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ISOLATION AND IDENTIFICATION OF MOLYBDENUM-REDUCING COLD-ADAPTED MARINE BACTERIA ISOLATED FROM BERNARDO O'HIGGINS RIQUELME BASE STATION, ANTARCTICA

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Abstract

Molybdenum (Mo) pollution is an emerging problem in some parts of the world. Traces of Mo can be found in the soil and snow even in the most remote part on earth, Antarctica. Bioremediation of Mo using microorganisms has been an up-and-coming alternative in cleaning up Mo from the environment. Mo reduction is a process that transforms sodium molybdate with an oxidation state of 5+ or 6+ to Mo-blue, a less toxic form of the compound. The objectives of this research are to screen, isolate and identify the best cold-adapted Mo-reducing bacterial strain isolated from marine water samples at Bernardo O'Higgins Riquelme Base Station, Antarctica. A total of 11 psychrotolerant strains were seen able to reduce Mo and further studied to determine their taxonomic position using phylogenetic analysis. Based on 16S rRNA identification, the strains were identified as *Shewanella* sp., *Staphylococcus* sp. and *Marinomonas* sp. This study suggests the potential use of the best Mo-reducing cold-adapted bacteria, strain *Marinomonas* sp. strain AQ5-A9, on the remediating of Mo in the Antarctic region.

INTRODUCTION

Heavy metals have harmful effect on the biological and ecological systems as they are nonbiodegradable but can be accumulated in living organisms, thus causing various negative effects even in relatively lower concentrations [1]. Numerous industrial activities and products are the prime examples of anthropogenic sources of uncontrollable heavy metal discharge into the environment, which give rise to heavy metal pollution [2-4]. These metals are leached out and transported through rivers and streams as dissolved species in water and may be deposited on river beds or leached deep into the soil, contaminating underground water [5-7].

Heavy metals including molybdenum (Mo) have been detected in Antarctic soil by Rowley et al. [8]. In recent years, the evidence of Mo found in Antarctic snow and lakes was further proven by Hong et al. [9] and Yang et al. [10]. Trace metals exist naturally on earth, however, anthropogenic activities in nearby countries including Chile could be among the predominant causes in snowballing traces of Mo in Antarctic as Chile is one of the largest Cu-Mo producers in the world and is relatively close to the Antarctic region [11]. Subsequently, the ever-increasing human visitions due to the establishment of research stations and flourishing tourism industry have also caused a significant rise in heavy metal pollutants in Antarctica [12, 13].

Scientists have turned towards bioremediation as a cheaper alternative to remediate heavy metal pollution as this approach is more ecologically sustainable than chemical and physical remediation. Bioremediation is described as the process of using resistant organisms such as plants and microorganisms to remove or break down contaminants into less toxic forms via various mechanisms [14-17]. Mo bioremediation using indigenous

microbe from contaminated site has been conducted in various countries such as Austria and Malaysia has shown positive results [17, 18].

There is limited number of researches carried out to study the possibility of microbes for the remediation of Mo in cold regions. Previously, *Pseudomonas sp.* strain DRY1 is the first and only report of an Antarctic bacterium isolated from soil able to reduce molybdate to Mo-blue [19]. Yet, there are no current studies done on Mo reduction in Antarctic waters using marine bacteria as the bacterial composition in soil and marine environment significantly varies as well as their growth requirement and heavy metal resistance.

The aim of this study was to evaluate the resistance and biotransformation capabilities of different species of Antarctic marine bacteria on Mo and thus determing their bioremediation potential in cold regions. The objectives of this study were to screen, isolate and identify the best cold-adapted molybdenum-reducing bacterial strain from Antarctic waters. The knowledge obtained from this study could show the best potential strain in remediating toxic heavy metals in Bernardo O'Higgins Riquelme Base Station.

MATERIALS AND METHODS

Sample collection and media preparation

Marine water samples were collected nearby the Bernardo O'Higgins Riquelme Base Station, Antarctic (63º 19′ 15.41″S, 57º 53' 58″W) by Dr. Siti Aqlima Ahmad and Nicolás Ramírez-Moreno on January, 2019. The samples were maintained in a -80ºC freezer in Eco-Remediation Technology Laboratory, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. Low phosphate media (LPM) (pH 7.0) was prepared by adding (%) sucrose (1.0), $MgSO_4.7H_20$ (0.05), $(NH_4)_2SO_4$ (0.3), NaCl (5), $Na_2MoO_4.2H_2O$ (0.242), yeast extract (0.05) and $Na_2HPO_4.2H_2O$ (0.05) while low phosphate agar also has the same composition but with an additional ingredient of bacteriological agar (20.0). The pH of the media was adjusted to 7.0±0.2. The media and sucrose were autoclaved separately at 121ºC for 15 min [19].

Screening of Mo-reducing bacterial strain

All provided samples were incubated in LPM at 10ºC on a 150 rpm orbital shaker to identify the reduction potential. The blue intensity produced was observed at 865 nm using a UV-VIS spectrophotometer [19]. The samples that showed reduction activity were streaked on low phosphate agar to obtain a single colony. The Mo-reducing bacteria with the highest intensity of Mo-blue complex were chosen for this study.

Identification and characterization of Mo-reducing isolates

The morphological characteristics of colonies were studied by streaking the isolates on nutrient agar with 5% sodium chloride. The physiological properties of selected strains were then examined with gram-staining. For 16S rRNA sequencing, genomic DNA was extracted using NucleoSpin® Microbial DNA extraction kit (Macherey-Nagel GmbH & Co. KG, Germany), followed by PCR amplification that was performed using two universal primers; forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 5′-GGTTACCTTGTTACGACTT-3′ (Vivantis, USA). PCR amplification mixture consisted of 1 µL of DNA template, 1 µL of 5 mM for each forward and reverse primer, 12.5 µL of 2X *Taq* Master Mix (Vivantis, USA) with the addition of 9.5 µL sterile deionised water for a final volume of 25 µL. PCR was performed using a T100 Thermal Cycler (Bio-rad Laboratories, USA) under the following conditions; pre-denaturation at 94°C for 3 min; 29 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min; and a final extension at 72°C for 10 min with an incubation at 4°C [14]. The PCR products were confirmed with gel electrophoresis on 1% agarose gel stained with red ViSafe gel stain. PCR purification was performed using Qiagen Purification Kit following the manufacturer's protocol prior to sequencing process. Nucleotide sequences obtained were compared with the NCBI database to retrieve the 16S rRNA sequences of closely related published species and aligned with ClustalW. Multiple alignments, distance analysis, clustering and constructing phylogenetic tree were performed using MEGA-X software. The phylogenetic trees were constructed applying a maximum likelihood algorithm of neighbour-joining method with Kimura's two-parameter model by means of 1000 replicates bootstrap analysis [20]. Nucleotide sequences were deposited in the NCBI database.

RESULTS AND DISCUSSION

Screening of Mo-reducing isolates

Microorganisms can be classified based on various characteristics. Bacteria capable of reducing Mo were isolated from 11 water samples W4, W5, W6, W13, WB3, WB8, WB10, WB11, WB12, WB13 and B1 collected from Bernardo O'Higgins Riquelme Base Station. **Figure 1** shows the blue intensity from the Mo-blue produced by all 11 isolates on low phosphate (LPM) agar. **Table 1** shows the morphological characteristics of different Mo-reducing colonies appeared after five days of incubation on nutrient agar with various consistencies and optical densities. **Table 2** records further characterisation of all isolates using gram-staining and all the isolates were renamed using code AQ5-A from AQ5-A1 to AQ5-A11.

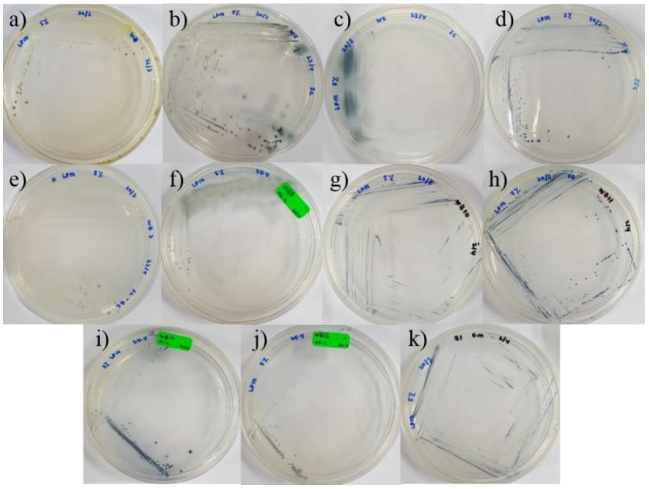

Figure 1. Blue colour intensity of Mo-reducing isolates streaked on LPM agar from 11 Mo-reducing samples after four weeks of incubation at 10ºC. (a) W4 (b) W5 (c) W6 (d) W13 (e) WB3 (f) WB8 (g) WB10 (h) WB11 (i) WB12 (j) WB13 (k) B1 0m.

Table 1. Morphological characteristics of Mo-reducing isolates

Sample	Pigmentation	Transparency	Consistency	Margin	Elevation
W4	Light yellow	Transparent	Mucoid	Entire	Convex
W5	Light yellow	Transparent	Mucoid	Entire	Convex
W6	White	Opaque	Buttery	Entire	Convex
W13	White	Opaque	Buttery	Entire	Convex
WB3	Orange	Opaque	Buttery	Entire	Convex
WB8	White	Opaque	Buttery	Entire	Convex
WB10	White	Opaque	Buttery	Entire	Convex
WB11	White	Opaque	Buttery	Entire	Convex
WB12	Light yellow	Translucent	Mucoid	Entire	Convex
WB13	Cream	Translucent	Buttery	Entire	Convex
B1	White	Opaque	Buttery	Entire	Convex

Table 2. Gram-staining of Mo-reducing isolates

Sample	Strain	Shape	Gram
W4	AQ5-A1	Rod	-
W5	AQ5-A2	Rod	-
W6	AQ5-A3	Cocci	+
W13	AQ5-A4	Cocci	+
WB3	AQ5-A5	Rod	-
WB8	AQ5-A6	Cocci	+
WB10	AQ5-A7	Cocci	+
WB11	AQ5-A8	Cocci	+
WB12	AQ5-A9	Rod	-
WB13	AQ5-A10	Cocci	+
B1	AQ5-A11	Cocci	+

Screening test was conducted by subjecting 11 pure strains to assess the Mo-reduction potential. The reduction capability of the isolates was confirmed by the formation of blue colour after nine days of incubation. As shown in **Figure 2**, strain AQ5-A9 has the highest Mo reduction with the highest blue intensity at 2.280 followed by AQ5-A2 and AQ5-A10 with the optical density of 2.210 and 2.100, respectively. A study by Ahmad et al. [19] using *Pseudomonas* sp. strain DRY1 isolated from Antarctic soil has shown its ability to reduce Mo optimally in 72 h.

Analysis of 16S rRNA partial sequence and phylogenetic trees

16S rRNA is by far the most common genetic marker in the study of bacterial phylogeny. 16S rRNA sequences are now typically used for classifying bacterial species as 16S rRNA universal primers that allow the amplification of gene sequences, which are widely available. Phylogenetic interpretations made from the resulting sequence data can be made through pairwise nucleotide similarity values of 16S rRNA gene sequences, thus allowing the placement of a given organism within a phylogenetic tree [21]. 16s rRNA partial sequencing and homology analysis of showed that strains AQ5-A1, AQ5-A2 and AQ5-A9 are closely related to genus Marinomonas, strain AQ5-A5 is similar to genus Shewanella while strains AQ5-A3, AQ5-A4, AQ5-A6, AQ5-A7, AQ5-A8, AQ5-A10, AQ5-A11 show the highest similarity to genus Staphylococcus (**Table 3).**

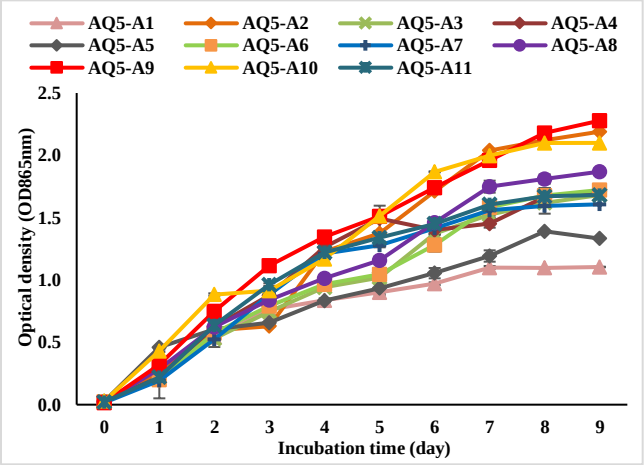

Figure 2. Molybdate reduction of 11 marine bacterial isolates in LPM. The error bars represent the mean ± standard deviation for three replicates, (n=3).

16S rRNA gene sequence analysis placed AQ5-A5 within the genus Shewanella; the strains showed the highest similarity to *S. vesiculosa* (100%) (**Table 3**). However, the phylogenetic tree (**Figure 3**) showed that strain AQ5-A5 was closely related but not genetically identical to *S. vesiculosa*. Analyses for strain AQ5-A5 were insufficient to classify them to species level with confidence, due to the low bootstrap scores obtained; hence they are referred to henceforth as *Shewanella* sp. strain AQ5-A5. The genus Shewanella was described as gram-negative γ-proteobacteria, non-spore forming, rod-shaped, motile by polar flagella, chemo-organotrophic, facultatively anaerobic and capable of respiratory and fermentative metabolism [22-24]. Infections caused by Shewanella are rare as they commonly serve as opportunistic pathogens. The prevalent species capable of human infection is *S. algae* that have implication in a case of human septicaemia in Japan [25, 26].

Their ability to utilise a variety of final electron acceptors in the absence of oxygen and various salt concentrations and barometric pressures allow them to survive in extreme environments [22, 27]. These traits allow Shewanella to be often found in high salinity and energy-rich environments where redox condition changes rapidly [25]. Shewanella are known to be widely distributed in diverse aquatic environments as they are isolated from various aquatic sources including the Black Sea [28]. This genus also has the capability to grow at low temperatures (<5ºC) and most Shewanella strains are psychrotolerant. The ability to grow in a low-temperature

environment provides additional advantage for this genus to thrive in cold environments like in the Polar Regions [27]. Various studies have shown that various Shewanella strains are isolated from Antarctic sea ice, coasts and lakes [22, 23, 27].

Table 3. Homology analysis of marine isolates from Bernardo O'Higgins Riquelme Base Station

Strain	Close representative/ Nearest phylogenetic group	Sequence alignment		Accession number
		No. of nucleotides	% identity*	
AQ5-A1	*Marinomonas primoryensis* strain NBRC 103029	863	98.61%	MN 749937
AQ5-A2	*Marinomonas primoryensis* strain NBRC 103029	823	99.03%	MN 749938
AQ5-A3	*Staphylococcus equorum* strain PA 231	815	99.63%	MN 749969
AQ5-A4	*Staphylococcus equorum* strain PA 231	826	99.80%	MN 750003
AQ5-A5	*Shewanella vesiculosa* strain M7	826	100.00%	MN 750004
AQ5-A6	*Staphylococcus equorum* strain PA 231	842	99.88%	MN 750005
AQ5-A7	*Staphylococcus equorum* strain PA 231	1000	99.80%	MN 750006
AQ5-A8	*Staphylococcus equorum* strain PA 231	852	99.76%	MN 750008
AQ5-A9	*Marinomonas polaris* strain CK13	859	99.30%	MN 750007
AQ5-A10	*Staphylococcus equorum* strain PA 231	822	100.00%	MN 750009
AQ5-A11	*Staphylococcus equorum* strain PA 231	808	99.88%	MN 750021

*****The percentage of identity with the 16s rRNA sequence of the nearest phylogenetic neighbour

In recent years, researchers have shown interest in Shewanella as this genus has shown a great potential in bioremediation of toxic substances [29]. It has the ability to use heavy metals and toxic substances as electron acceptors in respiratory states, thus altering their oxidation states that render them less toxic or results in the formation of solid colloids that expidite clean-up efforts. Some species of Shewanella have been reported to be able to reduce heavy metals such as chromium (Cr), uranium (U) and iron (Fe) [25, 30, 31]. However, to date, this is the first study on the potential remediation of Mo using Shewanella genus. This organism is easy to grow and shows a great potential in various environmental heavy metals pollutants especially Mo.

Phylogenetic analysis of 16S rRNA gene sequence of Antarctic isolates AQ5-A1, AQ5-A2, AQ5-A9 using the neighbour-joining algorithm confirmed the association of these marine strains to the genus Marinomonas. Homology analysis of strains AQ5-A1 and AQ5-A2 revealed their close relation with *M. primoryensis* strain NBRC 103209 with 98.61% and 99.03% sequence similarity respectively (**Table 3**). The phylogenetic tree in **Figure 4** showed that strains AQ5-A1 and AQ5-A2 are each other's close relatives as they formed a clade with each other. Isolate AQ5-A9 formed a clade with *Marinomonas polaris* strain CK13 with a bootstrap value of 98%. The close relationship between AQ5-A9 and *M. polaris* was further evident from a BLAST analysis of the 16S rRNA gene sequence of AQ5-A9, which exhibited 99.3% similarity with *M. polaris* (**Table 3**).

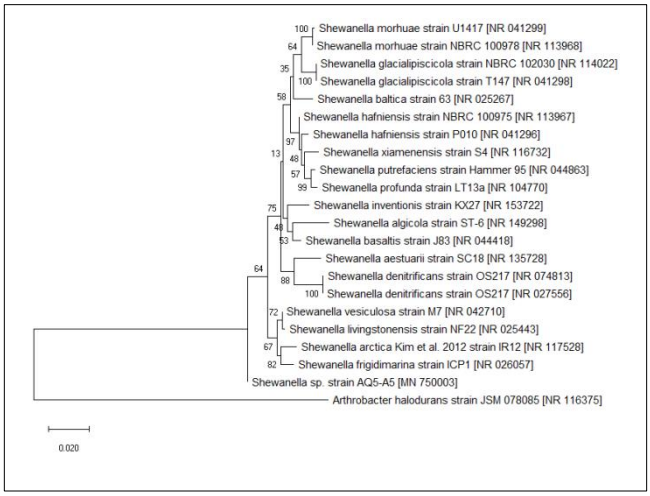

Figure 3. A phylogenetic tree (neighbour-joining method) based on 16S rRNA gene sequences showing genetic relationship between Antarctic isolates AQ5-A5 and other neighbouring species names of Shewanella genus. Species names are followed by the accession numbers. The numbers at branching points are the estimated confidence levels, expressed as percentages referring to bootstrap values, based on 1000 resamplings. The scale bar indicates the evolutionary distance between species, determined by measuring the lengths of the horizontal lines connecting two organisms. *Arthrobacter halodurans* is used as outgroup.

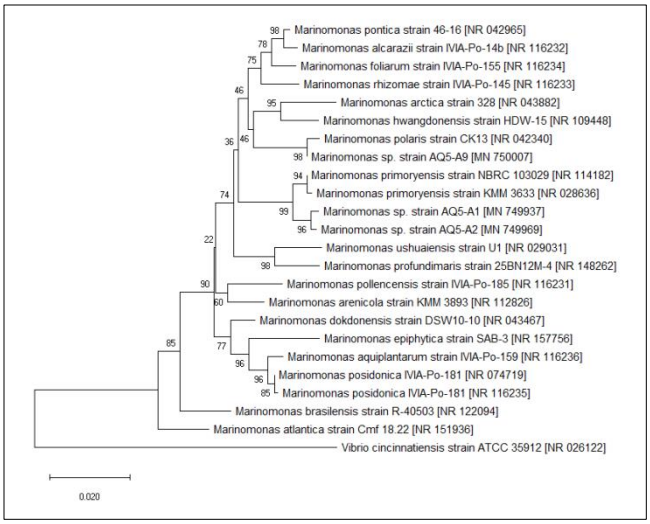

Figure 4. A phylogenetic tree (neighbour-joining method) based on 16S rRNA gene sequences showing genetic relationship between Antarctic isolates AQ5-A1, AQ5-A2, AQ5-A9 and other neighbouring species names of Marinomonas genus. Species names are followed by the accession numbers. The numbers at branching points are the estimated confidence levels, expressed as percentages referring to bootstrap values, based on 1000 resamplings. The scale bar indicates the evolutionary distance between species, determined by measuring the lengths of the horizontal lines connecting two organisms. *Vibrio cincinnatiensis* is used as outgroup.

Until now, only 32 species have been identified in this genus (http://www.bacterio.net/marinomonas.html). Marinomonas which belongs to the family Oceanospirillaceae is described as straight or curved Gram-negative γ-proteobacteria, motile and aerobic [32, 33]. The genus is halotolerant and widely distributed in various marine environments such as seawater, sea ice and marine animals [34-36]. Most species are unable to grow in no salt condition as they require Na^+ for growth and can live up in high salinity surrounding that can reach up to 12% (w/v) of NaCl [34]. A few researches have also proven those Marinomonas genuses are able to grow in cold environments such as in the polar region [32, 37, 38]. They can grow in a wide range of temperature between 4ºC to 37ºC [32].

Thus far, there are hardly any published researches on the ability of this genus on bioremediating inorganic toxic pollutants in marine environment. More than a decade ago, Takeuchi et al. [39] reported the bioaccumulating ability of heavy metal, arsenic (As), of *M. communis* in Japan. However, the mechanism of As accumulation remains unclear. In cold condition, bioremediation potential of this genus has been reported by Melcher et al. [40] in which *Marinomonas* sp. strain D104 isolated from the deep-sea subsurface sediment of the Makarov Basin in the Arctic Ocean was able to degrade polycyclic aromatic hydrocarbon (PAH). An unpublished data by Dong et al. [38] showed that strain D104 was also able to degrade a wide variety of PAHs such as naphthalene and pyrene. As yet, there is no research on Mo remediation using *Marinomonas* genus in the Antarctic.

16S rRNA gene sequence of Antarctic isolates AQ5-A3, AQ5-A4, AQ5-A6, AQ5-A7, AQ5-A8, AQ5-A10, AQ5-A11 analysed by using phylogenetic tree from the neighbour-joining algorithm confirmed the relation of these strains to the genus Staphylococcus. These strains showed the highest similarity (>99.5%) to *Staphylococcus equorum* strain PA 231 (**Table 3**). The phylogenetic tree in **Figure 5** showed that strain AQ5-A3, AQ5-A4, AQ5-A6, AQ5-A8, AQ5-A10 and AQ5-A11 formed a clade with each other and were closely related to *S. equorum* strain PA 231 with a bootstrap value of 84% while AQ5-A7 formed a clade with *S. equorum* subsp. linen strain RP29 and *S. equorum* strain PA 231 with a bootstrap value of 64%.

Genus Staphylococcus is virtually pervasive since numerous species and subspecies are being presented under this genus. This genus is described as Gram-positive bacteria under Staphylococcaceae family, cocci-shaped, form grape-like clusters, non-sporulating, non-motile and facultatively anaerobic [41]. Staphylococcus species differ considerably in their pathogenicity. Species such as *S. cohnii* and *S. cohnii* subsp. *urealyticus* which are frequently isolated in hospitals are responsible for many diseases such as pneumonia and urinary tract infection [42] while species such as *S. warneri* and *S. hominis* are not pathogenic as they do not possess any virulent gene [43].

Various means of microbes pioneering in Antarctica have been reported including natural dissemination processes such wind circulation, ocean currents, marine animals and migratory birds [44]. An increase of human activities in Antarctica has also caused an establishment of non-indigenous microbes observed in the past few decades. However, the origin of the Antarctic Staphylococcus isolates AQ5-A3, AQ5-A4, AQ5-A6, AQ5-A7, AQ5-A8, AQ5-A10 and AQ5-A11 is not yet known since only their genus have been identified. Until now, only a few species of Staphylococcus have been reported to be isolated from Antarctica. For example, Ah Tow and Cowan [45] were able to isolate *S. epidermis* from the soils of Dry Valley, a site that is greatly impacted by human occupation and in contrast, the species was not found in 'pristine' sites. Kashuba et al. [43] isolated ancient permafrost *S. warneri* and *S. hominis* that carry antibiotic resistant genes. Pantůček et al. [46] isolated *S. edaphicus* while Cameron [47] and Krikler [48] also isolated *S. aureus* though it was isolated from nasal swab of the isolated researchers in Antarctic and not from the environment.

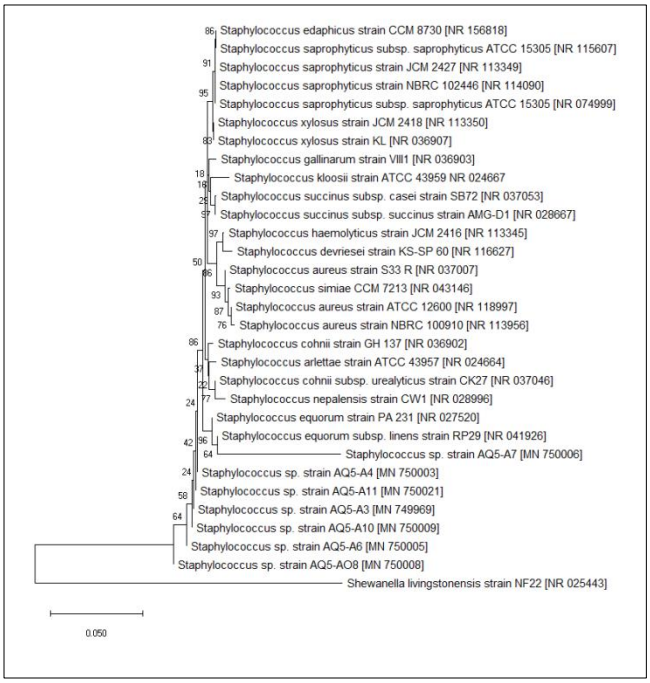

Figure 5. A phylogenetic tree (neighbour-joining method) based on 16S rRNA gene sequences showing genetic relationship between Antarctic isolates AQ5-A3, AQ5-A4, AQ5-A6, AQ5-A7, AQ5-A8, AQ5-A10 and AQ5-A11 and other neighbouring species names of Staphylococcus genus. Species names are followed by the accession numbers. The numbers at branching points are the estimated confidence levels, expressed as percentages referring to bootstrap values, based on 1000 resamplings. The scale bar indicates the evolutionary distance between species, determined by measuring the lengths of the horizontal lines connecting two organisms. *Shewanella livingstonensis* is used as outgroup**.**

Staphylococci are widespread in nature and commonly found living on the skin and on mucous membranes of humans, mammals or birds [49] . Based on **Figure 5**, these isolates are related to strains such as *S. equorum*, *S. gallinarum* and *S. xylosus*, which are often isolated from membrane of mammals and birds [41, 50, 51]. It is highly possible that Staphyloccocus strains isolated in this study are originated from the animals that largely inhabit the Bernardo O'Higgins Base Station such as the penguins and walruses since there are no known reports on the presence of Staphylococcus species in the mentioned animals' microbiome. *S. xylosus* has been described as nonpathogenic strain, but somehow serves as an opportunistic pathogen and its ability to form biofilm might explains its adaptability and persistence in harsh conditions and various niches [51].

Only a few studies have been reported on the potentiality of genus Staphylococcus in bioremedating toxic heavy

metals as the pathogenicity of most Staphylococcus species dissuades their applicability in environmental bioremediation. As a case in point, Elisangela et al. [52] managed to isolate *S. arlettae* strains from textile and tannery industrial effluents that were able to degrade azo dye, a common toxic by-product. However, Dinakaran et al. [53] claimed that *S. arlettae*, although from a different strain, contains potential virulence factors that might cause many negative implications. This suggests that further studies should be done on the origin, pathogenicity and biodegradation potential of Staphylococcus strains isolated from O'Higgins Riquelme Base Station as there is an abundance of this cold-adapted genus isolated from its marine environment.

CONCLUSION

16S rRNA partial sequencing suggests that all 11 isolates belongs to the genus Marinomonas, Shewanella and Staphylococcus. The strains isolated are a valuable collection for further taxonomic analysis, physiological characterization and screening. The potential of these psychrotolerant bacteria isolated from Antarctic propose the possibility of bioremediation of Mo and other pollutants in the polar region.

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