizosphere soils.

Bacterial class	Bulk	Rhizosphere	Bacteria class	Bulk	Rhizosphere
<i>Gammaproteobacteria</i>	19.60±0.61	19.15±2.15	<i>Planctomycetacia</i>	1.91±0.20	0.82±0.04
<i>Alphaproteobacteria</i>	17.84±0.20	13.60±0.80	<i>Deltaproteobacteria</i>	1.83±0.02	0.77±0.08
<i>Cyanobacteria</i>	1.55±0.20	20.86±5.02	<i>Unclassified Bacteria</i>	2.16±0.97	0.12±0.04
<i>Bacilli</i>	4.17±0.23	16.76±3.01	<i>Erysipelotrichia</i>	0.92±0.02	0.13±0.04
<i>Actinobacteria</i>	10.87±1.01	9.86±0.91	<i>Thermomicrobia</i>	0.48±0.15	0.42±0.05
<i>Sphingobacteriia</i>	6.36±0.52	3.88±0.47	<i>Gitt-GS-136</i>	0.75±0.08	0.15±0.01
<i>Betaproteobacteria</i>	5.90±0.45	4.33±0.72	<i>Phycisphaerae</i>	0.61±0.03	0.13±0.03
<i>Clostridia</i>	9.35±0.13	0.65±0.20	<i>Anaerolineae</i>	0.51±0.03	0.17±0.03
<i>Cytophagia</i>	3.62±0.18	3.20±0.49	Unclassified	0.49±0.08	0.01±0.00
<i>Gemmatimonadetes</i>	3.23±0.43	1.10±0.05	<i>Saccharibacteria</i>		
<i>Acidobacteria</i>	2.41±0.25	1.27±0.09	<i>Nitrospira</i>	0.34±0.05	0.08±0.01
<i>Flavobacteriia</i>	1.79±0.70	1.56±0.48	<i>Opitutae</i>	0.13±0.07	0.27±0.09
			<i>Bacteroidetes</i>	0.31±0.05	0.06±0.02
			<i>Incertae Sedis</i>		

Values (Mean ± SE) of bacterial classes highlighted in bold were significantly different (P < 0.05).

More than 70 bacterial classes were detected across all soil samples (Data not shown). The dominant classes (relative abundance > 10%) were: *Gammaproteobacteria*, *Alphaproteobacteria*, *Cyanobacteria*, *Bacilli* and *Actinobacteria* (Table 1). Compared with bulk soil samples, cucumber rhizosphere soil samples had lower relative abundances of *Alphaproteobacteria*, *Sphingobacteriia*, *Clostridia*, *Gemmatimonadetes*, *Acidobacteria*, *Planctomycetacia*, *Deltaproteobacteria*, *Erysipelotrichia*, *Gitt-GS-136*, *Phycisphaerae*, *Anaerolineae*, Unclassified *Saccharibacteria*, *Nitrospira* and *Bacteroidetes Incertae Sedis* (P < 0.05).

At the genus level, more than 580 bacterial genera were detected (Data not shown). Compared with bulk soil samples, cucumber rhizosphere soil samples had higher relative

abundances of *Agromyces*, *Bacillus*, *Dyadobacter*, *Lysobacter*, *Microbacterium*, *Novosphingobium*, *Phenylobacterium*, *Rhizobium*, *Shinella* and *Streptomyces* spp., but had lower relative abundances of *Rhodanobacter*, *Clostridium sensu stricto 1*, *Romboutsia*, *Sphingomonas*, *Terrisporobacter*, *Steroidobacter*, *Erythrobacter*, *Turicibacter*, *Mizugakiibacter*, *Aquicella*, *Castellaniella*, *Rhodopseudomonas*, *Microvirga*, *Blastococcus*, *Haliangium*, *Pseudolabrys*, *Taibaiella* and *Nitrospira* spp. ($P < 0.05$) (Table 2).

Table 2. Relative abundances (%) of main bacterial genera in bulk soils and cucumber rhizosphere soils.

Bacterial genus	Bulk	Rhizosphere	Bacteria genus	Bulk	Rhizosphere
<i>Bacillus</i>	2.10±0.09	13.95±2.39	<i>Cellvibrio</i>	0.21±0.01	0.63±0.20
<i>Lysobacter</i>	3.85±0.38	6.02±0.42	<i>Simiduia</i>	0.48±0.03	0.34±0.19
<i>Rhodanobacter</i>	4.09±0.43	1.71±0.56	<i>Mesorhizobium</i>	0.43±0.06	0.38±0.02
<i>Clostridium sensu stricto 1</i>	3.53±0.08	0.15±0.04	<i>Dyadobacter</i>	0.02±0.01	0.76±0.14
<i>Streptomyces</i>	0.66±0.15	2.95±0.32	<i>Salinimicrobium</i>	0.71±0.39	0.03±0.01
<i>Pseudomonas</i>	1.23±0.48	1.98±0.24	<i>Phenylobacterium</i>	0.25±0.06	0.48±0.03
<i>Romboutsia</i>	2.47±0.24	0.30±0.08	<i>Nitrospira</i>	0.50±0.09	0.22±0.02
<i>Rhizobium</i>	0.57±0.03	2.18±0.29	<i>Agromyces</i>	0.22±0.04	0.49±0.06
<i>Pseudoxanthomonas</i>	0.63±0.04	2.01±0.34	<i>Aquicella</i>	0.60±0.08	0.12±0.05
<i>Sphingomonas</i>	2.15±0.20	0.26±0.12	<i>Parapedobacter</i>	0.24±0.04	0.46±0.24
<i>Microbacterium</i>	0.82±0.09	1.29±0.05	<i>Gaiella</i>	0.57±0.12	0.13±0.04
<i>Terrisporobacter</i>	1.92±0.12	0.14±0.04	<i>Castellaniella</i>	0.56±0.08	0.10±0.04
<i>Devosia</i>	0.99±0.01	0.89±0.09	<i>Rhodopseudomonas</i>	0.45±0.06	0.19±0.02
<i>Shinella</i>	0.20±0.05	1.66±0.25	<i>Microvirga</i>	0.56±0.08	0.08±0.03
<i>Paenibacillus</i>	0.25±0.06	1.24±0.30	<i>Blastococcus</i>	0.45±0.07	0.16±0.05
<i>Steroidobacter</i>	1.04±0.04	0.42±0.04	<i>Brevundimonas</i>	0.31±0.05	0.27±0.04
<i>Chitinophaga</i>	0.66±0.09	0.73±0.22	<i>Dokdonella</i>	0.23±0.02	0.34±0.07
<i>Erythrobacter</i>	1.06±0.05	0.30±0.01	<i>Altererythrobacter</i>	0.36±0.06	0.17±0.01
<i>Novosphingobium</i>	0.56±0.03	0.72±0.04	<i>Amycolatopsis</i>	0.00±0.00	0.53±0.15
<i>Ohtaekwangia</i>	0.38±0.05	0.86±0.24	<i>Pedobacter</i>	0.41±0.09	0.10±0.02
<i>Achromobacter</i>	0.84±0.27	0.36±0.04	<i>Haliangium</i>	0.40±0.03	0.11±0.02
<i>Ureibacillus</i>	0.40±0.03	0.79±0.30	<i>Pirellula</i>	0.37±0.06	0.14±0.02
<i>Sphingopyxis</i>	0.48±0.04	0.68±0.13	<i>Pseudolabrys</i>	0.38±0.02	0.13±0.00
<i>Pseudoduganella</i>	0.06±0.00	1.04±0.62	<i>Mycobacterium</i>	0.38±0.08	0.12±0.03
<i>Turicibacter</i>	0.92±0.02	0.13±0.04	<i>Taibaiella</i>	0.34±0.06	0.13±0.04
<i>Flavobacterium</i>	0.22±0.10	0.80±0.36	<i>Bryobacter</i>	0.35±0.06	0.12±0.01
<i>Planctomyces</i>	0.72±0.10	0.30±0.01	<i>Arachidicoccus</i>	0.16±0.03	0.30±0.11
<i>Nocardioides</i>	0.52±0.12	0.47±0.09	<i>Nitrospira</i>	0.34±0.05	0.08±0.01
<i>Mizugakiibacter</i>	0.84±0.04	0.14±0.04	<i>Raoultella</i>	0.33±0.21	0.08±0.01

Values (Mean ± SE) of bacterial genus highlighted in bold were significantly different ($P < 0.05$).

Root secretions act as chemical signals by regulating the structure and activity of rhizosphere microorganisms (1). In this study, we found that rhizosphere was rich in some potential plant-beneficial microorganisms, such as *Bacillus* (31), *Lysobacter* (13), *Streptomyces* (19), *Rhizobium* (37) and *Microbacterium* (38). Previous studies reported that these genera contained isolates that can promote plant growth and suppress plant pathogens and thus play an important role in agriculture. For example, some species of *Bacillus* spp. are active antagonists against filamentous fungi (31). Similarly, *Streptomyces*

spp. can metabolize plant residues and root exudates and control some plant disease through the production of secondary metabolites and extracellular enzymes (19). Further, we found that cucumber rhizosphere soil had higher bacterial abundances of *Dyadobacter* (20) and *Agromyces* (35), which may affect the nitrogen fixation in soil. Therefore, cucumber itself could selectively recruit beneficial bacteria in the rhizosphere for its own benefit. However, further studies are needed to investigate the compounds, through which cucumber recruits the beneficial microorganisms.

Our previous studies have shown that phenolic autotoxins (*p*-coumaric acid, syringic acid and vanillin) changed cucumber rhizosphere bacterial community composition (42,44,45,52). These phenolic autotoxins decreased the bacterial community diversity and the abundances of beneficial microorganisms, such as *Lysobacter* and *Bacillus* spp., suggesting that phenolic autotoxins might decrease the ability of cucumber to recruit beneficial microorganisms in the rhizosphere.

Compared with bulk soil, the rhizosphere soil of cucumber had lower relative abundance of *Clostridium sensu stricto 1*, *Sphingomonas* and *Microvirga*. Lucy *et al.* (24) reported that the *Clostridium sensu stricto 1* could stimulate the plant nutrients uptake and produce plant hormones. Some species of *Sphingomonas* and *Microvirga* spp. degrade the phenolic acids and suppress plant diseases (2,28). The genera *Sphingomonas* and *Microvirga* of the phylum *Proteobacteria* have relative low abundances in C-rich environment (46). Therefore, it is possible that the competition for nutrients or inability to use carbon sources lead to the variations in above bacterial genera in the rhizosphere soil than in bulk soil.

CONCLUSIONS

Compared to bulk soil, the bacterial diversity decreased and its structure changed in rhizosphere soil of cucumber. Abundances of *Bacillus*, *Lysobacter*, *Microbacterium*, *Streptomyces* and *Rhizobium* spp., which contained potential plant-beneficial species, showed considerable increase but other taxa (*Clostridium sensu stricto 1*, *Sphingomonas* and *Microvirga* spp.) were decreased from bulk soil to rhizosphere, suggesting that the root niche plays an important role in bacterial composition.

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