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Mercury Resistance in Bacterial Strains Isolated from Tailing Ponds in a Gold Mining Area Near El Callao (Bolívar State, Venezuela)

María Mercedes Ball,[1] Pablo Carrero,[2] David Castro,[1] Luis Andrés Yarzábal[1]

[1]Laboratorio de Microbiología Molecular y Biotecnología, Departamento de Biología, Facultad de Ciencias, Núcleo de La Hechicera, Universidad de Los Andes, Mérida 5101, Venezuela
[2]Laboratorio de Espectroscopía Molecular, Departamento de Química, Facultad de Ciencias, Universidad de Los Andes, Núcleo de La Hechicera, Mérida 5101, Venezuela

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Abstract. Bacterial resistance to mercury (Hg) was investigated in strains isolated from Hg-contaminated tailing ponds located in the gold mining area of El Callao (Bolívar State, Venezuela). High frequencies of resistance were detected to both inorganic-Hg and organomercurials among these strains. A broad range of resistance levels was observed when determining minimal inhibitory concentrations of Hg^{2+}. Some strains were able to grow in liquid medium containing 25 μM Hg^{2+}, whereas others grew at 300 μM Hg^{2+}. Of 190 Hg-resistant strains tested, 58.2% were additionally shown to be resistant to ampicillin (40 mg/L), 33.3% to chloramphenicol (30 mg/L), 24.9% to streptomycin (30 mg/L), 23.3% to tetracycline (30 mg/L), and 1.6% to kanamycin (30 mg/L). Furthermore, we found that 20% of the Hg-resistant strains were simultaneously resistant to as many as four of these antibiotics, at the concentrations tested. The presence of large plasmids in 62.9% of 53 Hg-resistant strains screened prompted us to investigate the horizontal transfer of resistance determinants. Mating experiments were performed using *Escherichia coli* and *Pseudomonas aeruginosa* as recipient strains. The results obtained confirmed that indigenous Hg-resistant bacteria colonizing the tailing ponds can effectively transfer the phenotype to potentially pathogenic species.

In the last two decades, the Orinoco River basin has seen a dramatic increase in contamination over vast areas because of amalgamation with metallic mercury (Hg) during gold extraction from river sediments and/or high-grade ores. Typically, Hg from process waste is released in small tailing ponds, creeks, and/or rivers, resulting in a significant pollution of aquatic environments. Indeed, it is well known that ionic and organic forms of Hg are considered to be among the most powerful cytotoxic and neurotoxic agents for humans and animals [22]. In response to its presence and toxicity, microorganisms evolved different mechanisms to deal with high levels of mercurial species in the environment. Indeed, bacteria can reduce ionic Hg to its elemental form through the action of a cytosolic mercuric reductase encoded by the *mer A* gene of the *mer* operon; bacteria can also cleave the C-Hg bond of many organomercurials by means of an organomercurial lyase. The latter is encoded by an additional gene (*mer B*) in some *mer* operons and confers broad-spectrum resistance to mercuric compounds [3, 4]. Finally, the elemental Hg diffuses out of the cell and volatilizes. Additionally, elemental Hg may also become the substrate for a certain group of anaerobic bacteria, which may generate the most toxic form of this metal: methyl mercury (Me-Hg) [4]. Me-Hg formed this way can accumulate through the food chain and eventually poison humans inhabiting areas located far away from gold-processing sites.

In spite of its elevated toxicity, amalgamation with Hg remains the preferred procedure in extracting gold in the southeast region of Venezuela (Bolívar State). This is particularly true in the several dozen processing centers (''molinos'') located in the surroundings of El Callao. These centers crush, grind, concentrate, and amalgamate the ore extracted by artisanal miners. It

Correspondence to: L. A. Yarzábal; *email:* yluis@ula.ve

has been estimated that Hg emission in El Callao area can reach as much as 12 tons/y [21]. In 1989, Shresta et al. [19] reported the presence of high levels of Hg in the soil and sediments of a river located in the Bolívar State, pointing out the serious threat posed to human health by the almost uncontrolled use of this metal in mining prospecting. More recently, the United Nations Industrial Development Organization determined that the levels of Hg intoxication of gold miners and millers of El Callao area are among the most serious in the world [21]. Moreover, in recent years the number of persons intoxicated with Hg increased dramatically in the Bolívar State, particularly in the city of Puerto Ordaz and its surroundings, a hundred kilometers downstream from the gold-processing centers of El Callao [1, 9, 17, 20].

Even though Hg pollution has become a threat to public health in the Orinoco River basin, almost no information concerning this issue is currently available. Furthermore, no studies have been conducted concerning the effects of Hg on microbial communities and vice versa. The aim of the present study was to investigate the level of Hg pollution in seven different tailing ponds at processing centers located in the area surrounding El Callao (Bolívar State, Venezuela), and its relationship with bacterial communities colonizing such ponds. We further report the preliminary characterization of Hg resistance in bacterial strains isolated from these ponds. Additionally, we studied the patterns of metal and antibiotic resistance of Hg-resistant strains, as well as their plasmid profiles. Finally, we investigated the horizontal spreading of resistance determinants from indigenous strains to potentially pathogenic recipient bacterial species.

Materials and Methods

Sample Collection and Hg Detection. Water samples were collected aseptically in plastic containers at seven different small tailing ponds near El Callao (Venezuela) (latitude: 7 21′ 00″, longitude: −61 49′ 00″), in October 2005. An eighth sample was collected from a small creek (Quebrada Nacupay) that collects the drainage waters coming from many tailing ponds located in its margins. The last sample was collected from a pristine-type small pond in the same area, selected to be considered as the control site (Site I). The samples were amended with a few drops of pure HCl and kept ice-cold in the dark. Mercury was detected on each sample by Atomic Absorption Spectroscopy (AAS), using a recently developed method [7].

Enumeration of Heterotrophic and Resistant Bacteria. A replica of each one of the samples, kept on ice until reaching the laboratory, was serially diluted with sterile saline and plated onto Petri dishes containing Luria-Bertani agar (LB). The plates were incubated at 30°C and colony forming units (CFU) were counted after 48-h growth. Resistant bacteria were enumerated by plating dilutions of water samples onto LB agar supplemented with 50, 100, 200, and 400 mg/L $HgCl_2$. The resistance to organic Hg was also tested by supplementing agar plates with 3, 5, 10, and 15 mg/L Me-Hg. Stock solutions of $HgCl_2$ and Me-Hg were sterilized by ultrafiltration, kept cold in the dark, and added to the medium after autoclaving and cooling. Colonies isolated from each site and showing resistance to, at least, 100 mg/L $HgCl_2$ in solid medium, were selected on the basis of different colony phenotypes for further studies. The strains were grown on LB broth, mixed with 40% glycerol at a 1:1 volume ratio, and stored at −80°C.

Minimal Inhibitory Concentrations. The determination of the minimal inhibitory concentration (MIC) of $HgCl_2$ was estimated in LB broth. For this, the selected resistant strains were reactivated on LB agar supplemented with 50 mg/L $HgCl_2$, and inoculated in LB broth. The cultures were grown overnight (o/n) and further inoculated onto 96-multiwell plates containing 200 μL of LB supplemented with 0, 25, 50, 100, 200, 300, and 400 μM Hg^{2+}. Each well was inoculated with 5 μL of the o/n grown cultures and the plates were incubated at 30°C for 24 h. The lowest concentration of Hg^{2+} that caused no visible growth was considered as the MIC of the metallic compound.

Susceptibility Tests. All strains showing resistance to, at least, 100 mg/L $HgCl_2$ in agarized medium, were tested for additional antibiotic resistance, by streaking colonies onto LB agar plates containing one of the following: ampicillin (40 mg/L), streptomycin (30 mg/L), kanamycin (30 mg/L), chloramphenicol (30 mg/L) and tetracycline (30 mg/L).

Mercury Volatilization Assay. The ability to catalyze the volatilization of Hg was tested for each one of the Hg-resistant strains by using the nonradioactive X-ray film method described by Nakamura and Nakahara [13].

Plasmid Isolation and Analysis. All selected Hg-resistant strains were screened for the presence of large (potentially conjugative) plasmids. Mini preps were made following the protocol described by Kotchoni et al. [11], using o/n cultures grown in LB broth containing 50 mg/L $HgCl_2$. Plasmid preparations were subjected to electrophoresis in 1% agarose gels at 5 V/cm. The plasmid bands were visualized on an ultra violet (UV) box after following ethidium bromide staining of the gels.

Conjugation Experiments. The capacity of some Hg-resistant strains, isolated from sites A, B, C, D, E, and G, to transfer the metal-resistance to Hg-sensitive recipient strains was tested as follows: Hg-resistant strains harboring large plasmids (>30 kbp) (see above) were grown overnight in LB containing 50 mg/L $HgCl_2$. The next morning, cultures were diluted 1:10 in fresh LB and incubated for a further 8 h. Four different cultures, corresponding to strains (donors) coming from the same pond, were mixed in equal proportions (v/v). Each one of these suspensions was immediately mixed in a 1:1 ratio (vol/vol) with a recipient strain of (a) *Escherichia coli* (derivative of *E. coli* PA601), or (b) *Pseudomonas aeruginosa* (PAO38) [10], both exhibiting resistance to 100 mg/L rifampicin (Rif). Thereafter, 10-μL aliquots of each conjugation mixture were dropped on top of LB agar and incubated for 16 h at 30°C. The mixed growth was scraped off the surface of the agar, suspended in 500 μL LB, and plated on selective agar containing 100 mg/L Hg and 100 μg/mL Rif. The plates were incubated for 24–48 h. Putative transconjugants were isolated on selective plates and tested for antibiotic resistance.

Results

A total of seven tailing ponds, located at seven distinct processing centers (“molinos”) were sampled in the

Table 1. Characteristics of the water samples at each location

Site name	Total heterotrophs CFU/mL	pH	Mercury concentration (μg/L)
A) Mina La Caratal	5.6×10^3	8	4.45 ± 0.12
B) Inversiones Oshin	6.4×10^4	8	62.58 ± 3.13
C) Molinos Farremon	5.7×10^3	8	17.19 ± 0.86
D) Molino J. Herrera	2×10^3	8	118.22 ± 5.91
E) Molino R. Farrera	4.9×10^3	8	24.5 ± 1.23
F) Molino Esquina Caliente	6.2×10^3	8	1.78 ± 0.09
G) Molino La Vanguardia	1.7×10^3	8	4.36 ± 0.22
H) Nacupay Creek	6.7×10^2	8	0.14 ± 0.01
I) Natural pond Nacupay	4.8×10^3	8	1.43 ± 0.07

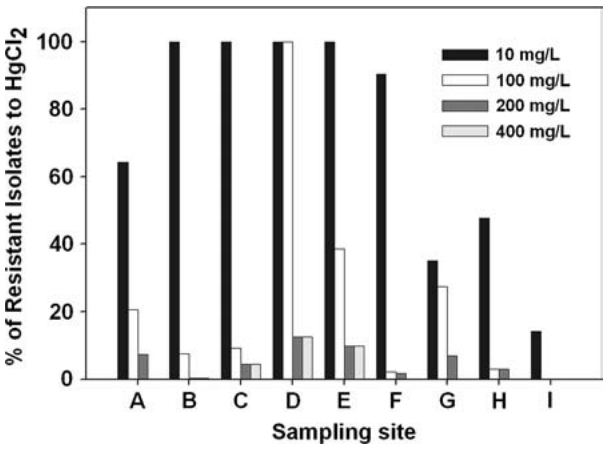

Fig. 1. Mercury resistance among bacterial strains isolated from different locations. The proportion of strains resistant to 10, 100, 200, and 400 mg/L $HgCl_2$ in each site is shown.

area surrounding El Callao during October 2005. The concentration of total soluble Hg in the water samples collected at these sites ranged from 1.78 μg/L to 118.22 μg/L, as determined by AAS (Table 1). The total number of heterotrophic, cultivable bacteria in each one of these ponds varied between 1.7×10^3 and 6.4×10^4 CFU/mL (Table 1). As expected, the highest frequencies of Hg-resistant bacteria were recorded in the most polluted ponds (i.e., sites B and D). Strikingly, 100% of the strains isolated from the pond showing the highest Hg concentration (site D, 118.22 μg/L Hg) were resistant to, at least, 100 mg/L $HgCl_2$ (Fig. 1). On the other hand, only 14.2% of the strains isolated at the control site (Site I) were resistant to 10 mg/L Hg. Surprisingly, water samples collected at this site showed a Hg concentration above the usual levels of unpolluted waters (<0.2 μg/L in areas free of contamination).

It is well known that resistance values obtained by growing strains in Hg-supplemented agarized media may cause an underestimation of this parameter. Thus, we calculated the MIC of Hg^{2+} in liquid medium. For this, 53 strains showing resistance to, at least, 100 mg/L in experiments using Hg-supplemented LB agar were incubated in LB broth containing $HgCl_2$ concentrations ranging from 6.8 mg/L (25 μM Hg^{2+}) to 81.5 mg/L (300 μM Hg^{2+}). The results show that the majority of the strains tested (73.58%) were able to grow in the presence of 100 μM Hg^{2+} (27.2 mg/L); on the other hand, only 1 of 53 strains tested (1.88%) was resistant to 300 μM Hg^{2+} (81.6 mg/L $HgCl_2$). Furthermore, the resistance to Me-Hg was also shown to be widely distributed among these strains; indeed, the proportion of resistance obtained when growing the same strains in the presence of 3, 5, 10, and 15 mg/L Me-Hg were 71.15%, 59.61%, 48.08%, and 30.77%, respectively. Finally, 52 of 53 strains (98.1%) were able to mediate Ag-reduction of an X-ray film after 60-min incubation, a reaction monitored by the formation of dark spots on the film (not shown).

Frequently, bacterial Hg-resistant determinants are genetically linked with antibiotic resistance genes [4, 8]. This was investigated by studying the antibiotic resistance exhibited by 190 Hg-resistant strains towards fixed concentrations of five antibiotics. The results obtained when testing these strains are shown in Fig. 2A. Ampicillin- and chloramphenicol-resistances were the most frequent among bacteria colonizing different ponds, showing global proportions of 58.2% and 33.3%, respectively (Fig. 2B). Furthermore, the results show that 20% of the Hg-resistant strains were simultaneously resistant to at least four different antibiotics (Fig. 2C).

To test the possible transfer of Hg resistance genes by conjugation, all strains resistant to 100 mg/L $HgCl_2$ (n = 53) were screened for the presence of large plasmids and further tested for horizontal transfer of Hg-resistance determinants in mating experiments. The results obtained confirm that 75.9% of the strains contained at least one plasmid; of these, 62.9% harbor plasmid bands with apparent sizes >30 kbp (not shown). When performing mating experiments, we detected Hg^RRif^R transconjugants in five of six mating experiments with *E. coli* PA601 (Rif^R) as the recipient strain, and two of six mating experiments, when using *P. aeruginosa* PAO38 (Rif^R). Antibiotic resistance was also detected in transconjugants (not shown).

Discussion

No reports have been published to date concerning both the extent of Hg contamination and its effects on the microbial communities colonizing fresh waters in the

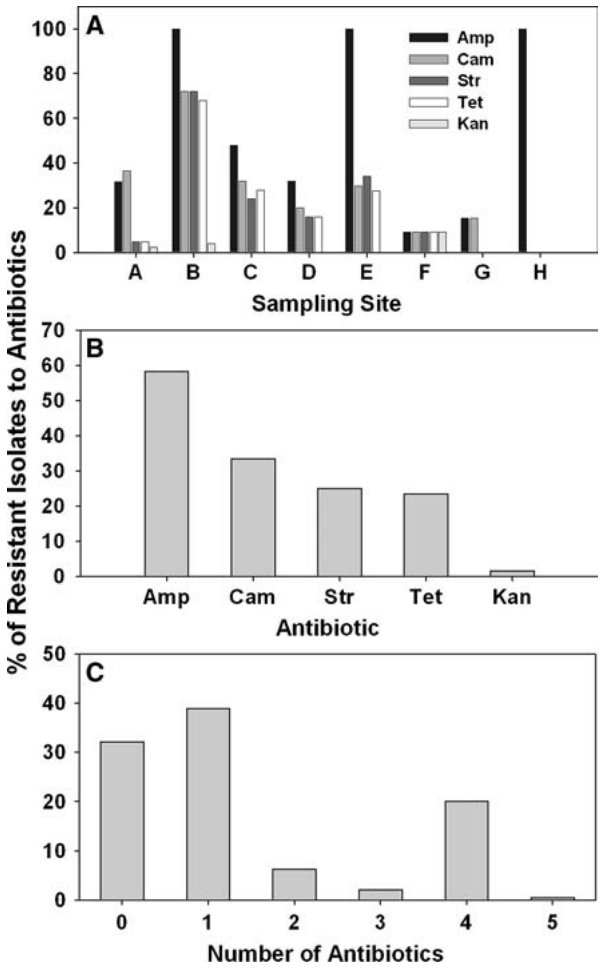

Fig. 2. **A** Levels of antibiotic resistance among Hg-resistant strains isolated from different ponds. The proportion of strains resistant to ampicillin, chloramphenicol, streptomycin, tetracycline, and kanamycin is shown. **B** Global levels of antibiotic resistance among Hg-resistant strains isolated from seven tailing ponds (n = 190). The proportion of strains resistant to ampicillin, chloramphenicol, streptomycin, tetracycline, and kanamycin is shown. **C** Resistance to antibiotics among Hg-resistant strains. The proportion of strains resistant to 0, 1, 2, 3, 4, and 5 antibiotics simultaneously is shown.

Orinoco River basin (southern region of Venezuela), even though this region has been heavily polluted for decades as a result of mining prospecting and exploitation. Here we show that the levels of soluble Hg in the waters of tailing ponds, located at several gold-processing centers in the surroundings of El Callao, are significantly greater (i.e., between 10- and >600-fold higher) than those observed in uncontaminated freshwaters, which as typically less than 0.2 μg/L [14]. Because Hg enters these aquatic systems in a metallic form, these results reflect the chemical and biochemical modifications of the metal driven by abiotic and biotic factors. Indeed, the oxidation of metallic Hg in aquatic systems gives rise to the ionic form, Hg^{2+}, which in turn can be methylated by a certain group of bacteria producing methyl Hg [4], the most toxic form of the metal. Highly contaminated waters may then reach small streams and rivers throughout surface flow, groundwater connectivity, and/or direct discharge, resulting in a serious pollution of major water bodies. In a recent report, one of us (P.C.) detected up to 34.8 μg/L ionic Hg and 22.4 μg/L Me-Hg in water samples collected in rivers of El Callao region [7]. Me-Hg, a highly liposoluble compound, may accumulate in living beings and translocate through the food chain. Interestingly, the levels of Hg in the meat of fish species often consumed by the population inhabiting the same region have been shown to be significantly greater than the normal levels [14, 16], therefore posing serious threats to human health.

Contrary to our expectations, the results obtained while studying the waters coming from a small creek (Quebrada Nacupay, site H) were particularly intriguing: not only was the Hg concentration significantly low (0.14 μg/L), but also the frequency of Hg-resistant strains was below the ones observed in the rest of the sites included in the present study (Fig. 1). However this creek is frequently loaded with drainage waters proceeding from the several dozen tailing ponds excavated near its margins. It can be argued, however, that the constant flow of nonpolluted water and the elevated discharge from frequent rainfall events would act as natural attenuation factors.

We were also surprised by the elevated concentration of soluble Hg detected in a pristine-type, natural pond located more than 500 m away from the closest tailing pond, which was selected by our group to represent the control site (Table 1, Site I). The Hg concentration at this site was shown to be sevenfold greater than the reported levels of uncontaminated waters in the same region [14]. However, there are more than 80 processing centers in a small area near El Callao and we cannot exclude the possibility of cross-contamination of this pond via groundwater and/or surface flow, particularly as a result of periodical rainfall during the rainy season. Nevertheless, this result clearly indicates that more attention should be given to this issue in future studies, because the extent of Hg contamination may be more dramatic than expected.

A second objective of this study was to determine the impact of Hg contamination on the bacterial communities colonizing the tailing ponds in El Callao, particularly on the selection of Hg-resistant strains. Indeed, it is well known that in response to the introduction of toxic elements, particularly heavy metals, the microbial communities colonizing terrestrial or aquatic ecosystems

adapt either by expressing and/or by acquiring resistance determinants by horizontal gene transfer [5]. As expected, high frequencies of Hg-resistant strains were detected in the most contaminated sites; in some cases up to 100% of the strains assayed were shown to be resistant to 100 mg/L $HgCl_2$ in solid medium. The great majority of these strains were shown to be able to mediate Hg volatilization from aqueous solutions, as a result of Hg^{2+} reduction to Hg^0. Moreover, a significant proportion of these strains were also shown to be resistant to Me-Hg. Taken together, these results strongly suggest the presence of broad-spectrum resistance determinants among Hg-resistant strains thriving in these ponds.

Genes encoding for heavy-metal resistance are very often linked to antibiotic resistance genes on the same plasmid and/or transposon [4]. In agreement with that, our results show elevated percentages of antibiotic resistance, particularly to ampicillin, among Hg-resistant strains (Fig. 2A and B). Furthermore, a significant fraction of the Hg-resistant strains (i.e., 20%) were also shown to be simultaneously resistant to as many as four different antibiotics (Fig. 2C). Multiple resistance genes are most often located in mobile genetic elements [2, 4, 15], able to spread among the bacterial community very rapidly [6, 12]. Considering that 62.96% of the Hg-resistant bacterial strains harbored large, potentially conjugative plasmids, we were concerned by the potential acquisition of multiple resistances by human pathogens through conjugation with indigenous bacteria. To test this hypothesis, we designed a mating experiment using two different bacterial species, potentially pathogenic to humans, as recipients: *P. aeruginosa* and *E. coli*. The results obtained in these experiments confirmed the possibility of horizontal transfer of Hg- as well as antibiotic-resistance genes, from indigenous bacteria to potentially harmful bacteria. Hence, our results pose serious questions regarding public health threats, i.e., the potential appearance and co-selection of pathogenic bacteria with multiple antimicrobial resistances, because the access to processing centers and their tailing ponds is almost uncontrolled. Furthermore, the gold workers as well as the people inhabiting the area-children in particular-are frequently in contact with the water of polluted ponds. Moreover, the runoff waters coming from the processing centers reaches the Yuruari River, which supplies water to the population of El Callao and nearby communities [21]. Thus, the human population is exposed not only to the intoxication with soluble forms of Hg, but to the possible infection with multiple-resistant pathogenic bacteria that may arise by horizontal transfer of resistance determinants in these ponds. This is also true for domestic animals, particularly pigs, as has been shown by Palheta and Taylor [14] in a gold mining area in the Brazilian Amazon.

Our results show that the constant exposure to anthropogenic Hg in the region of El Callao has selected for metal-resistant bacteria in tailing ponds. However, further research is needed to determine more adequately the co-selection for antibiotic-resistant strains, particularly of pathogenic species to humans and animals. It has been proposed that bacterial-mediated attenuation mechanisms may result in a decrease of Hg levels in aquatic systems [3, 18]. The results of the present study clearly show the existence of indigenous bacterial strains with such a potential in El Callao tailing ponds. We believe that the introduction of bacterial-mediated detoxification processes may contribute to reduce the impact of Hg pollution in these ponds, and we are currently conducting further investigations toward this objective.

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