Microorganisms isolated from seabirds feathers for mercury bioremediation

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Abstract: Environmental pollution caused by mercury has received increasing attention in recent years. Several studies have warned of the high rates of biomagnification in superior levels of marine food networks affecting seabirds. Although seabird feathers are reported as bioindicators of mercury, the possibility of using the microbiota associated with them for the bioremediation of this metal has not been considered. Despite the potential of the seabird feather microbiota, the cultivable microorganisms from this sample matrix have not been identified. In this study, we isolated and identified the organisms in the feathers from three types of seabirds, two species of penguins (Pygoscelis antarctica and Pygoscelis papua) and the brown skua bird (Catharacta lonnbergi) through poisoned media a final concentration of 10 mg / L Hg2+ in the culture medium for the microbial consortia. Yeast isolates belonged to the genus Debaryomyces, Meyerocynza, Papiliotrema, and Rhodotorula, and fungi genera Leiotrametes, Penicillium, Pseudogymnoascus, and Cladosporium were identified. Adult bird feathers with high mercury concentrations can serve as a matrix to isolate microorganisms capable of removing mercury.

Key words: Antarctica, bioremediation, feathers, mercury, microorganisms.

Introduction

Mercury (Hg) is among the most severe pollutants due to its accumulation in food chains, resulting in risks to human, animal, and environmental health1–4. The atmospheric transport of this metal affects the most remote and cleanest areas of the planet, such as the polar zones, reaching even higher levels of deposition than in other parts of the world5–8, thus affecting the aquatic ecosystems of Antarctica9,10.

The elemental mercury (Hg0) and ionic mercury (Hg2+) that reach Antarctica fall on sediment and water bodies, while some elemental mercury remains dissolved in the water column. Another part of mercury is transformed by microorganisms, through the biomethylation process, to a more toxic organometallic compound, the methylmercury (CH3Hg+), which will be bioaccumulated and biomagnified along the marine trophic chain11. With a trophic magnification factor (TMF) of 4 to 8 for each step of the trophic level12,13, the amount of CH3Hg+ in predatory species can be up to 100 times higher than their primary food source so that birds, among other species, are the most exposed in the marine ecosystems of Antarctica14. In addition to its high position in the food chain, the bioaccumulation of Hg in birds is favored by factors such as its wide distribution, population variety, long life cycles, and its type of diet14,15.

Although in Antarctica there is no industrial development that contributes to mercury emissions4,6,16, its proximity to the southern hemispheres, the tourism, the pollution from logistics activities of scientific stations17–20 as well as contamination of natural origin from volcanic activity10,21 contribute to the increase of Hg in predatory seabirds from different locations in this continent14,22.

Complex communities of microorganisms are found in birds’ feathers whose composition can be influenced by exposure to heavy metals23. It is well known that organisms living in contaminated or toxic conditions have developed different mechanisms to adapt to high levels of various forms of mercury present in the environment and can be used for bioremediation or mitigation of the contaminant24–26. Bioremediation is an option that uses those strategies that microorganisms have developed to deal with Hg, with exceptional advantages that include high efficiency, low cost and environmentally friendly27. Therefore, it is essential to identify the microorganisms living in high-mercury environments28–30.

Current research primarily focuses on the ability of Antarctic seabird feathers to act as bioindicators of Hg31–33 or in the isolation of microorganisms from soils and water contaminated with Hg34,35,37–39. However, the microbiota associated with bird feathers having high mercury levels has not been fully described.

Therefore, the objective of this study was to identify the cultivable microorganisms from the feathers of three Antarctic seabirds known to biomagnify mercury, including the geenwo penguins Pygoscelis papua and chinstrap Pygoscelis antarctica and the skuas brown Catharacta lonnbergi, which inhabit the surroundings of the Pedro Vicente Maldonado Scientific Station in Antarctica, as a first step that can aid further mercury bioremediation studies.
Materials and methods

Sample collection

The present investigation corresponds to an exploratory study using a purposive (judgmental) sampling method. The seabird feather samples were collected during the scientific expeditions to Antarctica carried out by the Instituto Antártico Ecuatoriano (INAE) during the summer of 2013 and 2014. The sampling was carried out in the surrounding areas of the Ecuadorian Scientific Station Pedro Vicente Maldonado (PEVIMA), located in the South Shetland archipelago of the Antarctic Peninsula. The islands evaluated were Barrientos (n = 2 sites), Dee (n = 1 site) and Greenwich (n = 2 sites). Figure 1 shows the sampling sites.

The molting feathers were collected using a non-invasive method and following the guidelines of the Antarctic Treaty (1959), in which animal welfare is preserved and the capture of living individuals is avoided (34). Therefore, fallen feathers were randomly collected in nests and colonies of three bird species: *P. antarctica* and *P. papua*, corresponding to chicks and adults with feathers lengths measured between 3-6 cm, while *C. lonnbergi*, compared to juveniles and adults with feathers lengths measured between 15-30 cm. The description of the samples is shown in table 1.

The samples were collected in Ziploc bags and delivered to the PEVIMA station laboratory, where they were rinsed with deionized water, dried at room temperature, wrapped in aluminum foil, and kept in the freezer (-20°C) until they were analyzed at the Centro de Investigaciones Biotecnológicas del Ecuador (CIBE-ESPOL) in Guayaquil, Ecuador.

Isolation of microorganisms from feathers

The feathers were subjected to an individual cleaning process in which the barbs exposed to external conditions and/or feather age, which could influence the interpretation of the results, were removed35,36. The rachis was cleaned with hypochlorite solution (30%) for 30 seconds and immersed in 99% and 70% ethanol for 30 seconds each. Finally, they were rinsed with plenty of ultrapure water. The rachis of the feathers was then ground under liquid nitrogen (LN2) in a porcelain mortar and collected in 15-ml falcon tubes to enrich microorganisms in the feathers.

For the enrichment process of the samples, 1 gr of the crushed sample was weighed and placed in a 15ml falcon tube with 9ml of liquid culture medium. The culture media used were Peptone water (AP, Oxoid, Thermo Scientific, USA), Luria Bertani (LB, Oxoid, Thermo Scientific, USA), and Potato Dextrose Broth (PDB, Oxoid, Thermo Scientific, USA) that were previously autoclaved at 121 °C for 25 minutes. Then, the samples were incubated at 10 °C for seven days with constant shaking at 110 rpm (Innova 44R, New Brunswick, USA).

Then, to determine the tolerance of microorganisms to Hg, the reference standard of inorganic mercury, Hg2+ (HACH, Germany), was added, using aseptic techniques, at a concentration 10 times higher than that reported in certain feathers of Antarctic birds31 and taking into account the toxicity threshold for adverse effects in seabird feathers of between 5-30 mg / L as reported by some authors14,37. For this reason, a final concentration of 10 mg / L Hg2+ in the culture medium was used.
medium was used for the microbial consortia and 5mg / L Hg2+ for the isolates cultured from the microbial consortia. The three poisoned media without samples were used as a blank, and each treatment was performed in duplicate.

**Isolation and molecular identification of microorganisms present in the consortia feathers**

Seven days after adding Hg to the microbial cultures of bird feather consortia, 100ul of the culture were taken and dispersed in Petri dishes previously prepared with 57.5 g / L of Potato Dextrose Agar medium (PDA, Oxoid, Thermo Scientific, USA) plus 10 mg / L Hg2+, and incubated at 10°C for isolation of cultivable microorganisms. After this, the obtained strains were separated into yeasts and fungi according to their macro and micromorphology.

The DNA extraction from isolates was carried out using a rapid fungal DNA extraction protocol according to Cenis (1992)\(^\text{38}\). Molecular identification was performed by PCR amplification and sequencing of the internal transcribed spacer regions (ITS1, 5.8S, and ITS2) using ITS1 (TCCG-TAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers, the samples that did not amplify, a PCR of nesting using primers ITS3 (TCCGTTAGGAAGACGCTTATGC) and ITS4. The master mix for both PCR was: 1X PCR buffer, 0.2 mM dNTP, 1.5 mM MgCl2, 5 u / µL Taq polymerase (ThermoFisher, USA), 0.4 µM of each primer, and the DNA concentration comprised 15-20ng / ul. The program in the thermal cycler (Eppendorf, Mastercycler Nexus GSX1-6345, Germany) for the first set of primers consisted of initial heating of 1 minute at 94 °C, followed by 30 cycles of 1 minute at 94 °C, 1 min at 55 °C, 1 min at 68 °C, and a final extension for 3 min at 68 °C. For the second set of primers, the PCR conditions were: 6 minute at 95 °C, 30 cycles from 0:30 min to 95 °C, 0:30 min to 55 °C, 0:30 min to 72 °C, followed by a final extension of 0:30 min at 72 °C. Amplification was verified by electrophoresis of a 1.5% agarose gel in 1X TAE solution (Tris base, boric acid, and 0.5M EDTA, pH 8.0), loading 5 µL of PCR product with 1 µL of loading dye (Loading dye, Promega, USA) at 100 volts for 30 minutes. The size of each DNA fragment was estimated using a 100 bp DNA marker (cat. 15628050, Invitrogen™). Gel images were analyzed using the Gel Doc XR Imager program (Bio-Rad, Philadelphia, PA).

The obtained PCR products were sequenced by Sanger at Macrogen, Inc., an external laboratory in South Korea, according to Genetic Resource Access Contract No MAE-DNB-CM-2017-0059, material transfer agreement MAE-DNB-CM-2017-0059-000-ATM-0001, and sample export authorization No 074-17-EXP-FAU-DNB/MA granted by the Ministry of the Environment of Ecuador.

The chromatograms of the DNA sequences of the different isolates were visualized and edited manually using the program Finch TV version 1.4.0 (Geospiza Inc.) and then compared with the database of the National Center for Biotechnological Information (NCBI) using BLAST. The sequences were deposited in the GenBank public database (http://www.ncbi.nlm.nih.gov/GenBank), and the isolates collection resides in the Microorganism Culture Collection of CIBE at Escuela Superior Politecnica del Litoral (http://www.wfcf.info/ccinfo/index.php/collection/by_id/1151/).

**Results and discussion**

Seabirds’ bioaccumulation mercury has been reported from different locations in the Antarctic and including in petrel feathers *Pagodroma nivea* 0.54 ± 0.18 µg g−1 dry wt\(^\text{16}\), antarctic petrel *Thalassiscoa antarctica* 2.71 ± 0.25 mg g−1 dw\(^\text{10}\), Gentoo penguins *Pygoscelis papua* 1.83 ± 0.80 µg g−1 dw\(^\text{31,40-42}\), Chinstrap penguins *Pygoscelis antarctica* 1.53 ± 0.08 ug g−1 dw\(^\text{11,40,41}\), *Pygoscelis adeliae* 0.82 to 1.40 ± 0.13 µg g−1 dw\(^\text{31,40,41}\), *Aptenodytes forsten* 0.98 ± 0.2 µg g−1 dw; skua *Catharacta macormicki* 2.91 ± 1.93 µg g−1 dw\(^\text{39}\), *Catharacta lonnbergi* 2.66 ± 2.60 µg g−1 dw\(^\text{39}\) and gull *Larus dominicanus* 246.6 ng g−1\(^\text{°}\).

In this study, isolated species of seabirds used in this study corresponding to the following yeast genera were identified: *Debaryomyces*, *Meyerozyma*, *Papiliotrema*, and *Rhodotorula* also fungi genera: *Leiotrametes*, *Penicillium*, *Pseudogymnoascus*, and *Cladosporium*. The most abundant species in the bird consortia correspond to yeasts of the genus *Debaryomyces* followed by the fungi genus *Pseu- dogymnoascus* and *Penicillus*. Table 2 and table S1. Some of the yeasts belonging to the *Rhodotorula* and family *Saccharomycetaceae* and fungi between these *Cladosporium* and *Penicillus* have been reported with efficient accumulation strategies and biovolatilization of mercury regardless of their origin. They are considered suitable for application in remedial technology\(^\text{43-45}\).

Regarding the efficiency of the culture media for metal removal, they were AP and PDB, and the fact that only yeasts and fungi have been isolated may be due to these culture media being a broad spectrum range favoring the growth of these groups\(^\text{41}\).

On the other hand, the differences in the level of resistance to metals among genera and strains depend on different growth requirements (such as temperature, pH, and nutrients), biological function\(^\text{41}\), or pressure origin\(^\text{45}\).

One of the mechanisms responsible for removing mercury in the medium from cultivable isolated may be due to the biosorption capacity of the fungal cell wall, which contains polysaccharides with reactive functional groups, amino-, carboxyl, and phosphate. Of these, it is known that the carboxyl and phosphate groups carry negative charges that allow fungal cell wall components to be highly metal ion-re- taining\(^\text{41}\). The peptide links of nitrogen and oxygen could be accompanied by the displacement of protons, depending on the pH, which also favors the removal of the metal\(^\text{49,50}\). The number of available binding sites determines metal biosorp- tion\(^\text{41}\). On the other hand, fungal mycelium secretes many extracellular enzymes and acids that decompose metals and has a huge potential for degrading contaminants\(^\text{52,53}\).

Urik et al. (2014)\(^\text{43}\) indicated fungal mercury uptake increases linearly with increased initial media mercury concentration until a threshold concentration near 8.2 mg L−1. When the amount of mercury remaining in media with higher than threshold concentration decreased by 75 % or more, sorption via mercury immobilization on the fungal cell wall and bioaccumulation in the intracellular compartments play insignificant roles in mercury resistance strategy; hence the fungal necessity to trigger other detoxification mechanisms confirm that biovolatilization is the main mechanism of de- toxification of mercury by fungal strains. Also, some authors confirm that biovolatilization is the primary mechanism of the detoxification of mercury by fungal strains\(^\text{43}\).

Other fungal strains isolated from soil samples like *Aspergillus niger* removed more than 90% and proved an excellent mercury absorber. *Aspergillus flavus* strain and *Cladosporium* can eliminate more than 90% of 10 mg L−1 of initial mercury concentration in static culture for 7 days
Table 2. Molecular identification according to ITS region of the cultivable isolates.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Closest related taxon (blast search)</th>
<th>Similarity (%)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1AP3, 1PDB3</td>
<td>Debaryomyces hansenii (MK394104.1)</td>
<td>100</td>
<td>1: P. antarctica-1, Barrientos 2013</td>
</tr>
<tr>
<td>2LB3, 2PDB3</td>
<td>Papillotrema flavescens (FN429892.1)</td>
<td>100</td>
<td>2: P. papua-1, Barrientos 2013</td>
</tr>
<tr>
<td>3LB3, 3PDB3</td>
<td>Meyerozyma guilliermondii (MH968317.1)</td>
<td>100</td>
<td>3: Lomberg-1, Barrientos 2013</td>
</tr>
<tr>
<td>4AP3</td>
<td>Popillotrema terrestris (NG 062961.1)</td>
<td>100</td>
<td>4: Lomberg-1, Barrientos 2013</td>
</tr>
<tr>
<td>5PDB4</td>
<td>Rhodotorula mucilaginosa (KY104874.1)</td>
<td>100</td>
<td>5: Lomberg-1, Barrientos 2013</td>
</tr>
<tr>
<td>6PDB4</td>
<td>Clistosporium cycadisola (NR 156279.1)</td>
<td>100</td>
<td>6: Lomberg-1, Barrientos 2013</td>
</tr>
<tr>
<td>6PDBP4</td>
<td>Pseudogymnoascus paneraeum (MH864549.1 y MH864758.1)</td>
<td>100</td>
<td>7: P. papua-1, Barrientos 2013</td>
</tr>
<tr>
<td>4APR4</td>
<td>Leiodrastus flavida (KC589130.1)</td>
<td>100</td>
<td>8: P. papua-1, Barrientos 2013</td>
</tr>
<tr>
<td>5LRB4</td>
<td>Penicillium adaezietoides (LT559804.1, KT279815.1, NR_116660.1 y AP033-003.1)</td>
<td>100</td>
<td>1: P. antarctica-1, Barrientos 2013</td>
</tr>
<tr>
<td>4LB3, 4PDB4</td>
<td>Penicillium brevicompactum (KF465776.1)</td>
<td>100</td>
<td>2: P. papua-1, Barrientos 2013</td>
</tr>
<tr>
<td>5PDB4</td>
<td>Penicillium bialosaeae (MH854996.1)</td>
<td>100</td>
<td>3: Lomberg-1, Barrientos 2013</td>
</tr>
<tr>
<td>7RAP4</td>
<td>Penicillium sp. (MW018922.1)</td>
<td>100</td>
<td>4: Lomberg-1, Barrientos 2013</td>
</tr>
<tr>
<td>8AP4, 8PDB4, 8RPDB4</td>
<td>Penicillium sp. (MW018922.1)</td>
<td>100</td>
<td>5: Lomberg-1, Barrientos 2013</td>
</tr>
</tbody>
</table>

and have been reported with biovolatilization efficiency rendering them the most suitable for application in remedial technology43,54,55.

The precise fungal mercury volatilization mechanism is not currently elucidated, but it most likely involves some intracellular or extracellular reducing factor and/or methylation agent when considering mercury volatilization in dimethyl form56,57. However, it should not be ruled out that another mechanism of action of the cultivable isolates proposed by Kelly et al. (2006) where mercury deposition as HgS in microfungi dominates at low mercury concentrations58.

Findings focused on bioremediation, comparing the use of consortia (multiple or heterogeneous systems) with pure isolates (homogeneous systems), describe the advantages of living in the community. Many factors can influence passive and active mechanisms in the removal of metals, as well as considering the relationship with the use of carbon sources and biodegradation processes59, and they can withstand higher concentrations of heavy metals. The use of these represents a closer approximation to what occurs in nature. This also allows the development of experimental model systems, which can explain the lag between the biodegradation of pure cultures in situ60. While pure isolates can resist lower concentrations of mercury compared to consortia, and few mechanisms of action on metal could be focused on, like bioaccumulation, biosorption, bioprecipiting, and/or biovolatilization46,52,59.

Conclusions

Microorganisms isolated from bird feathers that biomagnify Hg in Antarctica are yeast genera identified: Debaryomyces, Meyerozyma, Papillotrema, and Rhodotorula.
also, fungi genus: *Leiotrametes*, *Penicillium*, *Pseudogymnoascus*, and *Cladosporium*. Of these, yeasts belonging to the genus *Rhodotorula* and family *Saccharomycetaceae* and fungi between these *Cladosporium* and *Penicillium* have been reported with efficient strategies of accumulation and biovolatilization of mercury and are considered suitable for application in remedial technology according to the re-port ed bibliography.

This study opens the opportunity for bioprospecting microorganisms isolated from other matrices, not mer-cury-contaminated water and soil, but bird feathers that bio-magnified this metal. However, is a need to evaluate in time and elucidate the mechanisms used for these microorga-nisms in mercury removal and include other factors such as growth requirements between these pH and temperature, tolerance indices to major concentrations, and evaluation of their potential as adsorbents a low-cost and environmen-tally friendly. Therefore, the bioremediation of mercury from microorganisms isolated from bird feathers is still a deve-lo-ping technology.

**Supplementary Materials**

Table S1. Cultivable isolates with source and Genbank accession number.

**Author Contributions**


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**Data Availability Statement**

The partial DNA sequences obtained in this study were deposited in the GenBank database. Accession numbers for each of the isolates are given in Table S1.

**Conflicts of Interest**

The authors declare no conflict of interest.

**Bibliographic references**


