Arsenic Uptake by Rice Is Influenced by Microbe-Mediated Arsenic Redox Changes in the Rhizosphere

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Supporting Information

ABSTRACT: Arsenic (As) uptake by rice is largely determined by As speciation, which is strongly influenced by microbial activities. However, little is known about interactions between root and rhizosphere microbes, particularly on arsenic oxidation and reduction. In this study, two rice cultivars with different radial oxygen loss (ROL) ability were used to investigate the impact of microbially mediated As redox changes in the rhizosphere on As uptake. Results showed that the cultivar with higher ROL (Yangdao) had lower As uptake than that with lower ROL (Nongken). The enhancement of the rhizospheric effect on the abundance of the arsenite (As(III)) oxidase gene (aroA-like) was greater than on the arsenate (As(V)) reductase gene (arsC), and As(V) respiratory reductase gene (arra), resulting in As oxidation and sequestration in the rhizosphere, particularly for cultivar Yangdao. The community of As(III)-oxidizing bacteria in the rhizosphere was dominated by α-Proteobacteria and β-Proteobacteria and was influenced by rhizospheric effects, rice straw application, growth stage, and cultivar. Application of rice straw into the soil increased As release and accumulation into rice plants. These results highlighted that uptake of As by rice is influenced by microbial processes, especially As oxidation in the rhizosphere, and these processes are influenced by root ROL and organic matter application.

INTRODUCTION

Arsenic (As) contamination and its health impacts are widespread around the world.1,2 Large areas of paddy soils are contaminated by As due to irrigation with As-tainted groundwater, mining, and other industrial activities. Rice is particularly efficient in accumulating As, compared with other cereal crops, as a result of its anaerobic growth conditions.3 This poses potential harm to people through ingestion of rice, especially in Southeast Asia where rice is consumed as the staple food.4−6 Rhizospheric chemical processes, such as iron oxidation−reduction and iron plaque formation on root surfaces, play an important role in affecting As uptake by rice plants, and they are largely influenced by oxygen release from rice roots.7,8 The aerated rice rhizosphere and O2-releasing root surface are usually coated with iron (Fe) and manganese (Mn) oxides, while As is precipitated with these oxides mainly as arsenate (As(V)).8,9

In addition to chemical processes, As speciation and mobility in soils, sediments, and natural water systems are mainly driven by microbial transformations.10−12 Anaerobic bacteria containing the respiratory reductase (ArrA) can use As(V) as the terminal electron acceptor in respiration and conserve energy from this process.13 Another pathway for microbial As(V) reduction lies in the widespread As detoxification by As(V) reductase (ArsC).14,15 Arsenite (As(III)) is more weakly bound to most soil minerals than As(V); thus, As(V) reduction results in As release into soil solutions, especially under anaerobic conditions such as paddy soil.16,17 On the other hand, some heterotrophic as well as chemoautotrophic microorganisms are
able to oxidize As(III) to As(V).\textsuperscript{18} Arsenite oxidation is suggested to facilitate As sequestration in metal oxides and is often used as a tool to remediate As-contaminated waters and soils.\textsuperscript{19} Arsenic-reducing and arsenic-oxidizing microbes often coexist in the environment, and their relative abundance and activity determine the geochemistry and fate of As in the environment.\textsuperscript{19–23}

Although As biotransformation in the rice rhizosphere can significantly influence As uptake into plants, little has been done on microbial reduction and oxidation of As in the rhizosphere and how it influences As uptake into rice plants. The rice rhizosphere is characterized by strong oxygenation through root radical oxygen loss (ROL) as compared to the bulk soil in relation to soil properties, microbial composition, and activities.\textsuperscript{24,25} Moreover, mucigel, polysaccharides, amino acids, and organic acids released to the rhizosphere from the roots promote microbial abundance and alter community structures.\textsuperscript{26} In fact, the biogeochemical cycling within the rice rhizosphere is so intense and complex that this environment is usually studied as an independent compartment.\textsuperscript{25} Arsenic biotransformation in the rice rhizosphere significantly influences As interaction with Fe and Mn minerals and its bioavailability to the plants. Therefore, the aims of this study were to investigate the microbial oxidation and reduction of As in the rhizosphere and how it influences As uptake into rice plants. The soil of both compartments.

**Soil DNA Extraction.** Total microbial DNA was isolated from roots, rhizosphere soil, and bulk soil samples from the pot experiment using a FastDNA SPIN Kit for Soil (MP Biomedicals, U.S.A.) and the FastPrep Instrument (MP Biomedicals, U.S.A.) following the manufacturer’s instructions.

**Quantification of 16S rDNA, arrA-like, arrA, and arsC Gene Copy Numbers.** Soil DNA was conducted in real-time quantitative PCR (iQ5 Thermocycler; BioRad, U.S.A.) to quantify 16S rDNA, arrA gene, and arsC gene abundance in each sample. For quantification of the arrA-like, arsC, and arrA genes, primers of ArAdeg1F/ArAdeg1R and ArAdeg2F/ArAdeg2R,\textsuperscript{18} amlt-42-f/amlt-376-r and smrc-42-f/smrc-376-r,\textsuperscript{31} and HAArrA-D1F/HAArrA-G2R\textsuperscript{32} were used, respectively.

Details are in the Supporting Information.

**Construction of the arrA-like, arrA, and arsC Clone Library.** DNA from the rhizosphere soil and rice roots (Yangdao growth without rice straw addition, each with four replicates) in the pot experiment were used for the construction of arrA-like, arrA, and arsC clone libraries. The cloned sequences were submitted to the NCBI database from JX489044-JX489133 (arrA gene from JX489044-JX489050, arrA-like gene from JX489051-JX489101, and arsC gene from JX489102-JX489133).

**Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis of arrA-like Gene.** PCR amplification of arrA-like gene was conducted with each forward primer labeled by 6-carboxyfluorescein (FAM). The labeled PCR products were gel-purified with the Wizard SV Gel and PCR Clean-Up System and then digested by the restriction enzyme TagI (Takara, Japan) at 37 °C for 3 h. Digestion products were determined with 3130XL Genetic Analyzer (Applied Biosystems, U.S.A.). Relative abundances of each individual terminal restriction fragments (T-RFs) were calculated based on peak areas in relation to total peak area. Peaks that occurred in at least three replicates and with a percentage >2% were listed.

**Statistical Analysis.** Effects of rice cultivar, rhizosphere/bulk soil, rice growth stage, and rice straw application on abundance and composition of arrA-like genes were evaluated using canonical correspondence analysis (CCA) in CANOCO for Windows 4.54. Abundances of functional genes were subjected to ANOVA in SPSS 13.0, and P < 0.05 was considered to be statistically significant.

**RESULTS**

**Arsenic in Plants and Iron Plaque.** Arsenic concentrations in roots were significantly higher than those in shoots and were significantly higher in shoots of cultivar NK than in cultivar YD (Table 1). Rice straw addition into the soil significantly increased As concentrations in roots of both samples was incubated in DCB (dithionite–citrate–bicarbonate) solution for extraction of iron plaque on the roots surface,\textsuperscript{26} and the other half was used for extraction of total microbial DNA on the root. Concentrations of As, Fe, Mn, and P in the extraction solution were determined by ICP-OES (Optima 2000 DV; Perkin-Elmer, U.S.A.) or ICP-MS. After DCB extraction, roots and shoots were freeze-dried, ground to powder, and stored in a −80 °C refrigerator. Total As concentration in shoots and DCB extracted roots were digested in HNO₃, using a microwave digestion system (MARS, CEM Microwave Technology Ltd., U.S.A.) and determined using ICP-MS.\textsuperscript{29}

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**RESULTS**
Table 1. Arsenic Concentration in Shoots and Roots and Translocation Factor (TF) of Yangdao and Nongken Grown with or without Rice Straw Application into Paddy Soil

<table>
<thead>
<tr>
<th></th>
<th>rice straw</th>
<th>root (without iron plaque)</th>
<th>translocation factor (TF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yangdao</td>
<td>no</td>
<td>9.2 ± 0.5 b</td>
<td>223 ± 17 b</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>10.3 ± 0.8 b</td>
<td>305 ± 47 ab</td>
</tr>
<tr>
<td>Nongken</td>
<td>no</td>
<td>15.0 ± 2.2 a</td>
<td>294 ± 37 ab</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>18.8 ± 1.2 a</td>
<td>351 ± 32 a</td>
</tr>
</tbody>
</table>

"Data are mean ± SE (n = 4). Different letters mean significant differences of As concentration and translocation factor between the treatments (P < 0.05).

16S rDNA and arsC, arrA, and aroA-like Gene Abundances. Quantitative PCR assays were conducted to quantify abundances of genes related to As transformation in the rhizosphere and bulk soil. Copy numbers of 16S rDNA and the arsC, arrA, and aroA-like genes were influenced by the rhizospheric effect, rice cultivar, straw addition, and growth stage (Figure 2; Table S2, Supporting Information). Abundance of arrA and arsC genes in the paddy soil were 10^7 and 10^8 copies g^-1 dry soil, respectively, and the aroA-like gene was usually higher than 10^9 copies g^-1 dry soil (Figure 2). Copies of 16S rDNA and the aroA-like gene were generally higher in the rhizosphere soil of cultivar YD than NK, regardless of treatments (Figure 2; Table S2, Supporting Information). Overall, bacterial abundance (16S rDNA) was higher in rhizosphere soil by 59.2% on average compared to bulk soil, while the copy numbers of the arsC, arrA, and aroA-like genes were increased by 50.8%, 20.8%, and 120.9% on average, respectively. This showed more enhancement in microbial abundances of As(III) oxidation (aroA-like gene) and less enhancement in microbial abundances of As(V) reduction (arsC and arrA genes). Rice straw addition into the paddy soil significantly elevated the abundances of 16S rDNA and the arsC, arrA, and aroA-like genes in both rhizosphere and bulk soil in all treatments (Figure 2; Table S2, Supporting Information). Relative gene abundances (Δ = aroA − arsC − arrA, to indicate the relative As oxidation potential) and concentrations of dissolved As in soil solution were in a linear relationship (R = 0.300, P < 0.05) (Figure 3).

Arsenate-Reducing Microbes in Rice Rhizosphere. Diversity of microbes in the rice rhizosphere associated with As reduction is shown in Figures S1 and S2 of the Supporting Information. Analysis of the S0 arsC cloned sequences from rhizosphere soil and rice roots allowed the identification of 35 unique OTUs based on a 97% cutoff (Figure S1, Supporting Information). The main classes of these sequences belonged to α-, β-, and γ-Proteobacteria, including Enterobacteriales, Rhizobiales, Sphingomonadales, Burkholderiales, and Xanthomonadales. Some of these are branched to the common rhizospheric bacteria, such as Rhizobiales and Burkholderiales. Distribution of the taxonomic groups of arsC from rhizosphere soil and rice roots were among most of the detected groups but were gathered in different small groups revealing the similarities and differences of microbes on rice roots and in rhizosphere soil. Fourteen different arrA clones were amplified from the rhizosphere soil but not from the roots. Because of the scarcity of the already known arrA sequences in the NCBI database, a detailed classification of these clones to an identified microbial species was not possible. These amplified arrA sequences in the rhizosphere soil were aligned to previously reported partial arrA sequences amplified from anaerobic aquifer sediments, bay sediments, river basins, and biofilm reactors (Figure S2, Supporting Information).

Arsenite-Oxidizing Microbes in Rice Rhizosphere. A clone library was established to show the major microbial taxa responsible for As oxidation in the rhizosphere. Analysis of the 65 aroA-like sequences from rhizosphere soil and rice roots allowed the identification of 53 unique OTUs based on a 97% cutoff (Figure S3, Supporting Information). The microbes were mainly Rhizobiales and Burkholderiales in α-Proteobacteria and β-Proteobacteria, including Phyllobacteriaceae, Bradyrhizobiaceae, Methylobacteriaceae, Rhizobiaceae, Sphingomonadales, Burkholderiales, and Xanthomonadales. Some of these are branched to the common rhizospheric bacteria, such as Rhizobiales and Burkholderiales. Distribution of the taxonomic groups of aroA from rhizosphere soil and rice roots were among most of the detected groups but were gathered in different small groups revealing the similarities and differences of microbes on rice roots and in rhizosphere soil. Fourteen different arrA clones were amplified from the rhizosphere soil but not from the roots. Because of the scarcity of the already known arrA sequences in the NCBI database, a detailed classification of these clones to an identified microbial species was not possible. These amplified arrA sequences in the rhizosphere soil were aligned to previously reported partial arrA sequences amplified from anaerobic aquifer sediments, bay sediments, river basins, and biofilm reactors (Figure S2, Supporting Information).

Table 2. Iron Plaque Composition on Roots of Yangdao and Nongken Grown with or without Rice Straw Application in Paddy Soil

<table>
<thead>
<tr>
<th></th>
<th>rice straw</th>
<th>Fe g kg^-1</th>
<th>Mn g kg^-1</th>
<th>P g kg^-1</th>
<th>As g kg^-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yangdao</td>
<td>no</td>
<td>48.3 ± 2.6 a</td>
<td>0.319 ± 0.010 a</td>
<td>2.88 ± 0.14 b</td>
<td>0.462 ± 0.021 a</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>44.2 ± 0.9 ab</td>
<td>0.325 ± 0.021 a</td>
<td>2.49 ± 0.29 bc</td>
<td>0.430 ± 0.027 a</td>
</tr>
<tr>
<td>Nongken</td>
<td>no</td>
<td>42.1 ± 2.2 b</td>
<td>0.263 ± 0.044 b</td>
<td>3.58 ± 0.231 a</td>
<td>0.463 ± 0.033 a</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>35.7 ± 3.4 c</td>
<td>0.258 ± 0.010 b</td>
<td>2.35 ± 0.13 c</td>
<td>0.469 ± 0.019 a</td>
</tr>
</tbody>
</table>

"Data are mean ± SE (n = 4). Different letters mean significant differences of Fe, Mn, P, and As concentration between the treatments (P < 0.05). Linear relationship of As and Fe is y = 0.0094x + 157.7, R^2 = 0.885."
detected in T-RLFP profiles in almost all the treatments. These T-RFs and their relative abundances were similar to in vivo digestion of the cloned sequences in the phylogenetic tree (Figure S3, Supporting Information). The most abundant T-RF 81 bp mainly branched to cluster 7 in the phylogenetic tree (Figure S3, Supporting Information). The percentages of 50 bp and 81 bp T-RF were generally elevated, while the percentages of 131 bp and 221 bp T-RF were lower in the rhizosphere soil than those in the bulk soil. An obvious example was that the clones with an in vivo T-RF of 50, 81, 110, and 321 bp given in the phylogenetic tree were mainly from the rice roots (Figure S3, Supporting Information), suggesting root enhanced microbes.

The canonical correspondence analysis (CCA) reflected the relative contribution of environmental factors (rhizosphere/bulk soil, rice cultivar, growth stage, rice straw application) to the T-RLFP profile of the araA-like gene (Figure 4). Global Monte Carlo permutation tests demonstrated that both the first and all axes combined explained a significant amount of the variability in the araA-like community structure ($P < 0.01$). The first and second axis accounted for 55.5% ($P < 0.01$) and 23.8% ($P < 0.01$) of the total variance, respectively. Rhizospheric effects explained most variation in the microbial community and negatively correlated with the first axis. Growth stage and rice straw application also explained parts of the community variation, followed by rice cultivar. The correlation of T-RF profiles with rhizospheric effects reflected by CCA also agreed with the pattern of the community profile in Figure S4 of the Supporting Information, with an increase in percentage of 50, 81, 110, 321 bp T-RF and a decrease in other T-RFs in the rhizosphere soil compared to those in the bulk soil (Figure 4).

DISCUSSION

The present study revealed that rice uptake of As was modulated by the dynamics of microbial functional groups/gene related to As reduction and oxidation in the rhizosphere. Environmental factors influenced the abundance and community of As-transforming bacteria thus determined As behavior in the paddy soil. Functional genes responsible for As reduction and oxidation in rhizosphere soil, such as arrA, arrC, and araA-like genes, were altered in comparison with bulk soil, of which the abundance of As(III)-oxidizing microbes (possess araA-like gene) were increased more as compared to bacteria abundance (16S rDNA) in the rice rhizosphere (Figure 2). Microbial As reduction and oxidation together determined the As speciation and abundance in the soil solution, shown by the relationship between relative gene abundances and concentrations of dissolved As in soil solution (Figure 3). Microbial oxidation of As(III) to As(V) was enhanced in the rhizosphere and then sequestered on Fe/Mn hydroxide/oxyhydroxide precipitate in rhizosphere soil and on rice roots (Table 2), which reduced As bioavailability and uptake into rice plants.

Arsenic bioavailability in anaerobic paddy soil is usually high due to microbial reduction of iron oxyhydroxides and As$^{17,33}$

Figure 1. Arsenite (a, b), arsenate (c, d), and total As (e, f) concentration in the soil solutions of rhizosphere and bulk soils of Yangdao and Nongken with or without rice straw application in tillering and heading stages. Bars represent standard errors ($n = 4$). CT-R, rhizosphere soil without rice straw application; CT-B, bulk soil without rice straw application; RS-R, rhizosphere soil with rice straw application; and RS-B, bulk soil with rice straw application.
Some As(V)-respiring bacteria have been isolated from sediments, alkaline and saline lakes, and hot springs, respiring As(V) under the anaerobic condition to gain energy. These amplified \textit{arrA} clones from rhizosphere soil were branched to \textit{arrA} sequences amplified from aquifer sediments, bay sediments, river basins, and biofilm reactors. The dissimilatory As(V) reduction gene \textit{arrA} was more likely present among the anaerobic microbes, which limited its wide presence in the rice rhizosphere. Instead, the As(V) detoxification reductase gene \textit{arsC} is more widespread than the \textit{arrA} gene among both anaerobic and aerobic microbes. In this study, the abundance and diversity of the \textit{arsC} gene was higher than those of the \textit{arrA} gene in rhizosphere soil (Figure 2; Figure S2, Supporting Information), showing its importance in As reduction. Some of the microbes were typically rhizospheric microbes, such as Rhizobiales and Burkholderiales. It has been previously suggested that \textit{arsC} contributed to As(V) reduction and mobility under highly aerobic conditions of limed mine tailings. Abundance of the \textit{aroA}-like gene in rhizosphere soil was one fold higher than that in the bulk soil, indicating elevated microbial As oxidation in the rice rhizosphere (Figure 2). Under sterilized conditions, As(III) was stable under both aerobic and anaerobic conditions, while oxygen alone barely oxidized As(III), suggesting the importance of microbially mediated As(III) oxidation. Arsenic sequestered in the rice rhizosphere was mainly in the form of As(V) by Fe hydroxide/oxyhydroxide in root iron plaque and soil around the root. Therefore, the increased microbial As(III) oxidation may contribute to such As sequestration in the rice rhizosphere. The rhizo-bag method was used to define the rhizosphere soil in this study. The rhizosphere may be more limited to millimeters around roots and more severe in influence by roots would be expected. The mass-flow of As from bulk soil during water assimilation into rice roots, together with the fast efflux of As(III) from roots, also contributed to the replenishment of dissolved As to the rice rhizosphere. Thus, the actual effects of As oxidation and sequestration in the rice rhizosphere are likely more profound than the observed (16.8% and 13.5% lower

![Figure 2](image_url)

**Figure 2.** Copy numbers of 16S rDNA (a, b), \textit{arsC} gene (c, d), \textit{arrA} gene (e, f), and \textit{aroA}-like gene (g, h) in the rhizosphere and bulk soils of Yangdao and Nongken with or without rice straw application in tillering and heading stages. Bars represent standard errors (n = 4).

![Figure 3](image_url)

**Figure 3.** Relationship of relative gene abundances ($\Delta = \text{aroA} - \text{arsC} - \text{arrA}$) and concentration of dissolved As in soil solution. $P < 0.05$.

![Figure 4](image_url)

**Figure 4.** Canonical correspondence analysis of \textit{aroA}-like gene terminal restriction fragment length polymorphism (T-RLFP) profiles. Plots show the relationship between As(III)-oxidizing bacteria communities and environmental factors (rhizosphere/bulk soil, cultivar, growth stage, and rice straw application). Arrows indicate the direction and magnitude of each factor associated with bacterial communities.
than in bulk soil solution for YD and NK, respectively), shown by the fact that As accumulation on the root surface alone was higher than that accumulated in the whole plant. The major groups of As oxidizing bacteria were α-Proteobacteria and β-Proteobacteria in the rhizosphere and were mainly rhizospheric microbes (Figure S3, Supporting Information), while undefined cluster 1 and cluster 7 was enhanced on roots (Figure S3, Supporting Information). The diversity of the aroA-like gene revealed in this study is similar to what has been observed in many other diverse environments, such as mining-impacted soils and As-contaminated lake sediments, whereas in geothermal environments, aroA-like microbes were dominated by Aquifaecia. Arsenic oxidation and reduction (ArsC) share some microbial groups, such as Bradyrhizobiaceae and Rhizobiaceae, and also some different groups (Figures S1 and S3, Supporting Information).

The rhizosphere had the greatest influence on the bacterial community in the soil, followed by rice straw application, rice growth stage, and rice cultivar (Figure 4; Figure S4, Supporting Information). The rice rhizosphere is the crucial microenvironment influencing As bioavailability and uptake into rice plants. The rice rhizosphere was different in soil physical–chemical properties, shown by the elevated Eh and the lower soil pH (Figures S5 and S6, Supporting Information). Arsenic-oxidizing microbes were usually aerobic and thus were more likely to be elevated in the rice rhizosphere. The higher abundance of the aroA-like gene in the rhizosphere of cultivar YD than NK (Figure 2; Table S2, Supporting Information) was likely due to greater O₂ release. Total As sequestered on iron plaque (As concentration × root weight) of cultivar YD was higher than cultivar NK, and this could contribute to the lower As concentration in cultivar YD. Previous studies have suggested that rice genotypes with higher ROL accumulate lower overall As. However, these studies did not take into consideration the influence of ROL on microbial As transformation; whereas microbial As oxidation also contributes to As retention around the roots. High root ROL would be a useful trait for selecting genotypes to grow in As-contaminated areas. Furthermore, the input of rice straw into paddy soil significantly increased the microbial activity and increased As release to the soil solution and uptake into rice plants. Microbial degradation of organic matter usually couples with the reduction of Fe(III) minerals and As(V), which together enhances As release to the soil solution and elevates As bioavailability. Therefore, it was suggested that organic matter input into As-impacted paddy soils should be handled with care.

In conclusion, microbial As transformation in the rice rhizosphere has been overlooked in previous research when considering As bioavailability and its uptake into rice plants. As(III)-oxidizing and As(V)-reducing bacteria coexist in the rice rhizosphere, and their relative activities determine the As dynamic in the rhizosphere soil solution. In the rice rhizosphere, the abundance of As-oxidizing bacteria was more elevated than the As-reducing bacteria in comparison with their abundances in bulk soil. Arsenic oxidation under the activity of As-oxidizing microbes, together with the oxic soil condition in the rice rhizosphere, resulted in As sequestration around rice roots and in rhizosphere soil, which limited As uptake into rice plants. To reduce the As accumulation in rice plants, rice cultivars with high oxygen loss ability are preferred, while organic matter input into As-impacted paddy soil should be handled with care.

### REFERENCES


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