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Biodegradation of diphenylarsinic acid to arsenic acid by novel soil bacteria isolated from contaminated soil

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Abstract Microorganisms capable of degrading diphenylarsinic acid (DPAA) were enriched from contaminated soil using the soil-charcoal perfusion method. Two novel bacterial strains, L2406 and L2413, that can degrade DPAA in a mineral salt medium supplemented with DPAA as the sole carbon source were isolated. Based on comparative morphology, physiology, and comparison of the 16S rRNA gene sequences, both were presumed to be species closely related to *Ensifer adhaerens*. As the metabolites, phenylarsonic acid (PAA) was determined by liquid chromatography-mass spectrometry analysis as well as three unknown peaks all of whose molecular weights were estimated to be 278. The increase of $m/z = 16$ from DPAA in the unknowns suggests monohydroxylation of DPAA at the 2-, 3- and 4-positions. The ability of strains L2406 and L2413 to degrade DPAA was suppressed in iron insufficient conditions, e.g. less than 7.2 μM iron in the culture medium. These facts strongly suggest the following hypothesis: Monooxygenase works at the initial degradation step of DPAA degradation by the isolates; and direct hydrolysis from DPAA to PAA is not likely to occur. In addition, release of arsenic acid from PAA by strain L2406 was confirmed by liquid chromatography-inductively coupled plasma mass spectrometry. From these results, strain L2406 was considered to be capable of degrading DPAA to arsenic acid via PAA when DPAA was supplied as the sole carbon source.

Keywords Biodegradation · Diphenylarsinic acid · *Ensifer adhaerens* · LC–MS · Phenylarsonic acid

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Introduction

A large amount of phenylarsenic compounds was produced as chemical warfare agents such as Clark I (diphenylchloroarsine) and Clark II (diphenylcyanoarsine) during World Wars I and II. After the wars, most of these warfare agents were dumped into the sea or buried in the earth in several parts of Europe, China and Japan, and they have been causing water and soil contamination (Kurata 1980; Evans 1997; Glasby 1997; Pitten et al. 1999; Daus et al. 2008). It is known that diphenylchloroarsine and diphenylcyanoarsine can be chemically transformed via hydrolysis and oxidation in the environment.

Consequently, diphenylarsinic acid (DPAA) has often been detected as the major accumulated arsenical intermediate around contaminated sites (Haas et al. 1998; Hanaoka et al. 2005).

In 2002, heavy health hazards to several inhabitants due to DPAA-contaminated drinking well water occurred in Kizaki region of Kamisu City, Japan (Ishii et al. 2004). The environmental contamination of DPAA was attributed to the illegal dumping of DPAA itself. DPAA was also detected in the groundwater used for the irrigation of paddy fields around the contaminated area, and phenylarsonic acid (PAA) and bis(diphenylarsine)oxide (BDPAO) were present at a much lower concentration (Ishizaki et al. 2005). Baba et al. (2008) extracted arsenicals from contaminated paddy field soil collected in the Kizaki region and detected DPAA, PAA, methylphenylarsinic acid (MPAA), dimethylphenylarsine oxide (DMPAO) and diphenylmethylarsine oxide (DPMAO) along with inorganic arsenicals as the main species. More recently, Arao et al. (2009) showed that PAA and MPAA concentrations decreased and DMPAO concentration increased under the flooded conditions in a pot experiment with rice using the contaminated soil, while the concentrations of these species remained unchanged under the upland conditions.

Although the dynamics of phenylarsenic compounds in environmental conditions are being intensively investigated as described above, only a few reports regarding the participation of microorganisms in their transformation are available. Bacterial degradation of organoarsenic warfare compounds has been reported by Köhler et al. (2001), who isolated several bacterial strains capable of degrading triphenylarsine and triphenylarsine oxide. Nakamiya et al. (2007) tried to isolate DPAA-degrading microorganisms from toluene-utilizing ones and obtained *Kytococcus sedentarius* strain NK0508. To our knowledge, strain NK0508 is the only bacterial isolate previously reported to grow using DPAA as the sole carbon source. DPMAO was determined as the transformation product, and *cis*, *cis*-muconate and arsenic acid as the metabolites.

In this study, the authors aim to describe novel DPAA-degrading bacteria enriched from DPAA-contaminated rice field soil using the soil-charcoal perfusion method (Takagi and Yoshioka 2000; Takagi et al. 2009) with a medium containing DPAA as the sole carbon source. From a successfully enriched culture, two novel DPAA-degrading strains were isolated. In addition, the metabolites were estimated by liquid chromatography-mass spectrometry (LC–MS) and liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICPMS) analyses, and a metabolic pathway is proposed.

Materials and methods

Chemicals

DPAA and PAA used for isolation of DPAA-degrading bacteria were purchased from Wako (Osaka, Japan). Analytical standards used in LC-ICPMS analysis were described in Baba et al. (2008).

Growth media

The mineral salt medium (MM) used in this study contained 0.5 g NH_4NO_3, 1.0 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 5 mg $FeSO_4{\cdot}7H_2O$, 10 ml vitamin solution (Yamazaki et al. 2008) and 10 ml trace element solution (500 mg EDTA·2Na, 10 mg $ZnSO_4{\cdot}7H_2O$, 5 mg $MnSO_4{\cdot}H_2O$, 30 mg H_3BO_3, 24 mg $CoSO_4{\cdot}7H_2O$, 5 mg $CuSO_4{\cdot}5H_2O$, 5 mg $Na_2MoO_4{\cdot}2H_2O$ and 50 mg $Ca(OH)_2$ per liter) per liter. MM was supplemented with 20 μM DPAA or 6.3 μM PAA (a final concentration) and autoclaved at 121°C for 20 min. The trace element solution and the vitamin solution were filter-sterilized and added into MM after autoclaving. To examine effect of iron concentrations, MM was supplemented with 0, 0.70, 3.6, 7.2, 18 and 36 μM of $FeSO_4{\cdot}7H_2O$. Difco™ R2A agar medium (Becton, Dickinson and Company, NJ, USA) (Reasoner and Geldreich 1985) was used for isolation and subculturing of the DPAA-degrading bacteria.

Enrichment culture

The soil-charcoal perfusion method (Takagi and Yoshioka 2000; Takagi et al. 2009) was used for the enrichment of DPAA-degrading bacteria. Four alluvial soil samples (RSI–IV, Table 1) were collected in November 2005 from different rice fields in Kamisu, where DPAA-contaminated well water (DPAA concentrations: 1.25, 2.40, 0.48 and 3.60 μM, respectively [Ibaraki Prefecture, Japan 2004]) was

Table 1 Properties of soil samples used in this study

Sample	Soil type	pH(H_2O)	pH(KCl)	Total C [mg-C (g dry weight)$^{-1}$]	Total N [mg-N (g dry weight)$^{-1}$]	C/N
RSI	Alluvial	7.1	6.2	16.0	1.46	11.0
RSII	Alluvial	6.9	6.0	17.9	1.72	10.4
RSIII	Alluvial	6.8	5.9	16.8	1.80	9.3
RSIV	Alluvial	5.9	4.9	10.6	1.01	10.5

supplied for irrigation, and were used as the source of bacteria. After mixing with a small amount of Charcoal A100 (grain size 5–10 mm, BET specific surface area 95–115 m^2 g^{-1}, pH (H_2O) 7.8, Toyo Denka, Kochi, Japan), 30 g dry weight of each wet soil sample was layered on a glass filter placed in the neck part of the perfusion apparatus (Audus 1946). Then, 200 ml of sterile perfusion fluid (0.5 g NH_4NO_3, 0.5 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$ and 5 mg $FeSO_4{\cdot}7H_2O$ per liter) supplemented with 20 or 80 μM DPAA was perfused at 25°C by air lift using an air pump. The perfusion fluid in the apparatus was substituted with fresh medium every 2 weeks. The DPAA concentration in the perfusion fluid was monitored by reverse-phase high performance liquid chromatography (RP-HPLC, described below) to determine enrichment of DPAA-degrading bacteria.

Isolation of bacteria

Isolation of DPAA-degrading bacteria from the enrichment culture was performed by the limiting dilution-culture method. An aliquot of the enrichment culture adequately diluted with sterile saline was subcultured in L-shaped glass tubes (long leg length, 120 mm; short leg length, 70 mm; internal diameter, 16 mm) containing 6 ml of MM supplemented with 20 μM DPAA. After successive Monod's shaking cultivation at 25°C, tubes showing reduction in the DPAA concentrations were determined by RP-HPLC. Single isolates from the tubes were obtained on R2A agar plates at 30°C for several days, and their capabilities to degrade DPAA were examined in MM supplemented with 20 μM DPAA in the L-shaped tube cultures.

Identification of isolates

The isolates were characterized based on comparative studies of morphology and physiology performed in TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan), and comparison of 16S rRNA gene sequences. The known primers named fD1 and rD1 (Weisburg et al. 1991) were used for 16S rRNA gene amplification. The detailed PCR conditions have been described in Harada et al. (2006). Cycle sequencing reactions were then performed using 5′-Texas Red-labeled primers with a Thermo Sequenase Dye Primer Cycle Sequencing Kit with 7-deaza-dGTP (GE Healthcare UK Ltd., Buckinghamshire, UK) (Harada et al. 2006). The cycle sequencing reaction products were analyzed with an SQ5500E DNA sequencer (Hitachi, Tokyo, Japan). The sequences determined were compared with those available in the GenBank database by the FASTA search engine using GENETYX-PDB Ver. 4.0 (Genetyx, Tokyo, Japan).

The partial 16S rRNA gene sequences of the isolates described in the present study and reference sequences of the related species were aligned in ClustalW and edited to be the same length. All gaps and missing data were deleted. Subsequently, neighbor-joining phylogenetic dendrogram was constructed in MEGA 4.0.2 (Tamura et al. 2007) using the Kimura 2-parameter correction and 1,000 bootstrap replications.

Instrumental analysis

The DPAA concentrations in perfusion fluid and culture media were determined by RP-HPLC using HP1100 (Hewlett-Packard, CA, USA) equipped with a Capcell Pak UG120 ODS column (3 × 250 mm, 5 μm particle size; Shiseido, Tokyo, Japan). The column temperature was set at 40°C. The mobile phase was 0.2% acetic acid solution and acetonitrile (1+1 by volume). The injection volume was 10 μl and the flow rate was 0.5 ml min^{-1}. The detection was based on UV absorption at 220 nm.

Intermediate metabolites from DPAA were estimated using an LC–MS (HP1100 LC–MSD, Hewlett-

Packard, CA, USA) equipped with an electrospray chamber in the positive-ion mode. The separation method was the same as above. In the MS part, the drying gas flow rate was set at 12 ml min^{-1}; the nebulizer pressure was 45 psig; the drying temperature was 350°C; the capillary voltage was 3.5 kV; and the fragmenter voltage was 100 V. Production of inorganic arsenic was examined by LC-ICPMS as described in Baba et al. (2008).

Nucleotide sequence accession number

The 16S rRNA gene sequences of strains L2406 and L2413 were deposited in DNA Data Bank of Japan under accession numbers AB513650 and AB513651, respectively.

Results and discussion

Enrichment of DPAA-degrading bacteria

Figure 1a shows changes in the DPAA concentration in the perfusion apparatus using four rice field soil samples (RSI–IV) as bacterial sources. When RSI was supplied, a fast decrease in the DPAA concentration was observed. The rate of reduction of the DPAA concentration was accelerated after the first replacement of perfusion fluid. This phenomenon suggests enrichment of DPAA-degrading bacteria in the apparatus, but the reduction of DPAA was suppressed after the second replacement. In contrast, DPAA concentrations in the apparatus using RSII, III and IV soil samples were relatively stable except initial minor decreases presumably due to adsorption of DPAA to soil and charcoal. Bacterial enrichment was then performed again using RSI as a bacterial source and MM supplemented with 20 or 80 μM DPAA. Significant decreases in the DPAA concentration were again observed, particularly in MM supplemented with 20 μM DPAA, compared with the perfusion apparatus supplied with RSIV soil as a negative sample (Fig. 1b). In this attempt, the rate of DPAA reduction was fastest in the first perfusion period, and gradually decreased by replacement of perfusion fluid. The activity of DPAA-degrading bacteria may be partially inhibited by more toxic arsenic metabolites, as Köhler et al. (2001) demonstrated that the degradative capacity of bacteria against triphenylarsine oxide did not correlate with

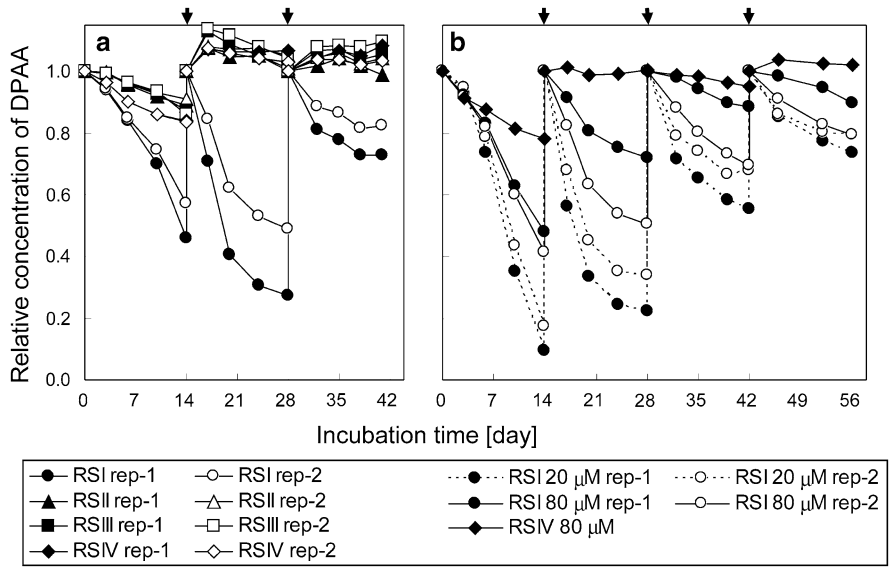

Fig. 1 Changes in DPAA concentration during enrichment of DPAA-degrading bacteria using a soil-charcoal perfusion system relative to that in the fresh perfusion fluid. *Arrows* indicate the time points of replacement of perfusion fluid. **a** First enrichment trial using four soil samples collected from DPAA-contaminated rice fields (RSI–IV) as bacterial sources. The initial DPAA concentration in perfusion fluid was adjusted to be 20 μM DPAA. The enrichments were performed in duplicate (indicated as rep-1 and rep-2) for each soil sample. **b** Second enrichment trial using RSI soil with two initial DPAA concentrations (20 and 80 μM, in duplicate). Enrichment with RSIV soil and 80 μM DPAA was also performed as a negative sample

high resistance against arsenite and arsenate ions. According to these results, DPAA-degrading bacteria were concluded to be enriched in the apparatus supplied with the RSI soil sample, and subsequent bacterial isolation from soil-charcoal layers in the apparatus was carried out. After repeating isolation of bacteria using R2A agar plates and performing assays of their degrading capability in MM supplemented with DPAA, two DPAA-degrading strains, L2406 and L2413, were obtained.

Identification of the isolated strains

The morphological and physiological characteristics of the isolates L2406 and L2413 were very similar. They belong to a species of aerobic, motile, Gram-negative, catalase-positive, oxidase-positive, non-spore-forming and rod-shaped bacteria which form cream circular colonies on nutrient agar at 30°C for 24 h. Cell sizes of strains L2406 and L2413 were 0.7–0.8 × 1.5–2.0 μm in an R2A medium. Only the consistency of the colonies differed: Strains L2406 and L2413 showed butter-like and viscid colonies, respectively, on nutrient agar. Sequence analyses of the isolates L2406 and L2413 determined respective 1,101 and 1,276 bp of the 16S rRNA genes. In comparison with the sequences available in the GenBank database by the FASTA, the gene sequences of strains L2406 and L2413 had 99.6 and 99.5% similarity to those of *Sinorhizobium morelense* strain Lc04 (AY024335) and *Ensifer adhaerens* strain LMG 20216^T (AM181733), respectively.

The phylogenetic dendrogram based on the 16S rRNA gene sequences of L2406, L2413 and related species of *Sinorhizobium* and *Ensifer* is presented in Fig. 2. Considering recent taxonomical consolidation of the genera *Sinorhizobium* and *Ensifer* (Judicial Commission of the International Committee on Systematics of Prokaryotes 2008), both strains L2406 and L2413 are presumed to be species closely related to *Ensifer adhaerens*, although a few differences in their 16S rRNA gene sequences were determined.

Metabolic pathway of DPAA

Strain L2406 was cultured in MM supplemented with 20 μM DPAA for 14 days (25°C). The culture medium was directly analyzed by LC–MS after centrifugation. As shown in Fig. 3a, the total ion chromatograph (TIC) obtained by scanning between m/z 100 and 350 displayed a peak of DPAA at $t_R = 5.4$. The mass spectrum of the peak showed the $[M+H]^+$ ion at m/z 263 (Fig. 3c), which is a molecular-related ion of DPAA. Three unknown peaks also appeared on the TIC, at $t_R = 4.0$, 4.3, and 4.6 (Unknown I, II, and III, respectively). The mass spectra of the three unknown peaks all showed $[M+H]^+$ ions at m/z 279 (Fig. 3e–g). The increase of

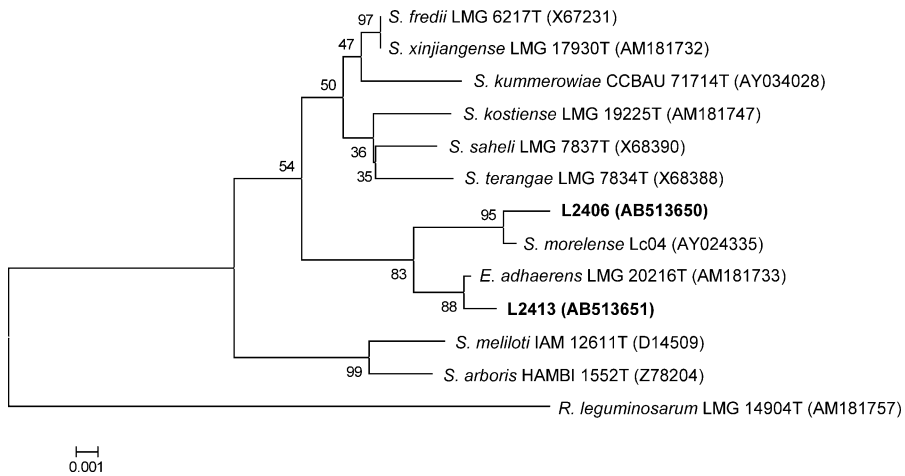

Fig. 2 Phylogenetic dendrogram based on 16S rRNA gene sequences of strain L2406, strain L2413 and the related *Sinorhizobium* and *Ensifer* species. The GenBank accession numbers are provided in *parentheses*. The *numbers* at the branch points are bootstrap values based on 1,000 trials. The *bar* indicates 1 nucleotide substitutions per 1,000 nucleotide positions. The *Rhizobium leguminosarum* strain LMG 14904^T 16S rRNA gene sequence (accession number AM181757) was used as an outgroup

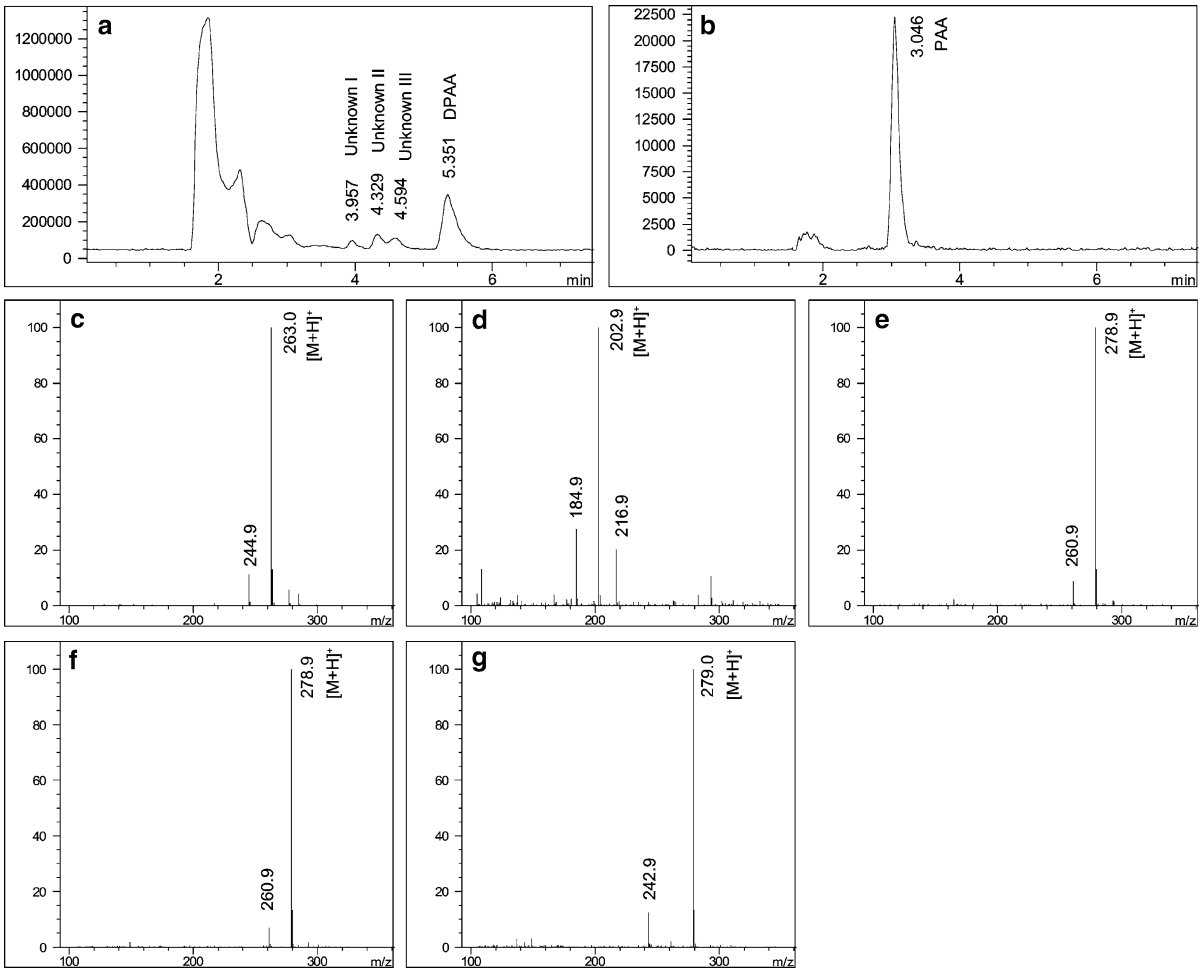

Fig. 3 LC-MS analysis of the metabolites of DPAA by strain L2406 in the mineral salt medium. The 14-day culture medium was directly analyzed after centrifugation. **a** Total ion chromatogram obtained by scanning between m/z 100 and 350. **b** Selected ion monitoring at m/z 203. (**c**–**g**) Mass spectra of DPAA, PAA and the unknown peaks I, II and III, respectively

m/z = 16 from that of DPAA in the unknowns strongly suggests monohydroxylation of DPAA at the 2-, 3- and 4-positions. In addition, selected ion monitoring at m/z 203 indicated formation of PAA in the culture medium (Fig. 3b, d). This fact indicates that elimination of a phenyl group occurs due to hydrolysis. These metabolites were also detected in the culture medium of strain L2413 (data not shown).

It is generally known that monohydroxylation is mediated by monooxygenase. Possession of monooxygenases by Rhizobiaceae species including *Ensifer* has been reported, although their functions vary (Keister et al. 1999; Kühn et al. 2006). Since monooxygenase is a hemoprotein, iron concentration in growth medium is likely to affect the activity, as Dinkla et al. (2001) showed that activity of toluene monooxygenase in *Pseudomonas* strains decreased under iron insufficient conditions. In the present study, the effects of iron concentration on degradation of DPAA by strains L2406 and L2413 were then investigated at 0–36 μM iron in 6 ml of MM containing 20 μM DPAA as the sole carbon source. Cultivation was performed for 7 days using L-shaped tubes. As a result, degradation of DPAA by the strains depended on iron concentrations between 0 and 7.2 μM (Fig. 4). In the cultures supplemented with 7.2 μM or more iron, the acceleration effect was no longer observed. This fact strongly suggests the

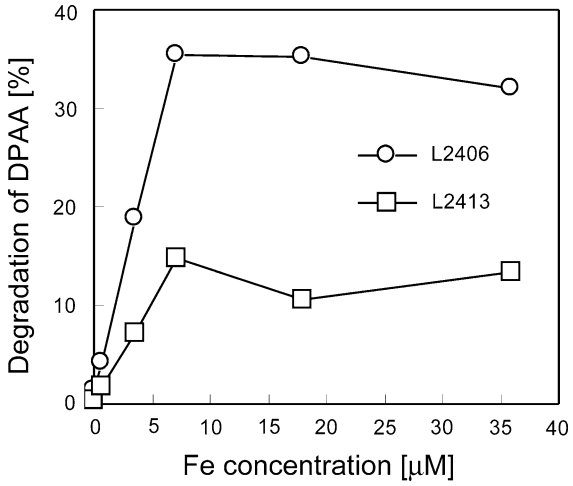

Fig. 4 Degradation rates of DPAA by strains L2406 and L2413 after 7-day cultivation in MM containing different iron concentrations. Each value corresponds to the average of duplicate analyses

following hypothesis: Monooxygenase works at the initial step of DPAA degradation by the isolates; and direct hydrolysis from DPAA to PAA is not likely to occur.

Changes in the concentrations of DPAA and the metabolites (PAA and the sum of the three unknowns) in the L2406 and L2413 culture media initially supplemented with 20 μM DPAA are presented in Fig. 5. The concentrations of the unknowns were speculated by calculation using the peak area of DPAA as the reference in TICs. The capability of strain L2406 to degrade DPAA (Fig. 5a) was superior to that of strain L2413 (Fig. 5b) in MM. During the cultivation, the DPAA concentration continuously decreased, while accumulation of PAA was likely to plateau. PAA seems to be one of the intermediate products and can be converted into other arsenic species. Recovery rates of arsenic in the 4-week cultures with strains L2406 and L2413 were calculated to be 77 and 96%, respectively.

The strain L2406 was, therefore, inoculated into MM supplemented with 6.3 μM PAA as the sole carbon source and changes in the PAA concentration were monitored by LC-MS. The concentration of PAA decreased to 5.0 and 3.8 μM after 2 and 4 weeks of incubation, respectively. Furthermore, LC-ICPMS analysis to determine arsenical species in the 2-week culture medium was conducted and formation of arsenic acid was determined (Fig. 6). These facts clearly indicate that strain L2406 is capable of degrading DPAA to arsenic acid via PAA when DPAA is supplied as the sole carbon source. Arsenic acid concentration in the 2-week culture medium was 0.9 μM, meaning that the recovery rate of arsenic was calculated to be 94%. The proposed metabolic pathway of DPAA by the strain L2406 is shown in Fig. 7. Although strain L2413 was also examined similarly, degradation of PAA was slight and release of arsenic acid was not confirmed.

Release of arsenate from phenylarsenic compounds by microorganisms has rarely been reported. As regards bacterial species which can transform DPAA to arsenic acid in aerobic conditions, strain L2406 is the only isolate reported other than *Kytococcus sedentarius* strain NK0508 obtained by Nakamiya et al. (2007). They determined DPMAO as

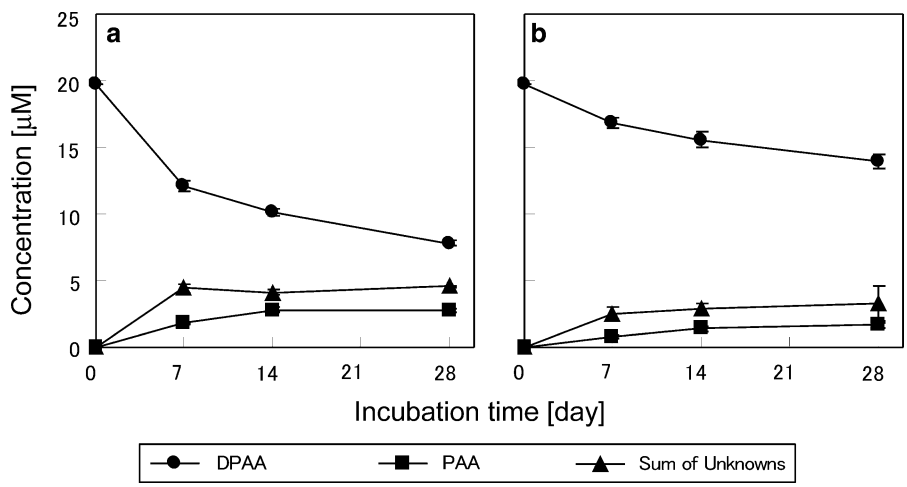

Fig. 5 Changes in concentration of DPAA, PAA and the sum of the three unknowns in the **a** L2406 and **b** L2413 culture media. The concentration of the unknowns was speculated by calculation using the peak area of DPAA in the total ion chromatograms as the reference. Each *point* and *bar* corresponds to the average of triplicate analysis ± SD

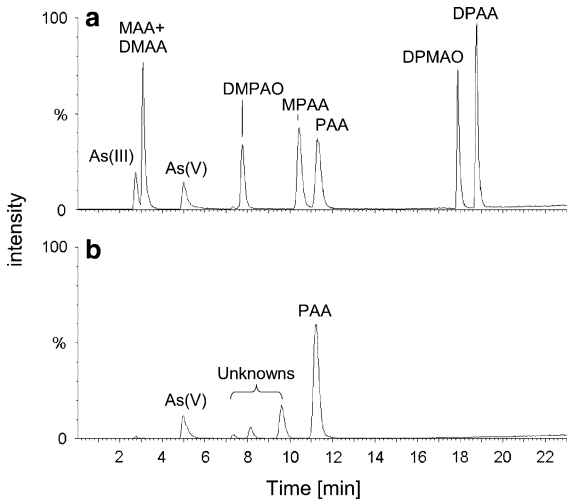

Fig. 6 LC-ICPMS chromatograms obtained from; **a** the standard solution containing 20 mg l^{-1} of each of arsenious acid (As(III)), monomethylarsonic acid (MAA), dimethylarsinic acid (DMAA), arsenic acid [As(V)], dimethylphenylarsine oxide (DMPAO), methylphenylarsinic acid (MPAA), PAA, diphenymethylarsine oxide (DPMAO) and DPAA; and **b** the 2-week culture medium of strain L2406 in MM supplemented with 6.3 μM PAA as the sole carbon source

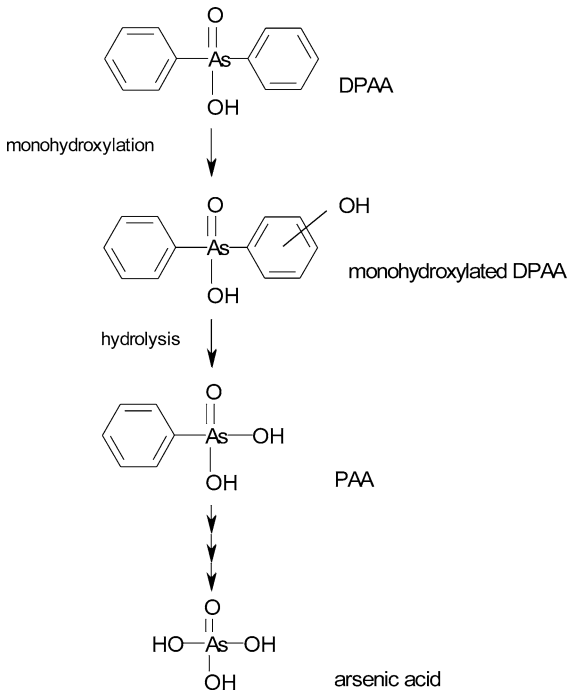

Fig. 7 Proposed metabolic pathway of diphenylarsinic acid (DPAA) by the strain L2406 isolated in this study

the transformed product, and *cis*, *cis*-muconate as the metabolite in addition to arsenic acid, but did not detect hydroxyl DPAA and PAA determined in the present study. In a microcosms study using a percolation apparatus, release of arsenous acid, arsenic acid and soluble organoarsenic compounds from organoarsenic warfare agent contaminated soil was observed (Köhler et al. 2001). Under anaerobic conditions, Stolz et al. (2007) demonstrated rapid biotransformation of 3-nitro-4-hydroxybenzene arsonic acid (roxarsone), a phenylarsenic animal-feed additive, by *Clostridium* sp. strain OhILAs. The subsequent main products were 3-amino-4-hydroxybenzene arsonic acid and inorganic arsenic. Further investigations into enzymatic or genetic aspects of bacterial release of inorganic arsenic from phenylarsenicals are needed for understanding the environmental fates of phenylarsenic compounds.

The present study highlights two novel strains L2406 and L2413 isolated as bacterial strains capable of degrading DPAA. The new isolates provide an opportunity to investigate a possible application in remediation strategies for environmental contamination with phenylarsenic compounds.

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