Environmental Science & Technology

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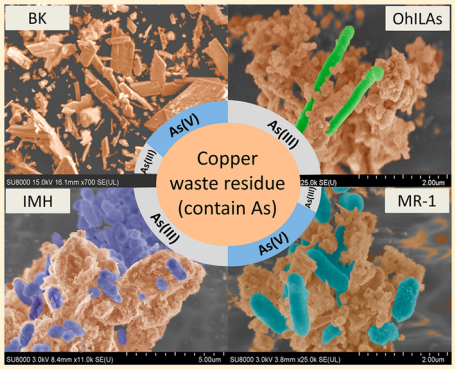
Arsenic Biotransformation in Solid Waste Residue: Comparison of Contributions from Bacteria with Arsenate and Iron Reducing Pathways

Haixia Tian, Qiantao Shi, and Chuanyong Jing*

State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

Supporting Information

ABSTRACT: Arsenic- and iron-reducing bacteria play an important role in regulating As redox transformation and mobility. The motivation of this study was to compare the contributions of different As- and Fe-reducing bacteria to As biotransformation. In this work, three bacteria strains with different functional genes were employed including *Pantoea* sp. IMH with the *ars*C gene, *Alkaliphilus oremlandii* OhILAs possessing the *arr*A gene, and *Shewanella oneidensis* MR-1, an iron reducer. The incubation results showed that *Pantoea* sp. IMH aerobically reduced 100% of As(V) released from waste residues, though total As release was not enhanced. Similarly, strain OhILAs anaerobically reduced dissolved As(V) but could not enhance As release. In contrast, strain MR-1 substantially enhanced As mobilization because of iron reduction, but without changing the As speciation. The formation of the secondary iron mineral pyrite in the MR-1 incubation experiments, as evidenced by the X-ray absorption near-edge spectroscopy (XANES) analysis, contributed little to the uptake of the freed As. Our results suggest that the *ars*C gene carriers mainly control the As speciation in the aqueous phase in aerobic environments, whereas in anaerobic conditions, the As speciation should be regulated by *arr*A gene carriers, and As mobility is greatly enhanced by iron reduction.



INTRODUCTION

The redox transformation and mobilization of arsenic (As) in the subsurface are generally regulated by microorganisms.[1] Although diverse microorganisms play a role in the As biogeochemical cycle, dissimilatory Fe(III)-reducing and As(V)-reducing bacteria have motivated strong interest and extensive experimental studies over the past decade.[2] Previous investigations highlight the importance of these bacteria in the biotransformation and partitioning of As in solid and aqueous phases, using synthetic iron oxides or soil/sediment samples. However, few studies have explored and compared the consequences of these bacterial As(V) and Fe reduction pathways in metallurgical solid wastes. Indeed, the massive amount of such solid waste with extremely high As content poses a pressing challenge,[3] and the ever increasing amount of waste underscores the urgent necessity in understanding and predicting As biotransformation in different microbial pathways and the role of microbial resistance to toxic metals.

Fe(III)-reducers such as *Shewanella* sp. ANA-3 and sp. alga BrY can initiate As release by reducing As-bearing Fe(III) oxides.[4,5] Recent studies, though, highlight the importance of biogenic secondary iron minerals such as magnetite, which could sequestrate the freed As and subsequently constrain As release.[6,7] The formation of such secondary minerals depends on delicately coupled conditions such as the As/Fe ratio,[2,6] the availability of organic carbon,[8] and the presence of As(V)-reducing bacteria.[9]

As(V)-reducing bacteria enzymatically reduce As(V) to more toxic As(III) via two mechanisms: aerobic detoxification encoded by the *ars*C gene and anaerobic respiration by the *arr*A gene.[10] Most studies focus on the As mobility mediated by *arr*A carriers in anaerobic conditions,[7,11−14] and their results suggest that the reduction to As(III), the more mobile As species, prevents the readsorption of freed As on minerals.[9] However, it has remained unclear whether bacteria could directly reduce solid-bound As(V).

In some shallow groundwater or vadose zones of aquifers rich in dissolved oxygen, a high percentage of As(III) is often detected,[15] suggesting that aerobic As(V) reducers may play a role in As biotransformation. However, limited reports are available concerning the contributions of these *ars*C carriers to As(V) reduction and mobilization. For example, a possible *ars*C carrier, *Clostridium* sp. CN8, can reduce aqueous As(V) to As(III) but cannot facilitate the release of adsorbed As(V).[16]

The desire to further understand As biotransformation and mobilization mediated by *ars*C and *arr*A gene carriers and Fe(III)-reducing bacteria motivates our study. The purpose of this study was, therefore, to compare the extent of As redox

Received: September 22, 2014
Revised: January 15, 2015
Accepted: January 21, 2015
Published: January 21, 2015

DOI: 10.1021/es504618x
Environ. Sci. Technol. 2015, 49, 2140−2146

transformation and release because of two As(V)-reducing bacteria, *Pantoea* sp. IMH (harboring the *ars*C gene) and *Alkaliphilus oremlandii* OhILAs (*arr*A gene), and a Fe(III)-reducing bacterium *Shewanella oneidensis* MR-1, in a solid waste residue. The study will shed new light on As mobilization and on evaluating the potential risks of waste residue.

MATERIALS AND METHODS

Sample Collection and Bacteria Strains. The solid waste residue was collected in a copper-smelting company in China. The residue was generated in the high-density sludge (HDS) process for the treatment of copper-smelting wastewater containing up to 3.5 g/L As(III) at pH around 1. This HDS process requires increasing the pH with hydrated lime and subsequently adding iron salts to form precipitates with As. The precipitate residue generally contains high contents of Cu, Pb, Zn, Ca, Cd, Fe, and As.[17,18] To determine the metal content in the solid waste, triplicate samples were freeze-dried and were digested with a microwave digestion system (MARS, CEM Corporation, U.S.).[19]

Three bacteria strains from different genera, *Pantoea* sp. IMH, *Alkaliphilus oremlandii* OhILAs, and *Shewanella oneidensis* MR-1, were employed in the present study. *Pantoea* sp. IMH has the *ars*C gene and can effectively reduce As(V) to As(III) aerobically.[20,21] *Alkaliphilus oremlandii* OhILAs has the *arr*A gene and can reduce As(V) under anaerobic conditions.[22] Though *Shewanella oneidensis* MR-1 has the *ars*C gene, it can only anaerobically serve as an Fe(III)-reducer rather than As(V)-reducer.[9] The culture media for the three strains are listed in Table S1 of the Supporting Information, and their specific components are also detailed in the Supporting Information (SI). Moreover, for OhILAs and MR-1, all manipulations were carried out under an anaerobic atmosphere in a glovebox (100% N_2). The procedures in this study were carried out using aseptic techniques.

Microbial Incubation. The bacteria were grown to late-log phase in the culture medium at 30 °C. Then, the cells were harvested by centrifugation at 10 000*g* (Eppendorf 5424, Hamburg, Germany) for 3 min and were washed by fresh culture medium three times. The cells were then inoculated into the corresponding culture medium (500 mL) with different contents of solid waste residue, which were determined on the basis of the maximum levels of solid waste that bacteria could tolerate in our preliminary experiments. The metal-resistant experiment is described in the SI. The final residue content was 10, 1, and 2 g/L, respectively, for IMH, OhILAs, and MR-1. In the incubation experiment of strain OhILAs and solid waste residue, As(V) was not added in the chemically defined medium (CDM). The solid waste residue was autoclaved prior to incubation to avoid the effect of other bacteria.

The IMH samples were incubated in conical flasks closed with sealing film and were shaken at 150 rpm for 37 h. The OhILAs and MR-1 incubations were carried out in anaerobic bottles closed with rubber stoppers and were placed in the darkness without agitation for 120 h. All incubations including controls were performed in triplicate at 30 °C. Controls were identical to the incubation experiment except without inoculation. The control was labeled as BK1 for IMH, BK2 for OhILAs, and BK3 for MR-1. At the end of the incubation, the pH was 8.8 for the Luria Broth (LB) medium (IMH and MR-1) and 9.1 for the CDM medium (OhILAs). Such a small difference in pH (8.8 vs 9.1) should have negligible effect on the As mobilization.

The suspension of all the above incubations was periodically sampled to determine the cell number and the concentrations of aqueous As(III), As(V), Fe(II), total Fe, SO_4^{2-}, HS^-, and other metals (Ca, Cu, Zn, Pb, Cd). The solids at the end of the incubation course were collected by centrifugation at 8000*g* for 10 min and were washed with autoclaved deionized (DI) water three times for analysis using scanning electron microscopy (SEM), X-ray powder diffraction (XRD), and X-ray absorption near edge structure (XANES) spectroscopy.

Sample Analysis. The concentrations of As(III) and As(V) were determined using high-performance liquid chromatography (HPLC) coupled with atomic fluorescence spectrometry (AFS, Haiguang, China).[19] The detection limit is 0.7 μg/L for As(III) and 1.7 μg/L for As(V). Ferrous iron was quantified using the colorimetric 1,10-phenanthroline method.[23] Sulfate was determined using a DX-1100 ion chromatograph (Dionex, Sunnyvale, CA) with an AS11-HC Ion Pac column. Dissolved sulfide was monitored using a sulfide electrode (9616BNWP, Thermo, U.S.), and H_2S (g) was determined by a H_2S gas detector (WT-80, China). Total iron and other metal concentrations were analyzed using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500a, U.S.) and inductively coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer, U.S.). Dissolved Fe(III) was calculated by subtracting ferrous from total iron. The cell number was counted with a hemocytometer. The SEM and XRD analyses are detailed in the SI.

As and Fe K Edge XANES Analysis. The solid samples were freeze-dried under vacuum and were sealed between two layers of Kapton tape for analysis. The As and Fe K edge XANES spectra were collected at beamline 14W at the Shanghai Synchrotron Radiation Facility (SSRF), China. Spectra were acquired from −50 to 300 eV relative to the As K edge of 11 867 eV and Fe K edge of 7112 eV. The fluorescence signals were collected using a Lytle detector. Standard reference chemicals, $Na_2HAsO_4{\cdot}7H_2O$, $NaAsO_2$, AsS, As_2S_3, ferrihydrite, goethite, $FeSO_4$, Fe_3O_4, and FeS_2, were also analyzed. The spectra were analyzed using a linear combination fit in the Athena program in the IFEFFIT computer package.[24] The data processing and fitting approach are similar to that used in our previous study.[19]

Statistical Analysis. The software IBM SPSS Statistics, version 20.0, was used to perform the statistical analysis. An independent-sample *t* test was applied to ascertain possible significant differences between the microbial samples and the control samples. Significance was considered when $p < 0.05$.

RESULTS AND DISCUSSION

Characterization of Solid Waste Residue. The acid digestion results listed in Table S2 of the Supporting Information indicated that the residue mainly contained 9.5 mg/g As, 27.5 mg/g Fe, and 216 mg/g Ca. The XANES analysis suggested that the Fe phase was composed of nearly equal amounts of ferrihydrite (50.7%) and goethite (49.3%), while the ratio of As(III)/As(V) was 0.3 (23.7/76.3, Table 1). Such composition and speciation in the residue are direct results of the high-density sludge process to remediate As smelting wastewater where lime, ferrous sulfate, and air were added.[18] Thus, the primary phase in the residue was gypsum as detected by XRD, and its existence of gypsum was not affected by culture media (Figure S1 of the SI).

As Release and Speciation. The soluble As speciation and concentration, normalized to account for differences in the solid/liquid ratio, showed contrasting behaviors during the incubation in the presence of the three bacteria (Figure 1). The two As(V) reducers, *Pantoea* sp. IMH (*ars*C gene carrier) and *Alkaliphilus*

DOI: 10.1021/es504618x
Environ. Sci. Technol. 2015, 49, 2140−2146

Table 1. Linear Combination Fitting of As and Fe K Edge XANES Spectra for Samples

	percentage, %						
std	raw	BK1	IMH	BK2	OhILAs	BK3	MR-1
As(III)	23.7	26.1	33.2	23.6	36.5	22.7	56.6
As(V)	76.3	73.9	66.8	76.4	63.5	77.3	43.4
ferrihydrite	50.7	56.1	56.4	56.6	43.6	55.9	38.2
goethite	49.3	43.9	43.6	43.4	37.7	44.1	39.6
$FeSO_4$	–[a]	–	–	–	18.7	–	–
FeS_2	–	–	–	–	–	–	22.2

[a]"–" indicates no detection.

oremlandii strain OhILAs (*arrA* gene carrier), completely reduced dissolved As(V) to As(III) within 20 h but liberated much less total soluble As than *Shewanella oneidensis* MR-1, the iron-reducing bacterium. Although not respiring As(V),[9] strain MR-1 released up to 6.9 mg/L, corresponding to 3.5 mg/g As(V) in 40 h (Figure 1c), which is equivalent to a release of 0.9 mg/g against the control (Figure 1d). In addition, a significant decrease ($p < 0.01$) in As(III) release was observed after 40 h in the MR-1 incubation (Figure 1c), which is likely due to As(III) readsorption to secondary biogenic Fe minerals.[6,7]

Reduction of dissolved As(V) was coupled with the growth of the As(V) reducers and reached 100% when the cell growth was at the exponential phase (Figure 1a, b). Actually, IMH resulted in a higher As(V) reduction rate (0.11 mg/g-h) than OhILAs (0.03 mg/g-h) because of its rapid growth under aerobic conditions.

Compared to control samples with no cells, As(V)-reducing bacteria did not result in the liberation of appreciable As into solution (Figure 1d). In agreement with our results, a possible *ars*C carrier, *Clostridium* sp. CN8, was able to effectively reduce dissolved As(V) but could not accelerate the release of adsorbed As from iron oxides.[16] Indeed, at the end of our incubation experiments, about 6% and 2% less As were detected, respectively, in IMH and OhILAs incubations compared to their controls (Figure 1d). This slight decrease in As(III) mobility may be attributed to As(III) biosorption on IMH ($\times 10^{11}$ cells) and OhILAs ($\times 10^{9}$ cells).[25] Though a previous study[26] suggested that the attachment of bacterium cells (S. *putrefaciens*) to mineral surfaces could promote As(V) desorption and thereby facilitate As(V) reduction, this situation did not occur in our study.

Microbial Release and Reduction of Fe(III). Dissolved Fe(II) and Fe(III) concentrations were monitored during the incubation with these three bacteria strains (Figure 2). IMH released <0.6% of Fe in the residue and did not reduce the dissolved Fe(III) to Fe(II) (Figure 2a). Compared with the control, IMH slightly enhanced ($p < 0.01$) total Fe release, possibly because of disaggregation of minerals in the residue induced by IMH colonization and physical penetration into the mineral surface.[27] This speculation was justified by our SEM characterization (Figure S2 of the SI), which showed that the microbial incubation induced the crushing of As-bearing minerals from a large lamellar assembly (about 20–50 μm, Figure S2a–c of the SI) to small particles (about 100 nm, Figure S2d–f of the SI).

Similar to IMH, strain OhILAs liberated about 4% of total Fe, a slight increase ($p < 0.01$) compared with the control (Figure 2b). Interestingly, about 23% of soluble Fe(III) was reduced after 40 h, and the Fe(II) concentration remained unchanged thereafter. The Fe(III) reduction was possibly mediated by strain OhILAs, and this premise was evidenced by additional experiments on ferrihydrite reduction in the presence of OhILAs (Figure S4 of the SI). However, Fe(III) reduction was inhibited after 47 h, as suggested by the fact that in the presence of approximately 0.8 mg/g dissolved Fe(III), the Fe(II) concentration stopped increasing (Figure 2b). This inhibition in Fe(III) reduction

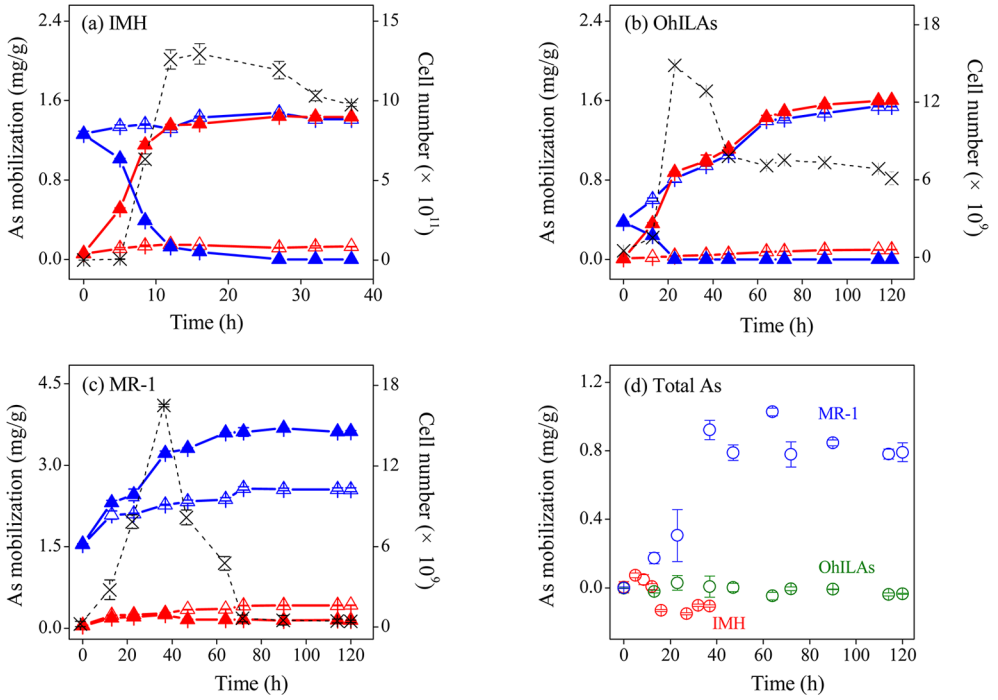


Figure 1. Change of dissolved As(V) (blue), As(III) (red), and cell number (black) as a function of incubation time in the solid waste residue mediated with (a) IMH, (b) OhILAs, and (c) MR-1. Closed (open) symbols represent inoculated (control) samples. Total released As concentrations against control are shown in panel d. Error bars represent the standard deviations of three replicates.

DOI: 10.1021/es504618x
Environ. Sci. Technol. 2015, 49, 2140–2146

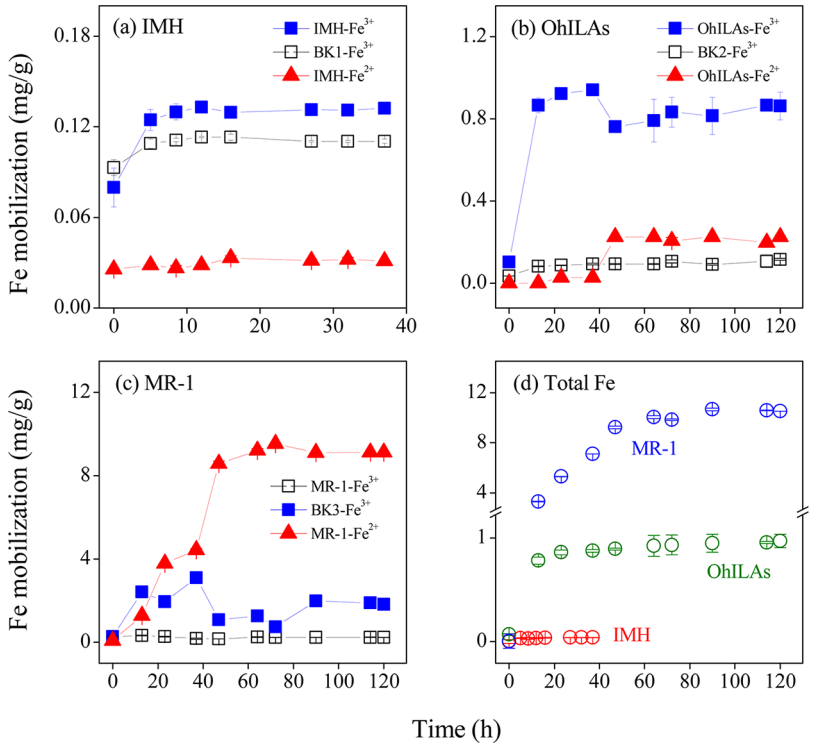


Figure 2. Change of dissolved Fe(III) (blue closed squares), Fe(II) (red closed triangles), and Fe(III) in control (black open squares) as a function of incubation time in the solid waste residue mediated with (a) IMH, (b) OhILAs, and (c) MR-1. Total released Fe concentrations against control are shown in panel d. Error bars represent the standard deviations of three replicates.

activity could be attributed to the release of high concentrations of heavy metals from the solid waste residue (Table S3 of the SI), which is toxic to the cells and suppresses the microbial Fe(III) reduction.[28,29] Compared with OhILAs, MR-1 and IMH liberated higher concentrations of heavy metals (Table S3 of the SI), mainly because of the different cultures used: CDM medium for OhILAs, and LB medium for MR-1 and IMH. LB medium contains more chelating agents in high concentrations than CDM, which subsequently extracted more heavy metals.

On the other hand, although more heavy metals were released in the MR-1 and IMH incubations, no significant toxic effect was observed on the activities of MR-1 and IMH (Figures 1 and 2). These two bacteria contain the *arsC* gene in a family of *ars* operon, which could confer a high resistance to heavy metals.[30] This trait of the high resistance of *ars*C carriers enables such bacteria to play an important role in metal mobilization in industrial solid waste. Nevertheless, for those dissimilatory As(V) reducing bacteria lacking an *ars* operon, their limited resistance to heavy metals would constrain their contributions to As(V) reduction in industrial solid waste.

Not surprisingly, as an Fe(III) reducer, strain MR-1 significantly ($p < 0.01$) promoted Fe mobilization, as evidenced by the fact that about 33% of Fe in the residue was dissolved as Fe(II) (Figure 2c). Compared with the two As(V) reducers, which only release <1 mg/g Fe against control, strain MR-1 could reduce Fe(III) to Fe(II) and result in 11 mg/g Fe release (Figure 2d).

The As liberation was significantly linearly correlated with Fe mobilization in MR-1 incubation ($p = 0.0003$, Figure 3), suggesting that the reductive dissolution of Fe(III) oxides in the solid waste residue contributes to As release, similar to the occurrence of groundwater As in geogenic areas.[2] For IMH and OhILAs incubations, no correlation was found in the

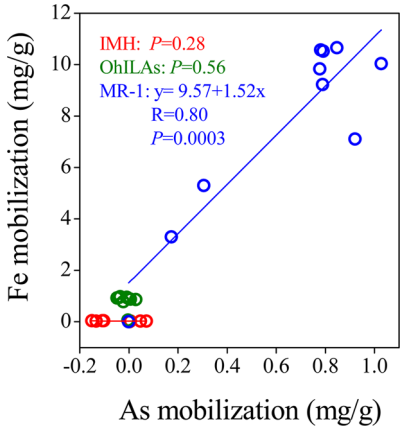


Figure 3. Correlation analysis between total released As and Fe in residue mediated by IMH, OhILAs, and MR-1.

mobilization of As and Fe ($p = 0.28$ for IMH, $p = 0.56$ for OhILAs).

Microbial Reduction of Sulfate. Sulfate was the only sulfur-containing species in the culture of IMH and OhILAs (Figure 4a, b). In contrast, in addition to sulfate, appreciable amounts of sulfide and H_2S gas were detected in MR-1 incubation (Figure 4c). Because MR-1 could not respire sulfate,[31] this indirect sulfate reduction might be induced by some reducing species metabolized by MR-1 in the LB medium. In the late period of MR-1 incubation (>90 h), however, soluble sulfide concentration decreased from 24 to 8 mg/g, which might be attributed to biogenic formation of secondary Fe−S minerals.[32,33] In addition, though HS^- existed in the MR-1 incubation, no As−S precipitate was expected because of the limited As(III) in the aqueous phase and the alkaline condition (pH = 8.4).[34] This

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Environ. Sci. Technol. 2015, 49, 2140−2146

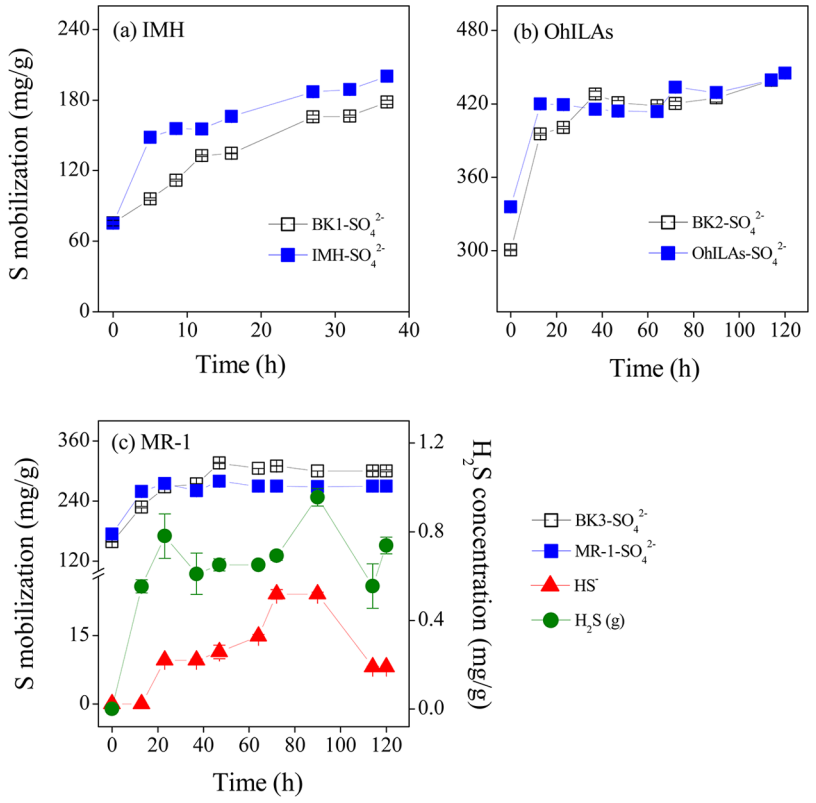

Figure 4. Change of dissolved sulfate (blue closed squares), sulfide (red closed triangles), H_2S gas (green closed circles), and sulfate in control (black open squares) as a function of incubation time in the solid waste residue mediated with (a) IMH, (b) OhILAs, and (c) MR-1. Error bars represent the standard deviations of three replicates.

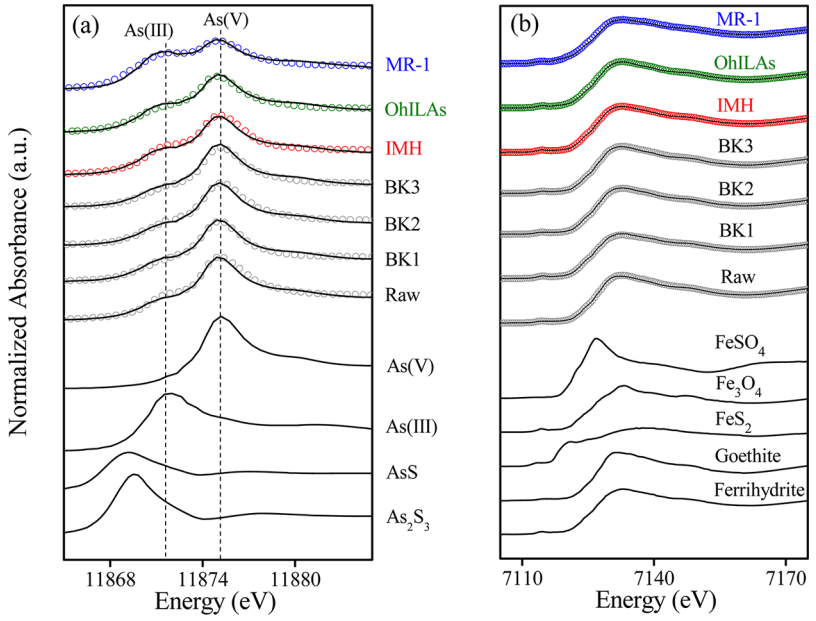

Figure 5. Observed (points) and linear combination fitting (lines) XANES spectra for (a) As and (b) Fe for solid residues at the end of incubation. Spectra for standard references are also shown for comparison.

conclusion motivated us to further explore the As and Fe speciation in the solid phase.

As and Fe Speciation in the Solid Phase. Synchrotron-based measurement of As speciation by XANES confirmed that, at the end of incubation when dissolved As speciation and concentration reached equilibrium, As(III) and As(V) were found in the solid phase, but not As−S compounds (Figure 5a). Interestingly, the ratio of As(III) to total As after incubation was higher than that in the raw waste residue (Table 1). This increase in As(III) ratio should be the result of concurrent processes including reductive dissolution of Fe(III), liberation and biotransformation of As(III/V), and sequestration of As on biogenic Fe minerals.

DOI: 10.1021/es504618x
Environ. Sci. Technol. 2015, 49, 2140−2146

The results of Fe K edge XANES analysis are shown in Figure 5b and Table 1. No significant change in Fe phases was observed in IMH incubation, whereas 18.7% $FeSO_4$ was detected because of the slight Fe(III) reduction in OhILAs incubation (Figure 2b). In the MR-1 culture, 22.2% FeS_2 was found, which could well explain the decrease in soluble sulfide concentration near the end of the incubation course (Figure 4c). Moreover, though FeS_2 is regarded as an effective adsorbent to remove As,[35,36] the formation of FeS_2 in our study had no significant influence on As mobility, probably because of its limited content, the high alkaline pH, and microbial competition. Furthermore, the As scavenging by FeS_2 precipitates might be masked by the amount of As released by Fe(III) oxide reductive dissolution.[37]

Researchers found that microbial Fe(III) reduction may have two contrasting consequences on the fate of associated As: mobilization[2,5,38] and sequestration,[7−9,39−41] depending on their experimental conditions. The primary driving force to sequester As during Fe(III) reductive dissolution is the formation of secondary Fe-minerals such as magnetite,[6,42] siderite, and vivianite.[39,41] These biogenic Fe-minerals' formation mainly depends on the microbial Fe(III) reduction rate, which is affected by experimental conditions including the bacteria, As/Fe ratio,[6] and the amount of organic carbon source.[8,43] In our study, Fe(III) dissolution in the MR-1 incubation resulted in a concurrent As mobilization because the secondary Fe(II) (hydr)oxide formation was inhibited because of the low content of Fe in the raw residue (2.75 wt %) and the sufficient organic carbon source in the culture medium. Though pyrite was detected (Table 1), its content and its ability to take up dissolved As were relatively low.

Environmental Implications. Microorganisms, including As(V)- and Fe(III)-reducing bacteria, play a key role in As biotransformation and mobility. The current study provides an example of three bacteria strains with different As(V) reducing mechanisms. The *ars*C gene carriers mainly regulate the As species in the aqueous phase in aerobic environments, while under anaerobic conditions, As species should be mediated by *arr*A gene carriers and As mobility is greatly enhanced by Fe(III) reductive dissolution.

The *arr*A gene carriers are proposed to be able to directly reduce the adsorbed As(V), because the ArrA location at membrane or periplasm should facilitate its possible contact with and reduction of adsorbed As(V). In our study, the addition of an *arr*A gene carrier during the 120 h incubation did not induce appreciable As release. This observation suggests that there should be no in situ reduction of adsorbed As(V), and desorption or release is the prerequisite for the subsequent microbial reduction. The As mobility in the presence of *arr*A gene carriers might have previously been overestimated.

Our study indicates that *ars*C gene carriers play an important role in As redox transformation under oxic conditions. This conclusion underscores the fact that high ratios of As(III) to total As are often detected in some groundwaters rich in dissolved oxygen. The existence of *ars*C gene carriers provides a plausible explanation for the occurrence of high As(III) in paddy soils and subsurface groundwater.[44,45] Meanwhile, by comparing the As biotransformation mediated by *arr*A and *ars*C gene carriers, we found that *ars*C gene carriers have a higher metal resistance and a faster rate of As(V) reduction. These observations have important implications for the fate and transport of As in industrial solid waste with extremely high metal contents.

Our results highlight a potential As release from industrial solid wastes that may have been underappreciated previously. Generally, potential As release is considered negligible in the oxic environment because most As(V) and Fe(III) reducers are of no avail under this oxidation condition. However, strain IMH with the *ars*C gene has high resistance to heavy metals and can rapidly reduce As(V) to more toxic As(III) aerobically. Thus, no matter whether in oxic or anoxic environments, safe disposal of As-containing solid waste residue is of paramount importance.

■ ASSOCIATED CONTENT

Supporting Information

The specific components of the culture media for the three strains, details of the metal-resistant experiment

, and SEM and XRD analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +86 10 6284 9523; fax: +86 10 6284 9523; e-mail: cyjing@rcees.ac.cn.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are very grateful to Dr. Haichun Gao for the donation of strain MR-1. We acknowledge the financial support of the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB14020201), the National Basic Research Program of China (2015CB932003), the National Natural Science Foundation of China (41373123, 41425016, 21337004), and RCEES (YSW2013A01).

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DOI: 10.1021/es504618x
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