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Identification of Antarctic Soil Bacteria Exhibiting Antiproliferative Activity Against a Colon Cancer Cell Line

Identificación de Bacterias de Suelo Antártico que Presentan Actividad Antiproliferativa Sobre una Línea Celular de Cáncer de Colon

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SUMMARY: Cancer is the second leading cause of death in the world and colorectal cancer is the only cancer that has shown a sustained increase in mortality in the last decade. In the search for new chemotherapeutic agents against cancer, extremophilic microorganisms have shown to be a potential source to obtain molecules of natural origin and with selective cytotoxic action towards cancer cells. In this work we analyzed the ability of a collection of Antarctic soil bacteria, isolated on Collins Glacier from the rhizosphere of *Deschampsia antarctica* Desv plant, to secrete molecules capable of inhibiting cell proliferation of a colorectal cancer tumor line. Our results demonstrated that culture supernatants from the Antarctic bacteria K2I17 and MI12 decreased the viability of LoVo cells, a colorectal adenocarcinoma cell line. Phenotypic and genotypic characterization of the Antarctic bacteria showed that they were taxonomically related and nucleotide identity analysis based on the 16S rRNA gene sequence identified the bacterium K2I17 as a species belonging to the genus Bacillus.

KEY WORDS: Antarctic bacteria; Antiproliferative activity; Colorectal cancer.

INTRODUCTION

The Antarctic continent is an extreme ecosystem, with very little anthropogenic intervention (Pidot *et al*. 2014). This unique ecological niche is a source of numerous extremophilic microorganisms, particularly previously undescribed psychrophiles, (Ramírez-Fernández *et al*., 2019). Given the adverse conditions in which they thrive, bacteria of Antarctic origin possess a broad potential to express a wide variety of molecules with biomedical utility, thanks to the developed high genomic plasticity that is associated with the production of secondary metabolites with unique functional groups in their structure, coupled with the development of diverse non-essential biochemical reactions (Giddings & Newman, 2015).

These secondary metabolites generally do not have a fundamental role in bacterial growth or reproduction and their function is mainly associated with the excretion of products accumulated during the imbalance or dysregulation of such metabolic pathways (Ruiz *et al*., 2010). However, this excretion property makes them an attractive source of new biomolecules with biotechnological uses. Among them, their use as anti-tumor agents against different types of cancer (Zarins-Tutt *et al*., 2016).

Currently, a great diversity of biomolecules of bacterial origin with anti-tumor properties have been described, nonetheless, when evaluating this type of biomolecules from Antarctic microorganisms, a limited description of microorganisms with anti-proliferative properties is observed. An interesting example is the ethanolic extracts obtained from the endophytic fungus *Microdochium phragmitis*, which presents a cytotoxic action

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on melanoma cells (Santiago *et al*., 2012). Likewise, the fungus *Oidiodendron truncatum* GW3-13 isolated from soil beneath lichens produces Chetracin B, a secondary metabolite with cytotoxicity towards several tumor lines of hepatic, gastric, pulmonary, and ovarian origin (Li *et al*., 2012). Moreover, physodic acid secondary metabolite from the Antarctic lichen *Hypogymnia lugubris* (Pers) inhibits the proliferation of a melanoma cell line (Cardile *et al*., 2017). Similarly, there are few examples of Antarctic bacteria, which present antiproliferative properties on various tumor lines with leukemia (Chen *et al*., 2013), fibrosarcoma (Mojib *et al*., 2011), HeLa cells, (Alem *et al*., 2020); breast cancer (MCF-7), glioblastoma (U251), lung cancer lines (NCI-H460), and kidney cancer (786-0) (Silva *et al*., 2020).

In this work, two Antarctic bacteria isolated from rhizospheric soil of *Deschampsia antarctica* Desv, capable of secreting molecules that inhibit *in vitro* the cell proliferation of a colon cancer tumor line, were identified.

MATERIAL AND METHOD

Obtaining Antarctic bacteria isolated from the rhizosphere of *Deschampsia antarctica* Desv plant. Fifty-five Antarctic bacteria isolated from the rhizosphere of *Deschampsia antarctica* Desv., collected in the vicinity of Collins Glacier (62º 22' S, 59º 43' W) located on King George Island, were used for the development of the present work. Soil samples were processed according to the protocols of Barrientos-Díaz *et al*. (2008). Briefly, 4 g of soil were mixed with 5 mL of sterile water and shaken for 10 min using a vortex. The supernatant and serial dilutions, with a dilution factor in base 10, were grown on plates with nutrient agar (Becton Dickinson - BD) diluted to 1/3 (nutrient agar 1/3; meat extract 1 g/L, peptone 1.67 g/L and agar 15 g/L). Cultures were incubated at 4 °C, 10 °C and 18 °C for 15 days. Bacterial growth was monitored every 24 h and colonies were transferred to the same medium used for isolation. Isolated Antarctic bacteria were stored at -80 °C in 50% glycerol until use.

Culture of mesophilic bacterial strains. *Escherichia coli* DH5α, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus aryabhattai* and *Bacillus murali* bacterial strains were growth on LB agar or LB broth and incubated at 37 °C for 24 h. For growth in broth, shaking at 120 rpm was used. The bacteria used belong to the culture collection of Laboratorio de Microbiología Molecular y Compuestos Bioactivos, Universidad Autónoma de Chile.

LoVo cell line and culture conditions. The LoVo ATCC® CCL-229™ tumor line (colorectal adenocarcinoma stage C, from a lymph node metastasis in the left supraclavicular region) was used in the evaluation of the antiproliferative activity of crude extracts of the 55 Antarctic bacteria. This cell line, which possesses mutations associated with the K-ras gene, came from the ATCC collection (Manassas, VA, USA) and was kindly donated by Dr. Manuel Gidekel.

LoVo cells were grown using DMEM medium high in glucose at 37 °C in 5% CO_2 atmosphere. Cells were grown to 80-90 % confluence and 0.25 % trypsin-EDTA 1X was used to subculture and maintain them in exponential growth phase. Independent experiments were performed from the same subculture of cells.

Obtaining culture supernatants and sterile crude extracts of the Antarctic bacteria. Bacteria-free supernatants were obtained from the pure cultures described above of the Antarctic bacteria. Briefly, an individual colony of each Antarctic bacterium was seeded in a 15 mL tube, with 5 mL of LB broth, and incubated at 18 °C under constant agitation at 150 rpm, until the culture reached the stationary phase of growth (OD_{600nm}= 1). The *E. coli* DH5a bacterium was initially used as a negative control and it was growth under the same conditions as the Antarctic bacteria; however, incubation was at 37 °C (its optimal growth temperature) and the OD600nm value was 1.1 for its stationary phase. Spectroscopic measurements of OD_{600nm} were performed in a SPECTRO UV-11 spectrophotometer with plastic cuvettes of 1 cm optical pitch. Subsequently, 15 mL tubes with bacterial growth were centrifuged in a HERMLE Z326K apparatus at 4610 x g for 10 min at the temperatures of 18 °C and 37 °C for the Antarctic bacteria and the negative control *E. coli* DH5a, respectively. Finally, crude extracts (EC) were obtained by sterilizing the culture supernatants by filtration with 0.22 μm membranes, which were stored at 4 °C for later use.

Evaluation of the antiproliferative effect of Antarctic bacterial extracts by MTT metabolic reduction assay. The antiproliferative activity of crude extracts (EC) on the LoVo cell line was evaluated by MTT metabolic reduction assay. However, the initial concentration of LoVo cells was determined in order to maintain exponential growth conditions during the experiment.

Transparent 96-well culture plates were seeded with 100 μL of a suspension of LoVo cells in Maintenance Medium supplemented with 10 % FBS and without antibiotics (MM) at the concentration of 2.3×10^5 cells/mL and were incubated 24 h at 37 °C at 5 % CO_2 atmosphere to facilitate cell adhesion. Subsequently, 50 μL of the MM was

removed from each well and 50 μL of the EC of each Antarctic bacteria was added. After 24 h of incubation at 37 °C at 5 % CO_2 atmosphere, the medium was aspirated, and the cells were washed three times with 200 μL of sterile 1X PBS. Then, 100 μL of MM and 10 μL of 12 mM MTT were added to each well. The plates were incubated for 3 h at 37 °C in 5% CO_2 atmosphere and finally 75 μL of medium was removed from each well and the same volume of DMSO was added. After incubating the plates for 30 min at 25 °C protected from light, the absorbance was determined at 540 nm on a Tecan infinite M200 PRO multi-plate reader. The compound 5-fluorouracil (5-FU) at a concentration of 300 mM was used as anti-cell proliferation control, in addition, MM-LB (maintenance medium supplemented with 10 % FBS without antibiotics and diluted 50 % with LB broth), EC ED (crude extract of *E. coli* DH5a) and MM5 (maintenance medium supplemented with 5 % FBS without antibiotics) were used as controls. In the first assays, EC ED was used as a control; however, EC TI (crude extract of the Antarctic bacterium TI12), with no antiproliferative activity on LoVo cells, was subsequently selected as a negative control.

The percentage of cell viability was determined with respect to cells incubated with the control EC (EC ED or EC TI), and the background value associated with the OD of MM with MTT was subtracted from all conditions. Experiments were performed in three independent assays with eight replicates.

Characterization of thermal stability of antiproliferative extracts EC K2 and EC MI. The crude extracts with antiproliferative activity EC K2 and EC MI were evaluated in a thermostability assay at different temperatures, to establish the optimal conditions to preserve their activity. Each extract was incubated 24 h at -80 °C, -20 °C, 4 °C and for 1 h at 90 °C and then the MTT metabolic reduction assay was performed as indicated above. EC TI, incubated at the same temperatures as antiproliferative extracts, and the extracts (EC K2, EC MI and EC TI) without incubation at different temperatures were used as controls. Additionally, MM-LB, MM5 and 300 μM of 5-FU were used as controls. The percentage of cell viability was determined with respect to cells incubated with EC TI and the experiments were performed with five replicates in three independent assays.

Phenotypic description of Antarctic bacteria K2I17 and MI12. Antarctic bacteria K2I17 and MI12 were phenotypically described according to growth on LB agar and their staining characteristics. The Gram stain classifies bacteria as Gram-positive or Gram-negative depending on the cell wall components, while the Shaeffer-Fulton stain visualizes the presence of endospores and their position in the cell. Slides of both stains were observed under a Motic BA 310E microscope at a total magnification of 1000X. Gram staining results were confirmed on selective and differential media mannitol salt agar (15 g/L agar, sodium chloride 75 g/L, D-mannitol 10 g/L, meat extract 1 g/L, casein pancreatic digest 5 g/L, animal tissue peptic digest 5 g/L, phenol red 0.025 g/L) and MacConkey II agar (agar 13.5 g/L, sodium chloride 5 g/L, crystal violet 0.001 g/L, casein pancreatic digest 1.5 g/L, gelatin pancreatic digest 17 g/L, animal tissue peptic digest 1.5 g/L, lactose 10 g/L, neutral red 0.03 g/L, bile salts 1.5 g/L), specific for the growth of Gram-positive and Gram-negative bacteria, respectively. *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) bacterial strains were used as controls and phenotypic identification of each isolate was performed in duplicate in three independent experiments.

Extraction of bacterial genomic DNA. Antarctic bacteria K2I17 and MI12 were grown in 2 mL LB broth as described above but incubated for only 24 h. The extraction of genomic DNA from pure cultures of Antarctic bacteria K2I17 and MI12 and *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus aryabhattai* and *Bacillus murali* was performed using the commercial PowerBiofilm® DNA Isolation Kit system, according to the manufacturer's instructions.

Quantification of DNA concentration and purity was determined by absorbance at 260 nm and with the ratio 260/280 nm, respectively, on the Tecan infinite M200 PRO multiplate reader. In addition, electrophoresis was performed with 10 μL of DNA, 2 μL of 6X loading buffer [glycerol 60 % (v/v), EDTA 60 mM (w/v), bromophenol blue 0.03 % (w/v), xylene cyanol FF 0.03 % (w/v), Tris-HCl 10 mM (w/v), ultrapure water, pH= 7.6] and 1 μL of GelRed™ 100X dissolved in DMSO was used as stain. Electrophoresis was run in an ENDURO™ Gel XL chamber for 30 min at 7 V/cm on 1% agarose gel with TAE buffer [40 mM Tris-acetate (w/v), 2 mM EDTA (w/v), H_2Od, pH=8.0].

The gel was visualized, and the image was digitized under UV light in a BIOTOP Fluor shot trans illuminator. 1 Kb Plus DNA Ladder was used as a molecular mass standard and 6X loading buffer was used as a negative control. Genomic DNA extractions were performed in five independent experiments.

Genotyping of Antarctic bacteria K2I17 and MI12 by detection of polymerase chain reaction (PCR) amplified repetitive elements. PCR amplification of naturally occurring repetitive elements interspersed in the bacterial genome was used to genotype K2I17 and MI12 bacteria. From the genomic DNA obtained as described above, ERIC

and BOX sequences were amplified to obtain genomic fingerprinting patterns (Versalovic *et al*., 1994).

The final reaction volume in the protocols with the ERIC primers (ERIC 1R 5'-ATG TAA GCT CCT GGG GAT TCA C-3' and ERIC 2 5'-AAG TAA GTG ACT GGG GTG AGC G-3') and BOX primer (BOX A1R 5'-CTA CGG CAA GGC GAC GCT GAC GAC G-3') was set to 25 µL and contained 2,5 µL of KAPA Taq C 5X buffer, 1.5 µL of 25 mM MgCl2, 0.5 µL of 10 mM dNTPs, 1 µL of 10 µM partitioner, 0.1 µL of KAPA Taq DNA Polymerase (5 U/µL) and 2 µL of DNA (50 ng/µL). The conditions for both PCRs were an initial denaturation cycle (95 °C for 3 min), followed by 30 extension cycles (denaturation at 95 °C for 30 s, pairing at 50 °C for 1 min and extension at 72 °C for 8 min) and finally 10 min at 72 °C, in a Labnet MultiGene OptiMax thermal cycler.

Genomic fingerprinting patterns of *B. cereus*, *B. subtilis*, *Bacillus megaterium*, *Bacillus aryabhattai* and *Bacillus murali*s strains were used as controls. Genomic DNA of *B. cereus* and *B. subtilis* were extracted as described above.

PCR products were analyzed by electrophoresis, using TBE buffer [50 mM Tris-borate, 2 mM EDTA, pH= 8.0]. The negative control consisted of the reaction mixture of each PCR, without DNA. Genotyping was performed in triplicate from three independent experiments.

Identification of the Antarctic bacterium K2I217 by sequencing of the *rrs* gene (16S rRNA). Taxonomic identification of this bacterium was performed by PCR amplification of the *rrs* gene, which codes for the 16S rRNA of the 30S subunit of the bacterial ribosome. From genomic DNA, amplification of a 1464 bp fragment was performed on a Labnet MultiGene Optimax thermal cycler with the universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3').

The final reaction volume for the protocol was set to 50 µL and contained 5 µL of KAPA Taq C 5X buffer, 3 µL of 25 mM MgCl2, 1 µL of 10 mM dNTPs, 2 µL of 10 µM partitioner, 0.5 µL of KAPA Taq DNA Polymerase (5 U/µL) and 2 µL of DNA with a concentration of 50 ng/µL. The PCR conditions were an initial denaturation cycle (95 °C for 2 min), followed by 30 extension cycles (denaturation at 95 °C for 1 min, pairing at 55 °C for 1 min and extension at 72 °C for 10 min) and finally 8 min at 72 °C, in a Labnet MultiGene OptiMax thermal cycler.

The PCR products were analyzed by electrophoresis and the DNA fragment (approximately 1500 bp) was extracted from the agarose gel with a sterile scalpel and weighed into a 1.5 mL microfuge tube. DNA extraction and purification on the agarose gel was performed with the commercial E.Z.N.A® Gel Extraction system according to the manufacturer's instructions. The purified DNA amplification of the *rrs* gene were reanalyzed by 1 % agarose gel electrophoresis to confirm their purity, and subsequently sent for sequencing to Macrogen Co (Korea).

Identification of *rrs* gene (16S rRNA) of bacterial K2I17 was performed by comparison of its consensus sequence in the NCBI BLAST nucleotide database (BLASTn).

Statistical analysis. Statistical analyses and graphs were performed with GraphPad Prism 6.01 software (GraphPad Software, La Jolla California USA, www.graphpad.com). For quantifiable and plotted variables, values were plotted with the EEM and differences were considered significant for α= 0.01 or α= 0.05 (indicated in each case). Analysis of the plotted results was performed with the Kruskall-Wallis nonparametric tests (rank test comparing medians), followed by Dunn's multiple comparisons (rank test comparing medians between groups) or the Mann-Whitney U test (rank test comparing medians between two groups).

RESULTS

In vitro antiproliferative activity of Antarctic bacteria against colon cancer cells.

The antiproliferative activity of crude extracts (EC) of Antarctic bacteria against LoVo cells (colorectal adenocarcinoma cell line) was determined by the MTT metabolic reduction assay. Of the 55 Antarctic bacteria tested, 3.6 % secreted bioactive molecules into the culture medium that inhibited cell proliferation. Fig. 1 shows that the ECs of K2I12 (EC K2) and MI12 (EC MI) bacteria decreased the percentage of viability in LoVo cells at 24 h. The viability values were 5.1 % and 7.6 % for EC K2 and EC MI respectively and 99.1 % for the negative control corresponding to the crude extract of *E. coli* DH5a (EC ED). When the antiproliferative effect of EC K2 and EC MI was compared with the control EC ED, the difference in the reduction of cell viability was statistically significant (****$p<0.0001$; α= 0.01).

Fig. 1 also shows that LoVo cells incubated with the crude extract of the Antarctic bacterium TI12 (EC TI) had 93.3 % of viability. The TI12 bacterium belongs to the Antarctic bacteria collection and the viability percentage obtained with its crude extract had no difference with the negative control EC ED (ns, $p>0.01$; α= 0.01). Moreover, when

the viability of the EC K2 and EC MI groups was compared with that of EC TI, it had the same degree of statistical significance (****p<0.0001; α= 0.01) as the comparison made with the respective control EC ED. The results above allowed the validation of EC TI as an extract without antiproliferative activity and using it as negative control under similar culture conditions as the other Antarctic bacterial extracts.

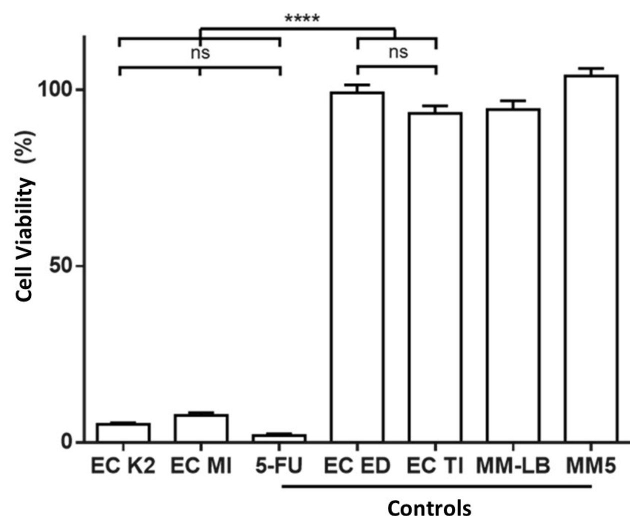

Fig. 1. Evaluation of the antiproliferative effect of crude EC K2 and EC MI extracts on LoVo cells. Cell viability was determined at 24 h by MTT metabolic reduction assay. EC K2 and EC MI: crude extracts of Antarctic bacteria K2I17 and MI12, respectively. Controls: crude extracts of *E. coli* DH5a (EC ED) and TI12 (EC TI), 5-FU: 5-fluorouracil 300 mM, MM-LB: maintenance medium supplemented with 10 % FBS without antibiotics and diluted 50 % with LB broth and MM5: maintenance medium supplemented with 5 % FBS without antibiotics. Statistical comparisons were performed with the Kruskal-Wallis test and a Dunns' multiple comparisons post analysis for an a= 0.01; ****p<0.0001. Results show the mean ± standard error of the mean of three independent experiments with eight replicates.

The decrease in viability caused by EC K2 and EC MI was comparable to the 1.9 % induced by 300 μM 5-fluorouracil (5-FU), a gold standard drug used in the treatment of colorectal carcinoma (CRC), with no significant differences (ns, p>0.01; α= 0.01) found between the three groups (Wyatt & Wilson, 2009). Finally, the controls MM-LB (maintenance medium supplemented with 10 % FBS without antibiotics and diluted 50 % with LB broth) and MM5 (maintenance medium supplemented with 5 % FBS without antibiotics) did not affect the viability of the LoVo cell line.

Antarctic bacteria are psychrophilic microorganisms adapted to grow at temperatures below 0 °C, for this reason, the extracts (EC K2 and EC MI) that inhibited the proliferation of the LoVo tumor line were subjected to a thermo stability test, to establish the optimal conditions for the conservation of their activity.

The range evaluated covered temperatures of -80 °C, -20 °C and 4 °C, used in the laboratory for the preservation of biological samples, and 90 °C, related to the techniques reported for the semi-purification of bioactive molecules present in cell-free culture supernatants. Table I show the incubation conditions of the crude extracts before evaluating their antiproliferative effect on LoVo cells and the viability percentages obtained in the MTT metabolic reduction assay at 24 h and the viability values corresponding to the MM5, 5-FU and MM-LB controls.

The Figure 2 shows that EC K2 and EC MI extracts maintained their antiproliferative activity against LoVo cells when extracts were incubated 24 h at -80 °C, -20 °C, and 4 °C. The percentages of cell viability of EC K2 and EC MI extracts incubated at the three temperatures were equal or less than 10 % (Table I). Moreover, the differences with the negative control group EC TI, with viability values above 98 %, remained significant (****p<0.0001; α= 0.01) at the temperatures of -80 °C, -20 °C and 4 °C. However, there was no antiproliferative activity of EC K2 and EC MI extracts when heated for 1 h at 90 °C, and no statistical difference (ns, p>0.01; α= 0.01) was observed between 99.9 % viability of EC K2 and 100.8 % of EC MI, with respect to 103.2 % of the negative control group EC TI (Fig. 2).

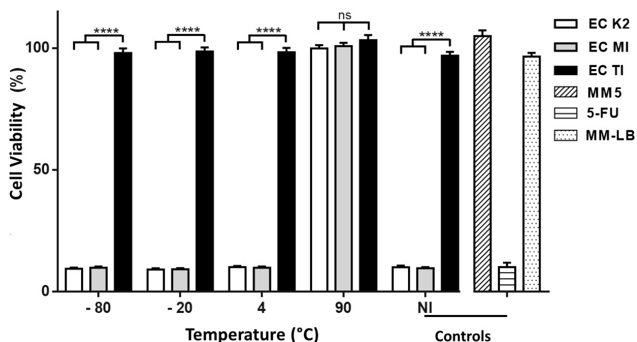

Fig. 2. Evaluation of temperature effect on anti-proliferative activity of crude EC K2 and EC MI extracts on LoVo cells. The crude extracts were incubated beforehand, 24 h at -80 °C, -20 °C, 4 °C and 1 h at 90 °C, and viability was determined at 24 h by MTT metabolic reduction assay. EC K2 and EC MI: crude extracts of Antarctic bacteria K2I17 and MI12, respectively. Controls: NI: crude extracts not incubated at the temperatures evaluated, EC TI: crude extract of Antarctic bacteria TI12, MM5: maintenance medium supplemented with 5 % FBS without antibiotics, 5-FU: 5-fluorouracil 300 mM, MM-LB: maintenance medium supplemented with 10 % FBS without antibiotics and diluted to 50 % with LB broth. Statistical comparisons were performed with the Kruskal-Wallis test and a Dunn's multiple comparisons post analysis for an a= 0.01; ****p<0.0001. Results show the mean ± standard error of the mean in three independent experiments with five replicates.

Table I shows that the viability percentage values of the EC K2, EC MI and EC TI groups incubated 24 h at temperatures of -80°C, -20 °C and 4 °C were similar to the results obtained for the same ECs without incubation at the temperatures evaluated. Furthermore, Figure 2 shows that the degree of significance between the differences was equal in all conditions. The MM5 and MM-LB control groups, with 104.9 % and 96.5 % cell viability, respectively, showed no antiproliferative effect; however, the positive control with 300 µM of 5-FU decreased viability to 10 %.

Table I. Effect of incubation conditions of Antarctic bacteria K2I17 (EC K2) and MI12 (EC MI) extracts on LoVo cells viability.

Incubation conditions		Cell Viability[1] (%)		
Temperature	**Time**	**EC K2**	**EC MI**	**EC TI**[2]
-80	24	9.4	9.8	98.0
-20	24	9.1	9.2	98.6
4	24	10.0	9.8	98.3
90	1	99.9	100.8	103.2
NI	0	10.0	9.5	96.9
Controls		**MM5**[3]	**5-FU**[4]	**MM-LB**[5]
		104.9	10.0	96.5

[1] percentage of viability determined with the MTT metabolic reduction assay at 24 h (with respect to cells incubated with the negative control EC TI). [2] EC TI is a crude extract of the Antarctic bacterium TI12, with no antiproliferative activity on LoVo cells (negative control). [3] MM5 is maintenance medium supplemented with 5 % FBS without antibiotics. [4] 5-FU is 5-fluorouracil (anti-cell proliferation control). [5] MM-LB is maintenance medium supplemented with 10 % FBS without antibiotics and diluted 50 % with LB broth.

Phenotypic and molecular identification of Antarctic bacteria with *in vitro* antiproliferative activity on colon cancer cells.

Colonies of K2I17 and MI12 bacteria were observed to be flat and irregular, cream-colored, with lobulated edges, rough surface and a creamy consistency, when seeded by depletion on LB agar. Microscopic analysis showed that both isolates were Gram-positive long bacilli, as shown in Figure 3. In addition, Shaeffer-Fulton staining showed the presence of ellipsoidal endospores (green structures) that deformed the cells in the central position (Fig. 3).

The results obtained with Gram staining were confirmed by seeding the K2I17 and MI12 Antarctic bacteria on the selective and differential media mannitol salt agar (for Gram-positive bacteria) and MacConkey II agar (for Gram-negative bacteria). Table II shows that there was no growth on MacConkey II agar. Moreover, there was no growth on mannitol salt agar, a culture medium that is selective and differential for Gram-positive staphylococci. The bacteria used as controls: *E. coli* (Gram negative) and *S. aureus* (Gram positive), did grow on the media corresponding to each bacterial type. The analysis of the obtained results suggests that the Antarctic bacteria K2I17 and MI12 could belong to the genus *Bacillus* (Stephenson & Lewis, 2005).

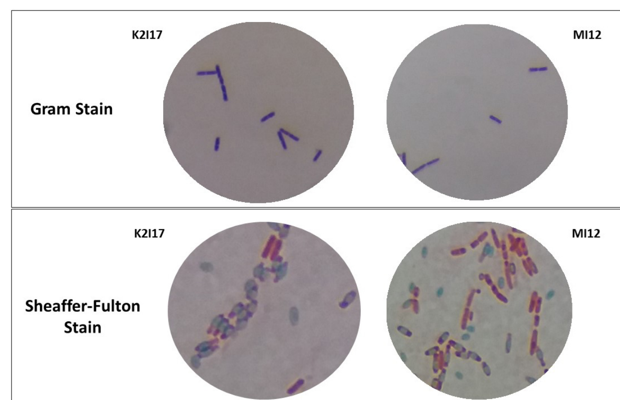

Fig. 3. Morphological characterization of Antarctic bacteria K2I17 and MI12. Morphology was observed with Gram and Shaeffer-Fulton stains. Gram staining showed violet-colored Gram-positive bacillary forms and Shaeffer-Fulton staining showed ellipsoidal endospores (green structures) that deformed the bacillus in the central position. The photographs were taken at a total magnification of 1000X and are representative of three independent experiments with two replicates.

Table II. Determination of Gram positive/negative characteristics of Antarctic bacteria K2I17 and MI12 using selective and differential growth medium.

Antarctic Bacteria	*MacConkey* II Agar	Mannitol Salt Agar
K2I17	-	-
MI12	-	-
Control Bacteria		
E. coli (Gram-negative)	+	-
S. aureus (Gram-positive)	-	+
B. cereus (Gram-positive	-	-

+: growth; -: no growth

The use of the BOX and ERIC primers by polymerase chain reaction (PCR) made it possible to generate separate DNA fragments among repeated sequences (caused by insertions or deletions) of the genome and to generate a fingerprint of K2I17 and MI12 bacteria with a high level of taxonomic resolution (Kumar *et al*., 2014). The fingerprinting patterns showed amplifications of conserved sequences located at different intergenic positions, and in both orientations of the bacterial chromosome (Giongo *et al*., 2008).

The amplification products of BOX-PCR and ERIC-PCR, performed with genomic DNA from Antarctic bacteria K2I17 and MI12, showed polymorphism produced by variability in sequence repeats and by the distance between immediate copies. Figure 4A shows the electrophoretic pattern, with the BOX primer, of approximately 18 DNA fragments distributed in a range from 400 to 3000 bp. On the other hand, Figure 4B shows at least 21 DNA bands with molecular sizes that ranged from 300 to 9000 bp in ERIC-PCR. Analysis of Figure 5 revealed that the Antarctic bacteria K2I17 and MI12 are taxonomically closely related or could have the same phylogenetic clonal origin.

Our group has the strains *B. cereus* and *B. subtilis*, as well as DNA (identified by molecular biology techniques) of *Bacillus megaterium*, *Bacillus aryabhattai* and *Bacillus muralis*. In order to identify the Antarctic bacteria K2I17 and MI12, the obtained fingerprints using BOX-PCR and ERIC-PCR were compared with the patterns of the five strains of the genus *Bacillus* mentioned above. Figure 5 shows that the fingerprints obtained by BOX-PCR (Fig. 5A) and ERIC-PCR (Fig. 5B) for K2I17 and MI12 did not match those corresponding to the five species of the genus *Bacillus*; however, there is no absolute certainty using only fingerprinting methods. The specificity of the PCR reactions with the BOX and ERIC primers was demonstrated by the absence of nonspecifically amplified products in the lanes corresponding to the negative control shown in Figures 4 and 5.

Genotyping by BOX-PCR and ERIC-PCR showed that the K2I17 and MI12 bacteria were taxonomically closely related; therefore, only the K2I17 bacterium was identified by 16S rRNA gene sequencing. The 16S rRNA gene amplification and sequencing result identified it as a member belonging to the genus *Bacillus* when compared to the *rrs* gene consensus sequence in the NCBI BLASTn database. However, as the percent identity for a 0 e-value was equal to 99 % match with more than 10 species included within the genus Bacillus, it could not be identified to the species level.

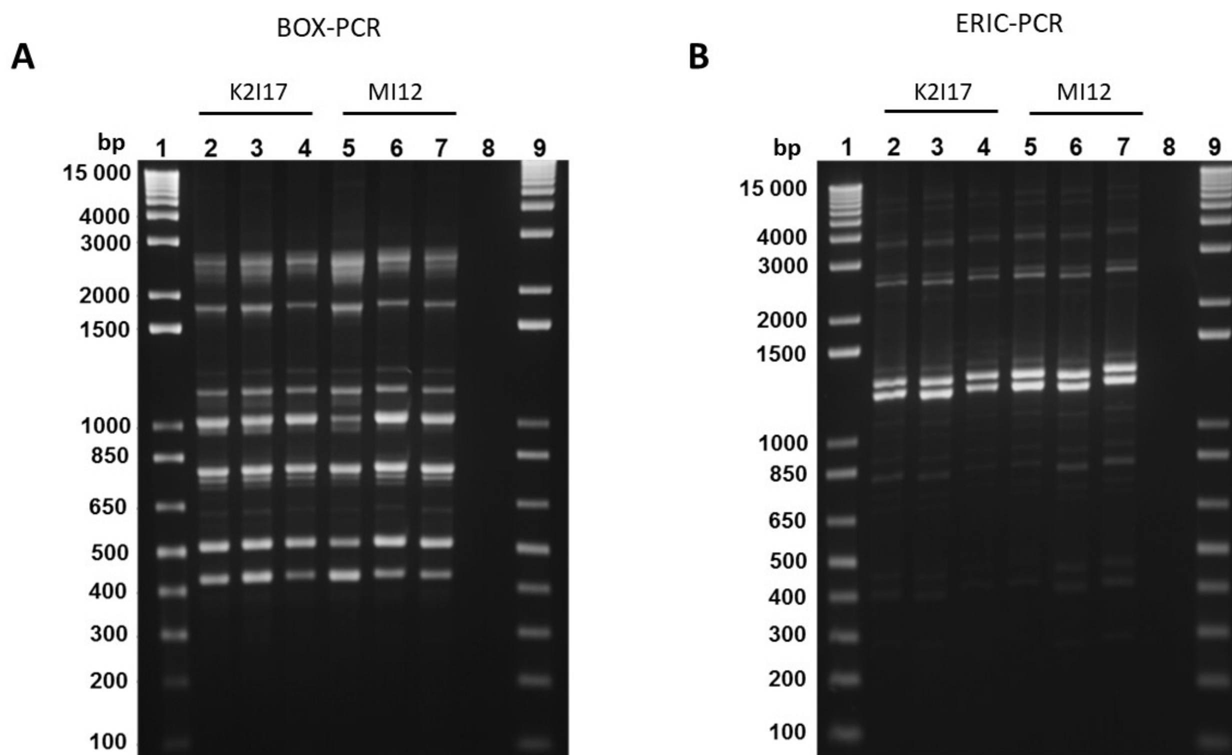

Fig. 4. Characterization of the genomic polymorphism of Antarctic bacteria K2I17 and MI12. The genomic product resulting from PCRs with the BOX (A) and ERIC (B) primers was visualized by 1% agarose gel electrophoresis. Genotyping of the Antarctic bacteria K2I17 and MI12 showed the same repetitive variability of sequences and equal distance between immediate copies of the genome. Lanes 1 and 9: 1 Kb Plus DNA Ladder molecular weight marker, lanes 2, 4 and 6: K2I17 bacteria, lanes 3, 5 and 7: MI12 bacteria, lane 8: negative control. Genotyping was performed in triplicate from three independent experiments.

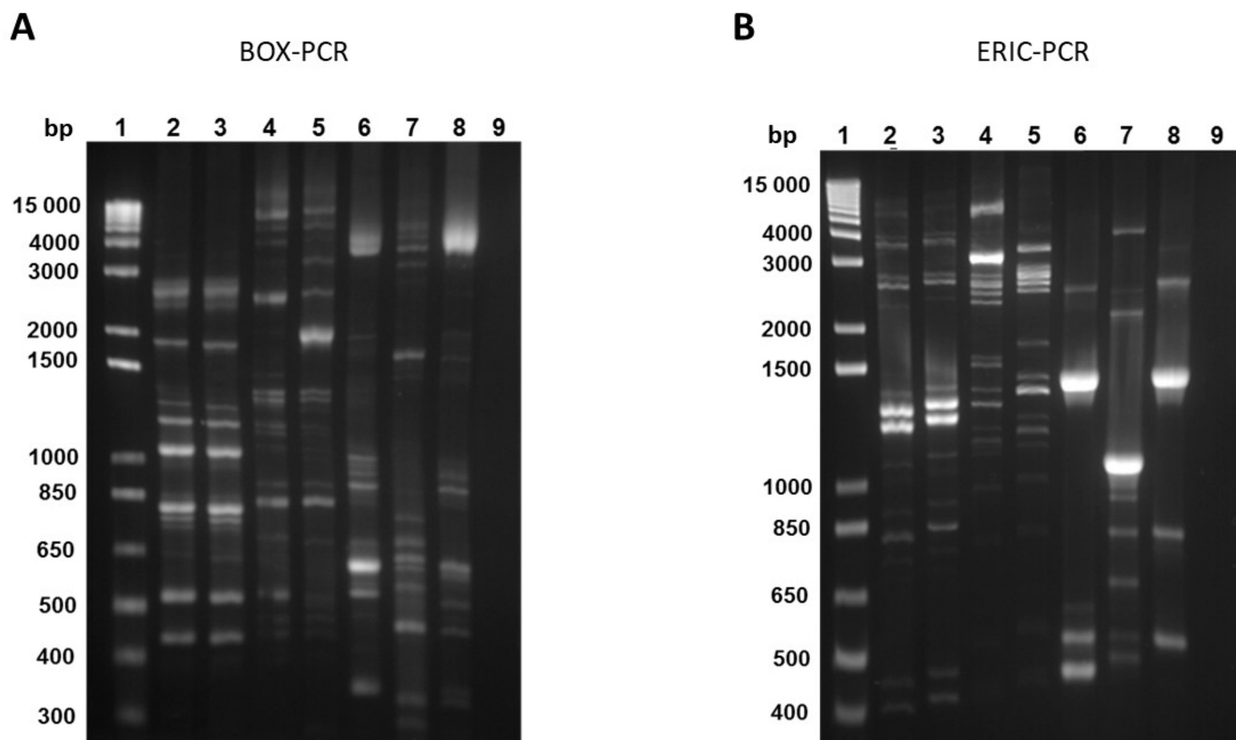

Fig. 5. Comparison of the genomic polymorphism of the Antarctic bacteria K2I17 and MI12 with representative strains of Bacillus genus. The genomic product resulting from PCR with the BOX (A) and ERIC (B) primers was compared with the profile of *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus aryabhattai* and *Bacillus murali*s strains by 1% agarose gel electrophoresis. Genotyping comparison showed different repetitive variability of the sequences and unequal distances between the immediate copies of the genomes. Lane 1: 1 Kb Plus DNA Ladder molecular weight marker, lane 2: K2I17 bacteria, lane 3: MI12 bacteria, lane 4: B. cereus, lane 5: B. subtilis, lane 6: B. megaterium, lane 7: B. aryabhattai, lane 8: B. muralis, lane 9: negative control. Genotyping was performed in triplicate from three independent experiments.

DISCUSSION

The 3.6 % of isolated Antarctic bacteria that excreted molecules with antiproliferative action against LoVo cell line was similar to the 4.4 % described in a study with the same purpose but focused on endophytic fungi of the plants *Deschampsia antarctica* Desv and *Colobanthus quitensis* (Kunth) Bartl (Santiago *et al*., 2012). Cell-free extracts obtained from K2I17 and MI12 bacteria decreased the proliferation of the LoVo cell line, to levels comparable to that induced by 5-fluorouracil, the "gold standard" drug for treatment of CRC (Katanyoo *et al*., 2018). The literature provides evidence that the rhizobiome is a potential source of antitumor compounds of bacterial origin (Park *et al*., 2017). On the other hand, some authors posit that viability assays based on metabolic functions and absorbance quantification, such as MTT, relate more closely to the integrated activity of a set of metabolic enzymes, such as NADH, and to a lesser extent to the proportionality of viable cells (Menyhárt *et al*., 2016). However, the method is considered moderately robust as a first approach for biological activity assessment and applicable in screening experiments involving a high number of samples (Menyhárt *et al*., 2016).

Morphological comparison of colonies belonging to Antarctic bacteria K2I17 and MI12 rise the possibility of them being the same bacteria, however, as they were obtained from different sampling sites they were studied separately. The aerobic or facultative anaerobic characteristics and the phenotypic results of Gram and Shaeffer-Fulton staining suggested that both belonged to the genus *Bacillus* as Gram-positive bacilli with ellipsoidal central endospores (Logan & Vos, 2009). The genus *Bacillus* is common in terrestrial ecosystems because of adaptive mechanisms, metabolic flexibility, and sporulation that provide it with a significant competitive advantage in the rhizosphere and harsh environments (Mappa, *et al*., 2021). Furthermore,

rhizosphere strains of the genus *Bacillus* (*B. subtilis* subsp. *subtilis* RG, *Bacillus amyloliquefaciens* AK-0) excrete cytotoxic metabolites towards different CRC and breast tumor lines (Ramasubburayan *et al*., 2015; Park *et al*., 2017).

As no phenotypic differences were found between the K2I17 and MI12 bacteria, they were genotyped with the aim of assessing whether they were phylogenetically related. The fingerprinting patterns confirmed the identical polymorphism in amplified sequence repeats and the distance of the immediate copies of the genome and suggested that both bacteria shared a phylogenetic clonal origin or belonged to the same bacterial species. The literature reports that certain bacterial phylogenetic clones agree in the genome and differ, for example, in characteristics such as the presence of plasmids, epigenetic modifications or proteome expression under different *in vitro* culture conditions (Jahn *et al*., 2015). As the phenotypic characteristics of both bacteria suggested their inclusion within the genus *Bacillus*, their fingerprint pattern was compared with that of strains of the same genus. The genomic profile of both bacteria did not match the patterns corresponding to *B. cereus*, *B. subtilis*, *Bacillus megaterium*, *Bacillus aryabhattai* and *Bacillus muralis*; however, the result did not rule out that they belonged to any of these species, due to the intrinsic taxonomic difficulty of the genus *Bacillus*. For example, comparing the sequence of genes and isoforms of enzymes central to metabolism shows that three species (*B. cereus, Bacillus thuringiensis* and *Bacillus anthrancis*) of this genus are not genetically distinct (Helgason *et al*., 2000). Gene sequencing studies with phylogenetic value have included in the genus *Bacillus* up to eight highly divergent phylogenetic lineages that make its taxonomy more complex compared to other bacterial genera (Koneman & Allen, 2006).

In bacterial taxonomy and phylogeny, the most widely used tool to identify species is the comparison of the *rrs* gene (16S rRNA gene) with specific bacterial sequence. In this sense, the percentage of identity of the *rrs* gene consensus sequence for K2I17 was higher than 99 % with more than 10 species of the genus *Bacillus* included in the NCBI BLASTn database, and confirmed the results suggested by the phenotypic characterization; nevertheless, it could not be specifically associated to any species within the genus. A particularity of the genus *Bacillus* is the difficulty in classifying strains to species when only *rrs* gene sequencing is used as a taxonomic tool; sometimes species-specific variable regions of closely related strains show percentages of identity between 99.2 % and 99.6 % (Mohkam *et al*., 2016). In addition, species included in some subdivisions made within the genus *Bacillus*, such as those in the *B. cereus* group, cannot be classified by *rrs* gene sequencing alone (Liu *et al*., 2015).

Extremophilic microorganisms have become a potential source for the isolation of new compounds with diverse applications in medicine and pharmaceutical industry (Giddings & Newman, 2015; Wang *et al*., 2019). Therefore, the halobiont formed by *Deschampsia antarctica* Desv is studied by our group with the aim of identifying molecules of biomedical importance (Berríos *et al*., 2013; Malvicini *et al*., 2018).

The study of rhizosphere bacteria in *Deschampsia antarctica* Desv has a main advantage, the intense microbial interaction and antagonistic relationships established in that ecosystem. The rhizobiome is currently considered a "hot spot" that favors the excretion of cytotoxic molecules for microorganisms to compete for space and nutrients (Hassani *et al*., 2018).

The extreme conditions of Antarctica suggested that rhizospheric bacteria of *Deschampsia antarctica* Desv plant could be a source of new molecules with biological activity, because the rhizobiome is under selective pressures that influence its evolution and adaptation to adverse niches. The hypothesis above was confirmed by demonstrating that *Bacillus* sp. K2I17, a bacterium isolated from this extreme ecosystem, secreted into the culture supernatant a molecule that inhibited the proliferation of a human colorectal cancer tumor line such as LoVo cells *in vitro*.

Fungi, actinomycetes and lichens that produce metabolites with antiproliferative effect on various cancer lines have been isolated from the Antarctic terrestrial ecosystem (Cardile *et al*., 2017). However, this is the first time that a rhizospheric *Deschampsia antarctica* Desv bacterium is reported as a secreter of cell proliferation inhibitory metabolites in colorectal adenocarcinoma tumor line.

AUTHORS' CONTRIBUTION

GC and LS: General drafting of the manuscript, data analysis and discussion.
AP, PO and GW: Experimental development of antitumor activity, phenotypic characterization and molecular characterization.
AP, GC, NC, LC and PC: Literature review, preparation of tables and figures. Text revision.
AG and MG: Manuscript review and facilitation of Antarctic bacteria collection.

DECLARATION OF INTEREST. The authors declare that they have no conflict of interest.

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PAVÓN; A.; CORSINI, G.; ORELLANA, P.; CALISTO, N.; NAVARRO, L.; WIESE, G.; CORTÉS-CORTÉS, P.; GIDEKEL, M.; GUTIERREZ-MORAGA, A. & SALAZAR, L. Identificación de bacterias de suelo antártico que presentan actividad antiproliferativa sobre una línea celular de cáncer de colon. *Int. J. Morphol., 41(1)*:286-296, 2023.

RESUMEN: El cáncer es la segunda causa de muerte en el mundo y el cáncer colorrectal es el único que presenta un aumento sostenido de la mortalidad en la última década. En la búsqueda de nuevos agentes quimioterapeúticos contra el cáncer, se ha propuesto a los microorganismos extremófilos como una fuente potencial para obtener moléculas de origen natural y con acción citotóxica selectiva hacia las células cancerígenas. En este trabajo analizamos la capacidad de una colección de bacterias de suelo antártico, aisladas en el glaciar Collins desde rizosfera de la planta de *Deschampsia antarctica* Desv, de secretar moléculas capaces de inhibir la proliferación celular de una línea tumoral de cáncer colorrectal. Nuestros resultados demostraron que los sobrenadantes de cultivo de las bacterias antárticas K2I17 y MI12 disminuyeron la viabilidad de la línea celular de adenocarcinoma colorrectal LoVo, en un ensayo de reducción metabólica de MTT. La caracterización fenotípica y genotípica de las bacterias antárticas, demostró que estaban relacionadas taxonómicamente y el análisis de la identidad nucleotídica en base a la secuencia del gen ARNr 16S identificó a la bacteria K2I17 como una especie perteneciente al género Bacillus.

PALABRAS CLAVE: Bacterias antárticas; Actividad antiproliferativa; Cáncer colorrectal.

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