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Psychrobacter sp: perchlorate reducing bacteria, isolated from marine sediments from Margarita Bay, Antarctica.

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Abstract* - Perchlorate (ClO_4^-) is an ion that occurs naturally in Antarctica, it is considered an emerging pollutant because it is a powerful endocrine disruptor that affects the functioning of the thyroid gland and the growth and development of humans and biota. The objective of this study was to characterize salt-tolerant bacteria that reduce (ClO_4^-) from marine sediments of Bahía Margarita, Antarctica, collected in the V Colombian Scientific Expedition to Antarctica "Almirante Tono" in 2019. The methodology used included three stages: [1] Isolation of the bacteria: for which broth and LB agar modified with seawater were used [2] Morphological and biochemical characterization of the isolated strains: through Gram staining, tests for catalase, oxidase and BBL Crystal; [3] Susceptibility tests (NaCl and ClO_4^-) and ClO_4- reduction test using selective electrode. The bacterial isolates grew at 10 °C for 7 days, tolerated NaCl concentrations up to 20% v/v and (ClO_4^-) concentrations up to 10,000 mg/L; with pH variations between 6.5 to 12.0. This contaminant was reduced by the isolated strains in percentages between 18% and 41%. The morphological and biochemical characterization of the isolated strains indicated that they were related to the genus Psychrobacter. In conclusion, salt-tolerant bacteria isolated from marine sediments in Margarita Bay, Antarctica are promising resources for bioremediation of (ClO_4^-) pollution in ecosystems.

Keywords- extreme environments, halotolerant bacteria, marine sediments, polar environments, toxicity.

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I. Introduction

Perchlorate (ClO_4-) is a persistent, toxic, inorganic chlorine compound present in all ecosystems. It originates naturally and anthropogenic [1], [2]. Its anthropogenic source is used as fireworks, ammunition, flares, in agriculture with the use of fertilizers and chlorine-based pesticides (Cl) and is used as rocket fuel. It originates naturally in the atmosphere, during storms, due to the reactions of Cl with ozone (O_3) [3], in volcanic eruptions, hypersaline environments, deserts and in remote places Antarctica [4].

Due to its high solubility, (ClO_4^-) is easily transported in groundwater and surface water [5]. This compound is classified as a potent endocrine disruptor that affects iodine fixation in the thyroid gland; therefore, it affects the growth and development of living beings [4], [6], [7] in low concentrations (24.5 µg L-1 of drinking water) [3]. This contaminant accumulates in foods

such as vegetables, cereals and fruits; likewise, traces of this contaminant can be found in human feces and breast milk [5], [6].

The effects of this contaminant vary according to the species [7]. Most vascular plants have the capacity to absorb and accumulate (ClO_4^-) in their plant tissues [8], which can affect the normal development and growth of plants [4], [9] and alter seed germination and affect chlorophyll content [10]. Likewise, (ClO_4^-) has antimetamorphic effects in the earliest stages of development of amphibians and some fish [11].

In humans, the intake of contaminated food and water causes hypothyroidism [4], [12], [13], [14] generating an increase in cardiovascular diseases; neurological, reproductive and immunological [15] Metabolism, development, and reproduction in humans can be affected by high concentrations of (ClO_4^-); due to the decreased transfer of thyroid hormones from the placenta to the fetus [12]. In men, the deterioration in the genetic material results in the reduction of testicular spermatogenesis [12]. In newborns and children, it can cause deterioration of the skeletal system and the central nervous system [4], [13], [16].

Due to the ecotoxicological effects that (ClO_4^-) has on the environment and on biota; and due to its persistence, its elimination or reduction in ecosystems is imperative; that is why they use physicochemical treatments for their degradation; but these are not very efficient, non-selective, incomplete, expensive and also generate brines that usually have a more toxic load than the same contaminant [17], [18]. Due to the above, the need arises to propose the use of biological treatments, which are efficient, selective, non-invasive and economical to achieve their reduction in ecosystems [6], [19], [20], [21].

The Antarctic ecosystem is a saline environment and the natural source of (ClO_4^-) production due to its atmospheric deposition; Concentrations of this pollutant between 91 ppm and 465 ppm have been reported in the South Shetland Islands and the Antarctic Peninsula [1], [2]. Antarctica, due to its extreme conditions, provides a favorable ecosystem for the development and proliferation of native bacteria capable of reducing (ClO_4^-) [22].

One of these ideal places in Antarctica to isolate (ClO_4^-) reducing bacteria is Bahía Margarita, which is located on the Antarctic peninsula and is a place that has some Antarctic Specially Protected Areas (ASPAs); such as Avian Island (ASPAs No. 117); in which, because they are protected areas, because they have naturally occurring concentrations of (ClO_4^-) and because of their hypersaline conditions; the isolation and characterization of native bacteria that reduce this contaminant is feasible [23], because it has been shown that the ability to reduce (ClO_4^-) is greater in bacteria tolerant to salt [6], [24].

Faced with this panorama, the need arises to characterize native bacteria of Bahía Margarita, Antarctica with the potential to reduce (ClO_4^-). This work aims to provide new knowledge of the characterization of salt-tolerant bacteria from Antarctica, promising for the bioremediation of ecosystems contaminated with this contaminant [2].

II. Material and Methods

A. *Sampling*

This study was conducted on Horseshoe Island in Margarita Bay, Antarctica (68° 30' 00" S 68° 30' 00" W); Marine sediment samples (0–10 cm depth) were collected in February 2020 during the 5th Colombian Scientific Expedition to Antarctica (2019–2020) (Fig. 1). Approximately 50 g of each sample was taken with sterile spatulas, placed in sterile Falcon tubes, and frozen at -20 °C until further processing in the laboratory [2]

B. *Isolation of perchlorate-reducing bacteria*

For isolation, 5 g of each sample were suspended in 45 mL of 0.85% (w/v) saline solution and shaken for 6 h. Subsequently, 1 mL of the homogenized sample was inoculated into tubes containing 5 mL of LB medium modified with seawater (LB NaCl) [2], [6], [24], [11]. Cultures showing growth were transferred to LB NaCl agar plates and incubated at 10°C for one week. Colonies with different morphologies were selected and repeatedly isolated until a pure culture was obtained.

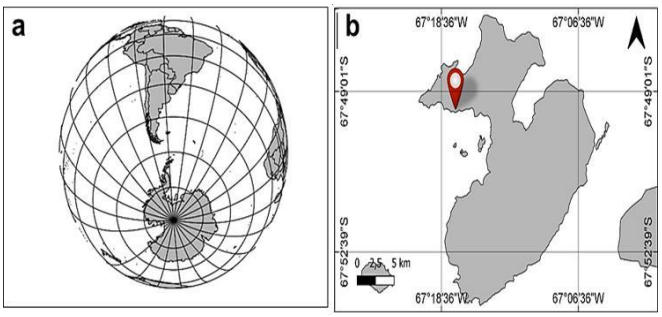

Fig 1: Geographic location of Antarctica (a) Horseshoe Island (b).

C. *Physiological characterisation of bacterial isolates*

Morphology and motility were determined using a light microscope (Nikon E100). Gram stain, catalase and peroxidase activity assays were performed according to the protocol described by [12]. The biochemical profile was determined with the BBL Crystal™ kit [2], [6], [24].

D. *16S rRNA gene sequencing and phylogenetic analysis*

Genomic DNA of the isolated bacteria was extracted using the DNAzol Kit (Invitrogen), according to the manufacturer's instructions. The 16S rRNA gene was sequenced using the universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [25] Amplification of 16S rRNA was performed following the protocol described by [25]. The polymerase chain reaction (PCR) products were sequenced using an ABI PRISM ® 3500 system (Laboratorio de secuenciación de ADN, Corporación CorpoGen Bogotá,

Colombia). The resultant 16S rRNA sequences were assembled using the sequence editor BioEdit (version 7.2.5) (Hall, 1999) and then compared with data from the Ribosomal Database Project II (RDPII), (http://wdcm.nig.ac.jp/RDP/html/index.html) [2], [6]. The evolutionary history was inferred using Phylogenetic reconstruction of the 16S rDNA gene using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates). The evolutionary distances were computed using the Kimura 2-parameter method. There were a total of 857 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [6], [26]. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains UTB-176, BBCOL-177, BBCOL-178, BBCOL-179 and BBCOL-180 are OQ457011, OQ457012, OQ457013, OQ457014 and OQ457015, respectively.

E. Susceptibility Testing

Sodium Chloride (NaCl) Susceptibility Test

All isolates were assayed for perchlorate susceptibility in LB broth in the presence of NaCl (3.5%, 5.0%, 7.5% and 20% v/v). The experiments were initiated by adding 20 µL of cell suspension (optical density (OD)=0.6) to 5 mL of LB broth [2], [6], [24].

Potassium perchlorate ($KClO_4$) susceptibility test

All isolates were assayed for perchlorate susceptibility in LB broth in the presence of perchlorate at concentrations of 700 mg/L, 5000 mg/L, and 10,000 mg/L [15]. Experiments were performed as described for the chloride susceptibility assay. After incubation for 14 days at 4 °C, the culture of each isolate was analysed on LB agar at their corresponding $KClO_4^-$ concentrations to confirm the viability of each bacterial isolate [2], [6], [24].

F. Evaluation of the reduction of potassium perchlorate

The experiments were performed using a $KClO_4^-$ concentration of 10000 mg/L in LB medium containing 3.5% NaCl. Inoculation of the isolates was as described for the chloride susceptibility assay and incubation was for 14 d at 4 °C. After incubation, the final $KClO_4^-$ concentration was measured using a Thermo Scientifc Orion-93 perchlorate electrode (Thermo Fisher Scientifc Inc., Beverly, MA, USA), according to the manufacturer's instructions. The diference between the concentrations before and after incubation was used to III. [2], [6].

III. Results

A. Morphological and biochemical identification

In this study, seven heterotrophic, aerobic cold-adapted bacteria were isolated from marine sediments sampled from Horseshoe Island, Antarctica (Table 1). Morphological and biochemical identification. In this study, five isolates from Antarctica were used. UTB-176 to UTB-180 were isolated from the sediment samples from Horseshoe Island of Margarita Bay; under aerobic heterotrophs conditions at 10°C. The isolates were gram negative coccobacillus. biochemical characteristics (for example, they did not ferment lactose). All presented positive catalase and positive oxidase activity and did not showed positive reactions for N-acetylglucosaminidase, nor nitrate reduction activity. The characteristics of these isolates are listed in table 1.

TABLE 1.
MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF ISOLATED STRAINS UTB-176 TO UTB-180

Characteristic	**UTB-176**	**UTB-177**	**UTB-178**	**UTB-179**	**UTB-180**
Molecular Identification	*Psychrobacter sp*	*Psychrobacter fozii*	*Psychrobacter fozii*	*Psychrobacter sp*	*Psychrobacter sp*
Source	Horseshoe Island	Horseshoe Island	Horseshoe Island	Horseshoe Island	Horseshoe Island
Color of colony	beige	Beige	beige	beige	beige
Morphology	cocobacilli	cocobacilli	cocobacilli	cocobacilli	cocobacilli
Halotorent	+	+	+	+	+
Psychrophilic	+	+	+	+	+
Motility	-	-	-	-	-
Gram straining	-	-	-	-	-
Endospore	-	-	-	-	-
Spore position	-	-	-	-	-
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Argenine	+	-	-	+	+

B. Phylogenetic analysis of the isolates

The results of the phylogenetic analysis showed that isolates UTB-176 to UTB-180 isolated from Horseshoe Island belong to the genus *Psychrobacter* UTB-176, UTB-179 and UTB-180 share 99% sequence identity with *Psychrobacter sp*, while UTB-177 and UTB-178 share 99% sequence identity with *Psychrobacter fozii* (Fig. 2).

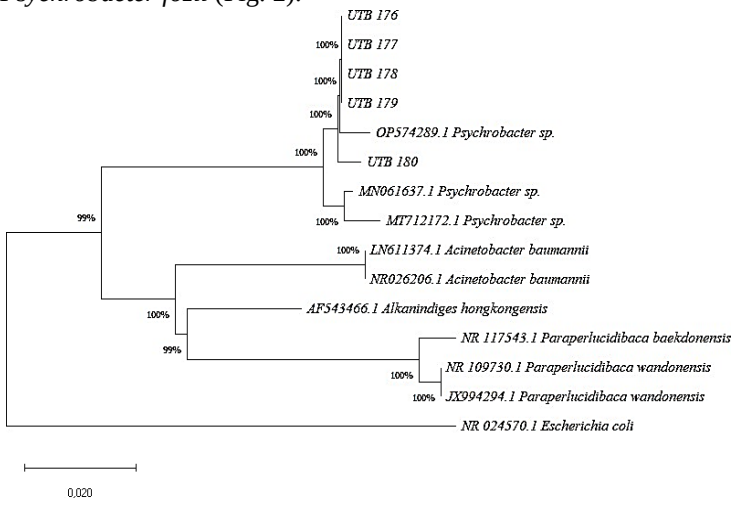

Fig 2: Phylogenetic reconstruction of the 16S rDNA gene using the Neighbor-Joining method to determine the taxonomic identity of the Antarctica *Psychrobacter sp.* strain.

C. Sodium chloride and perchlorate susceptibility assay

All the isolates grew in a culture medium with a high concentration of NaCl, reaching a tolerance of up to 20% NaCl. In the case of perchlorate, the concentration measured at Horseshoe Island ranged from 91 to 101 mg/L; *Psychrobacter sp* and *Psychrobacter fozii* can tolerate this range of perchlorate concentration in the environment. When these isolates were exposed to higher concentrations of $KClO_4^-$ formed biofilms. Therefore, these isolates showed the ability to survive at concentrations higher than those recorded in their habitats.

D. Evaluation of perchlorate reduction by the isolates

In this study, the isolated bacteria presented the biological capacity to reduce $KClO_4^-$- (Fig. 3) in concentrations of 10,000 mg/L for 7 d, the bacteria of the genus *Psychrobacter* presented reductions in perchlorate between 18 and 41%; isolated from marine sediments of Horseshoe Island, UTB-176 to UTB-180, reduced 40%, 18%, 18%, 41% and 39% of perchlorate respectively.

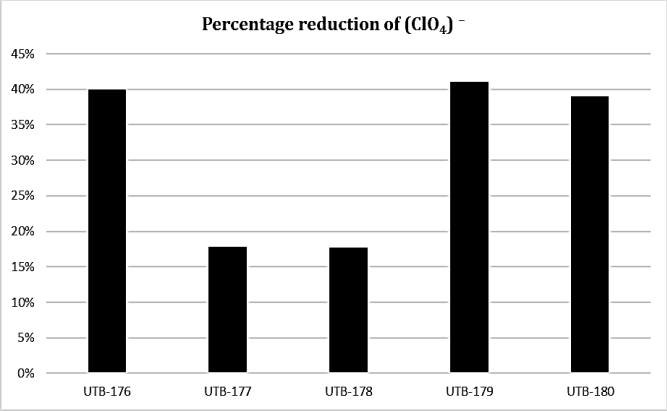

Fig: 3 Percentage reduction of $KClO_4^-$ concentration from marine sediment samples taken from Horseshoe Island Margarita Bay. Efect after 7d of contact at an optical density (OD) of 600 and optimal pH of 7.0±0.5 UTB-176, UTB-177, UTB-178, UTB-179, UTB-180.

IV. Discussion

A. Horseshoe Island

Isolates were taxonomically characterized based on 16S rRNA gene sequencing and phylogenetic analyses. The results showed that the five isolates belong to the genus *Psychrobacter*. They were characterized as aerobic, osmotolerant, and oxidase-positive bacteria and were psychrophilic or psychrotolerant. The *Psychrobacter* bacterium is found in a wide range of humid, saline and cold habitats, as well as warm and slightly salty habitats [2]. Our results are consistent with those of previous studies on microbial communities in various Antarctic habitats, where this genus was also isolated from Antarctic soils [27], [28], [29], [30], [31], [32], [33], [6]. However, the present study represents the first analysis of *Psychrobacter* species associated with marine sediments from Horseshoe Island [27], [34]. Environmental temperature and salinity gradients, along with geochemical processes on Horseshoe Island, are related to the isolation of this genus. *Psychrobacter* has previously been isolated from various environmental settings due to its diverse metabolic characteristics [27], [28], [32], [35], [36], [2].

B. Sodium chloride and perchlorate susceptibility of the bacterial isolates

Environmental factors, such as salinity, can influence bacterial growth by generating greater osmotic stress in microorganisms [2], [6], [24]. However, some bacteria can adapt to a low osmotic potential through mechanisms of salt accumulation in the cytoplasm [37]. The extreme conditions in the Antarctic continent have led to the development of salt tolerance mechanisms as a microbial survival strategy [38], [39], [40]. Therefore, the tolerance of the isolates in our experiments to a NaCl concentration of 20% can be attributed to the low availability of fresh water and the high salinity of the sampled ecosystem. Related isolates of *Psychrobacter* have been reported to be salinity tolerant; therefore, they have an adaptive advantage [2], [41], [42]. The ability of isolated species to tolerate a 20% NaCl concentration is promising for use as a biological system to reduce perchlorate in saline ecosystems essential ions [43], [6].

Perchlorate contamination of marine sediment and seawater, as well as other environmental matrices (for example, Antarctic soil), has resulted in the stimulation of bacterial growth and an increase in the number of bacteria that can resist and degrade these pollutants [1], [2], [6], [41], [44], [45], [46], [47].

C. Evaluation of perchlorate reduction by isolates

A variety of perchlorate-reducing bacterial species can reduce this contaminant; however, the percentage of reduction varies according to genus and the period of exposure to the pollutant. The rates of perchlorate reduction determined in this study were comparable to those reported by Acevedo-Barrios et al. [2], where *Psychrobacter*, *Idiomarina*, *Sporosarcina* and *Pseudomonas* genera were isolated from Caribbean hypersaline soils and reduced between 18% and 41% of 10,000 mg/L of $KClO_4^-$. In the present study, perchlorate reduction was performed by *Psychrobacter sp* and *Psychrobacter fozii.*

V. Conclusions

This study confrmed that native Antarctic bacteria isolated from sediment samples were tolerant to environmental concentrations of perchlorate and can tolerate higher concentrations of up to 10 000 mg/L. *Psychrobacter sp* and *Psychrobacter fozii*. were isolated from Horseshoe Island. Only a few studies have reported on the reduction of perchlorate by Antarctic microorganisms, our findings demonstrated that these isolated bacteria can reduce $KClO_4^-$, with reduction between 18% and 41%, thus providing a possibility for biotechnology

and the treatment of areas polluted by perchlorate. This salinity tolerance is promising for use as a biological system to reduce perchlorate in high-salinity ecosystems. It should be noted that there are no previous reports on the isolation of *P. cryohalolentis* and *P. lactis* from the Antarctic continent. Therefore, this study expands the existing knowledge regarding the presence of perchlorate-reducing bacteria in Antarctica.

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