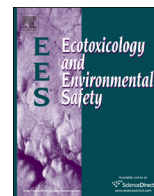




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A molecular study on bacterial resistance to arsenic-toxicity in surface and underground waters of Latium (Italy)



Domenico Davolos*, Biancamaria Pietrangeli

INAIL-Research, Certification, Verification Area, Department of Productive Plants and Human Settlements (DIPIA), Via Urbana, 167, 00184 Rome, Italy

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ABSTRACT

Latium, a region in central Italy, is known for its extensive volcanic areas that make a significant contribution to the arsenic (As) contamination of freshwater environments, even though some degree of As water pollution may be caused by human activities. The information available on indigenous As-resistant prokaryotes in aquatic environments of Latium is, however, still limited. In this study, we describe new bacteria that are resistant to arsenic toxicity and were isolated from the surface waters of Lake Vico and the Sacco River, two groundwater systems in Latium, as well as from bottled natural mineral water from the same region. The 16S rRNA gene sequence analysis for the As-resistant strains in lake and river waters points to a prevalence of β - and γ -*Proteobacteria*, while α -*Proteobacteria*, *Firmicutes* and *Bacteroidetes* are represented to a lesser extent. By contrast, solely γ -*Proteobacteria* were isolated from groundwater samples. The presence of *Actinobacteria* was documented exclusively in bottled mineral water. In addition, we conducted a DNA sequence-based study on the gene codifying *arsB*, an As(III) efflux membrane protein pump related to arsenic resistance, for all the As-resistant bacterial isolates. A phylogenetic analysis was carried out on the newly sequenced 16S rRNA genes and *arsB* in the present study as well as on an additional 16S rRNA/*arsB* dataset we obtained previously from Lake Albano, from the Tiber and from a well in Bassano Romano located in Latium (Davolos and Pietrangeli, 2011). Overall, the phylogenetic diversity of As-resistant bacteria in underground water was very limited if compared with lentic and lotic waters. Lastly, our molecular data support the hypothesis that the horizontal gene transfer of *ars* in As-containing freshwater environments is not limited to closely-related genomes, but also occurs between bacteria that are distant from an evolutionary viewpoint, thereby indicating that such genetic events may be considered a source of microbial resistance to arsenic-toxicity.

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1. Introduction

The presence of the highly toxic arsenic (As) in freshwater systems results from the mobility of this metalloid from the solid state to the aqueous state through various biogeochemical processes that occur on As-containing volcanic and sedimentary rocks (Aiuppa et al., 2006). Some As water pollution is, however, also presumably related to arsenic-containing waste derived from industrial or mining activities, as well as to the use of As compounds in agriculture and farm animals (Donati et al., 2005; Järup, 2003).

Arsenic contamination of aquatic systems constitutes a serious and widespread health problem (Ahmed Baig et al., 2010; Nordstrom, 2002). The World Health Organization and the European Union legislative frameworks on water for human

consumption have drawn up quality standards that do not allow more than 10 $\mu\text{g/L}$ of As. Indeed, it is generally accepted that a long-term intake of drinking water that contains As or exposure to water contaminated by this metalloid, even at low concentrations, may cause various diseases (e.g. Treas et al., 2012). The two most common inorganic arsenic species to which human populations might be exposed via water are arsenate As(V) and arsenite As(III). These two oxidation states of As have different toxicological effects and environmental impacts, with As(III) compounds proving considerably more harmful than As(V) compounds (Oremland and Stolz, 2003).

In recent years, attempts have been made to improve our knowledge of bacteria that display the ability to resist to As (V) and As(III) species (which enter the prokaryotic cells through membrane-associated phosphate transporters and aquaglyceroporins, respectively) given the potential of As-resistant prokaryotes to remove this toxic element from the aqueous medium. Indeed, strong evidence has emerged regarding As-resistant microbial species that can affect arsenic speciation and mobility (see Oremland and Stolz (2005)). Phylogenetically diverse bacteria

* Corresponding author. Fax: +39 697893304.

E-mail address: d.davolos@inail.it (D. Davolos)

resistant to As (both in the tetravalent and pentavalent form) have been found widely in various As-containing environments (Lara et al., 2012; Shakya et al., 2012, among others). By contrast, genes codifying for arsenic resistance have been investigated in a limited number of bacterial isolates (e.g. Sarkar et al., 2013; Sri Lakshmi Sunita et al., 2012). The best known mechanism of microbial As-resistance is related to the presence of *ars* operon located on plasmids or chromosomes. The *ars* gene system involves As (V) reduction to As(III) via ArsC, a cytoplasmic As(V) reductase, and the extrusion of As(III) from the cytoplasm by a membrane-associated arsenite efflux pump (ArsB permease or Acr3-type family; see Muller et al. (2007), and reference therein; Tsai et al., 2009).

Latium (central Italy) is a region that is characterized by extensive volcanic areas (including the Vulcini, Vico, Cimino, Sabatini and Albani Hills) that contribute greatly to As-contamination of freshwater environments (Angelone et al., 2009, among others), even if a certain amount of As water pollution may be ascribed to human activities. Consequently, people living in As-rich areas within this region may be exposed to arsenic via water and food (Cubadda et al., 2012).

Despite their importance in arsenic biogeochemistry and their potential for bioremediation strategies of this toxic metalloid from the aqueous medium, the resident As-resistant bacterial taxa in the freshwater of Latium remain largely unstudied. One exception is a recent study we carried out on As-resistant prokaryotes isolated from different freshwater sites (lake, river and well) located in this Italian region in which As levels were found not to exceed, or to be moderately above, $10 \mu\text{g L}^{-1}$. As we revealed in our previous study, some *ars* seemed to be acquired by horizontal gene transfer (HGT) events, even from phylogenetically distant taxa (Davolos and Pietrangeli, 2011). In order to more thoroughly assess the extent to which resistance to toxic arsenic and lateral gene exchanges of *ars* have occurred between freshwater bacteria, in this paper we examined DNA sequences from novel bacteria resistant to As(V) and As(III) species isolated from new freshwater sites in Latium, including the upper waters of lakes and rivers, two groundwater systems and bottled natural mineral water, where As selective pressure is believed to be present both naturally and, to a lesser extent, as a result of anthropogenic processes. Our study addresses three main issues. First, a 16S rDNA sequence analysis was conducted to gain an insight into the evolutionary

relationships of the bacteria examined. Second, for each arsenic tolerant microbial isolate, a PCR screening and, when detected (positive PCR reactions), a DNA sequence-based characterization of *arsB* were obtained to investigate the underlying mechanism of arsenic resistance. Third, on the basis of our 16S rRNA/*arsB* dataset obtained both in the present study and in a previous study recently published by us (Davolos and Pietrangeli, 2011), we investigated *arsB* mobility within As-resistant bacterial communities in various As-contaminated (approx. from 5 to $60 \text{As } \mu\text{g L}^{-1}$) freshwater sites of Latium.

2. Materials and methods

2.1. Study sites and sampling

Surface lake waters (epilimnion) were collected from Lake Vico, a small (12.93 km^2 ; max depth 48.5 m, mean depth 21.58 m) volcanic lake (510 m above sea level) located in the province of Viterbo, northern Latium, central Italy (Fig. 1), which has changed from an oligotrophic to a mesotrophic state (e.g. Dyer, 1994). Lake Vico is situated in an alkali-potassic Roman Comagmatic Quaternary age Province (see Casentini et al. (2010)), in which volcanic rocks and As-rich aquifer systems are the main source of As concentrations in water supplies (see Angelone et al. (2009) and references therein), particularly in springs and wells in the south-eastern area of the lake (Achene et al., 2010; Vivona et al., 2007). The concentration of As in the surface lake waters (pH around 8), according to recent geochemical studies, was around, or moderately above, $10 \mu\text{g/L}$. Assuming relatively aerobic conditions, As(V) oxy-anions such as H_2AsO_4^- and HASO_4^{2-} were likely to be the thermodynamically stable As species, whereas As(III) species such as H_3AsO_3 and H_2AsO_3^- were less prevalent.

Water samples were also collected from the uppermost oxic layer of the Sacco River, a small river (avg. discharge $16 \text{ m}^3/\text{s}$) situated in the south-east of Latium (Fig. 1), that is 87 km long and flows into the Liri River. Like many streams in the region, the Sacco is characterized by the intermittency of the water flow regime. The waters of this river are known to be heavily polluted by anthropic activities carried out in the vicinity. Although no detailed studies designed to investigate As occurrence in the river have, to our knowledge, been published, a recent survey reported values ranging between 3 and $8 \text{As } \mu\text{g/L}$ in three different fluvial water samples (pH=7.4–7.8; data extracted from www.latiumvolcano.it).

Two moderately oxidizing groundwater systems located in former oil refineries in Latium were also included in the study (Fig. 1). One of these systems contained $58.3 \mu\text{g/L}$ of As (pH=7.73), while the other contained $61 \mu\text{g/L}$ of As (pH=7.26; dissolved oxygen (DO)= 3.4 mg/L) (the pH and DO values were measured directly by probes in the field, while the As concentration values were obtained from samples; anonymous personal communication).

Lastly, two different bottled natural mineral waters, purchased from shops in Italy, one of which came from Latium (pH=5.8) and the other from northern Italy

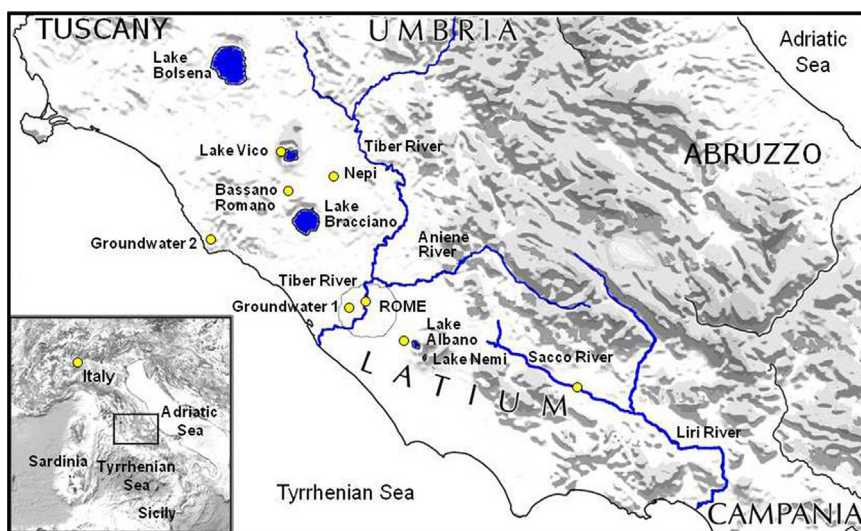


Fig. 1. Site map of the study area and the various sampling sites, including Lake Albano, the Tiber River and a well in Bassano Romano described by Davolos and Pietrangeli (2011), circled in yellow. The part of central Italy corresponding to the Latium region is zoomed in and shown in the inset. For the sake of clarity, the figure shows the sampling location in northern Italy solely in the small scale map of Italy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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