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Endophytic Bacteria in *Ricinus communis* L.: Diversity of Bacterial Community, Plant−Growth Promoting Traits of the Isolates and Its Effect on Cu and Cd Speciation in Soil

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Abstract: *Ricinus communis* L. shows certain tolerance to and good accumulation ability with heavy metals. Endophytic bacteria−enhanced phytoremediation is an effective method to improve heavy metal extraction efficiency. Here, for better application of castor in phytoremediation, the Illumina high−throughput sequencing was carried out to reveal the endophytic bacterial community in the tissues of castor grown in two locations, and traditional microbial cultivation was used to isolate endophytic bacteria from castor. The dominant bacterial phyla were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes* and *Acidobacteria*, and *Proteobacteria* was absolutely dominate in all castor tissues. There were significant differences in the composition of endophytic bacterial communities between castor grown in two sites, with obvious variation in the relative abundance of the dominant phylum. The samples from two sites also had their own unique dominant bacterial genera. The analysis of alpha diversity illustrated that the diversity and species richness of endophytic bacteria community in different parts of castor sampled in Tonglushan mining area were lower than those in Gangxia village, Yangxin county. In total, there were 44 endophytic bacteria strains isolated from the tissues of castor, of which 42 strains possessed three or more growth−promoting properties. Most of these isolates were tolerant to Cu or Cd to varying degrees. Eight isolates were selected for further Cu mobilization and soil incubation experiments. Strains TR8, TR18, TR21, YL1, YS3 and YS5 could well solubilize $Cu_2(OH)_2CO_3$ in medium. Strain YS3 had the best effect on increasing soil DTPA−Cu and DTPA−Cd contents by 8.4% and 6.9%, respectively. Inoculated endophytic isolates were conducive to the conversion of heavy metal forms from insoluble to relatively unstable, and could increase available phosphate content in soil (10.8–29.2%). Therefore, the plant growth−promoting endophytes screened from castor have great application prospects and can provide important support for the microbial−assisted phytoremediation of heavy metal−contaminated soil.

Keywords: *Ricinus communis* L.; endophytic bacterial community; Isolation; plant growth promoting characteristics; Cu and Cd

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Citation: Li, Q.; Fu, Q.; Zhu, J.; Sun, Y.; He, H.; Hu, H. Endophytic Bacteria in *Ricinus communis* L.: Diversity of Bacterial Community, Plant−Growth Promoting Traits of the Isolates and Its Effect on Cu and Cd Speciation in Soil. *Agronomy* **2023**, *13*, 333. https://doi.org/10.3390/agronomy13020333

Academic Editor: Marzia Vergine

Received: 23 December 2022
Revised: 18 January 2023
Accepted: 21 January 2023
Published: 23 January 2023

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1. Introduction

Plant endophytic bacteria are microorganisms that colonize healthy plant tissues by symbiosis. In the long−term evolution process, they have established a good interaction relationship with host plants, and play a critical role in regulating the growth and development of host plants. As an important element of the plant micro−ecosystem, plant endophytes are an indispensable part of plants, and host plants also provide a stable living environment for them [1,2]. Previous studies indicated that the inoculated endophytic bacteria with growth−promoting properties could mobilize mineral elements and increase their bioavailability in the rhizosphere, which could facilitate the uptake of these nutrients, promote the growth and development of the plant, and also alleviate the toxicity of various abiotic stresses to the plant [3–6]. Moreover, as one of the important components of microbial resources, understanding the community and structure of plant endophytes is

helpful to better understand the interaction between plants and endophytes. For different plants, the community composition of endophytes is often different, depending on the plant species, growth cycle and living environment [7–9].

Ricinus communis L. is an annual or perennial plant of the Euphorbiaceae family. The oil contents of castor seeds can reach 41–64% [10], and it is a raw material of industrial, medical and chemical products. Studies have shown that castor plants can grow normally in soil contaminated by various heavy metals because they have a certain tolerance to and good accumulation ability with different heavy metals such as Pb, Cd, Cu and Zn [11–15]. The castor plant has broad prospects in the phytoremediation of heavy metal−contaminated soil with the advantages of low soil fertility requirement, large biomass and high economic value. In addition to the ability of the plant to take up and translocate of pollutants, plant biomass is one of the decisive factors affecting the phytoremediation efficiency [16,17]. Bauddh and Singh [18] found that the biomass of castor bean is significantly greater than that of Indian mustard on Cd−contaminated soil; although the content of Cd in various parts of Indian mustard is higher than that in castor beans, the total amount of Cd extracted by the roots and shoots of Indian mustard from soil is significantly lower than that of castor bean plants.

Because of the limited ability of castor to translocate heavy metals to the above-ground part, it is necessary to explore effective methods to improve the accumulation of heavy metals in the above−ground parts of castor for utilizing castor to remediate heavy metal−contaminated soil. Under abiotic stress condition, it is one of the effective methods to strengthen the resistance of host plants to heavy metals, improve soil nutrient status and increase the bioavailability of soil heavy metals, and then improve the phytoremediation efficiency of heavy metals by host plants through inoculating exogenous endophytic bacteria with growth promoting properties [19–21]. At present, little is known about the community structure and composition of endophytic bacteria in castor, and it is still a question whether the plant growth promoting endophytic bacteria isolated from castor can be used to enhance the extraction of heavy metals by host plants. Therefore, the objectives of this study are: (1) to analyze the endophytic bacterial community structure and composition of castor which were grown in two regions by Illumina high−throughput sequencing; (2) to isolate endophytic bacteria with plant growth−promoting properties from the tissues of castor; (3) to explore the effects of the isolated endophytic bacteria on the mobility of soil heavy metal activity and soil available phosphorus.

2. Materials and Methods

2.1. Materials

Samples of castor were collected from Tonglushan Mining area, Daye (30°4′49″ N, 114°56′13″ E) and Gangxia village, Yangxin county, Huangshi (29°48′40″ N, 115°25′53″ E), Hubei province, China, respectively. Tonglushan is a copper mine with more than 3000 years of mining history, and the soil is mainly contaminated by Cu and Cd. Six healthy castor plants were randomly selected from two sampling sites and placed in sterile sample bags, then stored on the ice surface. After being brought back to the laboratory, the plants were immediately placed in a refrigerator at 4 °C, and the endophytic bacteria were isolated within 48 h. The soil surrounding the castor roots was collected, and the basic properties of the soil were measured and presented in Table 1.

Table 1. The basic characteristics of soils in two sampling sites.

Sampling Sites	Cu mg/kg	Cd mg/kg	pH	Available P mg/kg	OM (g/kg)	NH_4^+−N mg/kg	NO_3^-−N mg/kg
Tonglushan	693.8	4.3	8.10	4.4	7.0	3.8	3.4
Gangxia	833.0	9.5	7.76	22.4	39.5	5.8	59.2

The plant samples were washed with tap water and rinsed 5 times with deionized water, then washed with sterile water for 30 s. The plants were surface sterilized using 70%

ethanol for 2 min, then immersed in 2.5% NaClO (containing 0.1% Tween 80) for 5 min followed by transferring to 70% sterile ethanol for 30 s, and finally washed 3 times using sterile water. The plant samples were divided into two parts. One part was frozen with liquid nitrogen and quickly stored in a refrigerator at −80 °C for Illumina high−throughput sequencing, in which the root, stem and leaf samples from Tonglushan were named TR, TS and TL, respectively, and the samples from Gangxia village were named YR, YS and YL, respectively. The other part was used to isolate the endophytic bacteria immediately.

2.2. Illumina High−Throughput Sequencing of Endophytic Bacterial Community of the Castor

The total DNA of plant samples was extracted using FastDNA® SPIN Kit (MP Biomedicals, Santa Ana, CA, USA), and the concentration and purity of total genomic DNA were assessed. Then, the extracted DNA was amplified via two pairs of primers and two rounds of PCR [22–24]. The details of amplification were presented in Table S1. The amplified PCR products of each sample were tested by 2% agarose gel electrophoresis. The products were gel−extracted by AxyPrepDNA gel extraction kit (Axygen, San Francisco, CA, USA) and quantified by the QuantiFluor™ −ST Blue Fluorescence Quantification System (Promega, Madison, WI, USA), and mixed proportionally according to the required sequencing volume of each sample. The paired−end sequencing of purified amplicons was performed on Illumina's Miseq PE250 platform (2 × 250 bp) (Shanghai Majorbio Bio−pharm Technology Co., Ltd., Shanghai, China). All raw sequences were submitted to NCBI Sequence Read Archive (SRA) (BioProject ID: PRJNA893461).

The paired−end reads obtained by Miseq sequencing were assembled by Flash software (v1.2.11), while sequence quality was controlled and filtered using Fastp software (v0.19.6). Then, the sequences were clustered at 97% identity into operational taxonomic units (OTUs), and the OTUs obtained by clustering were analyzed by RDP classifier (v2.11), for which the comparison database was SILVA (v138). The rarefaction curves for OTUs were used to determine the sample sequencing depth, and Simpson, Chao1 and Coverages indices were used to characterize the diversity and richness of microbial community (Mothur, V.1.30.2). Based on Bray–Curtis distances, non−metric multidimensional scaling (NMDS) analysis was conducted using the R language (v3.3.1) vegan package to compare the differences in community composition among samples. Analysis of similarities (ANOSIM) was used to detect the significance of differences between groups.

2.3. Isolation of Endophytic Bacteria

The surface−sterilized plant tissues were fully ground together with 5 mL sterilized PBS buffer (pH 7.4), and 100 µL suspension was spread on an LB agar plate with the method of gradient dilution coating (10^{-1}, 10^{-2}, 10^{-3}). The stems of the castor were cut into slices with a sterilizing blade and were spread on LB agar plates. All plates were incubated at 28 °C for 3–5 d, and single colonies with different forms were picked and purified on LB agar plates. All details of the media used in the study were listed in Supplementary Materials Table S2.

2.4. Plant Growth−Promoting Properties of the Isolates

IAA production of isolates was measured in SMS medium containing 500 mg/L tryptophan and incubated at 30 °C for 2 d. After centrifugation, 1 mL of supernatant was mixed thoroughly with 2 mL of Salkowski's reagent and colored in the dark for 30 min at room temperature, in which the appearance of pink indicated the production of IAA, then the absorbance of the mixture was measured by colorimeter at 530 nm [25].

Siderophore secretion was determined according to the method described by Schwyn and Neilands [26]. Then, 1.5 mL of cell−free supernatant from incubation in SMS liquid medium for 48 h was mixed with an equal volume of Chrome azurol S (CAS) detection solution. The mixture was colored in the dark for 1 h at room temperature, then the absorbance was measured by colorimeter at 630 nm (λ) and the non−inoculated SMS liquid medium was used as the control (λ_0). The ability to produce siderophores is represented as $(\lambda_0 - \lambda)/\lambda_0$ and the higher the value, the higher the capacity for siderophore production.

Aminocyclopropane−1−carboxylate (ACC) deaminase activity was analyzed using the protocol described by Penrose and Glick [27]. In brief, the isolates were inoculated in DF medium and cultured at 30 °C for 24 h. After centrifugation at 8000× *g* for 10 min at 4 °C, cell pellets were collected and resuspended with an equal volume of ADF medium, then incubated to induce the activity of ACC deaminase of bacteria at 30 °C for 24 h. After that, the cell pellets were harvested and washed by 5 mL 0.1 mol/L Tris−HCl (pH 7.6) twice and stored at −20 °C for measurement of ACC deaminase activity. The total protein of bacterial cells was measured using the Bradford method. The unit of ACC deaminase activity is the amount of 1 μmol α−ketobutyrate generated by the ACC deaminase catalyzing ACC per hour (μmol α−KA/h).

The ability of nitrogen fixation was determined using Ashby's nitrogen−free agar medium. After subculturing 5 times, if the isolates were still growing on this medium, they were regarded as having stable nitrogen fixing activity.

NBRIP agar medium was used to estimate the capacity of inorganic phosphate solubilization of isolates according to the halos around the colonies. The strains possessing phosphate solubilization were inoculated in NBRIP liquid medium and cultured at 30 °C for 4 d. The supernatant was collected by centrifugation at 8000× *g* for 10 min at 30 °C, and then the pH and phosphorus concentration were measured. The concentration of soluble phosphate was determined by the molybdenum blue method [28].

2.5. The Tolerance of the Isolates to Cu and Cd

The tolerance of the isolates was determined in LB agar plate contained increasing concentrations of Cu^{2+} (80 mg/L–1200 mg/L) or Cd^{2+} (10 mg/L–250 mg/L). The plates were incubated at 30 °C for several days and the strains growing on it were recorded.

2.6. Molecular Identification of the Isolates

Based on the plant growth−promoting capacity and heavy metal tolerance of these isolates, eight endophytic bacteria were selected for further experimentation. The 16S rDNA gene of the eight endophytic bacteria was amplified using universal primers 27F and 1492R. The detailed reaction system and procedure of PCR amplification are shown in Table S3. The PCR products were sequenced by Tsingke Biotechnology Co., Ltd. (Wuhan, China). ContigExpress was used for splicing the sequence, and the inconsistent parts on both ends were removed. The splicing sequences were submitted to NCBI database (Accession numbers: OP310027-OP310034, accessed on 31 August 2022). The sequences were blasted with the model strains at the database using EzBioCloud (https://www.ezbiocloud.net/, accessed on 31 August 2022) [29] and the 16S rDNA sequences of model strains with the highest homology were obtained according to the homology between the isolates and model strains. The phylogenetic tree was constructed by the maximum−likelihood method using Mega software (v11.0).

2.7. Cu Mobilization Assay

The ability of Cu mobilization of the above−mentioned eight strains was analyzed according to the method of Sheng et al. [25]. Briefly, the bacterial suspension of the tested strains was inoculated in 10 mL sterilized SMS medium containing 10 mg $Cu_2(OH)_2CO_3$ and a medium with an equal volume of sterilized water was used as the control. These cultures were incubated at 30 °C with a rotation of 180 r/min. The samples were collected at different times. After centrifugation at 10,000× *g* for 10 min at 4 °C, Cu^{2+} concentration and pH values in the supernatant were determined.

2.8. Impact of the Endophytic Bacteria on Heavy Metal Speciation and Available Phosphate in Soil

Soil used for the incubation experiment was collected from Yangxin county, Hubei province (29°48′40″ N, 115°25′53″ E), China. The basic physicochemical properties of the soil were as follows: pH 7.88, OM 19.7 g/kg, Cu 380.6 mg/kg, and Cd 1.16 mg/kg. The tested soil was air−dried, ground and screened with a 10−mesh sieve. Then, 40 g soil was

added an appropriate amount of deionized water (maintaining 40% of the field capacity) to activate the soil microorganisms and incubated at 28 °C for 7 d. Next, the bacterial suspension (about 2×10^8 CFU/mL) of the eight isolates was added to the soil with the volume of 6 mL, respectively. The addition of bacterial suspension was divided into two parts, of which 4 mL was added on the first day and 2 mL on the eleventh day during incubation, respectively, maintaining the soil water content within 60–70% of the field capacity. The same amount of sterile deionized water was supplemented as the control (CK). Three replicates were set for each treatment. The soil samples were collected after culturing at 28 °C for 20 d. Soil available Cu and Cd were extracted by 0.005 mol/L DTPA (pH 7.3 ± 0.2) with a soil/water ratio of 1/2. Heavy metal speciation in soil was using BCR sequential extraction procedure [30]. Cu content in solution was measured by Flame Atomic Absorption Spectrometry (FAAS), and Cd content in solution was measured using Graphite Furnace Atomic Absorption Spectrometry (GFAAS). Soil available phosphate was extracted by 0.05 mol/L $NaHCO_3$ (pH 8.5) shaking at 25 °C for 30 min, and determined by the molybdenum blue method.

3. Results

3.1. The Composition and Diversity of Endophytic Bacteria in Ricinus communis L.

As shown in Table 2., the number of OTUs in the castor samples collected from Yangxin was 1124 (YR), 1061 (YS) and 902 (YL), respectively, and the Chao1 index was 654.8, 678.2 and 443.6, respectively, which were higher than those in the samples collected from Tonglushan mining area; Simpson index was 0.09 (YR), 0.19 (YS) and 0.20 (YL), respectively, which were lower than values of samples collected from Tonglushan mining area. These results indicated the diversity and species richness of the endophytic bacterial community in castor collected from Gangxia were greater than that collected from Tonglushan mining area, in which those in root samples were higher than those in stem and leaf samples.

Table 2. Observed OTUs and Alpha diversity indices in *Ricinus communis* L.

Sources	Samples	Sequences	OTUs	Simpson	Chao1	Coverage (%)
Tonglushan castor	TR	36,116	783	0.14	361.0	99.9
	TS	45,662	505	0.23	264.7	99.8
	TL	48,579	110	0.71	71.9	99.8
Gangxia castor	YR	52,468	1124	0.09	654.8	99.6
	YS	55,584	1061	0.19	678.2	99.4
	YL	63,409	902	0.20	443.6	99.5

The NMDS analysis (Figure 1A) showed that there were significant differences in the composition community of endophytic bacteria among all sample groups (r = 0.58, p = 0.001). To further test the inter−group differences in the endophytic bacterial community structure of the same plant tissue from two regions, the ANOSIM analysis was conducted based on the Bray–Curtis distance (Table S4). The results showed that there were significant differences between TR and YR (r = 0.50, p = 0.034), and TS and YS (r = 0.42, p = 0.034), indicating that there were significant differences in the endophytic bacterial composition of plants growing in the two soils. Moreover, ANOSIM analysis of different parts of plants in the same area also revealed that there were significant differences in the structure of endophytic bacterial community between tissues.

As given in Figure 1B., there were five phyla with relative abundance ≥1% in each sample, at phylum level, which are *Proteobacteria*, *Bacteroidota*, *Actinobacteriota*, *Firmicutes* and *Acidobacteria*. Among them, *Proteobateria* was the most dominant phyla with the relative abundance ranging from 69.8% to 97.0%, and its relative abundance in tissues was ordered as leaf > stem > root. The relative abundance of *Proteobacteria* in TR, TS and TL (82.5%, 90.7% and 97.0%) was higher than that in YR, YS and YL (69.8%, 82.3% and 84.3%), respectively. The relative abundance of *Bacteroidota* between the two groups showed an opposite trend, and the relative abundance of TR and TS (3.26%, 1.14%) was significantly lower than that

of YR and YS (11.9%, 10.3%). The relative abundance of *Actinobacteriota* and *Acidobacteria* in TR and TL was lower than that in YR and YL, respectively, but there was no significant difference in the relative abundance of TS and YS samples.

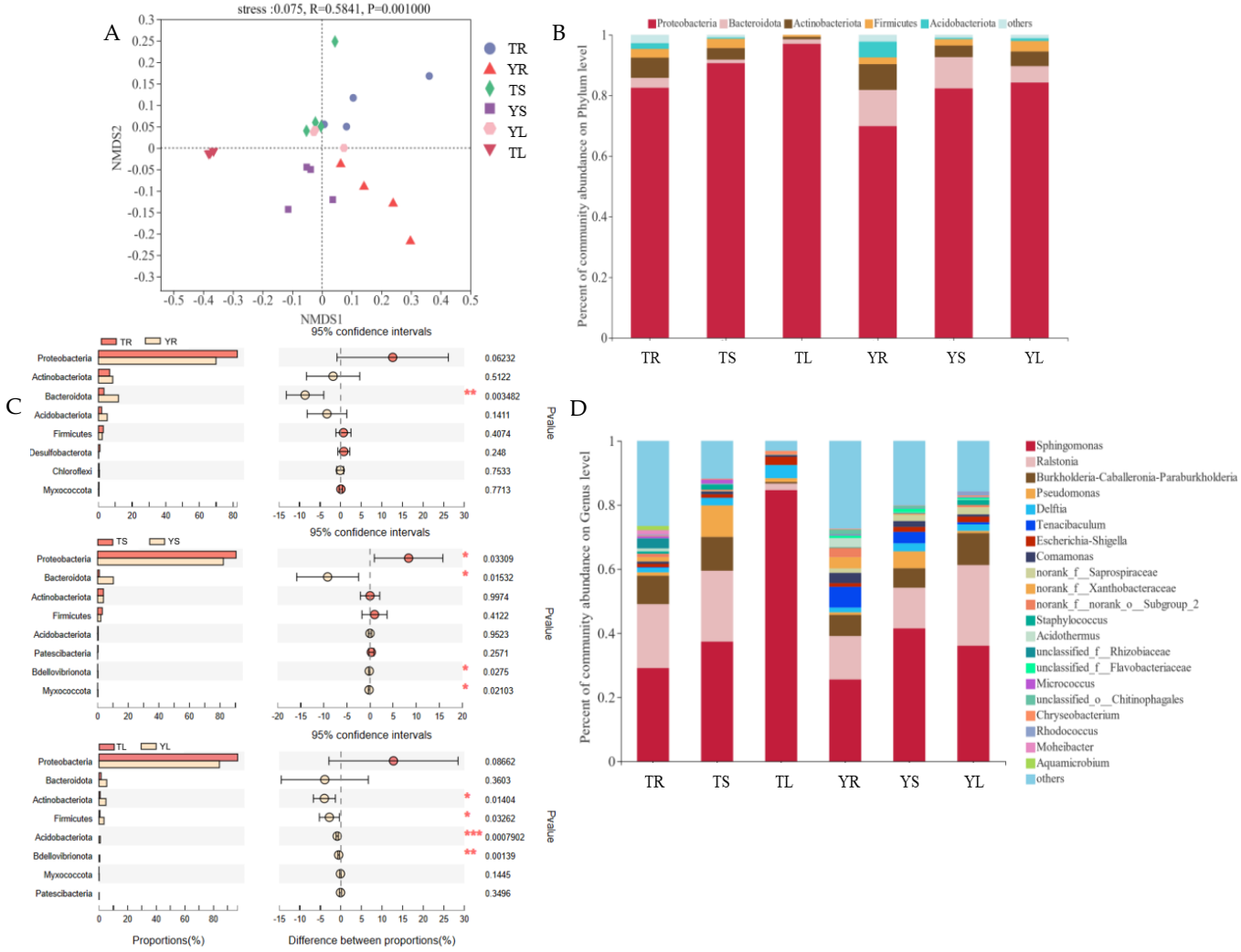

Figure 1. The composition of endophytic bacterial community in the castor. (**A**) The NMDS analysis of endophytic bacterial community; (**B**) Relative abundance of the endophytic bacterial community at phylum level; (**C**) Comparison of the relative abundance of endophytic bacteria between the same tissues at phylum level (* $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$); (**D**) Relative abundance of the endophytic bacterial community at genus level.

At genus level (Figure 1D), the bacterial genera in the six samples mainly included *Sphingomonas*, *Ralstonia*, *Burkholderia—Caballeronia—Paraburkholderia*, *Pseudomonas*, *Delftia* and *Escherichia—shigella*. Moreover, *unclassified_f_Rhizobiaceae* (3.1%), *Aquamicrobium* (1.3%), *Moheibacter* (1.3%) are the TR characteristic advantage genera; *Acidothermus* and *norank_f_Xanthobacteraceae* were found in TR (0.92%, 1.4%) and YR (2.8%, 3.5%). *Tenacibaculum*, *norank_f_Saprospiraceae*, *unclassified_f_Flavobacteriaceae* and *unclassified_o_ Chitinophagales* mainly existed in YR, YS and YL, and the relative abundance of *Tenacibaculum* decreased from underground parts to aboveground.

3.2. Isolation and Plant Growth—Promoting Characteristics of Endophytic Bacteria

A total of 44 endophytic bacterial strains were isolated from tissue of castor, including 22 strains from the root, 13 strains from the stem and 9 strains from the leaf. The growth—promoting traits and heavy metal tolerances of isolates are given in Table 3. It is

shown that all the isolates had the ability to produce IAA (4.0–140.1 mg/L), among which 18 strains produced more than 30 mg/L IAA. A previous study showed that when the microorganisms with ACC deaminase activity is no lower than 20 nmol α−KA/(mg·h), it is sufficient for the promotion of plant growth [27]. In this study, there were 30 strains of endophytic bacteria which could generate the ACC deaminase via using ACC, and the ACC deaminase activity of those strains was 11.6–233.9 μmol α−KA/(mg·h). There were 42 isolates that could grow normally in Ashby's nitrogen−free plate after subculturing 5 times, suggesting that these strains had potential nitrogen fixation ability. Moreover, there were 18 endophytic bacteria that could dissolve insoluble tricalcium phosphate, among which 10 isolated strains could increase soluble phosphate content in culture medium over 300 mg/L. The maximum content of soluble phosphate in NBRIP liquid medium inoculated strain TR16 could reach 495.5 mg/L. Furthermore, the tolerance of the isolates to Cu and Cd indicated that most of the endophytic isolates were resistant to both metals. The Cu^{2+} tolerance concentration of 17 isolates were up to 500 mg/L, of which strain TR16 could tolerate Cu^{2+} with the highest concentration of 1100 mg/L. The Cd^{2+} concentration tolerance of these strains ranged from 10 mg/L to 200 mg/L.

Table 3. Plant growth−promoting traits and heavy metal tolerance for isolated endophytic bacteria strains.

Strain	PGP Traits						Heavy Metal Tolerance	
				Phosphate Solubilization				
	IAA (mg/L)	Siderophores Production ($(\lambda_0 - \lambda)/\lambda_0$)	ACCD μmol α− KA/(h·mg)	Soluble P Concentration (mg/L)	pH in Supernatant	Nitrogen Fixation	Cu^{2+} (mg/L)	Cd^{2+} (mg/L)
TR1	51.8 ± 1.5	0.159	–	–	–	+	120	10
TR2	49.4 ± 0.2	0.264	73.7 ± 2.9	–	–	+	600	25
TR3	119.1 ± 15.4	0.245	138.1 ± 42.2	141.5 ± 8.0	5.0 ± 0.1	+	600	150
TR4	93.4 ± 19.4	0.232	107.4 ± 43.8	314.7 ± 71.2	4.7 ± 0.4	+	600	150
TR5	46.6 ± 12.3	0.141	38.6 ± 1.8	492.9 ± 14.4	4.3 ± 0.1	+	500	10
TR6	140.1 ± 9.5	0.098	27.6 ± 2.5	–	–	+	400	50
TR7	4.0 ± 0.0	0.161	–	416.2 ± 10.0	4.6 ± 0.1	+	500	10
TR8	63.5 ± 7.8	0.109	71.4 ± 7.6	190.7 ± 5.1	4.9 ± 0.0	+	600	100
TR10	73.1 ± 11.9	0.215	–	479.4 ± 26.2	4.5 ± 0.0	+	600	100
TR11	43.4 ± 0.1	–	35.9 ± 0.1	483.1 ± 23.5	4.4 ± 0.1	+	600	25
TR12	7.1 ± 0.0	0.289	109.6 ± 41.7	115.5 ± 29.0	5.0 ± 0.1	+	600	150
TR13	40.0 ± 1.2	0.130	–	–	–	+	500	25
TR14	32.6 ± 1.3	0.223	22.6 ± 2.5	–	–	+	300	10
TR15	13.0 ± 1.0	0.309	143.8 ± 6.1	130.1 ± 5.0	5.1 ± 0.0	+	600	150
TR16	6.6 ± 1.3	0.358	22.0 ± 2.0	495.5 ± 10.3	4.5 ± 0.0	+	1100	100
TR18	25.7 ± 1.6	0.291	47.4 ± 1.4	127.4 ± 7.3	5.0 ± 0.0	+	600	150
TR20	29.6 ± 2.6	0.241	37.9 ± 1.6	–	–	+	500	10
TR21	20.9 ± 5.2	–	233.9 ± 30.6	87.2 ± 4.1	5.0 ± 0.0	+	500	100
TR23	53.5 ± 9.0	0.339	50.2 ± 2.8	450.6 ± 7.4	4.4 ± 0.0	+	500	10
TS1	36.4 ± 1.4	0.236	23.8 ± 1.3	–	–	+	120	–
TS2	10.8 ± 0.7	0.092	–	–	–	+	120	25
TS8	11.9 ± 0.1	0.582	28.7 ± 0.4	–	–	+	40	25
TL1	6.8 ± 0.0	0.111	33.8 ± 0.2	–	–	+	120	10
TL2	5.6 ± 0.0	0.282	–	–	–	–	120	50
TR24	13.4 ± 0.5	0.250	–	467.9 ± 40.9	4.5 ± 0.1	+	120	50
YR1	50.3 ± 6.8	0.302	18.6 ± 0.4	–	–	+	120	–
YR2	28.5 ± 6.6	0.320	43.9 ± 3.6	–	–	+	600	10
YL1	8.1 ± 0.0	0.268	–	445.3 ± 2.1	4.5 ± 0.1	+	120	50
YL2	6.3 ± 0.0	0.241	–	85.6 ± 30.9	5.1 ± 0.2	+	80	50
YL3	4.9 ± 0.4	0.220	20.1 ± 3	–	–	+	120	–
YL4	38.3 ± 2.1	–	22.5 ± 0.4	–	–	+	120	10
YL5	22.0 ± 1.2	–	–	–	–	+	120	–
YL6	13.8 ± 0.0	0.130	–	–	–	+	120	50
YL7	6.2 ± 0.0	–	29.2 ± 0.0	–	–	+	120	25
YS1	33.1 ± 1.9	0.344	–	183.3 ± 12.2	4.9 ± 0.0	+	40	200
YS2	54.0 ± 3.1	0.347	19.3 ± 2.2	–	–	+	120	10
YS3	124.3 ± 3.4	0.306	21.5 ± 4.7	–	–	+	500	200
YS4	26.9 ± 6.4	0.344	41.5 ± 0.4	–	–	+	200	10
YS5	10.9 ±0.0	0.232	–	469.8 ± 9.2	4.7 ± 0.0	+	120	25
YS6	4.0 ± 0.0	–	–	–	–	–	120	25
YS7	15.3 ± 6.0	0.327	32.9 ± 2.9	–	–	+	120	–
YS8	18.1 ± 1.2	0.232	11.6 ± 3.7	–	–	+	300	10
YS9	9.0 ± 0.0	0.261	18.8 ± 0.5	–	–	+	120	25
YS10	42.3 ± 1.4	0.300	32.5 ± 11.1	–	–	+	120	10

"$(\lambda 0 - \lambda)/\lambda 0$)" represents the ability to produce siderophores, and the higher the value is, the higher the capacity for siderophore production will be. "ACCD" and "α− KA" mean ACC deaminase and α−ketobutyrate, respectively. "−" means the strain does not possess the corresponding growth−promoting ability. "+" indicates the strain has the ability to perform nitrogen fixation.

3.3. Characterization of Endophytic Bacterial Isolates

Eight strains with different growth−promoting characteristics selected from the above 44 isolates were identified using 16S rDNA sequencing, and their taxonomy was determined by sequence Blast analysis and phylogenetic tree construction. According to the results of sequence Blast (Table 4) and phylogenetic tree (Figure S1), strain TR4, TR18 and TR21 located in the same branch and belong to *Enterobacter*. Strain TR4 and TR18 were closely related to the model strain *Enterobacter kobei DSM* 13645^T, while TR21 was closely related to *Enterobacter huaxiensis* EB−24^T. The top−hit strain of TR8 was *Buttiauxella agrestis* ATCC 51602^T and TR8 was identified as *Buttiauxella*. Strain TR16 was characterized as *Acinetobacter*, which had the highest homology with the model strain *Acinetobacter pittii* CIP 70.29^T and was much closer on the phylogenetic tree. Strain YL1 had the highest similarity of 99.2% with *Bacillus altitudinis* 41KF2b^T and was assigned to *Bacillus* genera. Strain YS3 and *Proteus myxofaciens* ATCC 19692^T were clustered into an independent branch on the phylogenetic tree and YS3 was identified as *Proteus*. Strain YS5 had the closest genetic distance with the model strain *Metabacillus idriensis* SMC 4352−2^T and was characterized as *Metabacillus*.

Table 4. The 16S rDNA gene sequence similarity analysis of the eight isolates.

Strains	Accession Number	Closest Relative Strain	Similarity (%)	Genus
TR4	OP310027	*Enterobacter kobei* (CP017181)	99.8	*Enterobacter*
TR8	OP310028	*Buttiauxella agrestis* (JMPI01000079)	99.3	*Buttiauxella*
TR16	OP310029	*Acinetobacter pittii* (APQP01000001)	99.0	*Acinetobacter*
TR18	OP310030	*Enterobacter kobei* (CP017181)	99.8	*Enterobacter*
TR21	OP310031	*Enterobacter huaxiensis* (FYBI01000003)	99.3	*Enterobacter*
YL1	OP310032	*Bacillus altitudinis* (ASJC01000029)	99.2	*Bacillus*
YS3	OP310033	*Proteus myxofaciens* (LXEN01000172)	98.5	*Proteus*
YS5	OP310034	*Metabacillus idriensis* (AY904033)	99.8	*Metabacillus*

3.4. The Mobilization of Cu by Endophytic Bacteria

At the initial cultivation (12 h and 24 h), Cu^{2+} concentration in the supernatant of inoculated treatments decreased to varying degrees (9.8–53.7%) compared with that of in the uninoculated treatment (Figure 2A), which could be attributed to the adsorption capacity of these strain for heavy metals (Sheng et al., 2008). In the eight treatments, inoculated endophytic bacteria, strains TR21, TR8, YL1, YS3 and YS5, showed obvious mobilization ability with $Cu_2(OH)_2CO_3$ (Figure 2A). After 144 h of cultivation, the concentration of Cu^{2+} in the supernatant of these five treatments reached 84.1 mg/L, 84.3 mg/L, 100.4 mg/L, 108.2 mg/L and 98.9 mg/L, respectively. Strain TR16 did not possess the ability to mobilize $Cu_2(OH)_2CO_3$ in medium. With the increase in culture time, the pH value of the supernatant decreased, but was still higher than that of the CK. In comparison to CK treatment (42.7 mg/L), the Cu^{2+} concentration in strain TR16 treatment was 16.7 mg/L, which decreased by 60.9%. Furthermore, the pH value of TR4 treated supernatant gradually decreased and then stabilized with the culture time, while the concentration of Cu^{2+} in supernatant was lower than that in the control (CK1).

3.5. The Inoculation of Endophytic Bacteria on DTPA−Cu/Cd and Speciation in Soil

The effect of endophytic bacteria inoculation on DTPA extractable Cu and Cd in soil are shown in Figure 3. The inoculation of the eight endophytic bacteria could affect the bioavailability of soil Cu and Cd to varying degrees. Compared with the CK, strain YS3 treatment significantly improved the bioavailability of soil Cu and Cd most greatly, in which the content of DTPA extractable Cu and Cd increased by 8.4% and 6.9%, respectively. In addition to the treatment of strain TR4 and TR16, other inoculation strains also increased the content of DTPA−Cu in soil to some extent, but they had no significant effect on the content of DTPA−Cd in soil.

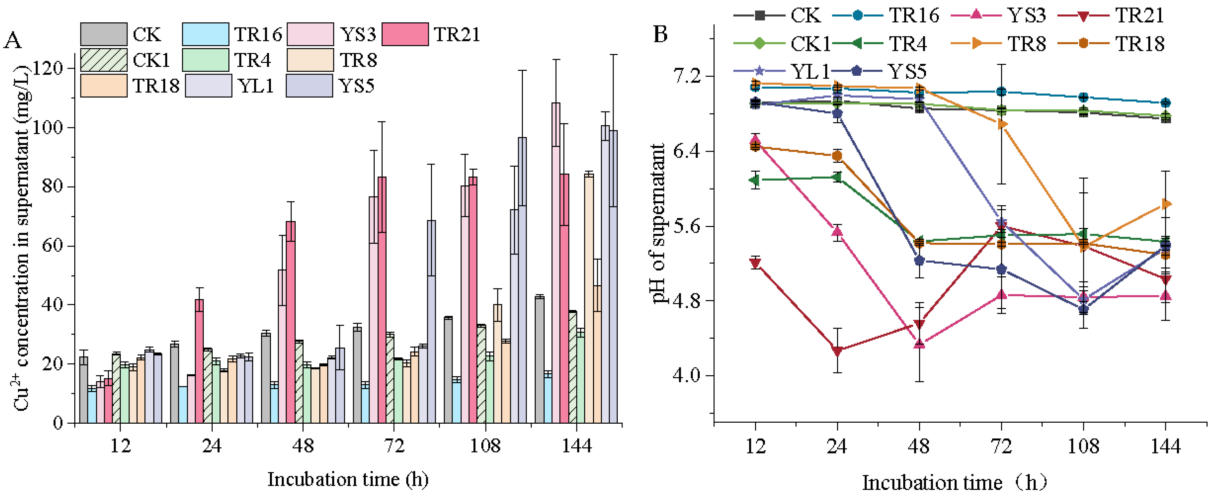

Figure 2. Cu mobilization and variances of pH values in supernatant. (**A**) Cu^{2+} concentration in supernatant; (**B**) pH of supernatant.

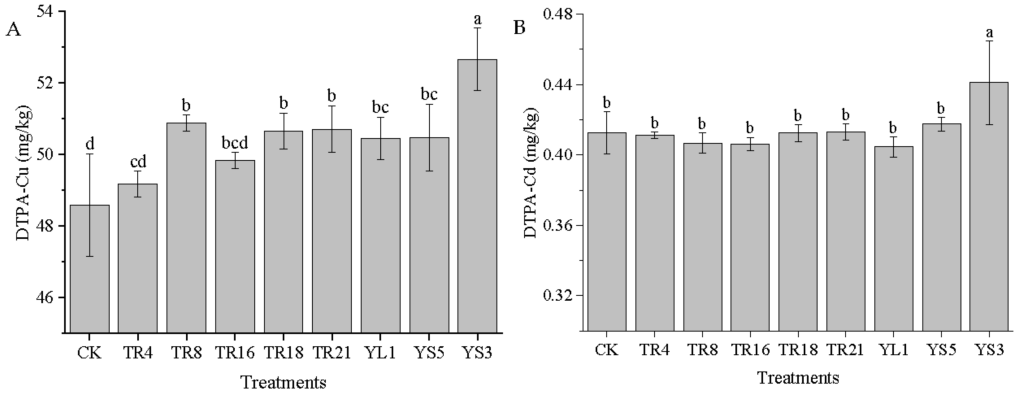

Figure 3. DTPA extractable Cu (**A**) and Cd (**B**) in soil. The different lowercase letters in the figures indicate significant differences ($p < 0.05$).

As given in Figure 4, Cu in soil mainly existed in the fraction of the reducible and the residual, while Cd mainly existed in the fraction of the acid extractable, the reducible and the residual. For Cu (Figure 4A), except for inoculation of strain YS5, other inoculation treatments had no significant effect on acid extractable Cu content. Compared with the control, YS5 treatment significantly increased acid extractable Cu and reducible Cu content in soil by 4.4% and 4.1%, respectively, but oxidable Cu content did not change significantly, indicating that inoculation of YS5 strain was conducive to the transformation of soil residual Cu to acid extractable Cu and reducible Cu, thus improving the bioavailability of soil Cu. In addition, strain YL1 and YS3 treatments also significantly increased the content of reducible Cu in soil.

For Cd (Figure 4B), in the treatments of strain TR8, TR16, YR8, TR21, YL1 and YS3, the content of acid extractable Cd, reducible Cd and oxidable Cd in soil increased by 12.5–58.1%, 7.4–33.0% and 102.7–141.1%, respectively, while the content of residual Cd decreased by 27.0–57.9%. The content of extractable Cd, oxidizable Cd and residual Cd in soil treated with strain TR4 had no significant change, but the content of reducible Cd significantly increased by 22.8%, while the content of residual Cd significantly decreased by 13.0%. YS5 treatment significantly increased the content of acid extractable Cd (23.3%) and oxidizable Cd (120%) in soil, and significantly decreased residual Cd content (21.7%), but had no significant effect on reducible Cd content. Therefore, the treatment of these eight endophytic bacteria strains was conducive to the transformation of soil heavy metal Cd from more stable form to unstable forms, such as acid extractable, reducible or oxidizable forms.

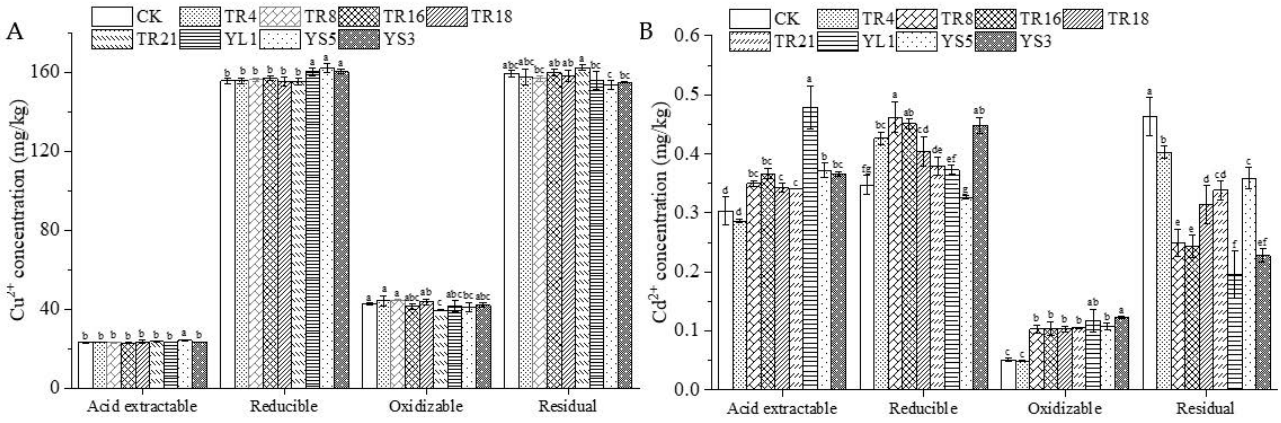

Figure 4. The chemical speciation of Cu (**A**) and Cd (**B**) in soil. The different lowercase letters in the figures indicate significant differences ($p < 0.05$).

3.6. The Inoculation of Endophytic Bacteria on Soil Available Phosphate

The effects of endophytic bacteria inoculation on soil available phosphate are shown in Figure 5. Compared with the no−inoculation treatment, the inoculation of eight endophytic bacteria increased the soil available phosphorus content to a certain degree. Except for the YL1 treatment, the soil available phosphorus content in soil significantly increased in the other seven treatments. Compared with the 12.8 mg/kg available phosphate content of the CK, the content of soil available phosphate increased by 11.4%, 19.3%, 16.6%, 13.0%, 29.2%, 15.7% and 10.8% in the treatments with TR4, TR8, TR16, YR8, TR21, YS5 and YS3, respectively. Among them, TR21 treatment had the best effect on increasing the content of soil available phosphate, reaching 16.5 mg/kg.

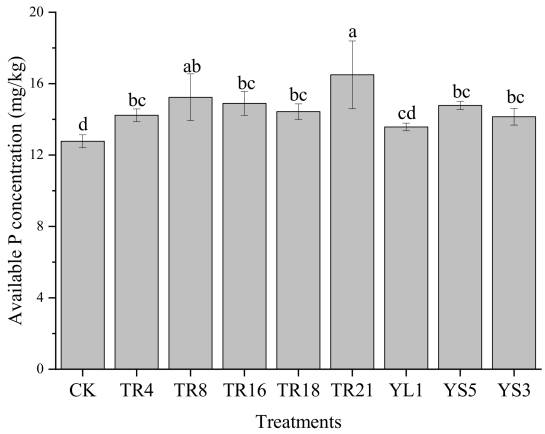

Figure 5. Available P concentration in soil with different inoculation of endophytic bacteria. The different lowercase letters in the figure indicate significant differences ($p < 0.05$).

4. Discussion

4.1. Differences in Diversity of Endophytic Bacterial Community of Castor Grown on Two Sites

Soil is one of the main sources of plant endophytic bacteria. Due to different physical and chemical properties, the microbial community composition in various soils is also different, which in turn affects the community structure of plant endophytic bacteria [1,31,32]. Metabolites secreted by different plant roots can recruit specific microorganisms and induce them to colonize on the root system, then part of these microorganisms can enter the plant tissue by invading the root system [2,33,34]. The plant periderm zone is an important selection barrier to control the composition of plant endophytic bacterial communities [35]. Wang et al. [9] reported that soil type and Cd level would affect the composition of the endophytic bacterial community in *Sedum alfredii Hance*. In the present study, Illumina

Hiseq high−throughput sequencing was used to study the community structure of endophytic bacteria in various tissues (roots, stems and leaves) of the castor grown in two soils (mine soil and farmland soil). It was found that the diversity and species richness of endophytic bacteria in various tissues of plants growing in farmland (Gangxia village, Yangxin county) were greater than those in mine (Tonglushan, Daye city) soil. These should be attributed to the different land use patterns, physical and chemical properties, heavy metal pollution levels and distribution of the two sampling sites [36]. The sampling site of Tonglushan mining area is a long−term abandoned mine site polluted heavily by heavy metals and has low vegetation cover with a harsh environment. The sampling site of Gangxia village belongs to farmland. Although the site is also contaminated by various elements to a certain extent, long−term crop planting and agronomic practices can provide a better living environment for the growth and reproduction of soil microorganisms [37,38]. The concentration of heavy metals in soil plays a critical role in regulating the structure of the plant endophyte community. High concentrations of heavy metals lead to a decline in the diversity and species richness of soil microbial community structures, thus affecting the composition of endophyte communities in plants [39]. Deng et al. [32] also showed that the bacterial community diversity and species richness in rhizosphere soil of *Elsholtzia haizhouensis* growing in Tonglushan mining area were lower than those growing in Macheng city, of which soil pH, nitrogen sources and heavy metals were the important factors affecting the composition of microbial communities.

Through Illumina Hiseq high−throughput sequencing, it was found that the main phyla of endophytic bacteria were *Proteobacteria*, *Bacteroidota*, *Actinobacteriota*, *Firmicutes* and *Acidobacteria* in the tissue of the castor grown on the two sites. Notably, the relative abundance of each phylum was different to varying degrees, of which the relative abundance of *Bacteroidetes* and *Actinobacteria* in the samples of two sites represented a diametrically opposite trend (Figure 1B,C). The results of ANOSIM analysis based on Bray Curtis distance (Table S4) also proved that there were significant differences in the composition of endophytic bacterial community in the castor grown on the two regions. Lv et al. [40] studied the composition of root endophytic bacterial community of five pea varieties grown in the same soil and found that the root endophytic bacteria of these five pea varieties had similar community compositions. Compared with the seed endophytic bacterial community, the root endophytic bacteria community composition was much closer to the soil bacterial community composition. Durand et al. [34] found that there was no difference in the community structure of endophytic bacteria in the seeds of two species of *Noccaea caerulescens* collected from two different regions. However, pot experiments indicated that the composition of endophytic bacteria communities in the roots, stems and leaves of *Noccaea caerulescens* grown in two soils was similar at the phylum level, but the relative abundance was significantly different, which is consistent with our present result. Moreover, the samples from the two places also had different specific dominant genera at genera level (Figure 1D). Consequently, it is suggested that the endophytic bacterial community composition of the same plant is affected obviously by the soil environment in which the plant is growing, which means the soil conditions are one of the driving factors to shape the endophytic bacterial community in plants.

4.2. Cu Mobilization of Eight Endophytic Bacteria Isolates in Medium

Recently, plant growth−promoting endophytic bacteria have attracted widespread attention in strengthening plant remediation of contaminated soil [41–43]. A previous study reported that the castor grown in Tonglushan mining area could grow normally on soil with severe Cu pollution, and accumulated more Cu in the tissues with strong tolerance to Cu [12]. In this study, 44 strains of endophytic bacteria were isolated from the tissues of the castor. Basically, most of the strains possessed three or more kinds of growth−promoting properties, and had different degrees of tolerance to Cu and Cd. These results suggest that the castor is a high−quality material source for screening endophytic bacteria with heavy metal resistance, and its endophytic bacteria contained a variety of

good characteristics with great development potential. Therefore, it is of great significance to isolate endophytic bacteria with growth promoting properties from the castor growing in various heavy metal−contaminated areas to find new functional microbial resources that can be used for phytoremediation.

The bioavailability of heavy metal is one of the important factors affecting the extraction efficiency of plants [44], in which carbonate bound heavy metals in the form of precipitation or coprecipitation are very sensitive to any change in soil pH [45]. Hence, the present study investigated the mobilization capacity of eight endophytic bacteria to insoluble Cu by using $Cu_2(OH)_2CO_3$ as the experimental material. The results demonstrated that in addition to strains TR4 and TR16, the strains TR8, TR18, TR21, YL1, YS3 and YS5 had strong mobilization capacity to Cu_2 $(OH)_2CO_3$ (Figure 2), and their capacity is obviously related to the change in pH in the culture solution, which should be attributed to the low molecular weight organic acids produced by the strain during the culturing process. Yang et al. [46] compared the total amount of organic acids secreted by two strains with different mobility for $CdCO_3$; the results showed that the total amount of organic acids secreted by *Pseudomonas fluorescens* GI with better Cd mobilization was much higher than that of *Bacillus cereus* HL, which was the reason why the two strains had significantly different Cd solubilization capacities. They further calculated the amount of $CdCO_3$ that can be solubilized by the concentration of secreted organic acid by using the linear fitting curve of low molecular organic acid to $CdCO_3$ mobility, and found that the fitting amount of dissolved Cd was basically equivalent to the actual amount of Cd solubilized by the strains. Li et al. [47] revealed that the addition of K^+ could significantly enhance the solubilization of *Agrobacterium sp*. to $CdCO_3$ by inducing the secretion of tartaric acid, 3−hydroxybutyric acid, fumaric acid and succinic acid, and the pH of the culture solution was also reduced to varying degrees due to the addition of K^+. Moreover, microorganisms also can release protons through the assimilation of NH_4^+, thus reducing the pH of the medium. Furthermore, although the Cu^{2+} concentration in the supernatant of the medium inoculated strain TR4 was still lower than that of the non−treated medium after 6 days of incubation, and the pH value decreased sharply, which might be related to the adsorption capacity of the strain itself for heavy metal ions.

4.3. Effect of the Eight Endophytic Isolates on Soil Cu and Cd Speciation

According to soil incubation experiment, it was further found that these eight endophytic bacterial strains had different influence on the availability of heavy metals in soil, of which YS3 had the best effect on increasing the content of DTPA−Cu and DTPA−Cd in soil. In the Cu mobilization experiment, strain YS3 also had a strong solubilizing ability on $Cu_2(OH)_2CO_3$. Moreover, the eight strain treatments also increased the content of available phosphate in soil. It is well known that soil pH value is the principal factor that affects the activity and fraction change of heavy metal in soil, and the metabolites secreted by plant endophytic growth−promoting bacteria, such as low molecular organic acids and siderophores, can reduce soil pH and activate soil heavy metals [25,48,49]. The eight isolates used in the present study had the growth promoting traits of producing IAA, siderophores or solubilizing phosphate. Wu et al. [50] showed that the inoculation of *Serratia Marcescens* with plant growth−promoting properties mobilized soil Cd, which was one of the direct reasons for the improvement in plant extraction efficiency, and it also increased the bioavailability of phosphate in soil. Liu et al. [51] revealed that inoculated endophytic bacteria Bacillus cereus reduced soil pH, increased soil extractable Cd and available P. Except for TR8 and TR16, the other treatments reduced soil pH value to varying degrees compared with uninoculated soil (Figure S2), which may be a reason for the increased availability of heavy metals in soil in this study. Furthermore, according to the analysis of the chemical speciation of soil heavy metal, the inoculation of endophytic bacteria could promote the transformation of soil heavy metals Cu or Cd from a stable residue form to a relatively unstable acid extractable form, reducible form and oxidizable form. The bioavailability of heavy metals in soil is closely related to their chemical speciation in soil.

The inoculation of microorganisms affects the composition of chemical forms of heavy metals in soil. Wang et al. [52] reported that the inoculation of two endophytic bacteria could promote the transformation of soil residual heavy metals to other three forms (acid extractable, reducible and oxidable), in which the content of soil oxidable V, Cr and acid extractable Cd was significantly increased.

5. Conclusions

A total of 44 strains of endophytic bacteria with great growth−promoting characteristics were isolated from the tissue of castor plants and most of the isolates were tolerant to various concentration of Cu^{2+} and Cd^{2+}. Eight endophytic bacteria with different growth−promoting properties were selected from the isolated strains for molecular identification. Among them, six strains were found to be able to solubilize $Cu_2(OH)_2CO_3$ in the medium, in which strains TR21, TR8, YL1, YS3 and YS5 could increase the concentration of Cu^{2+} in the supernatant, reaching 84.1 mg/L, 84.3 mg/L, 100.4 mg/L, 108.2 mg/L and 98.9 mg/L, respectively. Inoculating the soil contaminated by heavy metals with these eight endophytic bacteria to could not only improve the bioavailability of Cu and Cd, and change their chemical speciation, but also play a role in phosphate solubilization and increase the available phosphate content in the soil.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020333/s1, Table S1. The primers, reaction system and procedure of PCR amplification used in Illumina high−throughput sequencing; Table S2: Media and their formulas used in this study; Table S3. The primers, reaction system and procedure of PCR amplification used in molecular identification of the isolates; Table S4: Analysis of similarities endophytic microbial community in castor based on Bray–Curtis distance; Figure S1: Phylogenetic tree based on 16S rDNA sequence obtained by the Maximum likelihood (ML) method with 1000 replicates; Figure S2: Effect of inoculated endophytic bacteria on soil pH value.

Author Contributions: Conceptualization, Q.L. and H.H. (Hongqing Hu); validation, Q.L.; formal analysis, Q.L.; investigation, Q.L. and Y.S.; resources, H.H. (Huan He); data curation, Q.L.; writing—original draft preparation, Q.L.; writing—review and editing, H.H. (Hongqing Hu) and Q.F.; visualization, Q.L., H.H. (Hongqing Hu), Q.F. and J.Z.; supervision, H.H. (Hongqing Hu), Q.F. and J.Z.; project administration, Q.L. and H.H. (Hongqing Hu); funding acquisition, H.H. (Hongqing Hu). All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Joint Key Funds of the National Natural Science Foundation of China (U21A20237) and Chinese Postdoctoral Science Foundation (2021M691165).

Data Availability Statement: Data available from the author.

Conflicts of Interest: The authors declare no conflict of interest.

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