Insights into the effects of cadmium stress on endophytic bacterial community in the hyperaccumulating plant ryegrass

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ABSTRACT

We investigated the endophytic bacterial community of ryegrass under Cd stress. Results showed ryegrass could tolerate relatively high concentrations of Cd. The fresh weight and shoot length were not significantly affected, while physicochemical parameters, including total nitrogen (TN), total phosphorus (TP), soluble protein (SP), Ca, and Mg, were changed. Exposure to Cd disturbed the composition of endophytes at both phylum and genus levels. Pseudomonas accounted for relatively high proportion, was increased under Cd stress. Additionally, correlation analysis identified that physicochemical parameters, such as TP, TN, Ca and Mg, significantly affected the abundance and composition of endophytic bacterial genera, suggesting these parameters had potential roles in regulation of endophytic microbial communities. In summary, our study investigated the endophytic bacterial community of ryegrass, and characterized its response to Cd stress. These results may shed light on the process of improving the remediation of Cd by modulating endophyte community.

1. Introduction

Cadmium (Cd) is one of the most hazardous heavy metals and can cause multiple deleterious effects on both aquatic and terrestrial organisms [1–4]. Cd has been largely released and dispersed into multiple environmental media by mining, smelting, and the excessive use of pesticides and synthetic fertilizers. In fact, Cd is the most frequently detected heavy metal in soil, and approximately 7% of the sites investigated were found to be contaminated by Cd in a nationwide research survey in China [5]. The concentrations of Cd ranged between <0.1 and 184 mg/kg in areas investigated in several countries, including China, Pakistan, United States and 26 European countries [6–10]. Besides urban areas and mining zones, Cd has also been detected in cities. Cd contamination has been a worldwide environmental challenge for a long time, and the remediation of sites contaminated with Cd is an urgent issue that matters to both the human health and the safety of global ecology.

In addition to chemical or traditional physical strategies for remediation, phytoremediation is another promising way to remove heavy metals from soil, with advantages such as inexpensive and eco-friendly [11,12]. Previous research suggested that ryegrass (Lolium perenne L.) could be a potential species for bio remediation of soils polluted with Cd [13–15], which could accumulate Cd in both roots and its aboveground tissues. The germination of ryegrass seed was unaffected by Cd even at 1.25 mM, but it was totally inhibited at 6.25 mM [16]. Despite the fact that ryegrass can tolerate Cd at relatively high concentrations, some physicochemical parameters changed under Cd stress, including the shoot length, fresh weight, the contents of chlorophyll and soluble proteins, antioxidative enzymes and many relative functional genes responsible for the cellular metabolism or transport of Cd [14,17–19]. The physiological response of ryegrass to Cd stress not only indicates the toxic effects of Cd on cellular metabolism but may also help to decipher the molecular mechanisms for the ability of ryegrass to tolerate and accumulate high concentrations of Cd. Besides the physiological response to Cd, the microorganisms that live in ryegrass may also play key roles in its response to Cd stress.

Endophytes are microorganisms that inhabit the tissue within plants. The bacteria that inhabit this niche are sensitive to the growth conditions of the plant and can also affect its development. Researchers have isolated many endophytic microbes from ryegrass that can promote plant growth or improve the ability to remove pollutants [20,21]. Many studies have suggested that the endophytic bacteria can directly affect plant growth [22,23]. Thus, it is essential to recognize the structure of endophytic bacterial communities and understand how these members respond to stress. To our knowledge, no relative research has focused on the response of endophytic bacteria in ryegrass to Cd stress. Therefore, in this
study, we utilized pot experiments to explore the effects of Cd exposure on the endophytic bacterial community in ryegrass and to characterize the correlation between the bacterial community and other main elements, such as Mg, Ca, total phosphorous (TP), total nitrogen (TN), soluble sugar (SS), soluble protein (SP) and Cd, in leaves. It was expected that the results would help to better understand the Cd-tolerant mechanisms of ryegrass and shed light on the process of improving the efficiency of ryegrass in the remediation of Cd by modulating the endophytic community.

2. Materials and methods

2.1 Pot experiment

The pot experiment was performed under controlled conditions, with 65% to 75% relative air humidity and daily temperatures of 15°C to 32°C, at Agricultural Water and Soil Environment Scientific Field Observation Station of Chinese Academy of Agricultural Sciences, located in Xinxiang, Henan Province, China. Each plastic pot contained 7.5 kg of soil that had been air-dried and sieved with 2 mm mesh. The soil properties were shown in Table S1. Soil was spiked with Cd$^{2+}$ ($\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$) at concentrations of 5 mg/kg (CS) and 50 mg/kg (C50), equilibrated for one month before use. Ryegrass seeds were first germinated in nursery substrates that are composed of grass peat, perlite and vermiculite and then transplanted into the pots containing different concentrations of Cd with three replicates. Each pot contained 10 plants, and the plants were totally grown for 75 days under normal irrigation conditions.

2.2 Sampling and analysis of physicochemical parameters

The ryegrass was harvested after 75 days. The shoot length and fresh weight were measured manually. Aboveground parts were then used to determine the following physicochemical parameters.

(1) Determine the contents of SS and SP

The fresh leaves of ryegrass were cut into small pieces and used to test the contents of SS and SP. The SS was measured using a Microplate Soluble Sugar Content Assay Kit (Solarbio, Beijing, China), according to the manufacturer’s instructions. Briefly, 0.2 g samples were homogenized in 1 ml of distilled water, incubated at 100°C for 10 min, and then centrifuged at 8000 g for 10 min. The absorbance of the supernatant was measured at 620 nm. Glucose solutions (0.01–0.3 mg/mL) were used to generate the standard curve. The SP was measured with Coomassie brilliant blue G-250 assay. Briefly, 0.2 g samples were homogenized in 5 ml of distilled water and centrifuged at 8000 g for 10 min, and the content of SP was measured by reading the absorbance of the supernatant at 595 nm. The standard curve was prepared with bovine serum albumin solutions (0.02–0.1 mg/mL).

(2) Determine the contents of TN and TP

For TN and TP, the aboveground parts of ryegrass were dried at 110°C for 10 min and then at 70°C to constant weight, 0.3 g of dried ground material was digested in H$_2$SO$_4$ and H$_2$O$_2$ (30%) for 30 min, and TN and TP contents were determined using a continuous flow analytical system (AA3, Germany).

(3) Determine the contents of Ca, Mg and Cd

For Mg, Ca and Cd, 0.2 g dried samples were digested by an acid mixture (HNO$_3$·HClO$_4$, 3:1) and analyzed using atomic absorption spectrophotometry (Shimadzu AA-6300, Japan). The bioavailable fraction of Cd was determined with the diethylenetriaminepentaacetic acid (DTPA) method [24].

2.3 Isolation of total DNA from the endophytic microorganisms

The samples for isolation of genomic DNA from the endophytic bacteria were prepared as previously described [25,26]. Briefly, samples were flushed with tap water to remove attached debris and then thoroughly rinsed with distilled water, followed by surface sterilization with 75% ethanol for 3 min, a solution of sodium hypochlorite with 3% of available chlorine for 5 min, and a final rinse with sterile distilled water. The surface-disinfected samples were weighed and then ground using liquid nitrogen. The total DNA was isolated with FastDNA® Spin Kit (MP Biomedicals, USA), the concentration and quality of DNA were determined using a QuickDrop Spectrophotometer (Molecular Devices, USA) and analyzed by agarose gel electrophoresis. The ratios of absorbance at 260 nm to 280 nm of extracted DNA were between 1.8 and 2.0.

2.4 High throughput sequencing

Total genome DNA was isolated as described above. Library construction and sequencing were conducted at Shanghai Majorbio Bio-Pharm Technology Co., Ltd., using the Illumina MiSeq PE300 platform that generated 400 bp paired-end reads (Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China). As described previously [25], two sets of primers that target the V5-V7 region of 16S rDNA gene were used to eliminate the contamination by mitochondrial and chloroplast DNA. PCR was performed with the TransStart FastPfu DNA Polymerase system (TransGen Biotech AP221–02, China) and conducted on an ABI GeneAmp® 9700
PCR instrument. UPARSE (version 7.1 http://drive5.com/uparse/) was used to assign operational taxonomic units (OTUs) with a 97% similarity cutoff. The taxonomic affiliation of OTUs was assigned by the RDP classifier against the Silva database (http://www.arb-silva.de). Raw sequences were submitted to the SRA (Sequence Read Archive) database of NCBI with the accession numbers of SRR14572545 to SRR14572553.

2.5 Statistical analysis

Statistical significance analysis was performed using Student’s t-test (two-tail), assuming equal variance (p ≤ 0.05). Otherwise, the analysis was specified in the relevant text.

3. Results

3.1 Effects of exposure to Cd on plant growth and the physicochemical parameters

The bioavailable fraction concentrations of Cd in soil samples of C5 and C50 groups were 3.5 ± 0.2 mg/kg and 27.7 ± 1.1 mg/kg, respectively. As shown in (Table 1), Cd accumulated in the above-ground parts of ryegrass in a dose-dependent manner. The content of Cd was 8.4 ± 0.5 mg/kg (dry weight) and 17.5 ± 0.4 mg/kg (dry weight) in ryegrass exposed to Cd at 5 mg/kg and 50 mg/kg, respectively. Although ryegrass accumulated relatively high levels of Cd, there was no significant difference in the shoot length (SL), fresh weight (W) and content of soluble sugar (SS), suggesting that ryegrass had a relatively high tolerance to Cd stress. To further evaluate the effects of Cd stress on ryegrass growth, we determined the contents of SP, TN, TP, Mg and Ca. As shown in (Table 1), exposure to Cd significantly decreased the content of SP in ryegrass. Moreover, the contents of both TN and TP increased significantly in the group exposed to 50 mg/kg of Cd. TN, TP and SP are involved in many metabolic processes in plant cells and play vital roles in responding to environmental stress. Additionally, compared with the control group, the contents of mineral elements (Ca and Mg) in the leaves were also affected by exposure to Cd. In summary, these results suggested that ryegrass has a relatively high tolerance to Cd stress, though some physicochemical parameters were changed by Cd. These alternations not only displayed the toxic influence of Cd but also indicated the potential stress-resistant mechanisms of ryegrass.

3.2 The Endophytic bacterial abundance and diversity in ryegrass under Cd stress

To investigate the effects of Cd exposure on the endophytic community, 16S rDNA genes were sequenced using Illumina MiSeq. With totally generated 7829–24,131 sequences (Table S2), after subsampling the reads to equal depths, we obtained a total of 579 OTUs in all samples, with 343, 306, and 354 OTUs in Con, C5, and C50 groups, respectively (Figure 1(a)). The diversity and relative abundance of the endophytic bacterial community were evaluated by the indices of Shannon, Simpson, Chao and Ace. As shown in (Table 2), the richness indices of Ace and Chao were lower in the C5 samples compared with those in the control and C50 groups, but these differences were not statistically significant. The Shannon diversity index was higher in the C50 group than the other two groups, although not significantly, and consistently, the Simpson index was lower in C50 samples (not significantly), suggesting a higher microbial diversity.

A Venn diagram analysis showed that 145 OTUs were shared by these three groups and that 106, 77 and 117 OTUs were found exclusively in the Con, C5 and C50 groups, respectively (Figure 1(b)). The distinctive composition of OTUs in each group suggested that the endophyte of ryegrass was sensitive to Cd stress. Additionally, the 236 OTUs that only appear in the Cd exposure groups may have a high degree of resistance to Cd stress.

To further characterize the structure and composition of endophytic bacteria under Cd stress, we analyzed the relative bacterial abundance at different levels of taxonomic classification. As shown in (Figure 2(a)), Proteobacteria (52.1% – 64.9%), Actinobacteria (26.0% – 40.0%), Firmicutes (1.6% – 5.3%) and Bacteroidota (1.5% –4.8%) were the dominant phyla in all samples. At the genus level, Rhodococcus (19.4%-32.6%) and Candidatus Portieria (7.1%-23.3%) were the most two abundant groups in all the samples. Klebsiella (1.3% – 8.0%), Rickettsia (1.3%-6.2%), Pseudomonas (2.3%-5.2%) and Sphingomonas (2.6%-4.1%) also have high populations in these samples (Figure 2(b)). A statistical analysis showed that exposure to Cd disturbed the composition of endophytes at both the phylum and genus levels, as shown in (Figures 3 and S1). The proportion of Proteobacteria in the C5 group was significantly lower than that in the control samples, and the relative abundances of Myxococca, Verrucomicrobiota, Fibrobacterota, and Bdellovibrionota in the C50 group were significantly higher than those in the control

Table 1. Physicochemical parameters of ryegrass that is exposed to different concentrations of Cd.

|                | Con    | C5     | C50    |
|----------------|--------|--------|--------|
| Shoot length (cm) | 46 ± 7 | 38 ± 1 | 42 ± 1 |
| Fresh weight (g)  | 15 ± 2 | 16 ± 1 | 16 ± 1 |
| Soluble sugar (mg/g FW) | 8 ± 1 | 7 ± 0  | 10 ± 2 |
| Soluble protein (mg/g FW) | 55 ± 4 | 41 ± 3 * | 40 ± 1 * |
| Total nitrogen (mg/g DW) | 11.0 ± 0.1 | 15.6 ± 3.5 | 19.9 ± 0.1 * |
| Total phosphorus (mg/g DW) | 0.3 ± 0.1 | 0.5 ± 0.1 | 0.6 ± 0.1 * |
| Cd in shoot (mg/kg DW) | 0.6 ± 0.1 | 8.4 ± 0.5 * | 17.5 ± 0.4 * |
| Cd in root (mg/kg DW) | 0.6 ± 0.3 | 22.1 ± 2.6 * | 121.3 ± 12.1 * |
| Ca (mg/kg DW) | 3445 ± 71 | 3250 ± 20 * | 2847 ± 27 * |
| Mg (mg/kg DW) | 2893 ± 50 | 3245 ± 91 * | 3108 ± 107 * |

Data are presented as average ± SD of at least three replicates, FW represents the fresh weight, DW represents dry weight. * indicates statistically significant, p ≤ 0.05, versus control.
group (Figure S1). Exposure to Cd significantly changed the abundance of bacteria at the genus level, including Streptomyces, Exiguobacterium, Saccharothrix and Pandoraea, etc. (Figure 3). Among these genera that differed significantly in at least one Cd exposure group, Silicimonas and Nannocystis were not detected in control group, while increased in C5 and C50 groups. Kushneria and unclassified Pseudonocardia were exclusively detected in the control group. Thalassospira, unclassified Xanthomonadaceae, Pandoraea, Gordonia and Promicro monospora were only found in the control and C5 samples. Compared with the control group, Devosia, Bliri41 (no rank), Ohtaekwangia, Methylobacillus, Arenimonas, MND1 (no rank), Fibrobacteraceae (no rank), Bdellovibrio, Silicimonas and Nannocystis were exclusively detected in a dose-dependent manner in the samples that exposed to Cd, and Saccharothrix, Xanthomonadaceae (unclassified), Pandoraea, Pseudonocardia (unclassified), Kushneria, Promicromonospora and Ferrovibrio were dose-depen- dently decreased. In addition, there were some genera, such as Halomonas, Candidatus Hamiltonella and Raoultella, that showed a more complex response to Cd exposure. These populations were found in a U- or invert U- shaped pattern following exposure to Cd (Figure 3). In summary, these results confirmed that exposure to Cd could influence the composition of ryegrass endophytes, and the bacteria that were found at relatively low abundances were more susceptible to Cd exposure.

3.3 Correlation analysis for the physicochemical parameters and endophyte composition

To determine the potential effects of Cd as well as the physicochemical parameters of ryegrass on the composition of endophytic bacteria genera, a Spearman correlation heatmap analysis was performed. The top 50 genera were classified into three clusters. The genera in cluster I and cluster II had different trends toward these parameters. Most of the genera in cluster I positively correlated with Mg, TP, TN and Cd and negatively correlated with the contents of SP and Ca, in contrast, genera in cluster II showed opposite correlations (Figure 4). Altererythrobacter, Acidovorax, Methylobacillus, Aquimonas, Steroidobacter, Flavo bacteriaceae (unclassified), Cellvibrionaceae (no rank), Bliri41 (no rank), Sphingopyxis, Cellvibrio and Ohtaekwangia were the most susceptible populations to these parameters (Mg, TP, TN, Cd, SP and Ca). TN and TP correlated similarly with these genera, while Mg and Ca correlated oppositely to the genera in cluster I/II. However, they had similar correlations with bacteria in cluster III. SS significantly positively correlated with genera of Chryseobacterium, Devosia, Altererythrobac ter, Acidovorax, etc., and significantly negatively correlated with Sphingomonas, Rhodococcus and Thalassosira. SP significantly positively correlated with genera of Salinicola, Exiguobacterium, Streptomyces, Jatrophihabitans and Brevundimonas. Conclusively, these results figured out the variations in dominant populations that respond to the alterations of physicochemical characteristics, and indicated that, besides Cd, elements of N, P and Ca may also have vital roles that affect the composition of endophytic bacteria.

4. Discussion

It is well known that endophytic bacteria play crucial roles in maintaining the growth of plants and improving their fitness to abiotic stress [27], while there is little information on the response of endophytic bacteria in ryegrass to Cd stress. Data in the present study showed that exposure to Cd altered the composition of endophytic bacteria at both phylum and genus levels. Consistently, previous research studies also observed the alternation of the endophytic bacterial community of plants under Cd stress [28,29]. Proteobacteria, the dominant phylum of ryegrass, decreased under low Cd stress while increased under high Cd stress.
Figure 2. Endophytic microbial community composition of ryegrass under Cd stress. Microbial taxa on (a) phylum level, (b) genus level. Relative abundance less than 1% was classified as others.

(Figure 2(a)), and similar alteration was observed in the endophytic bacterial community of oilseed rapes under Cd stress [28]. However, it should be noted that the effects of Cd on endophytes belonging to Proteobacteria may vary in plant growing in different soil types [29]. In addition, phyla of Myxococcota, Verrucomicrobiota, Fibrobacterota, and Bdellovibrionota with low abundance were all increased under Cd stress (Figure S1). Similarly, Fibrobacterota was reported to be enriched in the endophytes of plant growing in mine soil (Cd 35.86 mg/kg) [29]. In fact, the bacterial community that increased under Cd stress would be expected to have specialized traits that enable themselves to survive and promote the plant to accumulated more Cd. For example, many studies characterized the Cd-tolerant and plant growth promoting abilities of bacteria belonging to Pseudomonas, these bacteria carry genes coding proteins that determine the cation-antiporter efflux system for cadmium resistance [30,31], produce indole-3-acetic acid, siderophore, ammonium and hydrogen cyanide that to promote the plant growth [21,22,32,33]. Many studies have reported that endophytic bacteria belonging to Pseudomonas were enriched under heavy metal stress [34–37]. In this study, we also observed that the proportion of Pseudomonas increased under Cd stress (at both two concentrations). Interestingly, Kukla, et al., identified Pseudomonas as the dominant genus in ryegrass [21], while our data demonstrated that Rhodococcus was the dominant genus in ryegrass (Figure 2(b)), the inconsistence may due to the fact that our study included all the endophytic bacteria and the previous one only focused on the cultivable bacteria. Moreover, bacteria belonging to Mycobacterium, Devosia and Bacillus also increased under Cd stress (though not all are significant), some of these bacteria could enhance the remediation of Cd
by plants growing in certain polluted soils [38,39]. As mentioned above, *Rhodococcus* was the dominant genus in ryegrass. The population of *Rhodococcus* thrived under low concentration of Cd, while decreased under high concentration of Cd (not significant) (Figure 2(b)). Besides the Cd-tolerant characteristic, bacteria belonging to *Rhodococcus* also play important roles in the global recycling of carbon, could promote plant growth, and degrade many organic pollutants (like PCBs) [21,40–43], suggesting that *Rhodococcus* may enhance the remediation of soils co-contaminated with Cd and organic pollutants.

Previous studies found significant correlations between physicochemical parameters and the composition of the bacterial community [44,45]. Consistently, we also found that the alternation of the bacterial community was correlated with the levels of mineral and nutrient elements (Figure 4). As these substances are important nutrients for bacteria, therefore, the alteration of these substances by Cd exposure may be one crucial factor in disturbing the community of endophytes. Moreover, the alternations of these physicochemical parameters under Cd stress may also indicate the potential stress-resistant mechanisms of ryegrass. Soluble protein in plants is an important indicator of physiological changes in metabolism and responds to a variety of abiotic stresses. Our study showed that Cd significantly decreased the content of soluble protein in the shoot of ryegrass, which is consistent with the previous findings [14,46,47]. Interestingly, unlike SP, the contents of total nitrogen and total phosphorus were significantly increased under Cd stress. Leite and Monteiro reported similar results that Cd stress increased the total N in the shoot of Tanzania Guinea grass [48], while invertible results were observed in rice supplied with different...
N fertilizers [49]. This may be due to the diverse and complex mechanisms involved in regulating the toxicity of Cd in plants [50]. Besides N and P, Ca was also involved in regulating the Cd toxicity in plants. Consistent with previous reports [51,52], we showed that Cd exposure significantly decreased the contents of Ca in ryegrass. It was reported that Cd could permeate through Ca channels in plant cells [53]; therefore, the reduction of Ca content in ryegrass under Cd stress may be due to the competition of membrane transporters. Moreover, we found that Cd exposure significantly increased the contents of Mg in ryegrass, which was inconsistent with the published research studies [51,52]. Although Mg is a macronutrient for plants, the role of Mg in regulating Cd toxicity in plants was under-researched [50,54]. Overall, since the effects of Cd on the contents of mineral nutrition may vary depending on the plant species, growth conditions and the available nutrition concentrations, therefore, more work needs to be done to investigate the interactions between Cd and mineral nutrients and, most importantly, to clarify the underlying molecular mechanisms.

5. Conclusion

In summary, we investigated the endophytic bacterial community of ryegrass under increasing Cd stress. Cd exposure disturbed the composition of endophytes at both phylum and genus levels. Bacteria belonging to *Pseudomonas*, etc., that have been reported to be Cd-tolerant or Cd-enriching are increased. Moreover, Spearman correlation heatmap analysis identified significant correlations between the physicochemical parameters and the composition of the bacterial community, suggesting the crucial roles of these parameters in regulating the bacterial community under Cd stress.
Overall, these results could help to better understand the Cd-tolerant mechanisms of ryegrass, and may shed light on the process of improving the remediation of Cd by modulating the endophyte community.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support this study are available in the SRA database (https://www.ncbi.nlm.nih.gov/sra) of NCBI with the accession numbers of SRR14572545 to SRR14572553.

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