Electronic Journal of Biotechnology 17 (2014) 1–5

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Antibacterial activity of the Antarctic bacterium *Janthinobacterium* sp. SMN 33.6 against multi-resistant Gram-negative bacteria

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ARTICLE INFO

Article history:
Received 25 April 2013
Accepted 15 November 2013
Available online 28 December 2013

Keywords:
Antarctica
Antimicrobial
Antibiotic resistance
Bacterial pigment

ABSTRACT

Background: The increment of resistant strains to commonly used antibiotics in clinical practices places in evidence the urgent need to search for new compounds with antibacterial activity. The adaptations that Antarctic microorganisms have developed, due to the extreme environment that they inhabit, promote them as a potential new source of active compounds for the control of microorganisms causing infections associated with health care. The aim of this study was to evaluate the antibacterial activity of an ethanol extract of the Antarctic bacterium *Janthinobacterium* sp., strain SMN 33.6, against nosocomial multi-resistant Gram-negative bacteria.
Results: Inhibitory activity against human Gram-negative bacterial pathogens, with concentrations that varied between 0.5 and 16 μg ml^{-1}, was demonstrated.
Conclusions: The ethanolic extract of *Janthinobacterium* sp. SMN 33.6 possesses antibacterial activity against a chromosomal AmpC beta-lactamase-producing strain of *Serratia marcescens*, an extended-spectrum beta-lactamase-producing *Escherichia coli* and also against carbapenemase-producing strains of *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. This becomes a potential and interesting biotechnological tool for the control of bacteria with multi-resistance to commonly used antibiotics.

1. Introduction

In the mid-twentieth century, before antibiotics were introduced, patients with bacteremia had low probabilities of survival. The discovery of antibiotics and development of new compounds reversed this pessimistic prognosis. In the last decade the number of antibiotic multi-resistant bacterial strains as etiologic agents of infectious diseases has increased at an alarming rate; challenging physicians to find an anti-infective therapy that ensures an effective result [1]. The selective pressure exerted by the use of antimicrobials reaches greater dimensions in hospital settings with bacterial pathogens acquiring new resistance mechanisms, facilitating the selection, accumulation, and dissemination of antibiotic resistance genes among the bacterial population [2,3].

This problem places an urgent necessity of finding new and more effective antibiotic drugs with activity against multi-resistant and, moreover, pan-resistant bacteria that has become a critical concern in public health [4,5]. Despite the emergency, the progress in the development of these agents has been slow and the number of approved new antimicrobials per year has been gradually decreasing [6,7].

Extremophile microorganisms produce molecules adapted to unusual life conditions and have been recognized as an important source of new biologic products [8]. Various studies have established that extremophiles represent a new and promising source to search for novel antibacterial molecules, although only a small fraction of these diverse biological compounds have been studied [9].

The aim of this investigation was to evaluate the inhibitory capacity of an ethanolic extract obtained from an Antarctic bacterial strain, identified within the Genus *Janthinobacterium*, against human bacterial pathogens isolated from Chilean hospitals, and multi-resistant to commonly used antibiotics in human medicine.

2. Materials and methods

2.1. Bacterial isolate

The bacterial strain SMN 33.6 used in this study was isolated in 2007, from a soil sample in Fildes Peninsula (62°12′S, 58°57′W) located in

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

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http://dx.doi.org/10.1016/j.ejbt.2013.12.001

Table 1
In vitro determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of ethanol extract of the strain *Janthinobacterium* sp. SMN 33.6 against multi-resistant nosocomial isolates.

Multi-resistant strains	Strain code	MIC (μg ml^{-1})	MBC (μg ml^{-1})
A. baumannii	AB-1 (OXA-58[a])	1	2
E. coli	EC-325 (ESBL)	0.5	2
E. coli	EC-241 (ESBL)	1	16
K. pneumoniae	KB-503 (ESBL)	16	16
K. pneumoniae	KB-495 (ESBL)	16	16
P. aeruginosa	P-145 (VIM-2[a])	1	16
S. marcescens	S-41 (AmpC)	2	2
S. marcescens	S-32 (AmpC)	0.5	2

[a] Carbapenemases; ESBL: extended-spectrum beta-lactamase; AmpC: chromosomal AmpC beta-lactamase.

King George Island, Antarctica. The sample was maintained at 4°C, inoculated in R_2A agar and cultivated at 15°C.

2.2. Amplification of 16S rRNA gene and phylogenetic analysis

To identify and characterize phylogenetically the strain SMN 33.6, DNA was extracted from a liquid culture according to the basic protocol described by Wilson [10]. The gene 16S rRNA was amplified by PCR, using standard primers 16S Fw (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S Rv (5′-CGGTTACCTTGTTACGACTT-3′). We used the reverse primers to obtain the complete and correct sequence of 16S rRNA CR [R] (5′-CTTGTGCGGGCCCCCGTCAATTC-3′) [11]. The PCR product was purified using the AxyPrep PCR Clean-up kit (Axygen Biosciences) and sequenced at Macrogen (Korea).

To determine the phylogenetic proximity to the SMN 33.6 strain, the molecular identification was done through a similarity profile of the gene sequence that encode for the 16S rRNA. The sequence was edited with the IT program Sequencher (Gene Codes Corporation) and the similarities were analyzed in the data base GenBank NCBI (National Center for Biotechnology Information). A phylogenetic tree was created using the Bosque program [12]. The method used corresponded to the maximum likelihood, using a GTR method.

2.3. Biochemical characterization of the SMN 33.6 strain

A morphologic and biochemical characterization was performed with API 20NE and API ZYM (Biomerieux) kits, according to the manufacturer's instructions, and growth parameters at different temperatures and pHs were also studied. Its behavior against commonly used antibiotics in clinical practice was also determined by an agar diffusion method according to the Clinical and Laboratory Standards Institute [13].

2.4. Chemical characterization of the ethanol extract

To obtain secondary metabolites of a polar soluble nature in ethanol, bacterial mass obtained from a solid culture medium (R_2A agar) was transferred to a 50 ml tube with absolute ethanol, in a ratio of 100 mg of bacterial mass in 1 ml of ethanol [14]. The cell mixture with ethanol was submitted to ultrasonication for 1 h using a Ney Ultrasonik 57H, in order to cause cell rupture and obtain soluble material [15]. Afterwards, the sample was separated by centrifuge and the supernatant was concentrated by lyophilization. Finally, the concentrate was resuspended in 70% ethanol.

The extract was analyzed by high performance liquid chromatography (HPLC) in a Jasco machine with a multi-wavelength detector and equipped with a C18 column (Kromasil 100-5C18 250 × 4.6 mm). The separation was done in a gradient mobile phase from 50:50 to 75:25 methanol:water, with a flow of 1 ml min^{-1} at room temperature. The volume of the injected sample was 100 μl and the running time was 20 m. The absorption spectrum, in the UVvisible range (200–800 nm) was studied using a Shimadzu UV-1603 spectrophotometer.

2.5. Janthinobacterium sp. SMN 33.6 antibacterial activity assay

The strains used in the initial assays of antimicrobial activity were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 14028 and *Kocuria rhyzophila* ATCC 9341 from the bacterial collection of the Laboratory of Investigation in Antibacterial Agents from the Department of Microbiology at the

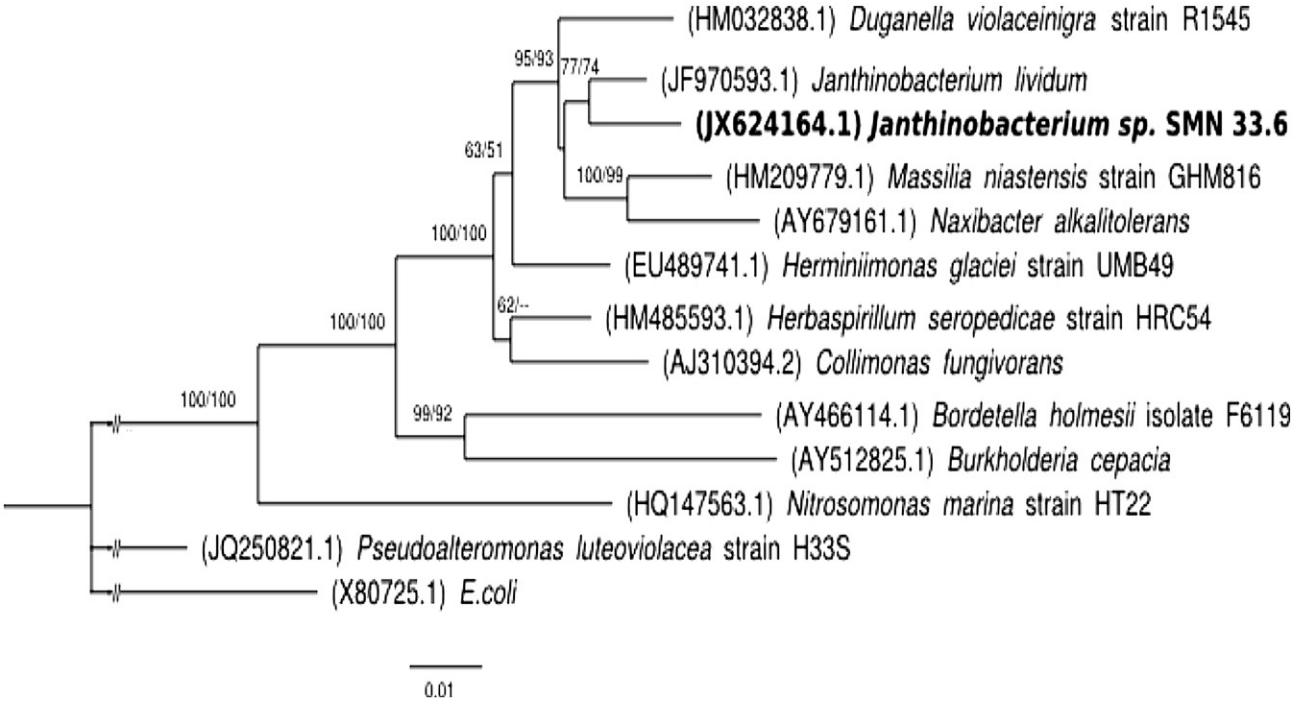

Fig. 1. Phylogenetic analysis based on maximum likelihood phylogenetic tree of 16S rDNA sequences showing the estimated phylogenetic relationships of strain SMN 33.6 with other *Janthinobacterium* and *Duganella* strains. Numbers at nodes are support values (bootstrap values).

Universidad de Concepcion, Chile. Multi-resistant strains were also included, and corresponded to strains isolated from clinical specimens in several Chilean hospitals (Table 1). The cultures were done in a nutritional medium LB at 37°C. Growth was evaluated by measuring the optical density at 600 nm with a Shimadzu UV-1603 spectrophotometer.

The screening of inhibitory activity was studied by the agar spot test described by Fleming et al. [16], with modifications, that involved culture of the strain SMN 33.6 on nutritive agar, in a tip shaped form until the growth reached 2–4 mm, then a second layer of LB agar (10 ml) with 50% agar was added, inoculated with 100 µl of the pathogenic bacterial strain to test, in exponential growth phase. The plates were incubated at 37°C for 24 h. The antibacterial activity was evaluated by the presence of an inhibition zone around the colony of the strain SMN 33.6.

The minimum inhibitory concentration (MIC) was evaluated with a standard method of serial microdilution in Mueller–Hinton broth (MH) using microtiter plates. Each assay was performed in triplicate and the value repeated at least two times was considered as the MIC. The bacterial inoculum used was 5×10^5 UFC ml^{-1}, obtained from a 0.5 McFarland turbidity standard (1.5×10^8 UFC ml^{-1}). Microtiter plates were incubated at 35°C for 24 h and MIC was registered as the lowest concentration without turbidity. The minimum bactericidal concentration (MBC) was determined, transferring broth from wells without growth to a MH agar plate without extract and incubating at 35°C for 24 h.

3. Results and discussion

From a total of 237 strains isolated on R_2A agar, from soil samples collected in King George Island, Antarctica (62°11′3.31″S, 58°56′1.18″ W), a violet Gram-negative bacteria was selected, and named as strain SMN 33.6. The initial assay of antibacterial activity demonstrated growth inhibition of *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *S. typhimurium* ATCC 14028 and *K. rhyzophila* ATCC 9341, which was verified by clear inhibition zones surrounding the SMN 33.6 strain colony (data not shown). The potent inhibition that the strain showed in the "Agar Spot Test" assay evidenced the production of compounds with antibacterial activity capable of diffusion in a solid medium and active against Gram-positive and Gram-negative bacteria.

The culture of SMN 33.6 on agar R_2A produced colonies with violet pigmentation, not observed in liquid cultures of the same medium, so the contact with a substrate could activate higher production of this characteristic pigment. The strain corresponded to an oxidase producing rod-shaped bacterium. The biochemical

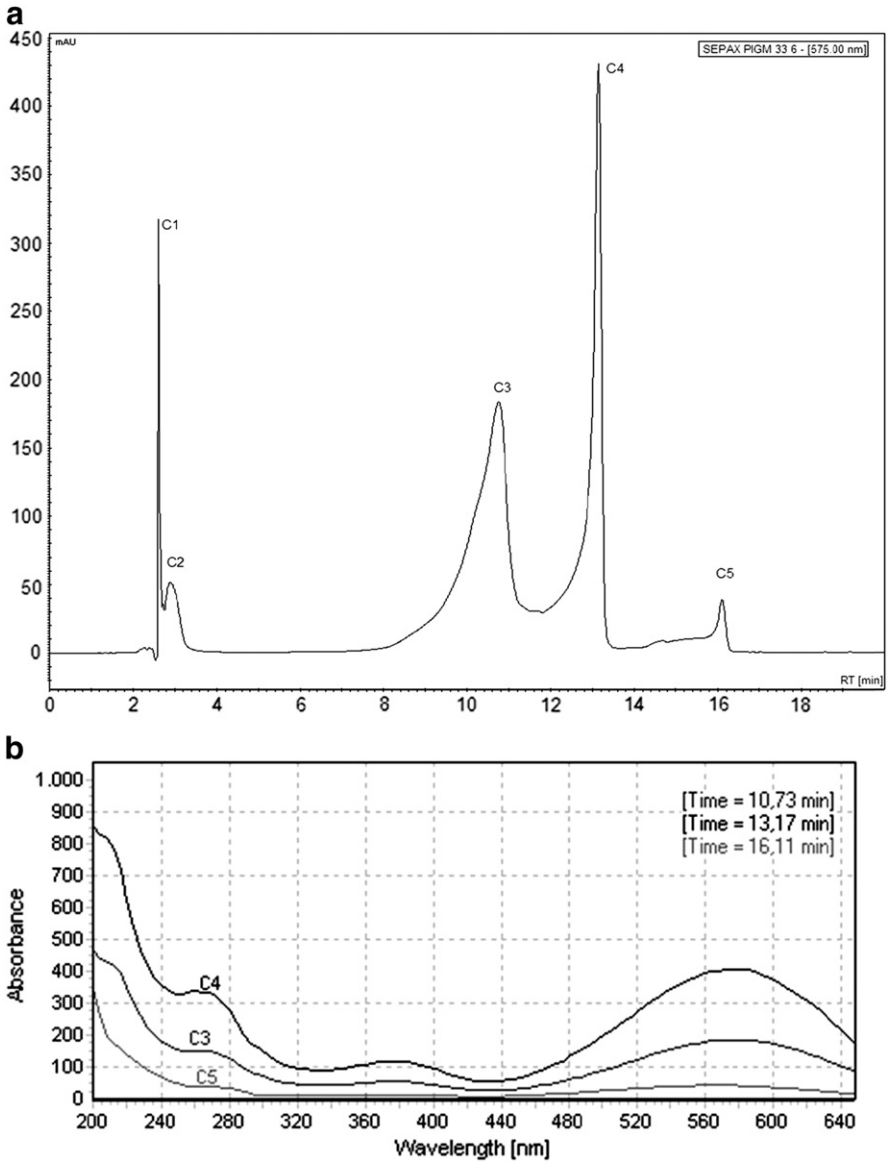

Fig. 2. Purification and characterization of bacterial pigment from *Janthinobacterium* sp. SMN 33.6. (a) HPLC of ethanol extract. The C18 column was used to separate the different peaks, and the compounds C3 and C4 (violacein) are the major peaks. (b) Absorption spectra of pigmented compounds, C3, C4 (violacein) and C5 with a maximum in the visible range at 576 nm.

assays with the API 20NE gallery showed positive activity to reduce nitrate, and assimilate glucose, arabinose, mannose, mannitol, maltose, potassium gluconate and malate. The assay with the API ZYM gallery demonstrated positive activity for alkaline phosphatase, naphthol-AS-BI-phosphohydrolase and α-glycosidase. The optimum growth temperature was 20°C which occurred during all the studied range of pH (5–9), pH 7 being the optimum.

In assays for molecular identification of SMN 33.6, a PCR product of approximately 1500 bp was amplified from the 16S rRNA gene, and a sequence of 1366 bp was obtained (Genbank Accession Number: JX624164). According to the homology analysis of nucleotide sequence, this strain exhibits a 97.4% similarity with the species *Janthinobacterium lividum*. However, there is also a high homology with strains from the genera *Duganella* (99%) and from this, the species *Duganella violaceinigra* is reported as a producer of the violacein pigment. This latest bacteria is a mesophilic bacteria with optimum growth between 28 and 30°C [17], while SMN 33.6 was not able to grow at these temperatures. The phylogenetic tree corroborated this similarity, grouping this strain with others of the Genus *Janthinobacterium* (Fig. 1). However, the results of API 20 NE present differences with information for the *J. lividum* NCTC 9796 strain in respect to the hydrolysis of aesculin and gelatin and assimilation of trisodium citrate, which would indicate atypical strains of the species [18,19].

The results of the behavior against some antibiotics indicate that SMN 33.6, presents susceptibility to cefotaxime, kanamycin, amikacin, gentamicin, tetracycline, nalidixic acid, ciprofloxacin and rifampicin. It possesses intermediate susceptibility to streptomycin and chloramphenicol, and is resistant to ampicillin, cephalothin, cefoxitin, cefuroxime and trimethoprim. Resistance to beta-lactam antibiotics could be explained by the production of an enzyme belonging to a new molecular class B metallo-β-lactamase, as was reported for an environmental strain of *J. lividum* by Rossolini et al. [20].

Strains of Genus *Janthinobacterium* with violet pigmentation have been isolated from Antarctica since 1981 [18], studying characteristics associated to its growth and physiology. On the other hand, various violet pigmented bacteria, obtained from temperate environments, have been studied in regard to the biological properties of the violet pigment they produced. This pigment is known as violacein which is an indole type of compound, derived from tryptophan metabolism and is produced by various bacteria such as *Chromobacterium violaceum*, *Pseudomonas luteoviolacea*, *Duganella* sp. and *Collimonas* sp., as well as, the already mentioned Genus *Janthinobacterium* [21,22]. This pigment is intracellular and the most important disadvantage to use it as a therapeutic compound is its poor solubility in water and biological fluids; therefore, it diffuses to a lesser degree than polar compounds in agar medium, so the activity obtained in the agar spot test could be due to a sum of antagonistic activities of compounds of other nature, such as diffusible peptides or enzymes [23,24,25].

The ethanol extract obtained from the strain SMN 33.6 biomass, has a violet coloration and presents absorption peaks at 575 and 375 nm and minimum at 431 nm. The chromatogram obtained by HPLC, shows the presence of five majority compounds, three of them colored, with maximum absorption close to 575 nm, which correspond to maximum absorption reported for violacein by Durán et al. [26]. The retention times are 11.7, 13.1 and 16.1 min (Fig. 2). However, the similitude in the absorption spectrum of these three compounds, and the difference in the time of retention, demonstrated that these compounds are different from each other. To identify violacein, the test described by Gillis and Logan [27], was carried out. Sulfuric acid was added at 10% (v/v), changing the coloration of the compounds, indicated as C3 and C4, and turning from violet to green. The C3 compound showed a maximum at 699 nm and a minimum at 489 nm of absorbance after the acid treatment, while the C4 compound had a maximum at 700 nm and a minimum at 497 nm. These results are consistent with violacein behavior which has a reported absorbance of 700 nm as a maximum and 502 nm as the minimum. By adding sodium hydroxide, the solution that contains violacein must turn into a green color and then to a reddish brown color. The C4 compound changed color as indicated, while the C3 turned immediately to reddish brown. Therefore, it is deduced that the C4 compound collected corresponds to violacein, which is 35% of the sample. The collected C3 compound corresponds to compounds derived from this one, such as oxyviolacein or deoxyviolacein [15,28]. However, the compound indicated as C5 did not change color with the acid and base treatments, indicating that this could be a new compound and future studies to identify and clarify its chemical structure are needed.

The violacein presents a wide antimicrobial spectrum since it has demonstrated activity against Gram-negative and Gram-positive bacteria, being more active upon these last ones, as well as antifungal activity against *Rosselinia necatrix*, a phytopathogenic fungus [26]. On the other hand, antimycobacterial activity in vitro against *Mycobacterium tuberculosis* [28], mild antiparasitic activity against *Trypanosoma cruzi*, and activity against tumor cells have been demonstrated; and when adding 10% deoxyviolacein, it also has antiviral activity against the *Herpes simplex* virus [29]. Lately, it has been demonstrated that the purple pigment also has antibacterial properties against methicillin resistant *Staphylococcus aureus*, proposing a possible binding to the bacterial DNA as mechanism of action [25]. These reports demonstrate a high biotechnological potential associated to the use of this compound in medicine, even though *C. violaceum* has been reported as an etiologic agent of serious infections in humans and it represents a difficult-to-treat entity, so special attention is required [30]. Violacein would not be involved in its pathogenicity; therefore, isolating new violacein producing strains or other molecules with antimicrobial activity, could contribute to the diversification in the application of natural products produced by bacteria.

The analyzed extract showed antibacterial activity over eight multi-resistant strains of Gram-negative bacilli isolated from clinical specimens in Chilean hospitals, exhibiting different MIC. The assayed species corresponded to an *Acinetobacter baumannii* strain and a *Pseudomonas aeruginosa* strain, both carbapenemase producers; two strains of *E. coli* and two strains of *Klebsiella pneumoniae*, producing extended-spectrum β-lactamases (ESBL) and two multi-resistant strains of *Serratia marcescens* producing a chromosomal AmpC beta-lactamase (Table 1). The MIC values ranged between 0.5 and 16 μg ml^{-1}. The minimum MBC varied between 2 and 16 μg ml^{-1} (Table 1).

The majority of the strains tested correspond to multi-resistant Gram-negative bacilli, that produce ESBL and carbapenemases, which are important human pathogens and main etiologic agents causing hospital-acquired infections in Chile and worldwide. ESBL producing strains and *A. baumannii* have been catalogued by the *Antimicrobial Availability Task Force* (AATF) of the *Infectious Diseases Society of America* (IDSA) as particularly problematic pathogens [5]. In recent years, a progressive increase in isolation of these bacteria has been observed in outbreaks in several hospitals, with an elevated morbidity and mortality. In the last decade strains of *A. baumannii* often exhibiting multidrug resistance have emerged as a significant clinical problem worldwide [31,32,33].

The extract showed a low MIC, 1 mg ml^{-1}, against the strain of *P. aeruginosa* tested, a VIM-2 carbapenemase-producer, which also presents multiple resistance to antibiotics and has limited therapeutic alternatives [34]. The metallo-enzymes, belonging to the IMP and VIM family are more frequent and are found disseminated worldwide. These have the capacity to hydrolyze all the penicillins, cephalosporins, and carbapenems [35].

S. marcescens is increasingly recognized as a cause of morbidity in nosocomial settings. It can act as an etiologic agent in several infections affecting humans. When transmitted to a patient, it colonizes wound surfaces, mucosal areas or indwelling devices [36]. The strains are resistant to rifampicin, penicillin, ampicillin, amoxicillin, tetracycline, amoxicillin–clavulanic acid, cefazolin, cefamandole, polymyxin B, colistin, fusidic acid, lincosamides, streptogramins, daptomycin, linezolid, and

cefuroxime [37]. The strain extract was capable of inhibiting growth of both strains of *S. marcescens* included in this study with an MIC of 0.5 and 2 μg ml^{-1}.

The activity of the ethanolic extract against *E. coli* strains cannot be explained for the violacein presence. De Azevedo et al. [38] have been reported a low acute toxicity of purified violacein against strains of this specie. Wang et al. [39] reported strong activities of violacein derivatives, deoxyviolacein and oxyviolacein against Gram-positive bacteria and antifungal activity. Oxyviolacein also showed activity against *Phytophtora capsici*, a pathogen of vegetable crops. This result suggest that there are different compounds present in the chromatogram which could be exerting such inhibitory activity or acting synergistically with violacein.

The results obtained in this study demonstrate the biotechnological potential of natural products obtained from Antarctic microorganisms as a tool for the control of hospital-acquired infections (infections associated with health care) which are difficult to treat. We demonstrate that the ethanolic extract of *Janthinobacterium* sp. SMN 33.6 could be useful in the growth control of bacterial pathogens, even against those multi-resistant strains isolated from hospitals in agreement with that reported by Huang et al. [25] but against Gram-negative bacilli. Future studies should be conducted concerning the identification of all the compounds present in the extract and the evaluation of their antibacterial activity, to clarify its nature, mechanism of action and regulation.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

This research was supported by grant number 07CN13PXT-264 from Innova-CORFO and grant number T-17-08 from INACH. The authors would like to thank personnel at INACH for their logistic support in Antarctica.

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