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Population Structure and Abundance of Arsenite-Oxidizing Bacteria along an Arsenic Pollution Gradient in Waters of the Upper Isle River Basin, France▽†

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Denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR (qPCR) were successfully developed to monitor functional *aoxB* genes as markers of aerobic arsenite oxidizers. DGGE profiles showed a shift in the structure of the *aoxB*-carrying bacterial population, composed of members of the *Alpha-*, *Beta-* and *Gammaproteobacteria*, depending on arsenic (As) and E_h levels in Upper Isle River Basin waters. The highest *aoxB* gene densities were found in the most As-polluted oxic surface waters but without any significant correlation with environmental factors. Arsenite oxidizers seem to play a key role in As mobility in As-impacted waters.

Arsenic (As) occurs naturally as a local geological constituent of the soils surrounding the Upper Isle River Basin (Massif Central, France) due to natural geochemical anomalies but is also released from Au/As deposits of disused gold mines (4, 11, 12, 33). Important variations in dissolved As concentrations are found in the Isle River and depend on the hydrogeological season, with maximum values in spring and summer generally detected during low-flow conditions (12, 22), and probably on temperature-controlled microbial As(V) reduction and/or microbial dissolution of solid As carrier phases (22). Two toxic inorganic forms of As are usually detected in aquatic system: arsenite, As(III), which is found mainly under anaerobic conditions and is more mobile than arsenate, As(V), which typically occurs under aerobic conditions and tends to associate with oxyhydroxides and clay minerals (11, 34). Although bacteria are known to play a key role in speciation, mobility, and bioavailability of As in the environment, they have never been considered in previous studies of As mobility in the Isle River system. Indeed, former investigations of As cycling were focused on geochemical studies (4, 11, 12, 22, 33).

As(III)-oxidizing bacteria can contribute to a natural attenuation of As pollution by decreasing its bioavailability and can help remove As from mine wastewaters through bioprocessing (1, 2). Many As(III) oxidizers have been isolated from various environments, especially mesophilic ecosystems (3, 5, 8, 16, 25, 27, 32, 38). They belong to more than 25 genera, mainly of the *Proteobacteria* phylum (3, 32, 38), and are related to organisms unable to oxidize As(III) based on 16S rRNA phylogeny. Diverse primer sets have been successfully developed to specifically target the functional *aoxB* gene (9, 14, 17, 25, 26), encoding the large molybdenum-bearing catalytic subunit of As(III)-oxidase (EC 1.20.98.1), an enzyme of the dimethyl sulfoxide (DMSO) reductase family. Using cloning-sequencing approaches, the *aoxB* gene has proven to be a reliable molecular marker for diversity studies of the polyphyletic aerobic As(III) oxidizers in As-impacted soil and water systems (17, 25). The genetic fingerprinting denaturing gradient gel electrophoresis (DGGE) technique is one useful tool for spatial, temporal, and geographical monitoring of complex bacterial population structure (23, 24). Quantitative real-time PCR (qPCR) provides reliable measurements of functional gene abundances and monitors their fluctuations in various ecosystems (6, 15, 21, 36). However, these tools need to be developed to study the *aoxB*-carrying As(III)-oxidizing community.

In this article, we demonstrate the feasibility of using qPCR and DGGE of *aoxB* genes for monitoring the structure, diversity, and abundance of As(III)-oxidizing bacterial populations in As-impacted waters and evaluate the impact of environmental factors on this community.

Surface water and groundwater were collected from eight locations upstream and downstream of disused mines of the Upper Isle River Basin. Sampling sites and physicochemical parameters of the waters are given in the supplemental material (see Fig. S1 and Table S1). DNA was extracted from filters (2 liters of water, 0.2 μm filtered) using the FastPrep spin kit for soil (Bio101).

For qPCR of *aoxB* genes, a CODEHOP set (28) combined the *aoxB*M1-2F forward primer (5′-CCACTTCTGCATCG TGGGNTGYGGNTA-3′) (25) with a new reverse primer, *aoxB*M2-1R (5′-GGAGTTGTAGGCGGGCCKRTTRTGDA T-3′), targeting the conserved motif HNRPAYNE (25) of bacterial and archaeal AoxB protein reference sequences. Nondegenerated primers, *aoxB*M1-2F-ND (5′-CCACTTCTGCAT CGTGGGCTGTGGCTA-3′) and *aoxB*M2-1R-ND (5′-GGA GTTGTAGGCGGGCCGGTTGTGGAT-3′), were designed for PCR-DGGE by replacing degeneracy of the primer pair *aoxB*M1-2F/*aoxB*M2-1R with the bases most frequently found

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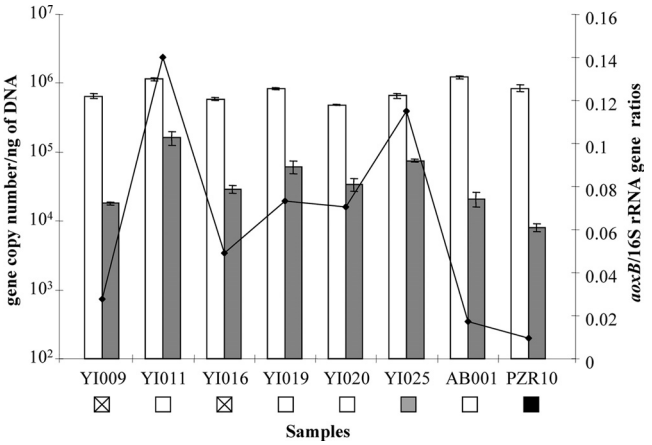

FIG. 1. Copy numbers of *aoxB* genes (gray bars) and 16S rRNA genes (white bars) in waters of the Upper Isle River Basin. Values are means ($n = 3$) ± standard errors (error bars) and are represented on a logarithmic scale. The *aoxB*/16S rRNA gene ratios (black curve) represent relative abundances of *aoxB* genes in the waters. White squares with a black cross, non-As-polluted (<10 μg · liter^{-1} As) or slightly As-polluted (15 μg · liter^{-1} As) waters; white squares, moderately (50 to 100 μg · liter^{-1} As) As(V)-polluted waters; gray squares, moderately (50 to 100 μg · liter^{-1} As) As(III)-polluted waters; black squares, highly (>1,000 μg · liter^{-1} As) As(III)-polluted water.

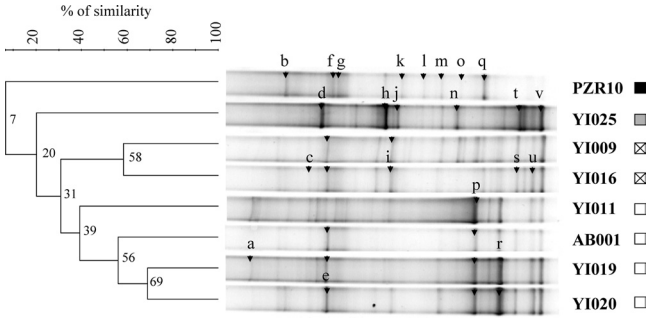

FIG. 2. UPGMA clustering analysis and DGGE profiles of *aoxB* gene fragments retrieved from waters of the Upper Isle River Basin. Arrows indicate excised and sequenced (primer *aoxB*M1-2F-ND) DGGE bands (for phylogenetic affiliation, refer to Fig. 3). As indicated in the legend to Fig. 1, the squares represent the levels of As concentration and speciation in water samples.

in aligned *aoxB* nucleotide reference sequences in order to avoid the multiple band patterns often produced when degenerated primers are used for DGGE analysis. A GC clamp (5′-CCGCCGCGCGGCGGGCGGGGCGGGGGCACGGG C-3′) was added to the 5′ end of the reverse primer *aoxB*M2-1R-ND. Both primer sets gave a specific fragment of *ca.* 550 bp from the DNAs extracted from the eight waters and the As(III)-oxidizing *Alpha*-, *Beta*-, and *Gammaproteobacteria* and *Chloroflexi* strains, previously used as positive controls (25). No amplification was obtained from non-As(III)-oxidizing bacteria harboring a homologous enzyme of the DMSO reductase family.

qPCR of *aoxB* and universal 16S rRNA genes and calculation of copy numbers were done as described in Cébron et al. (6). A linear calibration curve ($r^2 = 0.99$) was obtained over 7 orders of magnitude, ranging from 10^2 to 10^8 *aoxB* gene copies of a linearized plasmid carrying a *ca*. 1,100-bp *aoxB* gene fragment (see Fig. S2 in the supplemental material). Similar data were reported for other functional genes, such as those involved in denitrification (15) and nitrate reduction (21). No PCR inhibitor was found in the water DNAs by the detection of 2.9×10^6 ($\pm 6.5 \times 10^5$) copies of *aoxB* genes, as against 1.1×10^6 ($\pm 1.1 \times 10^5$) expected copies, in a reaction mixture containing 10^6 copies of the plasmid added to 2 ng of water DNAs. Copy numbers of *aoxB* genes retrieved from the waters ranged from 8.1×10^3 ($\pm 1.1 \times 10^3$) to 1.6×10^5 ($\pm 3.6 \times 10^4$) per ng of DNA (Fig. 1). Their relative abundances, i.e., ratios of *aoxB* gene copies relative to universal bacterial 16S rRNA gene copies, ranged from 0.01 to 0.14 (Fig. 1). No significant relationship with environmental factors was observed, which may be due to the small number of studied samples. Nevertheless, as observed for the *aoxB* gene copy numbers, the lowest ratio (0.01) was measured in the anoxic PZR10 groundwater, which could be expected since these genes are carried by aerobic bacteria, while the highest ratios (0.14 and 0.11) were found in the most As-polluted YI011 and YI025 oxic surface waters (Fig. 1). These ratios are only estimations of the proportion of As(III) oxidizers in the overall bacterial community. Assuming that As(III) oxidizers harbored one to two *aoxB* gene copies, the copy number of *aoxB* genes obtained by qPCR is close to the number of cells. But copy numbers of 16S rRNA genes cannot be correlated to cell numbers due to their variation from 1 to 15 copies per genome (10, 20). Nevertheless, the proportions of bacteria harboring *aoxB* genes in waters of the Upper Isle River Basin are realistic compared to proportions stated by Salmassi et al. (31), where As(III)-oxidizing bacteria represented 6% to 56% of the cultivable members of a biofilm of a highly As-polluted tributary of the Owens River (Hot Creek, California).

DGGE, performed on 8% polyacrylamide gels in a 55% to 65% urea-formamide denaturant gradient, generally showed a single-band profile for each As(III)-oxidizing strain, indicating the presence of a single copy of the *aoxB* gene. Bands migrated at distinct positions in the gel (see Fig. S3 in the supplemental material), even for strains belonging to the same genus (i.e., strains affiliated with the *Alcaligenes*, *Pseudomonas*, or *Thiomonas* genus) or the same species (e.g., *Alcaligenes* sp. YI013 and T12RB [25]) based on 16S rRNA gene similarities. The DGGE profile of "*Thiomonas arsenivorans*" DSM 16361^T (3) consisted of two bands corresponding to two distinct sequences, thus showing that "*T. arsenivorans*" carried two distinct copies of the *aoxB* gene. The deduced AoxB protein sequences were highly divergent (only 53.1% identity): one was properly positioned among the *Thiomonas* spp., while the other showed 91.7% identity with *Pseudomonas* sp. 72 (25). These data suggest the presence of multiple operons in "*T. arsenivorans*," probably with different regulations and/or roles. Two almost identical copies of the *aoxB* gene have been found in *Ancylobacter* sp. OL1 (26).

Different DGGE profiles of *aoxB*-carrying bacterial populations consisting of bands with various intensities and positions were obtained from the Upper Isle River Basin water DNAs (Fig. 2). Identical profiles were obtained from independent PCR runs on the same DNAs (data not shown), showing the reproducibility of this approach. Profile analyses were per-

formed with the Quantity One software program (Bio-Rad) and the implemented unweighted pair group method using average linkages (UPGMA) clustering method (35). The most complex DGGE profile (22 visible bands) was obtained for the slightly As-polluted (15 $\mu g \cdot liter^{-1}$) YI009 surface water, whereas the highly As-polluted (>1,000 $\mu g \cdot liter^{-1}$) PZR10 groundwater exhibited the simplest DGGE profile (12 visible bands) and thus the lowest *aoxB* gene richness. The highest similarity (69%) was found between the moderately As(V)-polluted (50 to 100 $\mu g \cdot liter^{-1}$) YI019 and YI020 surface waters. A high similarity (58%) was also found between the slightly As-polluted and non-As-polluted (<10 $\mu g \cdot liter^{-1}$) YI009 and YI016 surface waters, despite the geographic distance that separated them. Here, the lowest As concentrations (15 $\mu g \cdot liter^{-1}$) may have contributed to the presence of similar As(III)-oxidizing populations. The highest divergence (only 7% similarity) occurred between the surface waters and the groundwater PZR10, which showed the highest As concentration (1,846 $\mu g \cdot liter^{-1}$). This level of As may not be the only explanation for the divergent structure, since all the As(III) oxidizers isolated up to now are capable of oxidizing the highest As concentrations (3, 5, 8, 25, 38). The low dissolved oxygen concentration (1.2 $mg \cdot liter^{-1}$) and a negative E_h (−131 mV) measured in the PZR10 groundwater could impact the structure of this aerobic *aoxB*-carrying bacterial group. Divergences (20% similarity) were also found between the DGGE profile of the YI025 surface water, corresponding to mine drainage water, and the other surface waters, and this despite similar dissolved oxygen and total As contents. Here, the low E_h (26 mV) measured in YI025 water may explain a divergent population structure. The remarkable molecular-fingerprint differences between (i) the YI025 and PZR10 *aoxB*-carrying bacteria and (ii) the others could also be due to the dominance of the As(III) form and/or their mine drainage origin. Both the YI025 and PZR10 waters have specific geochemical signatures, with the highest iron and manganese concentrations, nitrate values below the detection limit, and approximately 100 $mg \cdot liter^{-1}$ of HCO_3^- versus an average of 35 for the other waters. Changes in environmental conditions may impact the structure of bacterial populations in rivers, leading to a selection of distinct physiological and phylogenetic bacteria (19, 29, 39). Taken together, our results reveal DGGE profile similarities and differences associated with the E_h and the As pollution level, thus suggesting a link between the As concentration/speciation and the structure/diversity of the *aoxB*-carrying bacterial population.

All nucleotide sequences obtained from dominant DGGE bands were affiliated with *aoxB* sequences, confirming the specificity of the developed DGGE approach. The neighbor-joining (30) tree based on Kimura two-parameter distances (18) of deduced AoxB sequences showed a large diversity of sequences related mainly to those from the *Betaproteobacteria* (55%) but also to those from the *Alpha-*, and *Gammaproteobacteria* (Fig. 3). Some were closely related to AoxB sequences retrieved from various geographically distant areas throughout the world, showing that the related *aoxB*-carrying bacteria are ubiquitous in As-polluted environments. The exclusive detection of proteobacterial AoxB sequences has been reported for mesophilic environments (9, 17, 25), while most of the nonproteobacterial AoxB sequences (*Chloroflexi*, *Aquificae*, *Deinococcus-Thermus*, and *Crenarchaeota* phyla) came from thermophilic strains or geothermal environment surveys (7, 14, 17).

Identical AoxB sequences were retrieved at different locations of the Upper Isle River Basin. Bands e, u, and v were omnipresent in the surface water profiles. Bands u and v were affiliated to *Hydrogenophaga* WA13 AoxB sequences (81.9 to 89.4% identity), and band e was phylogenetically located in a distinct branch of an *Alphaproteobacteria*-like AoxB sequence group formed only by sequences retrieved in this study (Fig. 3). Band i, a divergent AoxB sequence, was present in all but one (YI019) of the surface waters. Specific bands were common to the profiles of moderately As(V)-polluted AB001, YI020, YI019, and YI011 surface waters. Bands p and r were always present in these waters but were not detected in As(III)-polluted waters (Y1025 and PZR10). Band p was related to AoxB sequences of *Acidovorax* sp. GW2 and sp. 75 (97.1% identity), respectively, found in As-impacted mining sediments (China) (9) and soil (France) (25), and to operational taxonomic unit (OTU) LYB3 (99% identity), retrieved from Yangebup lake sediments impacted by As-containing industrial waste (Australia) (17). Band r clustered with sequences of *Ancylobacter* sp. OL1 (82.2 to 82.7% identity), a chemoautotrophic bacterium growing even under aerobic and denitrifying conditions. Band a appeared only in the profiles of the YI019 and YI011 tributary surface waters but was not detected in AB001 and YI020 surface waters collected in the river itself, suggesting that bacteria harboring this gene cannot survive or are found only at low density under the river conditions. It was closely related to AoxB sequences of *Gammaproteobacteria*-related OTU T12RSOL13 (97.3% identity) and *Pseudomonas* sp. D2OHCJ (97.7% identity), originating from two geographically distant As-impacted industrial soils of the Meuse Basin (Belgium) (25), and to AM clones (>90% identity) retrieved from an agricultural soil treated with As(III) in a column experiment (17), which suggests that bacteria harboring this gene could have originated from soils with a high natural As background level surrounding the river tributaries. The two most intense bands of the YI025 profile were specific to that water and could represent indigenous *aoxB*-carrying organisms: band d was affiliated with AoxB sequences of *Aminobacter* sp. 86 (81% identity), isolated from a disused mine soil (France) (25), and band h was affiliated with *Hydrogenophaga* spp. (82.7 to 92.4% identity).

The developed *aoxB* gene-targeting qPCR and DGGE tools proved to be efficient for evaluating diversity pattern and abundance of aerobic As(III)-oxidizing bacteria. Considering their abundance, broad distribution, and large-scale diversity, these bacteria are likely to play key roles in As transformation and mobility in the Upper Isle River Basin. Diversity and structure changes were highlighted, with dissolved As, E_h, and oxygen levels perhaps being important variables among several possible factors influencing As(III) oxidizer structure. A large-scale study integrating contrasted situations is now needed to demonstrate the impact of various environmental factors (organic carbon, dissolved oxygen levels, metal, and nutrient concentrations) on the structure and abundance of this functional group. If relationships can be established, *aoxB* gene-targeting tools may help to predict biological As(III) oxidation potential dur-

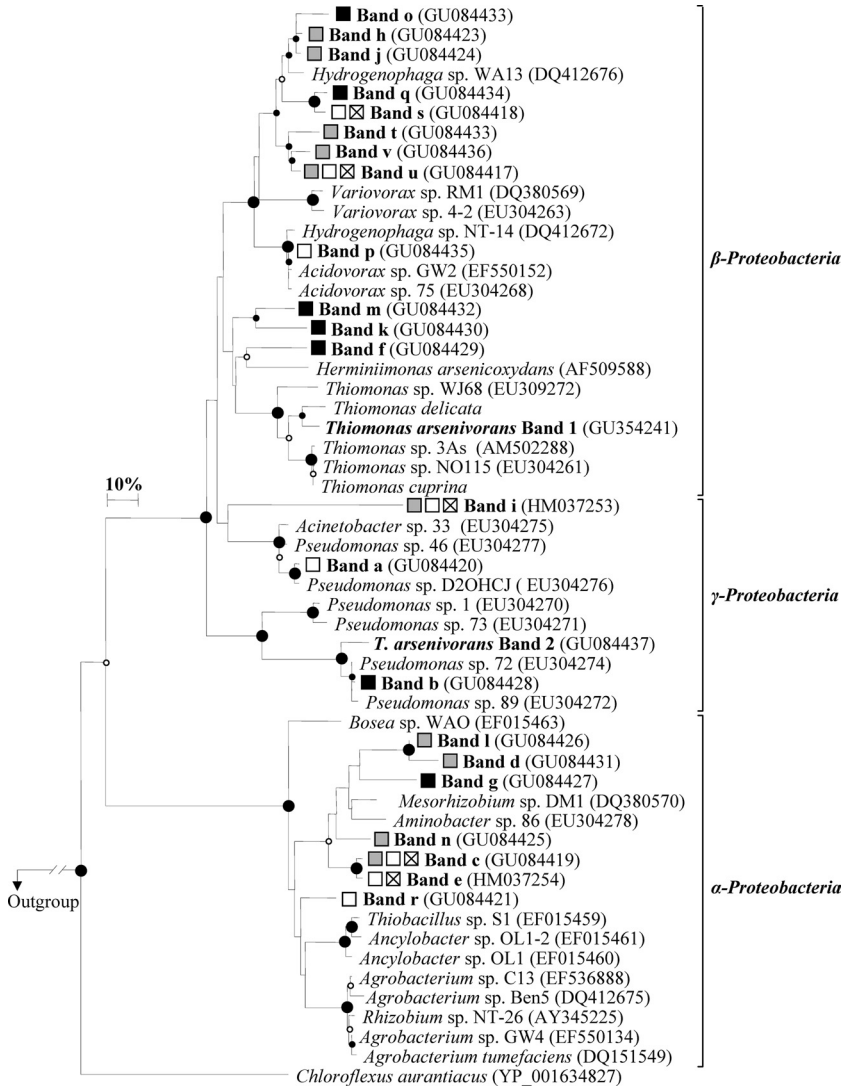

FIG. 3. Neighbor-joining phylogenetic tree of AoxB sequences (134 unambiguous amino acids) deduced from DGGE bands of the waters of the Upper Isle River Basin. The *Thermus* AoxB sequences (ABB17183 and BAD71923) were used as an outgroup. Circles at the branch nodes represent bootstrap confidence level percentages obtained from 1,000 replicates: large black circles, 95 to 100%; small black circles, 75 to 95%; small white circles, 50 to 75%. The scale bar corresponds to 10 mutations per 100 residues. As indicated in the legend to Fig. 1, the squares represent the levels of As concentration and speciation in water samples. Sequence manipulations and alignment were performed with the BioEdit software program (13). All phylogenetic programs are implemented in the Treecon software program (37).

ing bioremediation processes and serve as bioindicators of As bioavailability.

Nucleotide sequence accession numbers. *aoxB* gene sequences have been deposited under GenBank accession numbers HM037253, HM037254, GU354241, and GU084417 to GU084437 (DGGE experiments), GU133618 (*Thiomonas cuprina* DSM 5495^T), and GU133619 (*Thiomonas delicata* DSM 17897^T).

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