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Arsenite Oxidase *aox* Genes from a Metal-Resistant β-Proteobacterium

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The β-proteobacterial strain ULPAs1, isolated from an arsenic-contaminated environment, is able to efficiently oxidize arsenite [As(III)] to arsenate [As(V)]. Mutagenesis with a *lacZ*-based reporter transposon yielded two knockout derivatives deficient in arsenite oxidation. Sequence analysis of the DNA flanking the transposon insertions in the two mutants identified two adjacent open reading frames, named *aoxA* and *aoxB*, as well as a putative promoter upstream of the *aoxA* gene. Reverse transcription-PCR data indicated that these genes are organized in an operonic structure. The proteins encoded by *aoxA* and *aoxB* share 64 and 72% identity with the small Rieske subunit and the large subunit of the purified and crystallized arsenite oxidase of *Alcaligenes faecalis*, respectively (P. J. Ellis, T. Conrads, R. Hille, and P. Kuhn, Structure [Cambridge] 9:125-132, 2001). Importantly, almost all amino acids involved in cofactor interactions in both subunits of the *A. faecalis* enzyme were conserved in the corresponding sequences of strain ULPAs1. An additional Tat (twin-arginine translocation) signal peptide sequence was detected at the N terminus of the protein encoded by *aoxA*, strongly suggesting that the Tat pathway is involved in the translocation of the arsenite oxidase to its known periplasmic location.

Arsenic is present in various environments, is released either by natural weathering of rocks or by anthropogenic sources (e.g., mining industries and agricultural practices), and is found in the oxidation states +5 (arsenate), +3 (arsenite), 0 (elemental arsenic), and −3 (arsine). Contamination of drinking-water supplies with the inorganic soluble forms arsenite and arsenate has often been reported, and arsenic has been identified as a major risk for human health in different parts of the world (northeast India, Bangladesh, northwest United States) (31). The biogeochemical cycle of this element strongly depends on microbial transformation, which affects the mobility and the distribution of arsenic species in the environment (33, 41). Several bacteria involved in transformation processes comprising reduction, oxidation, and methylation of arsenic species have been described (8, 11, 26, 36, 40).

The toxicological effects of arsenic are related to its chemical form and oxidation state; the organic forms are the less toxic. Among inorganic forms, As(III) is reported to be on average 100 times more toxic than the less mobile As(V) (25). Several remediation processes have been described for arsenic removal (19) based on chemical oxidation of arsenite to arsenate followed by alkaline precipitation (5, 15–17, 24). The major drawbacks of these processes are that they generate additional pollution and are expensive. This has led to the exploration of alternative methods for arsenic remediation based on its biological oxidation. Several arsenite-oxidizing bacteria have been isolated, starting with an *Achromobacter* strain in 1918 (14). Since then, different arsenite-oxidizing bacteria, including several *Pseudomonas* strains (18, 42–44), *Alcaligenes faecalis* (29, 30), *Thiobacillus ferrooxidans* and *Thiobacillus acidophilus* (21), bacteria from the *Agrobacterium*/*Rhizobium* branch of β-Proteobacteria (36, 38), and bacteria of the *Thermus* genus (13), have been described. Recently, a bacterium belonging to the *Zoogloae* branch of β-Proteobacteria was isolated from an arsenic-contaminated environment (46). This strain, called ULPAs1, is able to efficiently oxidize arsenite to arsenate. Due to its high resistance to As(III) as well as its high tolerance for heavy metals, this strain is a good candidate for bioremediation of environments heavily contaminated with arsenic. The physiology, enzymology, and genetics of arsenite oxidation have remained largely uninvestigated so far. The only information available is the crystal structure of the arsenite oxidase isolated from *A. faecalis* strain NCIB 8667 (2, 12). Biophysical studies of this enzyme showed that it is composed of a large subunit of 825 residues and a small subunit of at least 134 residues. The large subunit contains a molybdenum binding site and a [3Fe–4S] cluster, whereas the small subunit harbors a Rieske type [2Fe–2S] site. The catalytic site of the enzyme was associated with the large subunit. Here we report (i) the isolation of knockout mutants of the arsenite oxidase of strain ULPAs1 generated by minitransposon insertion and (ii) the identification of the two genes encoding this enzyme in strain ULPAs1.

MATERIALS AND METHODS

Bacterial strains and media. Strain ULPAs1 (46) was routinely grown in a chemically defined medium (CDM), which was prepared as follows: 100 ml of solution A (81.2 mM $MgSO_4 \cdot 7H_2O$ [Sigma], 187 mM NH_4Cl [99.8%; Merck], 70 mM Na_2SO_4 [99%; Prolabo], 0.574 mM K_2HPO_4 [97%; Prolabo], 4.57 mM $CaCl_2 \cdot 2H_2O$ [99.5%; Merck], 446 mM sodium lactate [98%; Sigma]), 2.5 ml of solution B (4.8 mM $Fe_2SO_4 \cdot 7H_2O$ [99%; Prolabo]), and 10 ml of solution C (950 mM $NaHCO_3$ [99.5%; Prolabo]) were mixed and made up to 1 liter with water. The final pH of the medium was about 7.2. All solutions were prepared with purified water (Milli-Q system; Millipore) previously sterilized by autoclaving (at 120°C for 20 min). Solution A was sterilized by autoclaving (at 120°C for 20 min), and solutions B and C were sterilized by filtration (0.45-μm-pore-size filter; Millipore). For transposon mutagenesis, *Escherichia coli* strain S17-1 was cultivated on Luria-Bertani (LB) medium (Difco). Because strain ULPAs1 does

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not grow on LB medium and *E. coli* does not grow on CDM, matings were performed on a medium composed of solidified CDM to which 10% (wt/vol) LB medium had been added, a formulation on which both strains grew.

Determination of arsenic speciation. Arsenite transformation was determined with culture supernatants filtered through a sterile 0.22-μm-pore-size Durapore filter (Millipore). Arsenic species were separated by high-performance liquid chromatography on a reversed-phase polymeric resin (PRP-X100; inner diameter, 250 by 4.1 mm; particle size, 10 μm; Hamilton) and quantified by inductively coupled plasma-atomic emission spectrometry (ICP-AES) with a sequential Jobin Yvon JY 138 Ultrace spectrometer as described by Weeger et al. (46).

Transposon mutagenesis. The mini-Tn*5*::*lacZ2* transposon (9) was delivered by mobilization of the suicide vector pUT/miniTn*5*::*lacZ2* from the donor strain *E. coli* S17-1 (λ-*pir*) to the recipient strain ULPAs1. Conjugation was performed at 25°C. ULPAs1 derivatives harboring mini-Tn*5*::*lacZ2* were selected on CDM agar containing kanamycin (25 μg liter^{-1}) as well as 5-bromo-4-chloro-3-indolyl-galactoside (X-Gal; 40 μg liter^{-1}). Yellow colonies were transferred to microtiter plates containing liquid CDM with 20% glycerol and were stored at −80°C.

Selection of arsenite oxidase mutants. Arsenite oxidase mutants were isolated by transferring colonies from microtiter plates with a 48-pin stamp to CDM agar supplemented with kanamycin (25 μg liter^{-1}), X-Gal (40 μg liter^{-1}), and sodium arsenite (2 mg liter^{-1}). Blue colonies, representing arsenite-induced mutants, were selected after 72 h at 25°C, transferred to CDM agar plates supplemented with arsenite (100 mg liter^{-1}), and grown for 24 h at 25°C. Mutants were subsequently screened for oxidation of arsenite to arsenate by use of the $AgNO_3$ method (22). Agar plates were flooded with a solution of 0.1 M $AgNO_3$. A brownish precipitate revealed the presence of arsenate in the medium (colonies expressing arsenite oxidase), while the presence of arsenite (mutants lacking arsenite oxidase and control strains) was detected by a bright yellow precipitate (22). Mutants of interest were then checked for the transformation of arsenite in liquid cultures as reported by Weeger et al. (46).

Determination of MICs. Two protocols were used for determining the MICs. For the liquid method (7), nonamended CDM or CDM amended with arsenite or heavy metals in the 0.1 to 7 mM range was inoculated with cell suspensions from fresh precultures to a final density of approximately 10^6 CFU ml^{-1}. The following metal ions were tested: As(III), Cd(II), Hg(II), Se(IV), Mn(II), Cr(III), Sb(III), Ni(II), Hg(II), Cu(II), and Cr(VI). The resulting suspensions were incubated at 25°C for 3 days. The MIC was defined as the lowest concentration that completely inhibited growth (7).

Alternatively, the procedure described by Lim and Cooksey (23) was followed. Briefly, bacterial suspensions were transferred in triplicate from microtiter plates to solid CDM plates supplemented with increasing concentrations of arsenite. The MIC, defined as the As(III) concentration that inhibited confluent growth on plates after 3 days at 25°C, was determined.

DNA work. DNA manipulations were carried out according to standard protocols as described by Sambrook et al. (37). Total DNA of strain ULPAs1 was isolated by using the Wizard Genomic DNA purification kit (Promega). Templates for inverse PCR (I-PCR) were prepared from about 1 μg of total DNA digested with enzymes that did not cut the mini-Tn*5* element (*Kpn*I and *Sph*I), extracted with phenol-chloroform, and precipitated with ethanol. The digested DNA preparations (about 500 ng) were ligated overnight at 14°C in a total volume of 20 μl with 20 U of T4 DNA ligase (New England Biolabs). DNA flanking the mini-Tn*5* insertion was amplified by PCR with *Taq* DNA polymerase (Gibco-BRL) and with the minitransposon-specific primers 5′-AGATCTGATC AAGAGACAG-3′ (I-end) and 5′-ACTTGTGTATAAGAGTCAG-3′ (O-end) (9).

The PCR products obtained were purified and sequenced. Database searches and sequence analyses were performed by using the BLAST program (1) and the Genetics Computer Group package from the University of Wisconsin (10). Investigation of the signal peptide was performed by using the neural network SignalP prediction software (27, 28) (http://www.cbs.dtu.dk/services/SignalP/).

For Southern blot analysis, isolated DNA was digested with the restriction enzyme *Xho*I, which cuts inside the Km^r gene of the mini-Tn*5*::*lacZ2* transposon. The *Sma*I fragment of pUT/miniTn*5*::*lacZ2*, covering the major part of the transposon, was used as a probe.

RNA manipulations and reverse transcription-PCR (RT-PCR) experiments. RNA was prepared from ULPAs1 cultures grown in CDM to exponential phase. Cultures were induced by addition of 50 ppm of As(III) 1 h before extraction. Bacteria were pelleted by centrifugation, and total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were then treated with DNase I (Gibco-BRL) and quantified spectrophometrically at 260 nm.

DNA-free total RNA was reverse transcribed and subsequently PCR amplified with primer "a" (5′-AATGACACCTTCACGGCG-3′), annealing 48 bp upstream of the *aoxA* stop codon, and primer "b" (5′-AGCACTCGATCTTTTG CAG-3′), annealing 872 bp downstream of the *aoxB* start codon, by using a Titan one-tube RT-PCR system (Roche). Reverse transcription was carried out at 53°C for 30 min, followed by PCR amplification consisting of 10 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and elongation at 68°C for 45 s; 25 cycles with elongation time incrementally increased by 5 s in each cycle; and a final 10-min elongation step. RT-PCR products were examined by 2% agarose gel electrophoresis. DNA contamination of the mRNA was determined by PCR using *Taq* polymerase without reverse transcriptase.

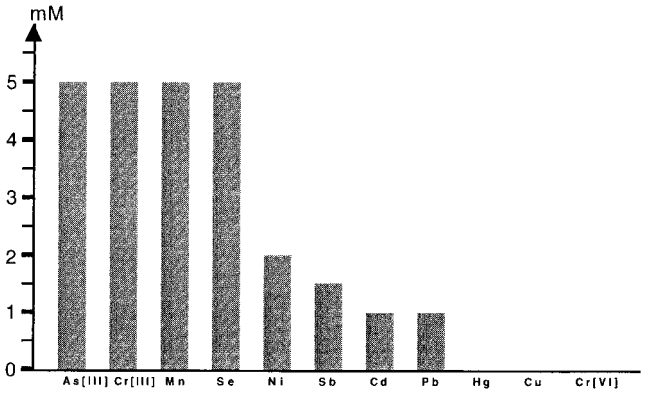


FIG. 1. MICs of chromium [Cr(III) and Cr(VI)], manganese (Mn), selenium (Se), nickel (Ni), antimony (Sb), cadmium (Cd), lead (Pb), mercury (Hg), and copper (Cu) for strain ULPAs1. Results are means of duplicate determinations.

Spheroplast preparation and dosage of arsenite oxidase activity. Bacteria grown in the presence of As(III) (100 mg liter^{-1}) were pelleted, resuspended in 20 mM Tris-HCl–0.1 mM phenylmethylsulfonyl fluoride–10 mM EDTA (pH 8.4) containing 20% sucrose, and treated with 0.5 mg of lysozyme ml^{-1} for 40 min at 25°C (2). Arsenite oxidase activity was determined based on the transfer of reducing equivalents from arsenite to 2,4-dichlorophenolindophenol (DCIP). Reduction of DCIP (60 μM) was monitored at 600 nm in the presence of 200 μM sodium arsenite in 50 mM morpholineethanesulfonic acid (MES), pH 6.0, at 25°C. Specific activity was defined as micromoles of DCIP reduced per minute per milligram of protein (2).

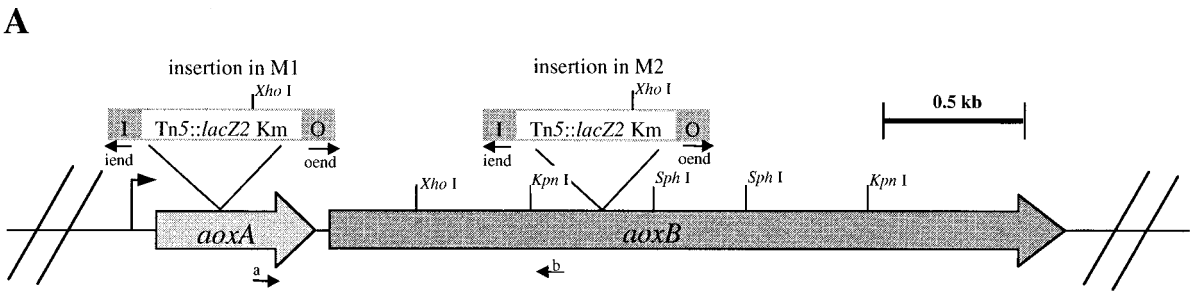
Nucleotide sequence accession number. The DNA sequences described in this report have been submitted to GenBank under accession number AF509588.

RESULTS

Arsenic and heavy metal multiresistance of strain ULPAs1. Sequencing of the 16S rRNA gene as well as fatty acid analysis indicated that strain ULPAs1 belongs to the β subclass of the Proteobacteria. Analysis of binary similarity data showed that the 16S rRNA sequence of strain ULPAs1 displays only 91 to 96% identity to sequences of representatives of known genera from the β-Proteobacteria (*Zoogloae* branch), suggesting that this strain belongs to a new genus (46; unpublished data).

Tolerance to arsenite and to various heavy metals was tested in order to explore the potentialities of strain ULPAs1 for use in bioremediation processes and to investigate its survival in a heavily polluted environment (Fig. 1). Strain ULPAs1 was resistant to many of the metals tested in addition to As(III) (MIC, 5 mM), namely, Se(IV) (MIC, 5 mM), Mn(II) (MIC, 5 mM), Cr(III) (MIC, 5 mM), Sb(III) (MIC, 1 mM), Ni(II) (MIC, 2 mM), Cd(II) (MIC, 1.42 mM), and Pb(II) (MIC, 1.2 mM). In contrast, strain ULPAs1 was sensitive to Hg(II), Cu(II), and Cr(VI).

Localization of arsenite oxidase. As previously observed for the arsenite-oxidizing strain *A. faecalis* NCIB 8667, arsenite



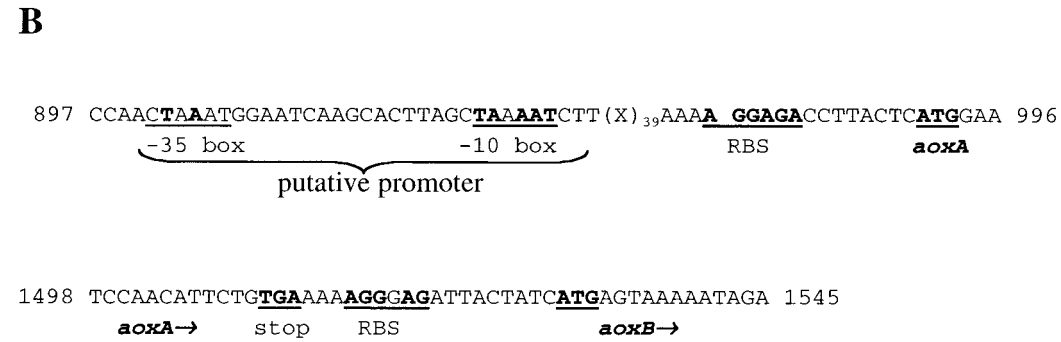


FIG. 2. (A) Genetic organization of the arsenite oxidase cluster in strain ULPAs1. Gene orientations are shown by arrows. Insertion sites of the mini-Tn*5*::*lacZ2* transposon are indicated. The locations of primers a and b, used for RT-PCR, are indicated. Restriction sites *Kpn*I, *Sph*I, and *Xho*I are shown. (B) Predicted structure of the promoter of the *aox* cluster. The −35 and −10 regions, ribosome binding sites (RBS), ATG codons, and stop codons are boldfaced.

oxidase activity was found to be associated with spheroplasts (1.4 μmol of DCIP min^{-1} mg of $protein^{-1}$). No activity was detected in the culture supernatant comprising the diluted contents of the periplasm. Activity was detected only when the cells were grown in the presence of arsenite, in accordance with previous results (46). This characteristic was exploited in our mutagenesis strategy.

Isolation of arsenite oxidase mutants. Mutagenesis was performed using the mini-Tn*5*::*lacZ2* transposon (9). ULPAs1-Km^r derivatives were selected after conjugation of strain ULPAs1 with *E. coli* S17-1(pUT/miniTn*5*::*lacZ2*). Blue colonies on arsenic-free CDM, corresponding to constitutive expression of the reporter gene *lacZ*, were discarded. The resulting collection of 4,000 yellow colonies was then screened on CDM plates supplemented with X-Gal and sodium arsenite. Of these transconjugants, 37 colonies showed arsenite-induced expression of β-galactosidase (blue colonies) and 10 strains showed reduced growth. However, no arsenite-sensitive mutants were detected when 100 mg of As(III) $liter^{-1}$ was used.

Mutants of interest were replicated on CDM agar supplemented with 50 mg of arsenite $liter^{-1}$, cultivated for 48 h, and tested for production of arsenate by use of the $AgNO_3$ method (22). Two mutants, called M1 and M2, showed loss of arsenite oxidase activity. In addition, these mutants, when cultivated at 25°C for 48 h in liquid CDM supplemented with 100 mg of arsenite $liter^{-1}$, did not transform arsenite to arsenate, as demonstrated by quantification of arsenic species in culture supernatants. In contrast, the wild-type ULPAs1 strain showed 100% transformation of arsenite under these conditions.

Identification of arsenite oxidase genes. Total DNAs of mutants M1 and M2, as well as DNA of strain ULPAs1, were extracted, digested with *Kpn*I or *Sph*I, and religated. The ligated DNA template was then amplified by I-PCR using primers corresponding to sequences from the ends of the transposon. For each mutant and each digest, unique PCR fragments of different sizes were found (Fig. 2A). Southern blot analysis was performed using an internal fragment of the mini-Tn*5*::*lacZ2* transposon. Transconjugants M1 and M2 displayed two hybridizing fragments of different sizes, while strain ULPAs1, used as a control, showed no hybridization (Fig. 2A). Taken together, these data suggested that each mutant was the result of a single insertion of the Tn*5* derivative into the genome of strain ULPAs1 and that the amplified PCR fragments truly reflected the organization of DNA in the ULPAs1 genome.

DNA regions flanking the mini-Tn*5*::*lacZ2* insertions were sequenced for both mutants M1 and M2 (Fig. 2A). Two open reading frames (ORFs) of 522 nucleotides (ORF1) and 2,478 nucleotides (ORF2), separated by 24 nucleotides, were identified and named *aoxA* and *aoxB*, respectively (Fig. 2B). A motif showing significant similarity to the σ^{70}-like 35/10 promoter consensus sequence was found upstream of *aoxA* but not of *aoxB*. The two ORFs were organized in the same direction, and no Pribnow box was detected between them. Good puta-

tive ribosome binding sites were found for both genes (Fig. 2B).

The amino acid sequences deduced from the DNA sequences of *aoxA* and *aoxB* correspond to two proteins of 174 and 826 amino acids (aa), respectively. Analysis of these sequences showed that they displayed strong similarity to the sequences of the two subunits of the arsenite oxidase enzyme of *A. faecalis* established by crystallography (12) (Fig. 3). The 134 aa of the C-terminal end of the putative AoxA protein displayed 65% identity with the small subunit of the enzyme of *A. faecalis*. The consensus sequence Cys-X-His-X_{15}-Cys-X_2-His, which coordinates the Rieske type [2Fe–2S] cluster in the small subunit of *A. faecalis*, was detected at equivalent positions in AoxA. However, the predicted AoxA protein of strain ULPAs1 was larger than the small Rieske subunit of *A. faecalis* (174 versus 134 aa). An additional sequence, characteristic of the Tat (twin-arginine translocation) secretion signal peptide (4), was detected at the N terminus of AoxA (Fig. 3A), with three distinct regions: (i) an N-terminal, positively charged sequence harboring the conserved motif S-R-R-X-F-L-K, (ii) a central region rich in hydrophobic amino acids, and (iii) a C-terminal sequence harboring a predicted cleavage site located, according to Nielsen et al. (27) and SignalP prediction software (28), between Ala33 and Ala34. However, the proline residue at position −6 relative to the signal peptidase cleavage site, found in the majority of Tat sequences so far, was replaced in the ULPAs1 AoxA sequence by a serine, as in the Tat sequence of the small subunit of the Ni-Fe hydrogenase from *Bradyrhizobium japonicum* (3).

The protein sequence deduced from *aoxB* (826 aa) showed 72% identity with the large subunit (825 aa) of the *A. faecalis* enzyme (Fig. 3B). No signal sequence was detected for this subunit. The [3Fe–4S] cluster binding motif Cys-X_2-Cys-X_3-Cys-X_{70}-Ser of *A. faecalis* and the majority of amino acids interacting with the molybdenum center are conserved in AoxB of strain ULPAs1. Differences were minor, namely, Ser238 (Thr in ULPAs1), Asn703 (Ala in ULPAs1), and Thr709 (Ser in ULPAs1). Moreover, residues His195, Glu203, Arg419, and His423, which were shown in *A. faecalis* to be involved in the fixation of the substrate As(III), were conserved in AoxB of strain ULPAs1.

Interestingly, close homologs of both *A. faecalis* and the ULPAs1 large and small arsenite oxidase subunits were detected in *Aeropyrum pernix*, an archaeal hyperthermophile isolated from a coastal sulfotaric thermal vent in Japan (20, 35). The AoxA sequence displays 44% identity with APE2563 (210 aa) from *A. pernix* (Fig. 3A). Moreover, a putative signal Tat peptide (Fig. 3A) was also found in APE2563. The proteins encoded by APE2556 of *A. pernix* and by *aoxB* also showed 44% identity (Fig. 3B).

The two genes *aoxA* and *aoxB* belong to the same operon. The existence of a transcript corresponding to the coexpression of *aoxA* and *aoxB* was inferred from RT-PCR experiments (see Fig. 2A; also Materials and Methods). A fragment of 0.85 kb corresponding to the intergenic region between *aoxA* and *aox*B was observed (Fig. 4, lanes 1 and 2). No DNA contamination of the mRNA was detected, as shown in lane C, corresponding to a PCR using *Taq* polymerase without reverse transcriptase.

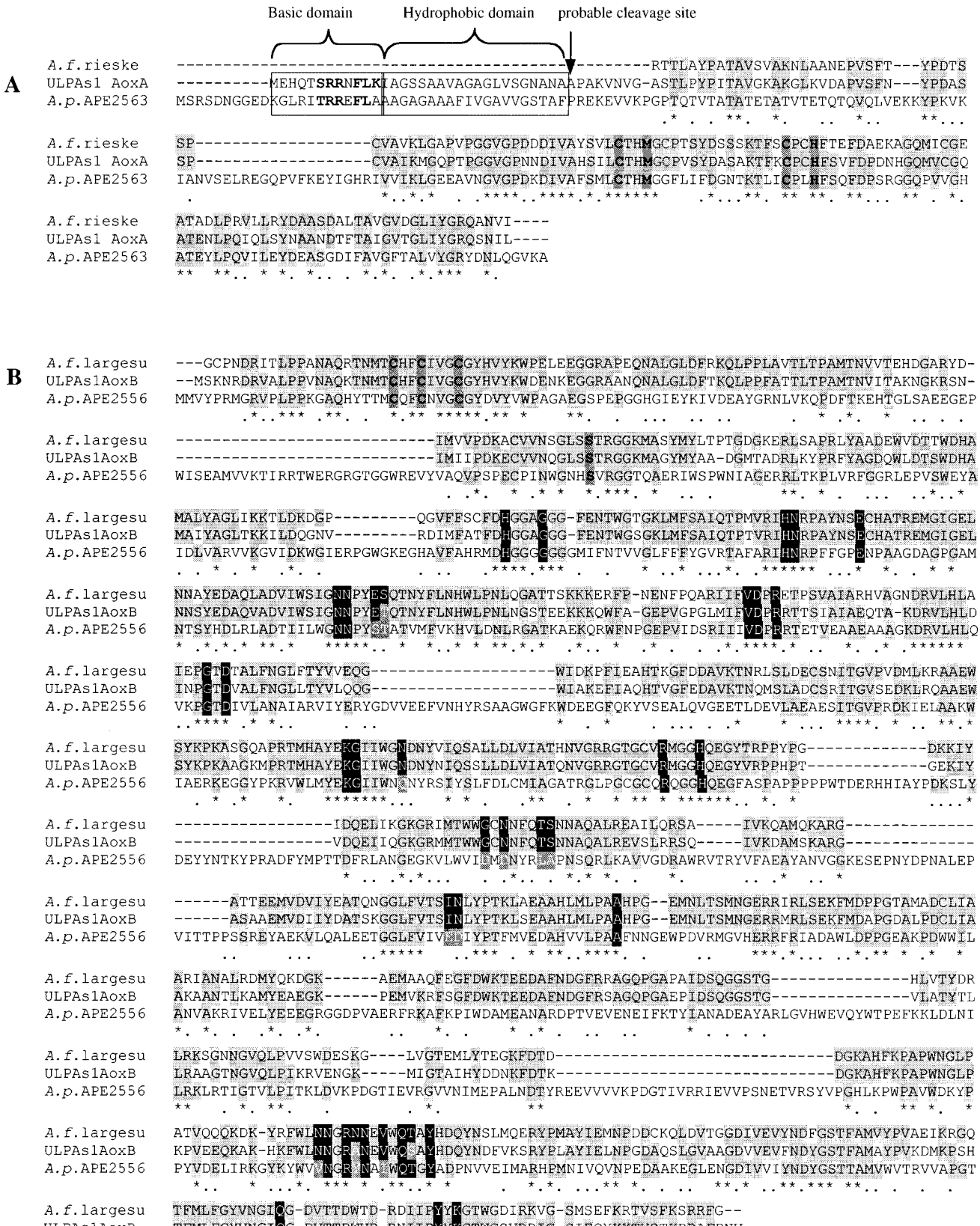
DISCUSSION

While much information about the molecular mechanism of arsenate reduction is available (6, 34), the mechanism of arsenite oxidation is still scarcely documented despite its importance for bioremediation. The arsenite oxidase of a strain of *A. faecalis* has been purified and its structure has been determined (2, 12), but no genetic information has been available so far. In this work, we identified the genes encoding a closely related 2-subunit arsenite oxidase from the metal-resistant β-proteobacterial strain ULPAs1 by using a transposon mutagenesis approach. Insertion of the mini-Tn*5*::*lacZ2* transposon in either *aoxA* or *aoxB* completely abolished arsenite oxidation, demonstrating that both genes are essential for arsenite oxidation in strain ULPAs1.

Importantly, comparison of the predicted AoxA protein of ULPAs1 with the small Rieske type subunit of *A. faecalis* NCIB 8667 led to detection of a Tat (twin-arginine translocation) secretion signal in the ULPAs1 sequence (2, 23), suggesting that the arsenite oxidase is exported to the periplasm via the Tat protein export pathway. This pathway is distinct from the general secretory system (Sec) (32) and transports folded proteins (including heterodimers) across the cytoplasmic membrane. In most cases the substrates of this pathway are proteins involved in electron transport which often bind redox cofactors (3, 4, 39, 45). This result is consistent with both the function of arsenite oxidase and its demonstrated periplasmic location in *A. faecalis* (2) and in strain ULPAs1. Moreover, a characteristic Tat signal peptide was also detected at the N terminus of the APE2563 gene product from *A. pernix*, which is homologous to AoxA, suggesting that the structure and function of arsenite oxidase are conserved in both procaryotic domains. This idea now needs to be confirmed by the study of a larger diversity of arsenite-oxidizing microorganisms.

The physiological importance of arsenite oxidation in strain ULPAs1 is still an open question. Whereas in some bacteria arsenite oxidase has been shown to be involved in energy metabolism (18), no difference in growth was observed for strain ULPAs1 in the presence versus the absence of arsenite. Moreover, no autotrophic growth occurred in the absence of an organic carbon source, even in the presence of As(III) (46). Thus, oxidation of arsenite does not seem to be directly implicated in energy metabolism in this strain.

FIG. 3. Sequence alignment of the two subunits of the arsenite oxidase of *A. faecalis* and the putative proteins deduced from the DNA sequences of strain ULPAs1 and *A. pernix*. (A) The small Rieske type subunit of *A. faecalis* (*A.f.* rieske) is compared to the putative protein encoded by *aoxA* (ULPAs1 AoxA) and the predicted protein encoded by *A. pernix* (*A.p.*APE2563). Residues belonging to the consensus Tat sequence are boxed. (B) The large subunit of *A. faecalis* (*A.f.*largesu) is compared to the putative protein encoded by *aoxB* (ULPAs1AoxB) and the predicted protein encoded by *A. pernix* (*A.p.*APE2556). Dark shading indicates the Fe-S clusters (inside the large and the small subunits); white letters on a solid background represent amino acids interacting with the molybdenum center (identical). Light shading, conserved amino acids; asterisks, positions where amino acids are identical; periods, positions where amino-acids are similar.


Basic domain
Hydrophobic domain
probable cleavage site
A
A.f.rieske
ULPAs1 AoxA
A.p.APE2563
B
A.f.largesu
ULPAs1AoxB
A.p.APE2556

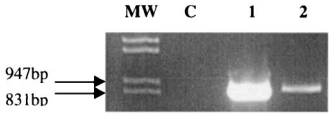


FIG. 4. DNA products derived from RT-PCRs of total RNA from a culture of strain ULPAs1 grown in the presence of arsenite (lanes 1 and 2). In lane 1, the concentration of RNA was 2 $\mu g \cdot \mu l^{-1}$; in lane 2, it was 0.2 $\mu g \cdot \mu l^{-1}$. The control reaction (C) without reverse transcriptase confirmed the absence of products derived from contaminated DNA.

On the other hand, different authors have proposed a role for arsenite oxidase in detoxification (2, 12). The derivatives of ULPAs1 that lack arsenite oxidase provided the opportunity to explore the relationship between resistance to As(III) and detoxification. When the wild-type strain ULPAs1 and the two mutants were compared by the method of Lim and Cooksey (23), the mutants expressed reduced resistance to arsenite: the MIC of As(III) was 2.66 mM for both mutants, while it was 5 mM for the wild-type strain. This result clearly indicates that arsenite oxidase plays a role in arsenite resistance. Since knocking out *aoxA* or *aoxB* did not completely abolish resistance to arsenic, it is likely that strain ULPAs1 possesses additional mechanisms of resistance to arsenite. An active arsenite efflux system, such as that described for many gram-positive and gram-negative arsenic-resistant microorganisms (6), is an attractive possibility worthy of future investigation.

Indeed, besides the 2 mutants (M1 and M2) characterized in the work presented here, 35 other mutants in which expression of the *lacZ* reporter gene was induced by arsenite were isolated, but their arsenite oxidase functions were not affected. This indicates the existence in strain ULPAs1 of other arsenite-regulated loci, whose characterization should provide further insight into the mechanisms used by this bacterium to cope with the presence of the most toxic form of arsenic in its environment.

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