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# Transformation of Organoarsenical Species by the Microflora of Freshwater Crayfish

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A study of the transformation of arsenic species by the microflora of the freshwater crayfish *Procambarus clarkii* was carried out. The study of the degradation of AB (arsenobetaine) was performed in aerobic conditions in two culture media (tryptic soy broth and saline medium) at two temperatures (30 and 8 °C). The microflora transformed AB into TMAO (trimethylarsine oxide), DMA (dimethylarsinate), MA (methylarsonate), and an unidentified compound (U1). The quickest transformations were carried out by microflora from hepatopancreas incubated in saline medium at 30 °C. The individualized study of other arsenic species [AC (arsenocholine), TETRA (tetramethylarsonium ion), TMAO, DMA, and MA] was also performed in saline medium. The only transformation observed was of AC into AB. The bacteria possibly responsible for AB degradation were isolated, identified by phenotypic and genotypic methods, and individually assayed for AB transformation. Only isolates allocated to the species *Pseudomonas putida* were able to metabolize AB.

**KEYWORDS: Arsenic species; arsenobetaine; crayfish; *Pseudomonas***

## INTRODUCTION

The degradation of arsenic species by the action of bacterial flora has been considered by various authors. The studies carried out on arsenobetaine (AB) biotransformation are the most numerous. At ambient temperature, this arsenic species can be transformed into arsenic species with a lower degree of methylation as a result of the action of microbes associated with seawater (*1*), marine sediments (*2*), seaweed (*3*), and animal tissues (*4*−*6*). This degradation varies in rapidity and the proportion of metabolites generated in accordance with the provenance of the flora isolated and the conditions employed in the assay (culture medium, aerobiosis or anaerobiosis, and temperature). The degradation pathway proposed starts with the cleavage of the carboxymethyl−arsenic bond of AB, generating trimethylarsine oxide (TMAO), with subsequent cleavage of methyl−arsenic bonds to form dimethylarsinate (DMA) and methylarsonate (MA). The biodegradation process ends with the formation of inorganic arsenic [AB → TMAO → DMA → MA → As(III)/As(V)] (*7*).

Recently, work done by Khokiattiwong et al. (*8*) has shown an alternative degradation pathway for AB, which begins with the cleavage of methyl−arsenic bonds, generating dimethylarsenoyl acetate (DMAA) [$Me_2As(O)CH_2COO^-$]. This metabolite had not previously been reported in studies on AB degradation processes. The assay, performed with a natural microbial population of marine origin (microbes associated with crab and/or seawater), shows that the AB degradation process is quick and complete, which explains the apparent absence of AB in marine waters. This degradation has subsequently been confirmed by Jenkins et al. (*9*).

The degradation of the other species of arsenic has been studied to a lesser extent. In aerobic conditions, studies have shown the degradation of arsenocholine (AC) via AB (*7*) or DMAA (*8*) to less methylated species. Other species such as TMAO, DMA, and MA are degraded with different conversion patterns under aerobic and anaerobic conditions (*7*). Species of greater complexity such as trimethylarsonioriboside (*10*), dimethylarsinoylethanol, and DMAA (*8*) are also biotransformed.

Little research has been done to identify the bacteria responsible for these transformations. Cullen and Reimer (*11*) indicated that certain Gram-negative psychrophilic bacteria (*Pseudomonas*, *Xanthomonas*, and *Achromobacter*) might be involved in biotransformation. Hanaoka and co-workers isolated various strains of the *Vibrio-Aeromonas* genera from marine sediment, which were capable of transforming AB to DMA under aerobic and anaerobic conditions (*7*, *12*). However, other strains of *Vibrio-Aeromonas* genera isolated from sediments were incapable of degrading AB (*13*). Therefore, identification at genus, species, and even strain level could be crucial for ascertaining degradative capability. Future studies in this interesting area should focus on isolation of the specific organisms responsible for the various biotransformations observed (*8*). The most important advance so far along these lines was achieved recently by Jenkins et al. (*9*), who identified

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*Paenibacillus* 13943 and *Pseudomonas* 13944 as strains capable of degrading AB to DMAA and subsequently to DMA.

The present paper reports a study on the effect that psychrotrophic and psychrophilic microflora may have on the degradation of arsenic species, with special emphasis on AB biotransformation. The microflora were isolated from red swamp crayfish *Procambarus clarkii*, a species whose commercial exploitation has generated a substantial food industry in the south of Spain. The bacterial strains possibly responsible for the transformations of arsenical species were isolated, identified, and individually confirmed as active AB-degrading strains.

## MATERIALS AND METHODS

**Arsenic Compounds.** A commercial standard solution of As(V) was used (Merck). The stock standard solutions of organoarsenical species were prepared by dissolving appropriate amounts of commercially available salts in deionized water (18.2 MΩ cm): MA (Carlo Erba, Milano, Italy), DMA (Fluka Chemika Biochemika, Madrid, Spain), AB (Hot Chemical Co., Tokyo, Japan), AC (Hot Chemical Co.), TMAO (Hot Chemical Co.), and tetramethylarsonium ion (TETRA; Hot Chemical Co.).

**Bacterial Strains and Culture Media.** The following strains provided by the Colección Española de Cultivos Tipo (CECT) were used as references: *Pseudomonas putida* 385, 4584, and 324 and *Pseudomonas fluorescens* 378. The bacterial strains characterized in this study and able to degrade AB have been deposited in the CECT. A tryptic soy broth (TSB; Scharlau Chemie, Barcelona, Spain) or TS agar (TSA; Scharlau Chemie, Spain) was used for bacterial counts, enrichment, and routine growth of the crayfish microflora. The assays of the transformation of arsenical species were performed in two media: saline medium and TSB. The saline medium consisted of 3% (m/v) NaCl (Panreac, Barcelona, Spain), 0.03% (m/v) $CaCl_2 \cdot 6H_2O$ (Panreac), 0.03% (m/v) KCl (Panreac), 0.001% (m/v) $FeCl_3$ (Scharlau Chemie), 0.05% (m/v) $KH_2PO_4$ (Panreac), 0.1% (m/v) $K_2HPO_4$ (Panreac), 0.05% (m/v) $MgSO_4 \cdot 7H_2O$ (Scharlau Chemie), and 0.1% (m/v) $NH_4Cl$ (Sigma, Sigma-Aldrich, Steinheim, Germany) in water adjusted to pH 7.5 with 4.5% (m/v) NaOH and 20% (v/v) HCl (*14*). In saline medium, arsenic species act as the sole carbon source for bacteria.

**Transformation of Organoarsenical Species by Total Crayfish Microflora.** The *P. clarkii* crayfish used in this study were obtained from the Fishing Brotherhood of the Albufera nature park (Valencia), where this product is distributed for marketing. After acquisition, the crayfish were stored at refrigeration temperatures (3−4 °C) for 3 days. The crayfish were then divided into three parts: hepatopancreas, tail, and remaining parts of the body (head and outer covering). Bacterial flora from these parts were extracted with 0.1% peptone water (Scharlau Chemie), using a stomacher (IUL Instruments, Barcelona, Spain). To enrich the total initial crayfish microflora, 5 mL of TSB was inoculated with the initial extracts obtained in peptone water and incubated for 4 days at 8 and 30 °C.

For degradation assays, a 0.5 mL aliquot of each enriched growth culture (tails, hepatopancreas, and remaining parts) was inoculated into 9.5 mL of fresh culture medium (TSB and/or saline medium) containing standards of arsenic species. The AB concentration employed was 10 $\mu$g mL$^{-1}$ as As, and the concentrations of the other arsenic species (DMA, MA, TMAO, AC, and TETRA) were 4 $\mu$g mL$^{-1}$ as As. The mixtures were incubated at 8 and 30 °C for a maximum time of 24 days. The various experiments that were carried out in the present study are shown in **Table 1**. As process blanks, noninoculated culture media containing the various arsenic species in the same concentrations as in the inoculated media were incubated at the same conditions as the samples.

**Arsenic Species Determination.** Aliquots of each culture medium (**Table 1**) were centrifuged (12000 rpm, 5 min; Heraeus Biofuge Pico centrifuge, Madrid, Spain), and the supernatant was filtered (0.45 $\mu$m) and analyzed using the methodology described by Súñer et al. (*15*). The species were separated by high-performance liquid chromatography (HPLC; Hewlett-Packard model 1100, Barcelona, Spain) using a column-switching system between a Hamilton PRP-X100 anionic exchange column and a Hamilton PRP-X200 cationic exchange column (both 10 $\mu$m, 250 mm × 4.1 mm i.d.; Teknokroma, Barcelona, Spain). The sample was injected onto the PRP-X200 column, and by using the switching valve (*15*), it was possible to separate DMA, MA, AB, and As(V) in the PRP-X100 column and TMAO, TETRA, and AC in the PRP-X200 column. The eluates of each column were quantified independently. Once the outlet of the PRP-X100 column had been thermo-oxidized, it was quantified by hydride generation atomic absorption spectrometry (HG-AAS; Perkin-Elmer 5000, Germany), whereas the PRP-X200 eluate was thermo-oxidized and quantified by HG-atomic fluorescence spectrometry (AFS; PSA 10.044 Excalibur PS, Analytical, Kent, England). Instrumental and analytical conditions for HPLC, HG-AAS, and HG-AFS are described in **Table 2**.

**Table 1.** Degradation Assays of Arsenic Species by Isolated Crayfish Microflora

| arsenic species added | $\mu$g mL$^{-1}$ as As | origin of flora | culture medium | incubation (°C) |
|---|---|---|---|---|
| AB | 10 | tail, hepatopancreas, remaining parts | TSB medium and saline medium | 30 and 8 |
| MA<br>DMA<br>TMAO<br>AC<br>TETRA | 4 | tail, hepatopancreas | saline medium | 30 and 8 |

Duplicate analyses were performed for each sample. The quantification was performed using aqueous standard calibration curves, given that there were no significative differences between the slopes of the calibration curves of aqueous standards and standards prepared with culture medium. The identification of peaks was done by comparison with the retention time of the standards and was confirmed by coinjection of standards. The results for time zero, i.e., the analysis of the medium performed immediately after inoculating the enrichment broth, established the concentrations of the species—other than those added—that existed in the matrix prior to the transformation study. The limits of detection were as follows: AB, 3.6 ng g$^{-1}$ dw; MA, 3.3 ng g$^{-1}$ dw; TETRA, 2.7 ng g$^{-1}$ dw; AC, 2.4 ng g$^{-1}$ dw; and TMAO and DMA, 0.9 ng g$^{-1}$ dw.

**Identification of the Bacterial Strains Possibly Involved in AB Degradation.** *Isolation of Bacterial Strains*. The reaction mixtures that showed AB degradation were pooled, and aliquots were plated in TSA and incubated at 30 °C for 48 h. Isolated colonies were selected from high dilution TSA plates, inoculated in fresh TSB, and incubated at 30 °C. The isolates were further submitted to a phenotypic and genotypic characterization and individually assayed for AB degradation as described below.

*Phenotypic Characterization*. All isolates were initially characterized by studying their morphology under Gram staining and catalase and oxidase production. The isolates were further identified by API 20 NE (BioMérieux, Lyon, France), in accordance with the manufacturer's instructions.

*Genotypic Characterization.* The identity of the isolates that showed AB transformation capability was confirmed by randomly amplified polymorphic DNA (RAPD)-polymerase chain reaction (PCR) analysis, amplified ribosomal DNA restriction analysis (ARDRA), and 16S rDNA sequencing, as described below.

*DNA Preparation.* Genomic DNA was extracted and purified using the cetyltrimethylammonium bromide method described by Wilson (*16*).

*RAPD-PCR Analysis*. The following primers were used to discriminate among different strains: OPL-01 (5′ GGCATGACCT 3′) and OPL-02 (5′ TGGGCGTCAA 3′) (*17*). PCR amplifications were carried out according to Saez et al. (*18*) in a Progene thermocycler (Techne, Cambridge, United Kingdom). The amplified DNA products were separated by electrophoresis on 1% (w/v) agarose gels (Pronadisa, Madrid, Spain) in 0.5× TBE buffer and stained with ethidium bromide (0.5 $\mu$g mL$^{-1}$; Sigma-Aldrich).

*ARDRA*. One isolate from each genotype defined by RAPD-PCR analysis was further characterized by restriction analysis of the 16S

**Table 2.** Instrumental and Analytical Conditions for HPLC-Thermo-oxidation-HG-AFS and HG-AAS

| | |
|---|---|
| | HPLC |
| cationic column | Hamilton PRP-X200 |
| anionic column | Hamilton PRP-X100 |
| mobile phase | cationic column<br>A: 100 mmol $L^{-1}$ [$(NH_4)H_2PO_4$], pH 4.5<br>B: deionized water<br>gradient program:<br>0–4 min: 10% A and 90% B<br>4–11 min: 40% A and 60% B<br>11–20 min: 10% A and 90% B<br>anionic column<br>C: 1 mmol $L^{-1}$ [$(NH_4)H_2PO_4$], pH 9.3<br>D: 20 mmol $L^{-1}$ [$(NH_4)H_2PO_4$], pH 9.3<br>gradient program:<br>0–5 min: 100% C<br>5.5–11 min: 100% D<br>12–20 min: 100% C |
| column switching valve | position 1: columns were not directly connected<br>position 2: direct connection between the two columns<br>$t$ = 0–1 min; position 1<br>$t$ = 1–4 min; position 2<br>$t$ = 4–20 min; position 1 |
| injection volume | 100 $\mu$L in column PRP-X200 |
| flow rate | 1 mL $min^{-1}$ |
| temperature | 10 °C |
| | Thermo-oxidation |
| oxidant | 1.29% (m/v) $K_2S_2O_8$ in 2.5% (m/v) NaOH; 1 mL $min^{-1}$ flow rate |
| reaction coil | 3 m × 0.3 mm i.d. |
| bath temperature | 155 °C |
| | HG-AFS |
| reducing agent | 1.5% (m/v) $NaBH_4$ in 0.7% (m/v) NaOH; 2.5 mL $min^{-1}$ flow rate |
| HCl solution | 1.5 mol $L^{-1}$; 6.0 mL $min^{-1}$ flow rate |
| carrier gas | argon; 300 mL $min^{-1}$ flow rate |
| dryer gas | air: 2.5 l $min^{-1}$ flow rate |
| hydrogen flow rate | 60 mL $min^{-1}$ |
| resonance wavelength | 193.7 nm |
| primary current | 27.5 mA |
| boost current | 35.0 mA |
| | HG-AAS |
| reducing agent | 1.25% (m/v) $NaBH_4$ in 0.7% (m/v) NaOH; 2.0 mL $min^{-1}$ flow rate |
| HCl solution | 3.25 mol $L^{-1}$; 2.0 mL $min^{-1}$ flow rate |
| carrier gas | argon; 45 mL $min^{-1}$ flow rate |
| wavelength | 193.7 nm |
| spectral band-pass | 0.7 nm |
| lamp power | 8.5 W (electrodeless discharge lamp) |

ribosomal DNA (rDNA). The 16S rDNAs were amplified by PCR using the eubacterial primers 27f and 1522r, according to Johnson (*19*). The amplified products were extracted from a 1% agarose gel and purified using GFX PCR DNA and the gel band purification kit (Amersham Pharmacia, Biotech Inc., Piscataway, NJ). The purified products were digested with *Hinf*I and *Alu*I (Roche Biochemicals, Mannheim, Germany) in accordance with the manufacturer's instructions (*20*). The digested products were analyzed in 3% high-resolution agarose gels (Ecogen, Barcelona, Spain) as described above.

*16S rDNA Sequencing*. The sequences of the purified 16S rDNA products were analyzed in an automated sequencer (ADN ABI 3700, Applied Biosystem, Foster City, CA), using the primers 27f, 685r, 530r, and 1220r (*19*). To determine the closest relatives of the partial 16S rDNA sequences retrieved, search analyses were conducted in GeneBank using the BLAST algorithm (*21*).

*Nucleotide Sequence Accession Numbers*. The partial 16S rDNA sequences of the representative isolates Q1, M, and L are deposited in GenBank under accession numbers AY450555, AY450556, and AY450557.

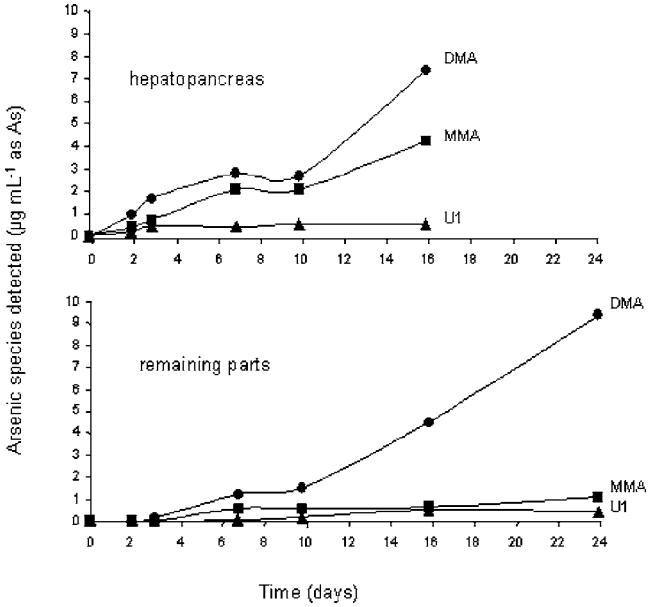


**Figure 1.** Arsenic species detected after AB conversion (10 $\mu$g $mL^{-1}$ as As) in saline medium at 30 °C by microflora from hepatopancreas and remaining parts of crayfish.

**Transformation of Organoarsenical Species by Individual Bacterial Isolates.** Cells were first grown in TSB at 30 °C until the stationary growth phase. The cells were then harvested by centrifugation (5000*g*, 10 min), washed in 0.085% NaCl solution, and suspended in the same solution to reach a final $OD_{660}$ of about 2. To evaluate the ability of each isolate to degrade AB, each cellular suspension (0.4 mL) was inoculated in saline medium (3.8 mL) containing 5 $\mu$g $mL^{-1}$ AB. The reaction mixtures were incubated at 30 °C for 21 days. Samples were taken at different times (0, 7, 14, and 21 days) during the incubation period to determine changes in viable population density of each strain. For this purpose, serial decimal dilutions were done in 0.1% tryptone water, plated in TSA, and incubated at 30 °C for 48 h. The corresponding samples were also taken to evaluate the transformation of different arsenic species as described above. In parallel, a control sample in which the inoculum was replaced by sterile water was monitored.

## RESULTS

**Transformation of AB by Total Crayfish Microflora.** TMAO, DMA, MA, and an unknown compound (U1) were the species detected in the culture supernatants generated by microbial transformation of AB (**Figures 1**−**3**) in the various experiments carried out (**Table 1**). Compound U1 was retained in the cation exchange column, with a retention time that did not coincide with that of any of the standards available in the laboratory (**Figure 4**).

In saline medium at 30 °C (**Figure 1**), after 16 days of incubation in the presence of hepatopancreas microflora, and after 24 days of incubation in the presence of microflora from the remaining parts, the sum of the metabolites obtained represented a value close to the initial quantity of AB in the medium (10 $\mu$g $mL^{-1}$, as As), so that it was considered that the AB had been completely degraded. The tail microflora did not give rise to AB degradation at 30 °C (data not shown). DMA, MA, and U1 were the major species, whereas TMAO was only detected up to day 7 and in concentrations below 0.100 $\mu$g $mL^{-1}$, as As.

In saline medium at 8 °C (**Figure 2**), transformation of AB was brought about by microflora of hepatopancreas, tail, and remaining parts. Complete transformation was not observed in any of the samples after 24 days of incubation. In all of the

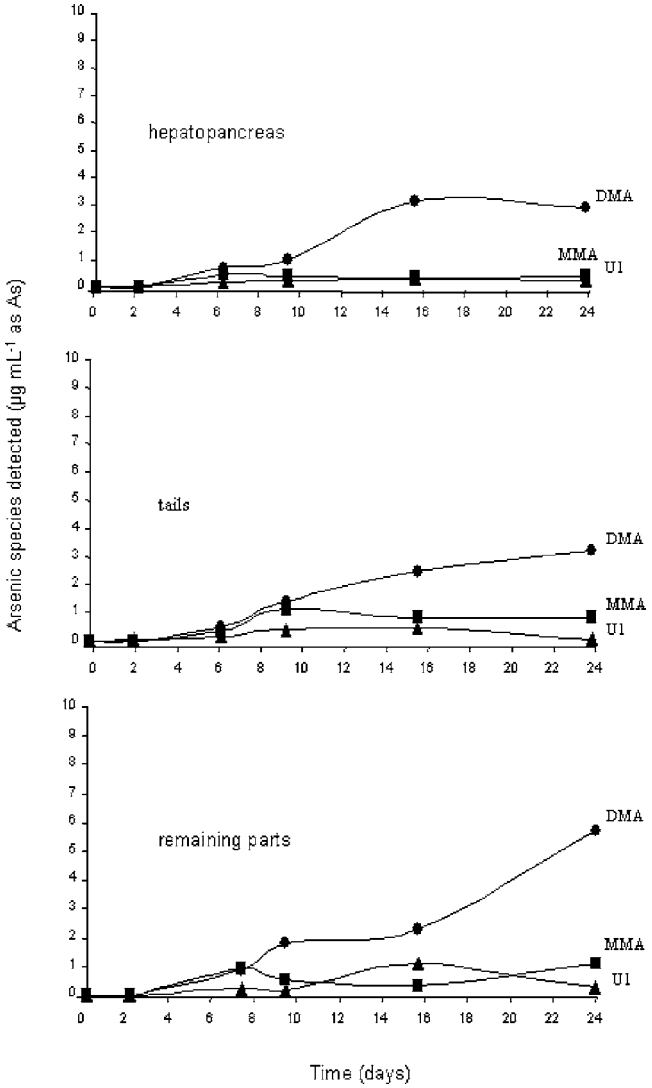


**Figure 2.** Arsenic species detected after AB conversion (10 $\mu$g mL$^{-1}$ as As) in saline medium at 8 °C by microflora from hepatopancreas, tails, and remaining parts of crayfish.

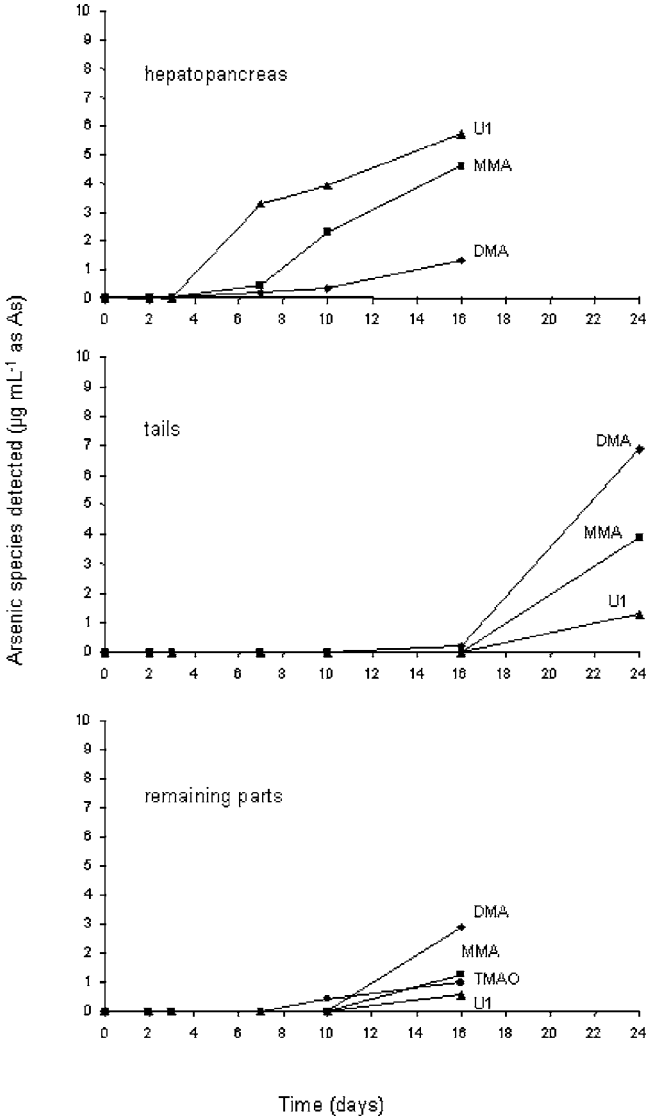


**Figure 3.** Arsenic species detected after AB conversion (10 $\mu$g mL$^{-1}$ as As) in TSB medium at 30 °C by microflora from hepatopancreas, tails, and remaining parts of crayfish.

conditions, the concentration of the metabolites generated followed the same order at 8 and 30 °C (DMA > MA > U1). TMAO was only detected in hepatopancreas and remaining parts at times less than 7 days and in concentrations below 0.050 $\mu$g mL$^{-1}$, as As.

In TSB medium at 30 °C (**Figure 3**), the AB was completely transformed in tails and hepatopancreas. DMA, MA, and U1 were the species detected in hepatopancreas and tails, whereas in remaining parts TMAO was also detected. In tails and remaining parts, the concentration of the metabolites generated followed the same order as that found in saline medium: DMA > MA > U1. In hepatopancreas, however, the order was U1 > MA > DMA, with U1 attaining a very high concentration (5.7 $\mu$g As mL$^{-1}$). When incubation was carried out in TSB medium at 8 °C, no significant degradation was observed in any of the samples corresponding to each part of the crayfish 24 days after inoculation (data not shown).

**Transformation of Other Arsenic Species by Total Crayfish Microflora.** The biodegradation of AC, TETRA, TMAO, DMA, and MA by the tail and hepatopancreas microflora was studied in saline medium at 8 and 30 °C (**Table 1**). The results obtained show that AC was the only arsenic species in which transformation had taken place after 24 days of incubation, generating AB as the only metabolite. The transformation of AC into AB occurred in samples corresponding to tails and hepatopancreas, and it began rapidly at both temperatures assayed, after 24 h of incubation (**Figure 5**). The formation of AB increased progressively with time until the added AC was no longer detected, which occurred on day 24 for the cultures incubated at 30 °C and on day 16 for those incubated at 8 °C. In the latter case, from day 16 onward, there was a decrease in the concentration of AB, which did not coincide with the appearance of arsenic species with a lower degree of methylation.

**Isolation and Identification of AB-Degrading Bacterial Strains from Crayfish.** For identification of the crayfish microflora possibly involved in AB degradation, samples were taken from the pooled reaction mixtures where AB transformations were previously demonstrated and were plated in TSA. After incubation at 30 °C for 48 h, a total of 11 colonies were isolated from the highest dilution plates as representatives of the microflora (**Table 3**). The isolates were initially identified on the basis of their morphology, Gram-staining properties, and biochemical tests. All were Gram-negative and oxidase positive rods and, therefore, identified at species level by the API20 NE system. In accordance with the phenotypic profiles obtained, the isolates were assigned to the species *P. putida* (five isolates),

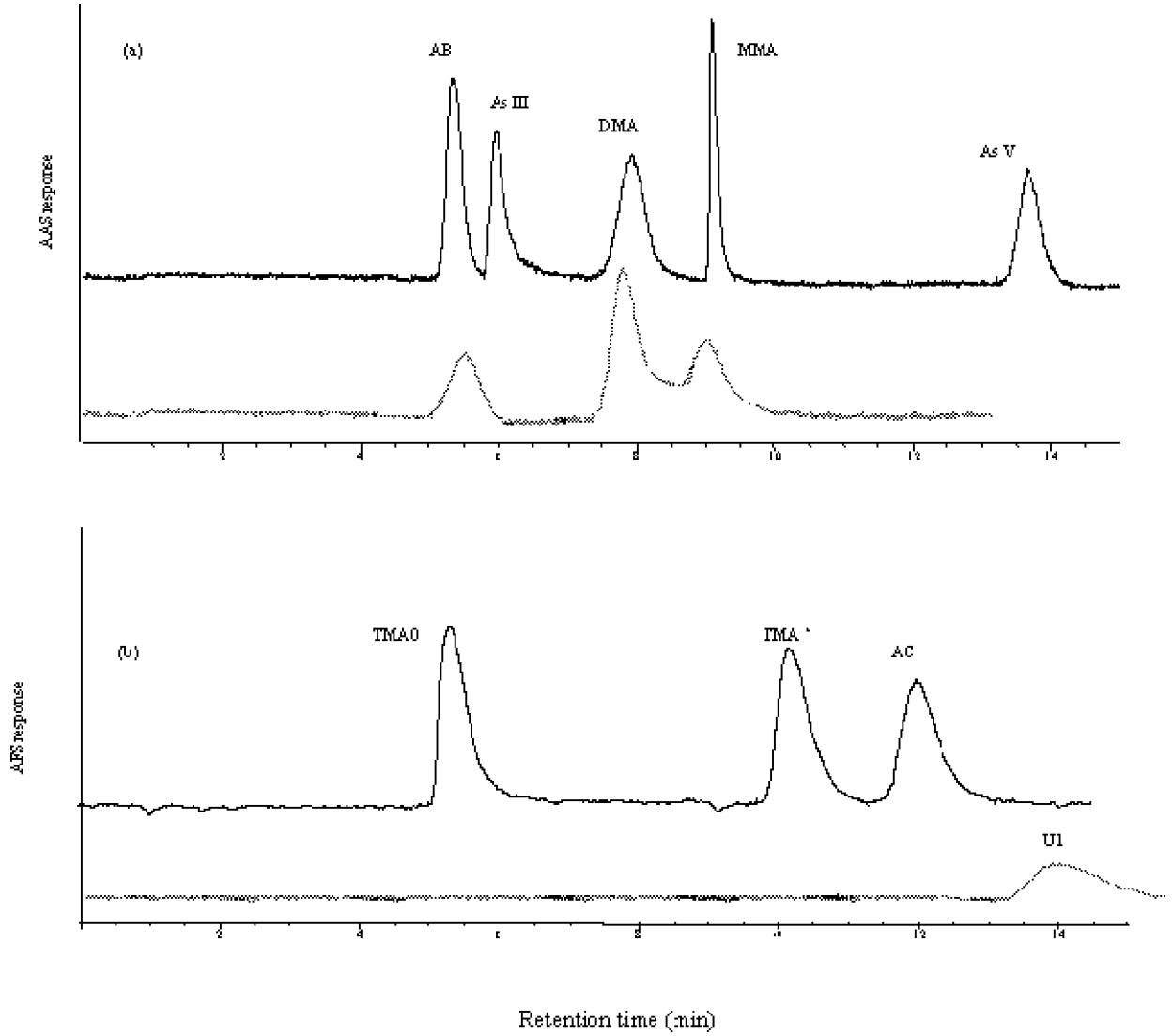


**Figure 4.** Separation of an arsenic species standard mixture of TMAO, TETRA, AC, AB, As(III), DMA, MA, and As(V) (0.1 $\mu$g g$^{-1}$ As each) (solid line) using the switching column system: (**a**) anion exchange column thermo-oxidation-HG-AAS and (**b**) cation exchange column thermo-oxidation-HG-AFS. AB conversion in saline medium at 8 °C by microflora from remaining parts (dotted line) obtained in (**a**) an anion exchange column with thermo-oxidation-HG-AAS detection and (**b**) cation exchange column thermo-oxidation-HG-AFS.

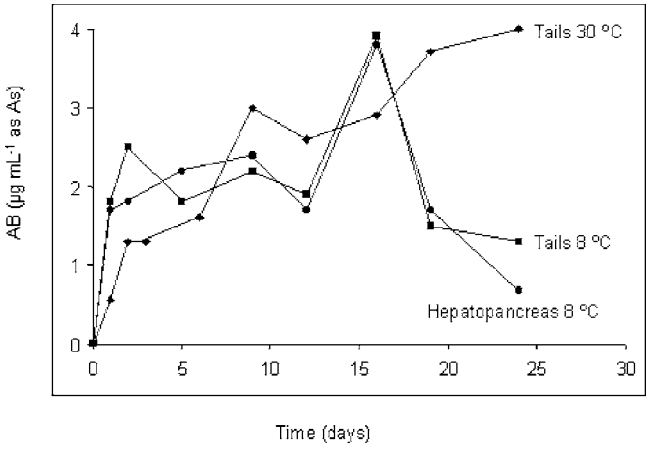


**Figure 5.** AB generated by transformation of AC (4 $\mu$g mL$^{-1}$ as As) in saline medium at 30 and 8 °C by microflora from hepatopancreas and tails.

*P. fluorescens* (one isolate), *Pasteurella* spp. (four isolates), and *Oligella ureolytica* (one isolate). Only *Pseudomonas* was isolated from the tail, while more heterogeneous flora were isolated from the hepatopancreas. Every isolate was individually assayed for AB degradation in mineral salt basal medium, as described below.

**Table 3.** Isolates from Different Parts of Crayfish, Phenotypic Identification, and Ability for AB Degradation

| isolates | species identification by API-NE (identification %) | AB degradation |
|---|---|---|
| L | *P. putida* (91%) | + |
| M | *P. putida* (97%) | + |
| Q1 | *P. putida* (92%) | + |
| Q2 | *P. putida* (92%) | + |
| R | *P. putida* (92%) | + |
| S | *Pasteurella* spp. (79%) | – |
| O | *Pasteurella* spp. (79%) | – |
| N | *Pasteurella* spp. (79%) | – |
| U | *Pasteurella* spp. (83%) | – |
| T | *Pseudomonas fluorescens* (87%) | – |
| P | *O. ureolytica* (68%) | – |

The genotype of the isolates with ability to transform AB was characterized to confirm the identification based on phenotypic properties and to discriminate between different strains. RAPD-PCR analysis was successfully applied for strain

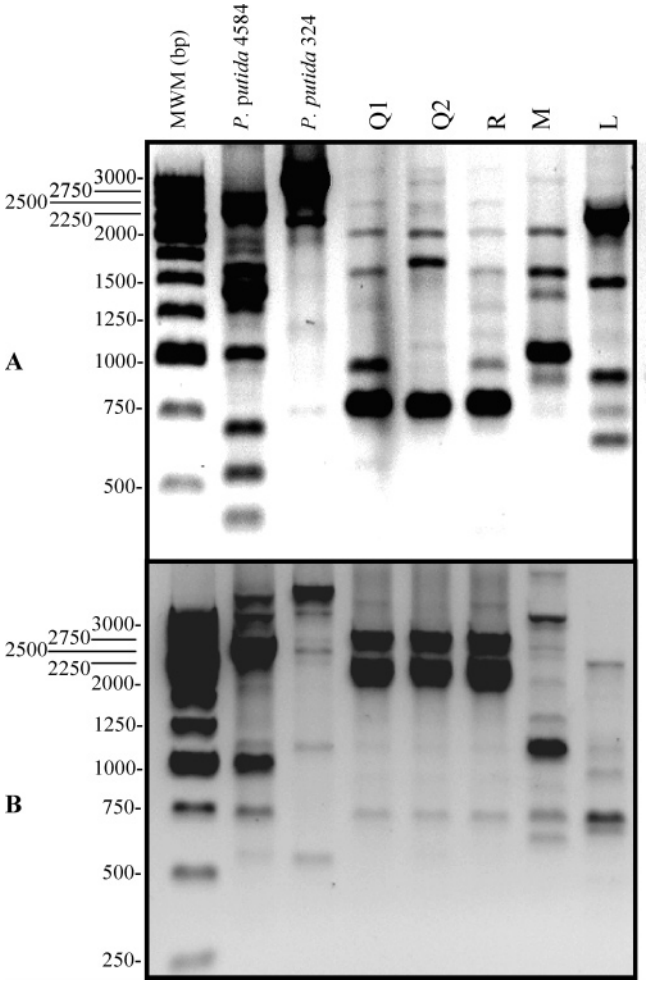


**Figure 6.** RAPD-PCR fingerprints obtained with the primers OPL-1 (**A**) and OPL-2 (**B**). The strain codes on the lanes correspond to those of the reference strains (see the Materials and Methods) and the isolates cited in **Table 3**. On the left (lane MWM), sizes in bp of DNA molecular weight marker XVI (Boheringer, Mannheim, Germany) are indicated.

typing with two different oligonucleotides, OPL1 and OPL2 (**Figure 6A**,**B**). In both cases, the three isolates (Q1, Q2, and R) from the hepatopancreas showed identical RAPD fingerprintings, representing a common genotype, while the other two isolates (M and L) from the tails showed unique RAPD fingerprintings. In accordance with these results, partial DNA sequences of the 16S rRNA genes of different isolates selected as representatives of each cluster (M, L, and Q1) were analyzed by restriction with two endonucleases (ARDRA) and sequenced. In both cases, ARDRA patterns of all isolates and the reference *P. putida* strains were identical (**Figure 7**) and clearly differed from those of *P. fluorescens*. The in silico analysis carried out with the 16S rDNA sequences of other *Pseudomonas* species also permitted confirmation of the suitability of this technique for species identification as previously described (*20*). 16S rDNA sequences of the isolates showed an identity among them of 99%. A high level of identity (96−98%) was observed between the sequence of our isolates and those of other *P. putida* strains from the GenBank. These results confirmed the identification based on phenotypic properties.

**AB Transformation by Pure Cultures of Different Isolates from Crayfish.** The individual ability of the various isolates for AB transformation was studied in mineral salt medium, which was inoculated with an overnight culture grown in TSB. The initial cell density in the assayed medium was, in every case, around $10^6-10^7$ cfu/mL. The bacterial counts increased slightly (0.5−1 log unit) after 7 days of incubation except for strains Q2 and N, whose counts remained constant or reduced (1 log unit). At the end of the incubation period, bacterial counts remained at levels of $10^6-10^8$ cfu/mL.

The only isolates that were able to generate other arsenic species from AB were those assigned to the species *P. putida* (**Table 3**), with strains isolated from tails (L and M) and from hepatopancreas (Q1, Q2, and R). The metabolites generated were DMA and MA. Strain L showed the highest activity, followed by strain M (**Figure 8**), with AB degradation that began slowly but attained 80 and 70%, respectively, after 21 days of incubation. DMA was the major metabolite in strain L, and MA was the major metabolite in strain M. These were also the strains that reached the highest levels ($10^8$ cfu/mL) after 7 days of incubation, which might partially explain the generation of higher amounts of degradation products from AB. Strains Q1, Q2, and R showed an ability to degrade AB, but the transforma-

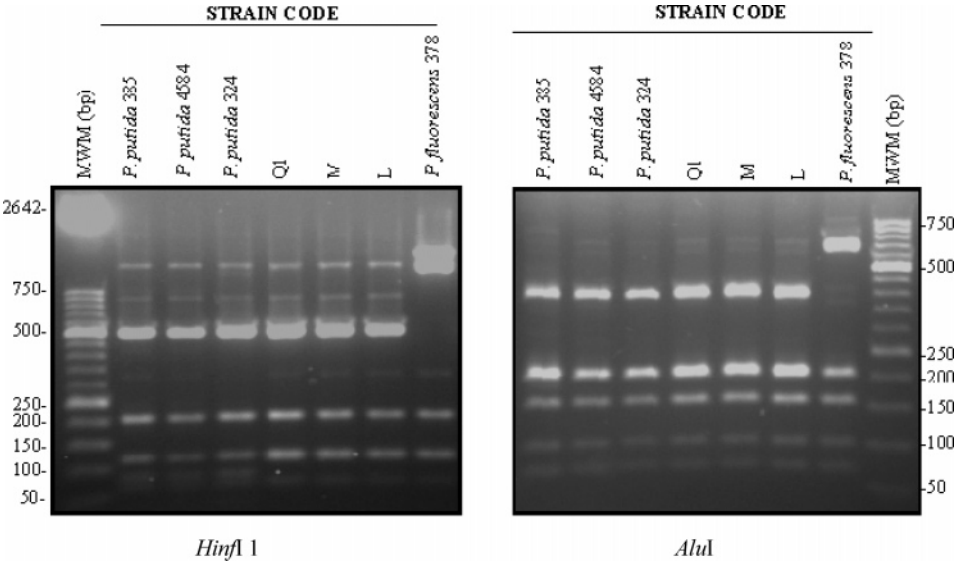


**Figure 7.** Patterns of *Hinf*I 16S and *Alu*I 16S rDNA restriction analysis of *P. putida* strains. The strain numbers on the lanes correspond to those of the reference strains (see the Materials and Methods) and the isolates cited in **Table 3**. Sizes in bp of DNA molecular weight marker XIII (Boheringer) are indicated.

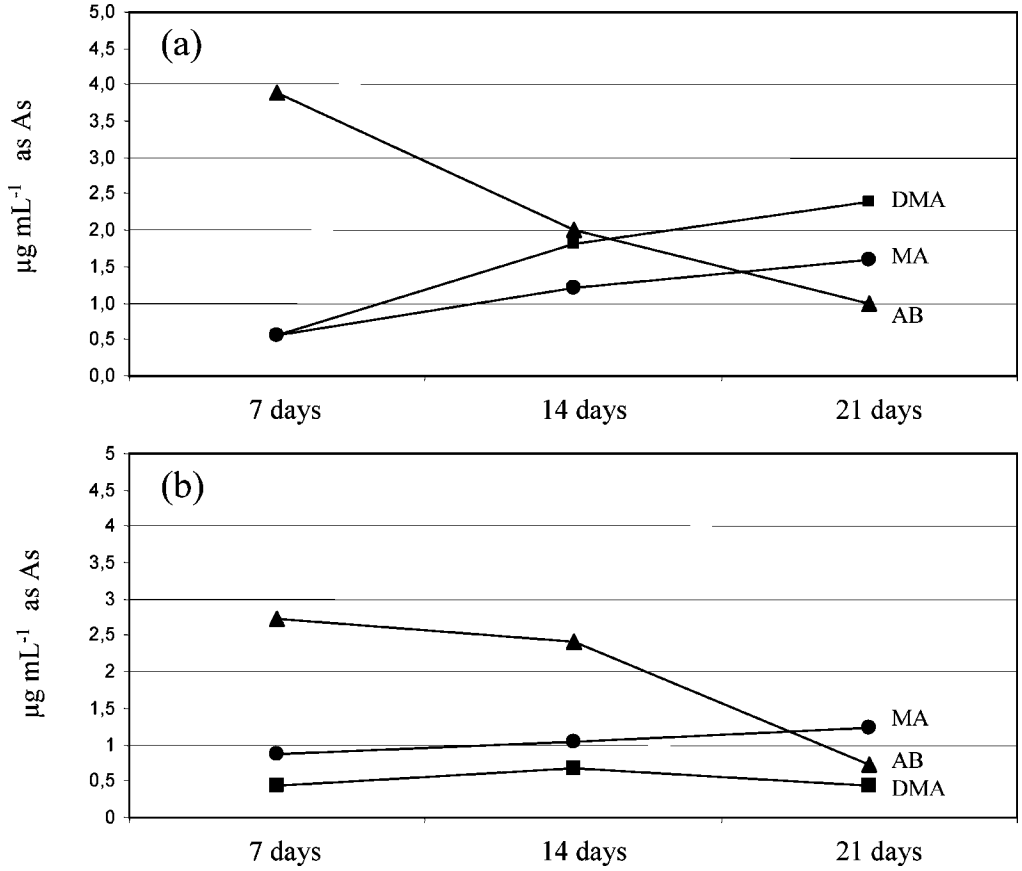


**Figure 8.** Arsenical species detected after AB conversion (5 $\mu$g mL$^{-1}$ as As) by pure cultures of strain L (**a**) and M (**b**) isolated from tails of crayfish. Incubation in aerobic conditions at 30 °C. Contents are expressed as arsenic.

tion was not very great, and after 21 days of incubation, between 84 and 87% of the AB, expressed as As, had not been degraded. In these three strains, MA was the most significant metabolite, 11−13% of total As, as compared with the 2% represented by DMA.

The strain of a closely related species also frequently found in seafood, *P. fluorescens* (strain T), did not show the ability to transform AB despite the fact that the culture population density reached in mineral salt medium was quite high and similar to those of *P. putida* strains (M and L), which displayed the highest degradation ability. Therefore, the ability to transform AB was associated with the species *P. putida*. This does not appear to be a strain-dependent property, as all of the different strains assigned to this species showed activity for AB transformation to some extent.

## DISCUSSION

**Transformation of AB by Total Crayfish Microflora.** In the present study, the microflora of the crayfish *P. clarkii* degraded AB to TMAO, DMA, MA, and U1. This unknown U1 might be DMAA, identified by Khokiattiwong et al. (*8*) and Jenkins et al. (*9*) as a metabolite of AB biodegradation. No standard of DMAA is available, and as the chromatographic conditions used by those authors are not comparable with the conditions employed in our study, it is not possible to compare the retention times of the signals obtained.

Assuming that U1 is DMAA, one could indicate the coexistence of the two degradation pathways for AB degradation proposed in the literature: (i) methyl−arsenic bond cleavage generating DMAA and subsequent cleavage of the carboxymethyl−arsenic bond (AB → DMAA → DMA → MA) and (ii) carboxymethyl−arsenic cleavage generating TMAO and subsequent cleavage of methyl−arsenic bonds (AB → TMAO → DMA → MA).

The appearance of TMAO in only some of the experiments and at short incubation times suggests that, although it is a product of degradation, its transformation into another metabolite in the degradation pathway is very rapid. This was observed previously by Hanaoka et al. (*3*) in the degradation of AB by flora present in seaweed in saline medium, where TMAO did not appear in the degradation pathway and DMA was the first metabolite found. These authors suggested that the microorganisms used the AB acetate group and the TMAO methyl group simultaneously, generating DMA. Other authors, however, detected TMAO as a major metabolite of AB (*4*, *5*), even after 12 (*9*) or 80 days of incubation (*7*). In our study, it was only in TSB medium at 30 °C that the microflora from remaining parts generated a noteworthy quantity of TMAO.

DMA is the major metabolite in the various experiments carried out in this work, which coincides with what is reported in the literature about the rapidity with which, once the AB has been degraded to TMAO or DMAA, these metabolites are transformed into DMA. We cannot explain the difference in the degradation pattern observed for hepatopancreas in TSB medium at 30 °C, where U1 > DMA.

In no case was As(V) generated during the 24 days of incubation, unlike the findings of other authors, who detected As(V) as an end product of AB degradation by microbes isolated from sediments (*2*, *7*) and suspended substances (*12*). It was recently shown that a strain of the genus *Paenibacillus* degraded 0.02% of AB to As(V) after 28 days of aerobic incubation at 25 °C (*9*). The type of flora involved might explain why the

degradation process did not generate As(V) in our study. The transition from AB to TMAO and from DMAA to DMA involves the release of the carboxymethyl moiety, which may be used by bacteria in aerobic pathways such as the tricarboxylic acids cycle, as proposed by Hanaoka et al. (*5*).

In TBS medium at 30 °C (**Figure 3**), the AB degradation was delayed in time in comparison with what was observed in saline medium (**Figure 1**). TSB is a medium rich in nutrients, whereas the saline medium contained only the arsenical species added as a carbon source, which might promote the expression of metabolic activities that allow their utilization as an energy source. In assays carried out in TSB at 8 °C, the use of a rich medium together with a low incubation temperature prevented any degradation. In the literature, there are no previous references for transformations of AB at 8 °C. The absence of degradation of AB at 8 °C suggests that, if there is a degradation of AB in crayfish during the time between their capture and consumption, it is probably slight and does not involve the appearance of inorganic arsenic, and so would not produce a negative impact on the safety of these foods from the viewpoint of health.

**Transformation of AC, TETRA, TMAO, DMA, and MA by Total Crayfish Microflora.** The degradation of AC to AB observed in saline medium in the present study is an oxidation process and, therefore, a reaction that permits the generation of electrons that might be used by microorganisms to obtain energy via the electron transport chain. AC is consumed completely, generating AB, which in turn decreases without the appearance of other products. There may be generation of trimethylarsine, a volatile compound that Kaise et al. (*4*) detected as a metabolite of AB aerobically incubated with microorganisms obtained from clams. Studies on AC biotransformation carried out by other authors have obtained results different from those obtained here. The biotransformation of AC to AB, TMAO, and DMA by microorganisms in sediments aerobically incubated at 25 °C has been reported (*22*). More recently, with flora isolated from shore crabs and seawater, Khokiattiwong et al. (*8*) showed complete transformation of AC into DMA after 24 h at 15 °C, without detecting AB as intermediate. The type of flora might also condition the process and consequently the differences observed.

Our results concerning the absence of microbiological degradation of TETRA, TMAO, DMA, and MA are similar to those obtained by Hanaoka et al. (*7*). They demonstrated the stability of TETRA, TMAO, DMA, and MA in the presence of microorganisms isolated from marine sediments in conditions of aerobiosis. Khokiattiwong et al. (*8*) also did not observe degradation for TMAO, DMA, and MA, although they did report a small transformation (15%) of TETRA to TMAO after 10 days of incubation. The fact that the microorganisms present in the various parts of crayfish are capable of carrying out transformation of AB to DMA and MA but incapable of transforming DMA when it is the initial substrate has not been explained. Uptake of DMA into the cells might possibly be an explanation.

**Identification of AB-Degrading Bacterial Strains from Crayfish.** Differences in the type of microorganisms involved in organoarsenical degradation might help to explain the origin of the various metabolites generated. Only the strains belonging to the species *P. putida* were able to metabolize AB in the conditions assayed. This is the first time that the active microorganisms have been identified and characterized at both species and strain level. In previous reports, only the genera of the microorganisms involved in such biotransformations were determined. Thus, Jenkins et al. (*9*) identified the genera of two strains (*Pseudomonas* 3944 and *Paenibacillus* 13943) as being possibly responsible for biodegradation of AB.

However, there are qualitative and quantitative differences between the degradative effect on AB carried out by the total crayfish microflora and the isolates of the species *P. putida*: (i) The degradation by individual isolates was quantitatively less significant than that carried out by the total microflora, and (ii) the degradation by *P. putida* strains did not generate U1 in the conditions assayed. An analysis of lysed-cell extract of the strains might help to explain these differences. The future use of alternative isolation conditions might promote the development and selection of other species probably less competitive than *P. putida* in TSA at 30 °C but also active in arsenical transformation and perhaps capable of generating U1. Overall, this might help to elucidate the potential for biotransformation of a more complex microbial ecosystem closer to what is found in nature and to foresee the possible toxicological effects of such transformations.

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