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ORIGINAL PAPER

Marine Sediment Recovered *Salinispora* sp. Inhibits the Growth of Emerging Bacterial Pathogens and other Multi-Drug-Resistant Bacteria

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Abstract

Marine obligate actinobacteria produce a wide variety of secondary metabolites with biological activity, notably those with antibiotic activity urgently needed against multi-drug-resistant bacteria. Seventy-five marine actinobacteria were isolated from a marine sediment sample collected in Punta Arena de La Ventana, Baja California Sur, Mexico. The 16S rRNA gene identification, Multi Locus Sequence Analysis, and the marine salt requirement for growth assigned seventy-one isolates as members of the genus *Salinispora*, grouped apart but related to the main *Salinispora arenicola* species clade. The ability of salinisporae to inhibit bacterial growth of *Staphylococcus epidermidis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacer baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. was evaluated by cross-streaking plate and supernatant inhibition tests. Ten supernatants inhibited the growth of eight strains of *S. epidermidis* from patients suffering from ocular infections, two out of the eight showed growth inhibition on ten *S. epidermidis* strains from prosthetic joint infections. Also, it inhibited the growth of the remaining six multi-drug-resistant bacteria tested. These results showed that some *Salinispora* strains could produce antibacterial compounds to combat bacteria of clinical importance and prove that studying different geographical sites uncovers untapped microorganisms with metabolic potential.

Key words: *Salinispora*, *emerging bacterial pathogens*, multi-drug-resistant bacteria, MLSA, Punta Arena de la Ventana

Introduction

The ESKAPE pathogens (Boucher et al. 2009; Pendleton et al. 2013) is an acronym used to designate a group of organisms formed by *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacer baumannii, Pseudomonas aeruginosa*, and species of *Enterobacter.* These bacteria usually cause infections in patients with immunosuppressed conditions and critical illnesses and are characterized by multiple antimicrobial resistance mechanisms (Pendleton et al. 2013; Partridge et al. 2018). Similarly, *Staphylococcus epidermidis* has recently been related to nosocomial infections derived from medical devices, like catheters, intracardiac valves, and needles due to biofilm (McCann et al. 2008; Buttner et al. 2015; Flores-Paez et al. 2015). Although there is a wide range of antibiotics for nosocomial infections, they are not effective in combating multi-drug-resistant bacteria present in the clinical environment.

Filamentous actinobacteria are well known for their ability to synthetize a great variety of antimicrobial, antifungal, antiviral, and anti-inflammatory molecules (Berdy 2012). Marine ecosystems encompass diverse genera of actinobacteria such as *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Saccharomonospora*, *Plantactinospora*, *Salinispora*, *Solwaraspora*, and *Streptomyces* among others (Maldonado et al. 2009; Jose and Jha 2017; Contreras-Castro et al. 2018). Marine actinobacteria have been primarily isolated from marine sediments around the world (Mincer et al. 2002; Gontang et al. 2007; Jose and Jha 2017) but also from

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other marine sources like sponges (Kim et al. 2006; Vidgen et al. 2011). Marine actinobacteria are prolific sources of unique novel bioactive compounds (Jose and Jebakumar 2013; Subramani and Sipkema 2019; Amin et al. 2020), and *Salinispora* (Maldonado et al. 2005a ; Jensen et al. 2015a) is the only marine obligate genus within the class *Actinobacteria* (Stackebrant et al. 1997). *Salinispora arenicola* and *S. tropica* (Maldonado et al. 2005a) and *S. pacifica* (Ahmed et al. 2013), are the only validly described species of the genus (at time of writing) and produce different bioactive molecules (Jensen et al. 2015b; Jensen 2016), like arenicolides, salinikinones, staurosporines, and salinisporamide A; the latter being a molecule for the treatment of multiple refractory myeloma that has completed phase 1 of clinical trials (Jensen et al. 2015b; Richardson et al. 2016).

There is evidence that certain compounds and their associated biosynthetic gene clusters may be fixed at the species level due to a strong selective advantage, which suggests that some secondary metabolites represent ecotype-defining traits for *S. tropica* and *S. arenicola*, although not for *S. pacifica*. The more metabolically diverse species is *S. pacifica* and these bacteria are currently undergoing series of nascent speciation events, which may lead to fixing pathways at the species level (Ziemert et al. 2014; Millan-Aguinaga et al. 2017). *Salinispora* strains isolated from distinct locations may produce new molecules, though an accurate identification of the isolate is compulsory (Goodfellow and Fiedler 2010). It is essential to evaluate bacterial growth inhibition by new marine *Salinispora* strains to find novel antibiotics for fighting the organisms of clinical importance and multi-drug-resistant bacteria. In the present work, obligate marine actinobacteria isolated from Punta Arena de la Ventana (PAV), the Gulf of California (GC), Mexico, were identified as species of the genus *Salinispora*, and its potential to inhibit the growth of *emerging bacterial pathogens* strains and multi-drug-resistant bacteria was evaluated.

Experimental

Materials and Methods

The procedure for selective actinobacteria isolation and preliminary characterization. Sediment was collected from 10 m depth from PAV, the GC, Mexico (N 24°03'40" W 109°49'52") and preserved at –80°C until processing. The isolation procedure was carried out as previously described (Maldonado et al. 2005b) with slight modifications. In brief, 1 g of wet sediment was transferred into a 15 ml universal tube, which contained 9 ml of salt solution (0.9% of artificial seawater; Instant Ocean, USA). A series of dilutions were then prepared up to 10^{-4}, and each dilution was used to inoculate a set of isolation plates. Two different media and two different conditions were tested. The first medium was GYM (Glucose Yeast Extract-Malt Extract Agar, DSMZ-Medium 65), and the second medium was GYEA (Glucose Yeast Extract Agar, Gordon and Mihm 1962). One set of the plates included 50 μg/ml of rifampicin (Sigma-Aldrich), and 50 μg/ml of nystatin (Bristol Myers Squibb), whereas the other set did not include any antibiotics or antifungal compounds.

All media were prepared with artificial seawater (Instant Ocean, USA). These media have been used to characterize and isolate members of the family Micromonosporaceae (Wiese et al. 2008; Maldonado et al. 2009; Maldonado and Quintana 2015; Carro et al. 2019). Isolation plates were incubated at 30°C (IncuMax^{TM} IC-320 Incubator, Amerex Instruments, Inc., USA) for at least eight weeks. To avoid desiccation, plates were folded using two plastic bags under a humid atmosphere in the incubator. The resulting cultivated actinobacteria were detected and selected based on typical colonial morphology as members of the *Micromonosporaceae* family (Genilloud 2015), namely, orange to dark brown or black colonies lacking aerial mycelium was picked up. Spore formation in *Salinispora* occurs when colonies change from orange colour and turn black (Jensen et al. 2015a). Pure cultures were grown on GYM (30°C, 7–14 days) and then inoculated onto artificial sea water-ISP media 1 to 7 (International Streptomyces Project media; Shirling and Gottlieb 1966), in order to observe the colonial morphology and phenotypic heterogeneity of the bacteria. ISP media are used to characterize not only *Streptomyces*, but also other *Actinobacteria* genera known to produce secondary metabolites, particularly antibiotics. The marine salt requirement was tested on the seventy-five isolates and recorded accordingly (Maldonado et al. 2005a).

DNA extraction and PCR amplification of 16S rRNA and MLSA genes of *Salinispora*. Genomic DNA was extracted using standard procedures reported previously (Maldonado et al. 2005b). Universal primers 27f and 1525r were used for the 16S rRNA gene amplification (Lane 1991). For Multi-Locus Sequence Analysis (MLSA) genes, the set of primers previously reported were used (Rong and Huang 2014). One set of extra primers for the gene *secY* (Adekambi et al. 2011) was modified and included for the MLSA studies. The full list of primers for MLSA is shown in Table I. The concentration of the PCR reagents was: 100 ng μl^{-1} of DNA template, 5 μl 10x DNA polymerase buffer, 1.5 μl $MgCl_2$ (50 mM stock solution, Bioline), 1.25 μl dNTP (10 mM stock mixture, Bioline), 0.5 μl of each primer (20 μM stock solution), 2 units of Taq polymerase (Bioline) made up to 50 μl with ultra-pure Milli-Q water. Amplification was achieved using a Techno 512 gra-

Table I
Primers for the MLSA amplification.

Gene	Primer sequence (5'-3')	Product size (bp)	Reference
atpD	ATPDF2 – CTTGCGGTGYATSGACCA	910	Rong and Huang 2014
	ATPDR3 – GAAGAASGCCTGYTCNGG		
gyrB	GYRBF – GAGGTCGTGCTGACCGTGCTGCACGCGGGCGGCAAGTTCGGC	781	
	GYRBR – ATGGCGGACGCCGACGTCGACGGCCAGCACATCAAC		
rpoB	MYCOF – GGYAAGGTCACSCCSAAGGG	730	
	MYCOR – ARCGGCTGCTGGGTRATC		
secY	SECYF – GGCATCATGCCCTACATCAC	797	Adekambi et al. 2011
	SECYR – AAACCGCCGTACTTCTTCAT		

dient machine using a protocol previously described (Maldonado et al. 2005a). PCR products were checked by electrophoresis (agarose, 1%) and purified using a QIAquick PCR purification Kit (QIAGEN, Germany). Purified products were sequenced using the commercial service of MACROGEN (Maryland, USA). The isolates' 16S rRNA gene sequences were compared against public databases using the BLAST option of the GenBank website (http://www.ncbi.nlm.nih.gov/). Manual alignment using SEAVIEW (Gouy et al. 2010) of the 16S rRNA gene sequences from the BLAST option was then employed to infer the phylogenetic position of each isolate. Phylogenetic trees were constructed individually for each gene to confirm phylogeny. Best Maximum Likelihood (ML) models were calculated using JModelTest v.2.1.10 (Guindon and Gascuel 2003; Darriba et al. 2012). With the best model, phylogenetic trees were constructed using Bayesian Analysis (BEAST v.1.8.4 (Suchard et al. 2018)), 30 million MCMC, 10% burn-in, 1000 sample frequency) and ML (phyML 3.0; (Guindon et al. 2010)), 1,000 bootstrap) and viewed with FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Six strains were selected for the MLSA after the 16S rRNA gene phylogeny and BOX-PCR analysis (the latter to reduce the number of strains to be studied; data not shown) and a concatenated sequence of 4,349 bp was built with all the genes mentioned previously. BOX-PCR was carried out according to the authors' protocol (Versalovic 1994). The BOX primer A1R (5'-CTACGGCAAGGCGACGTGACG-3') was used with 10% DMSO (v/v; Baker®) in the mixture reaction. For the amplification, the reaction began with a hot start of 10 min at 95°C, followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 45 s at 50°C and elongation for 2 min at 72°C, with a final elongation point of 10 min at 72°C (MultiGene™ Optimax, Labnet, USA). Based on the genomic fingerprinting observed by BOX-PCR, the strains that presented different patterns were selected for MLSA. Accession numbers are shown in Appendix 1 for the selected strains.

***S. epidermidis* and ESKAPE clinical isolates.** The *S. epidermidis* clinical isolates from ocular infection (n = 8) were obtained from patients at the "Instituto de Oftalmología Fundación Conde de Valenciana" (IOFCV), Mexico City, Mexico. The *S. epidermidis* prosthetic joint infection isolates (n = 10) were obtained from orthopedic infections from the "Instituto Nacional de Rehabilitación Luis Guillermo Ibarra Ibarra" (INR), Mexico City, Mexico. The ESKAPE group: *E. faecium, S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp. were obtained from wound, urine, and blood samples from patients of INR. The general characteristics of each isolate are shown in Table II. The antimicrobial susceptibility tests were carried out, analyzed, and interpreted by the Vitek 2 computerized system (software 0.8.01; 2017) using the sensitivity card for Gram-positive and Gram-negative bacteria, according to the criteria of the Clinical Laboratory Standards Institute (CLSI).

Cross-streaking plate technique for the growth inhibition test. The cross-streaking plate technique described previously (Quintana et al. 2015) was followed with two minor modifications: (a) the use of non-aerial mycelia forming actinobacteria (i.e., *Salinispora*) instead of *Streptomyces,* and (b) the addition of seawater to the GYM media for the bioassays. Biomass of ESKAPE bacteria and *S. epidermidis* that previously grew at 37°C for 18 h on GY broth (Glucose Yeast Extract) was used. Salinisporae were prepared according to a McFarland Nefelometer tube No. 5 (i.e., 1.5×10^9 CFU/ml). Fifteen microliters of each isolate were inoculated and dispersed in 2 cm of the left side of a Petri dishes, which were then incubated for three weeks at 30°C. To avoid desiccation, the plates were treated as mentioned above for humidity conditions in the incubator. After three weeks, 7 μl of a suspension from fresh cultures of ESKAPE bacteria or *S. epidermidis* clinical isolates biomass was spread out in perpendicular position 5 cm (right to the left) of the Petri dish growing the *Salinispora* (one different ESKAPE bacteria per

Table II
Characteristics of clinical isolates.

Clinical isolate	Source of infection	Antibiotic resistance
Staphylococcus epidermidis 146	Corneal ulcer	Oxacillin, ofloxacin, tobramycin, cefalotin, ceftriaxone, sulfisoxazole
Staphylococcus epidermidis 144	Corneal ulcer	Neomycin, gentamicin, ceftazidime, ceftriaxone, tetracycline, sulfisoxazole
Staphylococcus epidermidis 199	Corneal ulcer	Norfloxacin, ceftazidime, ceftriaxone, polymyxin B, sulfisoxazole
Staphylococcus epidermidis 2022	Corneal ulcer	Gentamicin, ceftazidime, ceftriaxone, tetracycline, sulfisoxazole
Staphylococcus epidermidis 1654	Corneal ulcer	Norfloxacin, ceftazidime, ceftriaxone
Staphylococcus epidermidis 2050	Conjunctivitis	Ofloxacin, tobramycin, gentamicin, norfloxacin, cefalotin, ceftazidime, tetracycline, sulfisoxazole
Staphylococcus epidermidis 2038	Endophthalmitis	Oxacillin, tobramycin, gentamicin, ceftazidime, ceftriaxone, tetracycline
Staphylococcus epidermidis 63	Endophthalmitis	Ofloxacin, tobramycin, gentamicin, norfloxacin, ceftazidime, ceftriaxone, polymyxin B, tetracycline, sulfisoxazole
Staphylococcus epidermidis 112IP	Hip	Oxacillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, rifampin, trimethoprim-sulfamethoxazole
Staphylococcus epidermidis 675IP	Knee	Oxacillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, tetracycline, rifampin, trimethoprim-sulfamethoxazole
Staphylococcus epidermidis 085IP	Hip	Oxacillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, tetracycline
Staphylococcus epidermidis 1302IP	Hip	Oxacillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, clindamycin
Staphylococcus epidermidis 583IP	Hip	Oxacillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, tetracycline, trimethoprim-sulfamethoxazole
Staphylococcus epidermidis 563IP	Hip	Oxacillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, tetracycline, trimethoprim-sulfamethoxazole
Staphylococcus epidermidis 536IP	Hip	Oxacillin, ciprofloxacin, levofloxacin, moxifloxacin, clindamycin
Staphylococcus epidermidis 274IP	Hip	Oxacillin, ciprofloxacin, levofloxacin, moxifloxacin
Staphylococcus epidermidis 587IP	Hip	Oxacillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, clindamycin
Staphylococcus epidermidis 848IP	Hip	Oxacillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin
Klebsiella pneumoniae	Urine sample	Ceftazidime, ceftriaxone, cefepime, doripenem, etapenem, meropenem,
		imipenem, amikacin, gentamicin, ciprofloxacin, rifamycin
Enterobacter cloacae	Wound infection	Ceftazidime, ceftriaxone, cefepime, doripenem, etapenem, meropenem, imipenem, amikacin, gentamicin, ciprofloxacin, rifamycin
Acinetobacter baumannii	Blood sample	Piperaciline, ceftazidime, ceftriaxone, cefepime, doripenem, etapenem, meropenem, imipenem, amikacin, gentamicin, ciprofloxacin, rifamycin
Pseudomonas aeruginosa	Blood sample	Piperaciline, ceftazidime, ceftriaxone, cefepime, doripenem, etapenem, meropenem, imipenem, amikacin, gentamicin, ciprofloxacin, rifamycin
Staphylococcus aureus	Wound infection	Oxacillin, gentamicin, ciprofloxacin, erythromycin, clindamycin, tetracycline, trimethoprim-sulfamethoxazole, penicillin, rifamycin
Enterococcus faecium	Urine sample	Erythromycin, clindamycin, tetracycline, penicillin, rifamycin

line, separated by 1 cm, or one different *S. epidermidis* isolate). Petri dishes were incubated for two extra days and checked visually. A positive score for the *Salinispora* against the ESKAPE group and *S. epidermidis* was considered when no growth or partial inhibition was observed in each line, although the comparison with a control plate also analyzed morphological variations of the affected bacteria without *Salinispora*. Forty-two *Salinispora* sp. were studied to inhibit bacterial growth of *S. epidermidis*, for which eight clinical *S. epidermidis* strains from ocular infection were used to carry out the cross-streaking plate technique technique. After the first assay, ten salinisporae were selected to test against the ESKAPE group: *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp.

Bacterial growth inhibition assay in a microplate. The ten selected salinisporae (9'4, 33'5, 9'2, 9'8, 9'17, 10'2, 14'1, 33'6, 33'9, and 34'12) were inoculated in 250 ml Erlenmeyer flaks containing 100 ml of liquid GYM and incubated for one month at 30°C with agitation (180 rpm; Thermo Scientific Q6000). The cultures were transferred to 50 ml universal tubes and centrifuged at 3,000 rpm for 5 minutes, collected, filtered through a 0.2-μm membrane and stored at –80°C until further use. *S. epidermidis* and ESKAPE bacteria were grown in tryptic soy broth (TSB, DIFCO) media overnight. The culture was diluted 1:200 with fresh TSB and 10 μl were inoculated in a flat-bottom 96-well microplate with 50–50% TSB supplemented with the supernatant obtained from the actinobacteria culture. The wells with TSB media were used as growth control. The plate was incubated at 37°C for 24 hours. Microbial growth was determined by optical density at 600 nm (OD 600) in a multi-scan spectrophotometer (Multiskan GO, Thermo Scientific). The results were analyzed using two-way ANOVA and Tukey tests. Graphs were created with GraphPad Prism 8.0.2.

Results

Actinobacterial isolation and identification. A total of seventy-five actinobacteria were isolated using two different media and the two different conditions. Seventy-one isolates were obligate marine actinobacteria, two non-marine obligate actinobacteria, and two non-obligate bacteria. Forty-two obligate marine actinobacteria were identified by the 16S rRNA gene sequencing and preliminarily characterized by the ISP media. Non-marine obligate actinobacteria were assigned to the genera *Micromonospora* (1), *Mycobacterium* (1), and non-marine obligate bacteria identified as *Erythrobacter* (1), and *Lutibacterium* (1), respectively.

Using the ISP media and based on the abundance of spore production, two different groups of salinisporae were formed (Appendix 1). The alignment analysis of the 16S rRNA gene sequences using BLAST showed that the closest genetic neighbors of the forty-two strains belonged to the genus *Salinispora* with a percentage of identity between 97–99%. The phylogenetic 16S rRNA gene tree of the forty-two strains with various *Salinispora tropica*, *S. pacifica*, *S. tropica* strains, and *Micromonospora* as an outgroup showed that the strains grouped inside the *Salinispora* clade, which confirmed that they belonged to this genus (Fig. 1). Strain 9’17 was outside the *S. arenicola* clade with posterior probability support. According to the preliminary characterization, phylogenetic analysis, and BOX-PCR profiles (data not shown), six strains were then selected for MLSA. MLSA not only confirmed that most of the isolates from PAV form a subclade within the *S. arenicola* clade, but also showed a high degree of differences amongst them. The clades formed in the concatenated analysis of the MLSA were supported by the ML analysis (Fig. 2). Moreover, MLSA assigned strain 9’17 within the *S. arenicola* group.

Determination of bacterial growth inhibition ability of *Salinispora*. Ten out of the forty-two strains of *Salinispora* sp. inhibited bacterial growth of all eight *S. epidermidis* strains studied. In the next assay, the supernatants of ten strains of *Salinispora* sp. that passed the first assay were tested against different bacterial species using different proportions of supernatant (25 or 50%) in the 96-well microplates. Optical density OD 600 was analyzed to define the statistical significance. Ten different clinical isolates of *S. epidermidis* from joint infections were tested. Only two supernatants from *Salinispora* sp. strain 9’4 and *Salinispora* sp. strain 33’5 showed the ability to consistently inhibit the growth of *S. epidermidis* clinical strains (concentration of 50%) from the two different sources of infection (ocular and prosthetic joint) with the statistical significance (Fig. 3a).

A third assay for testing *Salinispora* sp. (9’4 and 33’5) against *E. faecium, S. aureus, K. pneumoniae*, *A. baumannii, P. aeruginosa,* and *Enterobacter* spp. was done using the cross-streaking plate method and the microplate assay. The cross-streaking plate technique showed partial inhibition of bacterial growth and morphological changes of *E. faecalis* and *S. aureus* (i.e., *S. aureus* biomass change from typical opaque yellow to transparent). The microplate assay showed that supernatants from *Salinispora* sp. 9’4 and 33’5 inhibited the growth of ESCAPE bacteria with the statistical significance (Fig. 3b).

Discussion

Since the release and analysis of the whole genome sequencing of *S. tropica* (Udwary et al. 2007), it has been established that all members of the genus *Salinispora* can produce bioactive molecules at a similar level as *Streptomyces* does. In the present work, the selected organisms from a collection of obligate marine organisms of the genus *Salinispora* isolated from PAV, the GC, Mexico, were evaluated in terms of their antibacterial ability against emerging bacterial pathogens and multidrug-resistant bacteria. Two supernatants of selected *Salinispora* sp. inhibited the bacterial growth of *S. epidermidis* strains and *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp.

The preliminary characterization of the isolates recovered on ISP media 1 to 7 showed their high phenotypic heterogeneity. According to the spore production, two groups were formed. It is known that several secondary metabolites are expressed during germination (Cihak et al. 2017); thus, a difference in spore formation might lead to a different metabolic potential. Seventy-one out of seventy-five isolates required marine seawater for growth hence suggesting their assignment to the genus *Salinispora* (Maldonado et al. 2005). To our knowledge, this is the first time that such a large colony morphological study was performed on salinisporae isolates besides the description of the currently three species of the genus (Ahmed et al. 2013;

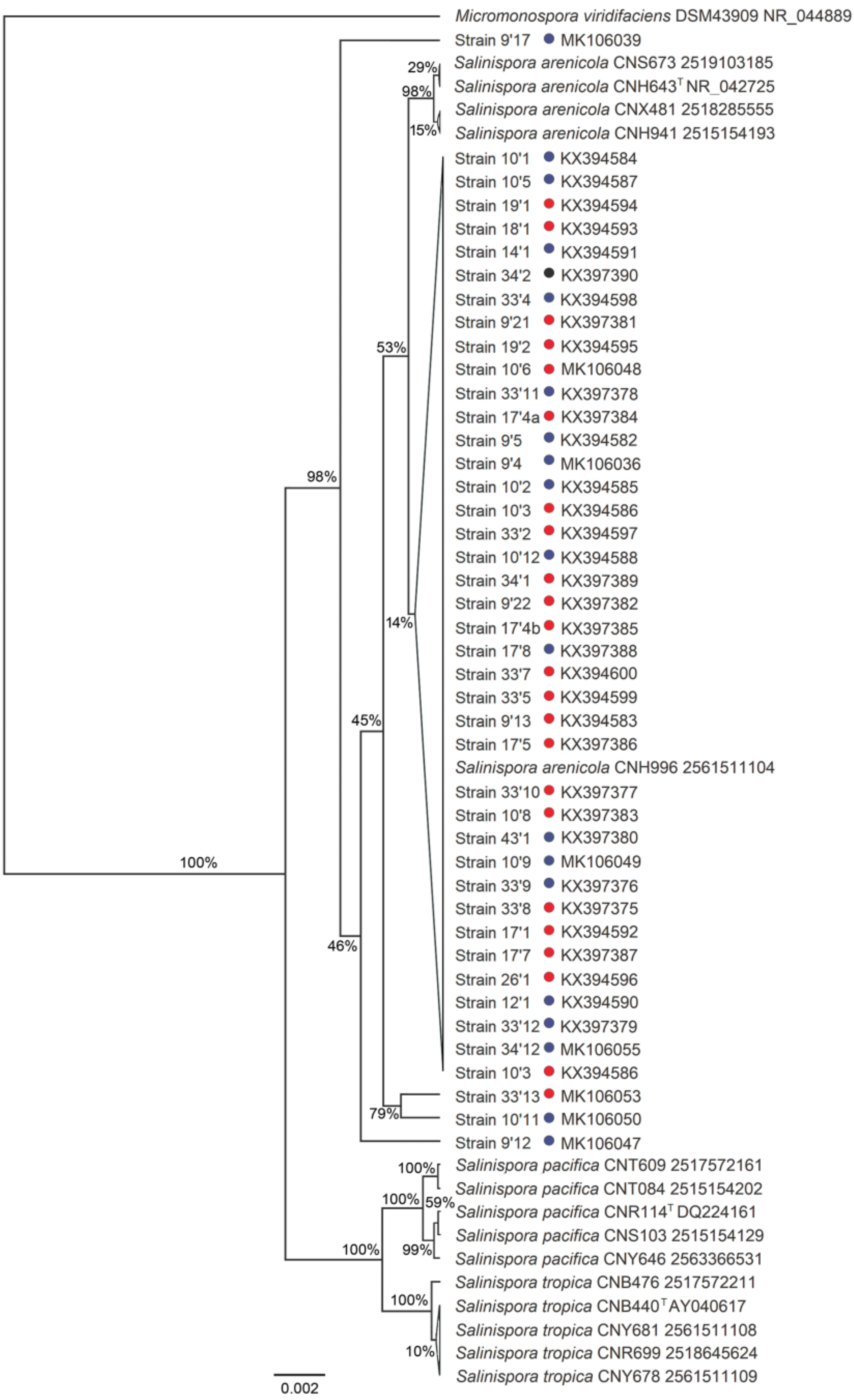

Fig. 1. The Bayesian inference tree of 1,175 bp of the 16S rRNA gene sequences from strains that composed the genus *Salinispora*, and the *Salinispora* strains isolated from Punta Arena de la Ventana sediments, with *Micromonospora viridifaciens* as outgroup. The posterior probability is indicated. Colored dots indicate groups previously determined by morphological properties. Blue: Group 1; Red: Group 2; Black: Undetermined.

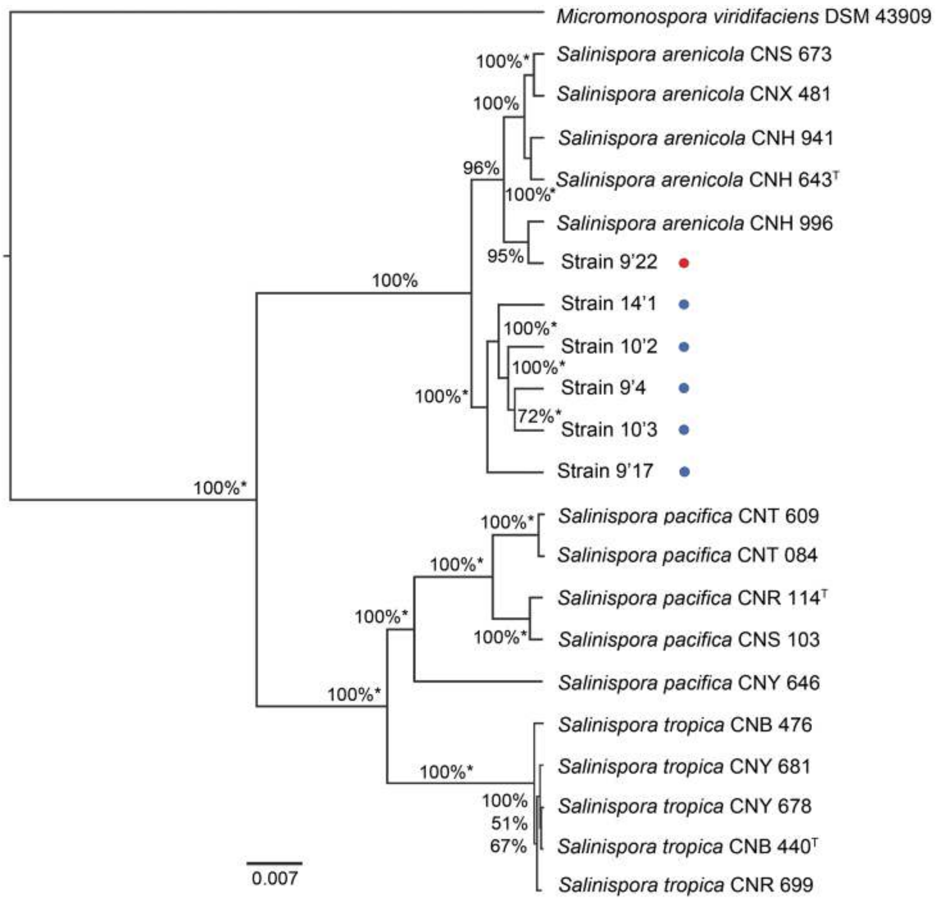

Fig. 2. The Bayesian inference tree of the 4,349 bp concatenated gene sequences (16S rRNA-*atpD*-*gyrB*-*rpoB*-*secY*) from the strains within the genus *Salinispora*, and the *Salinispora* strains isolated from Punta Arena de la Ventana sediment with *Micromonospora viridifaciens* as outgroup. The posterior probability is indicated. Colored dots indicate groups previously determined by morphological properties. The asterisk represents clades supported by ML. Blue: Group 1; Red: Group 2

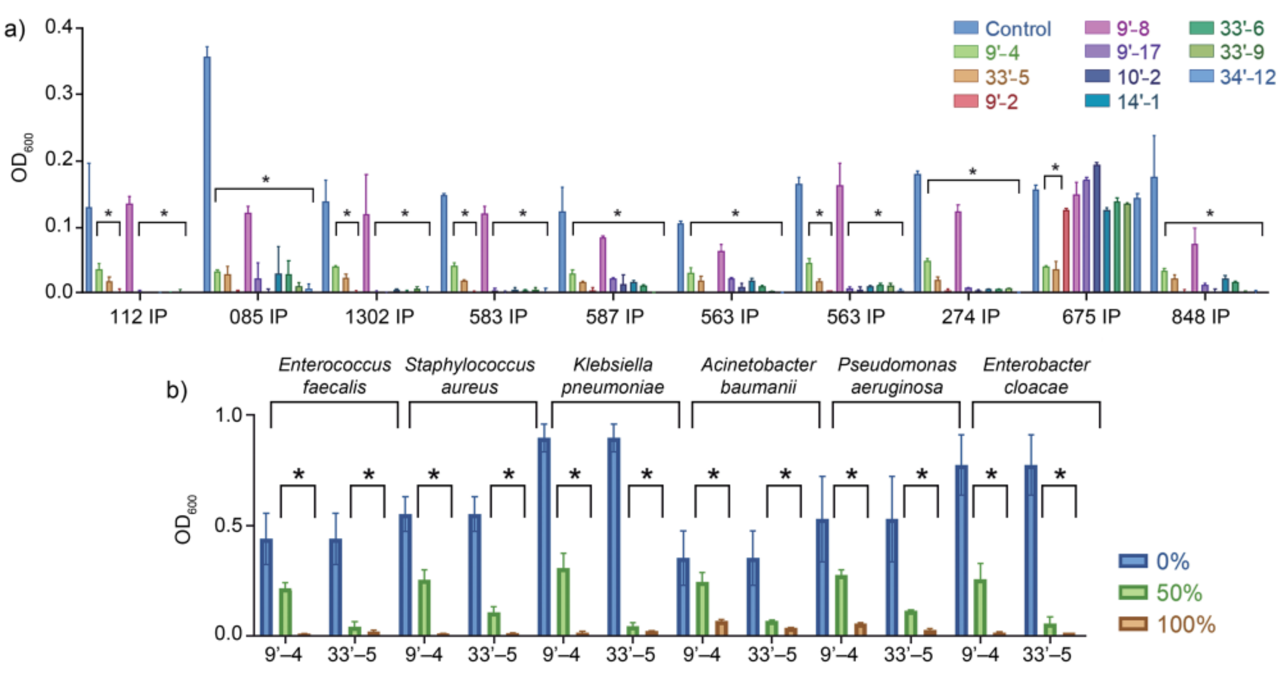

Fig. 3. The inhibition of the growth of *S. epidermidis* and ESKAPE bacteria.

a) the *Salinispora* sp. supernatants tested against ten isolates of *S. epidermidis* from prosthetic joint infections. b) inhibition of the growth of ESKAPE bacteria by the supernatants strains 9'4 and 33'5 of *Salinispora* sp. Significant differences compared with the control are marked with an asterisk ($p < 0.05$). Results of a) and b) are expressed as the average of triplicates, and the standard deviation is represented by error bars.

Maldonado et al. 2015). The 16S rRNA gene sequencing indeed confirmed their assignment to *Salinispora* spp. All the strains were found to be related to *S. arenicola* CNH996, which was originally isolated from the GC (Edlund et al. 2011; Millan-Aguinaga et al. 2019). Though, the selective isolation procedure was oriented to recover marine obligate microorganisms, other species as *Erythrobacter* sp., *Lutibacterium* sp., *Micromonospora* sp., and *Mycobacterium* sp. were also isolated and identified. According to the number of salinisporae isolated from a single sediment, PVA encompasses a high level of actinobacteria diversity that needs to be fully explored. Phylogenetic analysis supports the proposal that some strains recovered from PAV may represent novel species within the *Salinispora* genus, but a full polyphasic taxonomic approach is needed.

Although the 16S rRNA gene sequencing grouped the isolates to *S. arenicola* CNH996 which was previously isolated also from the GC, the ML analyses showed a different picture of the relationships between the sequences of our isolates and other sequences of *Salinispora* obtained from the databases (Fig. 1 and Fig. 2). The ML analysis performed on the strains selected confirmed the separation of a monophyletic group apart from other *S. arenicola* except for strain 9'22, which grouped again with *S. arenicola* CNH966. It is worth mentioning that despite the high levels of similarity found within *S. arenicola* strains, whole genome sequencing and a previous MLST study suggest that some *S. arenicola* strains are not "truly" *S. arenicola* but should be assigned to a different though still "unnamed" species (Millan-Aguinaga et al. 2017). The fact that a monophyletic group was formed with some of the strains from this study certainly supports the proposal that it may represent a novel species. Regarding Fig. 2, only *S. arenicola* CNH966 and CNH941 were reported to be isolated from the GC. Thus, the fact that our isolates were more related to *S. arenicola* than to *S. tropica* or *S. pacifica* provides solid ground for more studies on such marine sites along the GC peninsula to search strains with biotechnological potential capable of inhibiting pathogenic bacteria. *S. arenicola* CNH966 was isolated from a higher latitude (24°49.49' N, 110°35.16' W; around 115 km from the PVA sampling site); therefore, the geographic and phylogenetic variation could lead to different secondary metabolite production as it has already been suggested by the Jensen group (Jensen et al. 2007; Jensen 2015b).

The MLSA was well supported by the individual phylogeny of each gene fragment, and the phylogeny of the *secY* gene showed that it could be included with the "usual" MLSA genes to study, at least, members of the genus *Salinispora* (Freel et al. 2013). Interestingly, whole-genome phylogeny (Millan-Aguinaga et al. 2017) also showed that *S. arenicola* CNH941 belong to a different group than that of *S. arenicola* CNH996, and, as shown in Fig. 2, the MLSA analysis from our study also supported this relationship. *S. arenicola* strains (except for 9'22) identified in this study are clearly separated from the other two, that is, *S. arenicola* CNH966 and *S. arenicola* CNH941 based on the MLSA study thus suggesting its own and perhaps unique identity.

Salinispora sp. strains 9'4 and 33'5 showed the ability to inhibit the growth of eighteen clinical strains of *S. epidermidis* (from ocular and prosthetic joint infections) and ESKAPE pathogens. These two strains may produce antimicrobial molecules with a wide range of activity against Gram-positive and Gram-negative organisms because the ESKAPE group is composed of these two types of bacteria.

Salinispora sp. 9'22 and 33'5 were clonal, as confirmed by the fingerprinting with BOX-PCR (data not shown). They were separated from all the strains when assayed with MLSA, though grouped with *S. arenicola* CNH996 as previously mentioned for strain 9'22. *Salinispora* sp. 9'4 grouped with the other strains chosen. Some *Salinispora* inhibited the growth of bacteria from one bacterial genus, and some inhibited the growth of bacteria from more than two. The rest of the strains could be used in the inhibition of one specific bacterial genus. The strains that showed no inhibition of growth of other species (at least *S. epidermidis*) might be tested against fungi, viruses, or parasites. This also shows the importance of studying single strains of *Salinispora* to show their full metabolic potential.

The studies of *Salinispora* spp. have been centered around their cytotoxic and carcinogenic features due to important molecules like arenamides and saliniketals, although it is well known that *Salinispora* produces diverse forms of rifamycin (Kim et al. 2006), and molecules like cyclomarazines, which demonstrate inhibitory properties against *M. tuberculosis* (Weinhaupl et al. 2018).

The bacterial species used in the present work, like members of the ESKAPE group, are listed as priority organisms by the World Health Organization (WHO 2017). They are resistant to a whole range of commercial antibiotics, and there is a global initiative to discover, research, and develop new antibiotics to fight this multi-drug-resistant bacteria. To our knowledge, our report is one of the first in this area and was designed for searching *Salinispora* strains active against global priority organisms of medical importance.

Conclusions

Punta Arena de la Ventana is the furthest South Point of the GC ever studied and seemed to contain a high diversity of *Salinispora* species. This study supports the proposal that exploring distinct sites or ecological niches may end in the isolation of novel microorganisms producing new bioactive molecules. The species from PAV needs to be further explored for bioprospecting, ecology, and genetic potential. *Salinispora* sp. 9'4 and 33'5 inhibit the growth of emerging bacterial pathogens and other multi-drug-resistant bacteria, and among the latest, the priority pathogenic organisms, according to the WHO. Discovering of these abilities of *Salinispora* from PAV represents the first step of research and contribution to the global initiative. It is also a response to the urgent need to discover new antibiotics.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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