Arsenic interception by cell wall of bacteria observed with surface-enhanced Raman scattering

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The purpose of this study was to determine the interactions between arsenic (As) resistant bacteria and As, using surface-enhanced Raman scattering (SERS) and Fourier transform infrared (FTIR) spectroscopy. According to our 16S rDNA results, eight bacteria isolated from the environment can be identified to four genera (Arthrobacter, Pseudomonas, Sphingomonas, and Acinetobacter). The bacteria were separated into cell wall and protoplast in the study to assess the As(V) attack. The As(V) stress on bacteria could be identified with SERS, but not with FTIR. The bacteria in our study primarily resist As(V) through sequestration of As(V) by the cell wall. The change in SERS peaks and their relationships with cell wall suggested that As(V) mainly interacts with functional groups on the cell wall including polysaccharides and flavin derivates.

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1. Introduction

Arsenic-resistant bacteria play a dominant role in the biogeochemical cycling of arsenic (As). Elevated As concentrations are found in ground waters worldwide (Mandal and Suzuki, 2002). Although As is toxic, a number of bacteria have developed mechanisms of resisting high concentrations of As and even benefit from the As exposure (Oremland and Stolz, 2003). These mechanisms include detoxification through urs gene systems (Wang et al., 2009), alterations in transmembrane transportation to minimize arsenate uptake, and As redox transformation. To better understand the fate of As in the environment, extensive research has been performed in exploring As interactions with bacteria (Oremland and Stolz, 2003). At present, the mechanistic studies of As-bacteria interactions mainly employ characterization techniques in molecular biology and gene technology which are complicated in operation and time-consuming. In situ simple and rapid characterization methods are urgently needed.

Surface-enhanced Raman scattering (SERS) spectroscopy and Fourier transform infrared (FTIR) spectroscopy are two recent additions to physicochemical technologies for characterizing and identifying microorganisms (Amiali et al., 2007; Oust et al., 2006; Samelis et al., 2011; Schuster et al., 2000). SERS and FTIR spectra exhibit characteristics of bacteria cell components such as fatty acids, proteins, nucleic acids, and carbohydrates (Knauer et al., 2010; Yu and Irudayaraj, 2005). Previous studies demonstrate that the use of SERS and FTIR could be a viable approach for the characterization and identification of whole bacteria cells at the genus and species level (Dziuba et al., 2007; Wang et al., 2010; Yu and Irudayaraj, 2005). As far as we know, no SERS and FTIR study has been attempted at the genus level to investigate As-resistant mechanisms of bacteria. Therefore, the motivation of the research is to apply SERS and FTIR, in situ rapid spectroscopic techniques, for the first time, in the preliminary study of As-resistant mechanisms of bacteria.

The objective of this study was to use SERS and FTIR to determine their interactions with As. In this study, eight As-resistant bacteria isolated from contaminated soils and groundwater were characterized with 16S rDNA and Biolog tests for comparison. This article for the first time provides spectroscopic techniques for the study of As interactions with bacteria, which are crucial in As biogeochemical cycling.

2. Material and methods

2.1. Sample collection and bacterial isolation

Two soil and eight groundwater samples were collected from Inner Mongolia and Shanxi provinces of China. One gram of soil sample or 1 ml of groundwater sample was added into 10 ml of a chemically-defined liquid medium (CDM), and incubated at 30 °C with shaking (200 rpm) in the dark for 48 h. The CDM consisted of three kinds of nutrients (Chitpirom et al., 2009) supplemented with arsenite (NaAsO₂: 0, 5, 10, and 20 mM) or arsenate (Na₃AsO₄: 0, 5, 10, and 20 mM) at pH 7.0. The culture was expanded by subculturing twice. Each time 1% (v/v) culture was transferred to a fresh medium and incubated under the same conditions. The potential As-resistant bacteria were purified by the streak plate method on the CDM agar medium, and then each colony was kept at 4 °C.
2.2. Minimum inhibitory concentration (MIC) test

The bacteria were inoculated into Luria–Bertani medium (LB) supplemented with either As(III) (5–1000 mM) or As(V) (5–1000 mM) and incubated at 30 °C with shaking (200 rpm) in the dark for 48 h. Then, the OD_{600} value of the medium was determined using a spectrophotometer (HACH DR2800) and the MIC was defined as the lowest As concentration which completely inhibited the growth of the bacterium (Pepi et al., 2011).

2.3. Bacteria identification using 16S rDNA and Biolog plates

The bacterial 16S rDNA gene was amplified with universal primers, 27F and 1492R. The PCR products were purified using the Gel Extraction Kit (Omega, USA). Standard procedures were used for DNA cloning and manipulation. The DNA sequencing was performed by TSINGKE Company (Beijing, China). Sequence similarity was assessed using Blastn (http://www.ncbi.nlm.nih.gov/blast). Sequence alignments were performed using the Clustal X2 program with the sequences of GenBank database. Phylogenetic trees were generated from alignments with the neighbor-joining method using the MEGA version 5.0 software (Tamura et al., 2011).

Analysis was performed using the Biolog system to validate the 16S rDNA genus identification of isolates. Biolog GN2 and GP2 microplates (Biolog, Hayward, CA) were respectively used to identify Gram-negative and Gram-positive bacteria isolates. All protocols for preparation and identification of microorganisms are outlined in the Biolog manufacturer's protocol.

2.4. SERS analysis

The bacteria were inoculated into Luria–Bertani medium (LB) supplemented with As(V) (0, 40 mM) and incubated at 30 °C with shaking (200 rpm) for 10 min and washed twice with 0.6 M NaCl. Then, 20 μl bacteria sample was centrifuged for 1 min at 4000 rpm and the supernatant was replaced with deionized water. This washing procedure was repeated three times. The SERS measurements were conducted respectively on whole cells, protoplast, and cell walls.

The cell wall fraction was collected by the modified ultrasonic method (Knox and Brandsen, 1962; Walter et al., 2011). The collected biomass was re-suspended in deionized water and homogenized 5 s intermittently every 5 s in an ultrasonic cleaner (VWR, BS500A-DTH, 510 W maximum level). This repetitive ultrasonic procedure was continued for 20 min to fully separate cell walls from protoplast. The cell wall fraction was collected by centrifugation at 15000 g for 15 min at 4 °C. The supernatant was removed and the debris was washed twice with 0.8% NaCl.

The protoplast fraction was obtained using the modified lysozyme digestion method (Forsberg et al., 1970). The cell was treated with 0.1 M EDTA for the Gram negative bacteria before 5 ml lysozyme solution (1 mg/ml) was added and incubated with shaking at 30 °C until the formation of spheres was complete as observed under the microscope. The protoplast was separated from the suspension with centrifugation at 11000 g for 10 min and washed twice with 0.6 M NaCl.

The Ag nanoparticles (NPs) were synthesized by mixing 1 ml 0.01 M AgNO₃, 9 ml 1.7 M NH₂OH·HCl, and 0.3 ml 0.1 M NaOH and incubated at 30 °C with shaking (200 rpm) in the dark for 48 h. Then, the OD_{600} value of the medium was determined using a spectrophotometer (HACH DR2800) and the MIC was defined as the lowest As concentration which completely inhibited the growth of the bacterium (Pepi et al., 2011).

2.5. FTIR analysis

The bacteria sample preparation for FTIR measurements was the same as for SERS. After washing, 300 μl bacteria suspensions were used for collection of spectra.

FTIR spectra were recorded using an attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectrometer (Nicolet 6700) equipped with a liquid nitrogen-cooled mercury–cadmium–telluride (MCT) detector and an ATR Max II horizontal flow cell with 45° ZnSe crystal (PIKE Tech). FTIR signals were collected with 256 scans at a resolution of 4 cm⁻¹ at room temperature. Deionized water was measured and used as background, and subtracted from the sample spectra. To diminish the difficulties arising from unavoidable baseline shifts, autobaseline correction of the original spectrum was carried out (Oberreuter et al., 2002) and performed using instrument-provided software (Thermo Scientific OMNIC).

3. Results

3.1. Identification of As-resistant bacteria

Eight As-resistant bacteria were isolated from two As-free samples and six As-polluted samples. Bacteria were identified based on their biochemical characteristics and 16S rDNA sequences. Sequence analysis and Biolog test results (Table 1) indicate that the isolates are representatives of Arthrobacter, Pseudomonas, Sphingomonas, and Acinetobacter. The 16S rDNA gene neighbor-joining tree showing the genetic relationship between all isolates and reference species is illustrated in Fig. 1.

The As-resistance was tested, and the minimum inhibitory concentration (MIC) of As for eight bacteria is shown in Table 1. The MIC of As(III) and As(V) ranged from 10 to 40 mM and 160 to 500 mM, respectively. Among the eight bacteria, isolate P. sp. C can tolerate the highest concentration of As(III) (40 mM) and As(V) (500 mM).

3.2. FTIR analysis

Fig. 2 shows the FTIR spectra of whole cell samples of eight bacteria in the wavenumber range of 900–1800 cm⁻¹. These spectra exhibited characteristic peaks similar to reported data for different microorganisms (Gao and Choquer, 2009; Parikh and Choquer, 2006; Shephard et al., 2008). The assignment of FTIR bands is shown in Table S1. Although the spectra bear some similarities in peak position and number of main peaks, the differences in the peak height ratio at 1085 and 1046 cm⁻¹ (HR1085/1046) among these bacteria isolates are pronounced. The bands at 1085 cm⁻¹ and 1046 cm⁻¹ were assigned as symmetric phosphate and C–O–C stretching modes, respectively (Table S1). The HR1085/1046 was lower in Gram-positive bacteria (Arthrobacter sp. A and Arthrobacter sp. B; about 0.6) than in Gram-negative bacteria (P. sp. C, P. sp. D, P. sp. E, P. sp. F, Sphingomonas sp. G, Acinetobacter sp. H; range from 0.8 to 4.7).

To study the As(V) effect on whole cells, the FTIR spectra were collected after adding different As(V) concentrations (0, 20, 40 mM) in the bacteria culture medium. The spectra were similar to those of pure bacteria (data are not shown). The results suggest that the effect of As(V) on bacteria in our study could not be characterized with FTIR spectra.
3.3. SERS analysis

SERS was applied to assess the effect of As(V) on microbial cells. Fig. S1 shows the morphology of the whole cells with Ag colloids for SERS detection.

SERS spectra in the region of 530–1750 cm\(^{-1}\) of the cell walls, protoplast, and whole cells of bacteria are shown in Fig. 3. The corresponding band assignment is shown in Table S1. For the same bacteria, we can see that the additive SERS spectra of cell walls and protoplast were not equal to that of the whole cell. Significant differences of different genus bacteria are reflected in the spectra of whole cells, cell walls, and protoplast. The peak intensity of the band at 655 (or 662) cm\(^{-1}\) assigned as COO\(^{-}\) deformation decreases, and the band at 731 cm\(^{-1}\) assigned as adenine and DNA increases in the SERS spectra from protoplast compared with that from the cell walls. For the two Gram-positive bacteria (Arthrobacter sp. A and Arthrobacter sp. B), the peak intensity of the band at 1131 cm\(^{-1}\) assigned as carbohydrates deformation, and 1248 cm\(^{-1}\) as CH\(_2\) deformation in the cell wall increases compared with that in the protoplast.

Given its success in bacteria identification in recent reports, SERS analysis was employed to explore the interactions of As(V) and bacteria. Observable changes in the SERS spectra of cell walls were observed when 40 mM As(V) was added to the medium (Fig. 4). In addition, As(V) interactions with cell walls exhibited noticeable distinctions in SERS spectra for different bacteria. To describe these observable changes clearly, the SERS difference spectra was derived from As(V) exposed minus non-exposed control spectra in the region of 530–1750 cm\(^{-1}\) of cell wall of different bacteria with 40 mM arsenate (Fig. 5). The interactions of As(V) with Arthrobacter sp. A and Arthrobacter sp. B resulted in similar SERS changes, such that the intensities of the bands assigned as carbohydrates (573 cm\(^{-1}\)), COO\(^{-}\) deformation (658 cm\(^{-1}\)), C-N stretch (959 cm\(^{-1}\)), CH\(_2\) deformation (1248 cm\(^{-1}\)), and \_CH in plane or amide III (1327 cm\(^{-1}\)) decreased. However, the intensities of the bands assigned as flavin derivates or polysaccharides (731 cm\(^{-1}\)), C-N stretch (800 cm\(^{-1}\)), and COC stretch (897 cm\(^{-1}\)) increased. The presence of As(V) diminished the vibrations of CH\(_2\) def or amide III (1300 cm\(^{-1}\)), and enhanced the vibrations at band of 731 cm\(^{-1}\) for P. sp. C, P. sp. D, and P. sp. E.

Table 1

<table>
<thead>
<tr>
<th>Isolates(^a)</th>
<th>Closest cultured relative in GenBank (accession number, % similarity)</th>
<th>Closest cultured relative in Biolog database (SIM)</th>
<th>As concentration in the source sample (mg kg(^{-1}))</th>
<th>Source(^b)</th>
<th>As(V) resistance (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Arthrobacter sp. A-1 (EU264108, 98)</td>
<td>No ID</td>
<td>18.9</td>
<td>S</td>
<td>20</td>
<td>400</td>
</tr>
<tr>
<td>B Arthrobacter sp. GOL01 (AY940423, 99)</td>
<td>No ID</td>
<td>18.9</td>
<td>S</td>
<td>20</td>
<td>300</td>
</tr>
<tr>
<td>C Pseudomonas putida isolate Tg (S1275363, 99)</td>
<td>Pseudomonas putida (0.786)</td>
<td>0.171</td>
<td>W</td>
<td>40</td>
<td>500</td>
</tr>
<tr>
<td>D Pseudomonas putida Strain HIB B 1369 (GU186116, 99)</td>
<td>Pseudomonas putida (0.487)</td>
<td>0.171</td>
<td>W</td>
<td>20</td>
<td>160</td>
</tr>
<tr>
<td>E Pseudomonas paoe (DQ536513, 99)</td>
<td>Pseudomonas fluorescens (0.751)</td>
<td>0</td>
<td>W</td>
<td>20</td>
<td>400</td>
</tr>
<tr>
<td>F Pseudomonas sp. MY1106 (EU062808, 99)</td>
<td>Pseudomonas fluorescens (0.567)</td>
<td>0</td>
<td>W</td>
<td>10</td>
<td>160</td>
</tr>
<tr>
<td>G Sphingomonas yabuuchiae (NR038834, 98)</td>
<td>No ID</td>
<td>0.171</td>
<td>W</td>
<td>20</td>
<td>400</td>
</tr>
<tr>
<td>H Acinetobacter calcoaceticus (AM157426, 100)</td>
<td>Acinetobacter calcoaceticus /genospecies (0.611)</td>
<td>0.624</td>
<td>W</td>
<td>20</td>
<td>400</td>
</tr>
</tbody>
</table>

\(^a\) All bacteria cultured were numbered with the letter from A to H.

\(^b\) S, soil sample; W, water sample.

3.3. SERS analysis

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SERS spectra in the region of 530–1750 cm\(^{-1}\) of the cell walls, protoplast, and whole cells of bacteria are shown in Fig. 3. The corresponding band assignment is shown in Table S1. For the same bacteria, we can see that the additive SERS spectra of cell walls and protoplast were not equal to that of the whole cell. Significant differences of different genus bacteria are reflected in the spectra of whole cells, cell walls, and protoplast. The peak intensity of the band at 655 (or 662) cm\(^{-1}\) assigned as COO\(^{-}\) deformation decreases, and the band at 731 cm\(^{-1}\) assigned as adenine and DNA increases in the SERS spectra from protoplast compared with that from the cell walls. For the two Gram-positive bacteria (Arthrobacter sp. A and Arthrobacter sp. B), the peak intensity of the band at 1131 cm\(^{-1}\) assigned as carbohydrates deformation, and 1248 cm\(^{-1}\) as CH\(_2\) deformation in the cell wall increases compared with that in the protoplast.

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Fig. 1. Neighbor-joining tree based on 16S rDNA sequences showing the positions of all the bacteria cultured in our study. Bootstrap values (expressed as percentages of 1000 replications) are shown at the nodes.
Moreover, COO\(^{-}\) deformation (658 cm\(^{-1}\)) increased for *P. s. p. C* and *P. s. F*, decreased for *P. s. D*, in the presence of As(V). For *Sphingomonas* sp. G, both of COO\(^{-}\) deformation (658 and 1388 cm\(^{-1}\)) and flavin derivates or polysaccharides (731 cm\(^{-1}\)) increased. Besides, the addition of As(V) to *P. s. E* resulted in the enhancement of the COO\(^{-}\) deformation (658 cm\(^{-1}\)). In contrast, As(V) had negligible effect on the cell wall of *Acinetobacter* sp. H as evidenced by the SERS spectra (Fig. 5). On the other hand, no significant effect of As(V) on the protoplast spectra was detected (Fig. S2), indicating that As(V) may not enter into the protoplast. The SERS results suggest that As(V) may not enter into the protoplast. The SERS results suggest that As(V) may not enter into the protoplast. The SERS results suggest that As(V) may not enter into the protoplast. The SERS results suggest that As(V) may not enter into the protoplast.

4. Discussion

In this study, we cultured eight strains of As-resistant bacteria existing in both As-laden and As-free environments. They were identified as belonging to four genera capable of tolerating high As concentrations (Chitpirom, et al., 2009; Escalante et al., 2009). Among the four genera, *Pseudomonas* is the most widely reported genus detected in As contaminated soils (Achour et al., 2007; Escalante, et al., 2009; Huang et al., 2010), As-free soils (Jackson et al., 2005), and natural groundwater (Liao et al., 2011).

The MIC of As(III) and As(V) for each bacteria was determined in to be the range 10 to 500 mM; however, these values could not directly compare with other reports because of the differences in growth conditions (Chitpirom, et al., 2009; Jackson, et al., 2005; Liao, et al., 2011; Pepi, et al., 2011). To screen for bacteria that could regulate and change As oxidation states, As speciation was determined using HPLC–AFS. However, no redox transformation of As occurred (results are not shown). In agreement with previous reports, As resistance without a redox-based metabolism is relatively common (Chitpirom, et al., 2009). The As-resistant bacteria in our study may develop As resistance mechanisms by minimizing the amount of As entering the cell (Chowdhury et al., 2009).

FTIR has been demonstrated to be an effective alternative to traditional nutritional and biochemical methods to assess the effects of environmental factors on microbial cells. However, in our study, the effect of As(V) on eight strains of As-resistant bacteria can not be characterized by FTIR. Maybe it’s due to the minor change of the structure of bacteria brought by As(V).

SERS is a recent addition to the physicochemical spectroscopic technologies that have been applied to rapid characterization and identification of microorganisms. SERS and FTIR are complementary techniques, and can provide a highly specific vibrational spectroscopic fingerprint of cells (Maquelin et al., 2002). Ag NPs were used to enhance signals of Raman spectra, and the combination of Ag colloid and bacteria can influence the spectra of bacteria. Nanoparticles can either attach to the outside of the bacterial cell wall or enter the interior of the cell (Walter, et al., 2011), which may result in SERS spectral features from the cell wall alone, and from combinations of cell wall and protoplast. The inhomogenous information may constrain the application of SERS spectra to identify or assess the impact of environmental factors on bacteria. To solve this problem, Angela et al. obtained disrupted bacteria cells using ultrasound (Walter, et al., 2011) so that both cell wall and protoplast were able to associate with Ag NPs for SERS detection. However, this approach...
cannot differentiate which target component, cell wall or protoplast, would respond to the environmental stress. We separated cell walls and protoplast before SERS analysis so that the spectral information of cell walls and protoplast can be decoupled and collected.

Theoretically, the additive spectral information of cell walls and protoplast should be more than that of whole cell. However, it was not (Fig. 3), because in the process of collecting cell wall and protoplast, some components had been destroyed or decomposed. The Gram-positive strains *Arthrobacter* sp. A and *Arthrobacter* sp. B within the same genus exhibit almost the same SERS spectral peak intensities (Fig. 3).

As-resistant microorganisms are referred to multiple genus including *Bacillus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, *Comamonas*, *Enterobacter* (Chitpirom et al., 2009), *Campylobacter* (Wang et al., 2009), *Leptospirillum* (Tuffin et al., 2006), *Aeromonas*, *Aureobacterium*, *Escherichia*, *Micrococcus*, *Rhodococcus*, and *Stenotrophomonas* (Anderson and Cook, 2004; Clausen, 2000). Among these microorganisms, a well known efflux-based resistance mechanism is attributed to the presence of As resistance genes, such as *arsR*, *arsA*, *arsB*, *arsC*, *arsD*, *arsH*, *arsM*, and TnlFars. On the other hand, a wide spectrum of As-resistant microorganisms not involved in the As redox transformation has been isolated from various environmental sources. A common approach to study the As-resistant mechanism is to look for the As resistance genes (Canovas et al., 2003). However, for some bacteria, As detoxification can be the byproduct of normal metabolism, making it difficult to search for functional genes (Huang et al., 2010). From this perspective, cellular structure studies are important in understanding mechanisms of arsenic resistance. Here, the As(V) stress on bacteria was studied with SERS to explore the As-resisting mechanism. The noticeable changes in SERS spectra of cell walls (Fig. 5) in the presence of As(V) indicate that As(V) mainly acts upon the cell walls except in the case of *Acinetobacter* sp. H. Meanwhile, As(V) has negligible effect on protoplast (Fig. S2). This suggested that bacteria may resist elevated concentrations of As(V) because As(V) was mostly sequestered by the cell wall. Binding of metals onto bacteria cells is a well-known phenomenon. It has already been reported that functional groups such as carboxylic, amino, thiol, hydroxyl, and hydroxyl–carboxylic groups present in the bacterial cell wall can interact with metal ions (Mandal et al., 2008). In our study, the changes in SERS spectra upon As(V) attack suggested that As(V) mainly interacts with functional groups such as polysaccharides, flavin derivates, C–N, COO\(^{-}\), and CH\(_2\) on cell walls. However, the molecular-level structure of As(V) with the groups on the cell wall merits further study. The SERS spectra exhibited insignificant changes for As(V) stress on *Acinetobacter* sp. H (Fig. 5). The observation indicated that As(V) might interact with certain groups on the cell wall of *Acinetobacter* sp. H which are SERS-inactive. In conclusion, SERS can be applied to evaluate the effect of As(V) on bacteria structure changes.

![Fig. 4. SERS difference spectra in the region of 530–1750 cm\(^{-1}\) of cell wall of different bacteria with 40 mM arsenate. Note: the spectra were shifted vertically for easy differentiation.](image)

![Fig. 5. SERS spectra of a plot of As(V) exposed spectra minus unexposed spectra in the region of 530–1750 cm\(^{-1}\) of cell wall of different bacteria with added 40 mM arsenate. Note: the spectra were shifted vertically for easy differentiation.](image)
and served as the basic study of As resistant mechanism of bacteria. Compared with FTIR, SERS provides rich information in the preliminary study of bacterial arsenic-resistant mechanisms. The bacteria in our study mainly resist As through sequestering of As by the cell wall.

5. Nucleotide sequence accession numbers


Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jmimet.2012.03.018.

References