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Homology of *Escherichia coli* R773 *arsA*, *arsB*, and *arsC* Genes in Arsenic-Resistant Bacteria Isolated from Raw Sewage and Arsenic-Enriched Creek Waters

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The occurrence and diversity of the *Escherichia coli* R773 *ars* operon were investigated among arsenic-resistant enteric and nonenteric bacteria isolated from raw sewage and arsenic-enriched creek waters. Selected isolates from each creek location were screened for *ars* genes by colony hybridization and PCR. The occurrence of *arsA*, *arsB*, and *arsC* determined by low-stringency colony hybridization (31 to 53% estimated mismatch) was 81, 87, and 86%, respectively, for 84 bacteria isolated on arsenate- and arsenite-amended media from three locations. At moderate stringency (21 to 36% estimated mismatch), the occurrence decreased to 42, 56, and 63% for *arsA*, *arsB*, and *arsC*, respectively. PCR results showed that the *ars* operon is conserved in some enteric bacteria isolated from creek waters and raw sewage. The occurrence of the *arsBC* genotype was about 50% in raw sewage enteric bacteria, while *arsA* was detected in only 9.4% of the isolates ($n = 32$). The *arsABC* and *arsBC* genotypes occurred more frequently in enteric bacteria isolated from creek samples: 71.4 and 85.7% ($n = 7$), respectively. Average sequence divergence within *arsB* for six creek enteric bacteria was 20% compared to that of the *E. coli* R773 *ars* operon. Only 1 of 11 pseudomonads screened by PCR was positive for *arsB*. The results from this study suggest that significant divergence has occurred in the *ars* operon among As-resistant *E. coli* strains and in *Pseudomonas* spp.

The contamination of drinking water sources with arsenic (As) poses a potential threat to human health. Inorganic As, including the highly toxic trivalent form [arsenite; As(III)] and less toxic pentavalent As [arsenate; As(V)], is associated with increased cancer risk in a number of geographic areas (16, 40, 41). The toxicity of As is attributed to the substitution of As(V) for phosphate, affinity of As(III) for protein thiol groups, and protein-DNA and DNA-DNA cross-linking (23). Arsenic enrichment and pollution of environmental waters originate from either natural or anthropogenic sources. Sodium arsenite, monomethylarsonate (MMA), dimethylarsinic acid (DMA), and lead arsenate ($PbHAsO_4$) have been extensively used as herbicides and pesticides (13, 29, 30). Geological processes such as geothermal activity and weathering of As-containing rocks also contribute significantly to As enrichment of aquatic environments (15, 43).

Many bacteria have been isolated that exhibit resistance to lethal concentrations of arsenic (greater than 5 mM sodium arsenite) (8, 15), yet little is known about the genetics involved in As resistance (As^r) in environmental bacteria. Plasmids have been detected in some bacteria exhibiting high levels of resistance to arsenate, arsenite, and antimonate (5, 9, 19). In addition, As^r loci have also been found on the chromosomes of *Pseudomonas aeruginosa* and *E. coli*. The most-well-characterized genetic system for resistance to arsenicals is known as the *ars* operon. *Escherichia coli* and *Staphylococcus ars* operons have been thoroughly investigated at the genetic and biochemical levels (19, 33, 35). Additionally, similar homologs have been found during chromosomal sequencing of bacterial species, but the function of these is unknown (The Institute for Genomic Research <http://tigr.org/tdb/> and National Center for Biotechnology Information <http://www.ncbi.nlm.nih.gov/>). The *E. coli* plasmid R773 *ars* operon contains five genes, *arsRDABC*, encoding an arsenate reductase (ArsC) that reduces arsenate to arsenite, a membrane-bound anion-translocating ATPase (ArsA), and ArsB, an inner membrane protein that forms the anion-conducting channel (11). The *ars* operon functions as a detoxification mechanism by lowering the intracellular arsenic concentration, thus conferring resistance to As(V) and As(III). While ArsR is a *trans*-acting inducer-responsive repressor, ArsD is an inducer-independent protein controlling basal and upper-level expression.

Gram-negative plasmid *ars* operons share highly homologous sequences, yet are highly divergent from their gram-positive counterparts. Sequences homologous to the *E. coli* chromosomal *ars* operon are also highly conserved among enterobacterial genera (12). In addition, the recently discovered *P. aeruginosa ars* operon containing *arsRBC* appears to be conserved in *P. fluorescens*, but not in other arsenic-resistant *Pseudomonas* spp. (4). Open reading frames sharing homology with the *E. coli arsRDABC* have also been found on the *Acidiphilium multivorum* plasmid pKW301 (37) and the archeal *Halobacterium* sp. strain NRC-1 plasmid pNRC100 (10, 14).

In other metal resistance genetic systems, such as the copper resistance operon *pcop*, the enteric model represented by *E. coli* has been shown to be structurally and functionally equivalent to other copper-resistant systems (5, 31). We therefore used a well-characterized genetic system, the *E. coli* R773 *ars* operon, as a model to investigate the diversity of the *ars* operon in As^r bacteria isolated from raw sewage and natural waters.

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The overall goal of our study was to determine the prevalence of this genetic model in relating the As^r phenotype among selected populations of enteric and nonenteric bacteria. DNA-DNA hybridization was done under different stringency conditions to address the issue of diversity of *ars* genes in As^r isolates originating from natural waters. In addition, PCR was evaluated for detection of *ars* genes in enteric and nonenteric bacteria. A phylogenetic analysis of novel *ars*-like sequences detected in environmental enteric bacteria is also presented.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1. Isolates were obtained from water samples collected from Hot Creek, South Haiwee Drain 5, and Irvine Ranch Water District and Orange County Sanitation District raw sewage samples; all sampling sites are located in California. Samples were stored in sterile polyethylene bottles and transported on ice packs. Sewage samples were plated on mTEC (Difco) and mENDO (Difco). Creek waters were plated on 10 or 50% strength Difco plate count agar (PCA).

Arsenic-resistant bacteria were obtained by spread plating diluted samples onto PCA medium supplemented with 500 mg of As(III) and 1,500 mg of As(V) per liter. Selected isolates were subsequently streak purified twice on PCA (creek isolates) and Luria-Bertani-Miller (LB) agar (Difco) (raw sewage isolates) containing 500 mg of As(III) or 1,000 mg of As(V) per liter.

Creek isolates were grown at 30°C in broth similar to 50% PCA amended with 500 mg of sodium arsenite [As(III)] per liter. Raw sewage isolates were grown in LB broth supplemented with 500 mg of As(III) or As(V) per liter. Cultures were stored in 50% glycerol at −80°C.

DNA preparation. Total DNA was obtained by a modified guanidine thiocyanate extraction procedure (36). Overnight cultures (0.5 ml) were centrifuged (Eppendorf 5415C) at 14,000 × *g* for 5 min. Pellets were resuspended in 0.6 ml of guanidine thiocyanate lysis buffer (5.3 M guanidine thiocyanate, 10 mM dithiothreitol, 1% Tween 20, 0.3 M sodium acetate, 50 mM sodium citrate [pH 7.0]), 53 μl of cetyltrimethylammonium (CTAB [10%], 0.7 M NaCl), and 8 μl of 0.5 M NaCl. After incubation of the mixture at 65°C for 10 min, 50 μl of Glassmilk (Bio 101, Inc., La Jolla, Calif.) was added, and this combination was mixed for 15 min at room temperature. Following centrifugation for 1 min at 14,000 × *g*, pellets were rinsed three times with wash buffer (50% ethanol, 10 mM Tris-HCl [pH 7.5], 100 mM NaCl). DNA was eluted off the Glassmilk by resuspending the pellet in Tris-EDTA (TE [pH 8.0]) for 5 min at 50°C with periodic mixing. The supernatant was removed and treated with RNase at a final concentration of 50 μg/ml.

Species identification. Isolates were streak purified on Trypticase-soy agar (TSA) with or without 5% sheep's blood. Following incubation at 30°C for 24 h, GN Microplates (Biolog, Hayward, Calif.) were used to identify the species. A similarity index greater than 0.5 was considered a positive identification.

PCR amplification. Primers were designed for three of the structural genes *arsA*, *arsB*, and *arsC* of the *ars* operon from the *E. coli* pUM3 plasmid (7). The criteria used in the design of the primers included conservation of homologous sequences determined in multisequence alignments (data not shown) or inclusion of active sites for arsenic binding. Table 2 lists the primer sequences, the targeted regions for the specific gene, and the expected PCR fragment sizes. Primers were tested for cross-reactivity to other bacterial sequences by BLAST.

PCR was carried out with a Perkin-Elmer 9600 Thermocycler. Twenty-five or 50-μl reaction mixtures were composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 200 nM each primer, 0.625 U of *Taq* polymerase (Perkin-Elmer or Promega) per 25 μl, and 50 ng of template DNA. The protocol for each primer set consisted of an initial denaturation step (94°C for 3 min) followed by 30 to 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s (*arsA*-1) or 30 to 35 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s (*arsB*-1 and *arsC* −1). A final extension was done for 7 min at 72°C. The *E. coli* pUM3 purified plasmid served as a positive control for *arsA*-1, *arsB*-1, and *arsC*-1 primer sets. Negative controls included a deionized water reagent control, *Pseudomonas putida*, and *E. coli* JM109. The amplified products (5- to 10-μl aliquots) were separated on a 1× Tris-borate-EDTA (TBE)–1.5 or 2% agarose gel (0.5 μg of ethidium bromide per ml) by electrophoresis. The bands were visualized on a UV transilluminator.

Preparation of internal probe. PCR was used to construct internal probes to confirm the identity of the amplified DNA bands. Nested primers were designed to amplify internal regions of the *arsA*-1, *arsB*-1, and *arsC*-1 fragments, respectively. Plasmid DNA from *E. coli* pUM3 was first subjected to PCR with *arsA*-1, *arsB*-1, and *arsC*-1 primer sets, respectively, according to the PCR protocol described above. The fragments were purified by electrophoresis through a 2.5% agarose gel and buffered in 1× TBE, which was followed by excision of the corresponding band. DNA was eluted from the gel slice with a Genelute (Supelco) spin column according to the manufacturer's instructions. PCR with the internal primer sets I-*arsA*-1, I-*arsB*-1, and I-*arsC*-1, respectively, was then done. The amplified fragments were purified by agarose gel electrophoresis followed by band excision and Supelco spin column purification. Twenty-five to 50 ng of purified DNA was labeled with [^{32}P]dCTP by random priming with Klenow enzyme and random hexamers (Boehringer-Mannheim) according to the manufacturer's instructions.

Southern blot and hybridization. DNA was transferred to nylon membrane (MagnaCharge; MSI, Westboro, Mass.) by capillary action according to standard methods (2). DNA was fixed to the membrane by UV cross-linking at 1,200 J/cm^2 (FB-UVXL-1000; Fisher Scientific) and stored under a vacuum at ambient room temperature.

Membranes were hybridized by a method similar to that of Keller (17). Briefly, membranes were treated with prehybridization solution [50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 31 mM KH_2PO_4, 0.25% sodium dodecyl sulfate (SDS), 1× Denhardt's solution, and 100 μg of poly(A) per ml] and incubated at 42°C for 2 h, followed by hybridization at 42°C overnight with 1 to 5 ng of denatured ^{32}P-labeled probe DNA per ml. For moderate stringency, the filters were washed four times for 5 min each in 2× SSC–0.1% SDS. Low-stringency washes were done two times for 15 min each in 0.1× SSC–0.1% SDS at 50°C. The filters were exposed to X-Omat (Kodak) X-ray film at −80°C for 4 to 24 h.

Colony hybridization with *ars* operon probes. Colony lifts were done according to the method of Sambrook et al. (34). Colonies were transferred onto MagnaGraph nylon transfer membranes (0.22-μm pore size; MSI). DNA was fixed to the membranes by UV cross-linking. Negative controls included degrading *P. putida* and salmon sperm DNA. Probes for *arsA*, *arsB*, and *arsC* were constructed from PCR fragments amplified from *E. coli* pUM3. Radiolabeling was done with gel-purified PCR products (described above). Membranes were hybridized as described above with the following modifications: prehybridization and hybridization were done at 37°C and with washes at two stringencies: low (2× SSC–0.1% SDS at 37°C) and moderate (1× SSC–0.1% SDS at 50°C). The estimated percent mismatches for low- and moderate-stringency washes were 31 to 53 and 21 to 36%, respectively. Percent mismatch was calculated based on the method of Anderson (1). Filters were exposed to X-ray film for 2 to 4 days at −80°C. Colony lysis was confirmed with 0.2% methylene green staining of the membranes (25).

Cloning and sequencing. Isolates that were PCR positive for *arsB* and *arsC* were used to amplify a 1.5-kb fragment by using primers I-*arsB*-1-F and I-*arsC*-1-R. PCR products were separated on 1% agarose gel followed by excision of the corresponding band. The DNA was purified as described above. Purified *arsBC* fragments were cloned with the pGEM-T Easy Vector System (Promega, Madison, Wis.) according to the manufacturer's instructions. Ligation reactions were transformed into competent *E. coli* JM109 cells (Promega, Madison, Wis.). Blue and white screening was done on LB agar supplemented with 100 μg of ampicillin per ml, 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and 80 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. Plasmid DNA was extracted (Qiaprep spin kit; Qiagen) from overnight cultures of four white colonies per isolate. To verify the presence of the *arsBC* insert, PCR was used with I-*arsB*-1-F and I-*arsC*-1-R primers.

Automated sequencing was done on an ABI 377 by a dideoxynucleotide method at the Davis Sequencing Facility (Davis, Calif.). The m13(−21) forward sequencing primer was used to sequence the cloned PCR fragment. A single primer walk was performed on cloned *arsBC* fragments with the following primers: PW1 (5′-GAGCCACTCGGTATTCCTGTGAG-3′), specific for SHA1, SHA17, SHA35, and HCSH9; PW1-S29 (5′-TTTTGTCCTCAACCACTCG-3′), specific for SHA29; and PW1-B2 (5′-CTGGTTGGGTTCTTTGTCC-3′), specific for HCB2.

Phylogenetic analysis of *ars* operon genes. DNA sequences were aligned by using Clustal W (39), and phylogenetic trees were generated by PAUP* 4.0b4a (38) with an optimality criterion set to minimum evolution. The Kimura two-parameter model was used to estimate pairwise distances. Stepwise addition and TBR branch-swapping algorithms were used to construct phylogenetic trees. Following a heuristic search and bootstrap analysis with 100 samples, the final tree was assembled with TreeView (28). The following sequences (accession numbers in parentheses) were used as references: *E. coli* R773 (J02591), *E. coli* R46 (U38947), *E. coli* chromosomal *ars* operon (X80057), *Serratia marcescens* pR478 (AJ288983), *Yersinia enterocolitica* (U58366), and *Klebsiella oxytoca* pMH12 (AF168737), as well as the South Haiwee Drain isolates SHA1, SHA17,

TABLE 1. Bacterial strains analyzed for *arsA*, *arsB*, and *arsC*

Strain or isolate	Type/identification[a]	Phenotype[b]	*ars* genotype[c]	Total As of water sample (μg/liter)
E. coli strains				
	pUM3 *ars* operon subclone of R773	$As(III)^r$ $As(V)^r$ Sb^r	$arsA^+$ $arsB^+$ $arsC^+$	
	JM109 cloning host for pGEM-T vector		*ars* PCR negative	
Raw sewage isolates				<50
ECO1	*E. coli*	$As(III)^r$	$arsB^+$ $arsC^+$	
ECO2	*E. coli*	$As(III)^r$	$arsB^+$ $arsC^+$	
ECO7	*E. coli*	$As(III)^r$	$arsA^+$ $arsB^+$ $arsC^+$	
EN-1	Enteric	As^s	*ars* PCR negative	
EN-2	Enteric	As^s	*ars* PCR negative	
EN-3	Enteric	As^s	$arsC^+$	
EN-4	Enteric	$As(III)^r$ $As(V)^s$	$arsB^+$ $arsC^+$	
EN-5	Enteric	As^s	*ars* PCR negative	
ENIII-1	Enteric	$As(III)^r$ $As(V)^r$	*ars* PCR negative	
ENIII-2	Enteric	$As(III)^r$ $As(V)^r$	$arsC^+$	
ENIII-3	Enteric	$As(III)^r$ $As(V)^r$	*ars* PCR negative	
ENIII-4	Enteric	$As(III)^r$ $As(V)^r$	$arsA^+$ $arsB^+$ $arsC^+$	
ENV-1	Enteric	$As(III)^r$ $As(V)^r$	$arsB^+$ $arsC^+$	
ENV-2	Enteric	$As(III)^s$ $As(V)^r$	*ars* PCR negative	
ENV-3	Enteric	$As(III)^s$ $As(V)^r$	*ars* PCR negative	
ENV-4	Enteric	$As(III)^r$ $As(V)^r$	$arsB^+$ $arsC^+$	
ENV-5	Enteric	$As(III)^r$ $As(V)^r$	*ars* PCR negative	
G2	*E. coli*	$As(III)^r$	$arsB^+$	
G3	*E. coli*	$As(III)^r$	$arsB^+$	
G4	*E. coli*	$As(III)^r$	$arsB^+$	
G5	*E. coli*	$As(III)^r$	$arsB^+$ $arsC^+$	
G6	*E. coli*	$As(III)^r$	$arsB^+$ $arsC^+$	
G7	*E. coli*	$As(III)^r$	$arsB^+$ $arsC^+$	
G8	*E. coli*	$As(III)^r$	$arsB^+$ $arsC^+$	
G9	*E. coli*	$As(III)^r$	$arsB^+$ $arsC^+$	
G10	*E. coli*	$As(III)^r$	*ars* PCR negative	
G11	*E. coli*	$As(III)^r$	$arsB^+$ $arsC^+$	
G13	*E. coli*	$As(III)^r$	*ars* PCR negative	
G14	*E. coli*	$As(III)^r$	*ars* PCR negative	
G15	*E. coli*	$As(III)^r$	*ars* PCR negative	
G16	*E. coli*	$As(III)^r$	*ars* PCR negative	
G18	*E. coli*	$As(III)^r$	*ars* PCR negative	
Hot Creek Isolates				180
HCB2	*Enterobacter cloacae*	$As(III)^r$	$arsA^+$ $arsB^+$ $arsC^+$	
HCSH1	*Klebsiella* sp.	$As(III)^r$	$arsA_{hyb}^+$ $arsB_{hyb}^+$ $arsC_{hyb}^+$	
HCSH9	*Yersinia intermedia*	$As(III)^r$	$arsA_{hyb}^+$ $arsB^+$ $arsC^+$	
KOV2	*Acinetobacter calcoaceticus*	$As(III)^r$	*ars* PCR negative	
KOV3	*Xanthomonas oryzae*	$As(III)^r$	$arsB^+$	
KOV4	*Janthinobacterium lividium*	$As(III)^r$	*ars* PCR negative	
KOV5	*Pseudomonas corrugata*	$As(III)^r$	*ars* PCR negative	
South Haiwee Drain Isolates				740
SHA1	*Serratia fonticola*	$As(III)^r$	$arsA^+$ $arsB^+$ $arsC^+$	
SHA2	*Pseudomonas corrugata*	$As(III)^r$	$arsA_{hyb}^+$ $arsB_{hyb}^+$ $arsC_{hyb}^+$	
SHA3	*Acinetobacter* genospecies 15	$As(III)^r$	$arsA_{hyb}^+$ $arsB_{hyb}^+$ $arsC_{hyb}^+$	
SHA4	*Pseudomonas corrugata*	$As(III)^r$	$arsA_{hyb}^+$ $arsB_{hyb}^+$ $arsC_{hyb}^+$	
SHA6A	*Pseudomonas vescicularis*	$As(III)^r$	$arsB_{hyb}^+$ $arsC_{hyb}^+$	
SHA8	*Pseudomonas corrugata*	$As(III)^r$	$arsA_{hyb}^+$ $arsB_{hyb}^+$ $arsC_{hyb}^+$	
SHA10	*Pseudomonas corrugata*	$As(III)^r$	$arsA_{hyb}^+$ $arsB_{hyb}^+$ $arsC_{hyb}^+$	
SHA17	*Serratia fonticola*	$As(III)^r$	$arsA^+$ $arsB^+$ $arsC^+$	
SHA27	*Pseudomonas cichorii*	$As(III)^r$	$arsA_{hyb}^+$ $arsB_{hyb}^+$ $arsC_{hyb}^+$	
SHA29	*Serratia fonticola*	$As(III)^r$	$arsA^+$ $arsB^+$ $arsC^+$	
SHA35	*Serratia fonticola*	$As(III)^r$	$arsA^+$ $arsB^+$ $arsC^+$	

[a] *E. coli* refers to isolates plated on mTEC supplemented with arsenic. Enteric refers to isolates plated on mENDO amended with arsenic.
[b] $As(III)^r$, resistance to 500 mg of sodium arsenite per liter; $As(V)^r$, resistance to 1,000 mg of sodium arsenate per liter; $Sb(III)^r$, resistance to antimony.
[c] hyb^+, negative by PCR and positive by hybridization with the corresponding *ars* probe; +, positive by PCR.

SHA29, SHA35 (*Serratia fonticola*), HCSH9 (*Yersinia intermedia*), and HCB2 (*Enterobacter cloacae*).

RESULTS

Abundance and phenotypic As^r determination. The diversity of the *E. coli* R773 *ars* model was investigated in As^r bacteria originating from a variety of sources. We first established the abundance of the As^r phenotype in bacteria isolated from industrial or domestic raw sewage, two sites in Hot Creek, and one site in South Haiwee Drain 5. Resistance to arsenic was defined as observable growth in liquid broth at 500 mg of As(III) or 1,000 mg of As(V) per liter (19). Table 3 summa-

TABLE 2. PCR primers designed from the *E. coli* R773 plasmid encoded *ars* operon genes, *arsA, arsB*, and *arsC* and PCR primers for constructing internal probes for the *arsA*-1, *arsB*-1, and *arsC*-1 primer sets

Targeted region[a] (bp)	Name	Sequence (5′ to 3′)	T_m[b] (°C)	Product size (bp)
203–222	*arsA*-1-F	TCCTGGATTGTCGGCTCTTG	58	186
367–386	*arsA*-1-R	ATCTGTCAGTAATCCGGTAA		
266–285	I-*arsA*-1-F	CGTTGACCCTATTAAAGGCG	60	103
347–366	I-*arsA*-1-R	ATTCATCAAAAGCCGCAATC		
139–158	*arsB*-1-F	CGGTGGTGTGGAATATTGTC	59	219
336–355	*arsB*-1-R	GTCAGAATAAGAGCCGCACC		
166–184	I-*arsB*-1-F	CGACGGCAACATTTATCGC	62	181
327–344	I-*arsB*-1-R	AGCCGCACCATCGTTGGC		
46–67	*arsC* -1-F	GTAATACGCTGGAGATGATCCG	59	370
393–413	*arsC* -1-R	TTTTCCTGCTTCATCAACGAC		
99–118	I-*arsC* -1-F	TACCTTGAAAACCCGCCTTC	62	240
316–335	I-*arsC* -1-R	AACCACTTCAGAAGGACGGC		

[a] Referenced from the coding sequence (CDS) of the *E. coli* R773 *ars* operon.
[b] T_m, annealing temperature used in PCR cycle.

rizes the plating results for arsenate- or arsenite-resistant bacteria. The average abundance of *E. coli* was 4×10^4 CFU/ml on mTEC without arsenic supplementation. As(III) addition to the medium had no effect on decreasing the abundance of CFU. Interestingly, when raw sewage was plated on mTEC supplemented with As(V), a log increase in *E. coli* abundance was observed compared to that with no arsenic supplementation. The average number of CFU per milliliter for creek samples plated on 50% PCA without arsenic ranged from 3×10^3 to 9×10^3. The abundance of CFU growing on nonselective medium (50% PCA) compared to As(V) medium showed little change for the South Haiwee Drain sample. Moreover, As(III) addition to the medium inhibited the growth of bacteria by 1 log for all creek sites. Hot Creek sites 1 and 2 had measurable colony growth when plated on As(III) and As(V); however, there was less than 300 CFU/ml. We conclude that raw sewage provided a good source for the isolation of phenotypically As^r enteric bacteria. In addition, the South Haiwee Drain microbial community was more resistant to As than sites within Hot Creek, as would be expected based on the high total As in the waters of the South Haiwee Drain site.

Isolates were randomly chosen from mTEC, mENDO, and PCA plates and further investigated for *E. coli* R773 *ars*-like sequences. A mixed phenotype of As^r isolates was found for the enteric bacteria isolated on mENDO. Table 1 reports the phenotypes of isolates screened by PCR. One enteric bacterium (EN-4), although isolated originally on no arsenic, was able to grow on As(III), but not As(V). Some bacteria isolated on arsenate media showed $As(III)^r$, while others were $As(III)^s$. *E. coli*, South Haiwee Drain, and Hot Creek isolates were only tested for arsenite resistance. These isolates were routinely grown in broth cultures containing 500 mg of sodium arsenite per liter.

Homology of *ars* genes in environmental isolates to the *E. coli* R773 genetic model. The diversity of *ars* genes was investigated by DNA-DNA hybridization in a total of 84 gram-negative environmental isolates originating from two sites in Hot Creek and one site in South Haiwee Drain. We assessed how well the *E. coli* R773 *ars* operon described the genotype of phenotypically As^r isolates. The *arsA* probe included a region of the gene that encodes one of the three cysteine residues that form the As(III) binding site within the protein. Although there is no known active site for ArsB, we chose the probe based on conserved regions among the known enteric *arsB* genes found in the GenBank database. Primer construction for *arsC* was more restrictive due to methodological constraints (high G-C content), and therefore the first 45 bases were excluded in the design; unfortunately, this region contained Cys-14, which is required for protein activity.

In previous hybridization studies in our laboratory with the 370-bp *arsC* gene probe, high-stringency conditions resulted in less than 5% hybridizations among 50 creek isolates (unpublished data). Modifications to the wash conditions allowed increased detection of divergent *arsC*-like sequences. The results from this experiment formed the basis for investigation of *ars* genes under low- and moderate-stringency conditions. The results for the colony hybridization and effects of stringency are summarized in Table 4. For all *ars* probes, as stringency was relaxed, a 20 to 30% increase in hybridization was observed. There also appeared to be site-to-site variation in the distribution of divergent *ars*-like sequences. Interestingly, *arsA* was more prevalent and more conserved in South Haiwee Drain (total As, 740 μg/liter) isolates at moderate stringency. Hot Creek site 1 (HCSH) isolates exhibited a higher prevalence of *ars* genes at moderate stringency compared to site 2 (HCB) isolates. Site 1 is located in an area in which most of the geothermal activity occurs and approximately 50% of the dissolved As is As(III). At site 2, located approximately 1 km

TABLE 3. Bacterial counts on nonamended and As-amended media at various sampling locations

Location	Bacterial count (CFU/ml) on[a]:		
	No arsenic	As(III) (500 mg/liter)	As(V) (1,000 mg/liter)
Raw sewage[b]	4.0×10^4	5.7×10^4	5.4×10^5
South Haiwee Drain 5	3.3×10^3	4.7×10^2	4.2×10^3
Hot Creek Swimming Hole (site 1)	8.7×10^3	<300 (230)	<300 (285)
Hot Creek Bridge (site 2)	6.3×10^3	<300 (147)	<300 (183)

[a] Numbers in parentheses are the actual CFU per milliliter and are noted when counts were less than 300 CFU/ml.
[b] Samples plated on mTEC—selective for *E. coli*. Samples from other locations were plated on PCA amended with the corresponding arsenic concentrations.

TABLE 4. Summary of colony lift and hybridization with *E. coli* probes *arsA*-1, *arsB*-1, and *arsC*-1

Location (*n*)	% of hybridization at stringency[a]					
	arsA		*arsB*		*arsC*	
	Low	Moderate	Low	Moderate	Low	Moderate
South Haiwee (34)	79.4	70.6	94.1	38.2	94.1	64.7
Hot Creek Swim Hole (site 1) (26)	76.9	26.9	75.0	76.9	84.6	69.2
Hot Creek Bridge (site 2) (24)	87.5	16.7	87.5	58.3	75.0	54.2
Total isolates screened (84)	81.0	41.7	86.9	56.0	85.7	63.1

[a] Low, proportion of hybridizations at low stringency (31 to 53% estimated mismatch); moderate, proportion of hybridizations at moderate stringency (21 to 36% estimated mismatch).

downstream, almost 100% of the As had been oxidized to As(V), the less toxic form. Interestingly, *arsA* was significantly less diverse in the South Haiwee isolates than those originating from Hot Creek (Chi square; $P < 0.05$). It is concluded that at a higher As concentration, genetic diversity is lower for genes that affect high-level resistance to As, such as in *arsA*, the arsenite-translocating ATPase gene.

Use of PCR to detect *ars* genes associated with enteric bacteria and pseudomonads. PCR was evaluated for its usefulness to detect *ars*-like genes in the isolates listed in Table 1. Because low-stringency hybridization was required to detect divergent *ars*-like sequences, we hypothesized that PCR would only detect closely related sequences with less than 10% sequence divergence at the primer binding sites. Average levels of nucleotide divergence for known enteric *arsA*, *arsB*, and *arsC* genes are 0.22 (standard deviation [SD] = 0.03), 0.21 (SD = 0.06), and 0.19 (SD = 0.054) nucleotide substitutions per site, respectively. At this level of divergence within enteric *ars* genes, the use of nondegenerate PCR primers would most likely not capture divergent *ars* genes with greater than 10% sequence differences.

In general, *arsA, arsB*, and *arsC* genes were detected in only enteric bacteria and not in pseudomonads (Table 5). Eight isolates were found to contain *arsA* homologs. Hybridization with the I-*arsA*-1 internal probe showed that highly homologous DNA was detected in these isolates. Interestingly, *arsA* in *E. cloacae* (HCB2) exhibited an amplicon of similar size, yet failed to hybridize to the R773 *arsA* internal probe at high stringency. Decreasing the stringency of the final wash step resulted in the confirmation of the amplicon (data not shown). However, use of low-stringency colony hybridization also allowed detection of divergent *ars*-like sequences in many of the pseudomonads.

In contrast to our results for *arsA*, *arsB* (Fig. 1) was conserved in 69% of the raw sewage bacteria and 85.7% of the creek enteric bacteria (Table 5). However, the *E. coli* R773 *ars* operon was not a good model for describing the As^r genotypes of Hot Creek and South Haiwee Drain pseudomonads. Surprisingly, KOV3 (*Xanthomonas oryzae* AV) exhibited a faint 219-bp band following Southern blotting and hybridization with the *arsB*-specific internal probe. Successive PCR attempts failed to generate the 219-bp band.

Our investigation into the diversity of *arsC* resulted in similar findings to those with *arsB*. The total occurrence of *arsC* for all of the isolates was 42% (total $n = 50$). Southern blotting and hybridization with the internal probe I-*arsC*-1 verified that highly homologous DNA was amplified in the creek isolates (data not shown). All isolates that exhibited PCR positivity were enteric bacteria originating from raw sewage and creek waters. When examining the occurrence of *arsC* within As(III)^r *E. coli*, only 50% of the isolates were positive. This finding was much lower than we expected in light of the known sequence data for *arsC*. For the As^r enteric isolates originating from creek samples, *arsC* was observed in 86% of the isolates, which was much higher than that observed in the *E. coli* strains isolated from sewage. However, only seven isolates were investigated for the creek samples (Table 5). In contrast, *arsC* was not detected in any of the 11 creek pseudomonads, suggesting that divergence is too great to use the *E. coli* R773 *ars* operon as a model for *arsC*. We concluded that the PCR-negative As^r enteric and nonenteric isolates represent divergent groups of *ars* operons.

A significant difference (chi-square test; $P < 0.05$) in the distribution of the genotypes was found between raw sewage and creek waters. The *arsA* gene was generally conserved in enteric creek isolates and not in the raw sewage enteric isolates. We concluded that the low occurrence of *arsA* in raw sewage isolates resulted from the lack of a selective pressure for maintaining *arsA*.

Determination of diversity of *ars* genes among enteric isolates. The final investigation into the diversity of *ars* genes was done by phylogenetic inference of *arsB* sequences obtained from South Haiwee Drain and Hot Creek isolates. They were compared to known *arsB* genes obtained from the GenBank database. The *arsB* fragments of the creek *Serratia* spp. and *Yersinia* sp. were almost identical: the average sequence similarity for this group was 98%. However, they appeared to be of a divergent lineage compared to the recently published *Serratia marcescens* (accession no. AJ288983) and *Yersinia enterocolitica* (accession no. U58366) sequences. Phylogenetic analyses placed the creek *Serratia* and *Yersinia* spp. into a distinct cluster (Fig. 2). The *arsB* fragments of this group appeared to be more closely related to the *E. coli* R773 *arsB*; the average nucleotide sequence similarity to this group was 84%. The creek isolate HCB2 (*Enterobacter cloacae*) shared greater sequence similarity to *E. coli* R46 and *Klebsiella oxytoca arsB* genes—89 and 94%, respectively. In addition, HCB2 sequence similarity to the South Haiwee Drain cluster was about 81%. The overall topology of the tree indicated that clustering of *arsB* was not species dependent, as would be expected for a phylogeny in-

TABLE 5. Summary of PCR results grouped as enteric or nonenteric bacteria phenotypically resistant to arsenite or arsenate

Gene	% Positive		
	Nonenteric gene[a] ($n = 11$)	Enteric	
		Sewage ($n = 32$)	Hot Creek ($n = 7$)
arsA	0	9.4	71.4
arsB	9.1	69	85.7
arsC	0	46.9	85.7

[a] Isolates originated from Hot Creek samples.

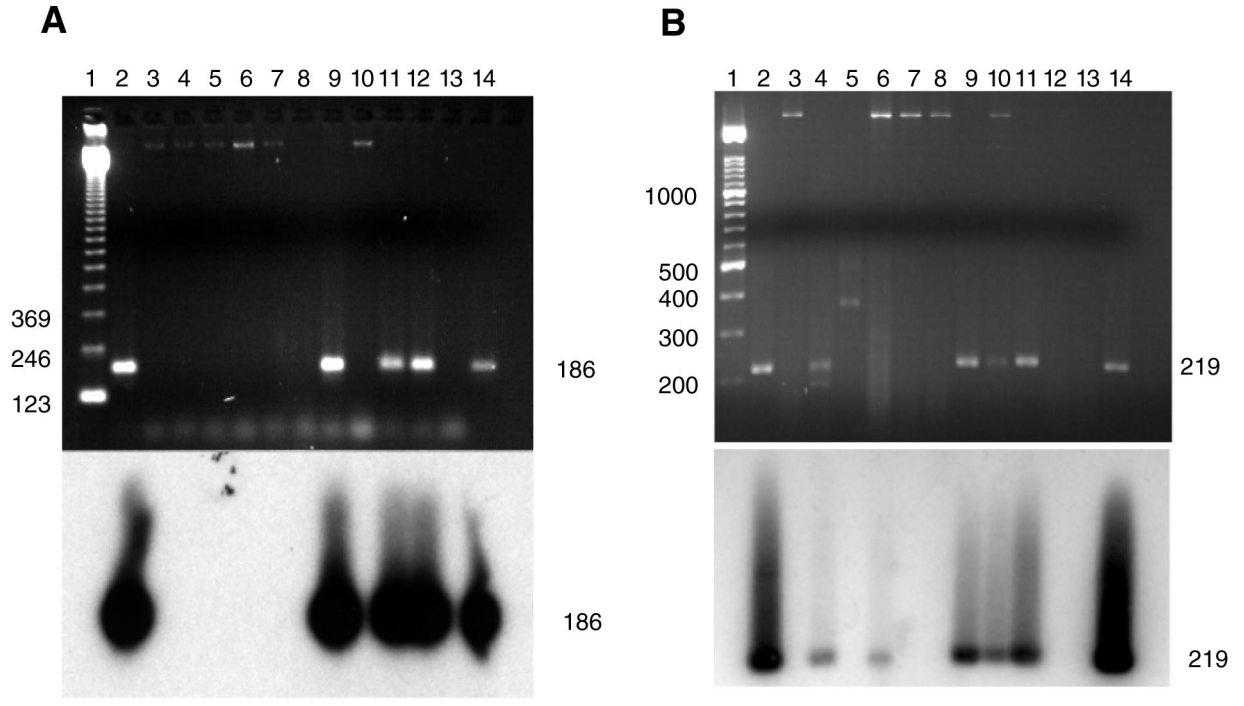

FIG. 1. (A) Ethidium bromide-stained agarose gel (top panel) and Southern blot analysis with the internal *arsA* probe (bottom panel). Electrophoretic analysis of PCR-amplified products was done with *arsA*-1 primers and genomic DNAs of South Haiwee Drain isolates. Lanes: 1, 123-bp ladder (GIBCO BRL); 2, *Serratia fonticola* (SHA1); 3, *Pseudomonas corrugata* (SHA2); 4, *Acinetobacter* genospecies strain 15 (SHA3); 5, *Pseudomonas corrugata* (SHA4); 6, *Pseudomonas vescicularis* (SHA6A); 7, *Pseudomonas corrugata (*SHA8); 8, *Pseudomonas corrugata* (SHA10); 9, *Serratia fonticola* (SHA17); 10, *Pseudomonas cichorii* (SHA27); 11, *Serratia fonticola* (SHA29); 12, *Serratia fonticola* (SHA35); 13, deionized water negative control; 14, *E. coli* pUM3-positive control. (B) Ethidium bromide-stained agarose gel (top panel) and Southern blot analysis with the internal *arsB* probe (bottom panel). Electrophoretic analysis of PCR-amplified products with *arsB*-1 primers and genomic DNAs of Hot Creek and *E. coli* isolates. Lanes: 1, 100-bp ladder (Boehringer/Mannheim Marker XIV); 2, *E. cloacea* (HCB2); 3, *Klebsiella* (HCSH1); 4, *Y. intermedia* (HCSH9); 5, *A. calcoaceticus* (KOV2); 6, *X. oryzae* (KOV3); 7, *J. lividium* (KOV4); 8, *P. corrugata* (KOV5); 9, *E. coli* (1ECO); 10, *E. coli* (2ECO); 11, *E. coli* (7ECO); 12, *P. putida*; 13, deionized water negative control; 14, *E. coli* pUM3 positive control.

ferred from 16S rRNA genes. However, it should be noted that the bacteria were identified by phenotype.

DISCUSSION

In recent years, the study of metal resistance in bacteria has led to the discovery of many metal-specific genetic models, including *czc* (Cd, Zn, and Co) (22) *cop* (Cu) (42), *mer* (Hg) (26), *cadA* (Cd) (24, 46; G. Nucifora, L. Chu, S. Silver, and T. K. Misra, Abstr. 88th Annu. Meet. Am. Soc. Microbiol. 1988, abstr. H-209, p. 179, 1988), and *chr* (Cr) (6, 21;C. Cervantes, H. Ohtake, and S. Silver, Abstr. 88th Annu. Meet. Am. Soc. Microbiol. 1988, abstr. H-213, p. 180, 1988). In addition, molecular approaches are more frequently being used to study microbial communities in metal-contaminated environments. PCR and gene probes are often used to characterize a community for the prevalence of a particular genetic model. Advantages to using molecular approaches included enhanced assessment of specific microbial populations in contaminated environments (32) and increased sensitivity in monitoring specific microbial populations during remediation efforts (18).

When investigating the diversity of *ars*, we used the *E. coli* R773 *ars* operon as a model for describing the genotype of phenotypically As^r bacteria. We first established the abundance of As^r bacteria in various water sources, including raw sewage and creek waters containing elevated levels of total arsenic. Raw sewage was used as a source of enteric bacteria, in which we hypothesized that *ars* genes would be relatively conserved in comparison to isolates from natural waters. Raw sewage exhibited an abundance of As^r enteric bacteria, despite low total As concentrations (<50 ppb; Y.-L. Tsai, personal communication.). We found that approximately 1% of the culturable *E. coli* species and enteric bacteria can resist the toxicity of As(III). However, all bacteria appeared to be resistant to As(V), because no reduction in the number of CFU compared to background CFU was obtained. Similar results were found for Hot Creek and the South Haiwee Drain. These results are similar to Hysman's and Frankenberger's (15) investigation of the abundance of As^r CFU in agricultural drainage waters and evaporation pond sediments. No CFU were observed on medium supplemented with ≥500 mg of As(III) per liter. In addition, Zelibor et al. (47) isolated $As(V)^r$ bacteria in well water samples. These isolates tolerated up to 2,000 μg of As(V) per ml. However, they did not test for As(III) resistance. Interestingly, bacteria indigenous to these monitoring wells did not precipitate or volatilize dissolved As. This suggests that the predominant resistance mechanism either involves a nonspecific physiologically based oxidation or reduction of As or is determined by an As-specific genetic system.

The difference in abundance between $As(III)^r$ and $As(V)^r$

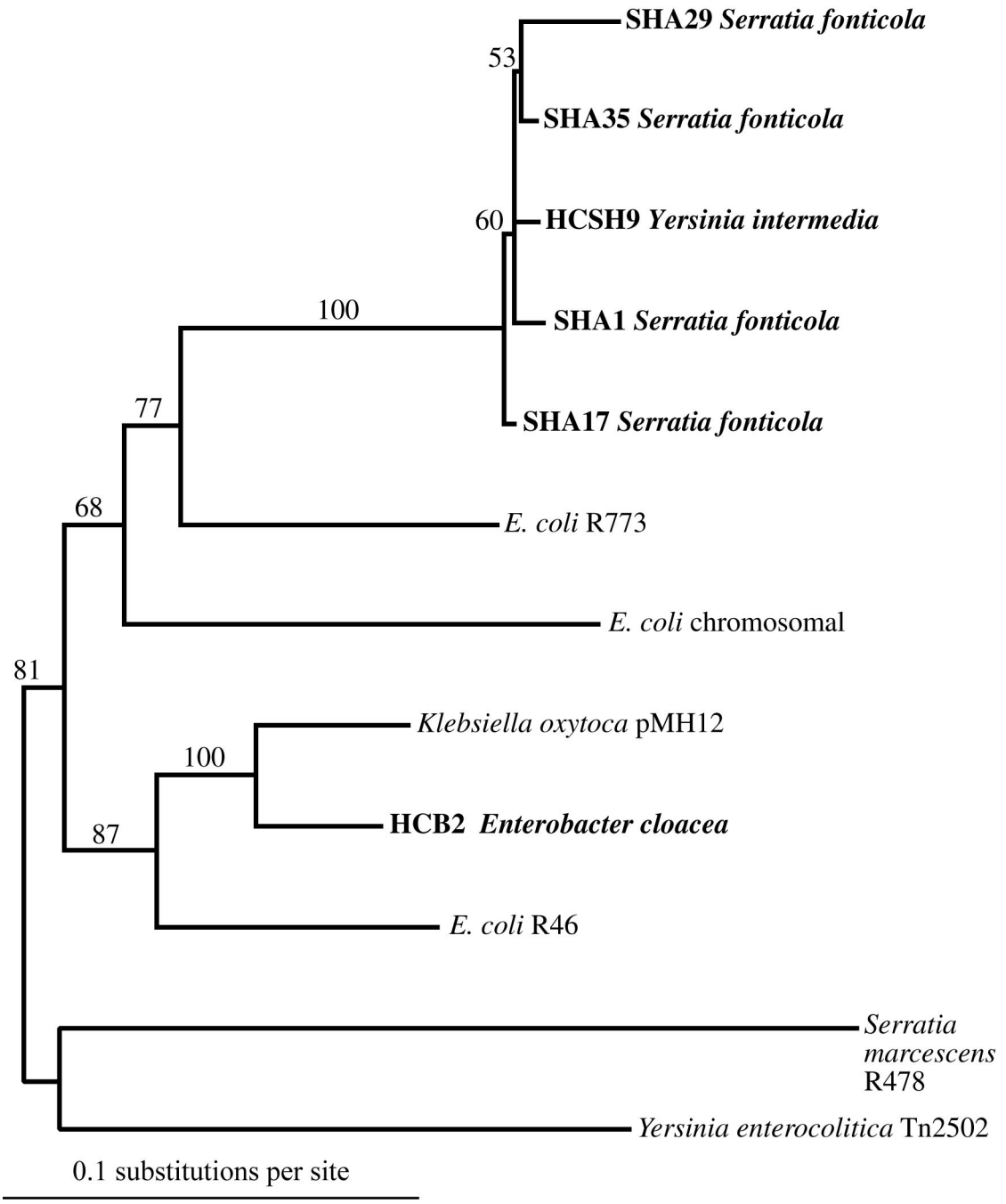

FIG. 2. Phylogenetic relationships among the *arsB* genes in enteric bacteria and Hot Creek and South Haiwee Drain isolates. The phylogenetic tree was constructed according to distance criterion (Kimura two-parameter substitution model with equal distribution of sites). The percentages of 100 bootstrap replicates that supported the branching order are shown above or near the relevant nodes. Clones from this study are indicated in boldface type.

phenotypes may be due to the expression of different mechanisms for the detoxification of arsenicals. Because As(V) is structurally similar to phosphate, in *E. coli*, As(V) can be taken up through the phosphate transport system (44, 45). In the *ars* system, intracellular As(V) is reduced to As(III) and pumped out of the cell by the ArsAB membrane-bound pump. Alternatively, physiological As(V)^r arises as a result of down regulatio of the inorganic phosphate transport system (*pit*), yielding a decrease in As(V) uptake and an insensitivity to As(V) toxicity (45). The apparent ubiquity of As(V)^r enteric bacteria suggests that physiological resistance may play a significant role in this observed phenotype. However, the proportion of physiological As(V)^r to *ars*-specific resistance cannot be determined from our results.

In a study detailed in reference 3, Barkay et al. investigated the diversity of mercury resistance genes of the *mer* operon.

Low-stringency hybridization with probes for *merA* permitted the detection of isolates that previously failed to hybridize with a Tn*21 mer* probe at high stringency (27). Similarly, when we investigated the diversity of *ars* within selected South Haiwee Drain and Hot Creek isolates, we found that colony hybridization at low and moderate stringency provided a useful tool for assessing diversity. Moreover, the relative frequencies of occurrence for *arsA*, *arsB*, and *arsC* were determined. The occurrence of *arsA* was much lower than that of *arsB* and *arsC*. On the other hand, *arsB* and *arsC* occurred in approximately equal frequencies. This suggests that either (i) *arsA* is more divergent than *arsB* and arsC, and the detection is therefore limited based on the current lack of *arsA* sequence data, or (ii) the *arsBC* genotype predominates in environments with low arsenic concentrations. The latter is supported in enteric bacteria for several reasons. Many known *ars* operons are composed of *arsRBC* (12). *Pseudomonas aeruginosa* PAO1 and *E. coli* K-12 have chromosomal *ars* operons composed of *arsRBC. Staphylococcus* spp. similarly have *arsRBC*. To date, there are only a few plasmid-associated *arsA* sequences: *E. coli* R773 and R46 and *Acidiphilium multivorum* pNCR-1. Because the arsenite-ATPase ArsA provides high-level resistance to arsenite, as was needed at the South Haiwee and Hot Creek sites, we propose that at low As concentrations, as in sewage, *arsA* is maintained in a few bacteria within a population, possibly on a plasmid or transposable element. The genetic determinant is then disseminated throughout a population of bacteria when a necessity arises for accelerated detoxification of As (i.e., during As enrichment events). The presence of *arsA*-like sequences in enteric isolates isolated from As-enriched waters supports this hypothesis. Our investigation of the diversity of the *ars* operon by PCR suggests that this technique is useful for detecting *ars*-like sequences in enteric bacteria. The high prevalence of the *ars* operon genes among the *E. coli*, mENDO, and creek enteric isolates is comparable with those in other studies that used filter hybridization with *ars* operon probes. Approximately 50% of As^r *E. coli* and *Klebsiella* spp. have been shown to contain homologs of the R773 *ars* operon, determined by hybridization with a 4.3-kb *ars* operon probe (20). Similarly, *ars* sequences have also been detected by Southern blotting and hybridization in the following bacteria: *E. coli* 40, *Shigella sonnei*, *Citrobacter freundii*, *Enterobacter cloacae*, *Erwinia carotovora*, *Salmonella enterica* serovar Arizonae, *Klebsiella pneumoniae*, and *Peudomonas aeruginosa* PAO1 (12).

The *E. coli* model for arsenic resistance appears to be an appropriate genetic system to use when screening for closely related isolates, when sequence identity is $\geq$80%. The enteric bacterium-related Hot Creek and raw sewage isolates showed strong PCR amplification products for *arsB* and *arsC*. Phylogenetic analysis of *arsB* fragments of South Haiwee Drain and Hot Creek isolates revealed significant divergence from the R773 *ars* model (Fig. 2). Possible mechanisms for this include horizontal gene transfer via plasmids or transposons. In addition, genetic recombination of *ars* genes and geographic isolation may have ultimately resulted in the formation of a distinct lineage of *ars* sequences.

The As^r pseudomonads listed in Table 1 showed little homology to the *arsA*, *arsB*, and *arsC* genes of the *E. coli* R773 model. In comparison to the *P. areuginosa ars* operon, the R773 *ars* operon exhibits significant differences at the nucleotide level. *Pseudomonas arsB* has 67% genetic similarity to *E. coli* R773 *arsB*. In addition, the *arsC* arsenate reductase gene shares only 29% sequence similarity to the *E. coli* R773 *arsC* gene. This high degree of sequence divergence is a limiting factor in the detection by PCR of distantly related sequences. Unrelated isolates did not show visible PCR amplification products. In some cases, smears were seen that indicated poorly matched primers to the target sequence. In addition, *X. oryzae* (KOV3) showed a weak hybridization signal with an internal probe for *arsB*, although no distinct DNA band was visualized on the agarose gel. The failure to detect *ars* genes in creek *Pseudomonas* spp. can be attributed to several factors, including limitations in the primer design and the high divergence of the target DNA. The use of degenerate primers may have allowed us to detect divergent *ars*-like genes. The *ars* primer sets were originally designed to amplify regions containing active sites or to encompass the most homologous sections of DNA indicated in multiple gene alignments with *E. coli* R773, R46, and chromosomal *ars* operons.

To detect divergent genes in nonenteric environmental bacteria, future research should focus on the development of hybridization methods based on model organisms that most appropriately reflect the environment to be studied. The abundance of pseudomonads in the aquatic environment makes them the most suitable for this type of methodology. *P. aeruginosa* and *P. fluorescens* are commonly found in aquatic environments. These species may provide better models for the development of PCR-based methods for the detection of As^r genes specific for the *ars* operon. The *E. coli* model will be most appropriately applied to environments in which fecal contamination is expected.

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