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Complex Regulation of Arsenite Oxidation in *Agrobacterium tumefaciens*

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Seminal regulatory controls of microbial arsenite [As(III)] oxidation are described in this study. Transposon mutagenesis of *Agrobacterium tumefaciens* identified genes essential for As(III) oxidation, including those coding for a two-component signal transduction pair. The transposon interrupted a response regulator gene (referred to as *aoxR*), which encodes an *ntrC*-like protein and is immediately downstream of a gene (*aoxS*) encoding a protein with primary structural features found in sensor histidine kinases. The structural genes for As(III) oxidase (*aoxAB*), a *c*-type cytochrome ($cytc_2$), and molybdopterin biosynthesis (*chlE*) were downstream of *aoxR*. The mutant could not be complemented by *aoxSR* in *trans* but was complemented by a clone containing *aoxS-aoxR-aoxA-aoxB-*$cytc_2$ and consistent with reverse transcriptase (RT) PCR experiments, which demonstrated these genes are cotranscribed as an operon. Expression of *aoxAB* was monitored by RT-PCR and found to be up-regulated by the addition of As(III) to cell cultures. Expression of *aoxAB* was also controlled in a fashion consistent with quorum sensing in that (i) expression of *aoxAB* was absent in As(III)-unexposed early-log-phase cells but was observed in As(III)-unexposed, late-log-phase cells and (ii) treating As(III)-unexposed, early-log-phase cells with ethyl acetate extracts of As(III)-unexposed, late-log-phase culture supernatants also resulted in *aoxAB* induction. Under inducing conditions, *aoxS* expression was readily observed in the wild-type strain but significantly reduced in the mutant, indicating that AoxR is autoregulatory and at least partially controls the expression of the *aox* operon. In summary, regulation of *A. tumefaciens* As(III) oxidation is complex, apparently being controlled by As(III) exposure, a two-component signal transduction system, and quorum sensing.

Arsenic (As) is a known carcinogen, occurring in the environment primarily as the oxyanion arsenate ($H_2AsO_4^-$, $HAsO_4^{2-}$) [As(V)] and arsenite ($H_3AsO_3^0$) [As(III)]. As(III) is more toxic and generally the more mobile species (reviewed in reference 6), and hence, there is significant interest in understanding the factors that control As speciation in the environment. Both abiotic and biotic factors are involved; however, it is now generally viewed that microbial As redox transformations are important, if not principal, drivers controlling As speciation in soils, sediments, and natural waters (6, 20).

Microbial As(III) oxidation and As(V) reduction activities are cellular strategies for either detoxification or for generating energy. A fairly detailed model explaining the genetics, regulation, and function of detoxification-based As(V) reduction is in place (32), but beyond that little is known. Genes encoding respiratory As(V) reductases have been cloned and enzyme characterizations are under way (recently reviewed in reference 32). Likewise, genes encoding As(III) oxidase have also recently been cloned and characterized (17, 29), and extensive enzyme characterization has been achieved (3), including crystal structure elucidation (8). As(III) oxidation in the heterotrophic β-proteobacterium *Cenibacterium arsenoxidans* is used as a detoxification mechanism (17), whereas As(III) oxidation can also enhance heterotrophic growth (39) or provide the sole source of energy generation for chemolithoautotrophic growth as shown for an *Agrobacterium-Rhizobium*-like organism isolated from a gold mine (30) and an obligate anaerobe isolated from Mono Lake, California (21). However, the genetic basis for the regulation of As(III) oxidation remains unknown. In the study summarized herein, we provide the first characterization of genetic determinants and mechanisms involved in regulating As(III) oxidation in a recently described (15) *Agrobacterium tumefaciens* soil isolate. Our data suggest that regulation of As(III) oxidation is complex, involving As(III)-sensing, two-component signal transduction, and quorum sensing.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are listed in Table 1. The *A. tumefaciens* strains were grown in a defined minimal mannitol medium (MMN) (35), and *Escherichia coli* strains were cultured in Luria-Bertani medium (28). Gentamicin (25 $\mu g \cdot ml^{-1}$) was added for selection of transposon-containing transconjugants, and tetracycline (10 $\mu g \cdot ml^{-1}$) and ampicillin (100 $\mu g \cdot ml^{-1}$) were included for selection of transformants when (sub)cloning PCR amplicons or when mobilizing plasmids into *A. tumefaciens*. As(III) (as NaH_2AsO_3) or As(V) (as NaH_2AsO_4) were included at designated levels to examine As transformation phenotypes, and 4-chloro-3-indoyl-β-D-galactoside (X-Gal, 40 $mg \cdot liter^{-1}$ agar medium) was added as required for detection of PCR amplicons cloned into either pTA2.1 or pCR-XR-TOPO or subcloned into pCPP30.

In experiments designed to examine the impact of quorum sensing on As(III)

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties or derivation	Source or reference
Strains		
Agrobacterium tumefaciens		
5A	Wild type, soil isolate, As(III) oxidizing	13
MSUAt1	*aoxR*::Tn*5*-B22	This study
Escherichia coli		
S17-1	Pro^- Mob^+, conjugation donor	30
JM110	*dam* and *dcm* deficient, cloning and expression host	Stratagene
TOP10	High-competency cloning host	Invitrogen
Plasmids		
pSUP102	Transposon vector	30
pCR2.1	PCR TA cloning vector	Invitrogen
pCR-XL-TOPO	PCR TA cloning vector for large PCR amplicons	Invitrogen
pCPP30	Broad host range, *tetA*	Michael Kahn, Washington State University
pDK401	pCPP30 with *aoxSR* PCR cloned from 5A	This study
pDK402	pCPP30 containing the *aox* operon as a 7,397-bp XbaI fragment PCR cloned from strain At5A	This study

oxidation, spent culture fluids were used in "add-back" experiments as a nonspecific source of quorum-sensing metabolites. For these studies, late-log-phase MMN-grown cultures were cleared by centrifugation, and the supernatants were filter sterilized and then added to fresh MMN broth (1:1 ratio). For control cultures, fresh MMN broth was mixed 1:1 with sterile distilled water. Both media mixtures were inoculated with 5A cells that had been prepared as follows: (i) grown to an optical density at 696 nm (OD_{595}) of 0.1, (ii) centrifuged and washed, and (iii) reinoculated into 100 ml of fresh media and grown again to an OD_{595} of 0.1. This allowed the use of cells that had not achieved late log phase or been exposed to accumulated quorum-sensing molecules and thus reduced/eliminated potential carryover of mRNA pools resulting from quorum-sensing-based gene expression. In additional experiments, cells prepared in the same way were treated with ethyl acetate extracts of culture supernatants obtained from late-log-phase cells. Ethyl acetate extracts were prepared by mixing equal volumes of ethyl acetate with late-log-phase culture supernatants (100-ml cultures) obtained in the same fashion as described above. The nonaqueous phase was transferred to a fresh container and the ethyl acetate removed by rotary evaporation at 37°C. Resulting residues were suspended in 1.5 ml sterile distilled water and added to 100-ml cultures of early-log-phase cells (prepared as described above). Control cells received 1.5 ml sterile water.

Transposon mutagenesis and detection of As(III)-oxidation mutants. Transposon Tn*5*-B22 (34) was used as we have described previously (37). Putative As(III) oxidation mutants were identified by subculturing Gm^r transconjugants (in duplicate) onto MMN-Gm agar that was modified to contain 30 μM potassium phosphate [excessive phosphate interfered with reliable As(V) detection] and amended with 1 mM As(III). One set of plates was flooded with 0.1 M $AgNO_3$ to detect the presence of As(V) (14) associated with As(III)-oxidizing transconjugants (dark purple-brown colonies) or the absence of As(III)-oxidation (yellow colonies), which would identify a mutant incapable of As(III) oxidation. Matched duplicate clones of the yellow colonies were subcultured to purity and the mutant phenotype verified by analytical techniques that directly measured As(V) formation (see below).

Mutants were then screened by Southern blot analysis using standard methods (28) to identify unique transposon insertions. Total DNA from each mutant was digested with EcoRI and HindIII in separate digests, blotted onto nylon membranes (GeneScreen Plus; NEN Life Sciences, Boston, MA), and then probed with the ~7.0-kb fragment from Tn*5*-B22 (containing most of the transposon) that was labeled using the digoxigenin high-prime DNA labeling kit (Roche Diagnostics, Indianapolis, IN). Transposon insertion sites of single insertion mutants were characterized by arbitrary PCR using the APAgene genome walking kit (Bio S&T Inc., Montreal, Quebec, Canada). Forward primers used for the first, second, and third consecutive PCRs in this protocol were 5′-GGCGACG TTAACCAAGCGGGCAGTACGGC-3′, 5′-GCCCAGTCGGCCGCACGATG AAGAGCAG-3′, and 5′-GGAAAACGGGAAAGGTTCCGTTCAGGACGC-3′, respectively. These primers represent sequences that are unique to the transposon, are progressively closer to the extreme end of the transposase arm, are separated by 67 to 80 nucleotides, and were designed to amplify the genomic DNA immediately flanking the transposon. The reverse primers were provided by the kit, and the PCR conditions were as described in the protocols supplied by the kit manufacturer. Mutant chromosomal DNA served as the template for the first round of PCR, and then amplicons from the first and second PCRs served as templates for the second and third PCRs, respectively. The products from the third PCR were separated by electrophoresis in 1.0% low-melting-point agarose gels containing Tris-acetate-EDTA buffer and purified using Agar ACE agar-digesting enzyme (Promega, Madison, WI). The purified PCR product was adenylated with *Taq* polymerase (Promega) and then cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and transformed into *E. coli* strain TOP10 (Invitrogen). Gene identification was based on sequencing of the cloned amplicons, using an ABI310 DNA sequencer (Applied Biosystems, Norwalk, CT) and synthetic primers complementary to the vector plasmid sequences flanking the multiple cloning site and to internal sequences. Homology searches of public databases were conducted using BLAST (2), and sequence alignments were completed using ClustalW (38).

Mutant complementation. All (sub)cloning work used standard molecular biology protocols involving transformations into chemically competent cells (28). Sample sequencing reactions verified the identity of cloned fragment(s). Primers P1 and P3 (Fig. 1) used for PCR cloning of the *aoxSR* genes were 5′-CCAATT CCGGCTACCTCGTCA-3′ and 5′-CCAGTGACCACGGATGTTCA-3′, respectively. Primer P1 was paired with P2 (5′-GGGACAGGTGTGGAGATCG C-3′) to PCR clone a genomic region containing *aoxSRAB-cytc*$_2$*-chlE* (partial). All strain-plasmid combinations were inoculated into MMN broth amended with 100 μM As(III), and then monitored for growth (OD_{595}, A_{595}) and As(III) oxidation (see below).

Gene expression analyses. Reverse transcriptase (RT) PCR was used to assess transcription of the genes identified in this study. For total RNA extraction, 10 ml of culture samples was transferred to cold centrifuge tubes and diluted with 10 ml of ice-cold 0.85% (wt/vol) NaCl containing 40 $\mu g \cdot ml^{-1}$ chloramphenicol. The suspension was centrifuged at 12,000 × *g* for 7 min at 1°C and the supernatant discarded. The cell pellet was suspended in 400 μl of the saline-chloramphenicol solution, transferred to a ice-cold 2-ml screw-cap tube, and then centrifuged again at 13,000 × *g* for 4 min at 4°C. The cell pellet was suspended in nuclease-free water (Promega), snap frozen in liquid nitrogen, and stored at −75°C. Total RNA was extracted from the frozen cells using a protocol used previously with environmental samples (19). RNA was treated with DNase using Turbo DNA-free (Ambion, Inc., Austin, TX) and then purified using the Ambion MEGAclear kit (Ambion, Inc., Austin, TX) following the manufacturer's instructions. DNA was verified to be absent by PCR tests containing 50 ng of the RNA preparation, *Tfl* DNA polymerase (Promega), and 0.4 μM concentrations of each primer. To monitor expression of the *aoxAB* genes, primers were designed to amplify a region spanning the junction of the terminal 331 nucleotides of *aoxA* (primer P4, 5′-GACGTTGCCTATCCCGATGAAGAT-3′) to nucleotide 126 of *aoxB* (P5, 5′-GTTTGTTGATTGGCCAGGTGTAGG-3′). For mon-

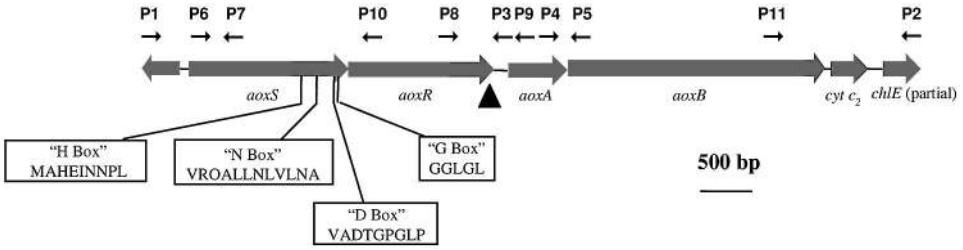

FIG. 1. Physical map of the *aox* locus in strain 5A and the Tn*5*-B22 insertion in mutant MSUAt1. The Tn*5*-B22 insertion (at nucleotide 1318 in *aoxR*) is shown as an inverted vertical arrowhead. Amino acid composition of primary structural motifs referred to as the H (aa 271 to 279), N (aa 375 to 386), D (aa 413 to 421), and G (aa 441 to 445) boxes are shown for *aoxS*. Primers used in the (sub)cloning and transcriptional analysis experiments (see text) are shown as P1 to P11; the amplification direction of each primer is indicated by an arrow. The GenBank accession number for the *aox* locus is DQ151549.

itoring expression of *aoxS*, primers amplified a region spanning nucleotidess 23 to 524 of *aoxS* (P6, 5′-CGACATTCCCTTGACGGTTC-3′; P7, 5′-CTAAGCC AGCATTCGTCGCAA-3′) (Fig. 1). To examine cotranscription of *aoxSR*, primer P6 was paired with P10 (5′-GATAACTGATGCAGAAACCTGGCG-3′). For *aoxRA* RT-PCRs, primer P8 (5′-ATCTGATATGCCACGGATCGG-3′) (Fig. 1), corresponding to the 3′ region of *aoxR*, was paired with P9 (5′-TCGA CCATGTTTCGACAG-3′) (Fig. 1), which amplifies from the 5′ region of *aoxA*. Finally, P11 (5′-ATGGACATCATCCCGACAAAGATCGG-3′) and P2 were used to examine cotranscription of the DNA spanning from *aoxB* to *cytc*$_2$. All primers were designed from sequences determined from the primer walking experiments described above. For RT-PCRs involving the 16S rRNA, primers 8F and 1392R were used as we have previously described (12). Unless otherwise noted, the RT-PCRs were conducted using the Access Quick RT-PCR system (Promega), including 0.4 μM concentrations of each primer and 5 ng of total RNA. All RT reactions were conducted at 48°C for 45 min. The PCR protocol for *aoxAB* amplification consisted of 94°C for 2 min and 30 cycles of 94°C for 30 s, 55°C for 1 min, and 68°C for 2 min. The final extension was 7 min at 68°C. For *aoxS*, the PCR program was 94°C for 2 min and 30 cycles of 94°C for 30 s, 53°C for 1 min, and 68°C for 2 min, with the final extension of 7 min at 68°C. For *aoxSR*, the PCR program was 94°C for 2 min and 30 cycles of 94°C for 30 s, 52°C for 1 min, and 68°C for 2 min, with the final extension of 7 min at 68°C. For *aoxRA* and *aoxBchlE* PCR, the annealing temperatures used were 51 and 55°C, respectively. In all cases, RT-PCR products were sample sequenced to verify the identity of the amplicon.

Arsenic chemical analysis. As(III) oxidation was measured using hydride generation-atomic absorption spectrophotometry as described previously (7). Briefly, culture aliquots were centrifuged and supernatants filtered (0.22 μm) into two separate 15-ml bottles (5 ml each). The first aliquot was acidified with 1 ml of 12.1 M HCl and stored at 4°C until analyzed for total As [As(t)]. In the second aliquot, As(V) was determined by measuring total As after removing As(III) by treatment with 1 ml of 2 M Tris buffer (pH 6) and 1 ml of 3% $NaBH_4$ (wt/vol in 0.1% NaOH) and sparging with N_2 for 7 min. Samples were acidified with 12.1 M HCl to achieve a final HCl concentration of 3 M and stored at 4°C. Prior to analysis, 1.0 ml of 11% KI (wt/vol in 3 M HCl) was added per 10 ml of the analysis mixture. The concentration of As(III) was determined by difference between As(t) and As(V).

RESULTS

Identification of As(III) oxidation mutants. Screening of the transposon mutants with $AgNO_3$ staining on As(III)-amended agar identified several *A. tumefaciens* 5A Gm^r transconjugants that were not capable of As(III) oxidation (identified as yellow colonies among dark brown colonies, results not shown). Southern blot probing of total genomic DNA digests with a transposon fragment identified likely siblings (results not shown) and directed subsequent characterization work on several mutants found to carry a single transposon insertion. One of the mutants is the focus of this report.

Arbitrary PCR anchored with transposase-specific primers identified the Tn*5*-B22 insertion site in mutant MSUAt1 (Fig. 1) as being near the 3′ terminus (nucleotide 1318) of an open reading frame (ORF) coding for a putative 442-amino-acid protein having 36% identity and 55% similarity to an NtrC-like response regulator from *Myxococcus xanthus* (GenBank accession no. AY337493). This ORF, referred to as *aoxR*, has the primary amino acid structural features common to response regulator partners of two-component signal transduction systems. These included the invariant Asp13 and Asp14 that comprise the "acid pocket" of orthodox receiver domains, an Asp58 which could participate in the phosphorylation signal from the cognate sensor (1, 22), and a Lys107 that is viewed to be important in translating the phosphorylation signal into a conformational change necessary for regulatory function (4, 22, 40). Primer walking experiments revealed a putative operon comprised of several flanking genes that included an upstream ORF (*aoxS*) coding for a 488-amino-acid peptide exhibiting features completely consistent with sensor histidine kinases, including perfect conservation of several signature motifs referred to as the H (amino acids [aa] 271 to 279), N (aa 375 to 386), D (aa 413 to 421), and G2 (aa 441 to 445) boxes (Fig. 1) (22, 36).

Immediately downstream of *aoxR* were two adjacent ORFs displaying 94% identity/97% similarity, respectively, to the recently identified *aroB* and *aroA* (predicted 175 and 845 amino acids, respectively) genes that code for the small and large subunits, respectively, of the As(III) oxidase purified and characterized from the α-proteobacterial As(III) chemolithoautotroph strain NT-26 (29, 30) (Fig. 1). Further downstream primer walking revealed two additional ORFs (Fig. 1): a c_2-type cytochrome isoform (49% identity/63% similarity to *Rhodobacter sphaeroides* isocytochrome c_2 across 127 inferred amino acids) (26), and a homolog to the *E. coli chlE* (44% identity/67% similarity; 67 inferred amino acids) that is involved in molybdopterin biosynthesis (18).

Complementation of the *aox* gene defect. Absence of As(III) oxidation in MSUAt1 and MSUAt1(pCPP30) was confirmed using analytical techniques that measured As(V) and As(t) and As(III) by difference (Fig. 2). Providing *aoxSR* in *trans* (PCR clone amplified with primers P1 and P3) (Fig. 1) failed to rescue the mutant phenotype (results not shown). However, the entire PCR-cloned *aox* region (*aoxS-aoxR-aoxB-aoxA-isocytc*$_2$-partial *chlE*; primers P1 and P2) (Fig. 1) resulted in complementation, with nearly quantitative As(III) oxidation achieved by late log phase (Fig. 2). These experiments con-

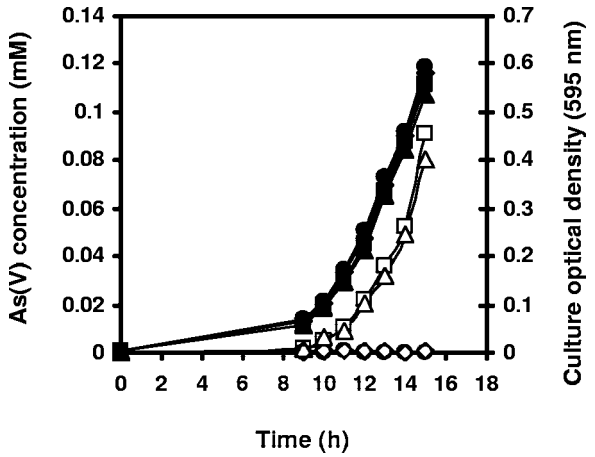

FIG. 2. Complementation of MSUAt1 with the cloned *aox* operon. Culture growth (optical density) is shown with black symbols, and As(V) concentration is shown with white symbols. □ and ■, wild-type strain 5A; ○ and ●, *aoxR* mutant MSUAt1; △ and ▲, *aoxR* mutant MSUAt1 containing pDK402, which contains the cloned *aox* operon; ◇ and ◆, *aoxR* mutant MSUAt1 containing the control plasmid pCPP30. Results are from one of two independent experiments demonstrating complementation by the *aox* operon PCR cloned (primers P1 and P2, see Fig. 1) in pCPP30 but not in MSUAt1 carrying the control plasmid pCPP30.

firmed that the Tn*5*-B22-interrupted gene was involved in As(III) oxidation in this organism and also suggested that these genes are all part of a common transcriptional unit.

As redox transformation phenotype. Our previous work showed that even though the wild-type strain exhibited an As(III)-oxidizing phenotype (15), it nevertheless contained an *arsC* (31). Therefore, we investigated the mutant to determine if the loss of As(III) oxidation capability would reveal an As(V) reduction phenotype. This was indeed the case (Fig. 3). $AgNO_3$ staining of the various strains on MMN agar containing 1 mM As(V) showed that MSUAt1 and MSUAt1(pCPP30) would reduce As(V) (Fig. 3). In contrast, no As(V) reduction was apparent with wild-type strain 5A or the mutant carrying

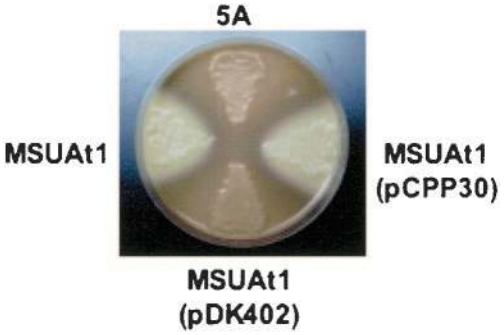

FIG. 3. As(V) reduction phenotype resulting from loss of As(III) capacity. As(V) reduction by mutant MSUAt1 and MSUAt1(pCPP30) is revealed by $AgNO_3$ staining. The presence of As(V) is indicated by dark brown color associated with agar inoculated with 5A and MSUAt1(pDK402), whereas As(III) is indicated by yellow and only found associated with agar inoculated with MSUAt1 and MSUAt1(pCPP30). Regions of the agar plate were inoculated as indicated.

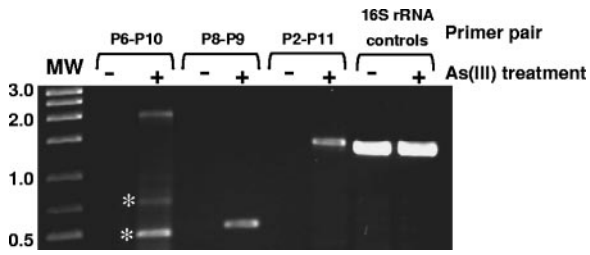

FIG. 4. RT-PCR analysis of the *aox* locus. RNA was extracted from As(III)-unexposed, early-log-phase cells and from As(III)-treated, late-log-phase cells and used as RT templates to demonstrate transcriptional linkage of genes identified at this locus. Lane assignments are based on culture treatment and primers used in the RT-PCRs (MW, molecular weight standards); primer numbers are linked to those shown in Fig. 1. 16S rRNA amplifications were included as controls. −, As(III)-unexposed, early-log-phase cells; +, As(III)-treated, late-log-phase cells; *, nontarget amplicons observed with RNA extracted from As(III)-treated, late-log-phase cells.

pDK402 (Fig. 3). In other experiments, we sought to determine whether the mutant was more sensitive to As(III) than the wild-type strain. In 24-h MMN-grown cultures amended with As(III) varying in concentration from 0 to 3 mM, there was little difference in growth phenotype between the mutant and wild-type strains. Both exhibited progressively poorer growth with increasing As(III), displaying half-maximum optical densities (A_{595} = ~0.27) at 1 mM As(III) and no growth at 3 mM As(III) (results not shown).

***aoxAB* transcriptional regulation. (i) Coordinated expression of *aox* locus.** Additional RT-PCRs were then conducted to obtain further evidence of an operon arrangement of the *aox* genes. Total RNA was extracted from As-unexposed, early-log-phase cells and from As(III)-exposed, late-log-phase cells and then used as templates with different primer sets that spanned the various genes found at this locus. Primers P6 and P10 (Fig. 1) generated a 1,966-bp amplicon corresponding to the *aoxS-aoxR* region of the operon along with two additional nontargeted cDNAs that persisted in spite of RT-PCR optimization efforts (Fig. 4). Primer P8 paired with P9 generated a 557-bp cDNA amplicon, thus demonstrating transcriptional linkage between *aoxR* and *aoxA* (Fig. 4). And finally, primers P2 and P11 generated a 1,439-bp product corresponding to *aoxB-chlE* region of this locus (Fig. 4). In each case, sequencing verified the identity of each amplicon, and no amplicons were observed in the absence of As(III), further demonstrating the coordinated expression of these genes in response to As(III) induction. In all cases, 16S rRNA was readily amplified, showing that the RNA from untreated cells was amplifiable (Fig. 4).

(ii) Involvement of a quorum-sensing-like response. Previous studies have shown that at least in some bacteria, As(III) oxidation does not occur until cultures have attained stationary phase (24) and is suggestive that quorum sensing may be involved in regulating As(III) oxidation. RT-PCR experiments (primers P4 and P5) (Fig. 1) were therefore conducted to detect and monitor *aoxBA* expression as a function of growth phase in the presence and absence of As(III) (Fig. 5A). In As(III)-treated cultures, an RT-PCR amplicon corresponding to the correct size (469 bp) was amplified from RNA extracted from cells harvested at all time points (Fig. 5B). Sequencing

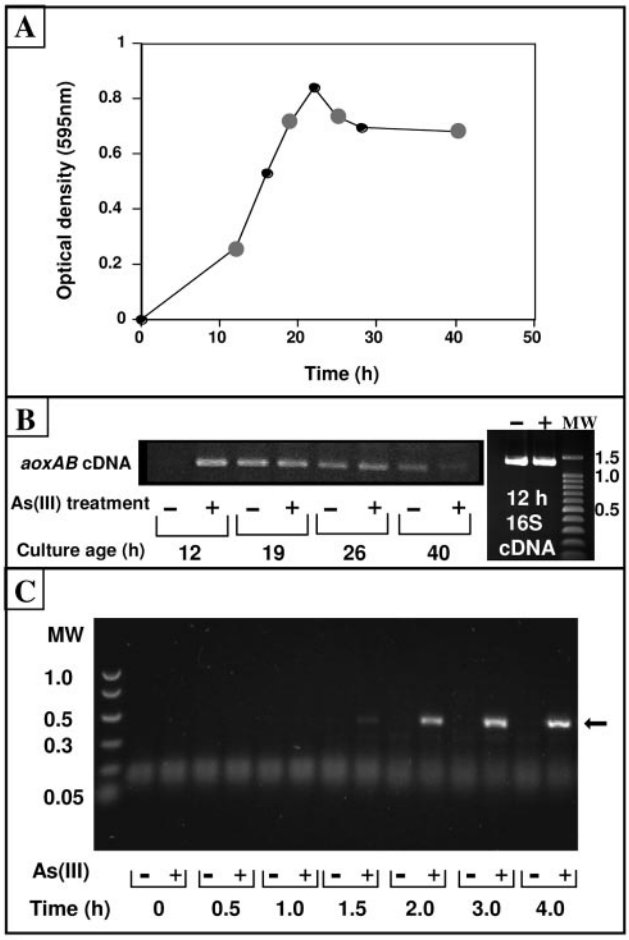

FIG. 5. Expression of *aoxAB* in *A. tumefaciens* 5A. (A) Growth curve of 5A, with RNA sampling points shown as large gray dots. Cell growth in As(III)-treated [100 μM As(III)] and control cultures was identical and is shown as a single response line. (B) *aoxAB* RT-PCR product amplified (primers P4 and P5, see Fig. 1) from total RNA extracted from As(III)-unexposed (− AsIII treatment) and As(III)-exposed (+ AsIII treatment) cells at time points corresponding to early log phase (12 h), late log phase (19 h), early stationary phase (25 h), and late stationary phase (40 h). (C) Reinduction of *aoxAB* in extended-stationary-phase cells with the addition of 75 μM As(III). The *aoxAB* RT-PCR product is shown as the primary amplicon and is indicated by an arrow. A weak, nonspecific product was also observed (~350 bp). For each gel, 5 μl of RT-PCR product was loaded in each lane. Molecular size standards are shown on the left (in kbp, lane MW).

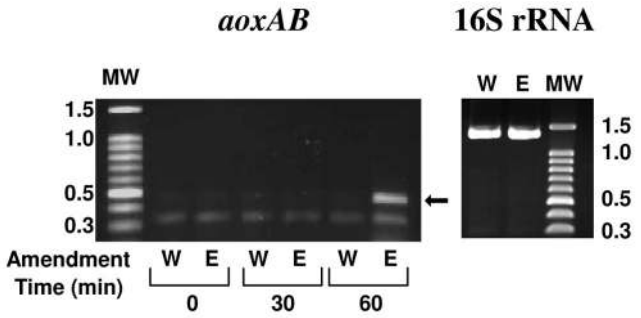

FIG. 6. Evidence of quorum-sensing control of *aoxAB* expression. Early-log-phase, As(III)-unexposed cells of wild-type strain 5A were inoculated into MMN broth that had been amended with either distilled water (W) or ethyl acetate extracts (E) of late-log-phase culture fluids. The arrow indicates *aoxAB* cDNA amplicon (primers P4 and P5). A weak, nonspecific product (~350 bp) was also observed in treated and control cells at all time points. Timing of cell samples is shown (in minutes). Molecular size standards are shown in the left lane (in kbp, lane MW). Five microliters of RT-PCR product was loaded in each lane.

verified that the fragment represented cDNA amplified from the region spanning the 3′ end of *aoxA* to the 5′ end of *aoxB* (results not shown). In As-unexposed cells, *aoxBA* mRNA was not detected in early-log-phase cells (Fig. 5B); however, it could be detected in late-log-phase cells as well as in stationary-phase cells (Fig. 5B). Interestingly, the RT-PCR product diminished as the culture incubations were extended further into stationary phase (Fig. 5B). However, the addition of As(III) to extended stationary cells (after 48 h in stationary phase) resulted in reinduction of *aoxAB* (Fig. 5C).

To obtain further evidence that quorum sensing was involved in As(III) oxidation, supernatants from late-log-phase cultures were filter sterilized and then used in add-back experiments. In early-log-phase cells suspended in a 1:1 mixture of filter-sterilized culture fluids and fresh MMN broth, *aoxAB* cDNA was detectable within 60 min, whereas *aoxAB* up-regulation was not observed in control cells suspended in fresh MMN broth diluted with an equal volume of sterile distilled water (results not shown). Ethyl acetate, which is commonly used to extract *N*-acylhomoserine lactones (AHLs) from *Pseudomonas aeruginosa* (23) and *A. tumefaciens* (42) cultures, was used to obtain further evidence that AHLs were the basis for the observed induction. In As-unexposed early-log-phase cells treated with the AHL extract, *aoxAB* induction patterns were identical to those observed with filtered supernatant add-back experiments (Fig. 6).

(iii) Involvement of AoxR. Experiments were then conducted to determine whether the AoxR protein is involved in regulating the *aox* gene cluster. Since the above complementation and RT-PCR experiments suggested that *aoxS-aoxR-aoxA-aoxB* are polycistronic (perhaps with other downstream genes), we explored whether AoxR might control expression of this operon (i.e., autoregulatory). Again using RT-PCR (primers P6 and P7) (Fig. 1), *aoxS* expression was examined in As(III)-treated, late-log-phase cells of wild-type and MSUAt1 strains. No *aoxS* cDNA was amplified from MSUAt1 RNA, but the expected 524-bp amplicon was obtained from wild-type RNA preparations (Fig. 7). To verify that the mutant RNA was of sufficient quality to yield cDNA, 16S rRNA primers were used in separate RT-PCRs with the same RNA preparations. The expected 1,385-bp cDNA amplicon was obtained from both the wild-type and mutant strains (Fig. 7).

DISCUSSION

Regulatory circuits, signals, and metabolites. In this study, transposon mutagenesis and subsequent experiments were used to identify regulatory elements involved in microbial As(III) oxidation. Data collected in this study are consistent with the interpretation that there are at least two separate

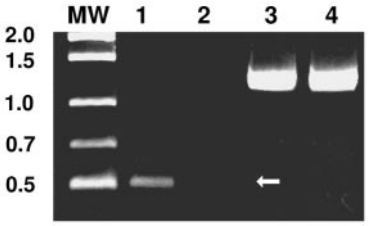

FIG. 7. AoxR-regulated expression of *aoxS*. Agarose gel showing cDNA products amplified from total RNA extracted from late-log-phase cells that had been treated with 100 μM As(III). Lane assignments: MW, molecular size standards in kbp; 1, 5A RNA amplified with primers P6 and P7; 2, MSUAt1 RNA amplified with primers P6 and P7; 3, 5A RNA amplified with 16S rRNA-specific primers; 4, MSUAt1 RNA amplified with 16S rRNA-specific primers. Five microliters of RT-PCR product was loaded in each lane. The *aoxS* cDNA is indicated by the arrow.

triggering metabolites that may be part of two separate regulatory circuits controlling expression of an operon containing the As(III) oxidase structural genes. One regulatory pathway that seems fairly certain involves an apparent two-component signal transduction system. Operon arrangements are standard for genes coding for two-component signal transduction protein pairs (1), and thus, the genomic organization of *aoxS* and *aoxR* is consistent with this common feature. Further, the inferred amino acid sequence of *aoxR* shares key amino acid assignments that are invariant among response regulators and that are critical in receiving the transduced environmental signal from the cognate sensor kinase (reviewed in references 4, 22, and 40). In addition, the inferred amino acid sequence of the upstream gene, *aoxS*, contains motifs that are appropriately located and perfect matches with key primary structural motifs in sensor kinases (Fig. 1) (22). Secondary structure analysis of AoxS (using SOSUI, http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) predicts a single membrane-spanning helix (amino acids 15 to 37) resulting in a 14-amino-acid residue exposed to the periplasm that could serve as a periplasmic signal receptor (Fig. 8). Future mutagenesis work will probe this putative periplasmic amino acid chain for its importance as a signal receptor as well as examine the importance of other regions of this protein or specific amino acids. For some signal transduction proteins such as FixL, signal receptors have been found to reside in the hydrophilic cytoplasmic portion of the protein (16), and thus, the sulfhydryl in Cys401 (Fig. 8) could be a receptor for the As(III) signal, with subsequent signal translation resulting in *aox* operon induction (Fig. 5B and C). As(III) is also the inducer signal for the *ars*-encoded detoxification-based As(V) reduction, which is regulated by the ArsR repressor (31). ArsR contains three cysteines that form an AsS_3 binding site. Upon binding As(III), ArsR undergoes a conformational change, resulting in release from the *ars* operon operator, allowing for transcription. It is important to note, however, that some ArsR proteins have been identified that do not contain the *E. coli* -type As(III) binding site (5), and therefore, there may be other regions of AoxS that may be essential for receiving the As(III) signal.

The discovery of a two-component signal transduction system involved in controlling As(III) oxidation is interesting from the perspective of its departure from the *ars* model but also because of the potential implications for regulation in nature. Wanner and colleagues (reviewed in reference 41) accumulated significant data that demonstrated "cross talk" occurring between two-component regulatory pairs. For example, PhoB, the response regulator for phosphate starvation-inducible genes, is a phosphorylation substrate for sensor kinases other than its normal partner, PhoR (41). Further, nonspecific PhoB phosphorylation reactions involving acetyl-P

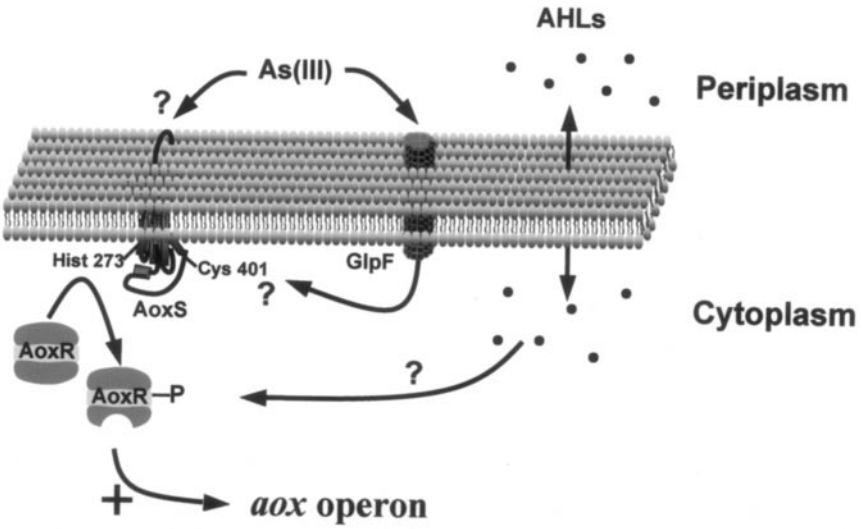

FIG. 8. Conceptual model depicting the predicted involvement of AoxS and AoxR in regulating expression of the *aox* operon. The putative input receiver domain is shown as a 14-amino-acid chain in the periplasm, potentially acting as the receptor for environmental signals such as As(III) or an autoinducer (depicted here as AHLs), which are shown equilibrating across the cytoplasmic membrane. Cys401 is shown as a potential cytoplasmic signal receptor, and Hist273 is depicted as the canonical phosphorylation site. Upon phosphorylation, AoxR acts as a positive regulator of the *aox* operon. As(III) entry into the cell is shown via an aquaglyceroporin, GlpF (28a).

or carbamoyl-P were also demonstrated (41). Therefore, if similar cross talk occurs between AoxR and other sensor kinases or if AoxR is subject to nonspecific phosphorylation reactions, then environmental regulation of As(III) oxidation in strain 5A (and other similarly genetically configured bacteria) could potentially be influenced by multiple environmental signals.

That *aox* regulation is indeed complex was demonstrated in other experiments that uncovered evidence that a quorum-sensing-based response is a second regulatory circuit (Fig. 5B and 6). In the absence of As(III), *aoxAB* expression was not detected in early-log-phase cells but was consistently observed in late-log-phase cultures. Furthermore, *aoxAB* RT-PCR products were obtained in early-log-phase cells incubated in filter-sterilized culture supernatants or ethyl acetate extracts derived from late-log-phase cultures (Fig. 6); these observations are hallmarks for quorum-sensing-controlled gene expression (10). It is conceivable that AoxR-based control of the *aox* operon in *A. tumefaciens* strain 5A could potentially derive from an AoxR conformational change brought about by binding to an effector molecular [e.g., As(III) or autoinducer]. The apparent down-regulation of *aoxAB* in our experiments (Fig. 5B) is not inconsistent with previous quorum-sensing work with *A. tumefaciens*, where autoinducer turnover was shown to occur soon after cells reached stationary phase (42). In the experiments conducted in the present study, As(III) oxidation was nearly complete by late log phase (Fig. 2), and thus, both inducer signals would likely be absent in late-stationary-phase cells. Reintroduction of As(III), however, resulted in *aoxAB* reinduction detectable within 90 min (Fig. 5C). Future experiments will examine which of the several AHLs produced by *A. tumefaciens* (42) are involved in controlling *aox* expression.

Complementation. Cloning experiments providing the wild-type *aoxR* allele (along with other genes, see below) in *trans* restored the mutant's capacity to oxidize As(III) and thus established a direct link between the mutation and the As(III) transformation phenotype. Subsequent experiments were performed to determine whether the defect in MSUAt1 was due to Tn*5*-B22 polar effects on *aoxAB* expression or perhaps to inactivation of AoxR. The data suggest that both explanations are at least partially relevant for explaining regulatory control of *aoxAB* and the mutant's As(III) oxidation phenotype. RT-PCR experiments (Fig. 4 and 5) demonstrating amplifiable cDNA spanning the *aoxS* and *aoxR* genes, *aoxR* and *aoxA* genes, *aoxA* and *aoxB* genes, and *aoxB* and *chlE* genes were in agreement with the complementation work which showed that MSUAt1 could be rescued by *aoxS-aoxR-aoxA-aoxB-cytc*$_2$*-chlE* (partial) but not by *aoxS-aoxR* alone. This argues that the transposon interrupted a large operon that includes at least *aoxS-aoxR-aoxA-aoxB-cytc*$_2$*-chlE* as well as other potentially downstream genes not identified in this study. Therefore, we conclude that the mutant redox phenotype is due in part to the polar effect of the Tn*5*-B22 insertion upstream of *aoxAB*. However, expression of *aoxS*, which is upstream of the Tn*5*-B22 insertion (Fig. 1), was absent in MSUAt1 (Fig. 7) and, thus, is consistent with the conclusion that AoxR is autoregulatory, such as is the case with ArsR that regulates detoxification-based As(V) reduction (33). However, we note that by using larger amounts of RNA in the RT-PCR mixtures ($\geq$50 ng), we could begin to detect *aoxS* cDNA (results not shown). This could be due to the truncated AoxR in MSUAt1 being less efficient as a transcriptional activator and is consistent with previous studies of other response regulators which have shown that the extreme 3′ region of these proteins is essential for DNA binding (9, 25) and, thus, transcriptional activation of targeted promoters. Alternatively, another regulator may be involved and facilitates expression at a lower level.

An operon-coordinated expression of *aoxAB* with a *c*-type cytochrome and molybdopterin biosynthesis genes would not necessarily be unexpected. *c*-type cytochromes have been implicated as physiological oxidants for the *Alcaligenes faecalis* and *Hydrogenophaga* As(III) oxidases (3, 40). And, molybdopterin is found in Mo-cofactored hydroxylases (13) such as the *A. faecalis* As(III) oxidase (3, 8). However, the complementing clone contained only a partial *chlE*, and thus, it would seem that *chlE* (and other potential operon components further downstream) may not be required for As(III) oxidation in this particular organism. Given the requirement of molybdopterin for several families of enzymes (13), this would seem to suggest that there is another molybdopterin biosynthetic pathway available to this particular organism. This would not be a precedent-setting observation, as gene/functional reiteration is well known in the *Rhizobiaceae*, particularly in *Agrobacterium* (11).

The function and requirement of As(III) oxidation in this organism is obscure. Our previous characterization of 5A concluded that it could not use As(III) as an energy source (15), and subsequent experiments with a range of media and culture conditions have again failed to demonstrate As(III) chemolithotrophy in this organism (results not shown). How this soil bacterium may have acquired the *aox* operon is also not yet known, but some type of lateral gene transfer seems reasonable and would not be inconsistent with the inheritance of blocks of genes that are then encountered as clusters of genes encoding As redox transformation-related activities. Such clustering was first described by Saltikov and Newman (27) for the As(V)-respiring *Shewanella* and then recently by Silver and Phung (32) for *A. faecalis*. We have identified additional loci that are required for As(III) oxidation in this organism (12a), and thus, it would seem that even though *aoxS-aoxR-aoxA-aoxB-cytc*$_2$ were sufficient for rescuing the mutant, these genes alone are not sufficient for converting a bacterium to an As(III)-oxidizing phenotype.

The inferred amino acid sequence of the As(III) oxidase structural genes discovered in *A. tumefaciens* strain 5A (Fig. 1) share significant homology (small subunit, 46% identity/58% similarity; large subunit, 44%/59%) with the *C. arsenoxidans* As(III) oxidase subunits (17), although highest identity was observed with the As(III) chemolithotroph NT-26 As(III) oxidase structural genes (discussed above) referred to as *aroAB* (29). For the *A. tumefaciens* As(III) oxidase genes described in the present study, the *aox* mnemonic was assigned to reflect the fact that there is very high degree of amino acid conservation among the *aox* and *aro* genes thus far identified, suggesting a common ancestry and thus arguing for a common mnemonic (32). Further, these genes code for the same enzyme function, so we suggest that these genes should share the same mnemonic assigned to the first officially characterized As(III) oxidase genes (17).

Finally, an interesting observation made in this study was

that the mutation also resulted in converting strain 5A to an As(V)-reducing phenotype (Fig. 3). This implies that under aerobic cultivation conditions both redox transformations may be occurring simultaneously in the wild-type parental strain, with perhaps the rate of As(III) oxidation exceeding As(V) reduction and the net outcome being an As(III)-oxidizing phenotype. Future experiments will aim to further examine this enigma and to determine how widespread this property may be among microbes in nature. Of interest is how both activities are regulated within a single organism and to what extent the same microbial population(s) may be pivotal with regard to controlling As speciation in a particular environment.

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