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CHARACTERIZATION OF ARSENIC-TOLERANT BACTERIAL CULTURES FROM THE LOWER LAGUNA MADRE OF SOUTH TEXAS

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Abstract.–Two forms of arsenic are found in the environment: As(V) and As(III), the latter being more toxic, water-soluble, and mobile. Microorganisms may increase the mobility of arsenic by reducing As(V) to As(III); however, detoxification and immobilization can occur via the oxidation of As(III) to As(V). The US EPA has set a minimum contaminant level of 10 parts per billion (ppb) for arsenic in drinking water. The research objective was to confirm the presence of arsenic-tolerant bacteria in the Lower Laguna Madre of south Texas. Sediment samples were collected and inoculated into growth media which contained either 2 mM As(III) or 2 mM As(V) to enrich for As(III)-tolerant and As(V)-tolerant bacteria, respectively. Twenty six (26) As(III)-tolerant and 12 As(V)-tolerant cultures were obtained. Most isolates were small white colonies of Gram-positive rods. Biochemical tests using commercially-made test strips showed that As(V)-tolerant isolates displayed greater resource usage compared to As(III)-tolerant isolates but overall, few cultures demonstrated a wide-range of biochemical capabilities. Isolates with distinct morphological and biochemical phenotypes were subjected to Polymerase Chain Reaction (PCR) amplification and sequencing of the 16S rRNA genes to identify the bacteria. Closest sequence matches were to the eubacterial genera *Mycoplasma*, *Salinispora, Frankia,* and *Pelodictyon.* These results suggest that the Lower Laguna Madre is inhabited by a diverse group of microorganisms able to tolerate toxic concentrations of different arsenic species.

Arsenic contamination is a significant problem in the environment because arsenic is toxic and carcinogenic. Although arsenic may exist in several oxidation states, it is typically found in two forms in the environment; arsenate (As(V)), which is typically insoluble, and arsenite (As(III)), which is water soluble and more toxic (Stumm & Morgan 1996). Insoluble As(V) often precipitates to the bottom of bodies of water making the arsenic immobile, but soluble As(III) is mobile and of much greater concern. The US EPA has set a Maximum Contaminant Level (MCL) of 10 ppb for total arsenic (As(III) + As(V)) in drinking water (USEPA 2001). A

1995 study showed sediment arsenic levels greater than 27,000 ppb in the Upper Laguna Madre (Barrera et al. 1995).

The Laguna Madre is a hypersaline estuary, one of only five in the world, with an average salt content ranging from 35 to 45 ppt; however, salt concentrations higher than 80 ppt have been reported (Quannen & Onuf 1993; Tunnell & Judd 2002; Whelan et al. 2005). The high salinity of the Laguna Madre is due to the fact that there are few freshwater inputs into the estuary and few outlets. The Lower Laguna Madre receives fresh water discharged from the highly-impacted Arroyo Colorado and from precipitation; however, the estuary is shallow (average depth 1.5 m) (Tunnell & Judd 2002). Salts concentrate during periods of drought and high rates of evaporation occur in the summer due to the warm regional climate of South Texas. Regional pollution has resulted in toxic chemical concentrations in water, sediment, and animals of the Laguna Madre (Davis et al. 1995). Among the contaminants at toxic concentrations is arsenic. Much of the arsenic detected in the Lower Rio Grande Valley of Texas and along the US-Mexico border is attributed to past usage of arsenical pesticides, but may also be related to sewage treatment discharge into regional waterways and non-point sources (Davis et al. 1995; Tunnell & Judd 2002).

Davis et al. (1995) reported that large amounts of arsenic were present in the Lower Laguna Madre; however, this study did not distinguish between insoluble As(V) and toxic, soluble As(III). Arsenic is also of concern in the Arroyo Colorado, a waterway in South Texas that is part of the natural drainage system for the Lower Rio Grande Valley and lies on the north of the Rio Grande Delta. The Arroyo Colorado is part of a floodway system, receives treated wastewater from several towns, runs through the Port of Harlingen, and discharges into the Lower Laguna Madre. Elevated arsenic levels have been detected in the Arroyo Colorado, most likely due to agricultural runoff (Wells et al. 1988). Thus, arsenic in the Laguna Madre presents a potential threat to the ecosystem

and may impact organisms in the lagoon. Some microorganisms may increase the mobility of arsenic by reducing As(V) to As(III) (Macy et al. 2000). However, aquatic plants and microorganisms may lessen the toxicity of arsenic by immobilizing it via oxidation, methylation, or accumulation (Oremland et al. 2002, Bentley & Chasteen 2002, Schmöger et al. 2000).

As(V) and As(III) can change oxidation state by chemical or biological processes. In marine sediments, As(V) can form a variety of insoluble mineral compounds that are structurally similar to phosphates. These occur when arsenate anions (AsO_4^{2-}) react with transition metals, such as iron and manganese (Smedley & Kinniburgh 2002). Reduction of metal-arsenate minerals can occur under low redox (reducing) conditions, thus liberating the arsenic as As(III). As(III) typically exists as soluble arsenic acids, such as H_3AsO_3, at marine pH values (Smedley & Kinniburgh 2002).

Biologically-mediated transformations of arsenic depend on the form of arsenic involved. As(V) may be reduced to As(III) by anaerobic sediment bacteria through dissimilatory anaerobic respiration (Newman et al. 1998, Macy et al. 2000). Plants can reduce As(V) to As(III) through a detoxification mechanism (Meharg & Hartley-Whitaker 2002) or the bioaccumulation of arsenic may take place in plant tissues (Davis et al. 1995). Arsenic can also be detoxified via biomethylation in microorganisms, algae, plants, and animals (Wang et al. 2004). Methylation detoxifies arsenic by making it volatile, converting it to gaseous forms (Qin et al. 2006). Reduction of As(V) to As(III) can potentially release arsenic bound to sediment particles or bound in minerals, and may impact the mobility of arsenic in the Laguna Madre ecosystem. Increased arsenic mobility will potentially impact plants and animals by introducing toxic, soluble As(III) into sediment pore waters and estuary waters. Conversely, As(III) oxidation is an important detoxification reaction since the resulting As(V) is less toxic and less bioavailable (Oremland et al. 2002).

The macro-ecology of the Laguna Madre has been extensively studied for decades. Several rare, endangered, threatened, and migratory animal species feed or nest in the Laguna Madre. Despite this, little is known about the microbial ecology of the Laguna Madre and the importance of microorganisms in nutrient and chemical cycling within the sediments of the ecosystem. Interest in microbial ecology has increased dramatically as the importance of microorganisms within sediments and subsurface environments are related to biogeochemistry, bioremediation, and biotechnology (Atlas & Bartha 1998; Nealson 1997). The interplay between biologically-mediated As(III) oxidation and biologically-mediated As(V) reduction is important in determining the fate of arsenic species in the Laguna Madre. In this research, culture-based and molecular methods were used to characterize bacterial populations that demonstrated tolerance to As(III) or As(V).

MATERIALS AND METHODS

Sample collection.–Sediment samples (approximately 50 g) were collected in March 2007 from the top 10 cm of sediment at four sites in the Lower Laguna Madre (Figure 1). Samples were transported on ice to the laboratory. The collection sites were: LMT-050 (N26°08'17.4",W97°10'41.0"), located near the South Padre Island Wastewater Treatment Plant; South Bay (SB; N26°02'48",W97°11'3.3"); site ABC (N26°10'09.7",W97°11'05.3") and LMT-051 (N26°10'09.7",W97°11'05.3"). All sites were south of the mouth of the Arroyo Colorado and have been described previously (Whelan et al. 2005).

Enrichment cultures for arsenic-oxidizing and arsenic-reducing bacteria.–Sediment from each site was homogenized and 1 g was placed into sterile 20-mL glass vials containing 10 mL of minimal liquid medium (Lowe et al. 2000). The final concentrations and composition of the medium was (per liter): CH_3COO Na [15 mM], $(NH_4)_2SO_4$ [0.9 mM], $K_2HPO_4 \cdot 3H_2O$ [0.57 mM], KH_2PO_4 [0.33 mM], $NaHCO_3$ [0.2 mM], $Na_2EDTA \cdot 2H_2O$ [7μM], H_3BO_3 [6μM], $FeSO_4 \cdot 7H_2O$ [0.6 μM], $CoCl_2 \cdot 6H_2O$ [0.5 μM], $Ni(NH_4)SO_4^{2-} \cdot$

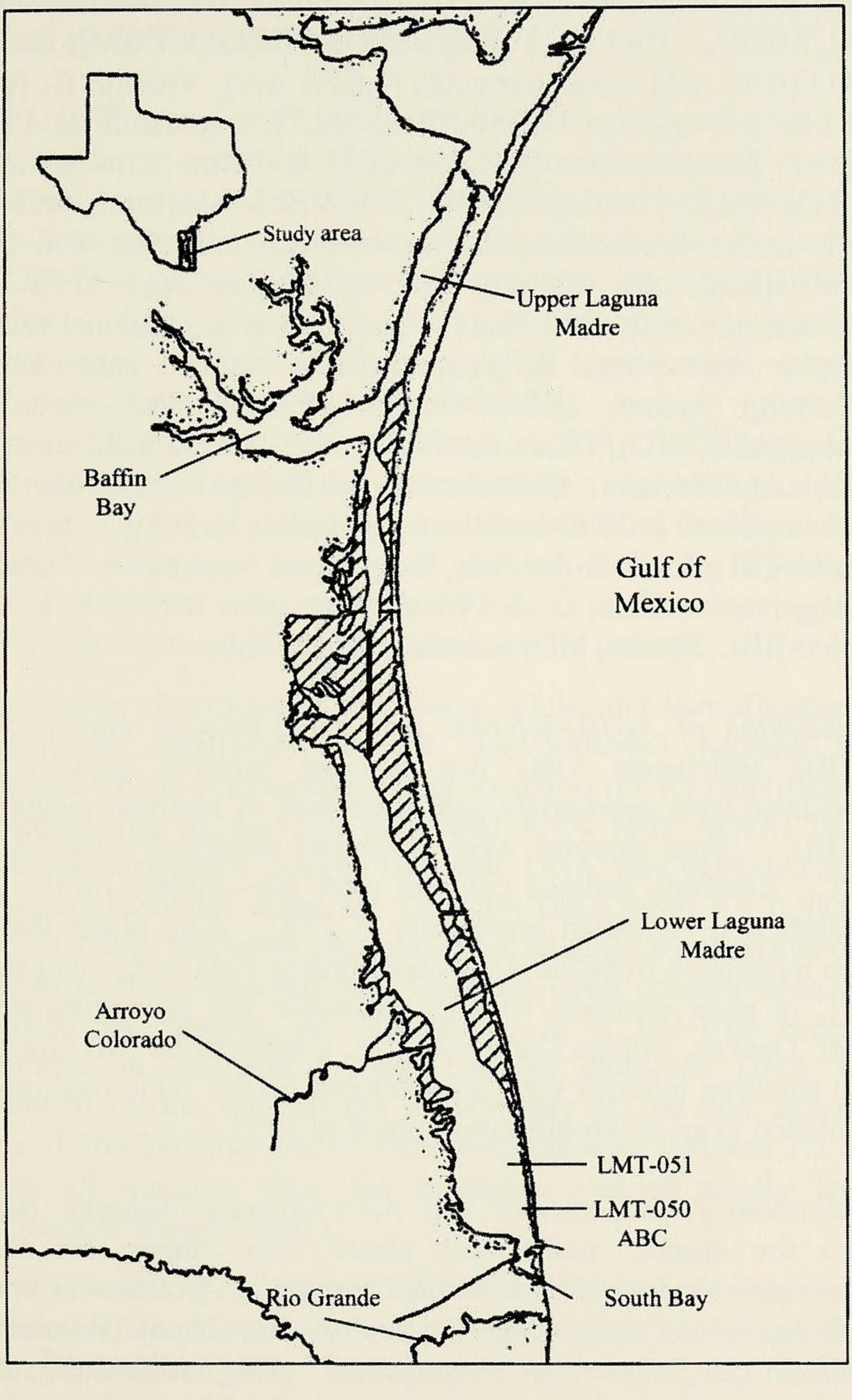

Figure 1. Map of the Lower Laguna Madre showing the sampling locations. (From Whelan et al. 2005).

$6H_2O$ [0.5 μM], $Na_2MoO_4 \cdot 2H_2O$ [0.4 μM], Na_2SeO_4 (anhyd) [0.15 μM], $MnSO_4 \cdot H_2O$ [0.13 μM], $ZnSO_4 \cdot 7H_2O$ [0.1 μM], $CuSO_4 \cdot 5H_2O$ [0.02 μM], casamino acids [0.01% w/v], Vitamin B_1 [0.001 mg mL^{-1}], L-arginine HCl [0.02 mg mL^{-1}], L-glutamic acid [0.02 mg mL^{-1}], L-glutamine [0.02 mg ml^{-1}], L-serine [0.04 mg mL^{-1}], $MgSO_4 \cdot 7H_2O$ [1 mM], $CaCl_2 \cdot 2H_2O$ [0.5 mM] and NaCl [3% w/v]. After sterilization, the medium was amended with either As(V) ($Na_2HAsO_4 \cdot 7H_2O$) or As(III) ($NaAsO_2$) to a final concentration of 2 mM. Vials with As(V) as the terminal electron acceptor were placed in glass canisters with an anaerobic gas generating system (BBL GasPak, Becton Dickenson Co., Cockeysville, MD). Vials containing As(III) were incubated in an aerobic environment. Cultures were performed in triplicate. Vials were incubated at 25°C until there was visible turbidity. Once there was visible growth in the vials, the samples were streaked onto the appropriate medium as described above plus 1.5% Bacto Agar (Difco-BBL, Sparks, MD) to isolate pure colonies.

Isolation of As(III)-tolerant and As(V)-tolerant bacteria.–The As(III) enrichment vials that showed bacterial growth were inoculated onto appropriate agar consisting of minimal media with As(III). These samples were incubated aerobically for 14 d at 25°C. Resulting isolated colonies were sub-cultured on the same medium and incubated aerobically at 25°C. Agar plates that had been inoculated from the As(V) enrichment vials were incubated at 25°C in glass canisters with an anaerobic gas generating system (BBL GasPak, Becton Dickenson Co., Cockeysville MD) for 30 d. The resulting cultures were sub-cultured on the same medium and incubated in an anaerobic environment at 25°C.

Random As(III)-tolerant and As(V)-tolerant bacterial isolates from the arsenic media agar plates were chosen for further investigation. Isolates were characterized by Gram-stain and by observed colony morphology (i.e., color, size, shape). Biochemical profiles for isolates were generated using API 20E® strips

(bioMérieux Inc., Durham, NC). Isolates were stored in 25% glycerol at -80°C for molecular studies.

API 20E® strips.–API 20E® strips include enzymatic tests for fermentation or oxidation of glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, melibiose, amygdalin, and arabinose, along with nitrate reduction to nitrite and nitrate reduction to nitrogen gas. API 20E® strips also test for the presence of β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophan deaminase, indole production, acetoin production (Voges – Proskauer), and gelatinase. API 20E® tests were performed according to the manufacturer's instructions. The number and types of positive tests were tabulated for the isolates and used to construct biochemical profiles of the As(III)-tolerant and As(V)-tolerant cultures. The API 20E® profiles were used to compare biochemical phenotypes amongst the isolates.

Molecular identification of isolates.–Molecular identification of the isolated As(III)-tolerant and As(V)-tolerant bacteria was performed by the Polymerase Chain Reaction (PCR) amplification and sequencing of the 16S ribosomal RNA (16S rRNA) genes (Sambrook & Russell 2001). Genomic DNA was extracted from the bacterial isolates using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Extracted genomic DNA integrity was verified on a 1% agarose electrophoresis gel. Genomic DNA concentration was quantified by reading the absorbance in a UV-VIS spectrophotometer at 260 nm. Purity of the extracted DNA was determined by the ratio of the absorbances at 260 and 280 nm. Ratios of 260/280 absorbance measurements were between 1.7 and 2.0 for all samples; thus, the extraction yielded mostly DNA (Sambrook & Russell 2001).

A sample (50 ng) of template genomic DNA was placed in a 0.5 mL thin-walled PCR tube with 25 μL of PCR Master Mix (Promega, Madison, WI). PCR primers used to amplify the 1505 bp target of the 16S rRNA gene were 5′- AGA GTT TGA TCC

TGG CTC AG – 3′ (forward) and 5′- ACG GCT ACC TTG TTA CGA CTT – 3′ (reverse) (Integrated DNA Technologies, Coralville, IA). The final concentration of each primer was 10 μM. The total PCR mixture volume was 50 μL. The PCR mixture was placed into a MyCycler PCR thermocycler (Bio Rad, Hercules, CA) and heated to 95°C for 6 min in order to initially denature the template DNA. After the initial denaturation, 40 cycles were run with the following conditions: denaturing at 95°C for 30 sec, annealing of the primers at 52°C for 30 sec, and primer extension at 72°C for 30 sec. A final extension at 72°C for 1 min was done and the PCR products were held at 4°C. An aliquot (5 μL) of the PCR products was visualized by electrophoresis on a 1% agarose gel. The remaining PCR product mixture was purified using a Wizard PCR Clean Up Kit (Promega, Madison, WI). Purified PCR products were used for DNA sequencing described below.

For sequencing, a second round of PCR was done using a commercially-available sequencing kit (Genome Lab DTCS Quick Start Kit; Beckman Coulter, Fullerton CA) according to the manufacturer's instructions. Dye-tagged dideoxynucleotides (ddUTP, ddGTP, ddCTP, and ddATP) were added to terminate elongation (Sambrook & Russell 2001). The resulting PCR product was loaded into an automated DNA sequencer (CEQ 8000 Genetic Analysis System; Beckman Coulter, Fullerton, CA). The sequences were compared to known bacterial sequences available in the National Center for Biotechnology Information Basic Local Alignment Search Tool (BLASTN) database (www.ncbi.nml.nih.gov/BLAST) to identify the microorganisms (Altschul et al. 1997).

RESULTS AND DISCUSSION

Pure colonies (n = 26) were successfully isolated from As(III) enrichments [Table 1]; 12 pure colonies were isolated from As(V) enrichments [Table 2]. Every isolate cultured in As(III) media was 100% Gram-positive [Table 1]. The As(III) Gram stain results were unusual because marine sediments usually contain mixtures of Gram-positive and Gram-negative bacteria (Atlas & Bartha 1998).

Table 1. Morphology of Laguna Madre bacteria isolated from As(III)-containing media. As(III)-tolerant bacteria were isolated from the Laguna Madre from different sites. Isolates were streaked onto minimal media supplemented with 2 mM As(III) and incubated aerobically for 14 d. Cells were Gram-stained and the colony morphology was visually observed and recorded. All isolates were Gram-positive and smooth in appearance.

Isolate	Sediment Source	Shape	Appearance
AS3 – A	South Bay	bacillus	white, small , round
AS3 – B	South Bay	bacillus	white, round
AS3 – C	ABC	bacillus	orange, round
AS3 – D	South Bay	bacillus	white, irregular
AS3 – E	South Bay	bacillus	ivory, small, irregular
AS3 – F	LMT-050	bacillus	clear, small, irregular
AS3 – G	LMT-050	bacillus	light yellow-orange, small, round
AS3 – H	LMT-050	bacillus	light yellow, small, round
AS3 – I	LMT-050	bacillus	pink, small, round
AS3 – J	ABC	bacillus	peach, small, round
AS3 – K	South Bay	coccus	transparent, small, round
AS3 – L	South Bay	coccus	white, small, round
AS3 – M	South Bay	bacillus	yellow/orange, small, round
AS3 – N	LMT-050	bacillus	ivory, small, round
AS3 – O	LMT-051	bacillus	white, small, round
AS3 – P	ABC	bacillus	white, small, round
AS3 – Q	South Bay	bacillus	white, small, round
AS3 – R	South Bay	bacillus	white, small , irregular
AS3 – S	South Bay	cocco-bacillus	white, small, round
AS3 – T	ABC	bacillus	orange, small, round
AS3 - U	ABC	bacillus	orange, small, round
AS3 - V	LMT-051	bacillus	pink/orange, small, round
AS3 - W	LMT-050	bacillus	ivory, small, round
AS3 - X	LMT-051	bacillus	peach, small, round
AS3 - Y	ABC	bacillus	ivory, tiny, round
AS3 - Z	ABC	bacillus	orange, small, round

The high percentage of Gram-positive bacteria may reflect the toxicity of arsenic, which may result in high selection pressure for Gram-positive organisms. Once inside a cell, As(III) disrupts protein folding and protein-DNA interactions (Norman 1998). Gram-positive cells have a thicker cell wall compared to Gram-negative cells. The thicker cell wall might make it more difficult for As(III) to enter the cell. The isolates enriched in As(III) medium were rod-shaped or bacillus (23/26), cocci (2/26) and

Table 2. Morphology of Laguna Madre bacteria isolated from As(V)-containing media. As(V)-tolerant bacteria were isolated from the Laguna Madre from different sites. Isolates were streaked onto minimal media supplemented with 2 mM As(V) and incubated anaerobically for 30 d. Cells were Gram-stained and the colony morphology was visually observed and recorded.

Isolate	Sediment Source	Gram Stain	Shape	Colony Appearance
AS5 - A	LMT-050	+	bacillus	transparent, small, round, smooth
AS5 - B	ABC	+	bacillus	transparent, small, round, smooth
AS5 - C	South Bay	+	bacillus	ivory, small, irregular, smooth
AS5 - D	LMT-051	–	cocco-bacillus	ivory, small, irregular smooth
AS5 - E	LMT-050	–	coccus	transparent, small, round, smooth
AS5 - F	LMT-050	+	coccus	ivory, round with filaments, smooth
AS5 - G	LMT-050	–	cocco-bacillus	ivory clear, small, irregular, smooth
AS5 - H	LMT-051	+	coccus	white, small,, round, smooth
AS5 - I	LMT-050	–	cocco-bacillus	white, small, irregular, smooth,
AS5 - J	LMT-050	–	bacillus	transparent, small, round, smooth
AS5 -K	LMT-050	+	coccus	ivory, small, irregular, filamentous
AS5 - L	LMT-050	+	coccus	transparent, small, round, smooth

cocco-bacillus (1/26) [Table 1]. The typical colony morphology of the As(III) isolates was smooth and round but varied in color and shape. The morphologies observed ranged from small, white punctiform colonies to irregular-shaped, colored colonies. Colony colors included pink and orange [Table 1].

The isolates cultured in As(V) were either Gram-positive (7/12) or Gram-negative bacteria (5/12) and were bacillus (4/12), cocci (5/12), and cocco-bacillus (3/12) [Table 2].

The colony morphologies also varied for the As(V) isolates. Colonies were mostly irregular and white, or almost transparent [Table 2]. Two of the As(V) isolates, AS5-F and AS5-K, were filamentous; both were isolated from LMT-050 [Table 2]. In general, isolates from the As(V) enrichments displayed slower growth rates than those isolated from As(III) media (data not shown). However, this was most likely due to incubation conditions – anaerobic for As(V) versus aerobic conditions for As(III) – and not arsenic toxicity.

For the As(III) isolates (n = 26) tested with API 20E® strips, three of the isolates were positive for β-galactosidase and six were positive for gelatinase; two were positive for mannitol and saccharose oxidation; one was positive for melibiose utilization. The remaining seventeen tests were negative for all As(III) isolates [Table 3].

Among the As(V) isolates (n = 12) tested with API 20E® strips, five were positive for β-galactosidase, two were positive for arginine dihydrolase, and seven were positive for gelatinase. In addition, three were positive for glucose, mannitol, rhamnose, saccharose, amygdalin, or arabinose oxidation. The remaining thirteen tests were negative for all As(V) isolates [Table 4].

The high number of negative tests made putative identification of the isolates difficult; however, the isolates from the anaerobic As(V) enrichment cultures appeared more metabolically diverse than those cultured in the aerobic As(III) enrichment cultures. The enzymatic flexibility that some As(V)-tolerant bacteria displayed may be due to the lower toxicity of As(V) relative to that of As(III) or As(III) may select for characteristics that were not included on the test strips and not observed.

There were a low number of positive API tests from the microorganisms isolated from As(III) enrichments [Table 3]. Among the As(III)-tolerant isolates tested, isolates AS3-J from site ABC and isolate AS3-K from South Bay each displayed only three positive tests which was the highest number of positive tests [Table 3]. Isolates AS3-J and AS3-K are likely to be different microorganisms because their morphology was different and their API profiles displayed two tests in common but differed in one [Table 3]. AS3-J and AS3-K were both able to oxidize mannitol and saccharose; however, only AS3-J was able to metabolize melibiose while AS3-K produced β-galactosidase indicating lactose utilization [Table 3]. Isolates AS3-G, AS3-H, AS3-T, AS3-U, AS3-W and AS3-Z tested positive for gelatinase [Table 3];

Table 3. Biochemical profiles of As(III)-tolerant bacteria. Twenty-six (26) As(III)-tolerant bacteria were isolated from the Laguna Madre sediment. The isolates' metabolic activities were tested using API 20E® strips. A plus sign (+) indicates that the isolate was positive for the test; a negative sign (–) indicates a negative reaction for the test. Only isolates that displayed at least one positive test are shown.

	Isolate									
	AS3-E	AS3-G	AS3-H	AS3-J	AS3-K	AS3-M	AS3-T	AS3-U	AS3-W	AS3-Z
ONPG	+	–	–	–	+	+	–	–	–	–
ADH	–	–	–	–	–	–	–	–	–	–
LDC	–	–	–	–	–	–	–	–	–	–
ODC	–	–	–	–	–	–	–	–	–	–
CIT	–	–	–	–	–	–	–	–	–	–
H2S	–	–	–	–	–	–	–	–	–	–
URE	–	–	–	–	–	–	–	–	–	–
TDA	–	–	–	–	–	–	–	–	–	–
IND	–	–	–	–	–	–	–	–	–	–
VP	–	–	–	–	–	–	–	–	–	–
GEL	–	+	+	–	–	–	+	+	+	+
GLU	–	–	–	–	–	–	–	–	–	–
MAN	–	–	–	+	+	–	–	–	–	–
INO	–	–	–	–	–	–	–	–	–	–
SOR	–	–	–	–	–	–	–	–	–	–
RHA	–	–	–	–	–	–	–	–	–	–
SAC	–	–	–	+	+	–	–	–	–	–
MEL	–	–	–	+	–	–	–	–	–	–
AMY	–	–	–	–	–	–	–	–	–	–
ARA	–	–	–	–	–	–	–	–	–	–
NO2	–	–	–	–	–	–	–	–	–	–
N2	–	–	–	–	–	–	–	–	–	–

Tests: ONPG, β-galactosidase activity; ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; CIT, citrate utilization; H2S, hydrogen sulfide production; URE, urease; TDA, tryptophan deaminase; IND, indole production; VP, acetoin production (Voges-Proskaur); GEL, gelatinase; GLU, glucose; MAN, mannitol; INO, inositol; SOR, sorbitol; RHA, rhamnose; SAC, sucrose; MEL, melibiose; AMY, amygdalin; ARA, arabinose; NO2, nitrate reduction to nitrite; N2, nitrate reduction to nitrogen gas.

moreover, AS3-T, AS3-U and AS3-Z all showed similar colony morphologies and are potentially the same organism despite being isolated from different locations in the Laguna Madre [Table 1].

The As(V) isolates from the sediment samples collected at LMT-050 had the highest number of positive API tests for all isolates tested [Table 4]. The LMT-050 population included AS5-A, AS5-

Table 4. Biochemical profiles of As(V)-tolerant bacteria. Twelve (12) As(V)-tolerant bacteria were isolated from the Laguna Madre sediment. The isolates' metabolic activities were tested using API 20E® strips. A plus sign (+) indicates that the isolate was positive for the test; a negative sign (–) indicates a negative reaction for the test.

	Isolate											
	AS5-A	AS5-B	AS5-C	AS5-D	AS5-E	AS5-F	AS5-G	AS5-H	AS5-I	AS5-J	AS5-K	AS5-L
ONPG	+	+	–	–	+	–	–	–	–	+	–	+
ADH	+	–	–	–	–	–	–	–	–	+	–	–
LDC	–	–	–	–	–	–	–	–	–	–	–	–
ODC	–	–	–	–	–	–	–	–	–	–	–	–
CIT	–	–	–	–	–	–	–	–	–	–	–	–
H2S	–	–	–	–	–	–	–	–	–	–	–	–
URE	–	–	–	–	–	–	–	–	–	–	–	–
TDA	–	–	–	–	–	–	–	–	–	–	–	–
IND	–	–	–	–	–	–	–	–	–	–	–	–
VP	–	–	–	–	–	–	–	–	–	–	–	–
GEL	–	–	+	+	–	–	+	+	+	–	+	+
GLU	+	–	–	–	+	–	–	–	–	+	–	–
MAN	+	–	–	–	+	–	–	–	–	+	–	–
INO	–	–	–	–	–	–	–	–	–	–	–	–
SOR	–	–	–	–	–	–	–	–	–	–	–	–
RHA	+	–	–	–	+	–	–	–	–	+	–	–
SAC	+	–	–	–	+	–	–	–	–	+	–	–
MEL	–	–	–	–	–	–	–	–	–	–	–	–
AMY	–	–	–	–	+	–	–	–	–	+	–	–
ARA	+	–	–	–	–	–	–	–	–	+	–	–
NO2	–	–	–	–	–	–	–	–	–	–	–	–
N2	–	–	–	–	–	–	–	–	–	–	–	–

Tests: ONPG, β-galactosidase activity; ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; CIT, citrate utilization; H2S, hydrogen sulfide production; URE, urease; TDA, tryptophan deaminase; IND, indole production; VP, acetoin production (Voges-Proskaur); GEL, gelatinase; GLU, glucose; MAN, mannitol; INO, inositol; SOR, sorbitol; RHA, rhamnose; SAC, sucrose; MEL, melibiose; AMY, amygdalin; ARA, arabinose; NO2, nitrate reduction to nitrite; N2, nitrate reduction to nitrogen gas.

E, and AS5-J, which were all isolated from the same sediment sample and have similar morphologies; however, they varied slightly in their biochemical profiles. The three isolates all tested positive for β-galactosidase and utilization of glucose, mannitol and rhamnose, but only AS5-A and AS5-J tested positive for arginine dihydrolase and arabinose metabolism, and AS5-E and AS5-J tested positive for amygdalin utilization [Table 4]. Even though AS5-A, AS5-E, and AS5-J isolates from LMT-050 were the most metabolically diverse, they all tested negative for gelatinase, which

overall had the highest number of positive reactions [Table 4]. The highest number of gelatinase-positive isolates were obtained in the anaerobically-grown As(V) isolates compared to the aerobic As(III) isolates. Only 6/26 As(III) isolates were positive for gelatinase whereas 7/12 of the As(V) isolates were gelatinase positive [Table 3 and 4]. Gelatinase is a digestive enzyme necessary to break down gelatin, a protein found in animal connective tissue, which is sometimes degraded by bacteria during biofilm formation (McNamara et al. 1997). The production of gelatinase is also used to differentiate and identify anaerobes (Whaley et al. 1982). The overall high number of positive gelatinase tests suggests that some of these organisms may be anaerobic decomposers of organic matter (McNamara et al. 1997).

Isolates (5/12) from the As(V) enrichments were positive for β-galactosidase activity which indicates that some As(V)-tolerant bacteria from the Laguna Madre can use lactose as a carbon source [Table 4]. This was unexpected because lactose is not a commonly used carbon source in marine environments as it does not easily absorb to sediment particles (Sansone et al. 1987). Acetate is more easily absorbed to sediment particles and is more readily available to sediment bacteria; thus, it was used as the principle carbon source in the isolation medium. No isolate was capable of nitrate reduction [Table 3 and 4] which was also unexpected because many facultative and anaerobic sediment bacteria are able reduce NO_3^-. NO_3^- is a better electron acceptor energetically compared to As(V) (Dowdle et al. 1996).

Results from the 16S rRNA gene sequencing showed an array of positive matches. Table 5 shows the top BLASTN result for the As(III) and As(V) isolates that were successfully sequenced. The top similarity match (% Match) is shown. Isolate AS3-E displayed high similarity to *Mycoplasma hyopneumoniae*. Isolate AS3-K showed good similarity to *Salinispora tropica* CNB-440, a marine Gram-positive bacterium with a high percentage of G+C bases in the DNA (Maldonado et al. 2005; Williams et al. 2005). Isolate

Table 5. As(III)-tolerant and As(V)-tolerant bacteria isolated from the Laguna Madre sediment. The 16S rRNA genes from the isolates were amplified by PCR, sequenced, and compared to other known bacteria using the BLASTN search engine and data-base. The isolates were matched to the highest percentage match. The top BLASTN matches and the percent sequence match are shown.

Isolate	Sediment Source	Best Match	Percent Match
AS3-E	South Bay	*Mycoplasma hyopneumoniae*	100
AS3-K	South Bay	*Salinispora tropica* CNB-440	92
AS5-B	ABC	*Frankia alni* ACN14A	100
AS5-E	LMT-050	*Pelodictyon luteolum*	95

AS5-B had high similarity with *Frankia alni* strain ACN14A which is a Gram-positive, nitrogen-fixing bacterium that can live symbiotically with some non-legume plants (Atlas & Bartha 1998; Normand et al. 2007.). Isolate AS5-E showed similarity with *Pelodictyon luteolum,* a photosynthetic, green-sulfur bacterium (Overmann & Tuschak 1997).

Although the 16S rRNA gene sequencing resulted in a variety of potential matches, the identity of the organisms is yet to be conclusively determined. This is because the sequencing results do not agree with the morphological and physiological data for the isolates. For example, isolate AS3-E was observed to be a Gram-positive, rod-shaped bacterium [Table 3] and showed high identity with *M. hyopneumoniae*. *Mycoplasma hyopneumoniae* is related to low G+C Gram-positive organisms, it lacks a cell wall and is associated with a mild, chronic form of pneumonia that affects pigs (Atlas & Bartha 1998; Minion et al. 2004). However, mycoplasma cells are typically small, coccus-shaped cells with a convex dense region in the center sometimes referred to a “fried egg” appearance (see Madigan & Martinko 2005). Thus, the observed morphology of this isolate does not agree with what would be expected for a mycoplasma cell and it is unclear why a swine pathogen would be present in Laguna Madre sediments. Recent research has involved inserting arsenic resistance genes as genetic markers for the

development of genetically-modified vaccines against *Mycoplasma hyopneumoniae* in infected swine (Matic 2008). What role this may play in arsenic tolerance is not yet known, especially for environmental strains of the bacterium.

Isolates AS5-B and AS3-K showed high similarity to *F. alni* and *S. tropica*, respectively. Organisms are Gram-positive, aerobic bacteria, which does agree with these observations [Table 3 & 4] yet these bacteria are typically filamentous and produce hyphae/mycelia when grown in laboratory media (Benson & Silvester 1993; Maldonado et al. 2005). This morphology was not observed for either isolate. The lack of agreement between the morphology and sequencing data may be due to PCR amplification of extraneous sequences or limited sequence information in the BLASTN database that did not allow for an ideal match. Thus, As(III)-tolerant and As(V)-tolerant isolates from the Laguna Madre appear to be diverse groups of yet unidentified microorganisms.

The results of this investigation provide insights into the potential for arsenic mobilization in the Lower Laguna Madre. Arsenic is carcinogenic and toxic, especially As(III); thus it is advantageous to have information into possible arsenic cycling in the Lower Laguna Madre due to recreational use of the lagoon and fisheries that occupy regions adjacent to the lagoon. The Laguna Madre is a rare hypersaline estuarine ecosystem, and it is important to ascertain whether arsenic and other contaminants are potentially harmful to the ecosystem. Arsenic was used as a pesticide for several decades and cannot be degraded like an organic pollutant; it can only be converted to different forms. Thus, once in the ecosystem, it will stay there in some manner. It is likely that the shallow water levels, low water flow, high evaporation rates, high salinity and pH of the Laguna Madre affect the concentration and speciation of the arsenic. To what extent is not yet known.

These results suggest that there is the potential for arsenic mobilization (i.e., reduction of As(V) to As(III)) in the Laguna

Madre due to the presence of several different arsenic-tolerant bacterial types isolated under arsenic-reducing (As(V)) conditions. These organisms displayed different metabolic abilities and different biochemical profiles, suggesting that they are not the same species. Results of the 16S rRNA gene sequencing experiments support this. Furthermore, these organisms were isolated from several locations in the Lower Laguna Madre with varying environmental conditions, which suggests that they might be widespread in the ecosystem.

If there is the potential for As(V) reduction to As(III), it is important to consider how this may affect the arsenic toxicity in the Laguna Madre and how this in turn will affect the biota in the ecosystem. One would think that bacterial cells reducing As(V) to As(III) would be making a toxic environment for themselves and be affected by their own metabolic products. Why this does not appear to affect the bacterial cells is not known but it necessitates the involvement of some type of arsenic tolerance. It is possible that the produced As(III) may re-oxidize by chemical or biological means, or that the cells may have some mechanism of resisting the produced As(III). Some bacterial cells have arsenic resistance mechanisms such as efflux pumps that keep arsenic out of the cell (Newman et al. 1998). Such mechanisms were not tested for in the current study.

The presence of As(III)-tolerant bacteria in the lagoon implies a possible mechanism for counteracting the mobilization of arsenic if these As(III)-tolerant organisms can also oxidize As(III) to As(V). Future studies will include the determination of the relative abundance and density of As(III)-oxidizing and As(V)-reducing bacteria in the Laguna Madre, comparing arsenic oxidation and reduction at different sites, conducting seasonal studies on arsenic transformations in the Laguna Madre, and the detection of genes associated with arsenic oxidation and arsenic reduction (Saltikov & Newman 2003; Murphy & Saltikov 2007). Additionally arsenic

reduction rates can be conducted in the future to conclusively demonstrate arsenic mobilization in the Laguna Madre.

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