JOURNAL OF BACTERIOLOGY, Nov. 2005, p. 7390–7396 Vol. 187, No. 21
0021-9193/05/$08.00+0 doi:10.1128/JB.187.21.7390–7396.2005

Expression Dynamics of Arsenic Respiration and Detoxification in *Shewanella* sp. Strain ANA-3

Chad W. Saltikov,* Richard A. Wildman, Jr., and Dianne K. Newman

California Institute of Technology, Department of Geological and Planetary Sciences, Mailstop 100-23, Pasadena, California 91125

Received 21 April 2005/Accepted 8 August 2005

Because arsenate [As(V)] reduction by bacteria can significantly enhance arsenic mobility in the environment, it is important to be able to predict when this activity will occur. Currently, two bacterial systems are known that specifically reduce As(V), namely, a respiratory system (encoded by the *arr* genes) and a detoxification system (encoded by the *ars* genes). Here we analyze the conditions under which these two systems are expressed in *Shewanella* sp. strain ANA-3. The *ars* system is expressed under both aerobic and anaerobic conditions, whereas the *arr* system is only expressed anaerobically and is repressed by oxygen and nitrate. When cells are grown on As(V), the *arr* system is maximally induced during exponential growth, with peak expression of the *ars* system occurring at the beginning of stationary phase. Both the *arr* and *ars* systems are specifically induced by arsenite [As(III)], but the *arr* system is activated by a concentration of As(III) that is 1,000 times lower than that required for the *arsC* system (≤100 nM versus ≤100 μM, respectively). A double mutant was constructed that does not reduce As(V) under any growth conditions. In this strain background, As(V) is capable of inducing the *arr* system at low micromolar concentrations, but it does not induce the *ars* system. Collectively, these results demonstrate that the two As(V) reductase systems in ANA-3 respond to different amounts and types of inorganic arsenic.

In sediments and groundwaters throughout the world, microorganisms affect the geochemistry of arsenic (As), which can lead to As contamination of drinking water supplies and poisoning of epidemic proportions (18, 20, 28). The mechanism for As release into drinking water typically involves the reductive dissolution of ferric (hydr)oxide minerals and/or the reduction of arsenate [$HAsO_4^{2-}$, or As(V)] to arsenite [H_3AsO_3, or As(III)] (19). Although both As(V) and As(III) strongly adsorb to ferric (hydr)oxides at the pH of most natural waters, microbial reduction of ferric (hydr)oxide and As(V) can liberate As(III) into sedimentary pore waters, which under the appropriate hydrological conditions can be drawn down into sandy aquifers, where As(III) is mobile in the aqueous phase (6, 7). In light of this, it is important to be able to predict when As(V)-reducing microorganisms will be active.

Two different arsenate reduction pathways exist in bacteria, namely, the *ars* and the *arr* systems. The *ars* genes are present in many bacteria and archaea and are diverse in their sequence and genomic organization (17, 21). The *Escherichia coli* and *Staphylococcus* sp. *ars* operons have been well characterized at the molecular level with respect to both their biochemistry and their regulation (17, 22). Because As(V) is structurally similar to phosphate (HPO_4^{2-}), it enters the cytosol via a low-affinity phosphate transporter (such as the Pit system in *E. coli*) (23, 29). When this happens, the cell employs a 12- to 15-kDa soluble reductase, ArsC, that couples the oxidation of thiols from either glutathione/glutaredoxin or thioredoxin to the reduction of As(V) to As(III) (17). A cytoplasmic membrane efflux pump, ArsB, extrudes As(III) from the cytosol, ridding the cell of As(III). ArsA, an ATPase subunit, interacts with ArsB to facilitate As(III) extrusion by using ATP hydrolysis to drive As(III) efflux through ArsB. The *ars* operon is induced by As(III) and antimonite [Sb(III)] (32). Two regulators, ArsR and ArsD, are repressors of the *ars* operon and control the basal and maximal levels of *ars* gene expression, respectively. Both ArsR and ArsD bind the same operator site immediately upstream of the *ars* operon, albeit with different affinities (2, 31, 33). ArsR exhibits a 100 times greater affinity for the *ars* operator site and requires 10 times less As(III) to relieve in vivo repression of the *ars* operon than does ArsD. The proposed model for As(III)-dependent regulation is that ArsR controls *ars* transcription when As(III) concentrations are low, whereas ArsD controls transcription in environments where [As(III)] is high (2). The *ars* system is expressed in *E. coli* and other organisms under both aerobic and anaerobic conditions.

The *arr* operon, in contrast, has only recently been identified (25), and relatively little is known about its regulation and biochemistry. The *arrAB* operon of *Shewanella* sp. strain ANA-3 encodes ArrA and ArrB, and both are required for respiratory As(V) reduction (25). Sequence analyses and biochemical studies of phylogenetically diverse As(V)-respiring bacteria indicate that ArrA is a large, ~95-kDa molybdenum-containing enzyme and that ArrB is an ~26-kDa enzyme containing several Fe-S clusters, and both of these proteins resemble enzymes in the dimethyl sulfoxide reductase family (1, 11). ArrA is thought to be the subunit that binds As(V) and reduces it to As(III), with ArrB serving as a conduit for electrons stemming from *c*-type cytochromes in the respiratory chain (12). The conservation of *arrA* and *arrB* is remarkably high, allowing their expression to be detected in the environment by reverse transcription-PCR (RT-PCR) with specific primers

* Corresponding author. Present address: University of California, Santa Cruz, Dept. of Environmental Toxicology, Santa Cruz, CA 95064. Phone: (831) 459-5520. Fax: (831) 459-3524. E-mail: saltikov@etox.ucsc.edu.

(15). Under conditions similar to those found in many sedimentary environments, where As(V) is adsorbed onto ferric (hydr)oxides and aqueous concentrations of As are low (e.g., nM to μM), expression of the *arrA* gene is required for the reduction of As(V) to As(III); the *arsC* gene is not involved in As(V) reduction under these conditions (15).

To quantify the geochemical impact of As(V)-reducing microorganisms in a given locale, it is necessary to be able to predict when they will be able to reduce As(V). Although many factors will influence the activity of these bacteria in the environment, it is essential as a starting point for being able to predict when bacteria will catalyze As(V) reduction in the environment to know what controls or represses the expression of the As(V) reductase genes such as *arsC* and *arrA*. Here we report the gene expression patterns for *arrA* and *arsC* in *Shewanella* sp. strain ANA-3 (24, 25), a model As(V)-reducing isolate, grown under a variety of environmentally relevant conditions.

MATERIALS AND METHODS

Growth conditions. *Shewanella* sp. strain ANA-3 was grown in a basal salts medium (TME) with the following composition: 1.5 g of NH_4Cl/liter, 0.1 g of KCl/liter, 0.6 g of $NaHPO_4$/liter, 0.5 g of yeast extract/liter, 10 mM HEPES, and 10 ml of trace elements/liter (10), adjusted to pH 7. Lactate was included in the medium at a 20 mM final concentration. Anaerobic medium was prepared by boiling under a stream of oxygen-free nitrogen (passed over a heated copper column), dispensed under nitrogen into Balch tubes which were then closed with stoppers, and sterilized by autoclaving. Electron acceptors were used at 20 mM for fumarate, nitrate, and trimethyl-*N*-amine oxide (TMAO) and 10 mM for As(V), unless otherwise indicated. All growth experiments were done in triplicate.

Overnight cultures of ANA-3 were grown aerobically at 30°C and 250 rpm. Cultures were diluted 1/100 in 10 ml of anaerobic medium and grown to an optical density at 600 nm (OD_{600}) of 0.1 (mid-exponential phase of growth), and 1 ml was harvested for RNA extraction (see below). For the analysis of aerobic conditions and the transcription of *arr* and *ars* operons, 10-ml cultures were grown in 250-ml Erlenmeyer flasks with continuous sparging of filtered (0.2 μm) air and shaking at 250 rpm. Once an OD_{600} of 0.1 was reached, 1 ml was harvested for RNA extraction as described below.

RNA extraction and processing. Cells were centrifuged for 10 min at 4°C and 14,000 rpm. Cell pellets were stored at −80°C. A QIAGEN RNeasy miniprep kit was used to extract the total RNA from cell pellets according to the manufacturer's instructions. Contaminating DNA was removed from 16 μl of RNA extract by the addition of 2 μl of 10× reaction buffer and 2 μl of DNase (Promega RQ1 DNase) and incubation for 30 min at 37°C. Reactions were terminated by the addition of 2 μl of stop buffer and heating for 10 min at 65°C.

cDNAs were synthesized using an Applied Biosystems TaqMan reverse transcription kit according to the following modified protocol. Each 20-μl reaction mixture consisted of 2 μl 10× TaqMan RT buffer, 4.4 μl 25 mM $MgCl_2$, 4 μl deoxynucleoside triphosphate mix, 1 μl random hexamers, 0.4 μl RNase inhibitor, 0.6 μl Multiscribe reverse transcriptase (50 U/μl), and 7.6 μl DNase-treated RNA sample. Reactions were incubated at 25°C for 10 min and 48°C for 30 min, followed by heat inactivation at 95°C for 5 min. Prior to quantitative PCR, cDNAs were diluted 1/4 in nuclease-free water.

Operon mapping. Mapping of the *arrAB* RNA transcript was performed on total RNA extracted from cells grown in the presence of As(V). Approximately 1 μg of DNase-treated RNA was used to synthesize cDNA using primer A, B, or 1 (see below). PCRs were performed with various combinations of the following primers to show the limits and interconnectedness of *arrAB* mRNA transcripts: 1, 5′-ATCAGCACCAAATGACAGGATA-3′; 2, 5′-TAAATCACCAATTACCCGTGCT-3′; 3, 5′-AACACGAACGACGGTATTCACT-3′; 4, 5′-AAGTTATGGGAAGGTGTTCGTC-3′; 5, 5′-TACACCTATTGTCGAGGGGATT-3′; 6, 5′-GCAACATAAAGCAGGATCGAAT-3′; A, 5′-GCTTCAGGTTTCAACTGCATAG-3′; and B, 5′-GATTCGCTGATGTTTAGTGATTG-3′.

Quantitative real-time PCR. The following primers were used for real-time PCR analyses: for *arrA*, Q-*arrA*-F4 (5′-AATGGTCAGATACCTCACCGCAC-3′) and Q-*arrA*-R4 (5′-GCTATTCCACACCCCTTTTTGC-3′); for *gyrB*, Q-*gyrB*-F1 (5′-ACGAGCGTGACAATAAGAATGA-3′) and Q-*gyrB*-R1 (5′-ACGTCTTTGTTTACTGGCGTTT-3′); and for *arsC*, Q-*arsC*-F1 (5′-GATTTACCATAATCCGGCCTGT-3′) and Q-*arsC*-R1 (5′-GGCGTCTCAAGGTAGAGGATAA-3′). PCR mixtures consisted of 4 μl diluted template (cDNA, standard ANA-3 genomic DNA, or water), 20 μl 2× SybrGreen *Taq* mix (Applied Biosystems), a 300 μM concentration of each primer, and nuclease-free water to make a final volume of 40 μl. PCRs were carried out in either an ABI 7300 or MJ Research Opticon 2 thermocycler in duplicate for triplicate cDNA samples and primer sets. The thermocycle profile was as follows: 95°C for 10 min, 40 cycles of 95°C for 30 s and 60°C for 1 min, and a final denaturation cycle to examine the DNA melting curves of PCR products.

The cycle thresholds (C_T) were determined for samples and genomic DNA standards. For each transcript, the C_T value was converted to a genomic DNA equivalent in nanograms by comparing the C_T of an unknown sample to standard curves prepared from ANA-3 genomic DNA (1, 0.1, 0.01, and 0.001 ng). The slopes for *arrA*, *arsC*, and *gyrB* standard curves ranged from 3.3 to 3.6 and were linear, with an R^2 value of 0.999. The *gyrB* gene (encoding DNA gyrase subunit B) was used to normalize the expression values for *arrA* and *arsC*.

Mutagenesis. An As(V) reduction-deficient strain of ANA-3 was generated by deleting the *arsC* gene from the *arrA* null mutant ARRA3 (25). Mutagenesis was performed similarly to procedures described elsewhere (25). Briefly, a mutant allele of *arsC* was generated using two sequential PCR steps. First, two 1-kb DNA fragments flanking the *arsC* gene were generated, using primers X-*arsC*-A (5′-GGACTAGTTAATGGTGCCCGTCGATATT-3′) and X-*arsC*-B (5′-[CCCATCCAGCATGCTTAAACA]GATATTTGCCATAACATGTCTCT-3′) for one fragment and X-*arsC*-C (5′-[TGTTTAAGCATGCTGGATGGG]AATGGACAAAGAGTCGCTCAA-3′) and X-*arsC*-D (5′-GGACTAGTTATAGCCACGCCTTCTGGTC-3′) for the other fragment (underlined sequence, SpeI site; the 21-bp fusion PCR linker, with a double-underlined internal SphI site, is shown in brackets). After gel purification of the two 1-kb PCR products using a Qiaquick gel extraction kit (QIAGEN, Valencia, CA), the products were mixed together and subjected to PCR using X-*arsC*-A and X-*arsC*-D, generating a 2-kb fused PCR product containing a deleted *arsC* allele. This fusion PCR product was cloned into the SpeI site of the suicide vector pSMV10, which confers resistance to kanamycin (Doug Lies, Caltech). The mutant *arsC* allele was introduced into ARRA3 by conjugation. Kanamycin-resistant colonies of ANA-3 were grown nonselectively in Luria-Bertani (LB) medium overnight and plated on LB agar plates containing 10% sucrose. Plates were incubated at room temperature (~22°C), and sucrose-resistant colonies were streak purified, checked for sensitivity to kanamycin and gentamicin, and screened by PCR for the *arsC* deletion. This strain of ANA-3 was referred to as ARM1 (*a*rsenate *r*eduction *m*utant).

RESULTS

***arrA* and *arrB*, a two-gene operon.** The *arrA* and *arrB* genes of *Shewanella* sp. strain ANA-3 are located adjacent to the *ars* operon on the chromosome (24). To test whether *arrA* and *arrB* constitute a simple two-gene operon, we used RT-PCR to determine how these genes are connected in mRNA (Fig. 1A). When cDNA was synthesized with either an *arrA*- or *arrB*-specific primer, PCR within the respective gene (primers 5 and 1 for *arrA* and primers 3 and 2 for *arrB*) or across *arrA* and *arrB* (primers 4 and 2) produced the predicted sizes. Because a PCR product was not observed from reactions with primers 6 and 1 from cDNA synthesized with primer 1, the mRNA start site must exist between *arsD* and *arrA*. To map the 3′ end of the *arr* mRNA, primers A and B, which flank a putative terminator downstream of *arrB*, were used to synthesize cDNA. PCR products were observed when using primers 3 and 2 on both A and B cDNA reactions (Fig. 1B, lanes 13 and 15). These results show that *arrA* and *arrB* are carried on the same mRNA and that either the predicted terminator is weak or the "real" terminator is located further downstream of the *arrAB* cluster. More detailed mapping efforts will be required to fully characterize the promoter and terminator regions. For the majority of the following experiments, we focus only on the expression of *arrA* and *arsC* as representative genes of the *arr* and *ars*

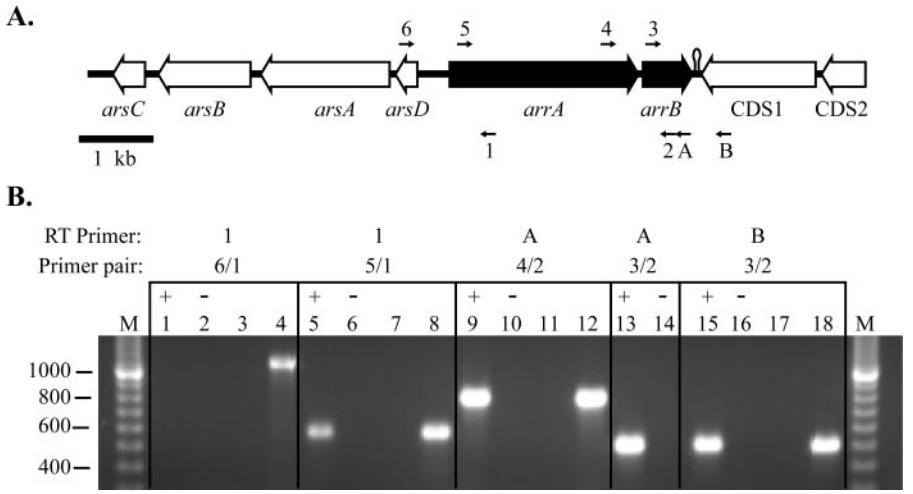

FIG. 1. Gene linkage of *arr* mRNA, determined by RT-PCR with RNA extracted from cells of ANA-3 grown on As(V). (A) Map of the ANA-3 *ars* and *arr* operons and two putative coding sequences (CDS1 and CDS2) of unknown function (NCBI accession no. AY271310). The positions of PCR and RT primers used to determine the gene linkages on the *arr* mRNA are shown as small arrows. (B) Agarose gel visualization of RT-PCR and control PCRs. Plus and minus signs denote RT and no-RT additions to cDNA reactions. Additional PCR controls consisted of either no template (water) (lanes 3, 7, 11, and 17) or ANA-3 genomic DNA (lanes 4, 8, 12, and 18). PCR products from primer pairs 6 and 1, 5 and 1, 4 and 2, and 3 and 2 are shown in lanes 1 to 4, 5 to 8, 9 to 12, and 13 to 18, respectively. RT-PCR products are shown in lanes 1, 2, 5, and 6 (RT primer 1), lanes 9, 10, 13, and 14 (RT primer A), and lanes 15 and 16 (RT primer B). M, 100-bp DNA size marker.

operons. When characterizing the *arr* and *ars* expression patterns of the *arrA* and *arsC* double mutant strain ARM1, *arrB* and *arsB* were used in place of *arrA* and *arsC*.

Effects of different electron acceptors on expression of *arrA* and *arsC*. We developed an RNA expression method based on quantitative real-time PCR to quantify the mRNA of either *arrA* or *arsC* in exponentially growing cultures prepared under various conditions. The DNA gyrase gene, *gyrB*, which encodes an essential DNA replication protein (4), was used as a reference to compare *arrA* and *arsC* gene expression. *gyrB* appeared to be constitutively expressed and unaffected by different electron donors and by arsenic and phosphate supplementation of the growth medium (data not shown). To determine how oxygen affected the expression of *arrA* and *arsC*, air-sparged cultures grown with or without arsenic [As(V) or As(III)] and cultures grown anaerobically on As(V) were examined for *arrA* and *arsC* gene expression. The expression patterns of *arrA* and *arsC* under these various conditions are summarized in Table 1. When ANA-3 was grown anaerobically on As(V), both *arrA* and *arsC* were highly expressed (~100-fold) compared to their expression under aerobic conditions without As. The expression of *arsC* increased 7-fold in cultures grown aerobically with 5 mM As(V) and 25-fold in aerobic cultures with 1 mM As(III) compared to aerobic cultures without As. In contrast, *arrA* expression was repressed under aerobic conditions, even in the presence of arsenic. These results suggest that *arsC* is induced in the presence of arsenic under both aerobic and anaerobic conditions and that *arrA* is maximally induced in the presence of arsenic under anaerobic conditions.

Given that *arr* is not expressed aerobically but is induced anaerobically with arsenic, we were interested in how growth on other terminal electron acceptors would affect *arrA* and *arsC* expression. Because strain ANA-3 can grow on at least eight different electron acceptors (24), we focused only on fumarate, nitrate, and TMAO as representatives. The expression values for *arrA* and *arsC* when ANA-3 was grown on these terminal electron acceptors alone and in the presence of either As(V) or As(III) are also summarized in Table 1. The expression of *arsC* in cultures containing fumarate, nitrate, or TMAO was similar to that in oxygen-containing cultures. However, a slight anaerobic induction of *arrA* was observed in anaerobic cultures in the absence of As(V) or As(III) compared to that measured in aerobic cultures. When As(V) or As(III) was included in anaerobic cultures of ANA-3, a significantly increased expression of *arrA* was observed for fumarate- or TMAO-containing cultures, but *arrA* was not induced in cul-

TABLE 1. Quantitative RT-PCR analysis using real-time PCR with RNAs extracted from ANA-3 grown on various electron acceptors concurrently with either As(V) or As(III)

Substrate and As source[a]	*arrA* expression[b]	Fold induction[c]	*arsC* expression[b]	Fold induction[c]
Arsenate	1.56 ± 0.05		1.86 ± 0.2	
As(III)	1.16 ± 0.2		1.27 ± 0.16	
Oxygen	0.012 ± 0.004		0.015 ± 0.005	
As(V)	0.015 ± 0.002		0.10 ± 0.02	7
As(III)	0.013 ± 0.002		0.37 ± 0.06	25
Fumarate	0.09 ± 0.03		0.04 ± 0.01	
As(V)	0.72 ± 0.09	7.8	0.44 ± 0.08	10.3
As(III)	0.92 ± 0.06	10.0	0.71 ± 0.04	16.9
Nitrate	0.05 ± 0.02		0.04 ± 0.00	
As(V)	0.08 ± 0.01		0.21 ± 0.11	5.1
As(III)	0.07 ± 0.01		0.68 ± 0.12	16.2
TMAO	0.02 ± 0.00		0.02 ± 0.00	
As(V)	0.43 ± 0.17	20.1	0.26 ± 0.09	13.6
As(III)	0.29 ± 0.03	13.5	0.61 ± 0.08	32.7

[a] As(V), 5 mM; As(III), 1 mM.
[b] Expression represents the ratio of the relative quantity (ng genomic DNA equivalents) of *arrA* or *arsC* transcript to that of the housekeeping gene *gyrB*. Data are means ± standard deviations.
[c] Induction was determined by normalizing the expression value for *arrA* or *arsC* to that for no-As conditions.

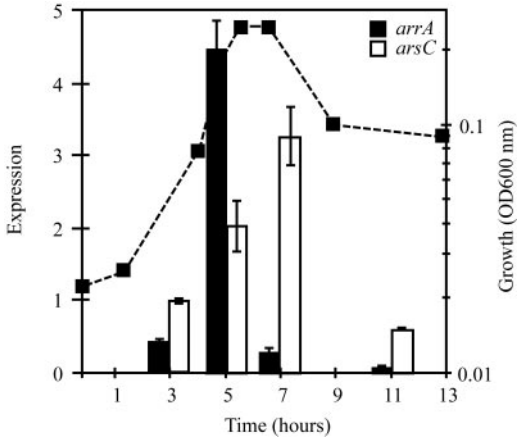

FIG. 2. Dynamics of gene expression for the As(V) respiratory reductase (*arrA*) and arsenic detoxification reductase (*arsC*) genes over different growth phases of a representative As(V)-grown batch culture. The expression of *arrA* (black bars) and *arsC* (white bars) was normalized to the expression of a housekeeping gene, *gyrB*, which encodes DNA gyrase. A representative growth curve (■) (from triplicate cultures) of ANA-3 grown on arsenate as a terminal electron acceptor is shown as an overlay.

tures grown on nitrate with either As(V) or As(III). In contrast, *arsC* was induced in the presence of both As(V) and As(III), regardless of the terminal electron acceptor. These results indicate that *arrA* is induced anaerobically in the presence of arsenic and that growth on nitrate represses the induction of the As(V) respiratory genes but not the arsenic detoxification genes.

Dynamic expression of *arrA* and *arsC*. Having determined that anaerobic conditions and As induce the expression of the *arr* operon, we predicted that *arrA* expression would be greatest during the exponential phase of growth in As(V)-respiring cultures and that the detoxification pathway would be induced as As(III) accumulated in the culture. To test this, we monitored *arrA* and *arsC* expression throughout different growth phases of an As(V)-respiring batch culture. The dynamic expression of *arrA* and *arsC* is illustrated in Fig. 2. Consistent with our predictions, the peak of *arrA* expression occurred during the exponential phase of growth, with the *arsC* expression peak following in the stationary phase.

Phosphate and arsenate effects on *arr*/*ars* transcription. The concentration of inorganic phosphate (HPO_4^{2-}, or P_i) in the environment is an important parameter to consider in the context of As cycling because P_i is a structural analog of As(V). As(V) and P_i compete for uptake via phosphate transporters (30) and adsorb similarly to sedimentary minerals such as iron oxides (5). We therefore sought to determine whether the P_i concentration would affect the expression of *ars* and *arr*. Our hypothesis was that increasing the concentration of P_i [increasing the P_i to As(V) ratio] would reduce *arr* and *ars* expression because P_i would out-compete As(V) for uptake into the cell. To test this, ANA-3 was grown on As(V) as a terminal electron acceptor in the presence of increasing concentrations of P_i. Figure 3A shows the physiological growth response to increasing levels of P_i. At equal levels of P_i and As(V), the growth rate was higher and the time to reach stationary phase was shorter than those for lower P_i-to-As(V) ratios. To ensure that this

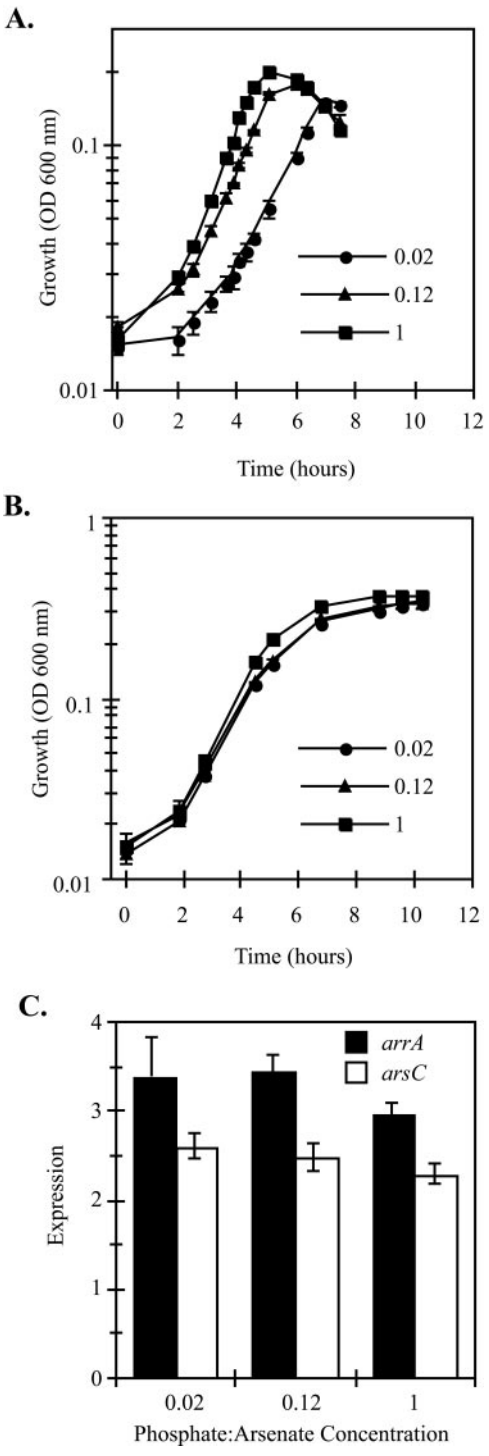

FIG. 3. Effects of inorganic phosphate (P_i) on As(V) respiration and *arrA*/*arsC* transcription. (A) Effect of different P_i concentrations on growth when ANA-3 is grown on 5 mM As(V). (B) Effect of increasing P_i concentrations on growth of ANA-3 when grown concurrently with fumarate (20 mM). Symbols for panels A and B represent P_i additions of 100 μM (●), 600 μM (▲), and 5 mM (■). (C) Effects of P_i additions on the expression of *arrA* (black bars) and *arsC* (white bars) relative to that of *gyrB* in As(V)-grown cultures harvested at the mid-log growth phase (OD_{600}, 0.1). Data points and error bars represent averages and standard deviations, respectively, of triplicate cultures.

growth difference was not due to phosphate limitation, we measured growth on fumarate for the different P_i concentrations used in Fig. 3A and found that the growth rate was constant (Fig. 3B). Surprisingly, the expression of *arrA* and *arsC* was not significantly different, regardless of the P_i-to-As(V) ratio (Fig. 3C). Nevertheless, the presence of more P_i did appear to offer some protection against the toxicity of As(V) (i.e., a higher P_i concentration resulted in higher growth rates and yields) (Fig. 3A).

Induction of *arr* and *ars* gene expression by As(III) and As(V). Because cultures of ANA-3 grown in mM concentrations of either As(V) or As(III) exhibited *arrA* and *arsC* induction in most of our experiments and because the As-to-P_i ratio did not affect *arrA* and *arsC* expression under the conditions tested, we sought to determine the minimal concentration of arsenic that would be required to induce the *arr* and *ars* systems. When the wild-type strain of ANA-3 is exposed to As(V), it is impossible to determine whether As(V) alone can induce gene expression because As(III) will be generated due to As(V) reductase activity. Therefore, we first investigated the potential for As(III) alone to induce *arr* and *ars* gene expression in fumarate-containing wild-type cultures. Figure 4A shows the expression profiles of *arrA* and *arsC* over a wide concentration of As(III). *arrA* gene expression was detected with 100 nM As(III) and increased almost 11-fold compared to no-arsenic controls. In contrast, the expression of *arsC* required 1,000 times more As(III) before its expression could be detected.

To determine whether As(V) alone could induce *arr* or *ars* gene expression, we constructed a double mutant (Δ*arrA* Δ*arsC*; strain ARM1) that cannot reduce As(V) under any conditions. Inductively coupled plasma-mass spectrometry (ICP-MS) analysis of As(III) and As(V) from filtrates of fumarate-grown cultures containing 10 mM As(V) revealed no detectable As(III) (data not shown). Using strain ARM1, we measured the expression of *arrB* and *arsB* in fumarate-grown cultures containing different As(V) concentrations. The results show that *arrB*, but not *arsB*, gene expression increases with increasing As(V) concentrations (Fig. 4B). The arsenite-dependent expression of *arrB* and *arsB* in the ARM1 strain (Fig. 4C) was similar to the expression of *arrA* and *arsC* in the wild type, indicating that deleting *arrA* and *arsC* did not alter the expression of the *ars* or *arr* operon. Although the detection limit for ICP-MS analysis is near 100 nM, it is possible that undetectable amounts of As(III) were present in cultures of ARM1 when grown with As(V). However, ARM1 exhibited similar growth rates when grown on fumarate and various As(V) concentrations, suggesting that this was not the case (data not shown). Moreover, had As(III) been present, presumably it would have been present at a constant amount, and thus if *arr* expression had been solely induced by As(III), we would have expected the trend to have been flat. Instead, *arrA* expression increased up to 10-fold compared to that with no As(V) at the highest As(V) concentration. In comparison to the As(III)-dependent expression level of *arrA* in the wild-type strain ANA-3, the expression level of *arrB* in ARM1 was 10-fold less, but the increases in *arr* expression with increasing concentrations of arsenic were similar for both ARM1 and wild-type ANA-3. It appeared that As(III) was a more potent inducer than As(V) of *arr* gene expression.

DISCUSSION

Bacteria reduce As(V) either to detoxify As from the cytoplasm or as a respiratory substrate for energy conservation reactions. Arsenate reduction via the detoxification route is attributed to the *ars* operon, which has been studied in great detail primarily in *E. coli* and *Staphylococcus aureus*. However, less is known about As(V) respiratory reduction, despite having numerous As(V)-respiring microbes in culture. We recently identified a gene cluster, *arrAB*, that confers the ability to respire As(V) on *Shewanella* sp. strain ANA-3 (25). Moreover, we also identified in ANA-3 an *ars* operon, *arsDABC*, and demonstrated that this system, although conferring resistance to As, is not required for As(V) respiration (24). The focus of this study was to investigate the expression dynamics of As(V) respiration and detoxification in ANA-3 under different environmental conditions.

We have shown that the *arr* transcript contains *arrA* and *arrB* on the same mRNA, constituting a simple two-gene operon, and that As(V) or As(III) and anaerobic conditions are the primary factors that induce the transcription of *arrA*. The expression of *arrA* is not induced aerobically, and nitrate represses transcription even in the presence of As(V) or As(III). In *E. coli*, nitrate is known to repress the expression of genes encoding electron carriers for alternative substrates with redox potentials lower than that of nitrate through the nitrate/nitrite sensor NarQ and the response regulator NarP (8). Given the standard redox potential of As(V)/As(III) (+130 mV) (13), which is lower than that of nitrate/nitrite (+420 mV) and oxygen (+820 mV) (14), by analogy to *E. coli* it is not surprising that nitrate represses the As(V) respiratory reductase. Moreover, the genome sequence for *Shewanella oneidensis* strain MR-1, a close relative of ANA-3, contains homologs of genes encoding NarQ and NarP, reinforcing the possibility that nitrate repression of *arr* transcription in ANA-3 involves homologs of NarQ/NarP. When ANA-3 was grown on the electron acceptor fumarate or TMAO, *arrA* expression was slightly induced compared to that in aerobically grown cultures. However, the presence of either As(V) or As(III) in these cultures greatly increased *arr* expression. These results demonstrate that *arr* is regulated by at least two control systems, one that senses the general redox state and another that induces expression in response to As(V) or As(III). The sensitivity of *arr* expression to nanomolar levels of arsenic shows that the proteins that regulate *arr* gene expression can sense arsenic at environmentally relevant concentrations such as those observed in some groundwaters of Bangladesh (up to 8.5 μM dissolved As) (6) or in pore waters of iron- and arsenic-enriched reservoir sediments in California (up to 17 μM) (9).

Because we found no significant changes in *arrA* and *arsC* transcription as a function of the P_i-to-As(V) ratio in our experiments, the amount of As(V) entering the cell under these conditions clearly was sufficient to induce *arrA* and *arsC* expression. In the environment, however, where the majority of P_i and As(V) is adsorbed onto solid-phase substances such as hydrous ferric oxides, the total aqueous concentrations of As(V) and P_i will be much lower (16). It is therefore possible that P_i will competitively inhibit As(V) uptake into the cell and thus decrease the expression of As(V) reductase systems under these conditions. Whether ANA-3 has a Pit homolog and the

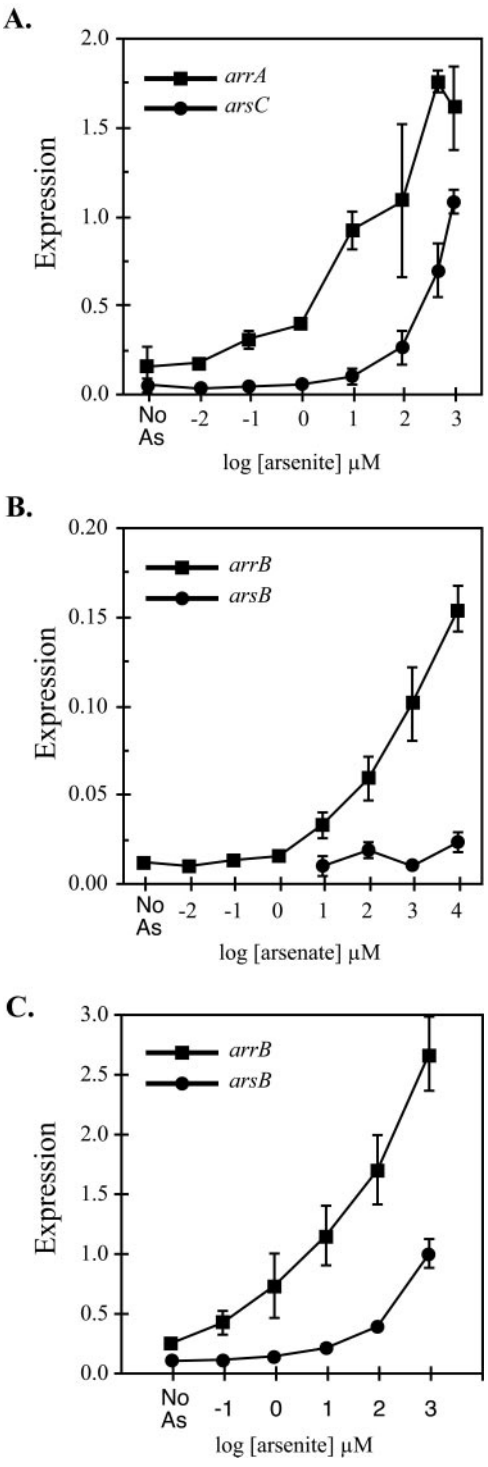

FIG. 4. Effect of increasing arsenic concentrations on expression of *arr* or *ars* operon in wild-type ANA-3 (A) and an As(V) reduction-deficient strain of ANA-3 (B). (A) Arsenite-dependent gene expression of the As(V) respiratory pathway compared to As detoxification in wild-type ANA-3. The expression of *arrA* (■) and *arsC* (●) relative to that of *gyrB* was determined as a function of increasing As(III) concentrations in cultures of wild-type *Shewanella* sp. strain ANA-3 grown concurrently with fumarate and the indicated As(III) concentrations. Data points and error bars represent averages of triplicate cultures and standard deviations, respectively. Arsenate-dependent (B) and arsenite-dependent (C) gene expression of the As(V) respiratory pathway was compared to As detoxification in an As(V) reduction-deficient strain (ARM1) of ANA-3. Because *arrA* and *arsC* are deleted in ARM1, the expression of *arrB* (■) and *arsB* (●) relative to that of *gyrB* was monitored as a function of increasing As(V) concentrations in cultures of ARM1 grown concurrently with fumarate and the indicated As(V) concentrations. Data points and error bars represent averages and standard deviations, respectively, of triplicate cultures.

extent to which As(V) competes with P_i for transport by the same machinery remain to be determined.

Nevertheless, our results showed that high concentrations of P_i promoted more robust growth of ANA-3 on As(V), suggesting that increased P_i in water supplies (e.g., due to eutrophication) might facilitate As(V) reduction by bacteria in environments where aqueous As(V) and P_i concentrations are both high.

In contrast to that of the As(V) respiratory system, transcription of the *ars* operon in ANA-3 is independent of the respiratory substrate (e.g., oxygen, fumarate, nitrate, or TMAO) and is expressed in the presence of As(III) but not As(V). However, *arsC* expression is greatest in As(V)-respiring cultures compared to oxygen-, fumarate-, nitrate-, or TMAO-grown cultures containing similar As(V) concentrations. The increased expression of the *ars* operon in As(V)-respiring cultures of ANA-3 is likely due to the accumulation of As(III) during growth on As(V). In other bacteria, As(III) has also been shown to be the main inducer of the *ars* operon (17). We found that 1,000 times more As(III) ($\leq$100 μM) is required to detect an increase in *arsC* transcription than that required for an increase in *arrA* transcription. This may be attributed to differences in the operator/promoter elements of the *arr* and *ars* operons such that *arr* becomes derepressed at lower As(III) concentrations than those required for the *ars* operon. Alternatively, ANA-3 may have several *arsR* genes encoding different regulators with various affinities for As(III) and/or the operator site. The latter hypothesis is supported by the existence of DNA sequences downstream of the *arrAB* cluster that contain two *arsR*-like genes (unpublished data). We are investigating the role of these putative regulatory elements in *arr* and *ars* transcription.

The temporal changes observed in *arrA* versus *arsC* transcription during growth on As(V) (i.e., maximum expression of *arrA* in exponential phase and maximal expression of *arsC* in early stationary phase) suggest that these staggered expression patterns may involve transcription factors that specifically sense either As(V) or As(III). The *arr* operon of ANA-3 was shown to be regulated in part by an As(V)-responsive transcription factor, and we are working to identify it. In *E. coli*, the ArsR and ArsD regulatory proteins are known to interact with As(III) and Sb(III) but not As(V) (2, 26), and therefore they are good candidates for As(III)-specific transcription factors. From a physiological perspective, it makes sense that the substrate for a reaction should induce the genes responsible for processing it. In addition to the substrate As(V), why then is the *arr* operon induced by As(III), the product of As(V) respiration? A hint may be taken from investigations of the induction of chemotaxis to Fe(III) and Mn(IV) oxides by *Geobacter metallireducens* (3). *G. metallireducens* undergoes chemotaxis towards dissolved Fe(II) and Mn(II), the products of respiration of Fe(III) and Mn(IV) (hydr)oxides, respec-

tively, rather than the substrates, Fe(III) and Mn(IV); this seems logical given that Fe(III) and Mn(IV) (hydr)oxides are expected to be much harder for bacteria to sense given their poor solubilities. Similarly, because As(V) is often adsorbed onto sedimentary minerals (27), it seems reasonable that ANA-3's responsiveness to As(III), the generally more mobile product of As(V) respiration, is an adaptive strategy used by the organism to poise itself to process As(V) should it encounter it.

In summary, our results suggest that the expression dynamics of As(V) respiration and detoxification play out as follows. Under anaerobic conditions the cell is primed to activate *arr* transcription should it sense nanomolar concentrations of either As(V) or As(III). During the early phases of growth, ANA-3 preferentially couples the reduction of As(V) to growth instead of detoxification by *arsC* because transcription of the *ars* operon is blocked. Over the course of growth, the demand to secrete As(III) increases as As(III) accumulates to toxic concentrations, and ANA-3 initiates production of its detoxification machinery by turning on transcription of the *ars* operon. Identification of the genetic and biochemical factors that influence the transcriptional dynamics of *arr* and *ars* operons will not only elucidate how genetic systems sharing a common substrate coordinate their expression but may be useful for the development of molecular tools for specifically sensing different forms of inorganic arsenic.

ACKNOWLEDGMENTS

This study was made possible by grants to D.K.N. from the Luce Foundation and the Packard Foundation. This material is based upon work supported by the National Science Foundation under a grant awarded in 2002 to C.W.S.

We thank Megan Ferguson (Caltech) for help with ICP-MS analysis and Jeff Gralnick and Doug Lies for helpful discussions.

REFERENCES

1. **Afkar, E., J. Lisak, C. Saltikov, P. Basu, R. S. Oremland, and J. F. Stolz.** 2003. The respiratory arsenate reductase from *Bacillus selenitireducens* strain MLS10. FEMS Microbiol. Lett. **226:**107–112.
2. **Chen, Y., and B. P. Rosen.** 1997. Metalloregulatory properties of the ArsD repressor. J. Biol. Chem. **272:**14257–14262.
3. **Childers, S. E., S. Ciufo, and D. R. Lovley.** 2002. *Geobacter metallireducens* accesses insoluble Fe(III) oxide by chemotaxis. Nature **416:**767–769.
4. **Drlica, K., and X. Zhao.** 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol. Mol. Biol. Rev. **61:**377–392.
5. **Gao, Y., and A. Mucci.** 2001. Acid base reactions, phosphate and arsenate complexation, and their competitive adsorption at the surface of goethite in 0.7 M NaCl solution. Geochim. Cosmochim. Acta **65:**2361–2378.
6. **Harvey, C. F., C. H. Swartz, A. B. Badruzzaman, N. Keon-Blute, W. Yu, M. A. Ali, J. Jay, R. Beckie, V. Niedan, D. Brabander, P. M. Oates, K. N. Ashfaque, S. Islam, H. F. Hemond, and M. F. Ahmed.** 2002. Arsenic mobility and groundwater extraction in Bangladesh. Science **298:**1602–1606.
7. **Islam, F. S., A. G. Gault, C. Boothman, D. A. Polya, J. M. Charnock, D. Chatterjee, and J. R. Lloyd.** 2004. Role of metal-reducing bacteria in arsenic release from Bengal delta sediments. Nature **430:**68–71.
8. **Iuchi, S., and E. C. Lin.** 1987. The *narL* gene product activates the nitrate reductase operon and represses the fumarate reductase and trimethylamine N-oxide reductase operons in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84:**3901–3905.
9. **Kneebone, P. E., P. A. O'Day, N. Jones, and J. G. Hering.** 2002. Deposition and fate of arsenic in iron- and arsenic-enriched reservoir sediments. Environ. Sci. Technol. **36:**381–386.
10. **Kostka, J., and K. H. Nealson.** 1998. Isolation, cultivation and characterization of iron- and manganese-reducing bacteria, p. 58–78. *In* R. S. Burlage, R. Atlas, D. Stahl, G. Geesey, and G. Sayler (ed.), Techniques in microbial ecology. Oxford, New York, N.Y.
11. **Krafft, T., and J. M. Macy.** 1998. Purification and characterization of the respiratory arsenate reductase of *Chrysiogenes arsenatis*. Eur. J. Biochem. **255:**647–653.
12. **Macy, J. M., J. M. Santini, B. V. Pauling, A. H. O'Neill, and L. I. Sly.** 2000. Two new arsenate/sulfate-reducing bacteria: mechanisms of arsenate reduction. Arch. Microbiol. **173:**49–57.
13. **Madigan, M., J. Martinoko, and J. Parker.** 2000. Metabolic diversity, p. 618. *In* P. Corey (ed.), Brock biology of microorganisms, 9th ed. Prentice-Hall, Upper Saddle River, N.J.
14. **Madigan, M., J. Martinoko, and J. Parker.** 2000. Nutrition and metabolism, p. 113. *In* P. Corey (ed.), Brock biology of microorganisms, 9th ed. Prentice-Hall, Upper Saddle River, N.J.
15. **Malasarn, D., C. W. Saltikov, K. M. Campbell, J. M. Santini, J. G. Hering, and D. K. Newman.** 2004. *arrA* is a reliable marker for As(V) respiration. Science **306:**455.
16. **Mucci, A., L. F. Richard, M. Lucotte, and C. Guignard.** 2000. The differential geochemical behavior of arsenic and phosphorus in the water column and sediments of the Saguenay Fjord estuary, Canada. Aquat. Geochem. **6:**293–324.
17. **Mukhopadhyay, R., B. P. Rosen, L. Phung, and S. Silver.** 2002. Microbial arsenic: from geocycles to genes and enzymes. FEMS Microbiol. Rev. **26:** 311–325.
18. **Nriagu, J.** 2002. Arsenic poisoning through the ages, p. 21–22. *In* W. T. Frankenberger (ed.), Environmental chemistry of arsenic. Marcel Dekker, Inc., New York, N.Y.
19. **O'Day, P. A., D. Vlassopoulos, R. Root, and N. Rivera.** 2004. The influence of sulfur and iron on dissolved arsenic concentrations in the shallow subsurface under changing redox conditions. Proc. Natl. Acad. Sci. USA **101:** 13703–13708.
20. **Oremland, R. S., and J. F. Stolz.** 2003. The ecology of arsenic. Science **300:**939–944.
21. **Rosen, B. P.** 2002. Biochemistry of arsenic detoxification. FEBS Lett. **529:** 86–92.
22. **Rosen, B. P.** 1999. Families of arsenic transporters. Trends Microbiol. **7:**207–212.
23. **Rosenberg, H., R. G. Gerdes, and K. Chegwidden.** 1977. Two systems for the uptake of phosphate in *Escherichia coli*. J. Bacteriol. **131:**505–511.
24. **Saltikov, C. W., A. Cifuentes, K. Venkateswaran, and D. K. Newman.** 2003. The *ars* detoxification system is advantageous but not required for As(V) respiration by the genetically tractable *Shewanella* sp. strain ANA-3. Appl. Environ. Microbiol. **69:**2800–2809.
25. **Saltikov, C. W., and D. K. Newman.** 2003. Genetic identification of a respiratory arsenate reductase. Proc. Natl. Acad. Sci. USA **100:**10983–10988.
26. **Shi, W., J. Dong, R. A. Scott, M. Y. Ksenzenko, and B. P. Rosen.** 1996. The role of arsenic-thiol interactions in metalloregulation of the *ars* operon. J. Biol. Chem. **271:**9291–9297.
27. **Smedley, P. L., and D. G. Kinniburgh.** 2002. A review of the source, behaviour and distribution of arsenic in natural waters. Appl. Geochem. **17:**517–568.
28. **Smith, A. H., E. O. Lingas, and M. Rahman.** 2000. Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. Bull. W. H. O. **78:**1093–1103.
29. **Willsky, G., and M. Malamy.** 1980. Characterization of two genetically separable inorganic phosphate transport systems in *Escherichia coli*. J. Bacteriol. **144:**356–365.
30. **Willsky, G., and M. Malamy.** 1980. Effect of arsenate on inorganic phosphate transport in *Escherichia coli*. J. Bacteriol. **144:**366–374.
31. **Wu, J., and B. P. Rosen.** 1993. The *arsD* gene encodes a second trans-acting regulatory protein of the plasmid-encoded arsenical resistance operon. Mol. Microbiol. **8:**615–623.
32. **Wu, J., and B. P. Rosen.** 1993. Metalloregulated expression of the ars operon. J. Biol. Chem. **268:**52–58.
33. **Xu, C., W. Shi, and B. P. Rosen.** 1996. The chromosomal *arsR* gene of *Escherichia coli* encodes a trans-acting metalloregulatory protein. J. Biol. Chem. **271:**2427–2432.