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Involvement of RpoN in Regulating Bacterial Arsenite Oxidation

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In this study with the model organism *Agrobacterium tumefaciens*, we used a combination of *lacZ* gene fusions, reverse transcriptase PCR (RT-PCR), and deletion and insertional inactivation mutations to show unambiguously that the alternative sigma factor RpoN participates in the regulation of As^{III} oxidation. A deletion mutation that removed the RpoN binding site from the *aioBA* promoter and an *aacC3* (gentamicin resistance) cassette insertional inactivation of the *rpoN* coding region eliminated *aioBA* expression and As^{III} oxidation, although *rpoN* expression was not related to cell exposure to As^{III}. Putative RpoN binding sites were identified throughout the genome and, as examples, included promoters for *aioB*, *phoB1*, *pstS1*, *dctA*, *glnA*, *glnB*, and *flgB* that were examined by using qualitative RT-PCR and *lacZ* reporter fusions to assess the relative contribution of RpoN to their transcription. The expressions of *aioB* and *dctA* in the wild-type strain were considerably enhanced in cells exposed to As^{III}, and both genes were silent in the *rpoN*::*aacC3* mutant regardless of As^{III}. The expression level of *glnA* was not influenced by As^{III} but was reduced (but not silent) in the *rpoN*::*aacC3* mutant and further reduced in the mutant under N starvation conditions. The *rpoN*::*aacC3* mutation had no obvious effect on the expression of *glnB*, *pstS1*, *phoB1*, or *flgB*. These experiments provide definitive evidence to document the requirement of RpoN for As^{III} oxidation but also illustrate that the presence of a consensus RpoN binding site does not necessarily link the associated gene with regulation by As^{III} or by this sigma factor.

Evidence of microbial arsenite (As^{III}) oxidation was first reported nearly a century ago (18). Subsequent progress has been sporadic, with work that identified some organisms capable of As^{III} oxidation (46, 48, 60) and then a study of a *Pseudomonas arsenitoxidans* strain reported to grow chemolithoautotrophically with As^{III} as a sole electron donor (23). Subsequent follow-up characterizations of this organism and this process failed to materialize; however, approximately 2 decades later, Santini et al. (52) described the isolation and initial characterization of a *Rhizobium*-like bacterium (strain NT-26) that could grow chemolithoautotrophically with As^{III} as a sole electron donor for energy generation and with CO_2 as a sole carbon source. Soon thereafter, and in part stimulated by the massive arsenic poisoning disaster in Bangladesh (2), a series of studies initiated the characterization of microbial As^{III} oxidation in natural environments, including geothermal springs (9, 11, 12, 17, 19, 24, 25, 35, 51) and soils (41); in mining-contaminated environments (6, 13, 40); and, most recently, in anoxic photosynthesis (21, 33). Likewise, progress has been made in the understanding of the biochemistry of the As^{III} oxidase enzyme (1, 14, 37).

Studies examining the genetics and regulatory control of As^{III} oxidation have advanced, particularly within the past decade. Muller et al. (43) cloned and described the genes from a betaproteobacterium (subsequently identified as *Herminiimonas arsenicoxydans* [44]) that encode the small (*aoxA* [now *aioB*]) and large (*aoxB* [now *aioA*]) subunits of the As^{III} oxidase enzyme. The As^{III} oxidase structural genes were later cloned from the above-mentioned *Rhizobium* NT-26 organism (53). The symbols for genes coding for functions associated with As^{III} oxidation have recently been changed from *aox*, *aro*, or *aso* to *aio* (36), and we will use this new *aio* gene nomenclature throughout this report and in the future.

Kashyap et al. (28) used random transposon mutagenesis to identify and characterize a two-component signal transduction pair, *aioS* and *aioR*, that is immediately adjacent to the *aioBA* genes in an *Agrobacterium tumefaciens* soil isolate, defining what was assessed at the time to be the *aio* operon. Parallel studies by Kashyap et al. (29) also identified a molybdate transporter and an Na^+/H^+ antiporter that were also found to be essential for As^{III} oxidase oxidation. Later, and using a similar transposon mutagenesis approach, Koechler et al. (31) also identified the *aioSR* two-component pair and molybdate transporter as being essential for As^{III} oxidation. While transposon mutation-based experiments (28, 31) indicated the role and importance of the sensor kinase AioS and its putative regulatory partner AioR (a bacterial enhancer binding protein), direct proof of these two proteins working together as part of a putative As^{III} signal perception and transduction cascade was just recently provided by Sardiwal et al. (54), who demonstrated the autophosphorylation of an AioS component and the AioS-specific phosphorylation of AioR. Recently, our work has expanded this regulatory model to now include a third component, AioX, which is a periplasmic As^{III} binding protein that is also essential for *aioBA* expression (39).

Koechler et al. (31) also isolated *rpoN*::Tn*5*::*lacZ2* and *dnaJ*::Tn*5*::*lacZ2* mutants that were defective in As^{III} oxidation, although those observations were not accompanied by complementation experiments to demonstrate that the loss of function with these mutants was due to the interrupted genes as opposed to polar effects of the transposon on the transcription of adjacent downstream genes. However, ancillary data presented by Koechler et al. (31) provided additional indirect evidence of the *rpoN* gene product (also referred to as σ^{54}, σ^N, and NtrA) being

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TABLE 1 Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer pair	Relevant marker(s) and/or characteristic(s) or sequence	Reference, source, or purpose
Bacterial strains		
Agrobacterium tumefaciens		
5A	Wild type, soil isolate, As^{III} oxidizing	41
5A(Δ*PaioB*)	*aioB* promoter region deletion mutant	This study
5A(*rpoN*::*aacC3*)	Gen^r; *rpoN* mutant by gentamicin cassette interruption	This study
5A(*rpoN*::*aacC3*)(pCPP30-*rpoN*)	Gen^r Tet^r; *rpoN*::*aacC3* complemented strain	This study
5A(P*rpoN*)	Km^r; 5A with pLSP-P*rpoN*	This study
5A(P*aioB*)	Km^r; 5A with pLSP-P*aioB*	Laboratory stock
5A(P*phoB1*)	Km^r; 5A with pLSP-P*phoB1*	Laboratory stock
5A(P*pstS1*)	Km^r; 5A with pLSP-P*pstS1*	Laboratory stock
Escherichia coli		
S17-1	Pro^- Mob^+; conjugation donor	57
Top10	High-competency cloning host	Invitrogen
Plasmids		
pCR2.1-TOPO	PCR TA cloning vector	Invitrogen
pCPP30	Broad host range; *tetA*	22
pJQ200sk	Gen^r *traJ oriT sacB*; suicide vector	49
pLSP-kt2lacZ	Km^r *oriV*; *lacZ* fusion vector used for *lacZ* fusion constructs	L. Pierson, Texas A&M University
pCR2.1-P*aioB*	pCR2.1-TOPO with P*aioB* region	Laboratory stock
pCR2.1-*rpoN*	pCR2.1-TOPO with *rpoN* region	This study
pJQ200sk-P*aioB*	pJQ200sk with deleted P*aioB* region	This study
pJQ200sk-*rpoN*::*aacC3*	pJQ200sk with *rpoN* gene interrupted with gentamicin cassette	This study
pCPP30::*rpoN*	pCPP30 containing the complete *rpoN* gene	This study
pLSP-P*rpoN*	pLSP-kt2lacZ containing *rpoN* promoter region	This study
pLSP-P*aioB*	pLSP-kt2lacZ containing *aioB* promoter region	Laboratory stock
pLSP-P*phoB1*	pLSP-kt2lacZ containing *phoB1* promoter region	Laboratory stock
pLSP-P*pstS1*	pLSP-kt2lacZ containing *pstS1* promoter region	Laboratory stock
Primer pairs		
rpoN-f/rpoN-r (2,124 bp)	5′-GGCGTTCTCATCACCGACCAC-3′/5′-TTTACCAGATACACGCACACTCAT-3′	For construction of *rpoN*::*aacC3*
PaioBMu-1f/PaioBMu-1r (540 bp)	5′-GCAGCGACGCCAGTTCCTT-3′/ 5′-CCCATCCACTAAACTTAAACAGTGTGGGGGTTCGGTTTTC-3′	For deletion of *aioB* promoter
PaioBMu-2f/PaioBMu-2r (547 bp)	5′-TGTTTAAGTTTAGTGGATGGGCTTCGACATTCAGTGGAGGAG-3′/ 5′-CGTTGGACAGGCGGCCGTAGATGA-3′	For deletion of *aioB* promoter
PrpoN-f/PrpoN-r (454 bp)	5′-GGCGTTCTCATCACCGACCAC-3′/5′-GATTGCGCTCCACCTCCTG-3′	For construction of P*rpoN*::*lacZ*
B22Gm-f/B22Gm-r (471 bp)	5′-ACAAAGTTAGGTGGCTCAA-3′/5′-TGGGTCGATATCAAAGTGC-3′	Gentamicin cassette-specific primer
P4/P5	5′-GACGTTGCCTATCCCGATGAAGAT-3′/5′-GTTTGTTGATTGGCCAGGTGTAGG-3′	For RT-PCR of *aioB*[a]
phoB1-2f/phoB1-2r (361 bp)	5′-TGTTTAAGTTTAGTGGATGGGAGGGGCGCCGGCTATTCAA-3′/ 5′-CGCTCTAGAGACACCGACGACCTCCCTCAG-3′	For RT-PCR of *phoB1*
flgBRT-f/flgBRT-r (313 bp)	5′-CAACATCGCCAACGCCAACACA-3′/5′-CCTCTTTACCGTCATCAGCATCAT-3′	For RT-PCR of *flgB*
nrtART-f/nrtART-r (400 bp)	5′-CCGGCAAGGTGACGCAGAA-3′/5′-TCCCCGGTGGTACAGGCAGTGAA-3′	For RT-PCR of *nrtA*
glnART-f/glnART-r (356 bp)	5′-AGGCCACTCTTCGGATTG-3′/5′-CATTGATGCCTTCGTGGTTGAT-3′	For RT-PCR of *glnA*
glnBRT-f/glnBRT-r (227 bp)	5′-CAAGCTCGATGAAGTGAAGG-3′/5′-CGATACGTCCGGTCTGTGC-3′	For RT-PCR of *glnB*
dctART-f/dctART-r (359 bp)	5′-CGACAAGGCCCATGAGCAGACCA-3′/5′-CAGCGCCGAGGACCACGAACAC-3′	For RT-PCR of *dctA*

[a] See reference 28.

important for *aioBA* expression. In the current study, with *A. tumefaciens* strain 5A, we focused on the importance of RpoN, by introducing specific and precise mutations that removed the putative RpoN binding site immediately upstream of the *aioBA* genes as well as insertionally inactivating *rpoN*. In addition, we examined the genome for additional putative RpoN-dependent promoters to investigate the role and importance of this sigma factor in As^{III}-dependent and As^{III}-independent expression. We provide unambiguous evidence that RpoN is required for As^{III} oxidation but apparently not for the expressions of other genes associated with a consensus RpoN promoter.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used for this study are listed in Table 1. The *A. tumefaciens* strains were cultured at 30°C in a defined minimal mannitol medium (MMN) (58) containing mannitol as a carbon and energy source and 50 μM phosphate, with aeration by shaking at 200 rpm. For some experiments, log-

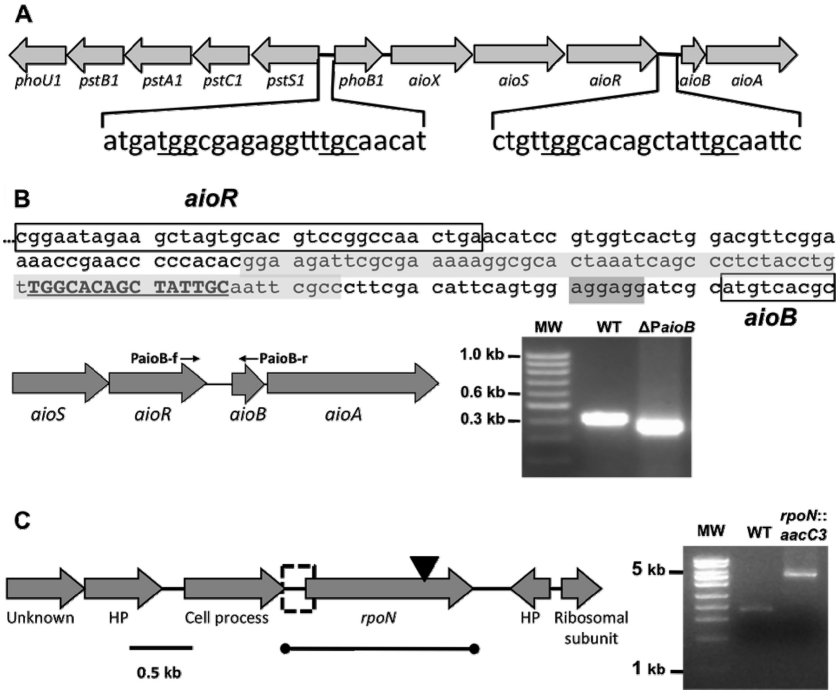

FIG 1 Physical arrangement and locations of genes involved in this study, and PCR-based evidence of mutation constructs. (A) Relative positions of the putative RpoN binding sites associated with the *aioB* gene and located between the *phoB1* and *pstS1* coding regions. (B) Sequence of the *aioB* promoter region that was deleted. The 3′ end of *aioR* and the 5′ end of *aioB* are boxed, and the ribosome binding site for *aioB* is highlighted in dark gray. The consensus RpoN binding site is highlighted with boldface uppercase type, and the region deleted (Δ*PaioB* mutant) is highlighted in light gray. In addition, PCR evidence to document the 67-bp deletion created in the *aioB* promoter region is provided. (C) Position of *rpoN* in strain 5A relative to the flanking genes, the approximate insertion point of the *aacC3* cassette (2.5 kb) (inverted black arrowhead) in the *rpoN* coding region (1.48 kb), the region of DNA that was PCR cloned and used for complementation of the *rpoN*::*aacC3* mutant, and the promoter region of *rpoN* used for the *rpoN*::*lacZ* reporter construct (dashed box). Also, PCR evidence documents the insertion of *aacC3* in the *rpoN* gene (4.6-kb construct). HP, hypothetical protein; WT, wild type; MW, molecular weight marker.

phase MMN-grown cells were washed via centrifugation and resuspended in MMN that lacked ammonium to simulate N starvation. *Escherichia coli* strains Top10 and S17-1 were grown at 37°C in Luria-Bertani (LB) medium. Bacterial growth was monitored via measurements of the culture optical density at 595 nm (OD_{595}) by use of a SpectraMax (Molecular Devices, CA) microtiter plate reader. Where indicated, *A. tumefaciens* growth media were amended with 80 μg ml^{-1} gentamicin (Gen) and/or 15% sucrose for the selection of double recombinants by using levansucrase selection (see below). Where required for mutant construction, *E. coli* cells were grown with 20 μg ml^{-1} Gen. The genome of *A. tumefaciens* 5A has been sequenced and deposited in the NCBI database (20).

Construction of *A. tumefaciens* 5A mutant strains. Primers used for the construction of *A. tumefaciens* deletion mutations, the insertional inactivation mutation, as well as the *lacZ* reporter fusions are provided in Table 1. The deletion of the RpoN binding site was constructed by crossover PCR as described previously (38) and by use of a levansucrase resistance strategy that we described previously (59). Briefly, the 497-bp C-terminal region of *aioR* was PCR amplified by using primers PaioBMu-1F and PaioBMu-1R (540-bp amplicon that includes 43 bp of the intergenic region). A second PCR amplified the region from 27 bp upstream of the *aioB* translational start site using primers PaioBMu-2F and PaioBMu-2R. A mixture of these two amplicons was used as the template for a third PCR that included primers PaioBMu-1F and PaioBMu-2R, yielding a product that contained a 67-bp deletion that removed the RpoN binding region of the *aioB* promoter (Fig. 1B). This product was ligated into pCR2.1-TOPO, yielding plasmid pCR2.1-P*aioB*. The 1,087-bp fragment was then subcloned into a BamHI- and XbaI-digested pJQ200sk vector, giving pJQ200sk-P*aioB*. This plasmid was transformed into *E. coli* S17-1 cells and then mobilized to *A. tumefaciens* 5A by conjugation. Merodiploids were selected on MMN agar plus Gen (MMN^{Gen} agar), with double crossovers then being selected on MMN agar containing 15% sucrose, which selects for the loss of the plasmid-borne *sacB* gene. Gentamicin-sensitive, sucrose-resistant colonies were screened and confirmed by PCR and sequencing, yielding the in-frame Δ*PaioB* deletion mutant.

Primer pair rpoN-f/rpoN-r was used to construct an insertion mutation in the *rpoN* coding region. The 2.1-kb amplified fragment was ligated and cloned into pCR2.1-TOPO, creating pCR2.1-*rpoN*. The *rpoN* gene harbored by pCR2.1-*rpoN* was then inactivated by the insertion of a gentamicin cassette (*aacC3*) at a ClaI site at bp 1378. The gentamicin cassette was prepared by the digestion of Tn*5*-B22 with BamHI and HindIII, polished with Klenow fragment (Promega) to create blunt ends, and ligated into the Klenow fragment-treated ClaI site. The *rpoN*::*aacC3* construct (4.6 kb) was then cloned into the BamHI and XbaI cloning sites of pJQ200sk, creating pJQ200sk-*rpoN*::*aacC3*, which was then transformed into S17-1 cells, followed by conjugation into strain 5A. Mutants wherein the wild-type allele was replaced by this construct were generated by using the above-described levansucrase selection strategy. For the complementation of the *rpoN*::*aacC3* insertion mutant, the same *rpoN* reading frame and upstream promoter region were PCR cloned by using primer pair rpoN-f/rpoN-r. The *rpoN* fragment from pCR2.1-*rpoN* was subcloned into the EcoRI cloning site of the pCPP30 vector, creating pCPP30-*rpoN*, which was transformed into S17-1 cells and then introduced into the *rpoN*::*aacC3* mutant by conjugation.

Arsenic chemical species analysis. As^{III} oxidation was assayed in the wild type and the *rpoN*::*aacC3* mutant by using HPLC-ICP-MS (high-performance liquid chromatography–inductively coupled plasma mass spectrometry) (Agilent 7500) to separate (ammonium carbonate mobile phase at a flow rate of 1 ml min^{-1}) and quantify As^{III} and As^{V} in culture supernatants. For calibration, standard solutions of 25, 50, 75, and 100 μM combined As^{III} and As^{V} were employed. Cell-free culture fluids

(0.22-μm filtration of centrifugation supernatants; Millipore) from cells grown in low-phosphate MMN containing 100 μM As^{III} were harvested at time zero and at the log and stationary phases.

Promoter activity analysis. The *aioB*, *rpoN*, *phoB1*, and *pstS1* promoter regions of the *A. tumefaciens* 5A genome were amplified by PCR using primers (Table 1) that incorporated EcoRI and BamHI restriction sites that allowed for directional cloning into *lacZ* reporter plasmid pLSP-kt2*lacZ* (Table 1). The resulting constructs were designated pLSP-P*aioB*, pLSP-P*rpoN*, pLSP-P*phoB1*, and pLSP-P*pstS1*, respectively, and were used for the analysis of promoter activity after the introduction of each construct into *A. tumefaciens* wild-type strain 5A via conjugation using *E. coli* strain S17-1. The *lacZ* reporter assays used cells grown in 50 ml of MMN with kanamycin under various conditions (with or without As^{III}) and incubated at 30°C on a rotary shaker at 200 rpm. After the various incubation times, 1-ml aliquots from each flask were collected and used for assays of β-galactosidase activity according to a method described previously by Miller (42).

Reverse transcriptase PCR analysis of gene expression. Cells were incubated in MMN with or without 100 μM As^{III} for 4 h. The total RNA of the cells (OD_{595} of ~0.2 to 0.3) was extracted and purified with an RNeasy kit (Qiagen) in accordance with the manufacturer's instructions. DNA contamination was removed by DNase I treatment and cleanup with a Turbo DNA-free kit (Ambion). The mRNA was then reverse transcribed into cDNA by using avian myeloblastosis virus (AMV) reverse transcriptase (RT) (Promega) with gene-specific primers (Table 1), according to the manufacturer's instructions, and with 500 ng RNA as the template in each RT reaction. PCR programs were standard, with primer-specific annealing temperatures, but all programs included final extension steps performed at 68°C for 5 min.

RESULTS

RpoN is required for As^{III} oxidation. Kashyap et al. (28) previously described an *aio* gene cluster comprised of *aioS-aioR-aioB-aioA-cyt-moeA* and provided RT-PCR evidence for these genes being expressed in a monocistronic fashion. Those authors also described the isolation of an *aioR* mutant having a Tn*5*-B22 transposon inserted into the 3′ region of the coding frame and which was interpreted as having polar effects on the transcription of the As^{III} oxidase-encoding genes *aioBA*. However, the intergenic region separating *aioR* and *aioB* (Fig. 1) was noted to be significant, perhaps accommodating another independently regulated promoter. This was consistent with a previous suggestion by Sardiwal et al. (54) and an earlier personal communication with J. M. Santini that a σ^{54}-dependent promoter was directly upstream of the *Rhizobium* NT-26 *aioBA* genes. An analysis of this region in *A. tumefaciens* strain 5A yielded a similar conclusion (Fig. 1A), and thus, experiments were designed to assess this possibility. Specifically, a deletion mutation was precisely constructed in the region directly upstream of *aioB* which included the entire predicted RpoN binding site but which excluded any of the flanking coding frames for *aioR* or *aioB* and likewise excluded the putative ribosome binding site for *aioB* (Fig. 1B); this mutant is referred to as the Δ*aioB* mutant. In parallel, the wild-type *rpoN* allele in strain 5A was replaced with an insertionally inactivated *rpoN* allele (*rpoN*::*aacC3*), creating an $RpoN^-$ mutant (Fig. 1C). Both mutations yielded organisms incapable of oxidizing As^{III} (Fig. 2A and B), but the complementation of the *rpoN*::*aacC3* mutant with a defined region of DNA that included only the *rpoN* reading frame and the upstream promoter region (Fig. 1C) reversed the defect back to wild-type As^{III} oxidation (Fig. 2A). Further analyses of the Δ*aioB* deletion mutant illustrated that the expression of *aioBA* was silenced (Fig. 2C). An analysis of *aioB*::*lacZ* and *rpoN*::*lacZ* reporters illustrated the expected As^{III}-dependent expression of *aioBA*, whereas *rpoN* expression appeared to be constitutive and not influenced by As^{III} (Fig. 3).

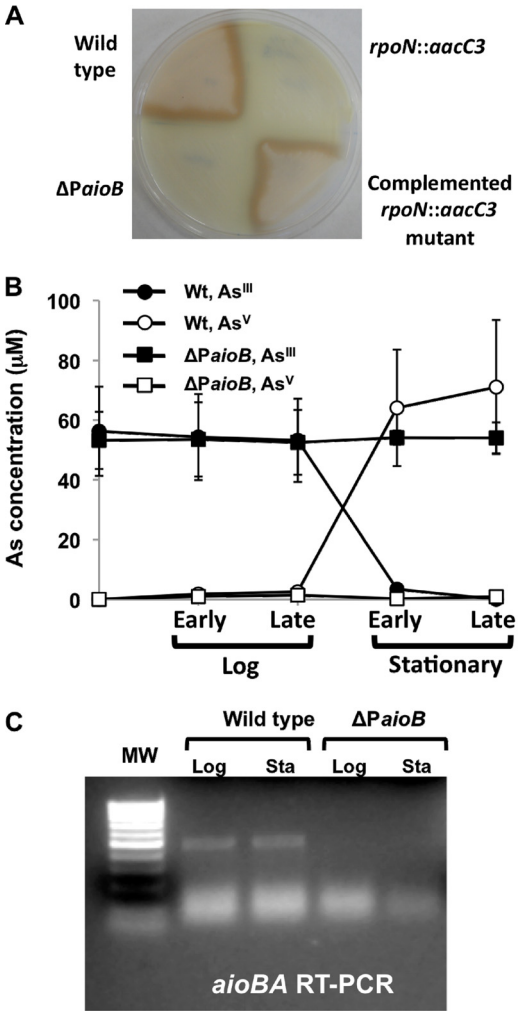

FIG 2 As^{III} oxidation phenotype of *A. tumefaciens* wild-type strain 5A and mutants described in this study. (A) Qualitative assessment of As^{III} oxidation using the $AgNO_3$ staining method for wild-type strain 5A, the Δ*aioB* mutant, the *rpoN*::*aacC3* mutant, and the rescued *rpoN*::*aacC3* mutant carrying pCPP30::*rpoN*. The brown precipitate indicates the presence of As^V. (B) HPLC-ICP-MS-based measurement of aqueous As^{III} and As^V in As^{III} oxidation assays with wild-type strain 5A and the Δ*aioB* mutant. Error bars represent the standard deviations of data from two replicate cultures. (C) RT-PCR monitoring of *aioBA* expression in the wild type and the Δ*aioB* mutant in the log and stationary phases of cells grown in the presence of 100 μM As^{III}.

An examination of the strain 5A genome for genes/operons known in other bacteria to be controlled by RpoN found putative RpoN binding sites associated with 16 genes coding for a variety of functions and which displayed the well-defined GG-(N10)-GC consensus RpoN binding site (Fig. 4). For all of these genes, the putative RpoN binding sites contained a perfectly conserved thymine at the 5′ end and a near-perfect consensus addition of a thymine at the 3′ end (Fig. 4). These genes code for functions associated with the phosphate stress response (*phoB1* and *pstS1*), flagellum construction (*flgB*), nitrogen metabolism (*nrtA*, *glnA*, and *glnB*), poly-β-hydroxybutyrate synthesis (*phbB*), and others (Fig. 4).

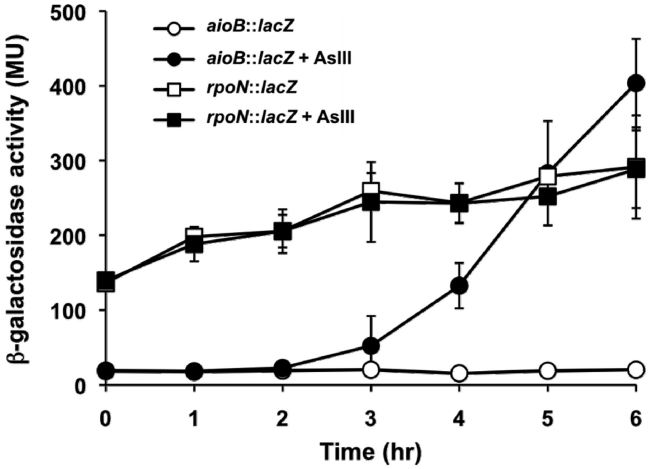

FIG 3 Reporter gene assays to track the expressions of *aioB* and *rpoN* as a function of time after the addition (or not) of 100 μM As^{III}. β-Galactosidase activity is presented as Miller units (MU), and error bars represent the standard deviations of data from two replicate cultures.

The importance of RpoN for the expression of a random sample of these genes was examined by qualitative RT-PCRs using RNA extracted from cells that were exposed to As^{III} or that were starved for nitrogen; the latter analysis was included because of the known association of RpoN with the regulation of N metabolism (e.g., see reference 16). Consistent with the experiments summarized in Fig. 2C, *aioB* expression was significantly upregulated in As^{III}-exposed wild-type cells but was not detectable in the *rpoN*::*aacC3* mutant regardless of the incubation conditions (Fig. 5A). Surprisingly, *dctA* was also upregulated by As^{III} and, like *aioB*, was silent in the *rpoN*::*aacC3* mutant. The *glnA* gene required RpoN for full expression, but it was found to be expressed under all incubation conditions and in both strains although at reduced levels in the *rpoN*::*aacC3* mutant and at further reduced levels in the *rpoN*::*aacC3* mutant incubated under N starvation conditions (Fig. 5A). Examples of genes where transcription appeared to be unaffected by the loss of functional RpoN included *phoB1*, *glnB*, and *flgB* (Fig. 5A). RT-PCR analysis showed that *phoB1* is upregulated by As^{III} (Fig. 5B). A quantitative *lacZ* reporter gene analysis of *phoB1* and the adjacent (but divergently transcribed) *pstS1* gene showed that both genes were dramatically induced by As^{III}; however, neither gene was affected by the *rpoN*::*aacC3* mutation (Fig. 5B). A significantly more expansive characterization of the As^{III} and phosphate coregulation of the *aio*, *pst*, and *pho* genes will be presented elsewhere (Y. S. Kang, B. Bothner, C. Rensing, and T. R. McDermott, submitted for publication).

DISCUSSION

The studies summarized in this report strengthen our understanding of transcriptional controls that regulate the expressions of key genes involved in As^{III} oxidation. The alternative sigma factor RpoN is essential for *aioBA* expression and As^{III} oxidation (Fig. 2), a conclusion supported by two independent lines of evidence. First, an insertional interruption of the *rpoN* coding region silenced *aioBA* expression and resulted in a null As^{III} oxidation phenotype. However, this could be reversed to the wild-type status with a defined DNA fragment containing the *rpoN* coding region and minimal flanking DNA. Second, the introduction of a precise deletion in a sequence that exhibited the well-known GG-(N10)-GC signature RpoN binding site (4) directly upstream of the *aioB* gene (but leaving the *aioB* translational start site intact)

Gene	RpoN binding site	Function
aioA	ctgt**tgg**cacagctat**tgc**aattc	Arsenite oxidase
phoB1	atga**tgg**cgagaggt**ttgc**aacat	Activator of phosphate stress responses
flgB	gaga**tgg**agaagtcta**tgc**aaccg	Flagella basal-body rod protein
ssuD	agcc**tgg**aaggggcg**gggc**ttggt	Alkanesulfonate monooxygenase
nrtA	aaac**tgg**cacgcctct**tgcc**tctt	Nitrate ABC transporter
gltB	tcaa**tgg**cgaccagcg**cgcc**tggc	Sulfite reductase
glnA	aatc**tgg**cacgcggca**tgc**atcta	Glutamine synthetase
glnB	gatc**tgg**cacaatacg**tgc**attct	Regulatory protein P-II for glutamine synthetase
nfoA	caag**tgg**ctcgagctt**ggc**gggac	NADH:flavin oxidoreductase
dctA	agtc**tgg**catgcgtat**tgc**acatg	C4-dicarboxylate transporter
yacG	gttg**tgg**aaaccaagg**tgc**gtttc	Zinc binding protein
phbB	tcaa**tgg**cggtgcgat**tgc**catcg	Acetoacetyl-CoA reductase
mmsB	cgag**tgg**agccggcgt**tgc**cactc	3-Hydroxyisobutyrate dehydrogenase
Peg.5251	agtc**tgg**agcacggcc**tgc**ctatc	TonB-dependent siderophore receptor
Peg.5626	gcca**tgg**cgcgcatcc**tgc**gcaca	Branched chain amino acid ABC transporter
Peg.2817	gctt**tgg**tacccatgt**tgc**ttcaa	Small heat shock protein

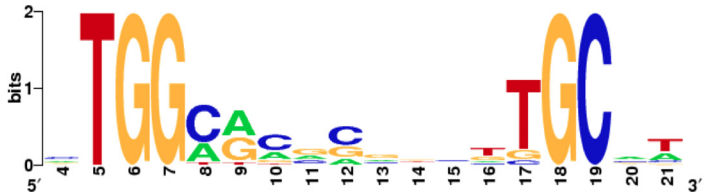

FIG 4 Alignment of the RpoN binding sites in the promoter regions from a sample of genes of the genome of strain 5A. Nucleotides highly conserved are highlighted in boldface type, and the consensus sequence depicting the relative conservation of each predicted position is shown at the bottom. CoA, coenzyme A.

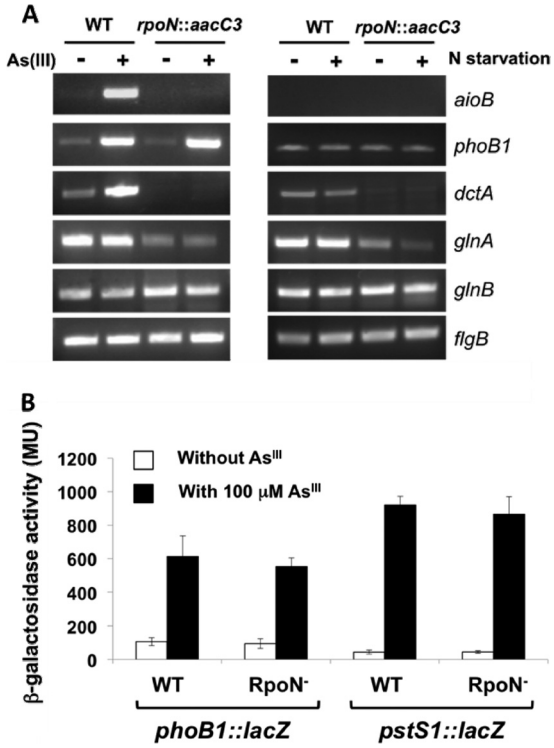

FIG 5 Influence of the *rpoN*::*aacC3* mutation on expressions of genes associated with an RpoN binding site. (A) Qualitative standardized RT-PCR expression analysis of genes (in the wild-type strain or the *rpoN*::*aacC3* mutant) associated with an RpoN binding site. Cells were grown in the absence (−) or in the presence (+) of As^{III} or under conditions with or without N. For each gene, RT-PCR amplicon volumes loaded into the gel were standardized. (B) β-Galactosidase activity (in Miller units) derived from the transcription of the *phoB1*::*lacZ* and *pstS1*::*lacZ* reporters in the wild type and the *rpoN*::*aacC3* mutant and as a function of cells grown in the presence or absence of 100 μM As^{III}.

also resulted in the silencing of *aioBA* expression and As^{III} oxidation. A recent random transposon-based mutation study with the organism *Herminiimonas arsenicoxydans* identified an *rpoN*::Tn*5* mutant as being defective in As^{III} oxidation (31). However, the necessary complementation experiments were not performed to confirm that the phenotype was actually due to the interrupted *rpoN* gene as opposed to transposon polar effects on the expression of *rpoX* (an *rpoS*-like sigma factor [61]) and other genes immediately downstream of what Duquesne et al. (13) depicted previously as an *H. arsenicoxydans* operon that contains *rpoN* (31). The precisely targeted mutation and complementation experiments in the present study provide definitive evidence that the *rpoN* gene product is essential for *aioBA* expression and, hence, As^{III} oxidation. This observation also allows a more definitive understanding of the As^{III} oxidase-negative phenotype of the *aioR*::Tn*5*-B22 mutant described previously (28). Specifically, the Tn*5*-B22 insertion in the extreme 3′ region of *aioR* in the *aioS-aioR-aioB-aioA* operon likely disrupted the DNA binding component of the regulator AioR and is consistent with AioR participating as a bacterial enhancer binding protein in conjunction with RpoN to facilitate the induction of *aioBA*. Kashyap et al. (28) also reported evidence that *aioSR* are cotranscribed with *aioBA* in what appears to be an operon arrangement. In this specific context, it is not presently clear why the loss of the RpoN binding site would then interrupt *aioBA* expression in strain 5A.

The requirement for RpoN as an essential regulator of As^{III} oxidation is not necessarily surprising given the wide variety of cellular functions that RpoN regulates (reviewed in references 34 and 56). Indeed, we found that the consensus GG-(N10)-GC RpoN binding-site signature associated with *aioB* was also found elsewhere in the genome (Fig. 4), being associated with a wide range of functions previously noted for other bacteria (34, 50, 56). Given such a range of functions, it is also not surprising that the expression of *rpoN* appears to be constitutive in *A. tumefaciens* strain 5A and is not influenced by a specific solute or ion such as As^{III} (Fig. 3), which is in agreement with previous studies (7, 26).

What was surprising, however, was the observation of a C_4-dicarboxylate transporter (*dctA*) (Fig. 4) upregulated by As^{III} (Fig. 5A). It is not immediately apparent why, or how, such a transporter might be involved (or not) in As^{III} oxidation or metabolism. Previously reported microarray studies also identified the As^{III} induction of a C_4-dicarboxylate transporter in *H. arsenicoxydans* (8). The Dct system in strain 5A is a secondary transporter operating without a periplasmic solute binding protein, whereas the As^{III}-inducible *dctPMQ* dicarboxylate transporter in *H. arsenicoxydans* is annotated as a tripartite ATP-independent periplasmic (TRAP)-type transporter (reviewed in reference 45). While these two C_4-dicarboxylate transporters are structurally different, their function is the same. Such shared regulatory and functional similarities would seem not to be a simple coincidence and suggest a potential involvement in some facet of arsenic metabolism. Rat and plant mitochondrial C_4-dicarboxylate transporters have been shown to facilitate the exchange of malate and phosphate (15, 27), with arsenate robustly substituting for phosphate in the shuttle (15). In the context of a prokaryote cell, such a mechanism could result in As^{V} removal from the cell and is an issue that we are examining in more detail.

The other instance where RpoN appeared to be important involved *glnA*. Not surprisingly, As^{III} had no influence on *glnA* expression in the wild-type or mutant strains. Also, *glnA* expression in the wild-type strain was observed under all conditions, although the level of expression varied depending on the incubation conditions. The *glnA* expression level was noticeably reduced in the *rpoN*::*aacC3* mutant under N-replete conditions, similar to what was found previously for *Xylella fastidiosa* (10), and was even further reduced when the mutant was starved for N (Fig. 5A).

In contrast to *aioB* and *dctA*, genes such as *phoB1*, *pstS1*, *glnB*, and *flgB* were examples where an association with a consensus RpoN binding site did not correlate with an RpoN requirement (Fig. 5), at least under the experimental conditions considered. Previous predictions suggested an RpoN involvement wherever the GG-(N10)-GC motif was present (30, 47), but others have documented observations similar to those reported here. For example, in *Pseudomonas putida*, the presence of the GG-(N10)-GC motif does not always imply RpoN-associated regulation (55), and the lack of functional RpoN in this organism was shown previously to be essentially irrelevant to bacterial environmental responses normally associated with RpoN-controlled regulator functions (5). Furthermore, there is evidence of RpoN potentially interacting with a repressor and, hence, participating in a negative regulatory sense (3, 32) or that the spacing between the GC doublet and the transcriptional start site might not always be optimal

(55). These types of explanations likely apply to *A. tumefaciens* strain 5A as well.

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