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Unsuspected Diversity of Arsenite-Oxidizing Bacteria as Revealed by Widespread Distribution of the *aoxB* Gene in Prokaryotes▽†

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In this study, new strains were isolated from an environment with elevated arsenic levels, Sainte-Marie-aux-Mines (France), and the diversity of *aoxB* genes encoding the arsenite oxidase large subunit was investigated. The distribution of bacterial *aoxB* genes is wider than what was previously thought. AoxB subfamilies characterized by specific signatures were identified. An exhaustive analysis of AoxB sequences from this study and from public databases shows that horizontal gene transfer has likely played a role in the spreading of *aoxB* in prokaryotic communities.

Arsenic, which is one of the most toxic metalloids, is distributed ubiquitously but not uniformly around the world. Levels of arsenic differ considerably from one geographical region to another, depending on the geochemical characteristics of the soil (natural contamination) and the industrial activities carried out in the vicinity (anthropogenic contamination) (22). In aquatic environments, arsenic occurs mainly in the form of the inorganic species arsenate [As(V)] and arsenite [As(III)]; the latter species, which is more bioavailable, is usually thought to have more-toxic effects on prokaryotes than As(V) (34). As(III) oxidation leads to the formation of the less available form As(V), which can either precipitate with iron [Fe(III)] or be adsorbed by ferrihydrite. The oxidation process may be mediated by microbial activities, which contribute to the natural remediation processes observed in contaminated environments (21, 26, 27, 34). Consequently, bioprocesses for the treatment of arsenic-contaminated waters have been developed based on the precipitation or adsorption of the As(V) produced by bacteria (4, 9, 21). Some well-known prokaryotes oxidize As(III) into As(V) under aerobic (e.g., *Herminiimonas arsenicoxydans*, *Thiomonas* spp., or *Rhizobium* sp. strain NT26) or anaerobic (e.g., *Alkalilimnicola ehrlichii*) conditions as part of a detoxification process (12, 17, 31, 32, 39). Some chemolithotrophs also use arsenite as an electron donor (e.g., *Rhizobium* sp. strain NT26 or *Thiomonas arsenivorans*) (5, 32). The aerobic arsenite oxidases involved in such processes are heterodimers consisting of a large subunit with a molybdenum center and a [3Fe-4S] cluster (AroA, AsoA, and AoxB) and a small subunit containing a Rieske-type [2Fe-2S] cluster (AroB, AsoB, and AoxA) (1, 13). The large subunit in these enzymes is similar to that found in other members of the dimethyl sulfoxide (DMSO) reductase family of molybdenum enzymes but is clearly phylogenetically divergent from the respiratory arsenate reductases (ArrA) or other proteins of the DMSO reductase family of molybdenum oxidoreductases, such as the new arsenite reductase described recently for *Alkalilimnicola ehrlichii* (25, 31, 40).

aox genes have been identified in 25 bacterial and archaeal genera isolated from various arsenic-rich environments, most of which belong to the *Alpha-*, *Beta-*, or *Gammaproteobacteria* phylum (7, 10, 12, 14, 23, 25, 29, 32, 37). Recent studies based on environmental DNA extracted from soils, sediments, and geothermal mats with different chemical characteristics and various levels of arsenic contamination have suggested that the distribution and the diversity of arsenite-oxidizing microorganisms may be greater than previously suggested (6, 10, 14–16, 18, 28, 29). The aim of this study was to investigate the presence of the *aox* gene in bacteria other than the *Proteobacteria* in order to improve our knowledge about the phylogeny and evolution of AoxB. With this aim, an environment with elevated arsenic levels showing considerable prokaryote diversity was chosen. The *aoxB* sequences studied here were amplified from the DNA of bacteria isolated from sediments collected at this site and from the DNA extracted directly from these sediments.

The study site is located in the vicinity of the Gabe-Gottes mine in the Sainte-Marie-aux-Mines valley (France) (48°12′51″N, 007°09′34″E) (15a). Water percolating from the mine walls accumulates in a creek, where sediments were collected in

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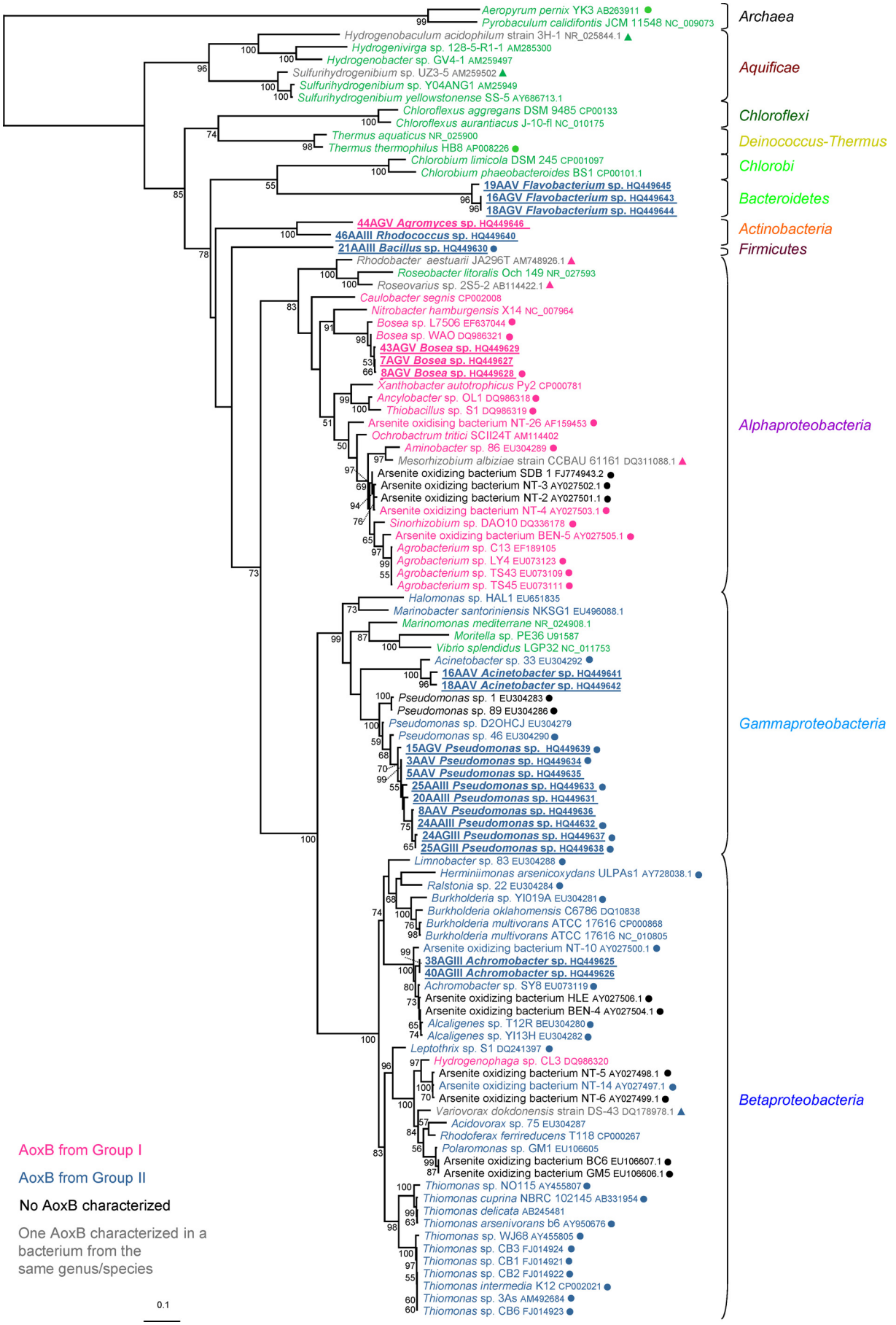
Aeropyrum pernix YK3 AB263911
Pyrobaculum calidifontis JCM 11548 NC_009073
Hydrogenobaculum acidophilum strain 3H-1 NR_025844.1
Hydrogenivirga sp. 128-5-R1-1 AM285300
Hydrogenobacter sp. GV4-1 AM259497
Sulfurihydrogenibium sp. UZ3-5 AM259502
Sulfurihydrogenibium sp. Y04ANG1 AM25949
Sulfurihydrogenibium yellowstonense SS-5 AY686713.1
Chloroflexus aggregans DSM 9485 CP00133
Chloroflexus aurantiacus J-10-fl NC_010175
Thermus aquaticus NR_025900
Thermus thermophilus HB8 AP008226
Chlorobium limicola DSM 245 CP001097
Chlorobium phaeobacteroides BS1 CP00101.1
19AAV Flavobacterium sp. HQ449645
16AGV Flavobacterium sp. HQ449643
18AGV Flavobacterium sp. HQ449644
44AGV Agromyces sp. HQ449646
46AIII Rhodococcus sp. HQ449640
21AIII Bacillus sp. HQ449630
Rhodobacter aestuarii JA296T AM748926.1
Roseobacter litoralis Och 149 NR_027593
Roseovarius sp. 2S5-2 AB114422.1
Caulobacter segnis CP002008
Nitrobacter hamburgensis X14 NC_007964
Bosea sp. L7506 EF637044
Bosea sp. WAO DQ986321
43AGV Bosea sp. HQ449629
7AGV Bosea sp. HQ449627
8AGV Bosea sp. HQ449628
Xanthobacter autotrophicus Py2 CP000781
Ancylobacter sp. OL1 DQ986318
Thiobacillus sp. S1 DQ986319
Arsenite oxidising bacterium NT-26 AF159453
Ochrobactrum tritici SCII24T AM114402
Aminobacter sp. 86 EU304289
Mesorhizobium albiziae strain CCBAU 61161 DQ311088.1
Arsenite oxidizing bacterium SDB 1 FJ774943.2
Arsenite oxidizing bacterium NT-3 AY027502.1
Arsenite oxidizing bacterium NT-2 AY027501.1
Arsenite oxidizing bacterium NT-4 AY027503.1
Sinorhizobium sp. DAO10 DQ336178
Arsenite oxidizing bacterium BEN-5 AY027505.1
Agrobacterium sp. C13 EF189105
Agrobacterium sp. LY4 EU073123
Agrobacterium sp. TS43 EU073109
Agrobacterium sp. TS45 EU073111
Halomonas sp. HAL1 EU651835
Marinobacter santoriniensis NKSG1 EU496088.1
Marinomonas mediterrane NR_024908.1
Moritella sp. PE36 U91587
Vibrio splendidus LGP32 NC_011753
Acinetobacter sp. 33 EU304292
16AAV Acinetobacter sp. HQ449641
18AAV Acinetobacter sp. HQ449642
Pseudomonas sp. 1 EU304283
Pseudomonas sp. 89 EU304286
Pseudomonas sp. D2OHCJ EU304279
Pseudomonas sp. 46 EU304290
15AGV Pseudomonas sp. HQ449639
3AAV Pseudomonas sp. HQ449634
5AAV Pseudomonas sp. HQ449635
25AAIII Pseudomonas sp. HQ449633
20AAIII Pseudomonas sp. HQ449631
8AAV Pseudomonas sp. HQ449636
24AAIII Pseudomonas sp. HQ44632
24AGIII Pseudomonas sp. HQ449637
25AGIII Pseudomonas sp. HQ449638
Limnobacter sp. 83 EU304288
Herminiimonas arsenicoxydans ULPAs1 AY728038.1
Ralstonia sp. 22 EU304284
Burkholderia sp. YI019A EU304281
Burkholderia oklahomensis C6786 DQ10838
Burkholderia multivorans ATCC 17616 CP000868
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Arsenite oxidizing bacterium NT-10 AY027500.1
38AGIII Achromobacter sp. HQ449625
40AGIII Achromobacter sp. HQ449626
Achromobacter sp. SY8 EU073119
Arsenite oxidizing bacterium HLE AY027506.1
Arsenite oxidizing bacterium BEN-4 AY027504.1
Alcaligenes sp. T12R BEU304280
Alcaligenes sp. YI13H EU304282
Leptothrix sp. S1 DQ241397
Hydrogenophaga sp. CL3 DQ986320
Arsenite oxidizing bacterium NT-5 AY027498.1
Arsenite oxidizing bacterium NT-14 AY027497.1
Arsenite oxidizing bacterium NT-6 AY027499.1
Variovorax dokdonensis strain DS-43 DQ178978.1
Acidovorax sp. 75 EU304287
Rhodoferax ferrireducens T118 CP000267
Polaromonas sp. GM1 EU106605
Arsenite oxidizing bacterium BC6 EU106607.1
Arsenite oxidizing bacterium GM5 EU106606.1
Thiomonas sp. NO115 AY455807
Thiomonas cuprina NBRC 102145 AB331954
Thiomonas delicata AB245481
Thiomonas arsenivorans b6 AY950676
Thiomonas sp. WJ68 AY455805
Thiomonas sp. CB3 FJ014924
Thiomonas sp. CB1 FJ014921
Thiomonas sp. CB2 FJ014922
Thiomonas intermedia K12 CP002021
Thiomonas sp. 3As AM492684
Thiomonas sp. CB6 FJ014923
Archaea
Aquificae
Chloroflexi
Deinococcus-Thermus
Chlorobi
Bacteroidetes
Actinobacteria
Firmicutes
Alphaproteobacteria
Gammaproteobacteria
Betaproteobacteria
AoxB from Group I
AoxB from Group II
No AoxB characterized
One AoxB characterized in a bacterium from the same genus/species
0.1

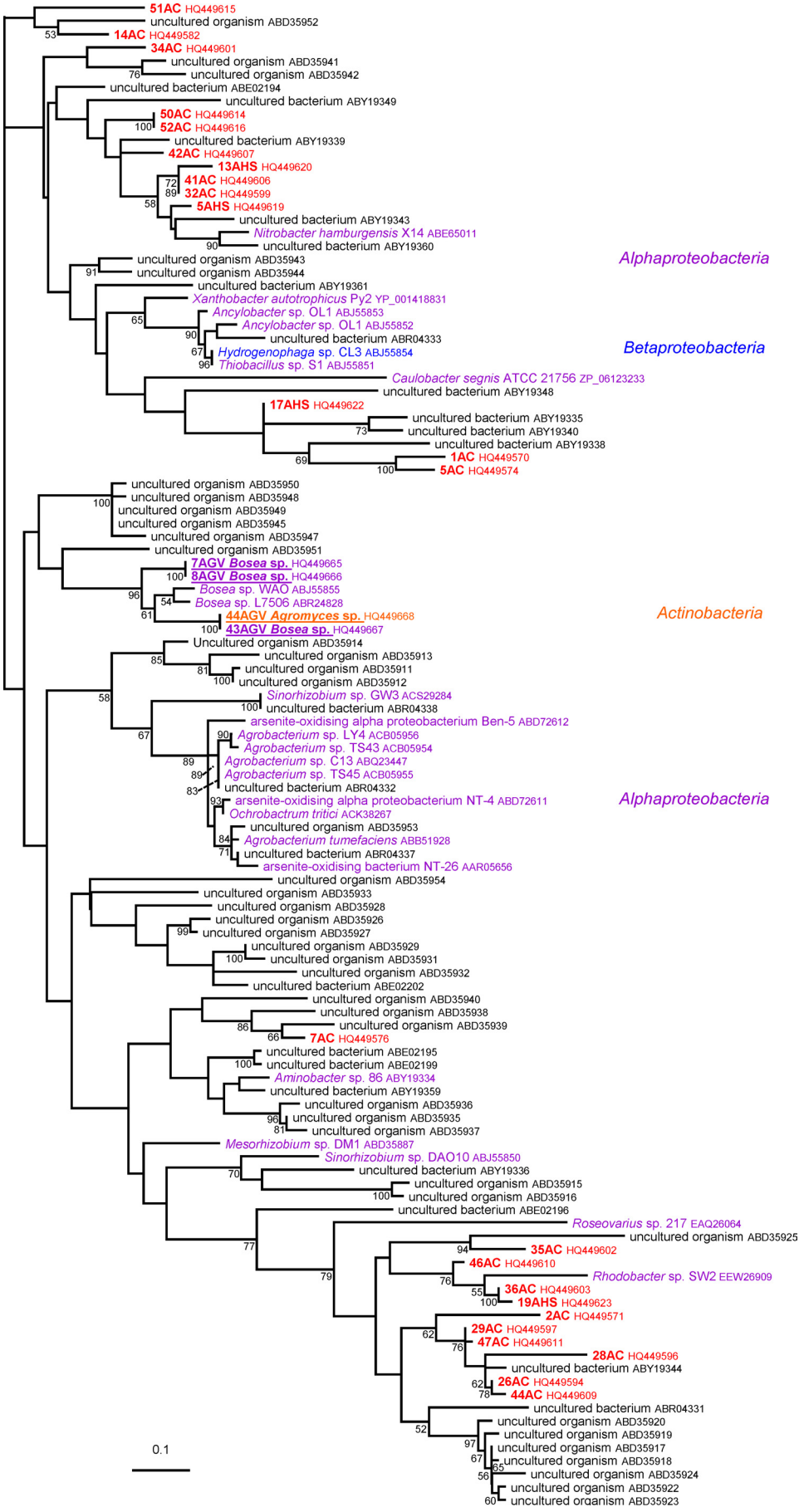
December 2007, 40 m downstream of the entrance to the mine, from a 30-m-long part of the creek. In these sediment samples, where the total arsenic level was 320 mg kg^{-1}, the As(V) species concentration was 10 times greater than the As(III) species (15a). The main arsenic species observed in sedimentary interstitial water were As(III) and As(V), in concentrations of 14.6 ($\pm$0.3) μg $liter^{-1}$ and 135 ($\pm$5) μg $liter^{-1}$, respectively. Such concentrations correspond to moderately high arsenic levels; our samples were either more severely contaminated than or similarly contaminated relative to most of previously studied sites at which arsenic oxidase-encoding genes have been detected (see Table S1 in the supplemental material) (3, 6, 8, 10, 14–16, 18, 29, 37). Moreover, the sediments at this site were previously found to show considerable bacterial diversity (15a). These characteristics made these samples suitable for investigation of the diversity of *aox* genes.

In the present study, *aoxB* genes were amplified from the total sediment DNA and from DNA extracted from several arsenic-resistant isolates. These bacterial isolates were grown for 18 days at 20°C on DR2A medium, for which the composition was as follows (per liter): 0.05 g each of yeast extract, peptone, acid hydrolysate of casein, dextrose, and soluble starch, 0.03 g each of dipotassium phosphate and sodium pyruvate, and 0.0024 g of magnesium sulfate (36) amended with either As(III) or As(V) [isolates named with the suffix III or V refer to the presence of As(III) or As(V), respectively] under aerobic or anaerobic conditions (isolates named with the prefix A refer to aerobic conditions) using the Anaerocult P system (Grosseron) (the middle letter, A or G, corresponds to the gelling agent, i.e., agar or gellan gum, respectively). Among the 220 isolates obtained and tested using BM1-2F/BM3-1R primers (see Table S2 in the supplemental material) to detect the presence of the *aoxB* gene, positive PCR amplification was obtained for 22 aerobe strains (see Table S3 in the supplemental material) and no *aoxB* amplification was observed for anaerobe isolates. 16S rRNA genes from these isolates were amplified and sequenced using primers W01/W02 (see Table S2). These 22 isolates belong to eight genera (*Achromobacter*, *Pseudomonas*, *Agromyces*, *Rhodococcus*, *Flavobacterium*, *Bosea*, *Acinetobacter*, and *Bacillus*), representing four bacterial phyla (*Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*) (Fig. 1 [underlined, bold names]; see also Table S3). To our knowledge, this is the first time *aoxB* genes have been reported to occur in *Bacillus*, *Rhodococcus*, *Agromyces*, and *Flavobacterium* spp. Positive arsenite oxidase activity was detected with the $AgNO_3$ method (35) using *H. arsenicoxydans* ULPAs1 as a positive control (39) in the case of 8 isolates, 6 of which belong to the *Pseudomonas* genus, 1 to the *Bacillus* genus, and 1 to the *Bosea* genus (Fig. 1 [underlined, bold names highlighted with circles]). The absence of activity in the other strains may be due to a lack of *aox* gene expression under the conditions tested, the lack of the small subunit, or a gene coding for a nonfunctional enzyme. Arsenite oxidation activity has been observed in previous studies in the *Pseudomonas*, *Acinetobacter*, *Achromobacter*, and *Bosea* genera (7, 28–30) (Fig. 1 [indicated with circles]).

The phylogenetic analysis of the 22 deduced AoxB amino acid sequences and their homologues present in the nonredundant protein database at the NCBI was performed. A preliminary phylogenetic analysis (data not shown) helped us to select only aerobic arsenite oxydase (AoxB, AroA, or AsoA) sequences and to exclude respiratory arsenate reductases (ArrA) or other proteins of the DMSO reductase family of molybdenum oxidoreductases, such as the new arsenite reductase recently described for *Alkalilimnicola ehrlichii*, which are clearly phylogenetically divergent from the AoxB/AsoA/AroA proteins, as previously shown (25, 40). The phylogenetic analysis of these AoxB amino acid sequences showed that most of them belonged to two major groups (see Fig. S1 in the supplemental material), as recently described (29). Group I sequences were found mainly in *Alphaproteobacteria*, whereas group II was composed mainly of betaproteobacterial sequences (Fig. 2 and 3; see also Fig. S1). This pattern suggests that these two groups probably originated from these two proteobacterial divisions. AoxB sequences belonging to none of these two groups were found in a few representatives of various bacterial divisions and a few archaeal phyla (see Fig. S1). Among the 22 AoxB characterized here, four were found to belong to group I. More specifically, three of them were amplified from isolates classified as *Bosea* sp. (*Alphaproteobacteria*) and one as *Agromyces* sp. (*Actinobacteria*) (Fig. 2). The 18 remaining AoxB sequences belong to group II. These sequences were amplified from isolates affiliated with *Betaproteobacteria* (*Achromobacter* sp.), *Gammaproteobacteria* (*Pseudomonas* sp.), *Actinobacteria* (*Rhodococcus* sp.), *Firmicutes* (*Bacillus* sp.), and *Bacteroidetes* (*Flavobacterium* sp.) (Fig. 3).

Comparisons between the 16S rRNA and AoxB phylogenies showed the existence of some striking inconsistencies between organism and gene evolutionary histories (Fig. 1, 2, and 3; see also Fig. S1 in the supplemental material). These can be ex-

FIG. 1. Maximum likelihood phylogenetic tree of the 16S rRNA sequences of our 22 isolates showing positive *aoxB* PCR amplification. This tree also includes 16S rRNA sequences deposited in public databases, corresponding to the organisms found (or close relatives) to carry the *aoxB* gene or closely related organisms. The tree was rooted in between *Archaea* and *Bacteria* domains. Phylogenetic analyses were performed on the 1,014 unambiguously aligned nucleic acid positions by using Treefinder (19). The evolutionary model GTR + Γ4 was used for this purpose, as suggested by the "propose model" tool available in Treefinder. Numbers at branches are bootstrap values obtained using the nonparametric bootstrap approach implemented in Treefinder (based on 100 replicates of the original data set). Only bootstrap values above 50% are shown. Bacteria harboring *aoxB* sequences corresponding to phylogenetic group I or group II or neither of these two groups are given in pink, dark blue, or green, respectively. The 22 bacterial isolates of this study are in boldface and underlined. All bacteria experimentally shown to be able to oxidize arsenite are labeled with a circle at the end of the name. Bacteria which were previously shown to be able to oxidize arsenite or harbor an *aoxB* gene, but for which no 16S sequence was available, were therefore not included in this tree. However, in this case, we included the closest relative of these strains for which a 16S gene was available (in gray), and the triangle color at the end of these names corresponds to the group to which the *aoxB* gene harbored by the strain belongs (pink, dark blue, and green corresponding to group I, group II, and neither of these groups, respectively). The names of bacteria which were described in the literature as able to oxidize arsenite but for which *aoxB* sequences were not available are presented in black. The scale bar represents the average number of substitutions estimated per site.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14AC HQ449582
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uncultured organism ABD35941
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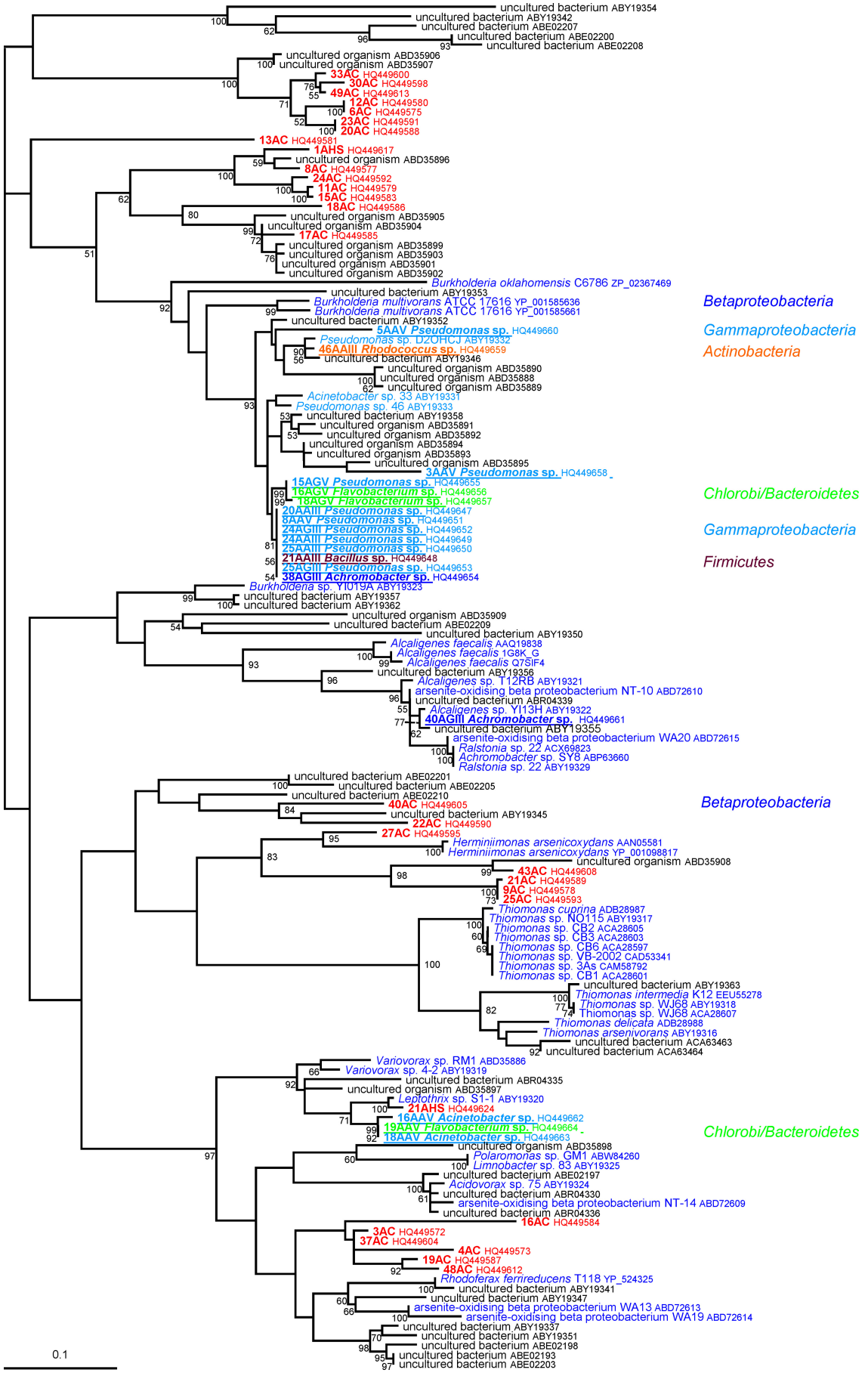
uncultured bacterium ABE02194
uncultured bacterium ABY19349
50AC HQ449614
52AC HQ449616
uncultured bacterium ABY19339
42AC HQ449607
13AHS HQ449620
41AC HQ449606
32AC HQ449599
5AHS HQ449619
uncultured bacterium ABY19343
Nitrobacter hamburgensis X14 ABE65011
uncultured bacterium ABY19360
uncultured organism ABD35943
uncultured organism ABD35944
uncultured bacterium ABY19361
Xanthobacter autotrophicus Py2 YP_001418831
Ancylobacter sp. OL1 ABJ55853
Ancylobacter sp. OL1 ABJ55852
uncultured bacterium ABR04333
Hydrogenophaga sp. CL3 ABJ55854
Thiobacillus sp. S1 ABJ55851
Caulobacter segnis ATCC 21756 ZP_06123233
uncultured bacterium ABY19348
17AHS HQ449622
uncultured bacterium ABY19335
uncultured bacterium ABY19340
uncultured bacterium ABY19338
1AC HQ449570
5AC HQ449574
Alphaproteobacteria
Betaproteobacteria
uncultured organism ABD35950
uncultured organism ABD35948
uncultured organism ABD35949
uncultured organism ABD35945
uncultured organism ABD35947
uncultured organism ABD35951
7AGV Bosea sp. HQ449665
8AGV Bosea sp. HQ449666
Bosea sp. WAO ABJ55855
Bosea sp. L7506 ABR24828
44AGV Agromyces sp. HQ449668
43AGV Bosea sp. HQ449667
Uncultured organism ABD35914
uncultured organism ABD35913
uncultured organism ABD35911
uncultured organism ABD35912
Sinorhizobium sp. GW3 ACS29284
uncultured bacterium ABR04338
arsenite-oxidising alpha proteobacterium Ben-5 ABD72612
Agrobacterium sp. LY4 ACB05956
Agrobacterium sp. TS43 ACB05954
Agrobacterium sp. C13 ABQ23447
Agrobacterium sp. TS45 ACB05955
uncultured bacterium ABR04332
arsenite-oxidising alpha proteobacterium NT-4 ABD72611
Ochrobactrum tritici ACK38267
uncultured organism ABD35953
Agrobacterium tumefaciens ABB51928
uncultured bacterium ABR04337
arsenite-oxidising bacterium NT-26 AAR05656
Actinobacteria
Alphaproteobacteria
uncultured organism ABD35954
uncultured organism ABD35933
uncultured organism ABD35928
uncultured organism ABD35926
uncultured organism ABD35927
uncultured organism ABD35929
uncultured organism ABD35931
uncultured organism ABD35932
uncultured bacterium ABE02202
uncultured organism ABD35940
uncultured organism ABD35938
uncultured organism ABD35939
7AC HQ449576
uncultured bacterium ABE02195
uncultured bacterium ABE02199
Aminobacter sp. 86 ABY19334
uncultured bacterium ABY19359
uncultured organism ABD35936
uncultured organism ABD35935
uncultured organism ABD35937
Mesorhizobium sp. DM1 ABD35887
Sinorhizobium sp. DAO10 ABJ55850
uncultured bacterium ABY19336
uncultured organism ABD35915
uncultured organism ABD35916
uncultured bacterium ABE02196
Roseovarius sp. 217 EAQ26064
uncultured organism ABD35925
35AC HQ449602
46AC HQ449610
Rhodobacter sp. SW2 EEW26909
36AC HQ449603
19AHS HQ449623
2AC HQ449571
29AC HQ449597
47AC HQ449611
28AC HQ449596
uncultured bacterium ABY19344
26AC HQ449594
44AC HQ449609
uncultured bacterium ABR04331
uncultured organism ABD35920
uncultured organism ABD35919
uncultured organism ABD35917
uncultured organism ABD35918
uncultured organism ABD35924
uncultured organism ABD35922
uncultured organism ABD35923
0.1

plained by four hypotheses: (i) hidden paralogies (i.e., gene duplications followed by differential gene losses), (ii) allele sorting, (iii) horizontal gene transfer (HGT), and (iv) tree reconstruction artifacts. In the case of AoxB, a few HGT events represent the most likely hypothesis to explain the presence of identical (or nearly identical) AoxB sequences in some distant lineages, such as group I AoxB sequences found in *Agromyces* and *Bosea*, two unrelated bacteria that belong respectively to *Actinobacteria* and *Alphaproteobacteria* (Fig. 2). Moreover, in addition to including sequences from *Alphaproteobacteria*, group I included one betaproteobacterial AoxB sequence (*Hydrogenophaga* sp. strain CL3) (Fig. 2), which indicates that this bacterium has very likely acquired its *aoxB* gene from *Alphaproteobacteria* via HGT. A similar process may account for the sequence observed in the *Agromyces* sp. isolated in this study (Fig. 2). In group II, some *Pseudomonas*, *Marinobacter*, *Halomonas*, and also *Firmicutes* and *Chlorobi*/*Bacteroidetes* AoxB sequences were intermixed with those from *Betaproteobacteria* (Fig. 3). This observation strongly suggests that the former acquired their *aoxB* gene from the latter by HGT. More convincingly, one of the two actinobacterial sequences characterized in this study belonged to group I, whereas the second sequence belonged to group II (Fig. 2 and 3), although the strains from which these sequences were amplified are closely related (Fig. 1). This suggests that these two *aoxB* genes were acquired by HGT from two unrelated proteobacterial donors. The great similarity observed between some AoxB sequences, such as those from *Bacillus* (isolate 21AAIII), *Flavobacterium* (isolates 16AGV and 18AGV) and *Pseudomonas*, indicates that the HGT events probably occurred quite recently (Fig. 3). HGT in prokaryotic communities has been proposed previously to explain the strong similarities observed between AoxB proteins from *Ralstonia* spp. and *Achromobacter* sp. strain SY8 (GenBank accession numbers ACX69823 and ABP63660) (20). In that previous study, it was suggested that *Ralstonia* has acquired the corresponding gene cluster by HGT from another betaproteobacterium (20). The proximity of these AoxB proteins in the AoxB phylogenetic tree (Fig. 3) strengthens this proposal to explain the presence of nearly identical AoxB sequences in these two *Betaproteobacteria*. Interestingly, the present work revealed for the first time that such HGT may have occurred between bacteria belonging to different classes or phyla.

Genomic islands (GEI) or plasmids may be involved in such transfers. This is, for instance, the case for *aoxB* genes found in *Thiomonas* (Fig. 3) that are localized in one genomic island (2). We searched for other examples of genomic islands containing *aoxB* belonging to group I (*Nitrobacter hamburgensis* X14 [*Alphaproteobacteria*]) (Fig. 2) or group II (*Thiomonas* strain 3As, *Thiomonas intermedia* K12, and *H. arsenicoxydans* [three *Betaproteobacteria*]) (Fig. 3) or outside these two groups (*Vibrio splendicus* [*Gammaproteobacteria*], *Aeropyrum pernix*, and *Pyrobaculum calidifontis* [*Archaea*], and *Thermus thermophilus* [*Thermus*/*Deinococcus*]) (see Fig. S1 in the supplemental material). With this aim, we used the RGPfinder (for regions of genomic plasticity [RGP]) tool of the Microscope platform (https://www.genoscope.cns.fr/agc/microscope/) (38). Interestingly, *aoxB* genes were present in RGP in *H. arsenicoxydans* as previously proposed (24), in *V. splendidus* and *P. calidifontis* (see Fig. S2 in the supplemental material). In addition, *aox* genes were carried on plasmids in *N. hamburgensis* X14 (plasmid 2, NC_007960) and *T. thermophilus* HB8 (pTT27, NC_006462). In the case of this microorganism, a transposase-encoding gene was found in the vicinity of the *aoxAB* genes (in green in Fig. S2 in the supplemental material). Finally, whereas the *aox* operon is present in a GEI in *Thiomonas* strain 3As (2), the orthologous genes were not found in an RGP in *Thiomonas intermedia*. In fact, the synteny between these two strains is restricted to the *aoxABD* and *ptxB-aoxRS* operons. More generally, the synteny of *aoxAB* genes is conserved among all bacteria for which a genome sequence was available (see Fig. S2). In contrast, synteny with the other *aox* genes, *aoxC* and *aoxD* or *aoxRS*, was observed only in few bacteria belonging to group II (*Thiomonas* sp. strain 3As and *H. arsenicoxydans*), whose *aoxB* genes are related (Fig. 3), and in *Xanthobacter autotrophicus*. Synteny of *aoxAB* with *aoxR* or *aoxS* was conserved in several bacteria belonging to group II or to group I. These observations suggest that the synteny conservation is not linked to the *aoxB* phylogeny observed or to a specific energy/carbon metabolism (heterotrophs versus autotrophs).

The phylogenic analyses support the hypothesis that HGTs have played a role in the widespread distribution of some *aoxB* genes in prokaryotes, meaning that the *aoxB* gene is not an efficient phylogenetic marker. Indeed, because of the discrepancies found to exist between the 16S rRNA and *aoxB* phylogenies, it is not possible to predict the taxonomic affiliation of strains based on the *aoxB* sequences alone. This precludes the use of approaches involving the direct amplification of *aoxB* in order to characterize the arsenite oxidizers from environmental samples. This is the case for the 56 *aoxB* sequences obtained in this study from the total DNA (shown in red in Fig. 2 and 3). As in the case of the sequences obtained from cultivated isolates, most of these deduced AoxB proteins belonged to group I (24 sequences) and group II (29 sequences). In contrast, only three of the characterized sequences were similar to sequences from *Chloroflexi* and *Thermus*, which branched outside these two groups (data not shown). Interestingly, most of our AoxB sequences belonging to groups I and II were found to be closely related to sequences from hitherto uncultured and/or unidentified bacteria or to constitute new subgroups (Fig. 2 and 3). This situation clearly shows that the diversity of AoxB is far from having been exhaustively investigated.

FIG. 2. Unrooted phylogenetic trees of the 86 group I AoxB sequences (152 unambiguously aligned positions). The 86 sequences were retrieved from the nonredundant database of the NCBI, deduced from *aoxB* genes from 4 isolated strains (in bold and underlined) or deduced from the 24 PCR products obtained from metagenomic DNA (in red). Phylogenetic analyses were performed using the maximum likelihood approach implemented in Treefinder (19). The LG + Γ4 model was used for this purpose, as suggested by the "propose model" tool available in Treefinder. Numbers at branches are bootstrap values determined using the nonparametric bootstrap approach implemented in Treefinder (based on 100 replicates of the original data set). The scale bar represents the average number of substitutions estimated per site.


uncultured bacterium ABY19354
uncultured bacterium ABY19342
uncultured bacterium ABE02207
uncultured bacterium ABE02200
uncultured bacterium ABE02208
uncultured organism ABD35906
uncultured organism ABD35907
33AC HQ449600
30AC HQ449598
49AC HQ449613
12AC HQ449580
6AC HQ449575
23AC HQ449591
20AC HQ449588
13AC HQ449581
1AHS HQ449617
uncultured organism ABD35896
8AC HQ449577
24AC HQ449592
11AC HQ449579
15AC HQ449583
18AC HQ449586
uncultured organism ABD35905
uncultured organism ABD35904
17AC HQ449585
uncultured organism ABD35899
uncultured organism ABD35903
uncultured organism ABD35901
uncultured organism ABD35902
Burkholderia oklahomensis C6786 ZP_02367469
uncultured bacterium ABY19353
Burkholderia multivorans ATCC 17616 YP_001585636
Burkholderia multivorans ATCC 17616 YP_001585661
uncultured bacterium ABY19352
5AAV Pseudomonas sp. HQ449660
Pseudomonas sp. DZOHCJ ABY19332
46AAIII Rhodococcus sp. HQ449659
uncultured bacterium ABY19346
uncultured organism ABD35890
uncultured organism ABD35888
uncultured organism ABD35889
Acinetobacter sp. 33 ABY19331
Pseudomonas sp. 46 ABY19333
uncultured bacterium ABY19358
uncultured organism ABD35891
uncultured organism ABD35892
uncultured organism ABD35894
uncultured organism ABD35893
uncultured organism ABD35895
3AAV Pseudomonas sp. HQ449658
15AGV Pseudomonas sp. HQ449655
16AGV Flavobacterium sp. HQ449656
18AGV Flavobacterium sp. HQ449657
20AAIII Pseudomonas sp. HQ449647
8AAV Pseudomonas sp. HQ449651
24AGIII Pseudomonas sp. HQ449652
24AAIII Pseudomonas sp. HQ449649
25AAIII Pseudomonas sp. HQ449650
21AAIII Bacillus sp. HQ449648
25AGIII Pseudomonas sp. HQ449653
38AGIII Achromobacter sp. HQ449654
Burkholderia sp. YI019A ABY19323
uncultured bacterium ABY19357
uncultured bacterium ABY19362
uncultured organism ABD35909
uncultured bacterium ABE02209
uncultured bacterium ABY19350
Alcaligenes faecalis AAQ19838
Alcaligenes faecalis 1G8K_G
Alcaligenes faecalis Q7SIF4
uncultured bacterium ABY19356
Alcaligenes sp. T12RB ABY19321
arsenite-oxidising beta proteobacterium NT-10 ABD72610
uncultured bacterium ABR04339
Alcaligenes sp. YI13H ABY19322
40AGIII Achromobacter sp. HQ449661
uncultured bacterium ABY19355
arsenite-oxidising beta proteobacterium WA20 ABD72615
Ralstonia sp. 22 ACX69823
Achromobacter sp. SY8 ABP63660
Ralstonia sp. 22 ABY19329
uncultured bacterium ABE02201
uncultured bacterium ABE02205
uncultured bacterium ABE02210
40AC HQ449605
uncultured bacterium ABY19345
22AC HQ449590
27AC HQ449595
Herminiimonas arsenicoxydans AAN05581
Herminiimonas arsenicoxydans YP_001098817
uncultured organism ABD35908
43AC HQ449608
21AC HQ449589
9AC HQ449578
25AC HQ449593
Thiomonas cuprina ADB28987
Thiomonas sp. NO115 ABY19317
Thiomonas sp. CB2 ACA28605
Thiomonas sp. CB3 ACA28603
Thiomonas sp. CB6 ACA28597
Thiomonas sp. VB-2002 CAD53341
Thiomonas sp. 3As CAM58792
Thiomonas sp. CB1 ACA28601
uncultured bacterium ABY19363
Thiomonas intermedia K12 EEU55278
Thiomonas sp. WJ68 ABY19318
Thiomonas sp. WJ68 ACA28607
Thiomonas delicata ADB28988
Thiomonas arsenivorans ABY19316
uncultured bacterium ACA63463
uncultured bacterium ACA63464
Variovorax sp. RM1 ABD35886
Variovorax sp. 4-2 ABY19319
uncultured bacterium ABR04335
uncultured organism ABD35897
Leptothrix sp. S1-1 ABY19320
21AHS HQ449624
16AAV Acinetobacter sp. HQ449662
19AAV Flavobacterium sp. HQ449664
18AAV Acinetobacter sp. HQ449663
uncultured organism ABD35898
Polaromonas sp. GM1 ABW84260
Limnobacter sp. 83 ABY19325
uncultured bacterium ABE02197
Acidovorax sp. 75 ABY19324
uncultured bacterium ABR04330
arsenite-oxidising beta proteobacterium NT-14 ABD72609
uncultured bacterium ABR04336
16AC HQ449584
3AC HQ449572
37AC HQ449604
4AC HQ449573
19AC HQ449587
48AC HQ449612
Rhodoferax ferrireducens T118 YP_524325
uncultured bacterium ABY19341
uncultured bacterium ABY19347
arsenite-oxidising beta proteobacterium WA13 ABD72613
arsenite-oxidising beta proteobacterium WA19 ABD72614
uncultured bacterium ABY19337
uncultured bacterium ABY19351
uncultured bacterium ABE02198
uncultured bacterium ABE02193
uncultured bacterium ABE02203
Betaproteobacteria
Gammaproteobacteria
Actinobacteria
Chlorobi/Bacteroidetes
Gammaproteobacteria
Firmicutes
Betaproteobacteria
Chlorobi/Bacteroidetes
0.1

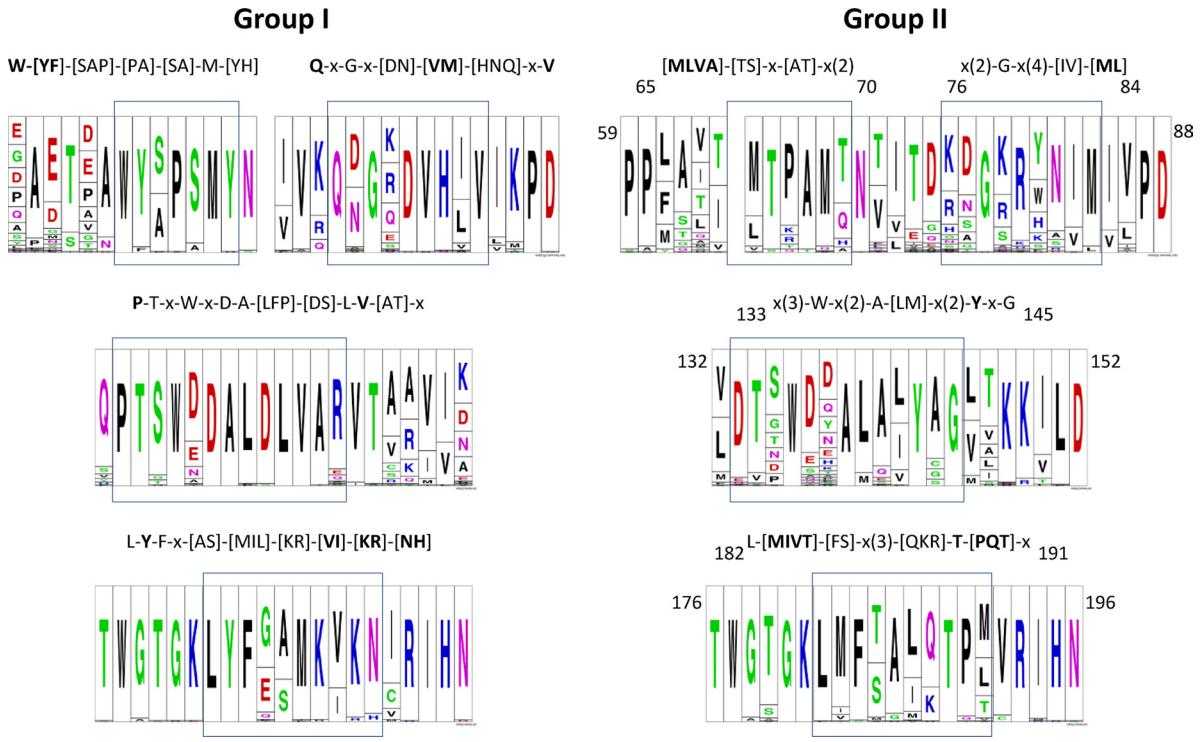


FIG. 4. Group I and group II-specific motifs deduced from AoxB sequence alignment with the WebLogo software program via the WebLogo website (http://weblogo.berkeley.edu/) (11, 33). Residues with which these motifs could be unambiguously defined are in bold. The absence of these motifs in AoxB sequences belonging to neither group I nor group II was checked visually. Amino acid numbering was based on the *Alcaligenes faecalis* protein sequence (UniProt accession number Q7SIF4).

The comparisons between 78 AoxB proteins characterized in this study, including the most divergent sequences, showed that residues present in the catalytic site [^{21}C-X_2-^{24}C-X_3-^{28}C-X_{70}-^{99}S] according to the betaproteobacterium *Alcaligenes faecalis* NCIB 8687 protein sequence numbering (13) (UniProt accession number Q7SIF4), and the hydrophilic channel near the Mo centers that was proposed to be the substrate binding site (from amino acid positions 195 to 203 according to the *A. faecalis* AoxB) were conserved in 76 of the 78 sequences studied (data not shown; see also Fig. S3 in the supplemental material). This suggested that when they are expressed, these AoxB proteins might function like the *A. faecalis* arsenite oxidase, for which a crystal structure was determined (13). Close comparisons between all the AoxB sequences belonging to groups I and II showed that four motifs differ between these two groups (Fig. 4; see also Fig. S3 in the supplemental material). These four regions extend from amino acid positions 65 to 70, 76 to 84, 133 to 145, and 182 to 191 (according to *A. faecalis* AoxB protein sequence numbering). They contain the motifs W-[YF]-[SAP]-[PA]-[SA]-M-[YH] and [MLVA]-[TS]-X-[AT]-X_2, Q-X-G-X-[DN]-[VM]-[HNQ]-X-V and X_2-G-X_4-[IV]-[ML], P-T-X-W-X-D-A-[LFP]-[DS]-L-V-[AT]-X and X_3-W-X_2-A-[LM]-X_2-Y-X-G or L-Y-F-X-[AS]-[MIL]-[KR]-[VI]-[KR]-[NH] and L-[MIVT]-[FS]-X_3-[QKR]-T-[PQT]-X in the case of group I sequences and group II sequences, respectively (according to the PROSITE pattern nomenclature) (Fig. 4). These motifs were not detected in other AoxB sequences and are therefore specific to each of these groups.

In conclusion, the results of these studies on a mildly arsenic-contaminated environment by using cell culture-dependent and -independent approaches show that the *aoxB* gene diversity is wider than described in previous studies. Comparisons between 78 AoxB bacterial sequences obtained from the Sainte-Marie-aux-Mines creek sedimentary microbial community and homologues previously deposited in public databases confirmed that most of the *aoxB* sequences detected so far belong to two subfamilies (groups I and II) harboring specific signatures. It is worth noting that the pattern of *aox* gene evolution was not strictly correlated with organism evolution and that AoxB was not found to be a suitable phylogenetic marker for studying the microbial diversity of arsenic-contaminated environments.

FIG. 3. Unrooted phylogenetic trees of the 97 group II AoxB sequences (260 unambiguously aligned positions). The 97 sequences were retrieved from the nonredundant database of the NCBI, deduced from 18 isolated strains (in bold and underlined) or deduced from the 29 PCR products obtained from metagenomic DNA (in red). Phylogenetic analyses were performed using the maximum likelihood approach implemented in Treefinder (19). The LG +Γ4 model was used for this purpose, as suggested by the "propose model" tool available in Treefinder. Numbers at branches are bootstrap values determined using the nonparametric bootstrap approach implemented in Treefinder (based on 100 replicates of the original data set). The scale bar represents the average number of substitutions estimated per site.

Nucleotide sequence accession numbers. Sequences were submitted to the GenBank database with the following accession numbers: HQ449625 to HQ449646 for 16S rRNA genes, HQ449647 to HQ449668 for *aoxB* from isolates, and HQ449570 to HQ449624 for *aoxB* from metagenomic DNA.

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